Further Investigations on Induction of Mammary Cancer in Mice by Isografts of Hypophyseal Tissue*

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SUMMARY

Hypophyseal isografts to sites remote from the hypothalamus in otherwise untreated female mice produce prolactin continuously. The excessive stimulation of the mammary glands by prolactin and progesterone in the graft-bearing animals may lead to mammary tumor formation.

The effect of isotransplantation of a single hypophysis at various sites (subcutaneous, intraperitoneal, spleen, ovary, kidney) in MTA-free (♀ O20 × ♂ IF)F1 female mice was investigated. Mammary tumor incidences of up to 80 per cent were observed in the experimental groups, as against a value of 2 per cent in the controls. Grafts of hypophyses of adult donors into the kidney were more effective than grafts into the spleen. Hypophyseal grafts of adult male donors were more effective than those of adult female donors. This sex difference was absent in the very potent grafts of infantile donors. Even grafts of a hypophysis of very old donors produced prolactin continuously and induced mammary tumors in the hosts.

Subcutaneous isografts of a single hypophysis frequently failed to “take,” but multiple subcutaneous grafts again proved efficacious. Multiple grafts into the kidney were more effective than single grafts.

Vaginal smear studies indicated that the effect of the hypophyseal grafts on mammary tumor formation was related to the amount of prolactin produced by the transplants in the course of time in the various experimental groups.

Many hypophyseal grafts showed progressive growth in the hosts. This phenomenon was most pronounced in the groups with a high mammary tumor incidence.

Heterotopic isografting of hypophyses into intact female mice has been shown to be an effective means to induce mammary tumors in mice free of the mammary tumor agent (MTA) (17, 29, 36, 37). The same method can also be used to accelerate mammary gland carcinogenesis in animals infected either naturally or experimentally with the MTA (17, 29–31, 37).

The mechanism involved seems to be the uninhibited release of relatively large amounts of prolactin by the grafts, causing excessive development of the mammary gland and, ultimately, tumor formation (36).

The object of this investigation was to study the effect of a number of possible variables in this experimental set-up, in an effort to determine which combination of factors is the most effective and economical in the induction of mammary tumors. The variables investigated were: (a) site of implantation of the hypophyses; (b) number of hypophyses implanted; (c) sex of the donors of the hypophyses; (d) age of the donors of the hypophyses.

The influence of the genetic and MTA factors, known also to be important in mammary tumor formation, was purposely avoided by studying the variables mentioned above in only one MTA-free F1 hybrid combination.

Preliminary reports have been given in short papers and abstracts (4–6).

MATERIALS AND METHODS

All experiments were performed on (♀ O20 × ♂ IF)F1 hybrids. Both parent strains are naturally
free of the MTA. The O2o strain was developed at our institute by the late Dr. R. Korteweg and was in the 107th–120th generation of inbreeding during the period of these experiments. Details on the mammary tumor frequency in normal animals of this strain and in animals infected experimentally with the MTA have been published (38). The IF strain was obtained from Dr. G. M. Bonser, Leeds, England, in 1950, and was inbred for a further eighteen to 32 generations in our institute during the period of these experiments. Details on the mammary tumor frequency in animals without and with the MTA have been published (35).

The animals were kept in glass boxes (17 × 11 × 12 cm.), three to a box, and were given commercial pellets and tap water ad libitum and some wheat twice a week. Environmental conditions such as temperature and light were rigidly controlled.

Grafting of the hypophyses was performed when the hosts were 6–8 weeks old, when normal estrous cycles had been established. All hosts remained intact—i.e., had their own ovaries and hypophyses in situ, and were kept as virgins. The implanted hypophyses were obtained from donors of the same hybrid combination, except in the few cases where old hypophyses of the female parent strain were used, as indicated in Table 5. Infantile hypophyses were obtained from animals 9–10 days old, adult hypophyses from animals 2–6 months old, and old hypophyses from animals above 21 months of age.

The donor animals were decapitated under light ether anesthesia, the whole hypophyses were removed (anterior plus posterior lobe) and transplanted immediately at the desired site in the ether-anesthetized hosts. In the experiment in which the hosts received only half of a hypophysis, one whole hypophysis was divided into two, and the parts were implanted into two successive recipients, no attention being paid to the fate of the posterior lobe.

The transplantations made under the kidney capsule, into the spleen, into the ovarian capsule, or intraperitoneally were performed by the lateral-abdominal approach. In the main experiment on grafts into the kidney with hypophyses from adult donors, half of the hosts received grafts in the right kidney, and the other half in the left one. No ultimate differences were observed between these groups. In all other experiments with grafts into the kidney, as well as with the grafts into the ovarian capsule, the left side was preferred because of a small technical advantage. In the group of animals into which four hypophyses were implanted, two to each kidney, either this operation was performed at one session, or the right and left grafts were made with an interval of 3–7 days.

In the animals which received subcutaneous grafts, the hypophyses were deposited in pockets made a small distance from the incision. In the multiple subcutaneous graftings, batches of five hypophyses together were implanted at the same time. This operation was repeated at weekly intervals until the desired total number was obtained (up to twenty hypophyses), whereby the order of sites of implantation was arbitrarily chosen as left axilla, right axilla, left groin, and right groin, respectively.

In the vaginal smear studies the smears were prepared according to the method of De Jongh and Laqueur (29). The evaluation of the smears and the method of diagrammatic representation have been described in an earlier publication (30).

All animals were inspected for mammary tumors or intercurrent disease at least once weekly. Mammary tumors were recorded as appearing on the day when first palpated.

