Studies on Establishment of the Neural Differentiation Pattern in Prospective Prosencephalic Area in the Chick (*Gallus domesticus*) Embryo

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(ABSTRACT)

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Differentiation Tendencies of Eye Potency Field in Early Chick *(Gallus domesticus)* Embryo*

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Paper includes a preliminary investigation on prosencephalic differentiation tendencies with special reference to differentiation of eye potency field at primitive streak and head process stages of chick embryo. The technique involves intracoelomic culturing of small ectodermal grafts measuring about 0.2 × 0.2 mm prepared from the presumtive prosencephalic area for 12 days and analysing them histologically. The study of 18 grafts reveals that loci for eye field appear anterolaterally on peripheral zone of presumptive prosencephalic ectoderm at primitive streak stage and shift medially during head process stage. Other prosencephalic differentiation tendencies are elaborated at head process stages.

THE organs forming potency maps of early chick embryo constructed by Rawles[1] and Rudnick[2,3] for medium head process and primitive streak stages were based upon differentiation of grafts having all germ layers. Neural differentiation tencies studied at these stages by Rao[4] were based on differentiation of ectodermal grafts in the absence of other germ layers; but it was not possible for him to specify exact loci of different structural elements of brain on these blastoderms because his grafts were large in size and rectangular in shape. The present investigation was undertaken to study the finer mode of changes of prosencephalic differentiation tendencies with special reference to the differentiation of eye potency field at Hamburger and Hamilton[5] stages 4 and 5 (primitive streak and head process-stages). The technique involved intracaelomic culturing of grafts prepared from the presumptive prosencephalic ectoderm during these stages.

Material and Methods

The hatchable red and white crossbreed leghorn eggs were obtained from Naya Bungalow Govt. Poultry Farm, Shillong. They were incubated at 37.5°C. Pans full of distilled water were kept inside incubators to maintain 60-70% humidity. For experiments Hara's[6] guidelines and Spratt's[7] fate maps were followed. The presumptive prosencephalic ectoderm was stripped free of underlying endoderm and mesoderm at H & H stages 4 and 5 and cut into 5 grafts—1 median (M) and 4 peripheral (A, B, C, and D) each measuring about 0.2 × 0.2 mm according to the operation plan shown in Fig. 1 The grafts were transplanted into coelom of another 2½ days old chick embryo and cultured for 12 days. The recovered grafts were fixed in Bouin's—picro—formol, dehydrated in butanol, embedded in paraffin wax and sectioned at 6 μm. The histological analysis was based on the criteria established by Hara[6] and Rao[4].

Results

The present investigation includes histological analysis of the 18 well-developed grafts. Among prosencephalic structures, tissues forming the telencephalic cortex (Fig. 2), pigmented and sensory layers of retina (Fig. 3), prosencephalic neural mass (Fig 4) and feathers (Fig. 5) were observed in the majority of grafts showing similar pattern as described by Hara[6] and Rao[4]. In few grafts cartilage was encountered along with the neural mass (Fig. 6) although clear olfactory complex could not be seen. Elements of pineal body and choroid plexus were rarely observed. More attention was paid to the study of tissues showing eye structures. In 10 out of 18 grafts studied eye

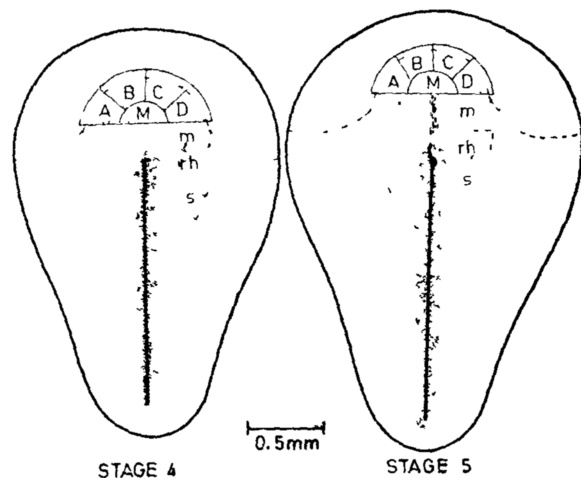

Fig. 1—Operation plan. Areas A, B, C, D and M represent grafts

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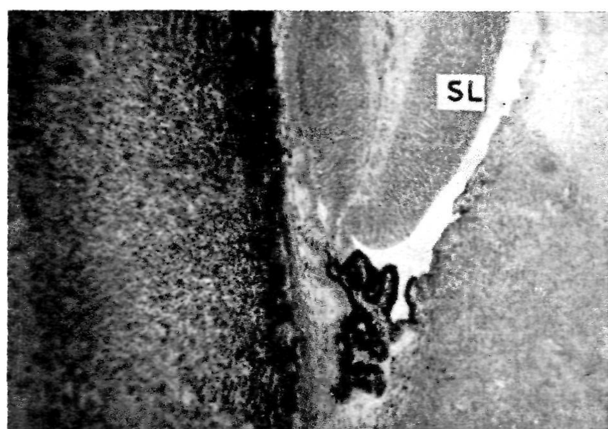
structures were observed. The histological analysis has been compiled in Table 1.

In the 1st set of experiments performed at H and H stage 4, 4 out of 7 grafts showed sensory layer of retina of which 2 were accompanied by pigmented layer. In the 2nd set of experiments at H and H stage 5, when length of the head process ranged from 0 to 0.30 mm, 3 out of 7 grafts showed sensory layer of retina of which 2 were accompanied by pigmented layer. One graft showed only pigmented layer. In the 3rd set of experiments when length of the head process ranged from 0.31 to 0.60 mm, 2 out of 4 grafts showed sensory layer, of which 1 was accompanied by pigmented layer. It can be noted that eye structures were encountered in 3 peripheral (AB, B and C) and 1 median (M) grafts in the 1st set of experiments and in peripheral grafts (A, B and C) in the 2nd set. In the 3rd set they were observed only in the median grafts. These results indicate the possibility that at H and H stage 4 the loci of eye vesicle rudiments first appear in the peripheral region, perhaps antero-laterally in the presumptive prosencephalic ectoderm, and then shift to its median region at H and H stage 5.



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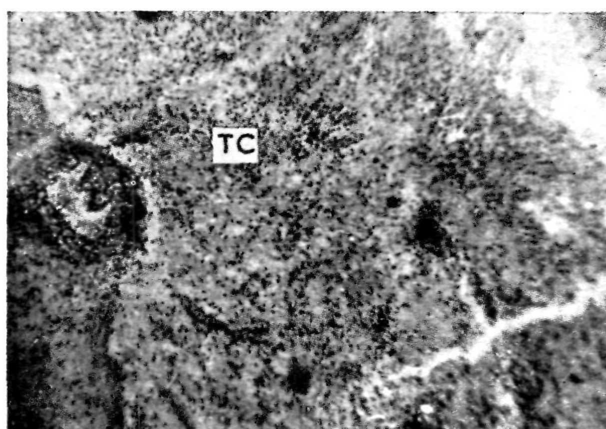

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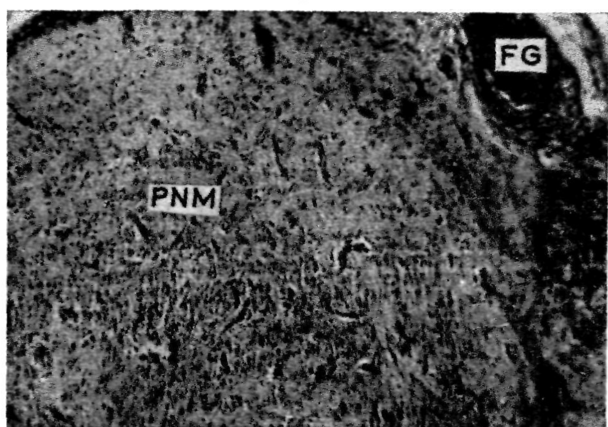

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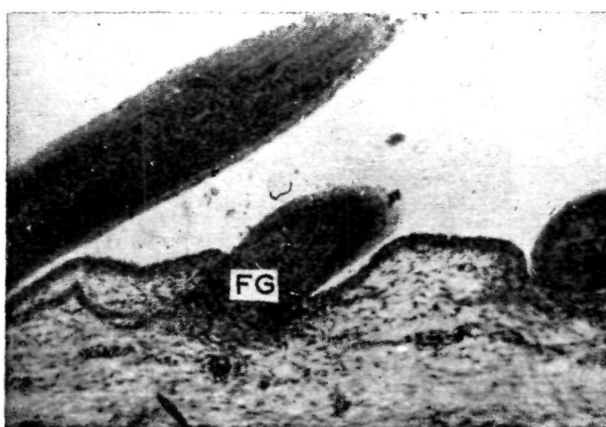

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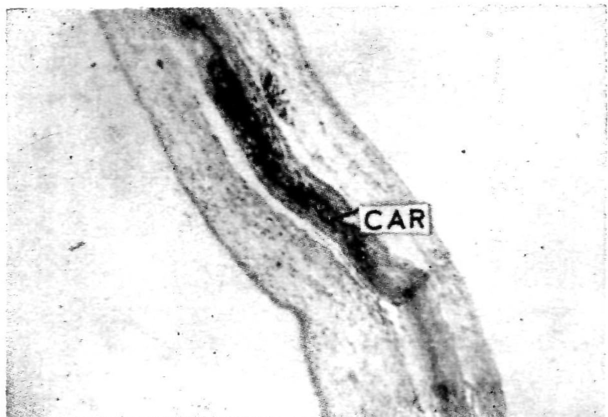

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Figs 2-6—Cross-sections of grafts showing : (3) telencephalie cortex(TC), (2) Sensory and pigmented layer of retina (SL, PL), (4) prosencephalic neural mass (PNM), (5) feather germs (FG) and (6) cartilage (CAR), × 400

TABLE 1 — HISTOLOGICAL ANALYSIS OF GRAFTS

Experimental Sets	Graft	Expt No.	Olfactory cortex	Telencephalic cortex	Choroid plexus	Pigmented layer of retina	Sensory layer of retina	Pineal body	Prosencephalic neural mass	Feathers	Epithelial vesicles
I	AB	1	—	+	+		+	+	—	—	—
Stage 4	B	2	—	—	—	+	+	+	—	—	—
No head process	M	3	?	—	—	—	+	—	+	+	—
	M	4	—	—	—	—	—	—	+	—	—
	B	5	—	—	—	—	—	—	+	—	—
	C	6	—	—	—	—	—	—	+	+	—
	C	7	—	—	—	+	+	—	+	—	—
II	A	8	—	—	+	+	—	—	—	—	—
State 5	A	9	—	—	—	—	—	—	+	—	—
Short head process	B	10	—	—	—	—	+	—	+	—	—
(0–0.30 mm)	M	11	—	+	—	—	—	—	+	—	—
	MB	12	—	+	—	—	—	—	+	+	—
	C	13	—	—	—	+	+	—	—	—	—
	C	14	—	—	—	—	+	+	+	—	—
III	M	15	?	+	—	+	+	—	+	—	—
Stage 5	M	16	?	+	—	—	+	—	+	—	—
Long head process	D	17	—	—	—	—	—	—	+	+	—
(0.31–0.60 mm)	C	18	—	—	—	—	—	—	+	—	—
										—	—

+Present; —absent; AB & MB were combined grafts. See Fig. 1

Discussion

Hara[6] and Rao[4] are the first workers to study the neural differentiation tendencies in early chick embryo by intracoelomic grafting technique. As reported by Rao[4], the neural differentiation tendencies are elaborated during head process stages. Similar observations have been made in the present investigation also. While referring to the differentiation tendencies of the eye, he writes, "Among the prosencephalic structural elements, those belonging to the eye (retina together with tapetum) are the most frequently encountered ones in the grafts from the median areas. In the lateral areas they are on the whole much less frequent." His median grafts were large covering more than half of the presumptive prosencephalic area. In the present investigation, 5 grafts were prepared from this area exploring the possibility of studying much finer mode of differentiation.

The organ forming potency maps of Rawles[1] and Rudnick[2] for medium head process and primitive streak stages show different loci for the eye rudiments. Rawles[1] cultured different pieces of blastoderm on the chorioallantoic membrane and showed that the eye potency field develops from the middle as well as lateral regions of the presumptive prosencephalic area. Her grafts, however, included all the three germ layers and so a clear picture of the eye potency field may not be possible because there was constant interaction between all the germ layers. The grafts in the present series of experiments were stripped free of endoderm and mesoderm and thus, they were purely ectodermal. The lens structures were never observed either by Rao[4] or in the present investigation. Rawles[1] found lens element in 1 out of 18 grafts but she did not show the loci of lens and eye vesicle rudiments separately. The eye potency field as shown in Rudnick's[2] fate map are located just lateral to the Henson's node in the region of presumptive mesencephalic area of Spratt[7]. The experiments in the present investigation reveal that the loci of the eye potency field appears laterally on the peripheral region of the Spratt's presumptive prosencephalic area during H and H Stage 4 and shift medially in front of the head process during H and H stage 5. More experimental data would, however, be required for determining the exact loci of the eye potency field. In a review on earlier work Rudnick[3] visualises a continuous medio-lateral separation of eye field in the anterior medullary plate at an early stage. It would be interesting to investigate the pattern of such morphogenetic behaviour.

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ABSTRACT

(1) The present work includes an investigation on the neural differentiation pattern in the prospective prosencephalic ectoderm of the chick blastoderm at the primitive streak and head-process stages by culturing its pieces intracoelomically as per the technique described by Hara (1961, 1970 and 1971).

(2) The endoderm and mesoderm were carefully separated from the prospective prosencephalic ectoderm. The freed ectoderm was then cut, with baseline anterior to the notochordal mesoderm, into various pieces according to the 6 operation plans. These pieces were transplanted into the coelom of 2 1/2 days old host embryos and cultured for 12 days.

(3) By open sandwich experiments, Hara (1961) demonstrated the differentiation tendencies in the competent ectoderm as induced by prechrodal and notochordal mesoderm. Subsequently, Rao (1968) studied the appearance and extension of neural differentiation tendencies in the prospective neurectoderm. As his grafts were large in size, the present investigation was carried out with much smaller grafts (size 0.2 x 0.2 mm or 0.4 x 0.1 mm) to find out the finer mode of differentiation tendencies.

(4) In the first 4 operation plans the grafts were excised from different central and peripheral areas of

the prospective prosencephalic ectoderm. In the other 2 operation plans 4 longitudinal grafts were prepared from one half, and 4 transverse grafts mostly from the other half of this region.

(5) A total of 1437 grafts were prepared from 448 donor blastoderms and transplanted into the coelom of 941 hosts. Of these, 206 hosts died (mortality 23%) From the 725 surviving hosts which carried 1241 grafts, 304 grafts were recovered.(recovery rate 25%) The grafts were analysed histologically to find out the differentiation tendencies.

(6) The results of the histological analysis may be summarised as follows.

(a) The grafts implanted according to the first 4 operation plans showed that the recovery of the central grafts was better than that of the peripheral grafts. The recovery and neuralization of the median longitudinal and posterior transverse grafts taken according to other 2 plans was better than the other peripheral grafts.

(b) The differentiation of all grafts taken at the primitive streak stage does not show any well defined pattern as the differentiation tendencies of the telencephalic, diencephalic and eye structures are mixed up. At this stage the grafts of the central region show better neural differentiation than those of the peripheral

region except in the first plan in which the central area was smaller in size. At the head process stage the pattern of differentiation was better defined. The eye structures (retina as well as tapetum) were found in an area measuring about 0.4 x 0.1 mm approximately 0.1 mm anterior to the head process. The structural elements of the telencephalic cortex were found mostly in the grafts of the anterior region, whereas diencephalic structures differentiated mostly in the grafts of the more posterior region.

(c) Structural elements of the lens were not observed in any graft.

(7) Interpreting the results in terms of activation - transformation hypothesis of Nieuwkoop (1952) the results of the present work are complementary to those of Hara (1961) and Rao (1968). It appears that as a result of first contact the prechordal mesoderm establishes in the prospective neural ectoderm a centrifugal neural (activation) field resulting into the induction and appearance of the prosencephalic differentiation tendencies. With the laying down of the head process, when the wave of activation is succeeded by a wave of transformation extending caudally and laterally transforming the prosencephalic differentiation tendencies in the more caudal parts of the prospective neural plate (Rao, 1968), the differentiation tendencies of telencephalic structures become localized to the anterior region of the prospective

prosencephalic area, and that of diencephalic and eye structure to its more posterior region as analysed in the present investigation.

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Dr. M.K. Khare
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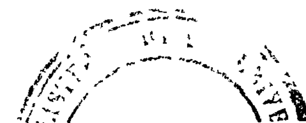
27th August, 1980.

I certify that the thesis entitled "studies on Establishment of the Neural Differentiation Pattern in Prospective Prosencephalic area in the Chick (*Gallus domesticus*) Embryo" submitted by Miss Sabitry Choudhury for the Degree of Doctor of Philosophy of the North-Eastern Hill University, Shillong embodies the record of original investigation carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any degree of any other University.

M K Khare
(M.K. Khare)
Head of the Department.



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INTRODUCTION

Chick's development has been one of the favoured subjects among embryologists since ancient times. As quoted by many authors (e.g. Needham, 1959; Balinsky,1976; Gilchrist, 1968) the development of chick was first described as early as in 342 B.C. by Aristotle as he observed it by naked eye. Detailed embryological descriptions of chick can be found in various monographs and text books (e.g. Lillie, 1908 revised by Hamilton, 1952; Patten, 1971; Romanoff, 1960). Analytical approach to the morphogenetic study of early chick embryo can be traced back to the work of Wilhelm His (1874), who pointed out that the flat undifferentiated blastoderm might be considered as an aggregate of organ forming areas, the potentiality of which is realised in subsequent developmental stages. Rauber (1876) was first to point out that the primitive streak of the chick blastoderm resembles the amphibian blastopore. With the help of injury experiments Peebles (1898) was first to report that anlage of entire head was situated anterior to the Hensen's node. The discovery of 'Organizer centre' by Spemann (1918) in amphibian embryos triggered a new phase of experimental investigations to understand causal relations of development.

As a result of various types of transplantation experiments, Spemann and Mangold (1924), Mangold (1933),

Nieuwkoop _et al_ (1952), among others, demonstrated the regionally different inductive capacities of the archenteric roof in amphibian embryos. Many workers attempted to find out similar inductive phenomena in other animals including the chick embryo. The progress of the work on the chick embryo, however, has been slow in comparison to that of amphibians due to technical difficulties (for review see Gallera, 1971; Hara, 1978). It was not until the beginning of 1930s when Waddington (1930, 1932) conclusively demonstrated with the help of _in vitro_ culture technique of Fell and Robison (1929) that the Hensen's node and anterior part of the primitive streak in the early chick embryo had inducing and organizing capacity. But he could not demonstrate that it was axial mesoderm as the inductive component like that of amphibians.

The possibility to test the inducing capacity of the mesoderm and to study the differentiation tendencies in the neural ectoderm of the primitive streak and head process chick blastoderms was explored by Nieuwkoop in early 1950s (unpublished), when he succeeded in mechanically separating the mesoderm from the ectoderm. He demonstrated that the pieces of the ectoderm anterior to the Hensen's node taken at the primitive streak stage and cultured intracoelomically for 10 days did not give rise to neural tissue while pieces from the same area overlying the head process at the

head process stage did give rise to the neural tissue.

Hara (1961) perfected the technique, and used it to investigate the induction of regional neural differentiation in the chick embryo. He performed open sandwich experiments comparable to that of Sala (1955) in Amphibia and demonstrated that in the chick embryo also the prechordal mesoderm induced prosencephalic differentiation, anterior part of the head process mesoderm induced mes-/rhombencephalic differentiation, and posterior part of the head process induced rhombencephalic and spinal cord differentiation in the competent ectoderm. Following this technique and Spratt's (1952) prospective neural area maps, Rao (1968) further extended the work on the appearance and extension of the regional differentiation tendencies in the prospective neural ectoderm and found that the self differentiating prospective mes-/rhombencephalic ectoderm isolated from the shortest head process blastoderm showed mostly prosencephalic differentiation tendencies, whereas from the head process blastoderm showed prosencephalic in addition to mes-/rhombencephalic differentiation tendencies. He demonstrated that the neural differentiation tendencies appeared and extended in the cranio-caudal sequence in the competent ectoderm. Hara (1978), based on his unpublished results, writes that prospective mes-/rhombencephalic ectoderm isolated from the long to

the definitive streak blastoderm which appeared to have only prosencephalic if cultured alone differentiated into mes-/rhombencephalic structures if it was recombined with the originally underlying prospective notochordal material. Therefore, it is reasonable to suppose that determination of the neurectoderm in the chick also involves at least 2 steps: activation and transformation like that of amphibians (see 'activation-transformation' hypothesis of Nieuwkoop, 1952).

Many authors, such as Stein (1933), Butler (1935), Dalton (1935), Rawles (1936) and Clarke (1936), with the help of chorio-allantoic grafting techniques and Rudnick (1938a and 1938b) with the help of in vitro technique attempted to study the differentiation of various parts of chick blastoderm including the localization and differentiation of the forebrain and eye structures. Rawles (1936) and Rudnick (1944) provided organ-forming maps of the head process and primitive streak stage blastoderms. But as the graft pieces prepared by all these workers consisted of all the three germ layers and inductive interaction could continue among them even after isolation of the grafts, it was difficult for them to pinpoint the exact localization of areas which differentiate into various structures. Hara (1961, p.10.) comments "In interpreting these results one must bear in mind that most of these grafts contained cells from

all three germ layers. It is, therefore, quite possible that the cells of one layer act after transplantation upon any of the other layers within the same graft. If this is actually the case one cannot expect to find in such three layered grafts an exact expression of self differentiation tendencies of any single germ layer at the stage of transplantation."

The graft pieces tested by Rao (1968) were relatively large in size - the length of the median grafts pieces being three-fourths of the width of the prospective neural area. Although he did provide a dynamic picture of the appearance and extension of neural differentiation tendencies in the prospective neural ectoderm it was not possible for him to describe the 'finer mode' of changes in the prospective prosencephalic, mesencephalic and rhombencephalic areas separately. In the present investigation, very small semicircular and rectangular pieces were isolated at different planes from the prospective prosencephalic ectoderm interrupting the process of neural induction at definitive primitive streak and head process stages, and cultured _in vivo_ intracoelomically. They were analysed histologically to find out their differentiation tendencies. There were certain questions such as: Where does the eye differentiation tendency appear at the primitive streak stage and shifts eventually during the head process stage? Whether or not the locus coincides with the eye potency field examined

by various workers (e.g. Clarke, 1936; Rawles, 1936; Rudnick, 1944) with other germ layers together? Is the lens placode able to differentiate within the prospective neural plate area? How do the self differentiation tendencies of the telencephalic and diencephalic appear and extend giving rise to the prosencephalic differentiation pattern? The present study is an attempt to answer these questions.

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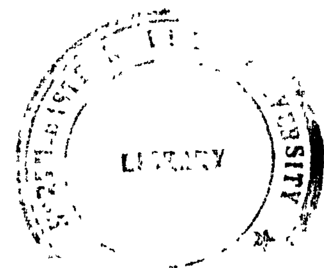
Chapter 2



REVIEW OF LITERATURE

Earliest description on the formation of chick brain from the blastoderm appears to be that of Kingsbury (1920, 1922, 1931) who considered that epichordal brain as opposed to prechordal extends from the medial floor of the midbrain backwards. Wetzel (1929) performed a series of experiments with vital staining technique and found that presumptive forebrain region lies anterior to the Hensen's node in the primitive streak stage. The antero-posterior length of this area does not appear to be more than 0.4 to 0.5 from his measurements. When an area anterior to the node was stained at definitive streak stage the stained material was found in the forebrain region. When node level was stained, the stained material was found in the area posterior to the forebrain. Soon after this work, when Waddington (1930, 1932), conclusively proved like that of Amphibians (Spemann, 1918), the presence of Organizer action in the chick embryo, workers started concentrating on varied experimental investigations leading to the analysis of inductive interactions, process and mechanism of neural induction in the chick embryo. The following résumé deals mainly with the contributions related to the process of Neural Induction.

Discovery of Organizer and Neural Induction in the Chick Embryo

Waddington (1930, 1932) used Fell and Robison's (1920) in vitro technique, removed endoderm from the Hensen's node and grafted it beneath the ectoderm of the area pellucida of another chick embryo and noted that it induced development of a secondary embryonic axis. He, thus, demonstrated the presence of 'Organizer' in birds and established a milestone in the study of chick development. He also showed that developing primitive streak can cause the competent ectoderm lying above it to differentiate into neural plate. He concluded that the grafts had the capacity to act as an inductor . The success led him (1933) to perform a series of experiments with different parts of the primitive streak. He performed 4 types of grafting experiments -

(1) Grafts of mesodermal portion of the primitive streak : Posterior two thirds of primitive streak of 18 hrs old blastoderm was grafted on the right side beneath the ectoderm of a host of similar age. No neural tissue was formed in the graft but there was a clear neural plate induced in the host ectoderm.

(2) Grafts of whole embryonic axis : This included a series of experiments (a) when a piece having part of head process at the head fold stage was grafted in the similar manner neural plate and notochord were induced.

(b) The part of the head process grafted with all the germ layers before the appearance of head fold induced neural groove and neural tissues in the host ectoderm. (c) The notochord along with attached neural plate from a 50 hrs old embryo induced neural plate immediately above the graft neural plate. (d) Embryonic axis with all the germ layers from the posterior part of the last somite of a 14 somite embryo induced neural groove and neural plate in the host ectoderm.

(3) Notochordal grafts : The notochordal grafts were prepared from 20-25 somite embryos, Notochord from the middle of the somites was grafted beneath the host ectoderm. No induction could be seen except thickening of the host ectoderm which had no resemblance with neural plate tissue.

(4) Grafts from the neural plate : The grafts prepared from the left or right wall of the neural tube induced neural plate. The grafts of the anterior part of the embryonic axis into the anterior part of the host blastoderm induced head structures whereas the posterior part of the axis never induced such structures. He did not find satisfactory inductions from the notochord. He found that the chick organizer like that of amphibians shows a tendency to complete itself in comparison to the fate of tissue which induced it; and to this extent it behaves like part of a harmonius equipotential system.

Thus, as a result of his experiments Waddington propounded two theses - (1) the inducing capacity is retained for sometimes by the derivatives of the primitive streak and (2) this capacity is still possessed by the differentiated neural tissue at a stage when it appears to have been already lost by the notochord. In early 1950s, Nieuwkoop (unpublished) clearly demonstrated the phenomena of neural induction by the head process.

