STUDIES IN THE PROCESSES OF DETERMINATION IN THE CHICK EMBRYO.

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Introduction.

In the higher animals, as a result of development, the simple unicellular zygote grows into a complex multicellular adult. This complexity is due to the determination and differentiation of the various structures.

Some centuries ago, when the concept of Preformation was the prevailing view of development, it was believed that the egg is a minature and preformed adult, and that the diversity which is exhibited during the development is present from the very beginning. Development was a mere process of unfolding of the creature. But in the early years of the last century the experimental work, especially of Driesch (1891), proved beyond doubt that the characters of the adult do not exist as such in the newly fertilised embryo, but on the contrary arise anew, by series of gradual interactions between the constituents of the egg among themselves and with the environment.

The word determination, or its german equivalent "Bestimmung" though occurring in the works of Roux, Wilson, Driesch and others, was not precisely defined in these works. Heider's definition of determination is given by Spemann (1938), thus - "Determination is the causation, the being caused as well as the having been caused - of the later fate of a part of the germ".

The word "segregation" occurs for determination in writings of the American School of Embryology, such as Hoadley, Lillie, Hunt, Willier and Rawles and others. Lillie (1929) defines segregation as the "process of origin of parts of/

of the embryo possessing irreversible prospective value as tested by self-differentiation". Thus he makes the power of self-differentiation a criterion of determination; but, as suggested by Waddington (1935), the capacity for self-differentiation does not mean irreversibility.

Though the concept of determination is regarded as the key to the understanding of embryonic development, there was a lot of confusion resulting from different usages employed in the literature. Harrison (1933) argued that a fertilised egg of Ascidian which is highly "determined" even at a very early stage, and is so sensitive to a slight disturbance in the cytoplasm, can in adult show perfect regeneration of the cut surface. Thus determination, according to him, must be reversible and hence the term loses its significance; but it was pointed out by Waddington (1940) that the processes which occur in regeneration may be different and thus should not be confused with those occurring during the formation of that organ in development.

The same confusion seems to have been caused by Spemann's (1938) explanation of the word determination. To quote his words "If in the two celled stage of the frog's egg the one lateral blastomere after destruction of the other, is able to produce half a neurula, this blastomere has been determined for half formation".

It was suggested by Waddington (1940) that the determination should be 'defined with reference to a particular process of development rather than to the production of a particular organ'.

One can define determination as Needham (1950) did, according/

according to which "determination is the fixing of the fates of parts of the embryo at a definite time in development".

At what stage is the embryo determined? And how is determination brought about? These were the problems confronting the investigators in experimental embryology.

The primary differentiation which takes place in the organism is the determination of the axis which marks the anterior and posterior ends of the embryo. It was shown by Jenkinson (1911) that in sea urchin this axis is determined when the egg is in the ovary. The end where the nucleus lies later on becomes the anterior end of the embryo while the opposite end where the yolk is situated, when present, becomes later on the posterior end of the embryo. In the frog also the axis was shown to be determined in the ovary (Huxley and de Beer 1934). It was further shown by Bellamy (1919, 1921) that the axis in the egg of the frog is determined by the position of the blood vessels in the ovary. The artery and the vein are situated at the opposite ends in relation to the developing oocyte. This would cause a gradient in oxidation and thus the end of the oocyte close to the artery becomes later the animal pole (that is the future head), while the opposite pole becomes the vegetal pole (that is the posterior end).

The embryo of a bird is available for study only after the egg is laid, which makes it difficult to study the earlier stages. However, in the pigeon's egg, Bartelmez (1912) showed that the main axis is determined in the oocyte by its attachment to the follicle.

The/

The next step in the differentiation is the determination of the plane of bilateral symmetry. It has not been described in hen's egg but, Bartelmez (1912) showed that in the pigeon's egg, after fertilization, a striking manifestation of bilaterality is shown by the periblastic ring and by the gramules in the segmental disc. In the frog it is determined by the entry of the sperm. The grey crescent, which is formed opposite to the entry of the sperm, marks the position of the future dorsal lip of the blastopore. The dorsal and ventral sides of the embryo are marked at this time; but apart from this differentiation there is yet no further differentiation of the embryo.