The animals were sacrificed either when a mammary tumor was observed or when moribund. The small number of animals which died before the age of 268 days (the age of the animal with the earliest mammary tumor observed in the various groups with hypophysal grafts) was disregarded in all calculations made. In the MTA-foster control groups the calculations were based on all animals surviving 151 days or more, since in these combined groups the first tumor appeared at that age.

At autopsy special attention was paid to the condition and size of the hypophysal grafts. Of a number of selected animals the grafts were fixed in Zenker-formol or Gendre and stained according to the Mallory method. The mammary tumors and other organs were fixed in Susa and stained with hematoxylin–azophloxin. The diagnosis of epithelial tumor of the mammary gland was confirmed histologically in all cases. Mammary glands of selected animals were studied by the whole-mount technic.

RESULTS

The success or failure of hypophysal isografting.

—When the effects of hypophysal isografting on mammary gland carcinogenesis under various experimental conditions were compared, it was essential to eliminate those animals in which the grafts do not "take"—called the "non-reacting" animals. At the beginning of these experiments it was thought that three criteria might be used independently to select the positive reactors—namely: (a) The conversion of the normal 4- to 5-day
estrous cycle of the hosts into a regular sequence of pseudopregnancy cycles shortly after isografting of the hypophyses, indicating the release of appreciable amounts of prolactin by the established grafts (36). This can be determined by taking vaginal smears daily for a certain period of time. (b) The finding of living grafts at autopsy. (c) The morphological picture of the mammary glands, at autopsy, indicating excessive hormonal stimulation.

It was found, however, that the latter two criteria could not always be used to distinguish between reacting and nonreacting animals. The mammary gland response was not reliable, since ca. 40 per cent of the old, untreated, control female mice also showed mammary gland pictures indicating abnormal hormonal stimulation. This aberrant reaction is owing to the presence of small, hormonally active (prolactin-producing) adenomatous foci in the hypophysis in situ. This hypophyseal change occurs only very late in life and thus has no appreciable influence on the mammary tumor incidence. A mammary gland picture at autopsy of old mice, indicating excessive hormonal stimulation in animals subjected to hypophyseal isografting, does not necessarily indicate functional activity of the grafts, at least not in the hybrid combination studied here. Only a negative reaction of the mammary gland could be used to eliminate nonreacting animals.

Furthermore, the finding of a living hypophyseal graft at autopsy is not sufficiently reliable as the sole criterion of reacting animals. With subcutaneous or intraperitoneal grafts of a single hypophysis, for instance, in which the site of implantation is ill defined, in some cases no grafts were found at routine autopsy, whereas the vaginal smear pattern still indicated the presence of a prolactin source. In these cases only a very laborious histological investigation probably would have revealed the grafted tissue. In other cases a macroscopic diagnosis was made impossible by the occurrence of leukemia or of adhesions at the site of operation. Again, reacting animals could not be selected in this way.

The only trustworthy method of selection seems to be that based on the change in vaginal smear pattern after the hypophyses were grafted. In all experimental groups, except those into which infantile hypophyses were implanted, the 4- to 5-day estrous cycle was converted to the pseudopregnancy type of cycle in the reacting animals within 1-8 days, largely depending on the stage of the estrous cycle at which the operation was performed. To establish routinely the "taking" of the grafts it was necessary only to take vaginal smears for the first 30 days after implantation. In doubtful cases smear-taking was extended beyond that period until a decision could be made. In all experimental groups a number of animals was selected at random to study the vaginal smear pattern throughout the entire lifetime.

From the combined material it can be concluded that, once the estrous cycle had changed into the pseudopregnancy type, it remained that way indefinitely, indicating that no grafts failed after an initially successful "take." Conversely, no instances were encountered in which the grafts started functioning after a prolonged delay.

In general the results of the vaginal smear studies shortly after hypophyseal isografting accorded with the autopsy findings. In only four cases were very small grafts found at sacrifice in animals which had failed to show an estrous cycle reaction. Two of these animals, given implants of an infantile hypophysis in the spleen (Experimental Groups v and w, respectively), showed no vaginal smear pattern reaction for 50 days and were sacrificed about 3 months after grafting. The third animal had received one hypophysis of a female donor subcutaneously (Exp. Group b), showed no vaginal smear pattern reaction for 7 months and was sacrificed at an age of 26 months. The fourth animal had been given an implant of a hypophysis from a male donor into the spleen (Exp. Group o), again showed no vaginal smear pattern reaction (for up to 3.5 months after implantation), and was sacrificed at an age of 25 months. In all four cases it is uncertain whether the grafts were completely inactive or produced only amounts of prolactin insufficient to affect the ovarian corpora lutea; the latter assumption seems to be the more probable one—nor is it known whether the grafts had assumed full activity between the moment of suspension of vaginal smear-taking and the time of sacrifice. It was decided to consider these four animals as nonreactors and to eliminate them from the calculations.

Table 1 shows the results of this part of the investigation. Only a number of the experimental groups are represented. In the groups not represented here, all isografts "took," and no correction was necessary.

The success or failure of subcutaneous hypophyseal isografts seems to depend largely on the number of hypophyses implanted, single grafts alone yielding a large number of failures. In the intraperitoneal, ovarian, spleen, and kidney grafts the site of implantation, as well as the age and sex of the donors, seems to play a role.

The ultimate fate of the surviving 37 animals in
which the grafts failed will be commented upon in
the next section.