Differentiation Capacity of Blastoderm

Murray and Selby (1930) found that dissections on unicubated blastoderms, before grafting, may have an inhibitory effect on differentiation whereas intact blastoderms at this stage may give rise to perfectly differentiated tissue when grafted. Hunt (1932) performed experiments to test morphogenetic potencies of the transverse levels of the chick blastoderm at the definitive streak stage. Whole blastoderm was divided into five transverse levels based on the areas determined by Wetzel (1929); and each piece was cultured on the chorioallantoic membrane. His analysis of the grafts was based on (i) the kind of tissue which differentiated in the grafts, (ii) the frequency of occurrence of a particular tissue and (iii) the greater degree of differentiation of certain tissues at particular levels. The study revealed that the nervous system tissue (brain) and eye structures developed from grafts containing anterior most part of

the node where notochord developed most frequently.

While studying the differentiation capacity of chick blastoderm at the definitive streak, head process and somite stages Willier and Rawles (1931) noted similarity in the development of the head process and the medullary plate of the chick embryo to that of Triton and suspected that medullary plate was induced by the notochordal strip of the head process.

Using the chorioallantoic grafting technique, Stein (1933) investigated the localization and differentiation of the presumptive forebrain and hypophyseal ectoderm. She performed two independent series of experiments at the primitive streak, head process stages including early head fold and the somite stages. Her experiments showed that the material from the level anterior to the Hensen's node at the definitive streak and Head Process stages gave rise invariably to the forebrain tissues. She did not mention posterior demarcation of the forebrain differentiation. She concluded that certain degree of determination of the forebrain occurs from the time of the definitive streak stage to the time when head process becomes morphologically indicated. She also found eye structures (sensory as well as pigmented layer of the retina in the differentiated grafts along with forebrain tissue), Commenting on the capacity of hypophysis differentiation she concluded

that it was limited to the levels anterior to the node in head process, head fold and somite stages. In younger implants, the frequency of occurrence of the hypophysis was less but it gradually increased from eight somite to 15 somite stages. She attributed the low rate of the occurrence of hypophysis material in younger stages to the failure of the brain and skin ectoderm to make proper contact and insufficient cell division in the grafts.

Butler (1935) with the help of the same technique tested developmental capacity of different regions of the unincubated chick blastoderm, She obtained full histological differentiation of all anterior tissues, viz., eye, midbrain as well as chorda in the grafts from the anterior region of the unincubated blastoderm. She noted widespread capacity for heart formation in the posterior and even anterior half. The heart appeared when a complete anterior of posterior half was transplanted. On the whole she found an increase in the percentage of fully differentiated grafts with older blastoderms.

During the same year, Dalton (1935) published his work trying to explain (1) what part was actually played by the primitive streak in the formation of the trunk region of the embryo. (2) what causal relations were responsible for embryonic segregation within the area pellucida and (3) at what time embryonic segregation concerned especially with the formation of embryonic

axis makes its appearance? Like earlier workers he also used chorioallantoic grafting technique. The age of the donors ranged from 8 hours of incubation to 4 somite stage viz. 8, 10, 12, 14, 16, 17, 18, and 19 hours respectively. In all the stages whole blastoderm was divided into 3 transverse levels. The physiological age of the embryos was determined by the ratio between the length of the streak and the length of the entire pellucid area which is constant for any given age. The hypoblast was removed from the transplants of stages up to definitive streak stage. At all the stages the transplants were cultured also with attached hypoblast and accordingly there was difference in the results. The differentiation was better when the grafts were transplanted by plantinum loop than by watch maker's tweezers. Results of his experiments provided a clear evidence of level to level restriction of structures and antero posterior arrangement of the area pellucida. (1) Level 1 (anterior to node) showed differentiation of nervous tissue and structures of anterior part of the embryonic axis and (2) Level 2 (node region) differentiated in posterior part of head, neck, notochord and anterior trunk parts, (3) Level 3 (rest of the pellucid area) differentiated into posterior trunk region. He observed that there was no differentiation of mesodermal structures in the absence of the hypoblast. The anterior part of the streak in absence of

the node was capable of self differentiation into axial structures confirming the results of Waddington(1933) that in the presence of the hypoblast the levels posterior to the node were capable of forming axial structures. As regard the central nervous system, there was an antero posterior arrangement of presumptive areas. Heart, liver and mesonephros differentiated from the posterior half of the third level and appearance of liver was found to be dependent upon the presence of differentiating cardiac muscle. Area capable of forming heart tissues was actually much larger than the area designated for presumptive heart by earlier workers. Heart was always found in association with the liver. These findings led him to conclude that embryonic segregation of all organs occurred during primitive streak formation as well as during the period of body axis formation.

Competence of the Responding System

Woodside (1937) attempted to investigate the part played by the responding system and also the developmental age of the host on the responding tissues in the presence of inducing tissues. He used Fell and Robison's (1929) watch glass technique to culture the grafts. He performed experiments on embryos ranging from the first appearance of short broad primitive streak to the 5 somite stages. He performed three sets of experiments according to the age of the host from 16-19 hrs incubation, (1) at the

first appearance of the short broad primitive streak to the long primitive streak stage, (2) at the early head process stage to the neural fold stage and (3) at one to five somite stages. (1). In the first group he flattened the host blastoderms on the culture medium, removed the endoderm from the ectoderm of the donor embryos at 16-19 hrs of incubation, prepared grafts from the ectoderm with the Hensen's node and sometimes without the Hensen's node and inserted them between the host ectoderm and endoderm. Induction of secondary embryos was found in the host ectoderm at the site of the implant. (2) In the second group of experiments hosts were taken from early head process stage to late neural fold stage. Induction of secondary neural tissues was found in younger hosts of this group. Neural fold hosts responded to inducting tissue by producing thickened epidermis (3) In the third group hosts varied from 1-5 pairs of somites. He demonstrated by this experiment that in this group the host ectoderm did not react to the presence of inducing tissue beneath it. Emphasizing on the role of the responding system and the period of competence, the author concluded that there was a transition from competent to non-competent and then to the determined phase of the ectodermal tissue of the host. He did not test the competence before 16 hrs; it was therefore not known that earlier stages were non-competent. The first appearance of morphogenetic differentiation was the short broad primitive streak

stage in which the epiblast was competent to form medullary plate. This competence was retained till the host was of neural fold stage. He mentioned the work of Umanski (1931) who reported that the chick medullary plate became irreversibly determined during head process formation. This investigation indicated that the epiblast as a whole was capable of forming medullary plate for a greater extent of time than the actual period of activity of normal induction in the region of the medullary plate itself. Irreversible determination of portion of the epiblast to form neural tissue did not prevent its formation elsewhere provided inducing stimulus was present. The ability of the epiblast to form induced neural tissue decreased with increasing age. The response was therefore inversely proportional to the developmental age of the host at the time of transplantation. Maximum response from the host was obtained in the first set of experiments at the stages earlier than those at head fold stage. The evocating node or node field was not necessary for the differentiation of the posterior part of the embryo except the notochord. Kallen (1958) also studied capacity of neural differentiation, but only from Stages 10 to 13.

Organ-Forming Potency Maps

Rawles (1936) used chorioallantoic grafting technique and performed various experiments to analyse the general organ forming areas at head process stages. She used three stages. Early head process stage (ranging

from invisibility to 0.24 mm), medium head process stage (0.24-0.6 mm) and head fold stage (just when head fold appears). She divided whole blastoderm by 5 transverse cuts and two longitudinal cuts thereby dividing the whole blastoderm into 18 pieces, 6 median and 12 laterals. She applied similar plan for all the three stages and cultured on the chorioallantoic membrane. Structural characteristics of differentiated grafts of levels were as follows :

Level A - The area anterior to the head process developed into epithelial tubes, vesicles, primordial germ cells and stratified epithelium.

Level B - the region of the head process anterior to the pit gave rise to tissues mainly of forebrain, sensory and pigmented layers of eye and thyroid in addition to midbrain, ganglia, notochord, heart, liver, feather, cartilage, bone and primordial germ cells.

Level C - Which included a part of the head process along with the node gave rise to ear and hind brain along with mid brain, ganglia, notochord, respiratory and various epithelial tubes in this level.

Level D - which included part of the streak posterior to the pit showed a decrease in nervous tissue as only hind brain and notochord tissues were prominent. Heart and mesonephros were chiefly found in association with other structures which also differentiated at levels A, B and C.

Level E - Which included the posterior region of the blastoderm is characterised by differentiation of intestine, skin, feather germs and primordial germ cells. No differentiation into any neural tissue was found. Level - F - which included the posterior most region of the streak and the area pellucida differentiated into intestine, epithelial tubes, vacuolated cells and epithelial pearls.

She concluded that the primitive streak and node were most active regions. Frequency of occurence of structures gradually declines both anteriorly and posteriorly. The frequency of differentiating structures were consistantly higher in the median grafts, distincty lower in the left grafts and lowest in the right grafts. The central nervous system showed a distinct medio-lateral gradation with regard to degree of differentiation attained as well as the size and frequency. Forebrain, eye, epiphysis were confined only to level B. Heart and liver were limited to lateral areas. Organ forming areas in the head process blastoderm tended to be restricted to a given level of the pellucid area and within each area developmental potencies diminished peripherally from the centre.

Clarke (1936) investigated regional differences in eye forming capacity of the early chick blastoderm. He also used chorioallantoic grafting technique and aimed his investigations to determine (1) the position, extent, and boundaries of the area which has eye forming

potencies; (2) the relative eye forming capacities within these areas and (3) the evidence for a changing organization of the eye potency field at various morphogenetic stages. The age of the donors used ranged from late streak to 12 somite stages. He studied differentiation capacity at 2 transverse levels. At the primitive streak stage the area pellucida was cut transversely at a level passing through the Hensen's node or at levels anterior or posterior to it; and at head process stage it was cut transversely anterior to the node. These two portions were cut twice to separate into 3 longitudinal pieces left, median and right. He performed experiments at following stages (1) 1-3 somite stage (ii) 4-8 somite stage and (iii) at 9-12 somite stages. The results indicated that the prospective eye forming potencies were present in median and lateral regions at the late streak and early head process stages in the anterior part of the node and anterior end of the notochord respectively. At the late streak stage eye forming area extends 0.06 mm anterior 0.02 mm posterior to the primitive pit and 0.2 mm laterally on each side of it. At head process stage the area extends 0.05 mm anteriorly, 0.1 mm posteriorly to anterior end of the head process and 0.2 mm on each side of it. The median region retains eye forming capacity until the eight somite stage. At the late streak and head process stages median region has greater eye forming capacity.

Within the eye forming area there is a medio lateral asymmetrical gradient. The left side is shown to be more active than the right. As a result of comparative study of the frequency of occurence of eye tissues in different grafts Clarke inferred that there was a changing organization of the eye potency field. There was an increase of eye material in lateral regions from the late streak to the head process stage and from head process to the early somite stages. There is a shift in the developmental potency from median to lateral regions. In normal development potentially single eye forming area becomes divided into bilateral primordia as a result of a stimulating influence exerted on the ectoderm by the mesoderm.

Rudnick (1938a) investigated differentiation of transverse pieces of definite streak and head process stages. She investigated the potentiality of the pieces by in vitro cultures. Pellucid area was divided transversely into four parts at the primitive streak stage and five parts at the head process stage. In some the three germ layers were intact whereas in others mesentoderm layer was removed. Cultures were maintained for 2-6 days. The node level and anterior region showed differentiation of medullary plate, body wall, notochord and axial mesodermal portions. The non axial parts formed coelomic vesicles. Heart was localized laterally and removal of

mesentoderm reduced incidence of chorda and heart structure in the endoderm. The second quarter of the streak did not give rise to any axial structure and showed no difference to the removal of mesentoderm. The posterior half of the streak formed large masses of erythroblasts and non specific cells. The experiments showed localization of axis at node and anterior level as the posterior levels did not show any axial differentiation.

In order to find a more radical explanation to the problem of early differentiation, Rudnick (1938b) performed a series of experiments with early pregrooved and grooved primitive streak stages. The cuts were made to isolate an anterior portion, material just antero-lateral to the streak and streak quadrant. The latter piece was sometimes subdivided into anterior and posterior part. These areas were tested both with and without entoderm. Plasma clot cultures were maintained for 2-6 days. In these experiments she did not find any effect of entoderm on the differentiation of upper layers. The anterior ectoderm formed medullary tubes and lateral pieces showed heart forming capacity in pregrooved stages and medullary plate in grooved stages. Formation of notochord and axial mesoderm was dependent on the organization of the streak. At definite streak stage anterior region of the blastoderm and anterior end of the streak was found to loose erythropoietic capacity.

During the formation and growth of the streak there was a progressive increase in the organ forming potency of embryonic structures. Full histological differentiation of above structures was possible even in the earliest stages investiagated.

Rudnick (1944, 1948) in her elaborate reviews traced the developmental history of the chick embryo from the earliest stages and discussed the basic problems like germinal movement during gastrulation, induction and regional organization of the medullary plate along with the mechanism of differentiation. She discussed main problems regarding the formation of the nervous system commencing in relationship with the medullary area to the underlying axial mesoderm. Based on earlier works and her own work she demarcated pear-shaped areas of medullary plate anterior to the Hensen's node. She supported the findings of Waddington and Schmidt (1933) and Woodside (1937) who reported that medullary plate arose as a result of induction by underlying mesoderm. Primitive streak and its derivatives possessed capacity to induce the medullary plate in ectoderm from various regions. Regarding formation of the eye field she (1948,p.768) referred to the work of Clarke (1936) and commented "There is a slight chance, for the eye that a tenuous strip median in the anterior medullary plate, too narrow to be tested by current methods, may from the first, be unable to form eye tissue, and that the diencephalic floor arises by enlargement of this strip.

The situation looks much more like a gradual separation of a continuous eye field into two laterally placed ones by the actual loss of ability of the medial cells". She distinguished three types of activity in the differentiation of nervous system from the medullary plate. (1) Secretory, responsible for the maintenance of the various cavities of the central nervous system (2) Mass movement within the whole epithelium responsible for formation of subsequent neural structures and (3) differentiation of individual cells.

Morphogenetic Movements and Shape of the Prospective Neural Plate Area

In the decades of 1940s and 1950s Spratt did extensive study with various techniques on the early chick blastoderm regarding various problems of organogenesis and localization of prospective organ forming areas, Spratt (1947) used following techniques to localize prospective neural plate at definitive streak stage. (1) Tracing the fate of carbon marked cells, (2) Observation of the presence of neural structures in isolated cultured pieces, (3) Tracing the fate of vitally stained areas in ovo, (4) Staining with vital stains the entire blastoderms, (5) Studying living blastoderms by dark ground illumination (6) Studying fixed and histologically stained blastoderms. For the localization of prospective chorda and somite mesoderm during regression

of the primitive streak in the chick blastoderm Spratt (1952) performed (1) Carbon marking and (2) Isolation experiments. By dark ground illumination and histological study of fixed blastoderms he found that at pre-streak stages the anterior half of the embryonic shield corresponded to the neural plate of the streak stages. His isolation experiments revealed that transection of the blastoderm inhibits the typical pattern of epiblastic movements with a consequent release of certain latent potencies. The results of the carbon marking experiment at definitive streak stage revealed that (i) the neural plate area lies in front of the node and (posterior wings are not contiguous with the sides of the streak) extends 0.4 mm behind, 0.55 mm anterior and 0.45 mm lateral to the pit. (ii) Non involuted mesoderm and prospective tail bud ectoderm extend forward to the node level. (iii) Anterior boundary of the neural area and the underlying head mesoderm is congruent. And (iv) the boundary between prospective fore brain and mid brain lies 0.2 mm in front of anterior edge of the node at definitive streak stage. He described that at the head process stage, the overall picture of definitive streak is maintained except following changes. (1) The posterior wings of the neural plate come closer to the sides of the streak as a result of involution of mesoderm from that area. (2) Width of the neural plate decreases from 0.9 mm - 0.7 mm. (3) The head process length is 0.4 mm (4) The area from

notochord tip to anterior edge of the neural plate is 0.4 mm (prospective fore brain area). (5) Primitive pit to posterior edge of neural plate is 0.4 mm. (6) Involution of all surface mesoderm is completed. (7) Prospective brain regions are elongated. Earlier, Pasteels (1936) also reported that during gastrulation movement prospective neurectoderm moves from more lateral region of the blastoderm towards the mid-line.

The results of isolation and explantation experiments were similar to the results obtained by him with other techniques. Transverse and longitudinal cuts were made both at definitive streak and head process stages.(1) When the cut passed 0.4 mm anterior to, 0.2 mm posterior to the pit the pieces differentiated into respective central nervous structures. (2) When the cut is 0.5 - 0.6 mm anterior to the pit, the anterior piece did not develop into neural structures, but the posterior piece forms complete neural axis.(3) When cut is 0.4 mm posterior to the pit, posteri or region forms no central nervous tissue. Both <u>in vitro</u> and <u>in ovo</u> staining methods also confirmed above results. Identical conclusions were drawn by observations on fixed and stained blastoderms. He discussed that the findings of earlier workers such as Rawles (1936), Rudnick (1938a, b) and his own findings (1947, 1952) all lead to similar unified picture regarding the localization of the neural plate; and concluded that the neural plate at definitive streak stage is ovoid in outline (the node lying in its

centre) and extends 0.5 mm ± anterior, 0.4 mm ± posterior and 0.45 mm lateral to the primitive pit, Subsequently Malan (1953) also showed with the vital staining experiments that prospective neurectoderm moved from more lateral regions to the mid-line of the blastoderm.

Induction of Regional Neural Differentiation.

When Hara (1961) undertook the investigation on the induction of regional neural differentiation by prechordal and presumptive chordal mesoderm, all earlier findings available were based mostly on the grafts tested which had three germ layers together. His work made a breakthrough in the field of Experimental Embryology. It was initiated by the experiments of Nieuwkoop (unpublished) performed in early 1950s that when a piece of prenodal ectoderm at primitive streak stage was cultured intra-coelomically in another embryo for 10 days it did not differentiate into neural tissue, while a similar piece separated at a later stage differentiated into neural tissue. Hara perfected the techniques of separation of germ layers and intracoelomic transplantation. He prepared open sandwiches by combining prechordal and presumptive chordal mesoderm with the competent ectoderm, comparable to that of Sala (1955) in Ambystoma Maxicanum, and cultured them in the coelom of another embryo. He isolated pieces of prechordal and presumptive chordal mesoderm from chick blastoderm at following 4 stages ranging from definitive primitive streak to the head

process stages (and in some cases at the head fold/
1 somite stage);

(I) Stage I : Corresponding to H & H Stage 4, the primitive streak stage.

(II) Stage II : Corresponding to H & H Stage 5, the young head process stage.

(III) Stage III : Corresponding to H & H Stage 5, the medium head process stage.

(IV) Stage IV : Corresponding to H & H Stages 6-7, the head fold and one somite stages.

The prechordal and presumptive chordal mesoderm inductors were combined with pieces of "Neutral" ectoderm (i.e. the ectoderm possessing no neural differentiation tendencies but being competant for neural induction) taken from blastoderm of stage slightly younger than the definitive primitive streak stage. He adopted a double control system, in which pieces of 'neutral' ectoderm, without addition of inductors, were used in the 'control A' series while ectodermal pieces taken from the area overlying the inductor material were used as 'control B' grafts.

He transplanted these open sandwiches as well as 'control A' and 'control B' grafts in the coelom of another 2 1/2 days old embryo and cultured them for 12 days. In the course of the investigation, a total of 61 sets of blastoderms were operated upon to provide the 296 grafts prepared in the manner described above.

Another 130 embryos served as hosts out of which 12 died within 12 days after the operation. The 180 survivals yielded 212 analysable grafts. Out of these 75 were expperimental grafts developed from the open sandwiches implanted, 45 were 'control A' grafts and 92 were 'control' B' grafts. None of the 'control A' grafts formed neural structures whereas 87/92 'control B' grafts formed neural structures. The stage wise results obtained were as follows:-

(I). Experiments with mesoderm of stage I.

(i) As the prechordal mesoderm was thinner and dispersed it was difficult to obtain sufficient mass of inductor material. The experimental grafts gave rise only to feather papillae, whereas 'control B' grafts formed prosencephalic neural material.

(ii) The experimental grafts with compact mesoderm exhibited prosencephalic and/or mesencephalic neural differentiation with high incidence of telencephalic components. Some of them also showed notochordal structures. The 'control B' grafts formed mesencephalic tissues with few telencephalic components. One case possibly contained rhombencephalic material also.

(II) Experiments with mesoderm of stage II

(i) Experimental grafts with prechordal mesoderm as inductor differentiated into prosencephalic (Telencephalic as well as diencephalic) and mesencephalic structures. 2 of the 3 grafts showing mesencephalic

structures also showed differentiation of notochord. 'control B' grafts showed mostly prosencephalic and in few cases mesencephalic structures. Prosencephalic material included telencephalic and diencephalic components.

(ii) Experimental grafts with young head process mesoderm as the inductor differentiated into varied range of structures such as prosencephalic mesencephalic, rhombencephalic, notochord, epidermis, cartilage and some tubules, the 'control B' grafts formed mesencephalic and/or rhombencephalic structures.

(III) <u>Experiments with mesoderm of stage III</u>.

(i) Experimental grafts using prechordal mesoderm as the inductor as well as 'control B' grafts showed differentiation similar to those in the experimental series of stage II described above.

(ii) Experimental grafts using anterior head process mesoderm as inductors gave rise to neural structures with predominant mesencephalic component. Rhombencephalic and notochord structures were also observed. The 'control B' grafts contained predominently mesencephalic material and very rarely prosencephalic materials.

(iii) Experimental grafts with posterior head process mesoderm as inductor material differentiated rhombencephalic as well as spinal cord material. The non-neuralized grafts formed feather papillae. The 'control B' grafts formed rhombencephalic and/or spinal cord materials.

(IV) Experiments with mesoderm of stage IV.

(i) One of the 4 experimental grafts with posterior head process mesoderm as the inductor was neuralized containing spinal cord and possibly rhombencephalic material. The 'control B' grafts were neuralized similarly as the neuralized experimental grafts showed notochordal structure.

Hara derived following conclusion from the structures differentiated in 'control B' and experimental grafts.

Conclusion from structures differentiated in 'Control B' grafts.

a) Ectodermal pieces isolated from presumptive mesencephalic area overlying the compact mesoderm of the definitive primitive streak stage, formed mesencephalic and in part also prosencephalic (mostly diencephalic) structures (partial lack of agreement with prospective significance).

b) Ectodermal pieces isolated from the presumptive prosencephalic area overlying the prechordal mesoderm of the young head-process stage formed prosencephalic structures. On the other hand ectodermal pieces isolated from the presumptive mes, and rhombencephalic (and possibly spinal cord) area overlying the young head process itself differentiated according to their prospective significance.

c) Ectodermal pieces isolated from the area overlying the prechordal mesoderm of the medium head process stage also formed prosencephalic structures. Ectodermal pieces taken from the anterior head process area (the presumptive mesencephalic and probably rhombencephalic area) and those from the posterior head process area (the presumptive rhombencephalic and spinal cord area) also differentiated according to their prospective significance.

Conclusion from structures differentiated in Experimental grafts:

The experimental grafts showed the regional neural differentiation in majority of grafts identical with that of 'control B' grafts.

(a) The compact mesoderm of the definitive primitive streak stage induced mesencephalic and complete prosencephalic formations. (The differentiation of the experimental and of the 'control B' grafts does not correspond).

(b) The prechordal mesoderm of the young head process stage induced prosencephalic differentiation (corresponding); whereas the young head process stage induced mes and rhombencephalic (and/or spinal cord) differentiation, in some cases accompanied by prosencephalic differentiation (not corresponding).

(c) The prechordal mesoderm of the median head process stage also induced prosencephalic differentiation.

The anterior head process of this stage induced mes and rhombencephalic (and/or spinal cord) differentiation, while the posterior head process induced rhombencephalon and/or spinal cord (all three parts corresponding).

As none of the ' control A' grafts formed neural tissues, most of the 'Control B'grafts formed neural structures and most of the experimental grafts showed neural induction as desctibed above, he concluded that the chordal mesoderm together with prechordal mesoderm plays a leading role in the regional determination of the induced neural formation.

Rao (1968) extended the work of Hara (1961) and investigated the appearance and extension of neural differentiation tendencies in the neurectoderm of the chick embryo. The two efficient techniques, the germ layer separation and intra coelomic transplantation refined by Hara (1961) made possible for him to interrupt the induction process and to see the sequence of changes in isolated ectoderm along with the differentiation tendencies in neurectoderm. He prepared grafts at 4 stages during H & H stages 4 to 5.

<u>Stage</u> I : He incubated eggs for 16-18 hours so that the length of the primitive streak was 1.66 mm. He separated the endoderm from the ectoderm, 0.17mm away from the pit and cut it into 6 rectangular pieces each measuring 0.17 X 0..48 mm. Of all the grafts, differentiated into neural structures, histological differentiation

was poor and along with prosencephalic structures feather papillae were also differentiated.

Stage II : For this stage he incubated the eggs for 18-19 hours so that the area pellucida was narrower than Stage I and streak length was 1.87 mm. This stage corresponds to the definitive primitive streak stage of Spratt (1952), He isolated 9 rectangular pieces from the endomesoderm free ectoderm, 0.14 mm anterior to the pit each measuring 0.14 x 0.44 mm. The opaque area anterior to the pit extended upto 0.56 mm. At this stage the grafts from all blastoderms were neuralized. Attachment was better than at previous stage. Recovery rate of median areas was better than the lateral areas and posterior median areas were better recovered than the anterior median grafts. Anterior grafts showed prosencephalic differentiation and the posterior most grafts showed prosencephalic differentiation along with mesencephalic structures.

Stage III : For stage III he incubated the eggs for 19-21 hrs. The streak length was same as in the stage II while the anterior opaque area extended to 0.68 mm. Short head process was visible measuring 0.31 mm from the pit. He isolated 9 grafts pieces from the endomesoderm free ectoderm 0.17 mm anterior to the pit each measuring 0.17 x 0.39 mm. Separation of endomesoderm was difficult near the node region. Recovery rate of lateral grafts was lower than those of

the corresponding median areas. In the two posterior levels prosencephalic along with mesencephalic and rhombencephalic tissues were differentiated.