It was shown by Waddington and Taylor (1937) that in the chick embryo at the primtive streak stage, the presumptive ectoderm could be converted to mesoderm. Abercrombie (1937) also described the conversion of epiblast and presumptive mesoderm into neural tissue in the chick. In Amphibia, results of a similar nature were obtained previously by Mangold (1925).

Spemann (1918) showed that in the amphibian embryo before gastrulation a piece of 'presumptive nerve cord will become ordinary skin and vice versa. The fate of this region had not irrevocably determined at the time of transplantation'. It was plastic but after gastrulation the tissues are no longer plastic and they cannot give rise to the organs other than those they are determined to give rise to. There is no morphological differentiation, yet chemically each tissue is determined. A term chemo-differentiation has been

suggested/

suggested by Huxley (1924), to this invisible or primary differentiation.

The classic discovery of organiser, and organiser centre of Amphibia, by Spemann (1918), opened a new field in the working of experimental embryology. And the attention of investigators was attracted to find out whether similar phenomena existed in other vertebrates, especially in the chick embryo. But as the chick embryo develops inside the shell, there were technical difficulties which had to be overcome. Thus, before dealing with the organiser in chick embryo, it is worthwhile describing the earlier techniques used in studying the development of the chick.

The earliest methods, employed in testing the determination of the tissue were the defect and injury or isolation methods. In the injury experiments, injuries made either in early or primitive streak blastoderm, were believed to lead to specific defects in later stages. Injuries were made by a variety of ways, either by hot needles, electrolytic device, or irradiation by the use of X-rays or ultra violet.

Thus Peebles (1898, 1904) and Kopsch (1927, 1934) made a series of experiments on the chick embryo in early stages; but as the early blastomeres have a considerable power of regulation, the results obtained from these experiments could not be relied upon.

Wolff (1936) made local defects in definite streak blastoderm by treating a part of it by X-rays and reported a number of abnormalities in the embryonic axis; but it was pointed out by Waddington (1952a) that production of abnormal/

abnormal embryos would not help to understand the causal processes of development.

A considerable number of experiments have been performed by the method of isolation. In this method a blastoderm is cut into two or more pieces and each piece is isolated to study its development.

Wetzel (1924, 1936), and Hoadley (1926a), used an *in situ* sectioning technique while Waddington (1930, 1932, 1935), Rudnick (1938a, b, c), and Spratt (1942, 1947a, b, etc.), cultivated the isolated pieces *in vitro*.

Hoadley (1926b), Willier and Rawles (1931a, b), Hunt (1931a, 1932), Dalton (1935) and others have tested various parts of blastoderm by means of chorio-allantoic grafts. In this technique, the portion of the blastoderm which is to be tested is grafted to the chorio-allantoic membrane of the chick embryo between the age of 8 to 10 days. But of this technique, it was pointed out by Waddington (1935) that the graft was placed in the vicinity of the chorio-allantoic blood vessels of the host, on which it nourished and thus the substrate on which the graft grew could not be neutral; moreover, the graft might not develop according to its normal fate due to the mechanical factors such as a small space. Grafts were made at other places other than chorio-allantois, such as coelom, by Hamburger (1938, 1939) but in this the host embryo was too old to study the mutual interactions. Thus these methods led to the search for another method by which it could be possible to explant the embryo on a neutral substrate. Tissue culture technique was first used to cultivate the entire chick embryos by McWorter and Whipple (1912). The

in vitro/

in vitro culturing technique was later on employed by Sabin (1919) on chick, and by Brachet (1913) and Maximov (1925), on mammalian blastodiscs; but they could not succeed in growing the embryos long enough to study their development. Using the watchglass technique, previously used by Fell and Robison (1929), Waddington (1930, 1932) introduced a new transplantation technique. It consisted in growing the chick blastoderm on plasma embryo extract and pushing the graft in between the epiblast and the endoderm of the host. Thus it was now possible to study the mutual interactions between the host embryo and the graft. More simple modifications in the culturing medium were later on reported by Spratt (1947d, e), who used agar base with albumen or yolk. However, the softness of agar on which the embryo lies, is not suitable for grafting experiments. Very recently a new ring technique for culturing the chick embryo has been introduced by New (1955). It consists essentially in supporting the explanted blastoderm on a piece of vitelline membrane stretched across a glass ring. The nutrient is fluid albumen. In the present work, the culturing technique of New (1955) and transplantation technique of Waddington (1932) has been employed.