Mammary tumor incidence in mice given iso-
grafts of hypophyses from adult donors.—The mam-
mary tumor incidences in the experimental groups
into which hypophyses of male or female adult
donors were successfully isografted subcutaneous-
ly or intraperitoneally are listed in Table 2, to-
gether with the value found in the control virgin
females.

Although only two mammary tumors were ob-
served in a total of 84 control virgin females, many
tumors were induced by the subcutaneous and
intraperitoneal hypophyseal isografts. In the

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**Table 1**

**Correction of the Experimental Groups by Elimination of the Animals with Unsuccessful Hypophyseal Isografts**

<table>
<thead>
<tr>
<th>Site of implantation</th>
<th>No. hypophyses</th>
<th>Donors</th>
<th>Initial no. animals</th>
<th>No. animals after correction</th>
<th>Exp. group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>with unsuccessful</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>grafts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.C.</td>
<td>1</td>
<td>Ø</td>
<td>Adult</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>1</td>
<td>Ø</td>
<td>&quot;</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>1X5</td>
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<td>16</td>
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<tr>
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<td>&quot;</td>
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<td>25</td>
</tr>
<tr>
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<td>Ø</td>
<td>&quot;</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>&quot;</td>
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<td>Ø</td>
<td>&quot;</td>
<td>4</td>
<td>2</td>
</tr>
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<td>Ø</td>
<td>&quot;</td>
<td>32</td>
<td>43</td>
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<td>&quot;</td>
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<td>&quot;</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>&quot;</td>
<td>1</td>
<td>Ø</td>
<td>&quot;</td>
<td>51</td>
<td>51</td>
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<tr>
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<td>Ø</td>
<td>&quot;</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>&quot;</td>
<td>1</td>
<td>Ø</td>
<td>&quot;</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Spleen</td>
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<td>25</td>
<td>24</td>
</tr>
<tr>
<td>&quot;</td>
<td>1</td>
<td>Ø</td>
<td>&quot;</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>Kidney</td>
<td>1</td>
<td>Ø</td>
<td>Old</td>
<td>21</td>
<td>20</td>
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</table>

*Animals sacrificed before the age of 268 days.

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**Table 2**

**Mammary Tumors in (♀ OpmX♂ IF)F1 Female Mice with Subcutaneous or Intraperitoneal Isografts of Hypophyses of Adult Donors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. hypophyses implanted</th>
<th>Sex of donors</th>
<th>No. animals with tumor</th>
<th>No.</th>
<th>Av. age (days)</th>
<th>Av. age at death (days)</th>
<th>Exp. group</th>
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<td>Controls (virgins) 84</td>
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<td>483</td>
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<td>1</td>
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<td>0</td>
<td>604</td>
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<td>1</td>
<td>Ø</td>
<td></td>
<td>3</td>
<td>2</td>
<td>702</td>
<td>c</td>
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<tr>
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<td>1</td>
<td>Ø</td>
<td></td>
<td>6</td>
<td>0</td>
<td>604</td>
<td>d</td>
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<td>Ø</td>
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<td>6</td>
<td>5</td>
<td>643</td>
<td>e</td>
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<td>Ø</td>
<td></td>
<td>4</td>
<td>4</td>
<td>712</td>
<td>f</td>
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<td>Ø</td>
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<td>2</td>
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<td>Ø</td>
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<td>13</td>
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<td>2</td>
<td>561</td>
<td>i</td>
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<td>Ø</td>
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<td>2</td>
<td>2</td>
<td>687</td>
<td>j</td>
</tr>
<tr>
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<td>Ø</td>
<td></td>
<td>2</td>
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<td>561</td>
<td>k</td>
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<tr>
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<td>2</td>
<td>2</td>
<td>687</td>
<td>l</td>
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groups with subcutaneous grafts the tumor percentage increased markedly when ten to twenty hypophyses were implanted, as compared with the values found in the groups receiving only one or five hypophyses.

No significant differences between male and female donors were evident in these relatively small groups. In the animals with subcutaneous hypophyseal isografts there seemed to be a relationship between the frequency of graft failures and the mammary tumor incidence ultimately observed in the animals with successful grafts—the groups with many graft failures showing low tumor frequencies (cf. Table 1, Exp. Groups b, c, and d). The number of animals with intraperitoneal grafts studied was too small to allow conclusions in this respect.

The mammary tumor incidences obtained by isografting a single hypophysis from adult donors into spleen, kidney, or ovary are given in Table 3. From the results in the groups with grafts in kidney or spleen it is evident that isografted male hypophyses were more active in mammary gland carcinogenesis than were female hypophyses, whereas grafts in the kidney were more active than those in the spleen, irrespective of the sex of the donor animals.

The grafts in the ovary showed a tumorigenic activity intermediate between those in spleen or kidney. A direct action of these grafts in the ovary on the adjacent ovarian tissue could not be established, either from the mammary tumor frequency or from the ovarian histology.

A marked correlation between a high mammary tumor frequency and a relatively low tumor induction time was evident in the Experimental Groups n, o, p, and q. The same groups show again the correlation discussed above between frequency of successful grafts (cf. Table 1) and the mammary tumor incidence in the animals with functional grafts.

Single hypophyseal grafts in kidney, ovary, or spleen were definitely superior to single subcutaneous grafts, especially when the number of failing grafts was also taken into account.

The presence of adhesions as a consequence of the surgical intervention, observed in some of the animals, did not influence the results. Special attention was paid in this respect to the groups with grafts in the spleen.