Stage IV : For Stage IV he incubated the eggs for 21-22 hrs. At this stage also streak length was the same as at Stage II while the anterior opaque area extended to 0.85 mm. Head process length was 0.47 mm which corresponds to the medium head process stage of Spratt (1952). He prepared 12 grafts from endomesoderm free ectoderm 0.17mm anterior to the pit each measuring 0.17 x 0.34 mm. At this stage he found difficulty in separating the germ layers. Anterior two levels showed non neuralized grafts along with neuralized ones. Lateral grafts showed higher neuralized grafts as in the previous stage. In the median level recovery rate and the number of neuralized grafts was higher than at the posterior most level.

Based on Hara's (1961) as well as his own experimental findings coelomic environment was taken as neutral. Based on his experimental results he suggested the following sequence of events.

(a) In all stages the relative number of grafts forming neural structures were lower in the lateral and anterior most median graft areas than in the more posterior median areas. They generally increased from Stage to Stage in all graft areas, ranging from 0% in the anterolateral areas at Stage I and II to 100% in the

posteromedian areas from Stage III onwards. Among the lateral areas in any one stage it was always the area located at the level of the prechordal mesoderm which showed the highest relative number of grafts forming neural structures.

(b) The anterior median grafts, representing a part of the prospective prosencephalic region, essentially showed prosencephalic differentiation only. Prosencephalic differentiation also occured in the more posterior grafts, representing a part of the prospective mesencephalon and rhombencephalon. In addition, more posterior neural structures appeared in these grafts and this became more pronounced with each successive stage.

(c) All the lateral grafts from all stages showed prosencephalic differentiation only, except for the lateral grafts adjoining anterior head process in Stage IV, in which mesencephalic differentiation was also encountered.

Rao summarised his results as follows. In all stages the relative number of grafts forming neural structures were lower in lateral and 'anterior' most median grafts than posterior median areas. They generally increased from stage to stage. Prosencephalic structures were noted in the lateral areas from the level of prechordal mesoderm and in median grafts representing prospective prosencephalic area and posterior to it. Interpreting the results in terms of activation-transformation hypothesis of Nieuwkoop (1952) propounded on the basis of

experiments carried out in amphibian embryos, he found results complementary to those of Hara (1961) leading to dynamic picture of the origin of the pattern of neural organization of chick embryo. As a result of first contact between neurectoderm and the most anterior prechordal mesoderm which exerts an 'activating' action a wave of activation spreads centrifugally through the ectoderm from the area of contact. As a consequence a neural field is set up in the neurectoderm leading to the prosencephalic differentiation tendencies in the ectoderm. During the formation of the head process the more posterior axial mesoderm (prospective notochordal material) is laid down craniocaudally in front of the node. At the same time the corresponding parts of the future neural plate shift from left and right to the midline where they come under the inducing influence of newly formed notochordal mesoderm which also possesses activating capacities; consequently the activation field gradually extends caudally. The activation field extends less far laterally than in the anterior regions possibly because the activating actions of the notochordal mesoderm is weaker than that of prechordal mesoderm but also becuase notochordal mesoderm is laid down later so its activating action starts later. This is followed by the transforming action of the notochordal mesoderm in the overlying ectoderm converting prpsencephalic differentiation tendencies for more caudal parts of the central nervous

system spreading less far laterally than in the more anterior regions.

Inducing Capacity of axial mesoderm and embryonic endoblast

Grabowski (1956) investigated by *in ovo* technique the effect of excision of Hensen's node on the early development of the chick embryo. He indicated that during primitive streak stages prospective notochordal material was located in the invaginating mesodermal layer anterior to the node but to what extent the notochordal material invaginated and became distributed around the node was not known accurately. Grabowski's work (1957) on the induction of secondary embryos in the early chick blastoderm by graft of Hensen's node again stressed more importance of axial mesoderm than the notochordal material in the process of induction.

Gallera (1971) reviewed the work on process of primary induction in birds. He referred to the work of Vakaet (1964; 65) that embryonic endoblast arising from primitive streak upto H & H Stages 5 had neural inductive capacities; and on the basis of his own work (Gallera and Nicolet, 1969) described that the first stimulus for neural induction would originate in the prospective embryonic endoblast. It is later reinforced by the inductive stimulus of chordamesoblast. In any event, once invaginated the embryonic endoblast cells loose their inducing capacity.

With the advent of electron microscopic studies knowledge on the process of neural induction has further advanced. Gallera et al (1968) obtained neural induction when millipore filters were inserted between grafted node and host ectoderm. EM studies revealed microvilli arising from the tissues and entering the filter. Giladi and wolk (1970) investigated the inducing capacities of primary hypoblast by transfilter induction studies. 25 ± 5 u thick TH Millipore filters were used. Culture medium was agar egg extract. They found two successive inducing capacities in the hypoblast. The first to appear and disappear was the induction of the primitive streak while the second is a prosencephalic induction. Primitive streak induction occured across the filter; but induction of a mature primitive streak with Hensen's node required a direct cellular contact between the two layers. Prosencephalic inducing power appeared in later stage and extended at the stage of head process formation.

Electron Microscopic and Biochemical investigations .

In recent years there is increasing thrust on the investigations on the mechanism of neural induction also. Several workers have undertaken SEM, TEM, and transfilter studies to understand the mechanism of neural induction (see Gallera et al, 1968; Gallera 1971; England 1973, 1974; England and Cowper 1976;) Contributions have also been made attributing high protein level in the hypoblast to its high inducing capacity (Eyal Giladi et al

1975) and on biochemical aspects leading to the understanding of molecular mechanisms (see for review Tiedemann 1976 and 1978) It is hoped that such research may provide us with a new insight to understand the phenomena of neural induction (process as well as mechanism) before long.

Chapter 3

MATERIAL AND METHODS

(a) General

The present investigation was carried out on White and Red Leghorn mixed breed eggs supplied regularly by "all weather" poultry farm maintained by the Govt. of Meghalaya at Bhoi, Nayabunglow situated about 18 kms from Shillong. They were obtained as fresh as possible every week and stored in a cold place (temperature never exceeding 14°C) and utilized within the same week. Eggs older than 5 days were always discarded.

The techniques prescribed by Hara (1961, 1970, 1971) were followed throughout the course of investigation. As far as possible asceptic conditions were always maintained.

Before operation the eggs were incubated in 'Tempo' incubators and after operation the host eggs were incubated in forced air drift type 'NSW' incubators. (Among Indian makes available these two incubators gave us satisfactory service for continuous incubation).

During operation a third incubator was used. The temperature of the incubators was maintained at 37.5°C (+1°C) and pans full of distilled water were kept inside each to maintain 60-70% humidity. Host eggs were incubated for 21/2 days by which time the anterior amniotic fold reaches almost at the level of vitelline

arteries. The donor eggs were incubated for 23-25 hours which includes about 3 hours for warming up, to get the primitive streak (Hamburger & Hamilton, H & H Stage 4) to head process (H & H Stage 5) blastoderms. Variation was always noted in the stage of blastoderms as reported earlier (Hamburger and Hamilton, 1951; Khare, 1975). Out of each set of the eggs incubated if the first donor egg opened was young, then other donor eggs of this set were left for some more time in the incubator to reach the desired stage. If the first donor egg opened was found to have crossed H & H Stage 4, other eggs of this set were taken out of the incubator and kept near the table lamp till they were used up for the operations. All operations were performed at room temperature (18-20°C).

BDH (ANALAR Grade) and E. MERCK (GR Grade) chemicals were used to prepare the physiological media for operations. The donor blastoderms were excised in 0.9% NaCl solution (Normal saline) The operations were performed in the following modified Locke Solutions (Yamamoto, 1949).

1) <u>Normal Locke Solution</u>

NaCl	M/6.2	- 94.5 ml
Kcl	M/6.2	- 3.5 ml
$CaCl_2$	M/9.3	- 2.0 ml
Glucose		- 0.2 g
* $NaHCO_3$		- 0.02 g

*(for buffering to maintain pH ± 8)

2) <u>Ca free Locke Solution</u>

NaCl	M/6.2	- 96.4 ml
KCl	M/6.2	- 3.6 ml
Glucose		- 0.20 g

(Non buffered pH ± 6)

The solutions were kept overnight in the incubators before use.

The agar bases for operations were prepared in the following manner: 2% solution of the bacteriological agar was prepared in normal saline in a pressure cooker and poured into sterilized petri dishes. As soon as it solidified, rectangular blocks were cut out from it and stored in normal saline in a refrigerator. For preparing agar bases some of these blocks were melted in a pressure cooker and the melted agar was poured in flame-sterilized watch glasses and each was covered immediately with another sterilized watch glass. It took about 24 hours for the agar to set properly for the operations.

Surgical steel instruments and Corning glasswares were used for the work. They were properly sterilized . Before operation the instruments and working table were wiped with 70% ethyl alcohol and the glasswares were rinsed by boiling glass distilled water. The sterilized physiological media, glasswares and instruments were also exposed to germicidal UV light for a short period as final sterilization measure before starting the operation.

The separation of germ layers of each blastoderm isolation of the graft pieces from it, transplantation of these pieces into host embryos was carried out under Meopta DM22 and Olympus VAII dissecting Stereoscopic binocular microscopes respectively. Tungsten needles were sharpened and polished according to the method prescribed by Tindall (1960). The instruments were every time sterilized in the 70% alcohol followed by rinsing in boiling distilled water and cold sterilized water during the course of the operations.

(b) Preparation of Hosts

The host eggs were taken out from the incubator and the egg shell was quickly wiped with a cotton swab soaked in 70% ethyl alcohol. The position of the embryo was marked in the egg shell over an electric candler and the egg was placed on a nest made of cheese cloth in a petri dish. Square window was prepared around the mark with a doctor's file without injuring the shell membrane. Drops of the Locke solution were put on the shell membrane. The air space at the broader end of the egg was punctured and the shell membrane was carefully removed taking care not to injure the underlying embryo. Due to collapse of air chamber the level of the embryo was slightly depressed below the window. The Locke solution was added drop by drop around the embryo and the egg was slowly rotated horizontally so that any part of the blastoderm

may not stick to the shell membrane. The stage of the embryo was noted, the window was sealed with a piece of cellophane tape and the egg was kept in the incubator.

(c) <u>Preparation of grafts</u> (FIGS : 3.1a. 3.1b.)

The underside of the shell of the donor egg was cracked and its contents were transferred gently in a finger bowl containing warm saline. The blastoderm was carefully cut out around its periphery with the help of foreceps and Deweker's scissors, separated from vitelline membrane and transferred by a wide mouth pipette in Locke's solution over the agar-base already set for operation. The watch glass with agar-base and the blastoderm was placed over a wooden castor dish filled with black wax. A microscope lamp was used for nearly horizontal beam of light. On proper adjustment in a dark field, as a result of total internal reflection at the glass-air-interface the living blastoderm glows up resulting into excellent contrast between its different parts.

The blastoderm was spread with the dorsal side facing upwards by tucking its edges with tungsten needles over the agar base. With the help of ocular micrometer measurements were recorded, and position of the prospective prosencephalic area was marked in the neurectoderm. The blastoderm was not turned upside down and again spread as nicely as possible by tucking its edges over the

Fig. 3.1a : Diagrammatic section through the operation dish. The main light beam is indicated by arrows.

Fig. 3.1b : Separation of the endo-mesodermal layer from the ectoderm. The figure shows a sagittal section at the level of primitive streak.

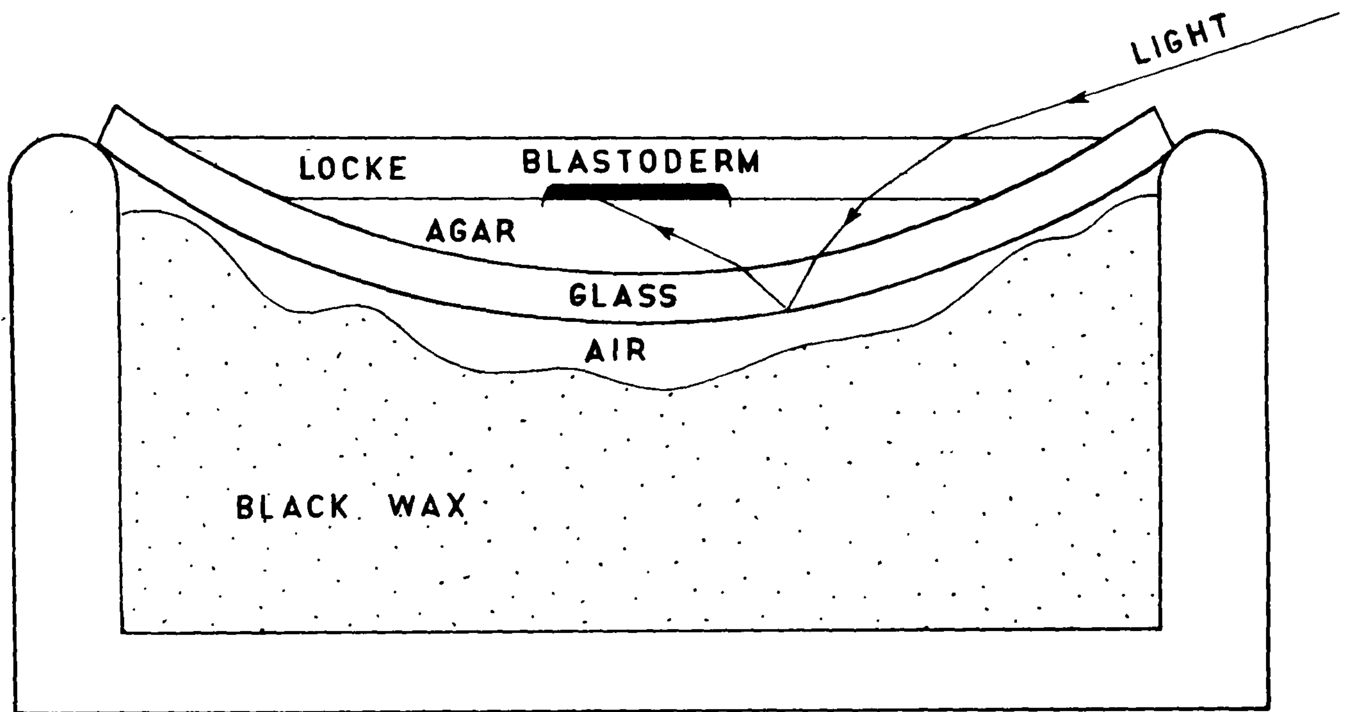


FIG 3.1 a

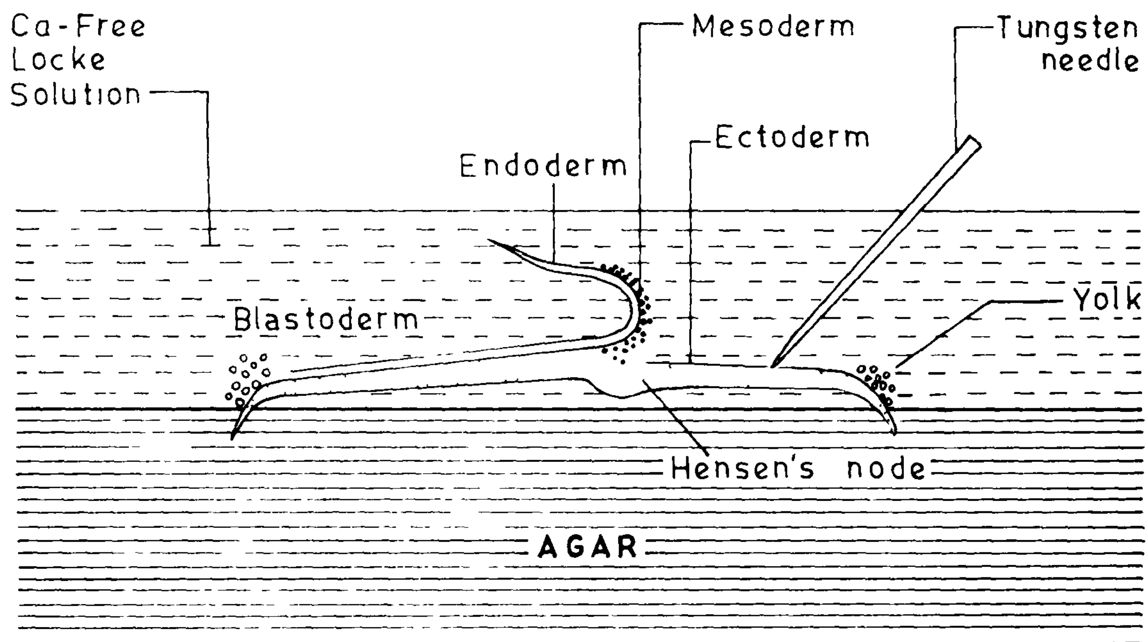


FIG 3.1 b

agar base. Locke Solution was replaced by Ca^{+} free Locke solution. Endoderm and mesoderm were removed from an area slightly larger than the prospective prosencephalic area outlined by Spratt, (1952). Ca^{+} free Locke solution was again replaced by normal Locke solution and the graft pieces were isolated by pressing the tungsten needles over the prospective prosencephalic ectoderm according to the operation plans as shown in Figures 5.1-5.3 and transplanted.

(d) Transplantation of the grafts (FIGS 3.2a, 3.2b.)

The cellophane tape of the host egg of the right age (in which amniotic folds have reached the level of vitelline arteries) was removed, and the level of the embryo was brought up by pouring the Locke Solution drop by drop around the embryo through the window in the shell. As soon as the embryo comes up at the level of the window, addition of one or two more drops of Locke solution forms a convex surface protruding over the window. The horizontal beam of light passing through this convex drop provides beautiful contrasting illumination to operate upon the host embryo. Small openings were made in the vitalline membrane and through these openings two slits were made in the somatopleure on each side of the embryo at the level of the vitelline blood vessels (large openings in the vitelline membrane cause herniation of the embryo). Each graft was now transferred gently by Spemann pipette over these cuts and with the

Fig. 3.2a : Operation on host embryo - situation of the host embryo with respect to the window during operation.

Fig. 3.2b : Operation on host embryo - sites of implantation of the grafts.

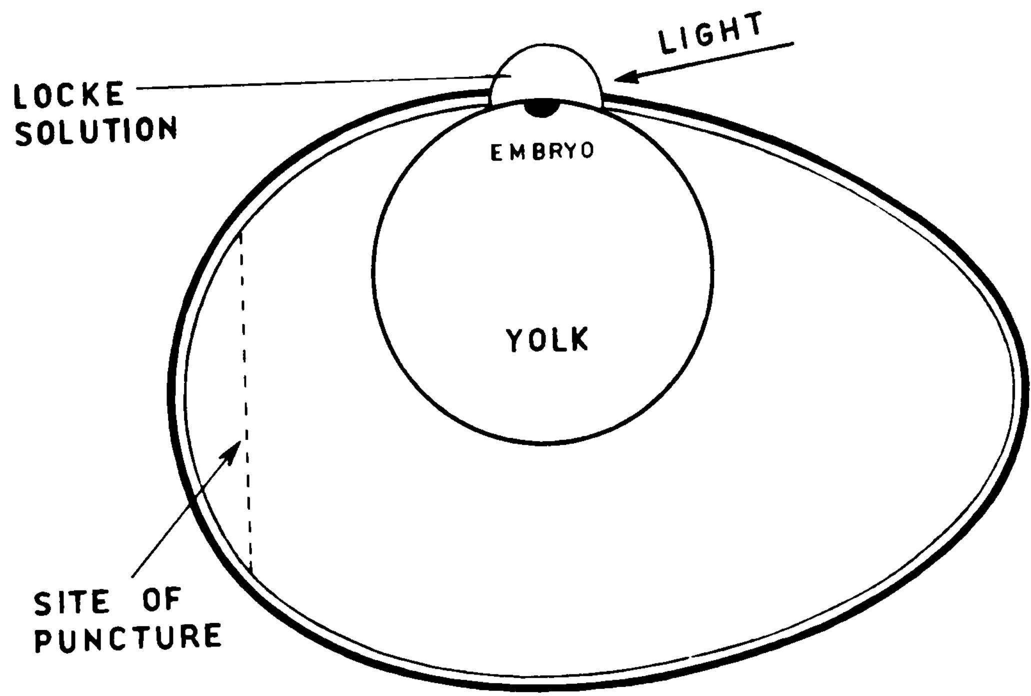


FIG 3.2a

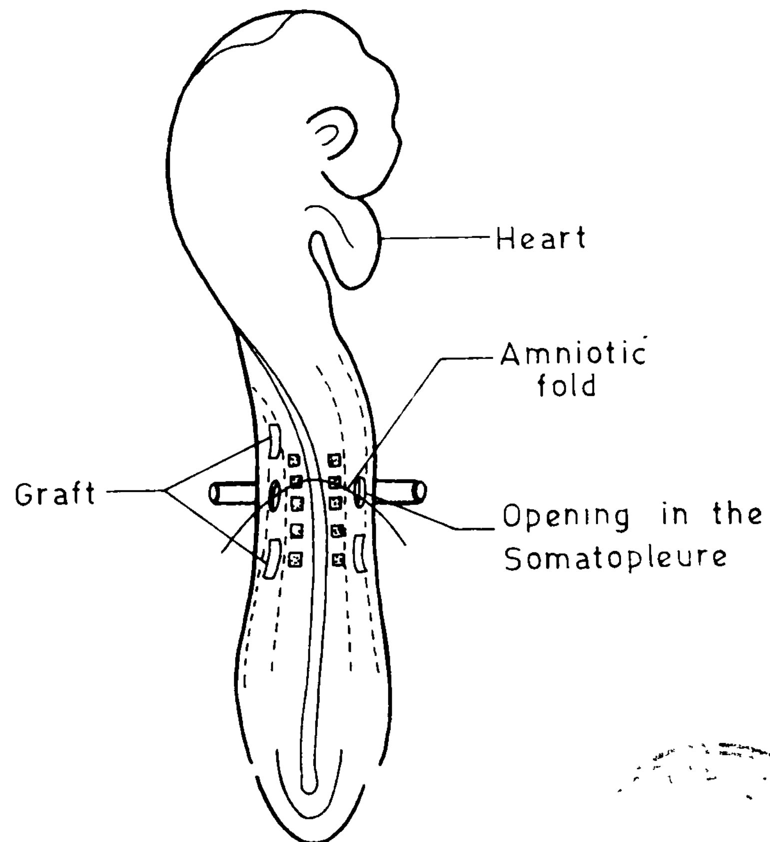
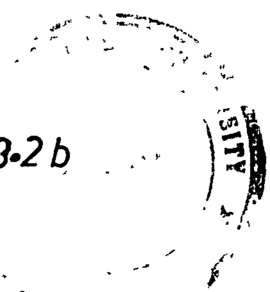


FIG 3.2b



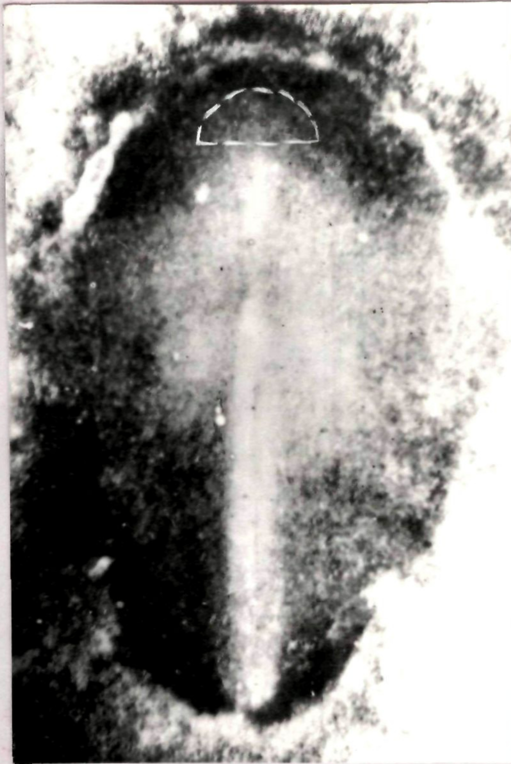
help of tungsten needles it was pushed inside the coelom of the host in such a way that it's inner surface lay flat against the somatopleure at a 3-somite distance from vitelline blood vessels. Sometimes 2 and sometimes 3 grafts were transplanted in a host. Like Hara (1961) and Rao (1968) the present author also found the left anterior coelom as the most favourable site for the attachment of the grafts, the next best place being right posterior coelom and third best being the left posterior coelom. After transplantation about 2 ml of albumen was taken out with a hypodermic syringe from the broad end of the host egg so that an artificial air chamber was prepared above the embryo. Few drops of Locke solution were now added around the host embryo and by gentle horizontal rotation of the egg insured that no extra embryonic part was stuck to the shell membrane. The window and the puncture at the broad end in the egg shell were now sealed with cellophane tape and the host egg was kept back in the incubator.

The entire operational procedure takes on an average 1 1/2 - 2 hours. The operated eggs were incubated for further 12 days without turning as the air chamber over the embryo excludes the possibility of its attachment with the shell membrane.

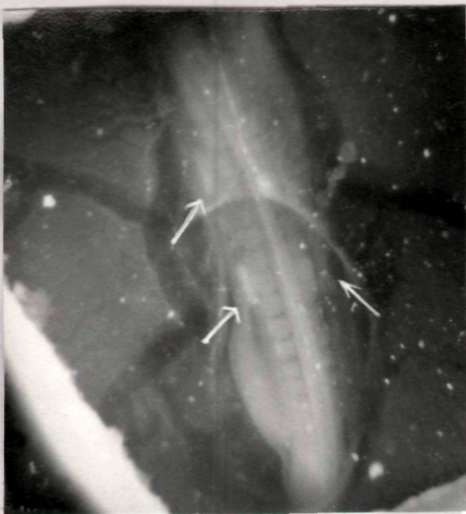
Plate 3.1a : Photomicrograph of a head process blastoderm showing the prospective prosencephalic area with dotted white line from where the grafts were taken.(x 24)

Plate 3.1b : Photomicrograph of grafts (shown by arrow) transplanted in 2½ old embryo. (x 16)

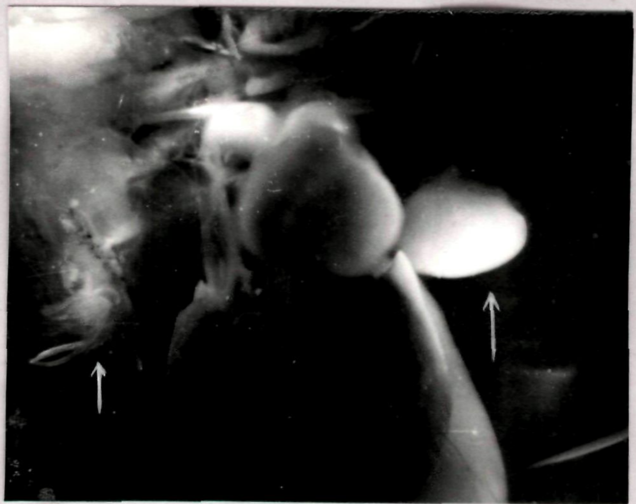
Plate 3.1c : Photomicrograph of the grafts developed in the host body cavity (shown by arrows) as on 12th day of transplantation.(x 4)



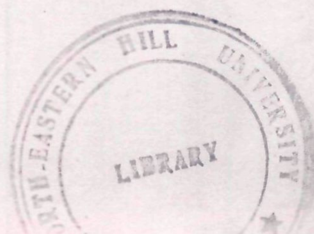
3·1a



3·1 b



3·1 c



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(e) Recovery of grafts

The operated hosts were sacrificed by decapitation and opened after 12 days. Position of graft and its attachment were noted. The grafts were removed along with some host tissue, fixed in Bouin's fluid, embedded in paraffin wax, sectioned at 6μ, and stained by Azan method for histological analysis.