Having described the techniques used to study the processes of determination in the chick embryo, the results of the application of the conception of the organiser (Spemann) to avian embryology will now be considered.

Hoadley (1927) and Gräper (1929) suggested that in the chick the endoderm has an organising influence. Wetzel (1924) on removing the node at an early stage could not get the development/

development of the posterior part and thus concluded that the node alone was an 'organiser'. Hunt (1929a, b, 31b, 32) by transplanting various regions of the blastoderm to the chorio-allantoic membrane and studying their differentiation supported Wetzel; although Headley (1926a, b), and Dalton (1935), using the same technique could also get good differentiation from the parts of the primitive streak other than Hensen's node, but the essential test of "organiser" is the induction of the secondary embryonic axis in the host, and by the method of intra-blastodermal grafting, Waddington (1930, 1932, 1933a, b) first showed that besides the node at least the anterior third of the primitive streak has an inducing capacity. Woodside (1937) supported him.

Using the culture technique, Waddington and his collaborators, brought to light many facts about the avian development. It was pointed out by Waddington (1933c) that the endoderm determines the formation of the primitive streak by controlling the tissue movements. Thus primitive streak is dynamically determined. In the chick, induction was shown to be performed by the immediate products of primitive streak such as head process and sinus rhomboidalis (Waddington 1933a).

As in Amphibia, the organising action of the avian primitive streak is not species specific for it was shown by Waddington and Schmidt (1933) that the primitive streak of the duck was functional when grafted into the blastoderm of the chick and vice versa. The primitive streak of chick was shown to cause induction even in mammalian blastoderm (Waddington 1934c, 1939).

Dealing/

Dealing with the Amphibian organisation centre Spemann (1931) showed that different parts of the organisation centre tend to induce different parts of the body; that is, they show regional differentiation. In the chick the same was shown to be true by Waddington and Schmidt (1933) and Waddington (1933a). Lopachov (1935, 1936), Holtfreter (1936), Lehmann (1936, 1938) seemed to support the view of qualitative differences in the organiser causing regional differentiation, while Waddington (1940) has attributed the regional character of the induced structures to the 'individuation field' of the living organiser. Moreover, he has suggested that the living organiser may transmit to the reacting ectoderm a basic evocator plus the modulator, the latter bringing about the specific differences. The point about the specificity of regional structures induced will be developed later on in connection with the specificity of inductors.

Having studied the various organiser grafts it was natural for the investigators to look for the causal analysis of induction.

An embryonic induction occurs as a reaction between the graft, the overlying ectoderm of the host and the host's organisation centre. Spemann (1931) showed that the organiser graft cannot induce the formation of a neural plate at all times. There is a certain period of development during which the host ectoderm can react to an inducing stimulus. The time at which the host ectoderm is in a reactive state was later on called as the period of competence by Waddington (1932), Needham, Waddington and Needham (1934). Thus it was demonstrated/

demonstrated in the chick embryo that, at the primitive streak stage, the whole of the ectoderm of area pellucida as well as of area opaca, Waddington (1934a) and the presumptive axial mesoderm, Abercrombie (1937), are competent to form the neural axis.

Woodside (1937), working on the chick, claimed that with increasing age the competence steadily falls off and disappears by the head fold stage.

Various suggestions were made to explain the phenomenon of induction. Child attributed the organising capacity of the dorsal lip to its intense metabolic activities; while the experiments of Spemann and Geinitz (1927), Holtfreter (1931) and others, seemed to suggest the importance of contact between the inducing and the induced tissues. Still further Needham (1931), Waddington (1932, 1933) and others believed that the induction was due to a chemical substance.

