Studies on Transplantations of Amphibian Anterior Pituitary into Chick Embryo. Analysis of Induction Capacity by Differential Solubility and Precipitation Method

by GAJANAN V. SHERBET

From the Department of Zoology, University of Poona

INTRODUCTION

SINCE the discovery by Bautzmann, Holtfreter, Spemann & Mangold (1932) that a diffusion of inducing substance takes place from the inductor into the competent reacting tissue, protein and ribonucleic acid have been favoured by candidates for the study of the nature of the inducing substance (see Brachet, 1960a, b).

Recently Mangold, Tiedemann & Woellwarth (1956) studied the inductive capacity of ox anterior pituitary in gastrulae of Triton alpestris. It was found to have considerable inductive power. They suggested that the gonadotropic hormones or the growth hormone might be the active principle of induction. These hormones are glycoprotein or simple protein in nature and hence it was felt desirable to study the inductive capacity possessed by fresh anterior pituitary gland by resorting to differential solubility and precipitation of the various glycoprotein, protein and ribonucleic acid (RNA) constituents of the gland.

MATERIALS AND METHODS

Fresh fertilized hens’ eggs obtained from a local poultry farm were incubated for approximately 18 hours at 37.5°C to get embryos at the primitive-streak stage. They were explanted in vitro by the method of New (1955). The glass-ware employed in these experiments was sterilized and the culture solutions were autoclaved.

The anterior pituitary gland of Rana tigrina was removed as described by Rugh (1952) and washed thoroughly in Pannett-Compton solution. Grafts of about 0.15 to 0.35 mm. in greatest dimension from either untreated (control) pituitaries or from treated pituitaries were made according to the method of

1 Author's address: Department of Zoology, University of Poona, Poona 7, India.
Following grafting the cultures were incubated for 18–20 hours.

Preliminary experiments showed that anterior pituitary gland possessed considerable inductive capacity. This inductive capacity was analysed by differential protein solubility and precipitation. The solvents used were half-saturated ammonium sulphate (0.5 SAS), 50 per cent pyridine and 2.5 per cent trichloroacetic acid (TCA), the use of which was suggested by Fevold, Lee Hisaw & Cohn (1940); Fraenkel-Conrat, Simpson & Evans (1940); Li, Simpson & Evans (1940, 1942); Li, Evans & Simpson (1943); White (1943); Long (1943); Fevold (1943); Li & Evans (1948); Li (1954, 1956) and Barnett, Ladman, McAllaster & Siperstein (1956). The employment of 0.5 SAS, 50 per cent pyridine and 2.5 per cent TCA was prompted by the fact that all the anterior pituitary hormones, e.g. FSH, LH, TSH, ACTH, STH and LTH, are precipitated by 0.5 SAS; 2.5 per cent TCA precipitated LH and 50 per cent pyridine extracted all the glycoprotein hormones (FSH, LH and TSH). These solvents were also found to affect the RNA content of the glands in a particular way, as could be seen from histochemical experiments performed during this work.

In the present investigations the anterior lobe of the gland was cut into two halves, from one piece control grafts were made. The graft pieces were taken from the postero-ventral region of the gland. The other half was kept immersed in 10 ml. of 0.5 SAS prepared by mixing 5 ml. of saturated ammonium sulphate in glass-distilled water with an equal amount of fresh Pannett-Compton solution, for a duration of 24 hours. Following this treatment the gland was transferred into fresh Pannett-Compton saline three to four times during 90 min. to ensure complete removal of ammonium sulphate. Pieces were then taken from the treated pituitary from a corresponding region, repeatedly washed in fresh Pannett-Compton saline and transplanted in between the epiblast and hypoblast of host chick embryos. It is worthwhile mentioning that the cut edge of the gland would have facilitated the penetration of the saline and further the separate washing of each graft before implantation would have allowed no trace of the solvents used to remain in the graft. In the same way control and experimental grafts were made from the gland after treatment with 50 per cent pyridine and 2.5 per cent TCA for 24 hours. Following post-operative incubation, the control and experimental embryos were fixed in Bouin's fluid, sectioned serially at 8 μ, stained in Delafield's haematoxylin and mounted in Canada balsam.