The condition most favorable for mammary tumor induction discussed so far—i.e., implantation of hypophyses from adult donors into the kidney—was also studied with the amount of hypophyseal tissue grafted as the variable. Two further experimental groups were studied: in the first the animals received half a hypophysis, in the second four hypophyses, two in each kidney. The results are given in Table 4.

As compared with the implantation of a whole hypophysis (Table 3, Exp. Group q) the isografting of half a hypophysis was insignificantly less effective in inducing mammary gland tumors. This relatively high activity again could be correlated with the fact that no graft failures were observed in this group.

In every respect the most successful group was that in which four hypophyses from adult male donors were implanted into the kidneys, two on each side. This group yielded the highest mammary tumor incidence at the lowest average tumor age, and also comprised the earliest mammary tumor observed in the MTA-free animals (268 days). No graft failures occurred.

All the animals in which the grafts failed (37 in total; cf. Table 1) and which survived for 268
days or longer originally belonged to the groups discussed above. The mammary tumor incidence in these animals was not significantly different from that in the controls (Exp. Group a). Two tumors were observed (5 per cent): one at an age of 785 days (in an animal of Exp. Group m), and one at an age of 606 days (in an animal of Exp. Group n), respectively. The average age of the 35 mice sacrificed without a mammary tumor was 736 days. Indirectly, this result again demonstrates the reliability of the selection method employed.

Mammary tumor incidence in mice isografted with hypophyses from infantile or old donors.—The mammary tumor frequencies found after isografting a single hypophysis from 9- to 10-day-old donors into the spleen, and after implantation of a single hypophysis from 21- to 28-month-old donors into the kidney are listed in Table 5. In the latter experiment hypophyses from animals of the hybrid combination, as well as those from the female parent strain, were used.

In the experimental groups given isografts of infantile hypophyses high mammary tumor frequencies were observed—higher than those seen in the groups in which hypophyses from adult donors were implanted into the spleen (cf. Table 3, Exp. Groups a and o). Moreover, the sex difference observed in the groups with the adult donors was absent when infantile donors were used.

Contrary to what was observed in all other experimental groups, the estrous cycle change in the hosts did not occur immediately after the grafting of infantile hypophyses. In these animals the regular sequence of pseudopregnancies was initiated only after a latency period of 10- to 20-days' duration, corresponding with two to four normal estrous cycles after transplantation. This phenomenon made it necessary to observe a control period of at least 50 days in these animals to determine with certainty whether or not the grafts were successful. This latency period, the cause of which might be sought either in the lesser quantity of hypophyseal tissue transplanted or in the difference in developmental stage between infantile and adult hypophyses, can explain only in part the relatively high average tumor ages in relation

### Table 4

<table>
<thead>
<tr>
<th>HYPOPHYSIS IMPLANTED</th>
<th>NO. ANIMALS</th>
<th>WITH TUMOR</th>
<th>WITHOUT TUMOR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>Per cent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>27</td>
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### Table 5

<table>
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<tr>
<th>SITE OF IMPLANTATION</th>
<th>TREATMENT</th>
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<td></td>
<td></td>
<td></td>
<td>No.</td>
<td>Per cent</td>
</tr>
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<td>Spleen 9-10 days</td>
<td>Q</td>
<td>24</td>
<td>20</td>
<td>88</td>
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<td>Q° (O20×1F)F1</td>
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<td>19</td>
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<td>Kidney 21-28 months</td>
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<td>35</td>
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<td></td>
<td>Q°</td>
<td>4</td>
<td>2</td>
<td>(50)</td>
</tr>
</tbody>
</table>
to the tumor incidences observed. This point will receive further consideration in the discussion of the long-term vaginal smear studies.

Hypophyses of old donors are also effective in mammary tumor induction. From the long-term vaginal smear studies, as well as from the histological aspect of the grafts at sacrifice of the hosts, it was evident that the old hypophyses fully retained the prolactin-producing capacity for at least 20 months after grafting, which is a rather striking fact.

Even with the small number of animals studied it is apparent that the mammary tumor incidence in the animals grafted with old hypophyses is rather low. This is owing at least partly to a secondary complication—the frequent occurrence of leukosis in the hosts at a relatively early age. Of the 28 animals grafted with hypophyses from old donors seven (25 per cent) developed leukosis at an average age of 402 days, leading to a low value for the average age at death of the animals sacrificed without a mammary tumor. The incidence of leukosis in the 82 control animals without mammary tumors was 11 per cent (nine animals), at an average age of 727 days.

The cause of this disease in the grafted animals is not clear. The donor animals were selected for the absence of manifest disease. Six cases of leukosis (7 per cent) at an average age of 697 days were observed in the 83 animals without mammary tumors given implants of adult hypophyses in spleen or kidney (Exp. Groups n, o, p, and q). Consequently, the leukosis does not seem to be related to the hormonal function of the grafts, unless it is assumed that this function of the old hypophyses differs qualitatively from that of adult grafted hypophyses. The high leukosis incidence and the low age at which it occurred in the animals given implants of old hypophyses seem to be related more specifically to the grafting of old tissue as such. The most acceptable explanation is either that occult leukosis of the donor animals became manifest in the host or that the leukotic processes originated in the grafted tissue after transplantation.

**Mammary tumor incidence in mice infected with the mammary tumor agent.**—As stated in "Materials and Methods" both parent strains of the (♀ O₉ × ♂ IF)F₁ hybrid are considered to be free of the MTA.