The phenomenon of embryonic induction was described as consisting of two aspects. Mere induction of the new axis was termed 'evocation" (Needham, Waddington and Needham, 1934), while the determination of regional character was termed 'individuation' (Waddington and Schmidt, 1933). Evocation could be brought about by even dead material; thus in the chick, Waddington (1933b, 1934b) demonstrated the capacity for induction in organiser killed by heat coagulation. In Amphibia, the results of similar nature were shown previously by various investigators namely, Bautzmann, Holtfreter, Spemann and Mangold (1932), and Holtfreter (1933). It was thus/

thus obvious that there is present in the organisation centre a specific chemical substance which diffuses in both dorsal and ventral directions to cause an induction (Waddington, 1930, 1932), (Abercrombie, 1937).

The evocation is the function of the graft. Individuation on the other hand is performed by the mutual interaction of the graft and the host, and was thought of as a "field phenomenon".

The field concept was first associated with the organisation centre by Spemann (1921), though he did not discuss its meaning. Gurwitsch (1922) and P. Weiss (1923) elaborately established the field character of the organisation. Waddington and Schmidt (1933) introduced the term 'individuation field' in connection with the chick organiser; and Abercrombie and Waddington (1937), while working on the chick, originally discussed the mutual influences of host, graft, and induction, in terms of an 'individuation field'. To quote their own words, (Waddington and Schmidt 1933, p. 555), 'An organisation centre is surrounded by a field, which may be called the individuation field, within which its unifying action makes itself felt'. It was further described as follows: (i) The field is strongest near the embryonic axis and falls off in intensity towards the edge of the area pellucida. (ii) It is regionally differentiated along antero-posterior axis of the embryo. (iii) More anterior parts have dominance over more posterior parts.

Thus it seems to correspond to a physical field such as an electro-magnetic field.

How/

How is individuation brought about? For example, the formation of the neural tube from the neural plate? It was suggested by Waddington (1942) that in Amphibia, the folding of the neural tube is brought about by changes in the surface tension of the cells. In the chick, as pointed out by Waddington (1950), the experiments of Abercrombie (1937), Abercrombie and Waddington (1937), and Waddington and Taylor (1937), seem to suggest the de-epithelization of epiblast before its conversion into mesoderm, but nothing is really known with certainty.

It is, however, likely that evocation is a chemical process and the attention of the investigators was next drawn to the exact nature of the chemical stuff involved. Waddington, Needham J. and Needham, D.M. (1933) found that ether extract of Amphibian eggs was inductive. Fischer and Wehmeier (1933) suggested that the evocator was identical with glycogen. They also obtained inductions through implantations of several other substances, such as muscle adenylic acid and thymonucleic acid. On the other hand, Waddington, Needham J. and their collaborators (1935) suggested that different synthetic polycyclic hydrocarbons related to sterols are inductive. Besides these, several investigators could obtain inductions through other substances. Waddington, Needham and Brachet (1936) reported induction through methylene blue. Barth (1934, 1935) in earlier work reported neural induction in Amphibia through crude or pure cephalin prepared from mammalian brain; later on, Barth (1939), Barth and Graff (1939) suggested that cephalin acted

as/

as neural inductor previously, probably due to its toxicity, while the naturally occurring organizer may probably be protein. Okada (1938), who reported inductions through calcium carbonate, silica, etc., also made a similar suggestion that the chemical substances may be releasing the evocator from the amphibian cells, thus indirectly causing inductions. Abercrombie (1939), working with chick embryo obtained induction through carcinogenic agent dibenzanthracene. Brachet and Rapkine (1939) stated that -SH group of protein causes induction. Recently Brachet (1944) has discussed extensively the role of proteins, nucleoproteins and nucleic acid in induction. On the other hand, Holtfreter (1944, 1945) maintains that most of the chemical substances, dead and foreign tissues, cause induction due to cytolysis.

Having known that neural induction can be brought about by a variety of chemical substances, it was essential to know whether there is any particular process setting the evocator at work in normal development or, in other words, to look for the causal connection between the metabolism and the induction.

Woerdmann (1933) using histochemical methods found that the cells of the animal pole of Amphibian embryo lose glycogen as they invaginate through the blastopore and thus suggested a connection between glycolysis and induction. Raven (1933) supported him, though Pasteels attributed glycolysis found by Woerdmann due to an artefact. Heatley (1935), Heatley and Lindahl (1937), later on showed by microchemical method that there issome loss of glycogen on the/

the part of the invaginating cells of the amphibian gastrula. Jacobson (1938a), working on chick embryo, found that the epiblast cells lose their glycogen as they invaginate to form the endoderm.