The RNA content of normal anterior pituitary and those treated with 0.5 SAS, 50 per cent pyridine and 2.5 per cent TCA was studied by histochemical staining with methyl green-pyronin.

For this purpose, normal and treated pituitaries were fixed in Carnoy or Serra. Here also the glands were cut into two halves and treated with the solvents for 24 hours. The method of washing the glands after treatment was similar for the pituitaries from which grafts were made and those fixed for
histochemical examination. Following fixation the glands were cleared in toluene and embedded in paraffin wax. The materials were sectioned serially at 4 μ. The slides of each set were serially numbered and alternate slides were treated with 10 per cent perchloric acid (PCA) at 4–5°C for 20 hours to extract RNA, and these slides served as controls for the histochemical experiments. The slides were stained by freshly prepared methyl green-pyronin mixture at pH 4-8 with acetate buffer (Brachet, 1953). These experiments were repeated in ten batches. In each batch were taken one control untreated pituitary and one each after treatment with 0·5 SAS and 50 per cent pyridine. The initial three batches also included pituitary treated with 2·5 per cent TCA but the sections failed to take stain, probably on account of a complete hydrolysis of both RNA and DNA and in subsequent experiments. Therefore, TCA treated pituitaries were excluded.

The histology of the anterior pituitary gland was studied by staining 6 μ thick sections of the gland with Mallory’s triple stain (aniline blue, acid fuchsin and orange G).

Measurements of volumes of the induced neural tissue and the graft were made from camera lucida outlines of sections of the tissue drawn on graph paper. The area measurements were made in every section in which the induced neural tissue and/or the graft appeared, and hence the figures for the various volumes presented as data are very accurate.

EXPERIMENTAL RESULTS

Grafting experiments

In the present experimental series 321 anterior pituitary grafts were made. Of these 197 grafts were experimental, that is made from pituitaries treated with the solvents, and 124 were made from untreated glands which served as controls. The results of the grafting experiments are presented in Table 1.

It is evident from Table 1 that treatment of the gland with 2·5 per cent TCA or 50 per cent pyridine strips it of all its capacity for induction. However, the gland retains its capacity to induce even after treatment with 0·5 SAS though the frequency of induction is decreased by the treatment.

The inductions obtained from both the control pituitary grafts and 0·5 SAS-treated pituitary grafts were classified as belonging to type A, B, C, D, E or F. The quality of induction, rather than the quantity of neural tissue induced, was used in classifying the inductions. Type A cases were those in which a secondary head structure (Text-fig. 1A) or a complete neural tube, whether regular or irregular, was formed. In some, designated as Type B inductions, medullary plates were formed, some of which though small in extent showed depression in the centre (Text-fig. 1B) giving the impression that had they been kept living longer they would have given rise to a regular neural tube. Those in which the induced medullary plate did not show any depression were considered as
### Table 1

Analysis of induction capacity of control and experimental anterior pituitary grafts

<table>
<thead>
<tr>
<th>Nature of experiment</th>
<th>Nature of graft</th>
<th>Total number of grafts</th>
<th>Graft not in the vicinity of the ectoderm</th>
<th>Grafts in the vicinity of the ectoderm</th>
<th>Grafts showing inductions</th>
<th>Total number of inductions</th>
<th>Percentage of inductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2·5% TCA</td>
<td>Control</td>
<td>36</td>
<td>17</td>
<td>19</td>
<td>– – 6 2 1 2 11</td>
<td>57·89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exptl.</td>
<td>62</td>
<td>48</td>
<td>14</td>
<td>– – – – – – – – – – – – –</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>50% Pyridine</td>
<td>Control</td>
<td>48</td>
<td>13</td>
<td>35</td>
<td>1 3 5 7 4 6 26</td>
<td>74·28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exptl.</td>
<td>71</td>
<td>48</td>
<td>23</td>
<td>– – – – – – – – – – – – –</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>0·5 SAS</td>
<td>Control</td>
<td>40</td>
<td>7</td>
<td>33</td>
<td>2 4 6 6 2 8 28</td>
<td>84·84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exptl.</td>
<td>64</td>
<td>22</td>
<td>42</td>
<td>– 1 1 6 1 13 22</td>
<td>52·38</td>
<td></td>
</tr>
</tbody>
</table>