To define more precisely the susceptibility to mammary gland carcinogenesis in the hybrid, both male and female animals were infected with the MTA by foster-nursing them from birth until weaning on (♀ C₃H × ♂ WLL)F₁ hybrid mothers, which harbor the agent. The female animals were kept as virgins; the males were castrated at an age of 6 weeks and thereafter treated continu-

<table>
<thead>
<tr>
<th>SEX</th>
<th>TREATMENT</th>
<th>No. ANIMALS</th>
<th>WITH TUMOR</th>
<th>WITHOUT TUMOR</th>
<th>EXP. GROUP</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No.</td>
<td>Per cent</td>
<td>Av. age (days)</td>
</tr>
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<td>40</td>
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<td>343</td>
</tr>
<tr>
<td>♀</td>
<td>Castration+estrone</td>
<td>44</td>
<td>29</td>
<td>66</td>
<td>353</td>
</tr>
<tr>
<td>♀</td>
<td>Controls, MTA-free, castration+estrone</td>
<td>36</td>
<td>2</td>
<td>6</td>
<td>621</td>
</tr>
</tbody>
</table>

Data on the mammary tumor incidences in these three experimental groups are presented in Table 6. The figures on the female MTA-free controls have been presented in Table 2 (Exp. Group a).

From the results it is apparent that the hybrid combination also shows a high susceptibility to the MTA. The tumor induction time after MTA infection proved to be shorter than that found after hypophyseal isografting. Indirectly, this experiment furnishes additional evidence that the hybrid is naturally MTA-free.

Nearly all male animals, both with and without the MTA, receiving long-term estrogen treatment developed large hypophyseal adenomas in situ, frequently necessitating sacrifice.

**Morphology of the mammary glands and mammary tumors.**—The mammary glands of nearly all mice with functional hypophyseal isografts

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TABLE 6

MAMMARY TUMORS IN (♀ O₉ × ♂ IF)F₁ MICE INFECTED WITH THE MAMMARY TUMOR AGENT OF (♀ C₃H × ♂ WLL)F₁ HYBRIDS
were studied, with the use of whole mounts of one gland of the second thoracic pair. These animals showed in principle the mammary gland picture already described in 1939 by Loeb and Kirtz (31). Although the main features of pregnancy development were present, the whole aspect was different from that observed in normal pregnancy, and very variable, even in individual glands. The ducts were often wide and filled with secretion; lateral buds were more numerous than in pregnancy; the lobules remained stunted, forming thick clusters of acini around the ducts; usually an irregularly distributed secretion of fat droplets and protein was present; and the amount of hemosiderin pigment was high.

The results of a more detailed quantitative investigation on the various aspects mentioned above will be published separately.

The epithelial mammary tumors in the animals with hypophyseal isografts were of the usual histological types found in mice, as described and classified by Dunn (10). In the hybrid combination studied here, as in many other mouse strains, the majority of the tumors were of type B. Type B was subdivided into three subtypes: AB, the partly acinar form; B, the more solid form; and B-pap., the markedly papillomatous tumor.

Of the 269 tumors studied, the incidences of the various types were: A, 1 per cent; AB, 44 per cent; B, 19 per cent; B-pap., 6 per cent; squamous, 23 per cent; carcinosarcoma, 4 per cent; and large-cell tumors, 3 per cent. None of the experimental groups yielded a spectrum of tumor types differing significantly from the average. The mean age of the animals with tumors of the squamous type was slightly higher than that of the animals with the other tumor types (577 and 521 days, respectively).

Of the 78 tumors found in the MTA groups (Exp. Groups aa and bb) 18 per cent were of type A, 36 per cent AB, 43 per cent B, and 3 per cent B-pap. The “miscellaneous” tumor types were lacking, presumably because of the lower tumor ages, as compared with those in the mice with hypophyseal grafts.

Often the tumors were markedly hemorrhagic. The incidence of hemorrhagic tumors was 45 per cent, both in the animals with hypophyseal isografts and in the MTA mice. In both groups the average tumor age of the mice with hemorrhagic tumors was somewhat lower than in the animals with non- or slightly hemorrhagic tumors. The significance of this phenomenon is obscure.

Long-term vaginal smear studies.—Vaginal smear studies were made not only to determine whether or not the heterotopic hypophyseal grafts were functional in each individual animal, as described above, but also to find out whether it would be possible to evaluate more precisely the quantitative function of these grafts over a long period. For this purpose vaginal smears were taken daily (never on Sunday, however) throughout the lifespan from a small number of animals chosen at random from each experimental group.

The gross changes in the vaginal smear pattern under the influence of the prolactin released from isografted hypophyses in intact female mice have been described in a previous paper (36). In that report it was explained how, after each ovulation, the corpora lutea formed through the intermediation of the hormones of the hypophysis in situ became active—i.e., produced progesterone under the influence of the continuously secreted prolactin, leading to a sequence of pseudopregnancies of 10- to 14-days’ duration each. There, too, it was stated that: “by the gradual changes in the vaginal estrous cycle—observed after transplantation of hypophyses—it was possible to evaluate the functional condition of the grafts not only qualitatively but also semi-quantitatively.” This conclusion was illustrated by charts showing that, under the influence of increasing numbers of subcutaneous hypophyseal grafts, the intervals between the short estrous periods tended to lengthen and that the signs of estrus in the vaginal smears became correspondingly less pronounced. These same phenomena were observed again in this investigation in the experimental groups receiving increasing numbers of subcutaneous isografts, and thus need no further elaboration here.

From the observations on animals of the experimental groups in which only a single hypophysis was implanted, it became apparent that here, too, a semiquantitative evaluation of the function of the grafts, according to site of implantation, and age and sex of the donors was possible, as will be illustrated by a number of charts. Except for the control female, all mice from which the smear graphs were selected for presentation were sacrificed with a mammary tumor.