In the meantime, it was reported by various investigators, namely Spemann and Geinitz (1927), Holtfreter (1931), Lehmann (1932), Raven (1933) and others, that in Amphibia, the parts which do not possess inducing capacity in the natural course can acquire it by merely remaining in contact with the organiser. Waddington and Taylor (1937), and Abercrombie (1937), found in the chick that the pieces of epiblast placed in the primitive streak can produce inductions. It was thus natural for some investigators, such as Woerdmann, Lindall, Raven and others, to presume a connection between invagination, glycolysis and induction.

Child's theory of metabolic gradients to explain morphogenesis stimulated some investigators to find out if there is any relation between the respiratory mechanisms of the organiser and the induction phenomenon. Brachet (1934), working on the amphibian embryos, showed that the output of carbon dioxide is considerably higher in the organiser than than in the ventral epidermis. As regards the oxygen uptake Brachet at first found the higher uptake in the organiser region, but with more sensitive manometric methods no difference was seen. Waddington, Needham and Brachet (1936), working on the isolated pieces of dorsal region and ventral epidermis of Triton cristatus did not find any difference in oxygen uptake. Boell and Needham (1939) further showed that/

that the rate of oxygen consumption of the 'dorsal lip region' of the amphibian gastrula is the same as that of the ventral ectoderm.

In the chick embryo, Rulon (1935), observed the reduction gradient, using Janus green, and correlated induction with the rate of reduction of vital dyes.

A gradient in cytochrome oxidase, with indophenol blue reaction, was obtained by Moog (1943a) in early chick blastoderm.

Shearer (1924) using a Barcroft differential manometer found a gradient of oxygen consumption in the chick embryo, though Phillips (1941, 1942) using a sensitive cartesian diver micro resperometer did not find differences of any significance in the head process blastoderm. It appears then that the organiser does not possess a higher respiratory metabolism than other regions although its Respiratory Quotient is higher than that of the other regions.

The concept of metabolic gradients is also found to be associated with differences in susceptibility to the action of injurious chemicals and physical agents. (Child, 1924, 1946). Much work has been done both on chick and Amphibia in this connection.

Buchanan (1926), in his study of the effect of hydrocyanic acid on the development of the chick embryo, found the anterior end to be the region of highest activity. Hymann (1927), using potassium cyanide, ammonium and sodium hydroxide found a simple primary antero posterior gradient in the primitive streak stage of the chick embryo. She also described/

described a medio-lateral gradient in susceptibility from the neural tube towards lateral plate. Hinrichs (1927) on exposing the chick embryos to the ultra-violet irradiation has confirmed a simple antero-posterior gradient in earlier developmental stages, though in later stages he suggested that the gradient is obscured.

In the frog, Bellamy (1919), Bellamy and Child (1924), have shown similar respiratory gradients. Although so much work was done with the susceptibility method by Child and his collaborators, Needham (1931) has suggested that "the susceptibility method is very complicated and conclusions from the experiments with it have to be drawn with caution".

Apart from the metabolic gradients, several investigators have described antero-posterior gradients of several biochemical substances along the primitive streak, in the chick. Jacobson (1938b) claimed a higher lipoid content at the node of the primitive streak. Moog (1943b, 1944) has described an antero-posterior gradient of acid and alkaline phosphatase in the chick embryo. Feldmann and Waddington (1955) suspected an antero-posterior gradient in the uptake of radio active methionine along the primitive streak. This would suggest the gradient in synthesis of protein.

Brachet (1940, 1944), using cytochemical methods demonstrated a gradient of sulphydryl and ribonucleoproteins in the early developmental stages of chick blastoderm, and suggested causal connection between ribonucleoproteins and induction. He presumed that the induction is brought about by/

by the transfer of microsomes, (which are richer in ribonucleoproteins), from the graft to the induced neural tube. However, Waddington and Sirlin (1954), and Sirlin, Brahma and Waddington (1956), using organiser grafts labelled with radioactive tracers in Amphibia could not find the specific transfer on a large scale; but they (Waddington and Sirlin, 1954) concluded that "In our experiments, however, the passage of labelled particles cannot be excluded, especially if only a small number are necessary to initiate a chain reaction such as is possible with induction". Pantelouris and Mulherkar (in press), working with chick blastoderm and using organiser grafts labelled with radio-active methionine came to a similar conclusion.