Chapter 4

CRITERIA FOR HISTOLOGICAL ANALYSIS

In principle the criteria established by Hara (1961) and further followed by Rao (1968) for the identification of the parts of central nervous system encountered in differentiated coelomic grafts as described below, has been followed. As an aid to the establishment of criteria isolates of different parts of the forebrain at H & H Stage 8 (4/5 somite stage) and H & H Stage 10 (10 somite stage) were cultured intra-coelomically for 12 days. The recovered grafts were examined histologically for comparison with the histological structures differentiated in the experimental grafts. Prior to this, a series of histological sections of 12-day old chick brain were also prepared for comparative study.

The recovered grafts contained various histological structures of brain or non-neural ectodermal derivatives such as epidermis with feather papillae or sometimes simple epithelial vesicles. The spatial configuration of the differentiated brain segments did not show the normal condition. As the experimental technique does not allow the noraml pattern of the closure of the neural tube, the majority of neural structures in the differentiated grafts are everted and become highly irregular; but the histological differentiation of the parts of nervous system reaches a level high enough to permit proper identification of corresponding brain areas or

sometimes remains similar to that of corresponding normal tissues. The study was concentrated on the differentiation of prosencephalic structures, but other structures wherever differentiated were also recorded. The microscopic criteria were as follows.

(1) <u>Prosencephalic structures</u>. (Plates 4.1a-4.7b)

<u>Telencephalic Cortex</u> : prosencephalic structures were identified by the presence of massive regions of homogeneously arranged nuclei corresponding to corpora striata of the normal telencephalon. A peculiar pattern of cellular arrangement found in some parts of prosencephalic (diencephalic) neural material suggest the pattern of puffed corn grain (as a puff is formed by cell bodies surrounding a fibrous core), the fibre bundle of which sometime appear to connect with the choroid plexus. This pattern and associated fibre tract are considered as an indication of the presence of telencephalic cortex.

<u>Choroid plexus</u> : The histological pattern of the choroid plexus in the grafts was similar to that found in the normal brain. The presence of choroid plexus in association with other prosencephalic structures formed a secondary criterion for the identification of prosencephalic structures. The prosencephalic choroid plexus was more extensively branched and larger than that differentiated in rhombencephalic structures. In the present study, however, the choroid plexus was observed in very few grafts.

Olfactory complex   Invaginated pseudostratified sensory epithelium (olfactory) and cuboidal respiratory epithelium (non sensory epithelium of the nasal cavity), surrounded by cartilage formed characteristic features of olfactory complex. Often granulation of the epithelial tissue is observed similar to the regions of external nasal apertures.

Eye structures : Eye structures are easily identified by the presence of sensory layer (having closely packed nuclei) often accompanied by pigmented layer, tapetum (having dark/black pigments). The pigmented layer sometimes is seen even without sensory layer. (retina)

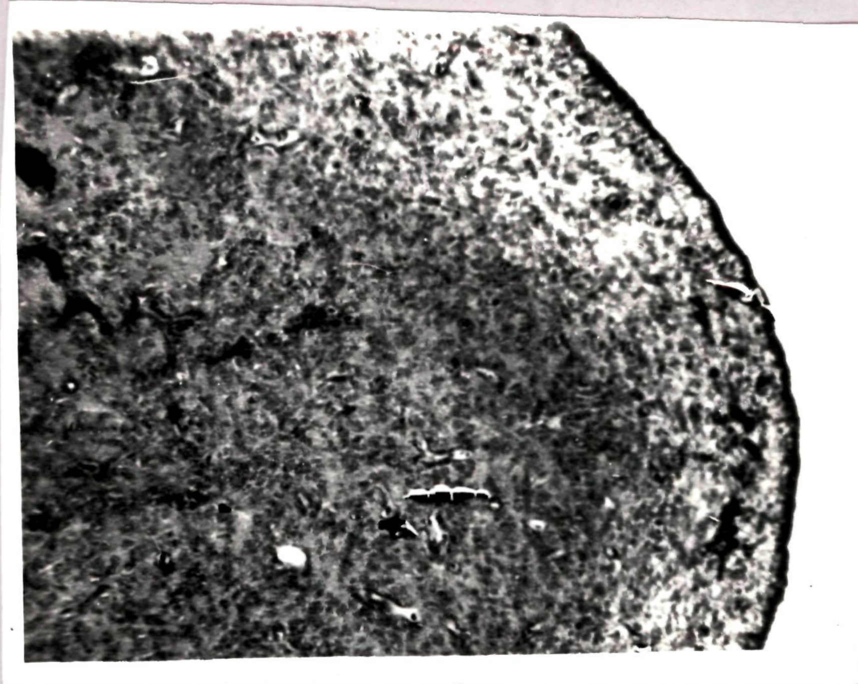
Pineal body : A structure consisting of many small hollow buds surrounding a large centrallumen, sometimes accompanied by melanin pigment is identified as pineal body.

(2) Mesencephalic structures.

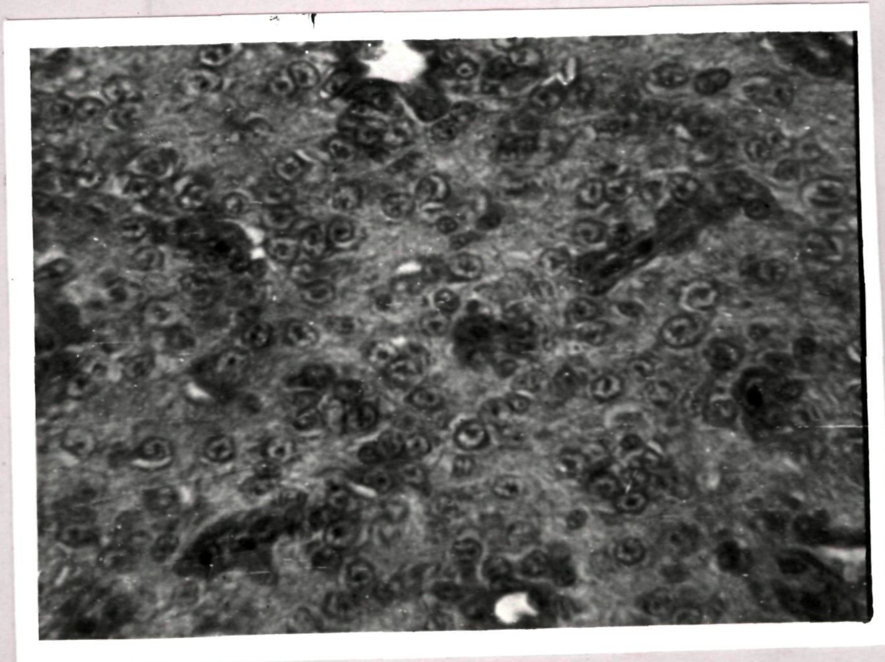
The typical laminated structure of the optic lobes with outer thicker and thinner inner nuclear layers (Cajal's layers) constitute a definite criterion for dorsal mesencephalic parts. The ganglionic cells, which are found in the deeper zone of tectum opticum in the normal development are sometimes encountered in grafts having mesencephalic portion. Large mesencephalic motor meurons form a definite criterion for ventral mesencephalic structures. The ganglionic cells representing

Plate 4.1a : Photomicrograph of a section of a graft showing telencephalic cortex - cellular arrangement being reminiscent of puffed corn grain. (x 100)

Plate 4.1b : Photomicrograph of a part of the above section under higher magnification (x 450).



4·1a



4·1b

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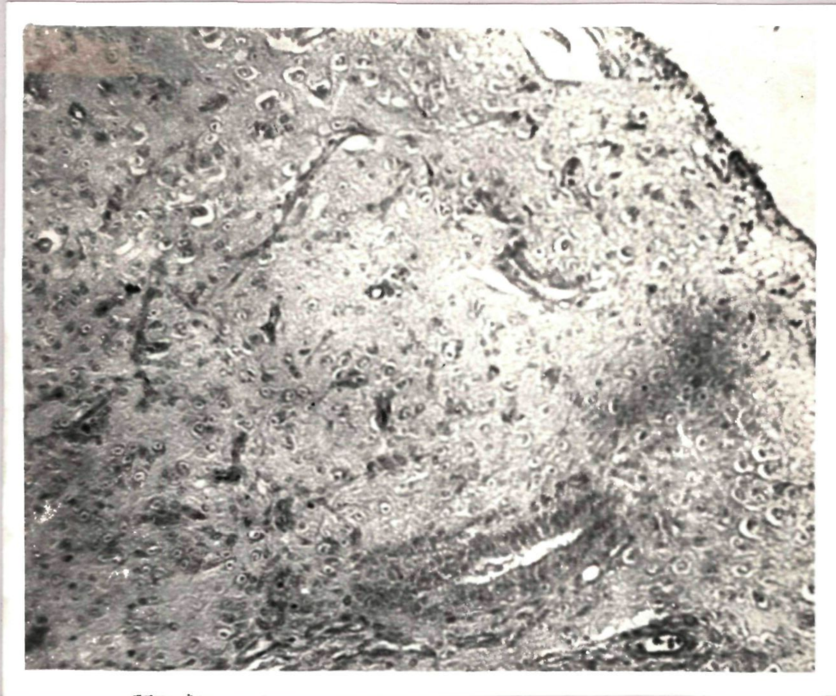
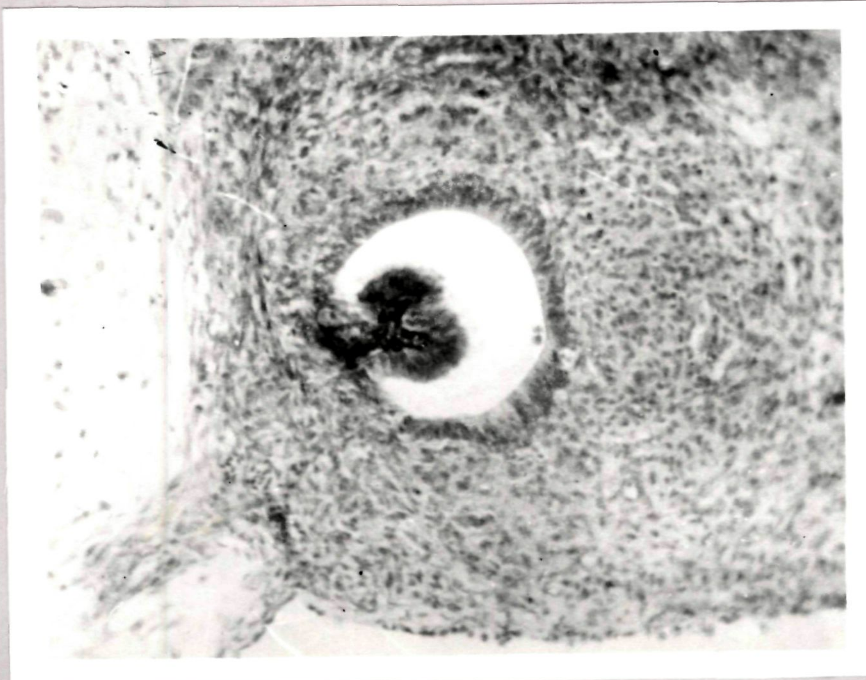
Plate 4.2a : Photomicrograph of a section of a graft showing prosencephalic neural mass (x 100).

Plate 4.2b : Photomicrograph of a section of a graft showing choroid plexus.



4·2a



4·2b

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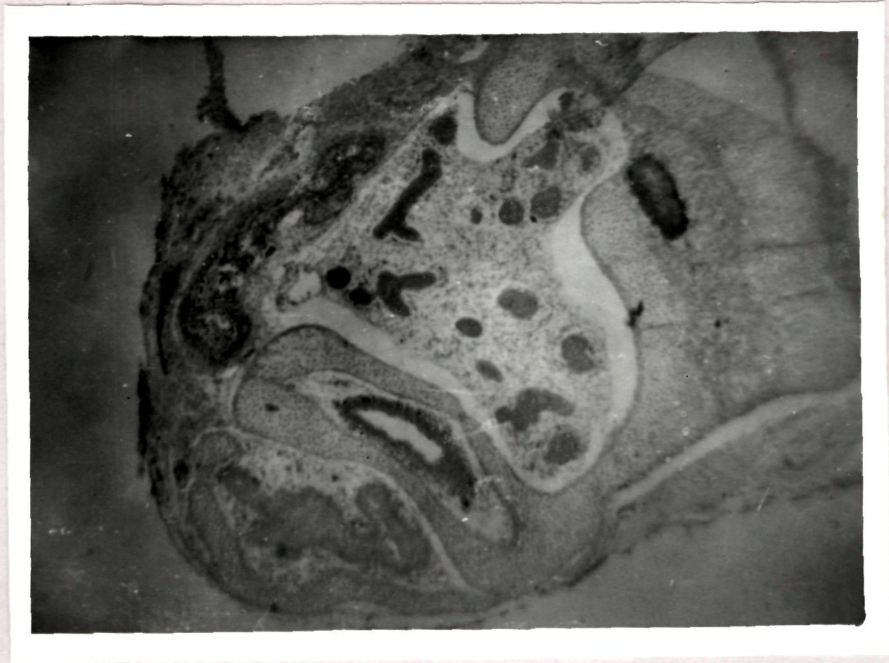
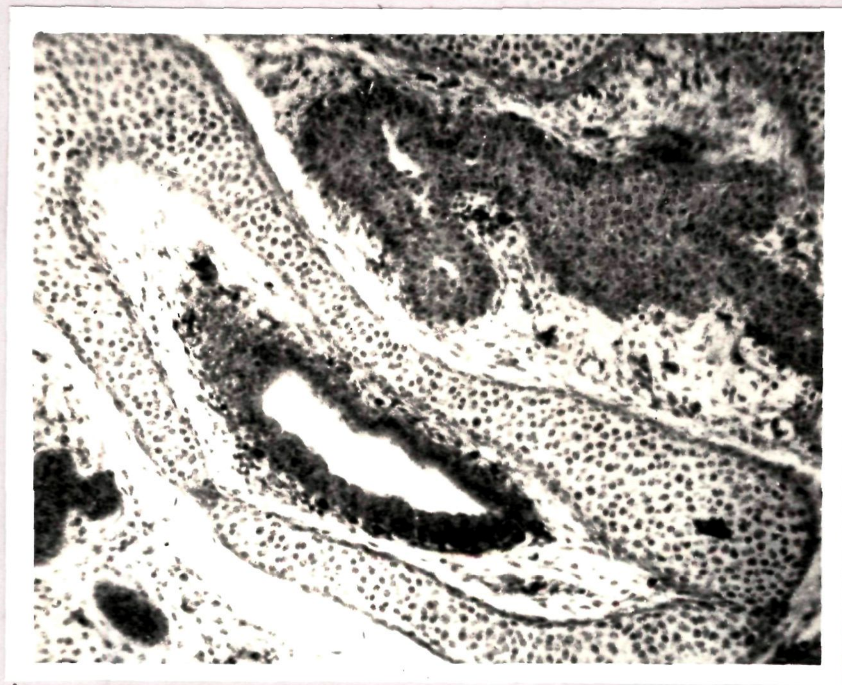
Plate 4.3a : Photomicrograph of a section of a graft showing olfactory complex (x 40)

Plate 4.3b : Photomicrograph of a section of a graft showing a part of olfactory complex (x 100)



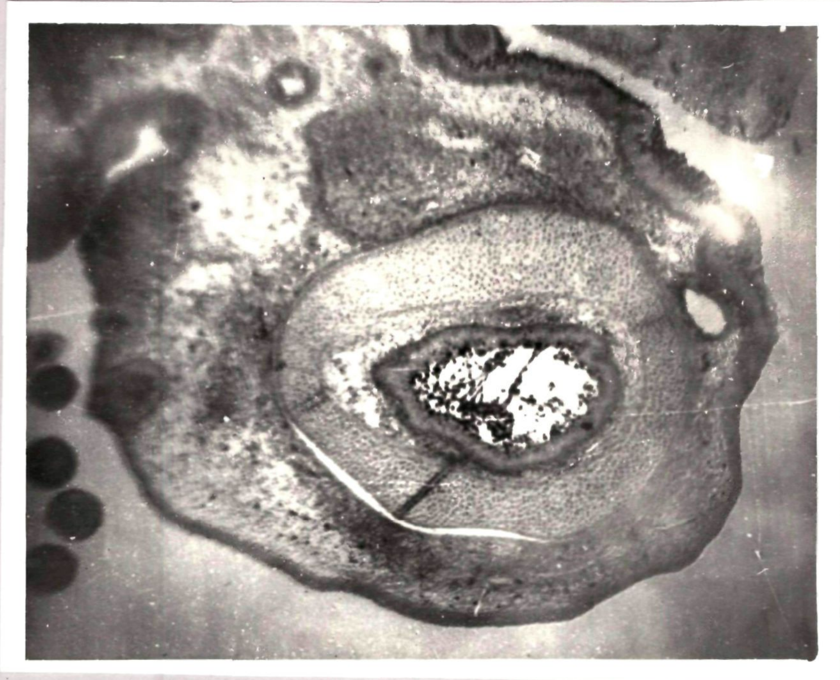
4·3a



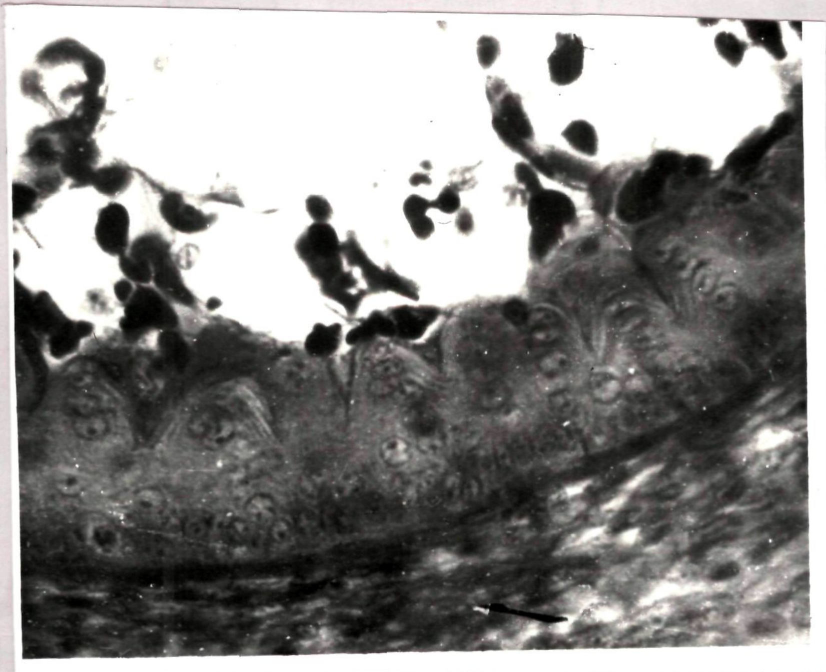
4·3b

Plate 4.4a : Photomicrograph of a section of a graft showing respiratory epithelium surrounded by nasal cartilage. (x 100)

Plate 4.4b : Photomicrograph of a part of the section of the above graft showing respiratory epithelium (x 450)



4·4a



4·4b

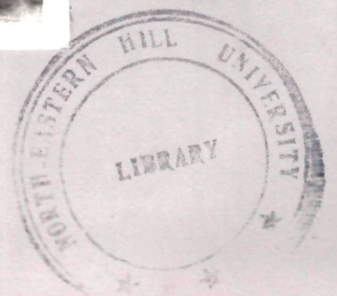


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Plate 4.5a : Photomicrograph of a section of a graft showing well differentiated sensory layer of the eye (retina). (x 40).

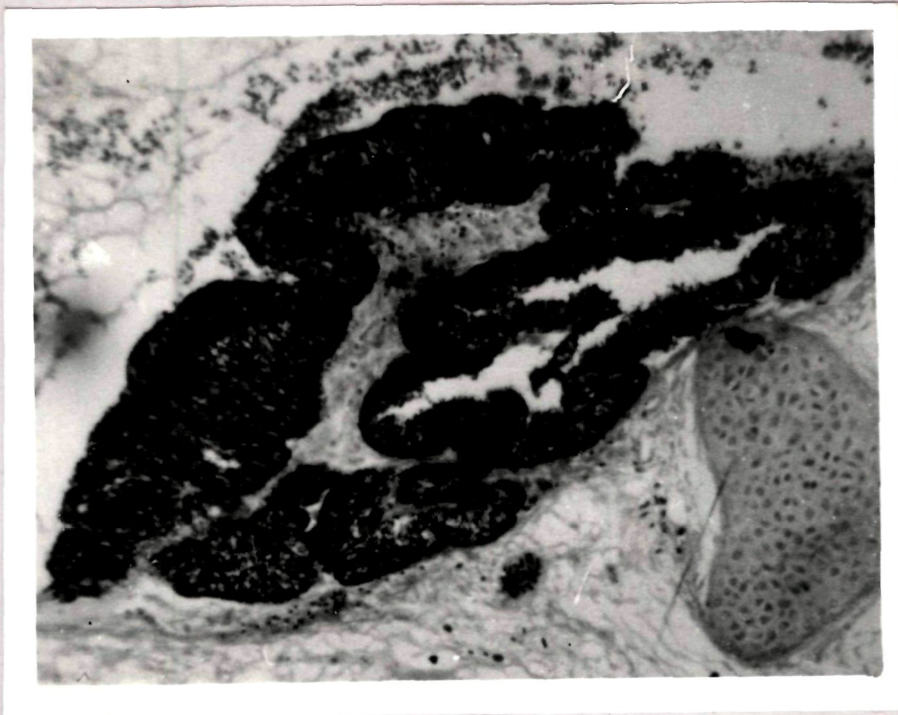
Plate 4.5b : Photomicrograph of a part of the above section showing the sensory layer of the eye (retina) (x 100)

4·5a

4·5b

Plate 4.6a : Photomicrograph of a section of a graft showing pigmented layer of the eye (Tapetum). (x 100).

Plate 4.6b : Photomicrograph of a section of another graft showing pigmented layer of the eye (Tapetum) (x 100)

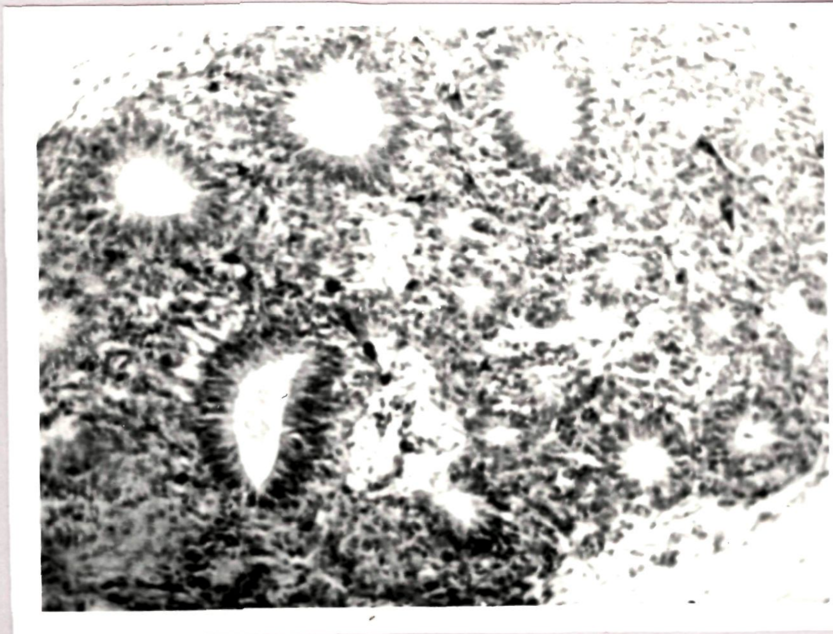


4·6a

4·6b

Plate 4.7a : Photomicrograph of a section of a graft showing pineal body - hollow central lumen surrounded by hollow buds. (x 100).

Plate 4.7b : Photomicrograph of a section of a graft showing feather germs (x 100)



4 · 7a

4 · 7b

dorsal mesencephalic elements and motor neurons representing the ventral element helps sometimes in classifying a graft as mesencephalic even in the absence of Cajal's layers.

(3) Rhombencephalic structures.

One of the important criterion for the identification of rhombencephalic structures is the presence of tall cylindrical cells of the ependymal layer similar to those present in the normal rhombencephalic portion.

Cerebellum and choroid plexus: The cerebral cortex can be recognised by densely packed nuclei showing high mitotic activity, although the cerebral tissues are not always observed in the grafts. The choroid plexus present in association with the carebral structures is considered to represent the plexus of the fourth ventricle.

Medulla oblongata and spinal cord : Although the normal topographic relations are considerably distorted, the alar and basal plate derivatives and the marginal zone can be made out as such.

No criteria has yet been formulated for the identification of medulla oblongata an spinal cord structures separately. All these structures are included under the category of rhombencephalic structures(Hara 1961, Rao 1968).

Chapter 5

EXPERIMENTS AND RESULTS

(a) OPERATIONAL PLANS

For the present investigation, graft pieces were isolated from the prospective prosencephalic ectoderm of the chick blastoderm at H and H stage 4 (definitive primitive streak stage) and H and H stage 5 (head process stages) according to the 6 operation plans B, C, D, F, G and H (Figs. 5.1, 5.2, and 5.3). The first 4 operation plans B,C, D and F were meant to provide 4 to 6 graft pieces from the semicircular central and peripheral areas of the prospective prosencephalic ectoderm with a view to localize the differentiation tendencies of various parts of the prospective prosencephalic area as precisely as possible. The last 2 operation plans G and H were meant to provide 4 longitudinal graft pieces from one half of the prospective prosencephalic ectoderm and 4 transverse graft pieces from the other half of this area. It was felt that by superimposing the results obtained from these two types of grafts a better picture of the neural differentiation pattern of the prospective prosencephalic ectoderm would emerge.