Chart 1 shows the estrous cycle history of one of the control animals (Exp. Group a), with 4- to 5-day cycles interrupted only occasionally by spontaneous pseudopregnancies (27, 28, 37). The regular pattern was maintained until an age of about 700 days, whereafter ovarian old age changes became evident, characterized by continuous sub-estrous periods (60).

Chart 2 gives the results after implantation of a hypophysis from an adult female donor into the spleen (Exp. Group n), showing the ideal picture of a continuous sequence of pseudopregnancies.
with an average interval of 12 days (43 cycles in 516 days). This sequence was maintained for more than 500 days, up to the moment the animal was sacrificed. Chart 3 shows the effect of a more active graft, that of a hypophysis of an adult male donor in the kidney (Exp. Group q). Initially there was again a clear-cut sequence of pseudopregnancies (average interval 13 days), with conspicuous estrous periods, which became gradually less pronounced, however, terminating in a more or less total disappearance of all estrous activity in the vaginal smears, indicating continuous progesterone activity.

Grafts of a hypophysis of male donors in the spleen and of female donors in the kidney gave vaginal smear graphs intermediate between those of Chart 2 and Chart 3. Corresponding pictures were seen in animals isografted with a hypophysis from old donors into the kidney (Chart 4). The age of the male donor animal was in this case 21 months (Exp. Group z).

The animals grafted with a hypophysis from infantile donors into the spleen yielded vaginal smear patterns deviating slightly from those discussed above. First there was the latency period in which the grafts did not yet produce enough prolactin to induce pseudopregnancies. Thereupon followed a period with clear-cut pseudopregnancies, as in the animals grafted with an adult hypophysis into spleen or kidney, then a period characterized by subnormal estrous peaks ending in a prolonged continuous diestrous stage (Chart 5, Exp. Group v). From these findings it is concluded that the grafts of infantile donors produced only relatively low amounts of prolactin initially, to develop a much greater activity later on.

Grafts of hypophyses in the ovary gave rather...
variable level of the grafts intermediate between those in spleen and kidney, respectively.

Subcutaneous grafts of a single hypophysis yielded pictures more or less like those illustrated in Chart 2. Occasionally, however, in these animals the regular pseudopregnancy sequence was interrupted by one or two normal 4- to 5-day estrous cycles, mainly in the period shortly after transplantation, indicating a near-threshold production of prolactin, which was at times too low to maintain the function of the corpora lutea. The number of animals in which this phenomenon could be studied was unfortunately too small to give more detailed findings.

With respect to the animals receiving grafts in the kidney of either one-half a hypophysis or four hypophyses the following is worth mentioning. In animals from Exp. Group t (half a hypophysis of an adult male donor) the vaginal smear pattern was indistinguishable from that of Exp. Group q (whole hypophysis of adult male in kidney, cf. Chart 3). In the mice of Exp. Group u, with two hypophyses of adult male donors in each kidney, the vaginal smears were nearly always anestrous, as illustrated in Chart 6, indicating very high levels of prolactin release from the beginning.

When the results of the long-term vaginal smear studies in the various experimental groups are compared with the mammary tumor incidences in these groups the correlation is clear: the higher the prolactin production by the grafts, the higher were the mammary tumor frequencies observed ultimately, and the lower the average tumor ages. This correlation is but slightly obscured by the fact that the prolactin secretion by the grafts increased gradually with time in many animals. This phenomenon is most clearly illustrated in Charts 3 and 5. Since no data on growth of the hypophyseal grafts with lapse of time in relation to the vaginal smear pattern are as yet available beyond those obtained at autopsy in animals sacrificed because of ill health or a mammary tumor, a more detailed investigation of this aspect seems clearly indicated. One apparent discrepancy in the results can, however, be explained by the increase in activity in the course of time—namely, the high average tumor age in the experiments on grafts of infantile donors. Here the initial low prolactin activity followed only later in life by a very strong activity leads to a high tumor incidence, but the average tumor induction times still are longer than in those groups in which the

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**Chart 5.**—Vaginal estrous cycle after implantation of one hypophysis of an infantile female donor into the spleen

**Chart 6.**—Vaginal estrous cycle after implantation of two hypophyses of adult male donors into each kidney

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grafs developed a stronger activity from the start (cf. Exp. Groups p and q against v and w in Tables 3 and 5).

One detail perhaps needs more comment. The vaginal smears observed at the estrous peaks in the animals carrying hypophyseal grafts were not identical with those seen in stages estrus 1 or estrus 2 in the untreated animals. Frequently no real cornification was seen, although leukocytes disappeared and marked desquamation of epithelial cells occurred. Of these cells, however, some were cornified, whereas others were small, oval, or triangular, and often retained a pyknotic nuclear remnant. The cytoplasm of this cell type contained masses of PAS-positive material. Histologically these cells were found to derive from mucified vaginal epithelium, which, on estrogenic stimulation, may shed mucus-filled cells. The same smear pictures were seen in mice which came into estrus.
following a spontaneous or induced pseudopregnancy, indicating that this effect is due to the influence of the preceding progesterone phase.

The hypophyseal grafts.—The hypophyseal grafts recovered from the hosts were studied both in respect of size and histology. Many grafts had developed to a size far beyond that of the amount of tissue implanted; others had remained small. Hypophyseal tumors developed from grafts of a single hypophysis measuring up to 8 × 8 × 8 mm. have been found.