A, Complete neural tube; B, Medullary plate with sinking; C, Medullary plate without sinking; D, Palisade; E, Neuroid type; F, Neuralization reaction.
A. Induction type A produced by a control pituitary graft. Volume of the induced neural tissue is approximately 400–500 × 10⁻⁶ cc.

B. Type B induction by control pituitary graft. Volume of induced tissue is 11.3 × 10⁻⁶ cc.

C. Neuroid thickening of sub-cephalic ectoderm (INT) to be noted. The graft (GR) represented very diagrammatically was from a control pituitary gland.

D. Type B induction produced by a graft from gland treated with 0.5 SAS for 24 hr. Volume of the induced tissue in this case is 7.13 × 10⁻⁶ cc.

E. Type D induction produced by graft form 0·5 SAS treated gland. Volume of the induced tissue is 10.3 × 10⁻⁶ cc.

F. Graft from gland treated with 2·5% TCA for 24 hr. has caused no induction.

G. Graft from gland treated with 50% pyridine for 24 hr. No inductive response is seen.

INT, Induced neural tissue; GR, Anterior pituitary graft; HD, Host embryo.

TEXT-FIG. 1
Type C. Type D inductions constituted those in which the ectoderm had produced thick palisades of neural tissue. Neuroid thickenings produced in some cases, largely in the sub-cephalic ectoderm, were classed as Type E inductions (Text-fig. 1C). A very large number of cases where the ectoderm in contact with the graft produced neuralization were classed Type F inductions. Types C, D and F are not illustrated.

Two cases of induction obtained from grafts from 0.5 SAS-treated pituitary are illustrated in Text-fig. 1D and E. Text-fig. 1D is a Type B induction. The volume of induced neural tissue in this instance was $7.13 \times 10^{-6}$ cc. Text-fig. 1E illustrates Type D induction. The volume of induced tissue in this case was $10.4 \times 10^{-6}$ cc.

Two illustrations are shown of cases in which the grafts from pituitaries treated with 2.5 per cent TCA or 50 per cent pyridine failed to evoke neural

### Table 2

<table>
<thead>
<tr>
<th>Season</th>
<th>Donor</th>
<th>No. of grafts made</th>
<th>Grafts in the vicinity of the ectoderm</th>
<th>No. of inductions</th>
<th>Percentage of inductions</th>
<th>Average vol. of induced neural tissue $\times 10^{-6}$ cc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-breeding</td>
<td>Female</td>
<td>38</td>
<td>23</td>
<td>18</td>
<td>78.3%</td>
<td>33.07</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>35</td>
<td>27</td>
<td>19</td>
<td>70.4%</td>
<td>10.69</td>
</tr>
<tr>
<td>Breeding</td>
<td>Female</td>
<td>21</td>
<td>16</td>
<td>14</td>
<td>87.5%</td>
<td>30.88</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Season</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-breeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of induction</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>No. of cases</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Percentage</td>
<td>5.56%</td>
<td>16.67%</td>
</tr>
<tr>
<td>Breeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cases</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Percentage</td>
<td>7.14%</td>
<td>7.14%</td>
</tr>
</tbody>
</table>

(Notation as in Table 1.)
tissue formation from competent host ectoderm (Text-fig. 1F and G). It should be noted that there is not even the slightest neuralization of the ectoderm.

It must be emphasized that in no case was any deleterious effect produced by the treated graft on the reacting ectoderm, which was normal and healthy in all cases. This is a definite indication of the fact that the solvents used were completely removed.