Earlier two more operation plans A and E were designed to provide 7 or even 8 semicircular graft pieces, 1 central, 3 median surrounding the central and 3 or 4 peripheral surrounding the median, from the prospective prosencephalic ectoderm with a view to test its differentiation tendencies as finely as possible.

But as it was difficult to isolate such small pieces (size ca 0.2 x 0.1 mm) precisely according to the operation plans and recovery of these grafts was also very discouraging, operations were not performed according to these plans.

The details of the 6 operation plans according to which the operations were performed are following.

Operation Plan B : According to this plan 5 graft pieces, 1 central semicircular (size 0.25 x 0.25 mm) and 4 arc shaped peripheral (0.25 x 0.25 mm), were isolated from the prospective prosencephalic ectoderm.

Operation Plan C : According to this plan also 5 graft pieces were isolated, 2 transverse from central semicircular area (size 0.1 x 0.5 mm and 0.15 x 0.55 mm) and 3 peripheral arc shaped (0.25 x 0.3 mm) surrounding the central median area.

Operation Plan D : According to this plan, 6 graft pieces were prepared, 3 from central median area (size 0.15 x 0.25; 0.15 x 0.28 mm and 0.15 x 0.25 mm) and 3 peripheral and arc shaped (size 0.25 x 0.25 mm).

Operation Plan F : According to this plan 6 graft pieces were prepared, 3 from central median area (0.2 x 0.25 mm) almost triangular in shape, and 3 from peripheral area (0.25 x 0.25 mm). The lines dividing the central and peripheral areas into smaller grafts was drawn from the centre of the base line to the periphery inclined at about 45°.

<u>Operation Plan G</u> : According to this plan 4 longitudinal grafts were prepared in the longitudinal axis from one half of the prospective prosencephalic area, the size of the grafts being 0.1 x 0.55 mm; 0.1 x 0.5 mm, 0.1 x 0.4 mm and 0.1 x 0.3 mm.

<u>Operation Plan H</u> : The grafts according to this plan were prepared transversely as far as possible from the other half of the same blastoderm from which grafts of plan G were prepared, the size of the grafts being 0.1 x 0.4 mm, 0.1 x 0.4 mm, 0.15 x 0.3 mm and 0.15 x 0.2 mm.

(b) <u>RECOVERY OF GRAFTS</u>

During the course of the present investigation, 448 blastoderms were operated upon to provide 1437 grafts. These were implanted in 941 hosts. Out of these 216 hosts died (survival 77%)before the completion of 12 days. The 725 hosts surviving up to 12 days yielded a total of 304 grafts out of 1241 grafts implanted in these.(recovery ca 25%) The data on the recovery of the grafts in the left anterior, right posterior, left posterior and right anterior coelom has been shown in Table 1. Attachment of recovered grafts has been shown in Table 2. Percentage of recovery of grafts has been rounded off to nearest complete number as shown in Table 5.3 and Figs. 5.1, 5.2 and 5.3. It would be clear from the fractions (denominator represents number of grafts implanted and numerator number of grafts recovered) shown in Table 3 that more number of operations

Table. 5.1

Recovery of Grafts.

Place of Implantation	Number of Grafts implanted	Number of Grafts recovered	Percentage of recovery
Left anterior coelom	528	157	29%
Right posterior coelom	307	43	14%
Left posterior coelom	302	63	21%
Right anterior coelom	300	41	14%

were performed at H & H stage 4 than at 2 substages of H & H stage 5. This is due to the fact that exact desired donor stage was not always available and operations had to be based subject to the availability of a particular stage. Such variations have also been reported earlier by certain workers such as Hamburger and Hamilton (1951) and Khare (1975). As Hara (1961) and Rao (1968) experienced the left anterior coelom was found to be the best site for the recovery of grafts in this investigation also.

As there was difficulty in getting the exact desired donor stage, there was also difficulty in getting the exact desired host stage for implantation of the grafts. Sometimes the amniotic fold had just crossed the level of heart, sometimes it was at the level of vitelline vessels and sometimes posterior to this level. Hosts younger than the first stage described above and older than the third stage (after it had reached the anterior level of hind limb buds) were always discarded. An analysis of the implantation of grafts at these different stages reveals that the recovery of grafts was best (50%) when the amniotic folds had crossed the level of heart. The recovery percentage was slightly less (48%) when the amniotic folds were at the level of vitelline vessels and lowest (37%) when the amniotic fold was reaching the level of posterior limb buds.

Table 5.2

Attachment of recovered grafts

Place of Implantation/ Place of attachment	Left anterior coelom	Right posterior coelom	Left posterior coelom	Right anterior coelom
Left anterior body wall	108	-	-	-
Right anterior body wall	-	-	-	29
Left posterior body wall	-	-	31	-
Right posterior body wall	-	31	-	-
Alimentary canal	10	2	4	2
Urinogenital system	-	7	12	1
Liver	29	2	8	6
Mesentry	6	1	8	1
Lung	4	-	-	2
Total	157	43	63	41

(c) ANALYSIS OF GRAFTS

(I) Analysis of grafts implanted according to different operation plans.

Like any other morphogenetic event the neural induction is also a dynamic process. Its appearance and extension in the prospective chick neuroectoderm is associated with the appearance and extension of the head process (Hara, 1961 ; Rao 1968). With a view to understand the dynamic picture of the neural differentiation pattern in the prospective prosencephalic ectoderm from H & H stage 4 to H & H stage 5 of the chick embryo, the differentiation of the grafts from this region has been studied at the 3 following stages of the donor embryos.

(i). H & H stage 4 (definitive primitive streak stage) when slight condensation of mesodermal cells is first observed anterior to the Hensen's node.

(ii). Early to middle H & H stage 5 (short to medium head process stage) when head process length ranges from 0.01to 0.3 mm.

(iii). Middle to late H & H stage 5 (medium to long head process stage) when head process length ranges from 0.31 to 0.6 mm.

The graft pieces have been named with capital and small letters. The capital letter indicates the operation plan and the small letter indicates the graft excised from that particular plan.

Table 5.3

Percentage of Recovery of Different types of Grafts.

Operation Plan	Grafts Transplanted	H and H Stage 4 HP: 0.0 mm		H and H stage 5			
				HP: 0.1-0.3 mm		HP: 0.31-0.6mm	
B	B m	7/17	41%	2/6	33%	4/19	21%
	B la	4/21	19%	4/12	33%	5/23	22%
	B lp	5/28	18%	6/20	30%	7/25	28%
C	C mm	2/10	20%	4/8	50%	1/8	12%
	C mp	5/11	45%	3/7	43%	6/8	75%
D	D mp	5/18	28%	2/3	66%	3/13	23%
	D ml	6/33	18%	2/5	40%	4/20	20%
F	F mp	3/27	11%	2/6	33%	1/10	10%
	F ml	5/63	8%	6/21	28%	3/25	12%
C/D/F combined	C/D/F ma	8/27	30%	6/20	30%	5/21	24%
	C/D/F l	13/51	25%	11/36	30%	11/51	21%
G	G t	12/56	21%	6/21	28%	10/19	53%
	G u	11/77	14%	7/28	25%	7/26	27%
	G v	6/32	19%	4/11	36%	5/9	56%
	G w	4/35	11%	1/6	17%	2/10	20%
H	H p	6/52	11%	2/9	22%	1/16	6%
	H q	8/52	15%	2/17	12%	4/34	12%
	H r	11/83	13%	5/25	20%	5/28	18%
	H s	13/76	17%	5/19	26%	6/27	22%

H and H : Hamburger and Hámilton

HP : Head Process.

At the time of operation every care was taken to record the size of the blastoderm and the grafts as accurately as pósSible. However, as Rao(1968) pointed out, slight variations were unavoidable due to inherent variability of the material and difficulties in measurement. At each of the 3 donor stages described above, the recovery of grafts and the histological elements differentiated within these grafts have been represented by figures and graphs. Figs 5.1, 5.2 and 5.3 show actual operation plans, graft areas in the prospective prosencephalic ectoderm, their sizes and the recovery percentage. Tables 5.4, 5.5, 5.6 and 5.7 and Figs 5.4, 5.5, 5.6, 5.7 and 5.8 show histological elements differentiated in the recovered grafts. The histological analysis has been carried out purely on the qualitative basis. Final inferences on the differentiation pattern have been drawn by combined analysis of results of the experiments performed according to different operation plans.

While analysing the histological structures main emphasis has been given to the differentiated elements of prosencephalon, viz., olfactory complex, telencephalic cortex, choroid plexus, sensory and pigmented layers of the eye, pineal body and prosencephalic neural mass. The structural elements of epithelial vesicles, feather germs and cartilage wherever differentiated were recorded, Donor-stage wise analysis is as follows.

As the graft pieces taken from the central area according to the operation plans C,D & F were different, experiments performed with these have been described separately; whereas description of experiments performed with the similar graft pieces taken from the peripheral region have been described together.

(i) H & H stage 4 (definitive primitive streak stage).

At this stage the area pellucida was more or less pear shaped. The primitive streak had a well defined primitive groove ending anteriorly in a primitive pit surrounded by Hensen's node. The primitive streak was sometimes bifurcated at the posterior end. The length of the streak measured from the pit to the posterior end was 1.66 mm (SD 0.29). Extending anteriorly to the node about 0.50 to 0.63 mm from the pit a somewhat opaque area can be distinguished from the rest of the blastoderm. Just anterior to the node slight condensation of the mesodermal cells was observed below the neurectoderm. The analysis of the grafts implanted from these blastoderms and results obtained can be summed up as follows:

Operation Plan B : The recovery of the median grafts B m was 7/17, anterior lateral grafts B la was 4/21 and posterior lateral grafts B lp was 5/28. All B m grafts were neuralized. Among others, 3 B la and 2 B lp grafts were neuralized. The feather papillae were noticed in 1 B m, 1 B la and 1 B lp grafts. Among eye structures,

Fig. 5.1 : Graft areas and the recovery percentage of different types of grafts implanted at the definitive primitive streak stage.

Abbreviations :

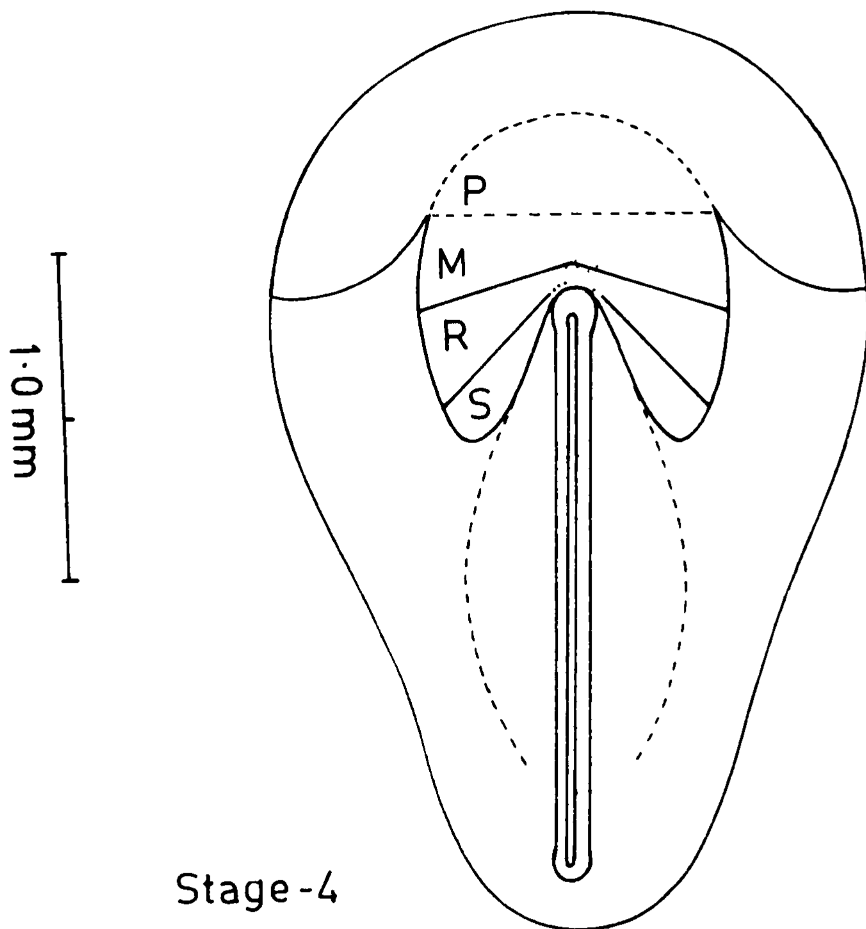
P ; Prospective prosencephalic area.

M : Prospective mesencephalic area.

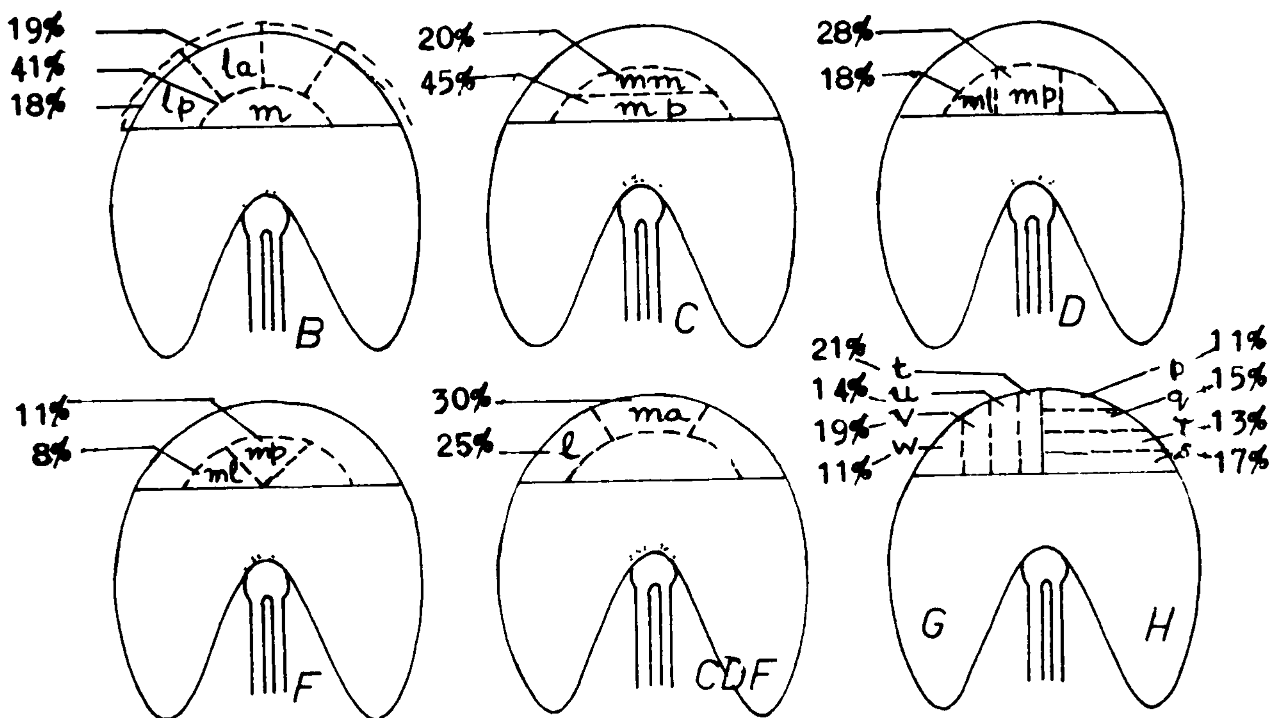
R : Prospective rhombencephalic area.

S : Prospective spinal cord area.

For graft areas see the text.



Definitive primitive streak stage.



B-H: operation plans   *F I G. 5·1*

sensory layer of the eye was observed in 1 B m, 2 B la and 2 B lp grafts. The pigmented layer was observed in 1 B la and 2 B lp grafts. 2 B m and 2 B la grafts showed telencephalic cortex.

Operation plan C (central grafts) : The recovery of median C mm grafts was 2/10, posterior median C mp graft was 5/11. All recovered C mm and C mp grafts were neuralized. None of these grafts showed eye structures or telencephalic cortex.

Operation plan D(central grafts) : The recovery of the posterior median D mp grafts was 5/18, median lateral D ml grafts was 6/33. All recovered D mp grafts were neuralized, whereas neuralization in D ml grafts was observed only in 4 out of 6 recovered grafts. The sensory layer of eye was observed in 2 D mp grafts only.

Operation plan F (central grafts): The recovery of posterior median grafts F mp was 3/27 and median lateral grafts F ml was 5/63, 2 F mp and 3 F ml grafts were neuralized. Of the eye structures sensory as well as pigmented layer of retina was encountered only in 1 F ml graft. The recovery of these grafts was very low due to very small size of the grafts.

C,D,F (peripheral grafts): The recovery of anterior median ma grafts implanted according to the operation plans C, D and F was 8/27 and lateral graft l was 13/51. 6 out of 8 ma and all l grafts were neuralized. Structural lelements of the eye were observed in 2 l grafts only.

Operation plan G : A decline was noted in the recovery of grafts in this as well as plan H depending upon the size of graft pieces implanted. Thus the recovery of grafts G t was 12/56, G u was 11/77, G v was 6/32 and G w was 4/35. Out of the recovered grafts 10 G t, all G u grafts, 5 G v and 2 G w grafts showed neuralization. Sensory layer of the eye was encountered in 4 G t, 3 G u and 2 G v grafts. Pigmented layer was seen only in 1 G t and 2 G u grafts. Telencephalic cortex was observed: in 1 G t and 2 G u grafts.

Operation plan H : The recovery of grafts H p was 6/52, H q was 8/52, H r 11/83 and H s was 13/76. Out of these 2 H p, 2 H q, all H r and 13 H s grafts were neuralized. The sensory layer was encountered in 5 H r and 3 H s grafts whereas pigmented layer was found in 3 H r and 1 H s graft. 1 H s graft showed Telencephalic cortex.

(ii) Early to middle H and H stage 5 (short to medium Head-Process stage).

At this stage the shape of the area pellucida remains similar to that of H and H stage 4. The length of the primitive streak was 1.60 mm (SD: 0.34). The opaque area extends anteriorly about 0.63 to 0.75 mm from the pit. The head process measures from 0.1 mm to 0.3 mm from the pit. The analysis of the grafts implanted at this stage can be summed up as follows.

Operation plan B : The recovery of the median B m grafts was 2/6, anterior lateral B la grafts was 4/12, the posterior lateral grafts B lp was 6/20. All these grafts showed neuralization. Eye structures, sensory as well as pigmented layers, were encountered in 1 B la and 3 Bl p grafts only. Telencephalic cortex was observed in 1 B m and 1 B la grafts.

Operation plan C (central grafts) : The recovery of median C mm grafts was 4/8, posterior median C mp grafts was 3/7. All C mm and C mp grafts were neuralized. Sensory layer of the eye was found to have differentiated nicely in 1 cmm and 1 cmp grafts, whereas the pigmented layer was found in 1 cmm graft only.

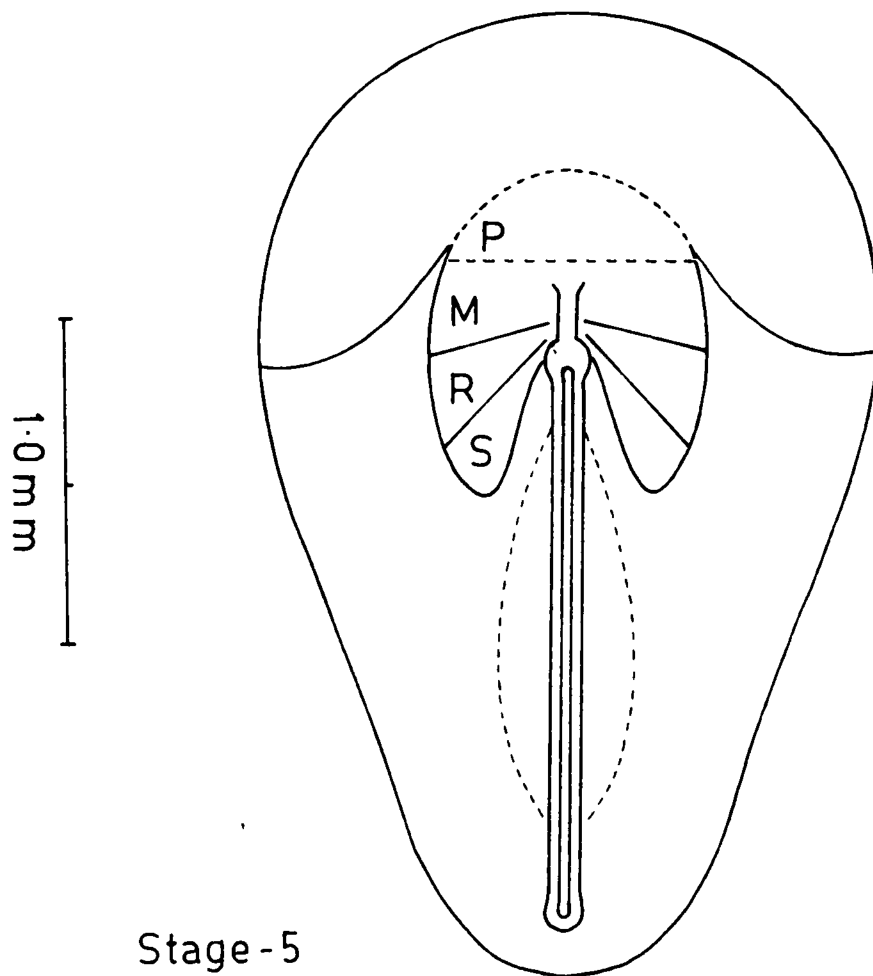
Operation plan D (central grafts) : The recovery of the posterior median grafts D mp was 2/3 and that of median lateral graft D ml was 2/5. All the D mp and D ml grafts were neuralized. The structural elements of the sensory layer of the eye were seen in 1 D mp graft only.

Operation plan F (central grafts) : The recovery of posterior median grafts F mp was 2/6 and median lateral graft F ml was 6/21. All the F mp and F ml grafts were neuralized. But somehow sensory and pigmented layers of eye were not encountered in these grafts.

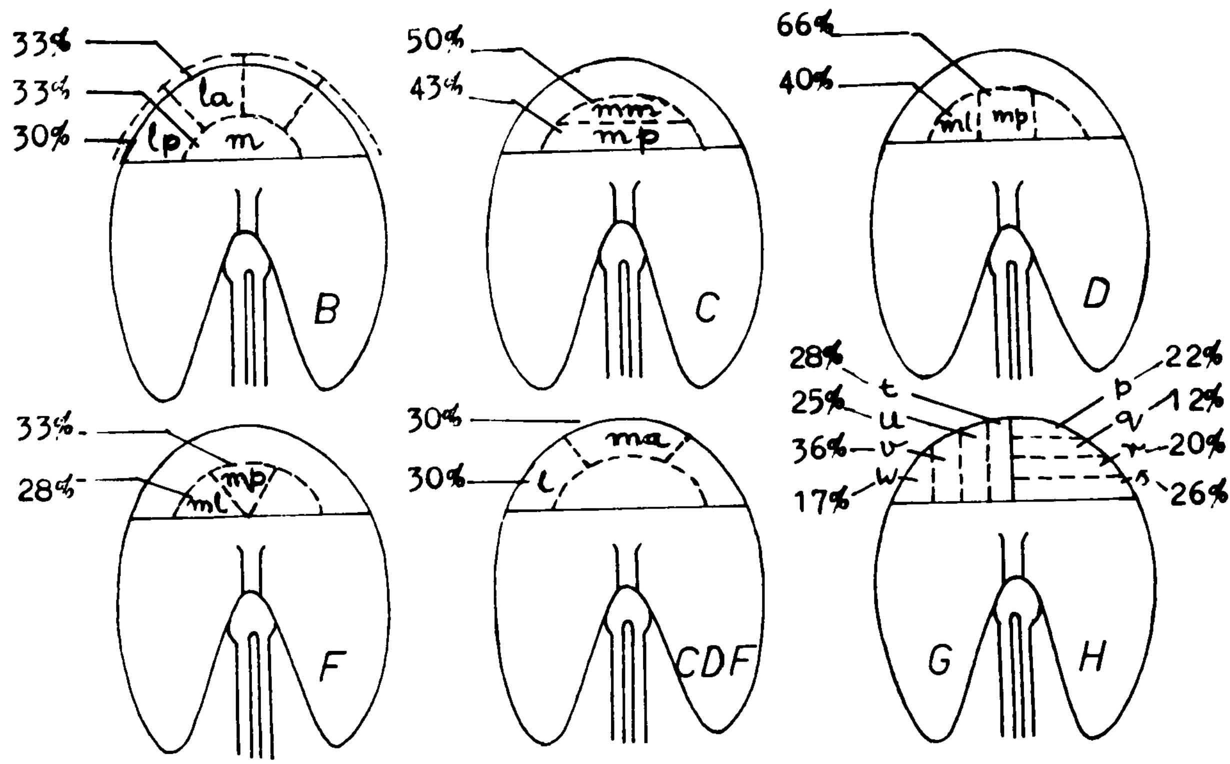
C,D,F (peripheral grafts) : The recovery of anterior median ma grafts was 6/20 and that of lateral l grafts was 11/36. All ma and 8 l grafts were neuralized. Structural elements of the telencephalic cortex were encountered in 2 ma grafts. The eye structures were absent inthese grafts.

Fig 5.2 : Graft areas and the recovery percentage of different types of grafts implanted at the median head process stage.

For abbreviationssee fig 5.1



Stage-5
Median head process stage.



B-H: operation plans

FIG. 5·2

Operation plan G : A decline in the percentage of recovery was noted here also in relation to size of the grafts. The recovery of grafts G t was 6/21, G u was 7/28, G v was 4/11 and G w was 1/6. All these grafts were neuralized. Sensory layer of the eye was present in 2 G t and 4 G u grafts whereas pigmented layer was found in 1 G t and 1 G v grafts.

Operation plan H : Operations according to this plan also showed a decline in recovery percentage which appear to be dependent on the size of the grafts. The recovery of the grafts H p was 2/9, H q was 2/17, H r was 5/25 and that of H s was 5/19. 1 H p, 1 H q and all H r and H s grafts were neuralized although they showed epithelial vesicles feather germs also. 1 H r graft showed sensory as well as pigmented layer of the eye.