Tumorous transformation of hypophyseal isografts has been noted before in mice (3, 14, 29, 36, 48) and rats (26). The cause of the progressive growth of the hypophyseal grafts still remains obscure. Strain differences seem to be involved (3, 14, 36), implying an influence of the genotype. Differences in growth of the grafts, depending on the sex of the hosts and the hormone dependency of the tumors after transplantation, may indicate that hormonal factors play a part (3). Contrary to what might be expected a priori, estrogens cannot be considered as primary causative factors, since in the hosts in which the grafts did develop into large tumors the hypophysis in situ remained normal in size and histological picture. However, preliminary observations on a number of strains of mice in this laboratory seem to indicate that there may be a correlation between the liability to develop hypophyseal tumors in situ after estrogen treatment and the liability of the hypophyseal grafts to become tumorous. The effects of the disconnection from the inhibitory influences of the hypothalamus as well as those exerted by progesterone certainly merit further study.

The data on the growth of the hypophyseal isografts which became available during the experiments described above are unsuitable for a complete analysis, since this investigation was primarily concerned with mammary gland carcinogenesis. Thus, the animals were sacrificed as soon as a mammary tumor was diagnosed; or, when not presenting a tumor, they were kept alive as long as possible, leading to a broad range of ages at sacrifice in the various experimental groups. Still, some tentative generalizing conclusions seem permissible.

1. The size of the grafts frequently increased progressively with age.
2. Isografts of both male and female hypophyses could become tumorous.
3. The growth of the grafts was dependent on the site of implantation. Marked growth was observed in the grafts in the kidney, less growth in the grafts in the spleen, and still less in the subcutaneous grafts.
4. Within the experimental groups very marked individual variations were found. The same variability was apparent in the animals with the multiple subcutaneous grafts, in which one batch of five hypophyses implanted might have grown to a much larger size than another batch implanted one week earlier or later.
5. Grafts of infantile donors in the spleen grew better than those of adult donors, especially when the difference in amount of tissue originally implanted was taken into account.
6. Isografts of hypophyseal tissue of old donors could also become tumorous.

With regard to mammary gland tumorigenesis under the influence of the growth of the hypophyseal grafts, the conclusions above may be summarized as follows:

1. In comparing the different experimental groups there was a positive correlation between growth of the hypophyseal grafts and the mammary tumor incidence.
2. Within the individual experimental groups the animals with a mammary tumor did not show larger grafts than those without a mammary tumor, when animals sacrificed at the same age were compared. In the whole material, however, animals without mammary tumors showed larger grafts than those with mammary tumors, because of the difference in age at sacrifice.
3. Tumorous transformation of the grafts was not a prerequisite for mammary gland carcinogenesis. Especially in animals in which an early mammary tumor was found, the grafts sometimes were still small.

Histological study of the grafts in a large number of selected animals showed that many transplants contained large, distended sinusoids filled with blood or fluid. Thus, the size of the grafts determined macroscopically or in serial sections was not a true measure of the amount of hypophysal cell tissue. Correction for this complicating factor failed to affect the conclusions based on the sizes of the grafts discussed above.

The anterior lobe was always the part of the hypophysis that “took” and usually proliferated markedly. This tissue showed cells of different color shades and sizes, sometimes with visible negative Golgi images. Although some scattered dark blue granulated cells and some highly degranulated acidophils could be recognized, the majority of the cells could best be defined as “chromophobes.” An exception was found in the grafts of old donors. Here areas of fully granulated, normal-looking acidophils were present.

In many grafts small remnants of the pars intermedia were found, generally showing cells
with a normal aspect of size and coloring. The incidences of grafts with intermediate lobe tissue varied in the experimental groups from 20 to 80 per cent. The highest incidences were found in the groups with the highest mammary tumor frequencies, perhaps indicating that the circumstances which favor graft function and growth also favor preservation of the intermediate lobe tissue. Within each individual experimental group no correlation could be found between presence of pars intermedia tissue and age of the animals, size of the grafts, and presence or absence of mammary tumors.

In a few grafts the intermediate lobe tissue consisted of very large cells, sometimes containing two nuclei. The abnormally large size of these intermediate lobes may be the result of the large size of the individual cells only and not of proliferation.

In only one of the grafts a small part could be identified as neural lobe tissue.

**DISCUSSION**

The hormonal conditions in the mice bearing isografts of hypophyseal tissue seem to be well definable. The heterotopic hypophyses, being released from the inhibitory regulatory influence of the hypothalamus, produce prolactin continuously, which in turn causes progesterone secretion by the ovarian corpora lutea. The mammary glands of the graft-bearing animals are subjected to a continuous, long-term, excessive stimulation by prolactin and progesterone in the presence of sufficient amounts of estrogens and all the other hormones involved in mammary gland growth. Ultimately this stimulation leads to the formation of mammary tumors.

The literature on the luteotrophic function of heterotopic hypophyseal grafts in rats and mice has been reviewed previously (36). Many more recent publications emphasize this basic fact by functional and histological investigations (1–3, 9, 14, 26, 29, 34, 39, 40, 44, 45).

The possibility that other hormones as well are secreted by the grafts needs some further discussion. Depending on the experimental conditions, production of gonadotrophin (FSH and/or LH) (8, 15, 19–22), adrenocorticotrophin (ACTH) (7, 12, 13, 15, 21, 24, 32, 33), thyrotrophin (TSH) (11, 15, 16, 24, 25, 32, 46), and growth hormone (20) by heterotopic hypophyseal grafts in rats or mice has been described. Many of these investigations concern relatively short-term experiments on hypophysectomized hosts with intra-ocular grafts, so some caution is necessary in extrapolating the findings to the experiments described above.