Inductions by control grafts were further analysed. For example, comparisons of the behaviour of grafts from donors in breeding and non-breeding seasons, as judged by the percentage of inductions and average volume of induced neural tissue, and of grafts from male and female donors in the same season were made. The analysis, given in Tables 2 and 3, indicates that pituitary from breeding season possesses greater inductive capacity than that from the non-breeding season, and further, in one season, either breeding or non-breeding, the female pituitary has more inductive capacity than the male.

Histochemical experiments

Histochemical staining by methyl green-pyronin mixture showed that a 24-hour treatment by 2.5 per cent TCA resulted in a complete hydrolysis of both RNA and DNA. In sections of the gland treated with 0.5 SAS, the cytoplasmic pyronin staining was not seen and the nuclei showed pyroninophily due to depolymerization of DNA. Digestion by 10 per cent PCA made no visible difference. Sections of gland treated with 50 per cent pyridine distinctly showed cytoplasmic staining by pyronin. The nuclei were stained bluish green by methyl green. These experiments seem to indicate that treatment of gland with 0.5 SAS removes the RNA, but treatment with 50 per cent pyridine does not affect the RNA content of the gland.

DISCUSSION

Mangold, Tiedemann & Woellwarth (1956) found that extract pellets of anterior pituitary of ox when implanted into the blastocoel of Triton gastrulae produced strong inductions. They stated, in discussing their results, that they suspect the gonadotrophic hormones and the growth hormone of being responsible. Preliminary experiments seemed to indicate that fresh anterior pituitary glands of Rana tigrina exert a considerable inductive influence. It was felt, therefore, that dissociation of the various fractions of the anterior pituitary hormones would be useful and accordingly grafts were made from fresh glands after various treatments as described.

The anterior pituitary gland contains both proteins (as glycoprotein or simple protein hormones and other cellular proteins) and RNA (Desclin, 1940; Dempsey & Wislocki, 1945) and by the method of analysis adopted we could only indicate the relative importance of the different factors involved.
The hormones as a factor

A histological study of the gland has revealed that in the postero-ventral region both the alpha and beta cells are present, which between them secrete the various hormones. Since the grafts were taken from this particular region we assume the presence of all the hormones in the grafts. Now, on the basis of the work, indicated in experimental results which suggested the use of these solvents, the observation that the gland retains the inductive capacity despite 0·5 SAS treatment whereas it loses all that capacity on treatment with 2·5 per cent TCA or 50 per cent pyridine, is interpreted to mean that either FSH or TSH might be responsible for causing the inductions.

Relationship between volume of graft and that of INT

It was felt during the present experiments that a relationship existed between the volume of the graft and that of the neural tissue it produced, with some exceptions. Indications that some such relationship might be present were earlier given by Waddington (1952).

![Text-fig. 2. Graph showing relationship between the volume of the induced neural tissue and that of the pituitary graft. The letters A, B, C, F, etc. stand for individual induction cases. BGA, IPQ and HM (continuous lines) are the non-breeding season graphs; OFV and WU are breeding season graphs.]

When the values of the volumes were plotted on a graph paper (Text-fig. 2) it was observed that they fell into a number of groups. In each group it was found that the size of the graft and the volume of the induced tissue were inversely proportional, and in each group the graph was a straight line and graphs of the groups of cases belonging to one season (breeding or non-breeding)
were parallel to one another. For example, the graphs BGA, IPQ and HM (continuous lines) which refer to grafts made in the non-breeding season were parallel to one another. So, also, were the graphs OFV and WU (broken lines) of breeding season grafts parallel to each other. The rate of decrease of neural tissue per unit volume increase in graft worked out with the help of this graph is constant in the non-breeding season (for BGA 0.611, IPQ 0.588 and HM 0.583). Similarly, this rate of decrease is constant for grafts made in the breeding season (for OFV 0.382 and WU 0.400).