(iii). Middle to late H and H stage 5 (long Head-Process stage)

The shape of the area pellucida does not show any marked change except slight increase in its boundaries. The length of the primitive streak measures 1.60 mm (SD: 0.33). The opaque area anterior to the node extends from about 0.75 mm to 0.92 mm. The length of head process ranges from 0.31 to 0.60 mm from the primitive pit. The breadth of the prospective neural area is somewhat reduced as reported by Spratt (1952). The following is the analysis of the grafts inplanted according to different operation plans at this stage.

<u>Operation plan B</u> : The recovery of median grafts B m was 4/19, anterior lateral grafts B la was 5/23 and posterior lateral grafts B lp was 7/25. All B m grafts, 2 B la and 4 B lp grafts were neuralized. Sensory layer was observed in 2 B m and 1 B lp grafts whereas pigmented layer was represented in 2 B m grafts. Telencephalic cortex was encountered in 1 B m and 1 Bla grafts.

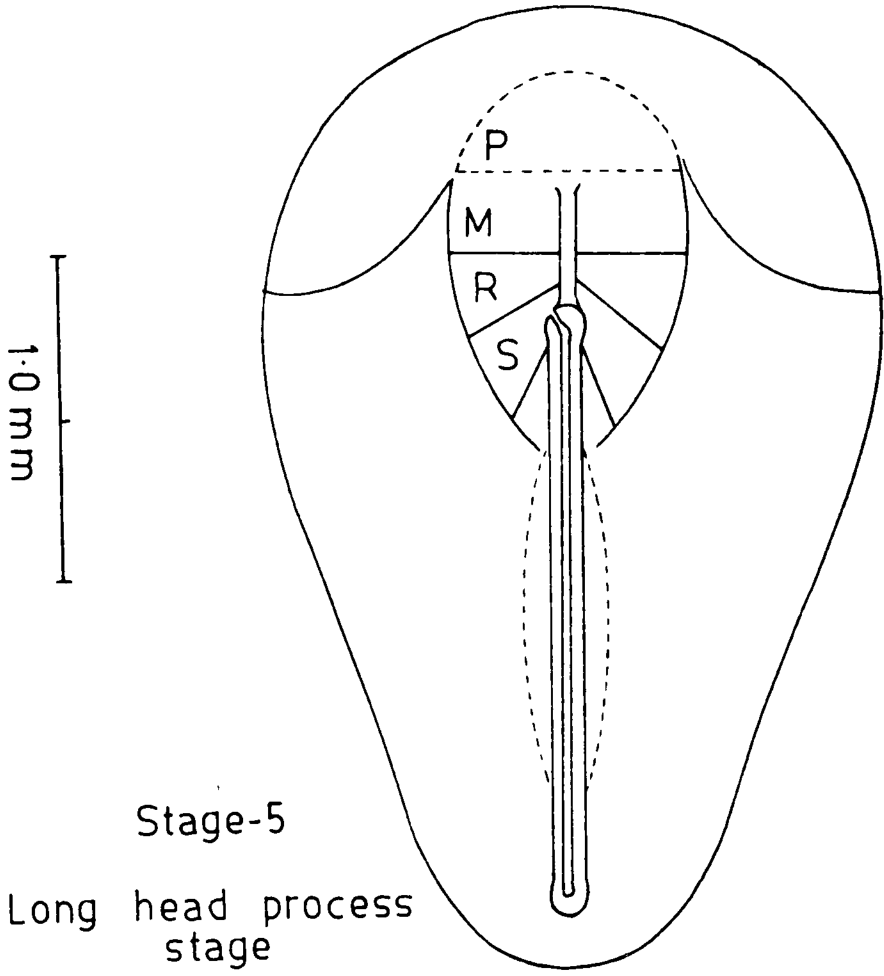
<u>Operation plan C (central grafts)</u> : The recovery of median C mm graft was 1/8 and posterior median C mp was 6/8. All C mm and C mp grafts were neuralized. Sensory layer was found in 2 C mp grafts but pigmented layer was found only in one of these. Telencephalic cortex was represented in 1 C mm graft.

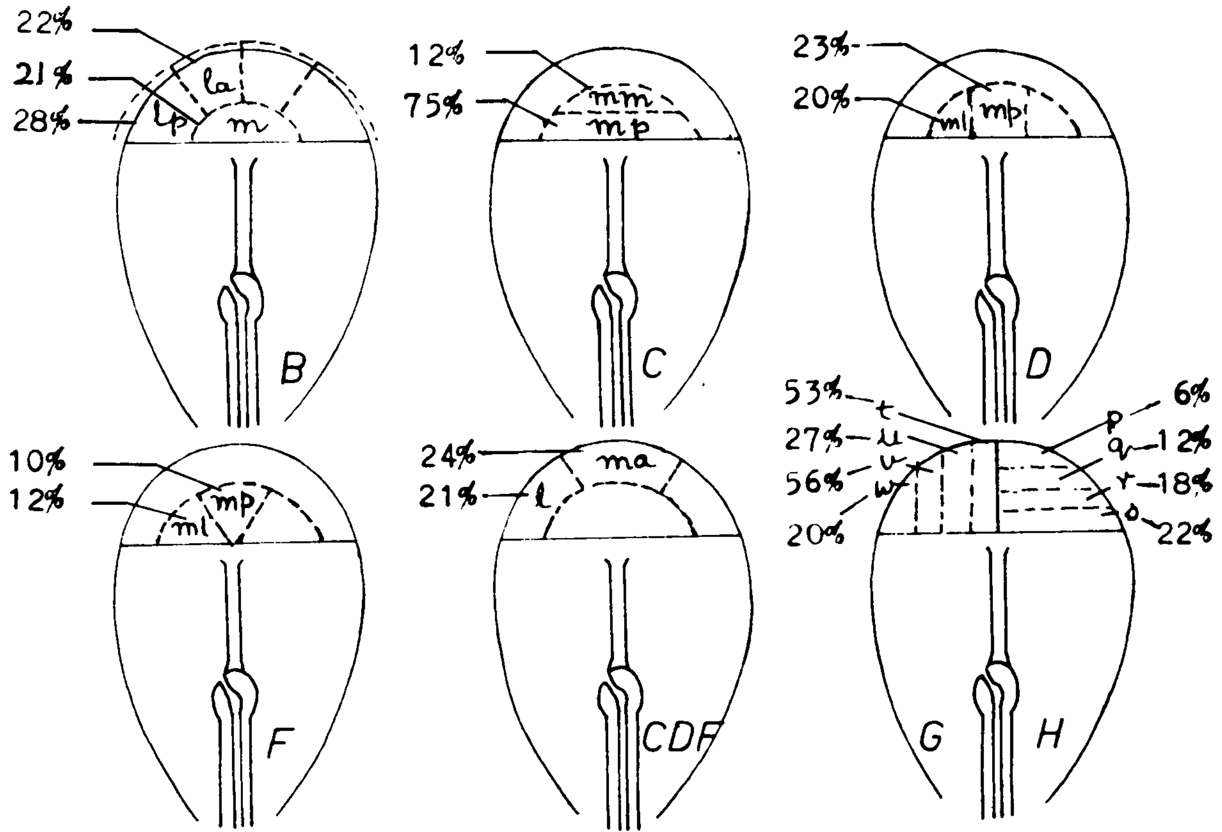
<u>Operation plan D (central grafts)</u>: The recovery of posterior median D mp grafts was 3/13 and median lateral D ml grafts was 4/20. All these grafts were neuralized. The sensory layer of the eye was observed in 1 D mp and 1 D ml grafts.

<u>Operation plan F (central grafts)</u> : The recovery of posterior median grafts F mp was 1/10 and median lateral grafts F ml was 3/25. All F mp and F ml grafts were neuralized but structural elements of the eye were not observed in them.

<u>C.D.F. (peripheral grafts)</u> : The combined analysis of anterior median ma and lateral l grafts of the plans C, D and F reveal that recovery of ma was 5/21 and that of

Fig. 5.3 : Graft areas and the recovery percentage of different types of grafts implanted at the long head process stage. For abbreviationssee fig 5.1.





B-H: operation plans *FIG. 5·3*

l was 11/51. All ma and l grafts were neuralized. Eye structures were absent in these grafts.

Operation plan G : There was a decline in the recovery rate in relation to the size of the grafts. The recovery of G t grafts was 10/19, G u was 7/26, G v was 5/9 and G w was 2/10. All the grafts were neuralized. Sensory layer of the eye was observed in 3 G t, 2 G u and 1 G v grafts. Telencephalic cortex was represented in 1 Gu graft only.

Operation plan H : The recovery of H p graft was 1/16, H q was 4/34, H r was 5/28 and H s was 6/27. Neuralization was observed in 2 H q, 5 H r and 4 H s grafts. Sensory layer of the eye was observed in 2 H r and telencephalic cortex was found only in one of these.

(II) Combined histological analysis of the grafts

On comparing the prosencephalic differentiation tendencies obtained in the 6 types of grafts cultured a composite picture of the differentiation pattern can be drawn. Based on the types of grafts implanted the analysis has been made in two groups. The grafts cultured according to the operation plans B, C, D and F have been analysed as one group and the grafts cultured according to the operation plans G and H have been analysed together.

Table- 5.4

Numbers of grafts out of the recovered grafts showing different types of structural elements.

Operation Plan	Donor's Head Process Length (mm)	Grafts Recovered	Structural element									
			OC	TC	CP	Ret	Tap	PB	PNM	FG	EP	CAR
	0.0	B m (7)	1	2	.	1	–	.	5	1	.	2
		B la(4)	.	2	1	2	1	1	2	1	.	.
		B lp(5)	.	.	.	2	2	.	2	1	.	.
B	0.01-0.30	B m (2)	.	1	.	.	.	.	2	1	.	.
		B la(4)	.	1	.	1	1	.	4	3	.	1
		B lp(6)	.	.	.	3	3	.	6	.	.	.
	0.31-0.60	B m (4)	.	1	.	2	2	.	4	1	1	2
		B la(5)	.	1	.	.	.	.	2	3	.	.
		B lp(7)	.	.	.	1	.	.	4	4	.	.

Number in parenthesis denotes the number of grafts recovered.

Abbreviations :
OC - Olfactory Complex
TC - Telencephalic Cortex.
CP - Choroid plexus
Ret- Retina
Tap- Tapetum
PB - Pineal Body
PNM- Prosencephalic Neural Mass
FG - Feather germ
EV - Epithelial vesicle
CAR- Cartilage.

Fig. 5.4 : Histograms representing percentage of various structural elements differentiated in the neuralized grafts of the plan B (graft areas shown by oblique hatching)

Abbreviations :

OC : Olfactory complex
TC : Telencephalic cortex
CP : Choroid plexus
Ret: Retina
Tap: Tapetum
PB : Pineal body
PNM: Prosencephalic neural mass
Ev/FG: Epithelial vesicle/feather germ,
PS : Primitive Streak HP: Head Process

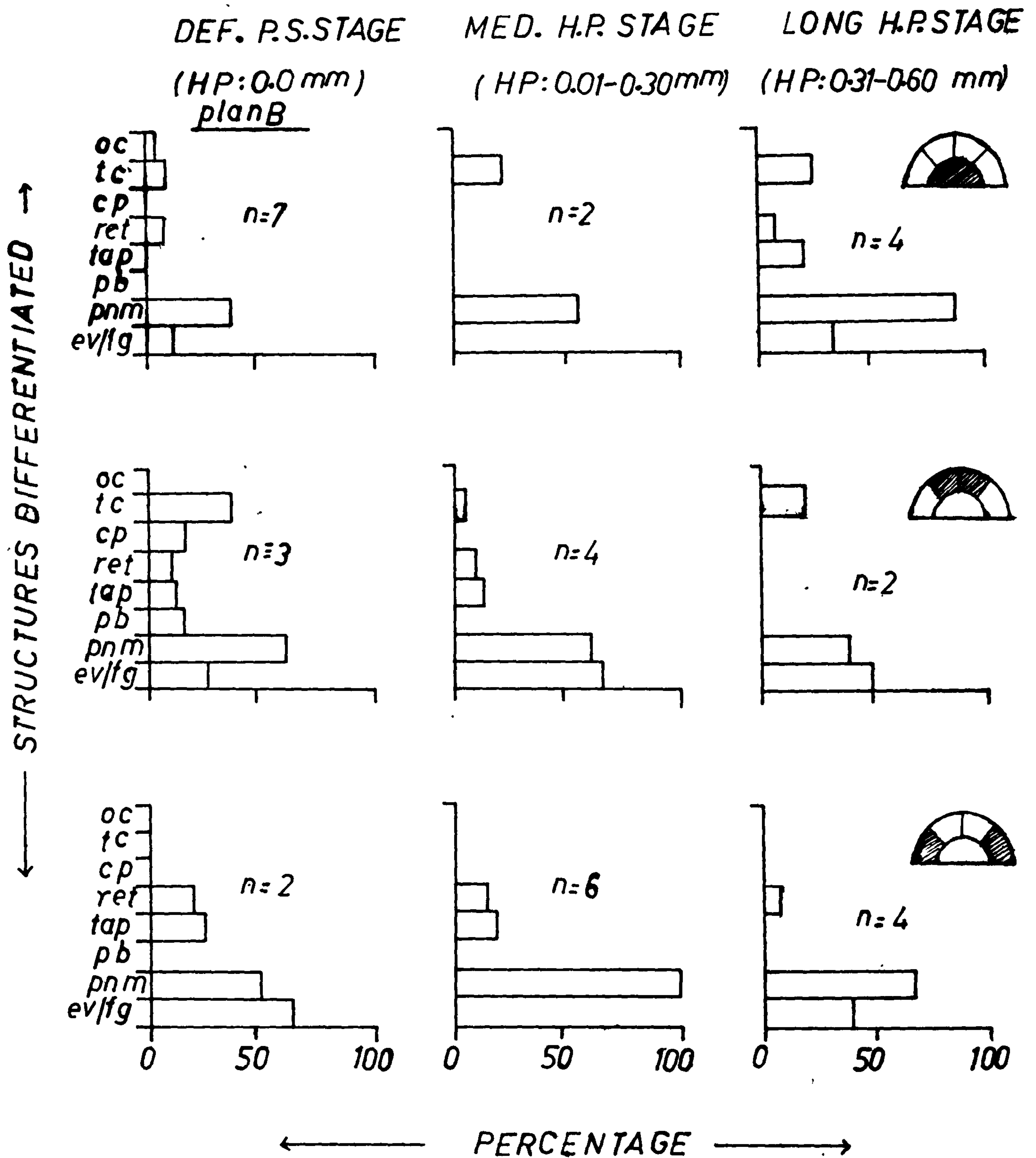


FIG. 5.4

Table 5.5.

Numbers of grafts out of grafts recovered showing different types of structural elements.

Operation Plan	Donor's Head Process length(mm)	Grafts recovered	Structural elements									
			OC	TC	CP	Ret	Tap	PB	PNM	FG	EP	CAR
C (Central grafts)	0.0	C mm (2)	.	.	.	.	.	.	2	1	.	.
		C mp (5)	.	.	.	.	.	.	5	1	.	1
	0.01-0.30	C mm (4)	.	.	.	1	1	.	3	.	.	1
		C mp (3)	.	.	.	1	.	.	2	.	.	1
	0.31-0.60	C mm (1)	.	1	.	.	.	.	1	.	.	.
		C mp (6)	.	.	.	2	1	.	6	1	.	.
D (Central grafts)	0.0	D mp (5)	.	.	.	2	.	.	5	1	.	.
		D ml (6)	.	.	.	.	.	.	4	1	1	1
	0.01 0.30	D mp (2)	.	.	.	1	.	.	2	.	.	.
		D ml (2)	.	.	.	.	.	.	2	.	.	..
	0.31-0.60	D mp (3)	.	.	.	1	.	1	3	2	.	.
		D ml (4)	.	.	.	1	.	1	4	.	.	.
F (Central Grafts)	0.0	F mp (3)	.	.	.	.	.	.	2	1	.	.
		F ml (5)	.	.	.	1	1	1	3	2	.	1
	0.01 0.30	F mp (2)	.	.	.	.	.	.	2	.	.	.
		F ml (6)	.	.	.	.	.	.	6	2	1	.
	0.31 0.60	F mp (1)	.	.	.	.	.	.	1	.	.	.
		F ml (3)	.	.	.	.	.	.	3	1	.	.
C,D,F (peripheral Grafts)	0.0	m a (8)	.	.	.	.	.	1	6	2	.	.
		l (13)	.	.	.	2	2	1	13	2	.	1
	0.01 0.30	m a (6)	.	2	.	.	.	.	4	1	1	.
		l (11)	.	.	.	.	.	.	8	5	.	.
	0.31 0.60	m a (5)	.	2	.	.	.	.	5	2	.	.
		l (11)	.	.	.	.	.	.	11	3	.	.

For numbers in parenthesis and Abbreviations see table 5.4.

Fig. 5.5. Histograms representing percentage of various histological elements differentiated in the neuralized grafts of the Central area of the plan C and D. (graft areas shown by oblique hatching).

For Abbreviations: see Fig 5.4.

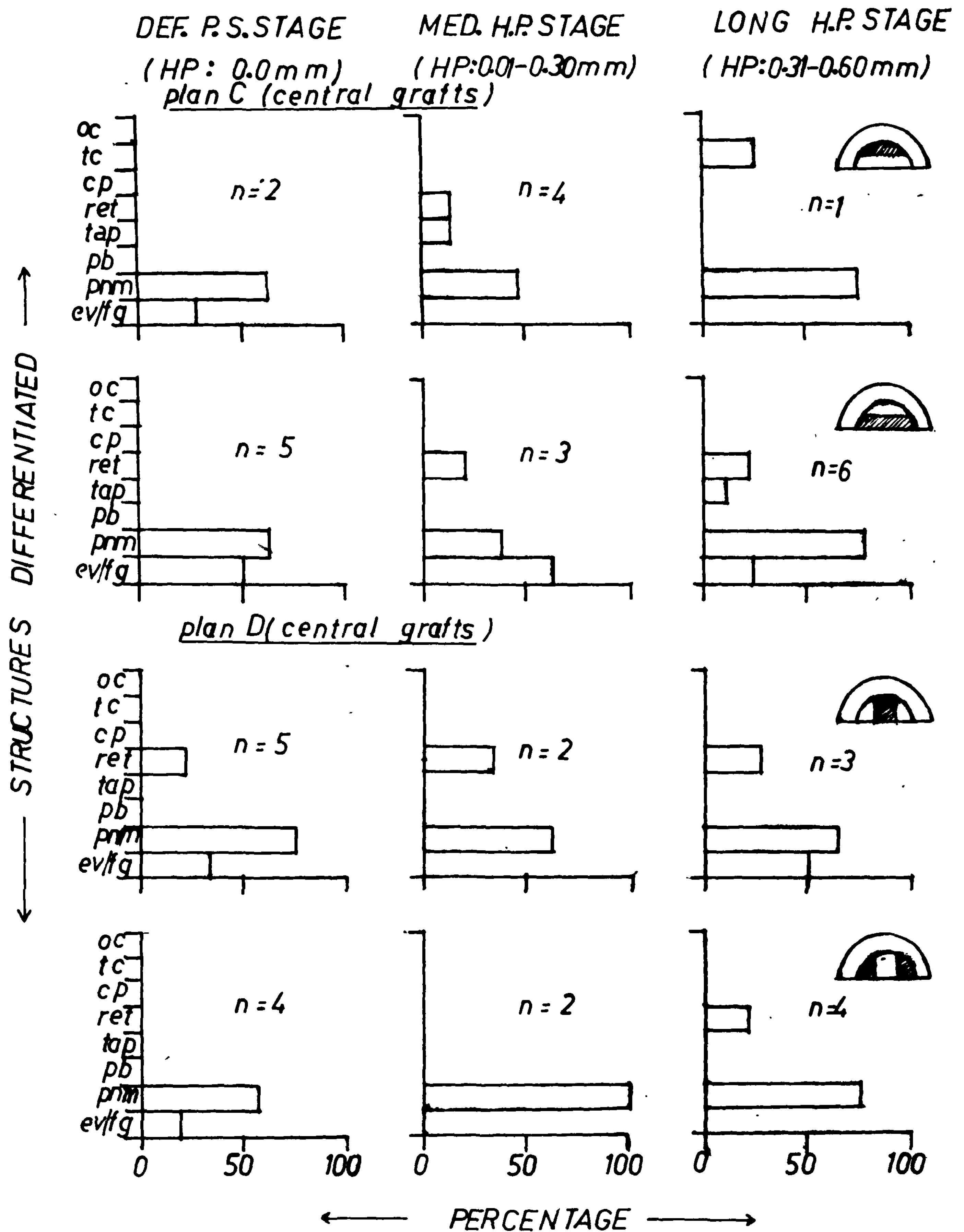


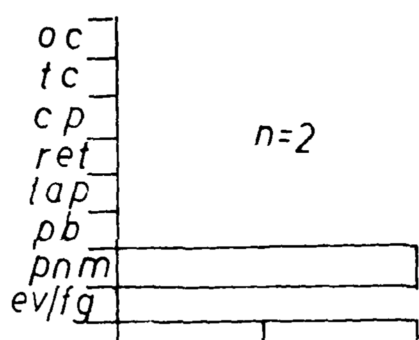
FIG. 5.5

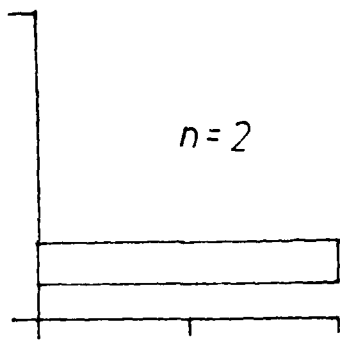
Fig. 5.6 Histograms representing percentage of various histological elements differentiated in the neuralized grafts of the central area of the plan F and those of the peripheral area of the plans C, D and F. (graft areas shown by oblique hatching)

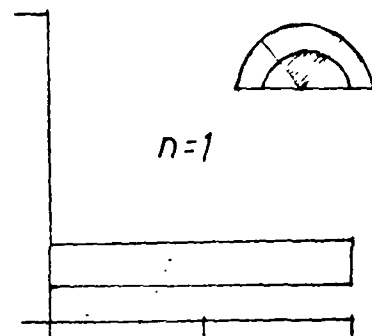
For abbreviationssee Fig,. 5.4.

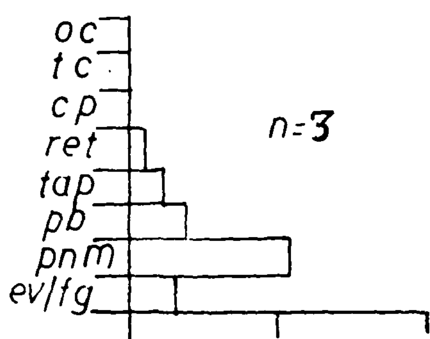
DEF. P.S. STAGE (HP: 0.0 mm) | MED. H.P. STAGE (HP: 0.01-0.30 mm) | LONG H.P. STAGE (HP: 0.31-0.60 mm)

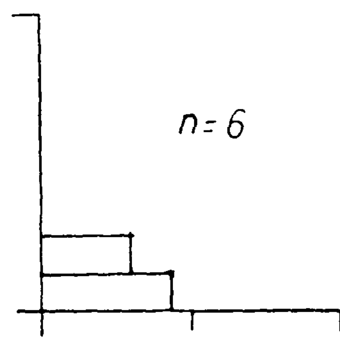
plan F (central grafts)

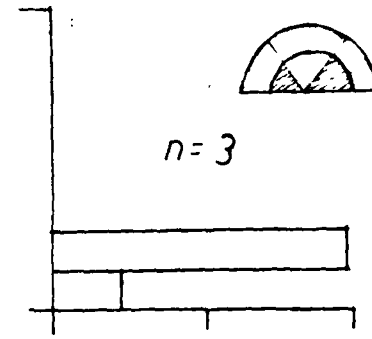




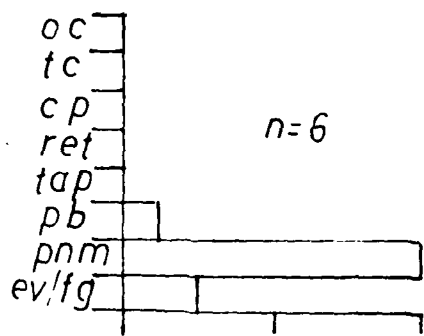


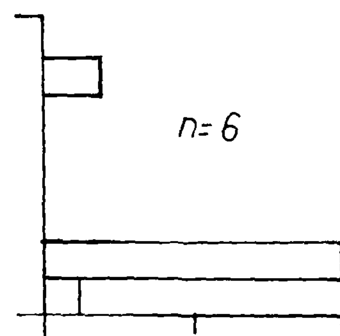


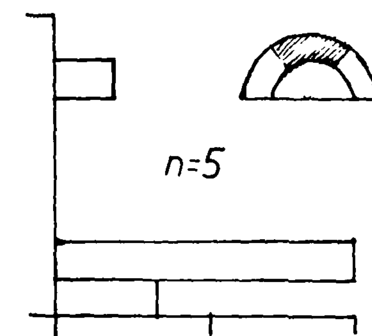


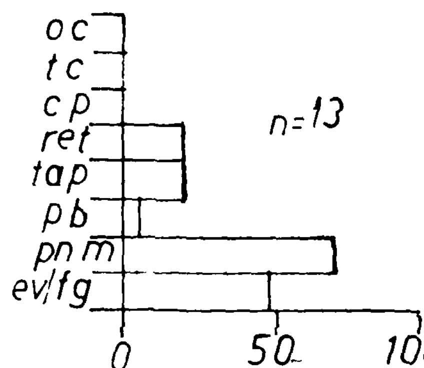


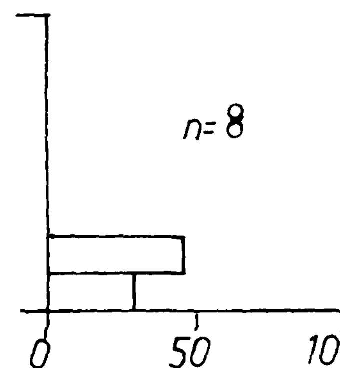
plans C, D, F (peripheral grafts)

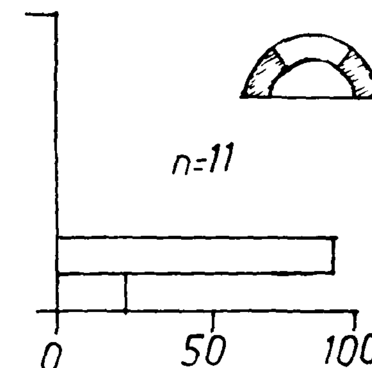












← STRUCTURES DIFFERENTIATED →

← PERCENTAGE →

FIG. 5·6

Table - 5.6

Numbers of grafts out of recovered grafts showing different types of structural elements.