The problem of the specificity of inductors attracted the attention of investigators, after Spemann (1931) had found the regional differences shown by the different levels of Amphibian organiser. The experiments of Holtfreter (1936), Lopachov (1935, 1936), and Lehmann (1936, 1938), supported the qualitative nature of the organiser to suggest the regional differentiation in Amphibia. Waddington (1938, 1940), seemed to propose among other possibilities, a different concentration of the same chemical substance or the presence of the basic evocator and the modulator in the organiser, the latter causing specific differences. However, Shen (1939) did not get regional differences by using the different concentrations of the sodium endo succinate salt of 1 : 2 : 5 : 6 - dibenzanthracene. On the other hand Chuang (1938) using Triton liver and mouse kidney could obtain/

obtain specific inductions in both the ectodermal explants and entire gastrulae; though in the latter, the specificity was not well marked due to the influence of the host. Chuang (1940) studying further the effect of heat on both Triton liver and the mouse kidney found that different periods of heating affect the nature of the induced structures. Thus he maintained that there must be specific substances for the inductions of neural or mesodermal structures. The experiments of Toivonen (1940, 1953a, b, 1954) and Barth and Graff (1943) also suggest the same.

The problem of regional differentiation has been taken up once more recently by Dalcq (1940, 1947), Nieukoop (1947, 1952), Waddington (1952b, 1956) and others. Dalcq (1947) working with Discoglossus, suggested that the regional differentiation is due to the quantitative differences in the organiser. Waddington and Yao (1950) emphasise the space-time relationship between the ectoderm and the archenteron roof, for causing the regional differentiation. They further pointed out that the anterior mesoderm is in the field of evocatory stimulus for a longer time than the posterior mesoderm and as such it will have a higher evocatory stimulus than the posterior one. Waddington and Deucher (1952) tried to prove this by prolonging the time contact between the presumptive neural plate and the archenteron roof in Triton, though with little success. Quite recently Waddington (1952b, 1956) has attributed the regional differentiation to the process of self-individuation, occurring in the neural tissue rather than

to/

to any specific substance in the organiser; yet nothing is known how the self-individuation is brought about.

Another way of studying the problem of specificity has been tried by observing the specific effect caused by specific substances on the embryo, before the differentiation of organs. It is suggested by Waddington (1956) that "the differentiation of tissues certainly involves the appearance of tissue specific proteins and it is probable that an alteration in protein metabolism is one of the most important results of induction". Thus Brachet (1944, 1946), obtained inhibition of neural induction by treating Amphibian gastrulae with benziminazole, which is supposed to inhibit the synthesis of nucleic acid. Waddington (1955), while studying the effects of some metabolic antagonists and cytotoxic substances on Amphibia found some specific effects on some tissues. But he, like Brachet, suggested that the process by which they act in the embryo is not known and thus nothing can be said about the specificity of the effects.

The effects of some purine antagonists were further studied in the chick and the newt embryos by Waddington, Feldmann and Perry (1955). Here again they reported some specific effects of some substances on certain tissues of both the embryos.

The same problem of specificity has been approached in quite a new way by employing serological tests. Thus Weiss (1947) prepared the antisera in guinea pig against the adult organs of the chicken, and on injecting them in the developing embryo of 3 to 8 days, obtained specific

stimulating/

stimulating effect on homologous organs. On the other hand, Ebert (1950) using the serological technique, reported the specific inhibitory effect of anti-heart and anti-brain sera on the definitive primitive streak and head process stage blastoderms, cultivated in vitro.

Clayton (1953) prepared antisera in rabbit against both the entire Newt gastrulae and its particular tissues, and found six different antigenic fractions, some of which are found only after gastrulation. Moreover, she obtained the specificity of some fractions of antigen to certain layers.

Yet in spite of several researches, the problem of specificity remains unsolved at the present moment.