Gonadotrophin secretion by heterotopic hypophyseal grafts has been observed thus far directly only incidentally in hypophysectomized male animals, whereas secretion of ACTH and TSH in subnormal amounts has been found much more regularly. It is important to note that all investigators agree that the quantitative level of the secretion of ACTH and TSH in the hypophysectomized hosts was inadequate for optimal function of the respective target organs.

In the present experiments in which nonhypophysectomized hosts were used, it seems unlikely that either FSH, LH, ACTH, or TSH secretion by the grafts played a major role in the mammary tumor induction. Routine histological investigations of the respective target organs failed to provide evidence for increased circulating levels of these hypophyseal hormones. If one keeps in mind the known negative feedback mechanisms between hypophysis and the target organs, it seems reasonable to assume that a limited secretion of the gonadotrophins, ACTH, or TSH by the grafts would be offset immediately by a decreased production of these hormones by the hypophysis in situ. An additional argument in this respect is that even grafted hypophyses may continue to be subjected to the inhibitory influences of the feedback mechanisms, as has been proved for the TSH secretion by grafted hypophyses (24, 46). If this principle is generally valid, any secretion of gonadotrophins, ACTH, or TSH by the grafted hypophyses in intact hosts will be kept in check by the dominating influence of the hypophysis in situ.

The situation in regard to growth hormone is somewhat more complex. Those investigators who found indications of growth hormone secretion by hypophyseal grafts in hypophysectomized hosts agree that here, too, the level of hormone production is suboptimal. A major role of the growth hormone secreted by the hypophyseal grafts on mammary tumor formation might be suspected in the cases in which multiple grafts were used, as in our previous investigation (36), but this seems unlikely in the present experiments with grafts of a single hypophysis or even part of a hypophysis, unless it can be proved that the growth hormone secretion by the grafts increases in time as appears to be the case frequently with the prolactin secretion. The available indirect evidence indicating that hypothalamic lesions may cause deficiency of growth hormone secretion by the hypophysis in situ suggests that growth hormone secretion in the intact animal may be regulated by the hypothalamus by a stimulatory mechanism analogous to that exerted by the hypothalamus.
on the secretion of gonadotrophin, ACTH, and TSH (41–43, 49). This would make the inhibitory regulation of the prolactin secretion by the hypothalamus the only exception to the general rule. The demonstration of a negative feedback mechanism for growth hormone secretion would be necessary, however, to complete the picture.

In summary, it is concluded that thus far there is no reason to assume that, in the changes in the hormonal equilibrium following heterotopic hypophyseal grafting in intact hosts leading to mammary tumor formation, other hypophyseal hormones besides prolactin are involved. The high levels of circulating prolactin and progesterone in the graft-bearing hosts may of course secondarily affect the other endocrine organs, as can be easily demonstrated by the conversion of the normal 4- to 5-day estrous cycle to the pseudopregnancy type of cycle after grafting, owing to changes in the secretion pattern of FSH and LH by the hypophysis in situ. A similar effect may explain the changes in adrenocortical function in hypophyseal graft-bearing mice described by Halberg and Haus (18).

The hormonal induction of mammary tumors in the graft-bearing animals must be considered to be the ultimate effect of all hormones combined acting on the mammary gland. The major change in the prolactin and progesterone secretion is thus primarily responsible for the tumor formation, but it is realized that these hormones can act only in conjunction with the other circulating hormones. This hypothesis of the primary role of prolactin and progesterone is strongly supported by the positive correlation found between the levels of prolactin (and secondarily progesterone) secretion, as demonstrated by the vaginal smear studies, and the mammary tumor induction time and frequency in the various experimental groups.

The present experiments as well as those of Liebelt and Liebelt (29) with intra-ocular hypophyseal grafts in mice also show convincingly that the prolactin effect is a systemic one, a point which could not be proved in the experiments with multiple subcutaneous grafts. An additional, but apparently small, local effect of the subcutaneous grafts on the mammary gland is not excluded, however.

The amount of prolactin produced by the grafts is determined not only by the conditions fixed at the beginning of the experiment, such as age and sex of the donors, site of implantation, and amount of tissue grafted, but also by the subsequent growth of the transplants. This potentiality for growth of the heterotopically grafted hypophyseal tissue, being determined by factors as yet unknown, explains at least in part the effectiveness of the grafts of hypophyses from infantile donors, where only small amounts of tissue are used. Preliminary investigations on the vaginal smear pattern in animals isografted with only small fragments of an adult hypophysis indicate that even these fragments "take," grow, and probably will induce mammary tumors in due course.

On the other hand the effect of the ultimately tumorous transformation of the grafts on mammary tumor formation must not be overestimated. First, it has not yet been proved that the amount of prolactin released by the grafts is directly proportional to the mass of the hypophyseal graft tissue. Even when this is the case, the grafts reach excessive sizes only late in life—owing to the exponential type of growth—too late to have a dominating effect on mammary gland carcinogenesis.

Besides the mammary tumors and the tumorous transformation of the hypophyseal grafts, no increase in the incidence of tumors of other types was observed in the experimental groups as compared with the controls, with the exception of the increased leukosis incidence in the animals grafted with old hypophyses. The explanation offered for this increase in leukosis incidence is at variance with the suggestion of Silberberg and Silberberg (47) that grafted hypophyses may exert a leukemogenic effect via the endocrine system.

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Further Investigations on Induction of Mammary Cancer in Mice by Isografts of Hypophyseal Tissue


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