Operation Plan	Donor's Head Process length (mm)	grafts recovered	Structural elements									
			OC	TC	CP	Ret	Tap	PB	PNM	FG	EP	CAR
G	0.0	G t (12)	.	1	.	4	1	.	9	4	.	1
		G u (11)	1	2	.	3	2	.	10	2	1	1
		G v (6)	.	.	.	2	.	.	5	3	.	1
		G w (4)	.	.	.	.	.	.	2	2	.	.
	0.01-0.30	G t (6)	.	.	.	2	1	.	5	3	1	3
		G u (7)	.	.	.	4	.	.	4	2	.	2
		G v (4)	.	.	.	.	1	.	3	3	1	3
		G w (1)	.	.	.	.	.	.	1	1	.	.
	0.31-0.60	G t (10)	.	.	.	3	2	1	8	3	.	2
		G u (7)	.	1	.	2	1	1	5	1	.	3
		G v (5)	.	.	.	1	.	.	3	3	1	1
		G w (2)	.	.	.	.	.	.	1	1	.	.

For numbers in parenthesis and abbreviations see table 5.4.

Fig. 5.7 : Histograms representing percentage of various histological elements differentiated in the neuralized grafts of the plan G (graft areas shown by oblique hatching)

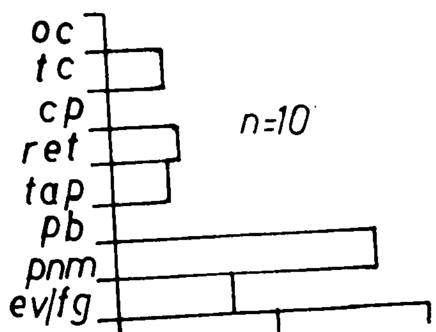
For abbreviations see Fig. 5.4

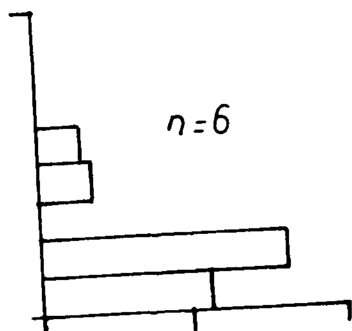
DEF. P.S. STAGE (HP: 0.0 mm) plan G

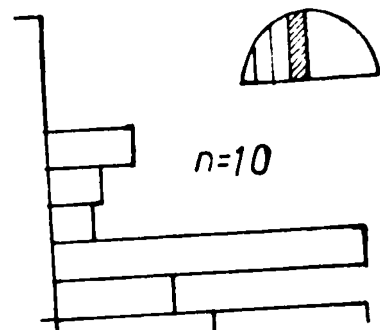
MED. H.P. STAGE (HP: 0.01-0.30 mm)

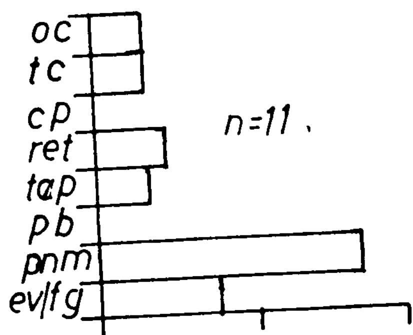
LONG H.P. STAGE (HP 0.30-0.60 mm)

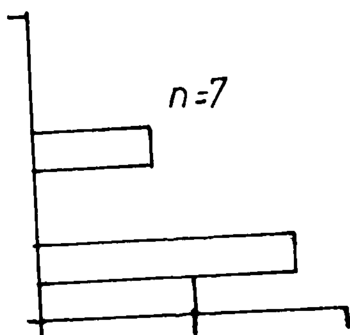
DIFFERENTIATED STRUCTURES

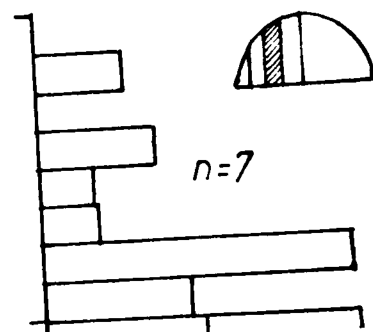


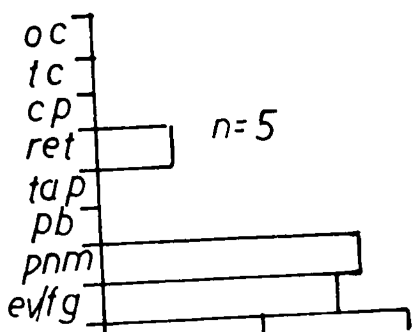


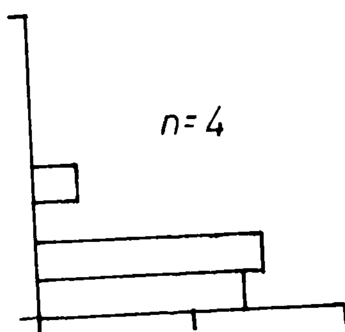


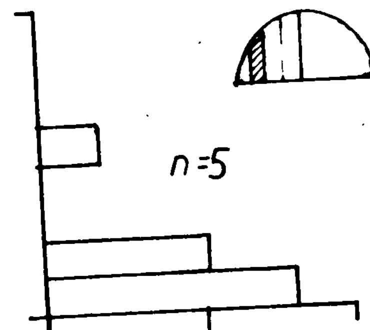


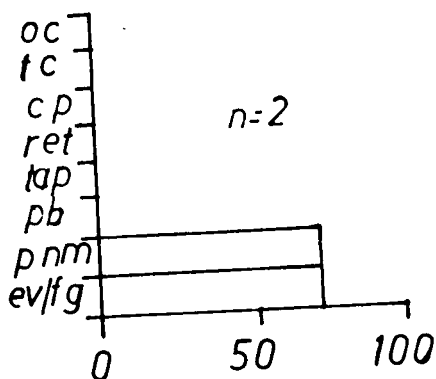


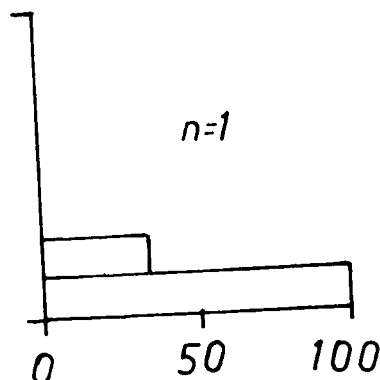


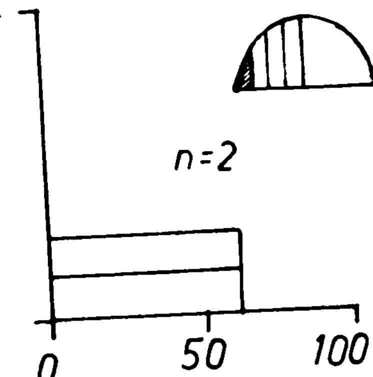












PERCENTAGE

FIG. 5·7

Table 5.7

Number of grafts out of recovered grafts showing different types of structural elements.

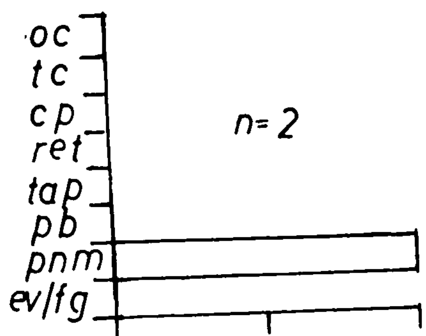
Operation Plan	Donor's Head Process Length (mm)	grafts recovered	Structural elements										
			OC	TC	CP	Ret	Tap	PB	PNM	FG	EP	CAR	DY
H	0.0	H P (6)	.	.	.	.	.	.	2	4	.	.	.
		H q (8)	.	.	.	.	.	.	2	.	.	.	.
		H r (11)	.	.	.	5	3	1	10	5	1	3	.
		H s (13)	.	1	.	3	1	2	12	6	1	2	.
	0.01-0.30	H p (2)	.	.	.	.	.	.	1	1	.	.	.
		H q (2)	.	.	.	.	.	.	1	1	.	.	.
		H r (5)	.	.	1	1	1	.	5	3	1	2	.
		H s (5)	.	.	.	.	.	.	5	2	1	.	.
	0.31-0.60	H p (1)	.	.	.	.	.	.	.	1	.	.	.
		H q (4)	.	.	.	.	.	.	2	2	.	.	.
		H r (5)	.	1	.	2	.	.	4	1	.	1	.
		H s (6)	.	.	.	.	.	.	4	1	.	1	.

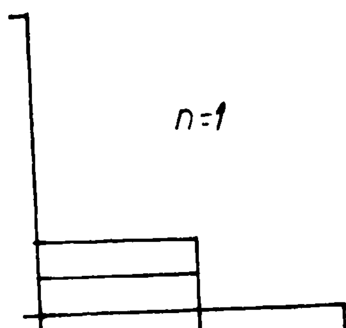
For numbers in parenthesis and abbreviations see table 5.4.

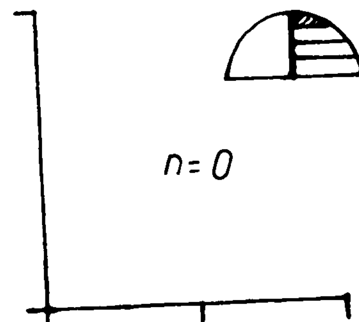
Fig. 5.8 : Histograms representing percentage of various histological elements differentiated in the neuralized grafts of the plan H (graft areas shown by oblique hatching)

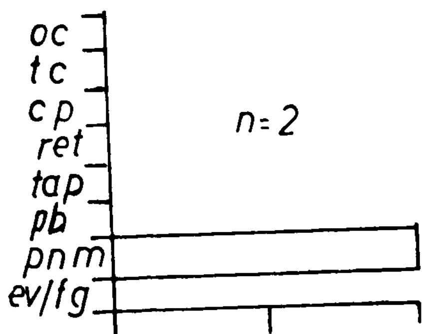
For abbreviationssee Fig. 5.4

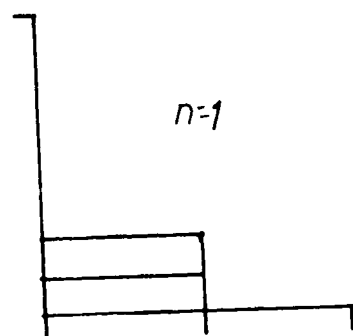
DEF. P.S. STAGE (HP: 0·0mm) plan H | MED: H.P. STAGE (HP: 0·01–0·30 mm) | LONG H.P. STAGE (HP. 0·31–0·60 mm)

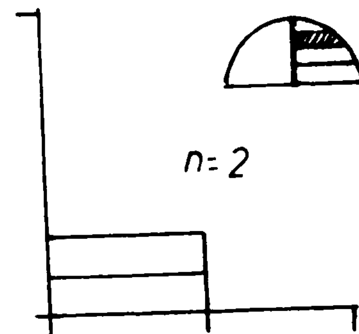


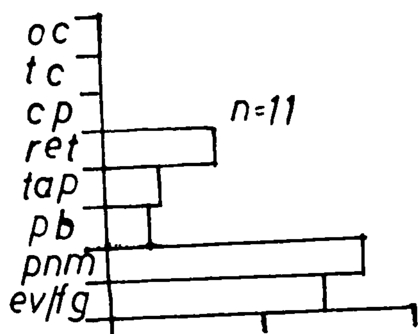


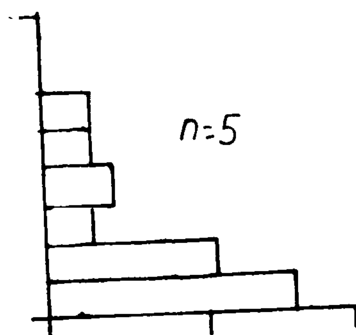


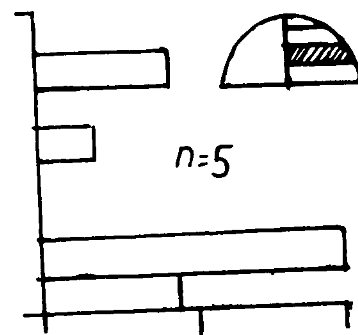


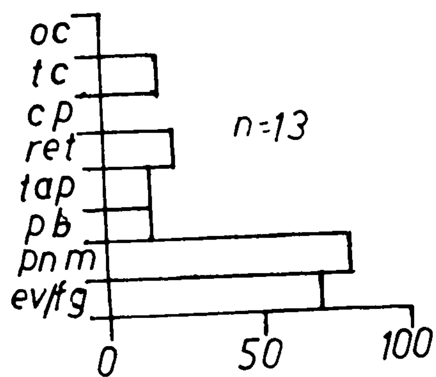


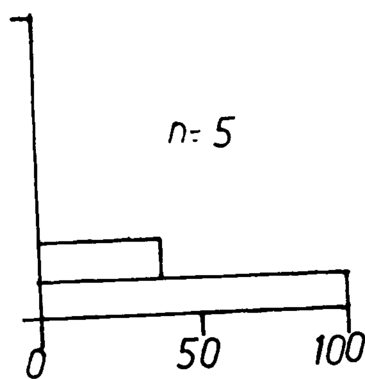


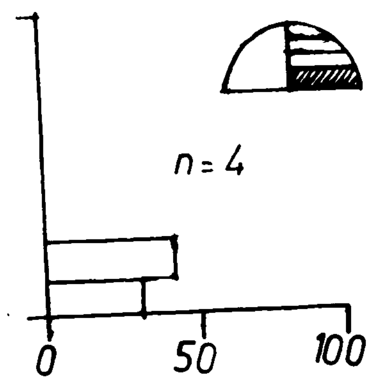












← STRUCTURES DIFFERENTIATED →

← PERCENTAGE →

FIG. 5·8

(a) Analysis of the grafts cultured according to the operation plans B,C,D and F (FIGS. 5.4, 5.5, 5.6)

The structural elements of the prosencephalon differentiated in these grafts at the H and H stage 4 (definitive primitive streak stage) show a mixed up differentiation pattern of the neural differentiation tendencies. The central as well as peripheral grafts of the operation plan B show differentiation of all prosencephalic structures, whereas these grafts implanted according to the operation plans C,D and F show that all these differentiation tendencies are more localized in the central area. The central area from where these grafts were isolated was larger in size than that of the plan B. The eye structures (retina as well as tapetum) were, however, found to be localized to the grafts of the central area in the plans C, D and F. Some segregation of the localization of prosencephalic differentiation tendencies has been observed at the H and H stage 5 when the underlying prechordal mesoderm spreads and the head process grows in length. The analysis of the grafts implanted according to the plan B revealed that the eye structures were mostly localized to the central area although the percentage of neuralization was high in the peripheral grafts. The telencephalic differentiation tendencies were localized to anterior median peripheral (B 1a) grafts and diencephalic differentiation tendencies

(represented by massive areas of homogeneous nuclei, termed as prosencephalic neural mass) were localized to central (B m) and posterior lateral peripheral (B lp) grafts. The eye structures (retina as well as tapetum) were found to be localized to the diencephalic areas. Structural elements representing olfactory complex were found only in 1 Bm graft at H and H stage 4, and few anterior peripheral (ma) grafts at early H and H stage 5. The formation of olfactory complex has been discussed elsewhere (see Discussion).

The grafts cultured according to the operation plans C, D and F at H and H stage 5 confirm these results showing the localization of diencephalic structures, retina and tapetum to the posterior part of the prosepective prosencephalic area, the eye structures being localized to the grafts of the central region only. The telencephalic cortex was, however, observed in very few anterior peripheral (ma) grafts of this plan.

(ii) <u>Analysis of the grafts cultured according to the operation plans G and H</u> (FIGS. 5.7, 5.8)

Although some pilot experiments indicated that the recovery percentage of the grafts implanted according to the operation plans G and H would be better, it was ultimately not found very encouraging. However, the analysis of the grafts recovered revealed a somewhat more clear picture of the prosencephalic differentiation tendencies as compared to the results of the other plans.

At H and H stage 4, the grafts G t, G u and G v cultured according to the plan G show differentiation of all prosencephalic structures, (telencephalic as well as diencephalic) although these tendencies appeared to be more localized in the areas of the grafts G t and G u. The frequency of differentiation of eye structures (retina as well as tapetum) was also observed in larger number of these grafts. At H and H stage 5, again there was better differentiation of the prosencephalic structures in the grafts G t and G u. Telencephalic cortex appeared together with pineal body in G u grafts. Structural elements of retina as well as tapetum were mainly localized to G t and G u grafts.

The operations performed according to the plan H revealed following pattern of differentiation. At H and H stage 4 the prosencephalic differentiation tendencies were found to be more localized in the graft areas H r and H s in the posterior part of the prospective prosencephalic area. The eye structures were well differentiated in the grafts of both these regions, the tapetum occuring mostly in the H r grafts. During H and H stages 5 these differentiation tendencies of the eye structures were found to have become localized mainly in the area of the grafts H r.

On superimposition of the results of analysis of the grafts of the 2 plans, G and H, it appears that at H and H stage 4 the eye potency field is localized

0.2 mm anterior to the primitive pit in an area measuring about 0.4 x 0.2 mm in the central median region of the prospective ectoderm in the area common to that of grafts G t, G u and G v of the plan G and H r and H s of the plan H. At H and H stage 5 these differentiation tendencies become localized in an area common to that of G t and G u of the plan G and H r of the plan H measuring about 0.4 x 0.1 mm stretched transversely in the prospective prosencephalic region about 0.1 mm anterior to the long head process. The telencephalic structures were clearly observed in G t and G u grafts and diencephalic mostly in the H r and H s grafts although G v, G w and H p and H q grafts also showed neuralization except in 1 case.

(d) DIFFERENTIATION PATTERN

The differentiation of neural structures in the grafts isolated at the primitive streak stage does not show any clear pattern of morphogenetic potencies of the prosencephalic structures. The telencephalic cortex as well as prosencephalic neural mass differentiate nicely in the grafts of the central area of the prospective prosencephalic ectoderm according to operation plans B, C, D and F. The graft area of the central region of plan F is smaller and so some of the prosencephalic structures encountered in the grafts of central area of the plans C, D and F seem to have differentiated in the grafts of the peripheral region according to this plan. The structures

developed in the grafts taken according to the operation plans G and H also reveal a similar pattern. The telencephalic cortex, prosencephalic neural mass and eye structures are more localized in the medial grafts of the operation Plan G and posterior grafts of the operation plan H.

At the head process stage the differentiation pattern of these structures is better defined. The telencephalic structures appear more in the grafts of anterior peripheral region and prosencephalic neural mass including eye structures in the more posterior region , of the prospective prosencephalic area. The eye potency field becomes localized in a transverse area measuring ca. 0.4 x 0.1 mm about 0.1 mm anterior to the head process. This pattern of differentiation agrees with the morphology of adult prosencephalon which has telencephalic region anteriorly and diencephalic region posteriorly. As the differentiation of the prosencephalic structures is more localized in the central area at the primitive streak stage and extends peripherally at the head process stage it seems that the activation and then transformation of the prospective prosencephalic ectoderm extends centrifugally under the inductive influence of underlying prechordal mesoderm which leads to the differentiation of ventro-dorsal pattern of the prosencephalon. This is also in accordance with the morphogenesis of the neural tube in which the medial

region of the neural plate becomes ventral part of future neural tube, and the lateral regions of the neural plate become lateral and dorsal parts of the neural tube at the time when the edges of the neural plate close dorsally to form the neural tube.

(c) CARTILAGE FORMATION

The cartilage formation was observed in some grafts, rarely in association with respiratory epithelium as olfactory complex. This may be due to secondary induction of the neural crest cells by host or graft tissue. (See Discussion).

Chapter 6

DISCUSSION

(a) Coelomic Environment, Recovery of grafts and Neuralization.

Gallera (1971) reports that chick embryos explanted at the primitive streak stage never develop beyond Hamburger and Hamilton (1951) stage 18 (age ca. 65-69 hrs). Wolff and Simon (1955) and Simon (1956) followed a method which allowed establishment of blood circulation in explanted blastoderms extending the duration of culture for another 2 to 3 or more days. The chorioallantoic grafting technique (Willier and Rawles 1931) does not allow differentiation of grafts for more than 10 days and there is always a chance of smaller grafts to slip away from the chorio-allantoic membrane. The intra-coelomic grafting technique of Hamburger (1938) improved and perfected by Hara (1961, 1970, 1971) allows development of grafts up to 12 days, when the differentiation of histological structures reaches a stage sufficiently advanced for proper analysis of histological differentiation. The longer intra-coelomic culturing is also possible but the large size of graft may impair the growth of the vital host organs such as heart and liver resulting in the mortality of the host. Although this technique has been followed by several workers (Rao, 1968; Viswanath and Mulherkar, 1972; Khare, 1972; Veini and Hara, 1975;), questions have been raised on the neutrality of the coelomic environment (see Gallera, 1971). Hara (1961) and Rao (1968) have

discussed this question in great detail. Hara (1961) found that competent ectoderm which has not been in contact with the mesoderm does not show any neural differentiation in the coelomic environment. Similarly, Rao (1968) found that antero-median and lateral grafts from early stages did not show any neural differentiation. In the present investigation also, the grafts expected to be neuralized were mostly neuralized. However, at the primitive streak stage the frequency of neuralization was less in the peripheral, particularly in the lateral grafts. The somatopleure of the host may influence the differentiation of underinduced ectodermal fragments, or the grafts which were expected to be completely neuralized at the moment of isolation differentiated partly or entirely into epidermal structures. He discussed that it was difficult to explain such cases; but the overall analysis of grafts by all workers who followed this technique does not show much deviation and provides a better picture of differentiation tendencies in comparison to other techniques.

It is evident from the experiments of Hara (1961) and subsequently that of Rao (1968) that the recovery of neuralized grafts is always better than that of the non neuralized grafts. Further, the recovery rates of lateral grafts were always found to be lower than those of the median grafts. Among recovery rate of the median grafts there was a decrease in postero-anterior direction. The recovery of grafts from

prospective prosencephalic area was always lower than mesencephalic area, and it was distinctly lower in the earlier stages (H & H stage 4 and 4^{+}) than at later stages. Neuralization of these grafts was also observed in the same order.

Size of the grafts seems to be an important factor for recovery of grafts. In the present investigation the recovery of graft pieces implanted smaller than 0.2 x 0.2 mm was very discouraging and this was the reason to abondon the experiments according to Plan A and E mentioned elsewhere. Earlier Holtfreter (1936) reported that very small grafts do not differentiate; and Tiedemann (1975) also mentioned that very small pieces differentiate badly or not at all.

(b). <u>Induction of Neural Plate</u>.

Work on the neural induction in the chick embryo has been reviewed beautifully by Gallera (1971) and Hara (1978). The prechordal and chordal mesoderm induces the overlying competent ectoderm to become Neural Plate, which differentiates into the nervous system. Morphological changes are not apparent, however, until several hours have elapsed, Holtzer (1968) interpreted that such morphological changes cannot take place until new and specialized molecules have formed within the cells. He called these as 'luxury molecules' in contrast to the 'ordinary' molecules which are meant only for the

maintenance of cell activity. The luxury molecules are highly specialized and occur in certain tissues at the time of differentiation, although nothing is properly understood about such mechanisms. Neuralizing and vegetalizing factors have been isolated and purified upto a certain stage (Tiedemann, 1976 and 1978). While on one hand investigators are concentrating to understand these molecular mechanisms, experimental morphologists have been attempting to understand the process of neural induction and pattern formation under the influence of inducing materials.

Spratt's (1952) exhaustive work on fate maps demarcating the prospective neural plate at the primitive streak and head process stages of the chick embryo have been of immense help to workers for the last 2 1/2 decades. These fate maps are universally accepted and followed by workers for various types of experiments on neural Induction. When Nieuwkoop in early 1950s (unpublished) isolated pieces of ectoderm anterior to Hensen's node at primitive streak stage and cultured them intra - coelomically for 10 days, they did not show neuralization but when isolated at later stages from the ectoderm overlying the head process, they did give rise to neural tissue. Hara (1961) extended this work and prepared open Sandwiches, comparable to that of Sala (1955) in Amphibia by combining neutral and competent ectoderm of the primitive streak stage with prechordal and chordal

mesoderm. He cultured these sandwiches intra-coelomically; and on the basis of histological structures differentiated in the recovered grafts concluded that prechordal mesoderm induces prosencephalic structures, anterior head process mesoderm induces mesencephalic and rhombencephalic structures, and posterior head process mesoderm induces rhombencephalic and spinal cord structures. Rao (1968) further continued the work and investigated the appearance and extension of neural differentiation tendencies in the neurectoderm by interrupting the process of neural induction in the primitive streak and head process stages, and culturing the pieces of prospective neurectoderm intra-coelomically. Based on the histological analysis of the structures differentiated in the grafts he concluded a cranio-caudal sequence of induction and discussed that this sequence upholds Nieuwkoop's (1952) Activation-transformation hypothesis. In the present investigation, differentiation tendencies of much smaller grafts of the prospective prosencephalic ectoderm have been studied. The experimental results reveal a centrifugal spreading of neural differentiation tendencies in the prospective prosencephalic ectoderm from the primitive streak to head-process stages.