A difference in the slope between the non-breeding season graphs and the breeding season graphs is also evident. The rate of decrease of neural tissue in the non-breeding season is greater than in the breeding season (0.583 to 0.611 and 0.382 to 0.400 respectively). Obviously a change in factors present in the graft alters this ratio. What possibly happens in the breeding season is that an increase in the active principle of induction takes place. And, presumably, the disadvantage incurred by an increase in size is offset to a large extent by an increase in the quantity of the active principle of induction in the breeding season grafts. It has already been indicated earlier in the paper that either FSH or TSH might be responsible for the inductions. Of the two it seems more likely that the gonadotropic factor (FSH) is the one which governs the relationship between the volumes of the induced neural tissue and graft.

This relationship thus seems to indicate a probable part played by FSH, though there has been nothing in these experiments to preclude a possible role of TSH. In this context we would recall the tentative hypothesis put forward by Mangold et al. (1956) that the gonadotropic hormones might become localized in the oocytes while they are still in the ovary. As the ovary is the target organ of FSH, this hormone might be localized in the ovarian follicles as they grow.

**RNA as a factor**

The histochemical finding that 0.5 SAS-treated pituitaries do not contain RNA while pyridine-treated ones do contain it, when considered along with their capacities for induction, makes one inclined to suggest that RNA by itself has no morphogenetic action. The normal pituitary induced more frequently that the 0.5 SAS-treated one. This seems to indicate that RNA has only a 'facilitating action'. This reasoning seems to agree with the view expressed by Brachet (1960a) that RNA has an important role in protein synthesis, though by itself it 'cannot induce morphogenesis, i.e., act as an organizer'. The present investigations also seem to indicate that the inducing substance of the anterior pituitary gland is, protein nature, either FSH protein or some other cellular protein affected in the same way as FSH by pyridine or half-saturated ammonium sulphate treatment.
SUMMARY

1. An attempt at analysing the inductive capacity of the anterior pituitary gland of *Rana tigrina*, in order to understand the nature of its active principle, was made, having recourse to differential solubility and precipitation of the hormonal and the ribonucleic acid factors using half-saturated ammonium sulphate, 50 per cent pyridine and 2.5 per cent trichloroacetic acid.

2. The study of the inductive capacity of the gland in its normal state and after a 24-hour treatment with the solvents was made by grafting experiments on chick primitive-streak embryos. The behaviour of control pituitary grafts taken from donors at different seasons, namely breeding and non-breeding, and those taken from female and male donors at the same season, and also the relationship found to exist between the volume of the graft and that of the induced neural tissue, have suggested that the active principle of induction is probably FSH glycoprotein, and that the RNA contained in the gland has only a facilitating action.

RÉSUMÉ

*Etudes sur les transplantations de l'hypophyse antérieure des amphibiens dans l'embryon de poulet. Analyse de la capacité inductrice par la méthode de solubilité différentielle et de précipitation*

1. Il a été tenté d'analyser la capacité inductrice de l'hypophyse antérieure chez *Rana tigrina*, afin de comprendre la nature de son principe actif; on a eu recours à la solubilité différentielle et à la précipitation des facteurs tant hormonaux que liés à l'ARN en utilisant le sulfate d'ammonium à demi-saturation, 50 per cent de pyridine et 2,5 per cent d'acide trichloracétique.

2. L'étude de la capacité inductrice de la glande dans son état normal et après 24 h. de traitement par les colorants a été faite en réalisant des expériences de greffe sur les embryons de Poulet au stade de la ligne primitive. Le comportement des greffons contrôlés provenant d'hypophyses prélevées en diverses saisons, notamment celles de reproduction et de repos sexuel, le rendement des greffons prélevés à la même saison sur des mâles et des femelles, et aussi la relation trouvée entre le volume de la greffe et celle du tissu neural induit ont suggéré que le principe actif de l'induction est probablement une FSH-glycoprotéine et que l'ARN contenu dans la glande exerce seulement une action de facilitation.

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