(c). Prosencephalic differentiation tendencies

The prosencephalic differentiation tendencies have been studied by a number of workers (Wetzel, 1929; Stein, 1933; Rawles, 1936; Spratt, 1952; Hara, 1961 and Rao, 1968). Wetzel (1929) with the help of *in vitro*

technique demonstrated that prospective forebrain region lies anterior to Hensen's node and extends 0.4 - 0.5 mm antero-posteriorly. By chorio-allantoic grafting technique Stein (1933) showed that the prospective forebrain area lies anterior to Hensen's node at the definitive streak stage ,anterior to head process at the head process stage. She mentioned that some determination of forebrain tissue occurs from primitive streak to the head process stage, she also found eye structures in these tissues but did not mention the posterior demarcation of forebrain area. Rawles (1936) found that area of the blastoderm having anterior part of the head process gave rise to tissues mainly of the forebrain whereas the area of the blastoderm just posterior to it having head process and part of Hensen's node gave rise to the hind brain tissues. She also found eye structures with forebrain tissues. With the work of Spratt (1952) demarcating clearly the areas of prosencephalon, mesencephalon and rhombencephalon subsequent workers could perform experiments more precisely than earlier workers to test the morphogenetic potencies and differentiation tendencies of various parts of prospective neural area. Hara (1961) demonstrated that prechordal mesoderm induces the appearance of differentiation tendencies of prosencephalic structures in the competent ectoderm and Rao (1968) showed that prosencephalic differentiation tendencies appear in the prospective neural ectoderm at the primitive streak and extend at the head process stages. Rao tested the differentiation

tendencies of the prospective prosencephalic and mesencephalic ectoderm with the help of graft pieces measuring 0.17 x 0.48 mm, 0.14 x 0.44 mm, 0.17 x 0.39 mm and 0.17 x 0.34 mm. He prepared grafts from the median as well as lateral regions of the prospective neural plate. Median grafts included a large part about three-fourths of the width of the prospective neural area. The lateral grafts included very small neural area. As a result of histological analysis of the grafts he found that the relative number of grafts forming neural structures were lower in the lateral and anterior most median grafts than in the posterior median areas.These tendencies increased from 0% in antero-lateral areas of stage I and II(early and definitive primitive streak stage) to 100% in the postero-median areas from Stage III (early head process) onwards. He found some prosencephalic differentiation of median grafts in the prospective mesencephalic and sometimes in rhombencephalic area also. He found eye structures both in the anterior as well as posterior median areas of prospective prosencephalic region in all stages of experimentation from the primitive streak to the head process stage. In the present study the differentiation tendencies of much smaller grafts (size 0.2 x0.2 mm to 0.1 x 0.4mm) isolated from prospective prosencephalic eectoderm at these stages suggest a mixed up pattern of prosencephalic differentiation tendencies at the primitive streak stage with high neuralization tendency in the

grafts of its central area. These differentiation tendencies are elaborated at the head-process stages, while the frequency of formation of the structural elements of the telencephalic cortex was found to be high in the grafts of the anterior region of the prospective prosencephalic ectoderm and that of diencephalic and eye structures were high in the grafts of its posterior region.

(d) Morphogenetic tendencies of the Eye Potency field.

The organ forming potency maps of Willier and Rawles (Rawles, 1936) show the localization of forebrain and eye potency field just anterior to the head process at the transverse level across the blastoderm. They have shown forebrain to arise from the anterior levels of this strip and eye from the posterior levels of this strip but they have not shown exact localization of these parts. Rudnick (1944) has elaborated the organ forming potency map at the definitive primitive streak stage. She has given two maps (1) One, based on the work of Hunt (1932) and supported by the description available from the work of Murray (1932), Dalton (1935) and Rawles (1936) and (2) the other, based mainly on the work of Pasteels (1935; 1936; 1937) with suggestions from Wolff (1936) and Yntema (1942) and embellishments from her own work.

These area maps provide a better picture of prospective prosencephalic area as compared to the

prospective area maps given by Rawles (1936) and show a clear boundary of future neural structures and localization of morphogenetic potencies of eye and certain other structures. The future forebrain and the eye areas are very clearly demarcated. However the demarcation of forebrain area has not been exactly supported by the carbon particle marking method of Spratt (1952). Certain workers have attempted to investigate precisely the localization and differentiation of eye potency fields in early chick embryo. Earliest description appears to be that of Stein (1933) who used chorio-allantoic grafting technique and investigated the localization and differentiation of prospective forebrain and hypophyseal ectoderm at the primitive streak and head process stages. She reported that material at the level anterior to Hensen's node at the primitive streak and head process stages invariably formed forebrain tissue including eye structures (retina as well as tapetum). Rawles (1936) tested a transverse strip of blastoderm 0.27 mm to 0.54 mm in width lying 0.26 mm to 0.4 mm anterior to the pit and divided this strip into a median (0.1-0-18 mm on each side of the pit) and two lateral pieces. Among the recovered grafts, (18 out of 33 median, 14 out of 32 left lateral and 10 out of 12 right lateral grafts) she found eye structures along with brain tissue in 83% of the median grafts showing mostly sensory layer. In one case she found lens also. In lateral grafts the pigmented layer was found in 7 out of 10 right and 4 out

14 left grafts although there was large amount of differentiated structures in these. She reported that degree of differentiation was unquestionably superior on the left side. With the help of same chorio-allantoic technique Clarke (1936) attempted to study the regional differences in eye forming capacity of early chick blastoderm ranging from late streak to 12 somite stages. He analysed that at the late streak stage the eye forming area extended 0.06 mm anterior and 0.02 mm posterior to the pit and 0.2 mm laterally on each side. At head process stage it extends 0.05 mm anteriorly 0.1 mm posteriorly and 0.2 mm bilaterally from the anterior end of the head process. He found that the eye forming capacity increased progressively in the lateral regions upto 12 somite stage. The median region retains eye forming capacity until 8 somite stage. As Rawles (1936), he also analysed that the differentiation in the left side was better than the right side. In her reconstructed organ forming maps Rudnick (1944) has demarcated eye area just anterior and lateral to the Hensen's node and lens area outside the neural boundaries.

As the pieces of blastoderm tested by all these workers had all the three germ layers together and there was continuous inductive interaction among these germ layers one could not expect to find an exact expression of self differentiation tendencies of any single germ layer from the stage of transplantation.

Rao (1968) interrupted the induction process by cutting pieces of neurectoderm only and tested their differentiation tendencies. Although he did find eye structures (retina as well as tapetum) mostly in the median prosencephalic grafts but he did not discuss this aspect in detail. He just mentioned, "Among the prosencephalic structures structural elements those belonging to eye (retina together with tapetum) are the most frequently encountered ones in the grafts of the median areas. In lateral grafts they are on the whole much less frequent."

In the present investigation according to first four operation plans more than 5 grafts were prepared from the prospective prosencephalic ectoderm. High frequency of eye structures was observed in the peripheral grafts of Plan B at primitive streak stage and in its central grafts at the head process stages. It was, however, high in the central grafts of the plans C, D and F area at both stages. The central area of these plans was larger than that of plan B. The grafts of plan G and H reveal a more clear picture. The eye potency field seems to occupy a transverse area (ca. 0.4 x 0.2 mm) at the primitive streak stage anterior to the compact mesoderm. At the head process stage the area becomes localized at ca 0.4 x 0.1 mm about 0.1 mm anterior to the head process.

(e) <u>Deviation from the prospective significance</u>

Commenting on the deviations from prospective significance, Rao (1968) reported that in all stages various grafts areas frequently yielded results which were not in complete accordance with the prospective area maps given for each stage. He asserts that the prospective area maps are only approximations. In some of his posterior grafts he found structures of more anterior neural areas. He explains this to be partly because the induction process was interrupted. Thus the originally evoked prosencephalic differentiation tendencies still had a chance to express themselves in the grafts of the rhombencephalic area. Similar results were obtained by Eyal Giladi (1954) in amphibians. Slight differences in age and size of donor blastoderms of any one stage may also cause deviation. Slight deviation from the boundaries of the graft areas during preparation of graft may also cause the deviations. In his stage II to IV he found prosencephalic differentiation in the median grafts of mesencephalic area in 74-85% cases which he was unable to explain except attributing it to the interruption of the induction process.

The present study also suggests that the prospective area maps are only approximations and final results of induction process. The histological analysis of the grafts in the present study show that central prosencephalic area shows more neuralization than

the peripheral at the definitive primitive streak stage, and that its frequency is higher in the central as well as peripheral grafts at the head process stage is due to the fact that the process of induction was interrupted while excising the grafts from the prospective prosencephalic ectoderm. There was not much deviation in the prospective significance as Rao found for other areas.

(f) Stage-wise results

The results obtained from the histological analysis of the grafts reveal following pattern of prosencephalic differentiation at the 3 donor stages.

(i) H and H stage 4 (definitive Primitive streak stage)

The anterior lateral as well as posterior lateral grafts implanted according to the operation plan B showed that the peripheral area from where these grafts were taken acquired incomplete neuralizing tendencies as feather germs were also present in many of these grafts. Presence of sensory layer of the eye in the grafts from the central as well as peripheral areas suggests that although eye differentiation tendency has appeared at this stage it is still not localized. The differentiation of the grafts taken according to the operation plans C, D and F show somewhat more clear picture, In these cases the eye structures were found mostly in the grafts taken from the central area which was undoubtedly larger than that of the plan B. The frequency of neuralization is also higher in the grafts

taken from this central area. However, the frequency of neuralization was better in the posterior lateral grafts than the anterior median grafts taken from the peripheral area of the plan B. This was also confirmed by the results obtained from the experiments performed according to the plan H where out of 6 p and 8 q grafts only 3 were neuralized showing that the anterior peripheral region has not acquired that much neuralization. Again, the recovery and neuralization of the grafts taken from the median region according to the operation plan G was better than that of the grafts taken from the lateral regions. The frequency of eye structures was high in the Gt, Gu as well as in the Hr and Hs grafts. It was very low in the Gv grafts. The area common to these grafts according to these two plans is also common to the central graft areas of the plans C, D and F. The structural elements of the telencephalic cortex were observed in very few Gu and other grafts.

(ii). H and H stage 5 (Early to medium head process stage)

At this stage an increase in the degree of neuralization was observed in the peripheral grafts of the prospective prosencephalic ectoderm. This was revealed by the fact that the central grafts taken according to all the plans were invariably neuralized; and peripheral grafts also showed neuralization to a greater extent. Eye structures were observed mostly in the grafts of the prospective prosencephalic ectoderm

overlying the region anterior to the head process. This is more obvious from the analysis of the Gt, Gu, Gv and Hr grafts. The eye structures were not encountered in Bm grafts, though they were present in the grafts of the central area of the plans C, D and F which is common to the area common to graft areas Gt, Gu and Hr. The pigmented layer was much better differentiated in these grafts. The width of Hs grafts is 0.1 mm. As the eye structures were absent in Hs as well as Bm grafts, and they were present in Hr as well as grafts of the central area of the plans C, D and F, which is larger than that of Bm graft, the superimposed picture suggest the localization of eye structures only in the area of Hr grafts. Some segregation of telencephalic and diencephalic differentiation tendencies was observed in the anterior and posterior areas respectively.

(iii) <u>H and H stage 5 (medium to long head process stage)</u>

By the time the head process reaches full length the prospective prosencephalic area becomes somewhat narrower than at the earlier stages. Almost all grafts show neuralization at this stage. The central as well as postero-lateral grafts taken according to the operation plan B show eye structures. These areas are common to the graft area Hr of the plan H which also show eye structures. The peripheral grafts taken according to the operation plans C, D and F sometimes show epithelial vesicles and feather germs also perhaps due to inclusion of extra neural areas in these. Overall differentiation pattern is very clear at this stage

than at the primitive streak stage. The anterior peripheral grafts according to the operation plans B, C, D and F show telencephalic structures; and those from the lateral peripheral as well as from the central area of these plans show mostly diencephalic differentiation tendencies. This pattern is confirmed by the grafts taken according to the operation plans G and H also. The median grafts of the operation plan G show both telencephalic,diencephalic as well as eye structures whereas posterior transverse grafts of the plan H show only diencephalic and eye structures.

It would be worthwhile to analyse these results in the light of work done and results obtained by Rao (1968). Discussing the stage wise results Rao referred to the work of Hillman and Hillman (1965) who found patches of mesodermal cell (prechordal mesoderm) in front of node as early as 12 to 13 hr. (H & H stage 3). His stage I was 5 to 6 hrs older when mesodermal patches extend further anteriorly and laterally and reached the posterior border of his graft A. That is why only 2 out of 9 grafts from this anterior region could form prosencephalic structures. He referred to the works of Nieuwkoop (1952) in amphibians and Hara (1961) in chick that activating influence may spread into ectoderm over considerably large areas than the area of actual contact with the inductors. At the primitive streak stage (Stage II) he found that majority of

anterior (A) and posterior (B) grafts from prospective prosencephalic area formed prosencephalic structures indicating that activating influence of prechordal mesoderm has extended anteriorly into area A in most cases. As in stage I at this stage also the lateral graft areas are uneffected by activating influence. He gives two reasons (1) either prechordal mesoderm exerts a stronger activating action than the prosepective notochordal mesoderm (2) or notochordal mesoderm has been laid down later or both. At the stage III (short head process stage) the prechordal mesoderm is located approximately below the graft area B. At this stage as 1 out of 10 of his anterior lateral, 3 out of 9 of his posterior lateral and 1 out of 9 of his lateral grafts from mesencephalic area (LA, LB and LC) were neuralized, He concluded that pear shaped neural field has extended centrifugally. At the stage IV (long head process stage) the anterior tip of prechordal mesoderm is located at the border between areas B and C. The posterior border of prechordal mesoderm coincides with posterior border of B areas. At this stage while the LA, LB and LD grafts taken from lateral regions show prosencephalic differentiation only some LC grafts show mesencephalic differentiation also. The neuralization of LC is also more than LD because transforming influence has not reached this region by this time. Similar was the result with LC grafts at the earlier stage.

The findings in the present investigation reveal that differentiation of the prosencephalic structures is mixed up with stronger differentiation tendencies in the central part of the prospective prosencephalic area at the primitive streak stage, and extends centrifugally in the prospective prosencephalic ectoderm at the head process stage when the localization of telencephalic differentiation is fixed in its anterior part and diencephalic differentiation in its posterior parts, and that of eye structures (retina as well as tapetum) in a transversely stretched area measuring about 0.4 x 0.1 mm ca. 0.1 mm anterior to the tip of the head process. The centrifugal spreading of the neuralizing tendencies in the competent ectoderm agrees well with the centrifugal differentiation pattern reported by Rao (1968). As none of the mesencephalic structures were found in the grafts of the prospective prosencephalic region and the area just posterior to it differentiates into mesencephalic structures (Rao, 1968, Khare personal communication), the differentiation tendencies clearly appear to be in accordance with the prospective significance of the area as demonstrated by Spratt (1952).

(g) General Remarks

Rudnick (1948), in a detailed review on prospective areas and differentiation potencies in the chick blastoderm mentioned, " for the chick, in the stages when ectoderm is being underlain by mesoderm, separation of the two layers for experimental test has not been

technically possible. The critical, direct test of differentiating capacity of the same region of ectoderm before and shortly after mesodermal contact has not been performed. Only indirect approaches can be cited and they have led to new questions rather than to satisfactory answers" (p.765). In his invaluable book Epigenetics of Birds (1952, p.1) Waddington writes "it is particularly unfortunate that separation of mesoderm from the ectoderm in the gastrulation stage is almost impossible to achieve". Nieuwkoop in 1950s (unpublished) overcame this problem by mechanically separating the germ layers and then testing its pieces by intracoelomic grafting technique. As already mentioned elsewhere, this opened up a new line of research enabling the workers (e.g. Hara, 1961; Rao, 1968) to study the differentiation tendencies of different parts of the germinal layers of chick blastoderm.

The prospective area maps of chick blastoderm provided by Spratt (1952) and organ forming potency maps of Rawles (1936) and Rudnick (1944) reflect the final outcome of the induction process. The graft pieces tested by Rawles and Rudnick consisted of 3 germ layers and the inductive interactions could continue even after isolation. The role of prechordal and notochordal mesoderm in the process of the neural induction in early chick embryo is now well understood (Hara, 1961; Rao, 1968). For a better understanding of the neural induction in the chick embryo a comparison with that of amphibians is

necessary. Among amphibians, it is well established, that during gastrulation the future archenteric roof material moves inward round the blastoporal lip in a caudo-cranial direction, while at the same time neurectoderm extends cranio-caudally. During these movements the prechordal and prospective notochordal mesoderm comes into contact with the overlying ectoderm and immediately exerts its inducing influence. Thus, the induction in amphibians spreads essentially in a caudo-cranial direction through the ectodermal layer(See review, Saxen and Toivonen 1962). Nieuwkoop (1952) proposed the Activation-Transformation hypothesis to demonstrate the process of neural induction in amphibian embryos. Rao (1968) interpreted his results in the light of this hypothesis, but Hara (1978) discusses that a more direct information based on critical experiments which could demonstrate that activation process preceeds the process of transformation in the 'chick embryo' is still not available. Based on earlier morphological studies of Spratt (1947, 1955, 1957a,b) and Vakaet (1962) that the prospective notochordal material is laid down in front of the node in a cranio-caudal sequence during the backward migration of the Hensen's node and the shortening of the streak, and on the results of his own experiments Rao (1968) concluded a cranio-caudal induction process in the chick embryo. Rao, (1968 p. 228) summarises the overall picture of neural induction as follows. "The activating action of the prechordal material in front of

the node sets up an activating field in the overlying ectoderm. This field gradually spreads further anteriorly as well as laterally, and with the appearance of the notochordal anlage also extends caudally in subsquent stages. Throughout the activation field the ectoderm acquires prosencephalic differentiation tendencies. At the same time transforming influences emanate from the prospective notochordal mesoderm. In the ectoderm overlying this mesoderm the transforming action suceeds the activating action, gradually changing the prosencephalic differentiation tendencies of the ectoderm into tendencies for more posterior region of the central nervous system. The more posterior prosepctive notochordal mesoderm has a stronger transforming influence so that more and more posterior tendencies will appear successively in more posterior areas. The fact that more posterior notochordal mesoderm is laid down later than the more anterior mesoderm leads to a lagging behind of the induction process in the more posterior regions of the neurectoderm. This finds expression in the initially less extensive lateral spreading of both activating and transforming influence in the posterior part of the prospective neural plate."

The differentiation leading to regional segregation thus depends on the accurately timed successive actions of activating and transforming influence upon the overlying competent ectoderm.

The present investigation reveals that the differentiation of telencephalic and diencephalic structures appears and extends in the prospective prosencephalic ectoderm centrifugally from the primitive streak to the head process stages under the inductive influence of the underlying prechordal mesoderm. The mediolateral differentiation of the prosencephalic structures also accounts for the ventrodorsal pattern formation of this region. The study agrees well with the results obtained by Rao(1968) and can be said to complement each other as well as that of Hara (1961).

Like that of Rao (1968) the present author has also found a high recovery rate of the median graft area and higher relative number of neuralized grafts than the peripheral graft areas. This clearly suggests that the inductive action originates from the central (median) prechordal mesoderm. Whether the inductive action extends in the prosencephalic ectoderm or in the peripheral mesoderm which in turn induces overlying ectoderm or whether both processes are in action cannot be explained in the present study. Nieuwkoop (1952) demonstrated that folds of competent ectoderm implanted in the dorsal midline of amphibian embryos become neuralized up to a distance equal to half the width of the neural plate under the influence of host mesoderm by spreading of inductive action in the folds in the absence of mesoderm.

The inductive capacity declines mediolaterally in the mesoderm as well as in the neurectoderm as studied in amphibians (Leussink, 1971) and also in the notochordal and lateral mesoderm of the chick embryo (Hara, unpublished).

Although the grafts are capable of self organisation and never show complete differentiation pattern (Rao, 1968), they can certainly be analysed for deriving a definite differentiation pattern. Moreover, as the size of grafts implanted by Rao was very large, self differentiation of many neural structures was seen in these. We found some grafts which showed only one type of prosencephalic structure but such grafts were very few in number.

Formation of cartilage in the grafts has been discussed by Rao (1968). In the present study also certain cases of cartilage formation were observed without nasal epithelium . It might have been induced by host tissues or graft induced reduplication of the somatopleure. Nasal cartilage is induced by the nasal placode which is known to be induced by prosencephalon (Orts llorca and Ferrol, 1961). The other secondary induced structures such as eye lens were not found in our grafts. Rao (1968) also did not find any lens structures in his experiments.

The localization and differentiation of eye potency field in the early chick embryo has been

exhaustively investigated by(Clarke, 1936; Rawles, 1936; and Rudnick 1938a, 1944 and 1948). Clarke (1936) reported that at late streak stage eye forming area was situated around primitive pit and at the head process stage just anterior to the head process. In the organ forming maps, Rawles (1936) showed eye structures differentiating in the median as well as lateral grafts taken from areas situated transversely just anterior to the head process. In her organ forming maps Rudnick (1944) showed eye potency field at the anterolateral angles at some distance from the Hensen's node at the primitive streak stage. Based on their preliminary investigation Choudhury and Khare (1978) reported a medial inward shifting of the eye potency field from the peripheral region during primitive streak and head process stages, but the number of grafts studied were not sufficient to provide a definite conclusion. Infact, the neural field itself becomes narrower at the head process stage (Spratt, 1952). The present study, based on the analysis of the large number of smaller grafts (more than 5)isolated from the prospective prosencephalic ectoderm at different planes, reveals that eye forming area appears in the prosencephalic ectoderm overlying just anterior to the compact mesoderm at the primitive streak stage. At the head process stage this field becomes localized ca 0.1 mm anterior to the head process in a transverse area measuring about 0.4 x 0.1 mm. Based on the work of

Clarke (1936), Rudnick (1948, p.768) commented, "There is slight chance for the eye, that a tenuous strip median in the anterior medullary plate too narrow to be tested by current methods, may from the first be unable to form eye tissue, and that the diencephalic floor arises by enlargement of this strip. The situation looks much more like a gradual separation of a continuous eye field into two laterally placed ones by the actual loss of ability of the medial cells to perform a certain histogenetic task". As discussed above this medio lateral separation of the eye field occurs at a later stage (Clarke, 1936). It is thus, not possible to comment on such separation in the present investigation. Rudnick (1944) shows prospective area of lens outside the neural field. Rawles (1936) found lens only in 1 out of 18 grafts. Hara (1961), Rao(1968) and the present author never found lens structures in the differentiated grafts.

In recent years, role of primary hypoblast in prosencephalic induction before the laying down of prechordal mesoderm has also been discovered (Vakaet, 1964, 1965; Gallera, 1971; Eyal Gyladi, 1971) and morphogenetic

changes during neural induction have been better investigated with the help of SEM,TEM and transfitter techniques (for reference see Gallera, 1968; England, 1973; Eyal Giladi, 1975; Rasilo and Leikola, 1976; England and Cowper, 1976). Although these works do not come under the direct purview of the present investigation, but a picture of the mechanism of prosencephalic induction and differentiation does emerge from these. These studies suggest that the prosencephalic induction is first triggered by the primary hypoblast and then elaborated further by the inducing capacity of the prechordal mesoderm. Information is also pouring in that some specific proteins (Gallers, 1973) may be responsible for such induction phenomena. Search for specific molecules (Tiedemann, 1975, 1978) may provide us with some definite clue to the mechanism of neural induction and pattern formation.

SUMMARY

SUMMARY

(1) This thesis includes an investigation on the establishment of neural differentiation pattern in the prospective prosencephalic ectoderm of the chick embryo at the primitive streak and head process stages. The technique of dark field illumination and intra coelomic transplantation described by Hara (1961, 1970, 1971) have been followed.

(2) The endoderm and mesoderm were carefully removed from the ectoderm of prospective prosencephalic area. Spratts (1952) fate maps were followed for measurements and demarcation of the neural area. 8 operation plans A, B, C, D, E, F, G, H were designed to isolate pieces of the prospective prosencephalic ectoderm from its different areas. Of these plans A and E were meant to provide 7 or 8 semicircular grafts, plans B, C, D and F were meant to provide more than 5 semicircular grafts from each and plan G to provide 4 longitudinal grafts from one half of and plan H to provide 4 transverse grafts from the other half of the prospective prosencephalic area. After few pilot experiments plans A and E were not followed because their graft size was very small (0.2 x 0.1 mm) and recovery was very discouraging. The size of grafts taken according to plans B, C, D and F was about (0.2 x 0.2 mm) and those of plan G and H ranged (0.2 x 0.1 to 0.4 x 0.1) mm.

The graft pieces were transplanted in the coelom of 2½ days host embryos and cultured for 12 days. The grafts recovered were analysed histologically to find out the differentiation tendencies - with the help of critera described in chapter III.

(3) By open Sandwich experiments, Hara (1961) demonstrated the differentiation tendencies in the competent ectoderm as induced by prechordal and notochordal mesoderm. Subsequently, Rao (1968) studied the appearance and extension of neural differentiation tendencies in the prospective neurectoderm. As his grafts were large in size, the present investigation was carried out with much smaller grafts (size 0.2 x 0.2 mm or 0.4 x 0.1 mm) to find out the finer mode of differentiation tendencies.

(4) In the first 4 operation plans the grafts were excised from different central and peripheral areas of the prospective prosencephalic ectoderm. In the other 2 operation plans 4 longitudinal grafts were prepared from one half, and 4 transverse grafts mostly from the other half of this region.

(5) A total of 1437 grafts were prepared from 448 donor blastoderms and transplanted into the coelom of 941 hosts. Of these, 206 hosts died. (mortality 23%) From the 725 surviving hosts which carried 1241 grafts, 304 grafts were recovered. (recovery rate 25%) The grafts were analysed

histologically to find out the differentiation tendencies.

(6) The results of the histological analysis may be summarised as follows:

(a) The grafts implanted according to the first 4 operation plans showed that the recovery of the central grafts was better than that of the peripheral grafts. The recovery and neuralization of the median longitudinal and posterior transverse grafts taken according to other 2 plans was better than the other peripheral grafts.

(b) The differentiation of all grafts taken at the primitive streak stage does not show any well defined pattern as the differentiation tendencies of the telencephalic diencephalic and eye structures are mixed up. At this stage the grafts of the central region show better neural differentiation than those of the peripheral region except in the first plan in which the central area was smaller in size. At the head process stage the pattern of differentiation was better defined. The eye structures (retina as well as tapetum) were found in an area measuring about 0.4 x 0.1 mm approximately 0.1 mm anterior to the head process. The structural elements of the telencephalic cortex were found mostly in the grafts of the anterior region, whereas diencephalic structures differentiated mostly in the grafts of the more posterior region.

(e) Structural elements of thelens were not observed in any graft.

(7) Interpreting the results in terms of activation-transformation hypothesis of Nieuwkoop (1952) the results of the present work are complementary to those of Hara (1961) and Rao (1968). It appears that as a result of first contact the prechordal mesoderm established in the prospective neural ectoderm a centrifugal meural (activation) field resulting into the induction and appearance of the prosencephalic differentiation tendencies. With the laying down of the head process, when the wave of activation is succeeded by a wave of transformation extending caudally and laterally transforming the prosencephalic differentiation tendencies in the more caudal parts of the prospective neural plate (Rao, 1968), the differentiation tendencies of telencephalic structures become localized to the anterior region of the prospective prosencephalic area, and that of diencephalic and eye structure to its more posterior region as analysed in the present investigation.

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