With this background in mind, an attempt will be made in the present paper to study the processes of determination in the chick embryo.

Materials and Methods.

Fertilised fresh eggs, obtained from the Poultry Research Centre in Edinburgh, were incubated in an electrically regulated incubator at 37.5°C, to get the desired stage of development. (Definitive primitive streak) shown in Fig. 1.

All the instruments, containers, etc., were sterilised in an oven at 150°C for one hour and the solutions autoclaved. The experiments were done in a sterile room to avoid undue contamination.

The embryos were cultivated in vitro by the technique described by New (1955). It essentially consists in supporting the explanted blastoderm on a piece of vitelline membrane, stretched across a glass ring. The nutrient is fluid albumen. The grafting operations were performed with fine steel knives.

After about 22-24 hours of culturing, some of the entire specimens were stained with dilute Delafield's haematoxylin; they were then cleared in cedar wood oil and photographed. Sections were serially cut at 10 μ. They were stained in Delafield's haematoxylin and differentiated in acid alcohol. In all 303 grafts were made and 270 histologically examined.

The experiments consisted in cutting a single blastoderm of a definite primitive streak stage of development, (the length of the streak varied from 1.7 to 2.1 mm) into a certain number of median and lateral squares each of about 0.3 mm area. Tungsten needles were used for cutting the blastoderm/

Fig. 1. Whole mount of a chick blastoderm in a definitive primitive streak stage.

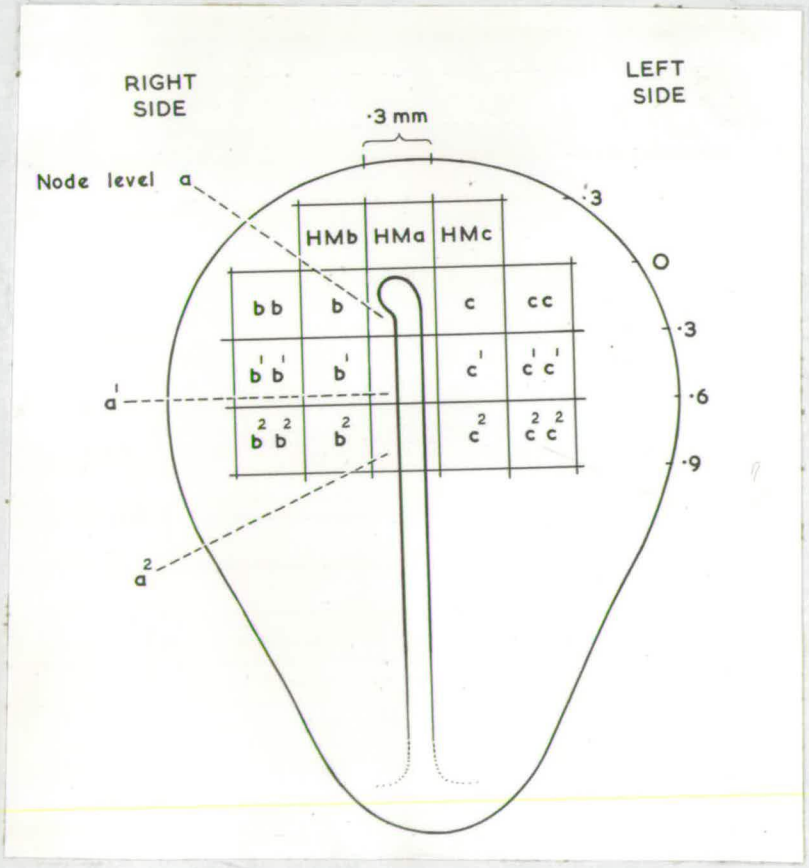

Fig. 2. Scale drawn map of a primitive streak stage blastoderm (ventral view) showing the scheme of grafting made in the experiments.

Description and Results of Experiments

(In the description of the specimens, the letters HMb, HMc, etc., refer to the level of the donor blastoderm from which the graft was made. The protocol number of the operation is given afterwards).

HMa level.

This level represents a square of about 0.3 mm. in front of the node level (a) of the primitive streak. It is the region of the presumptive forebrain. Some of the grafts are described below.

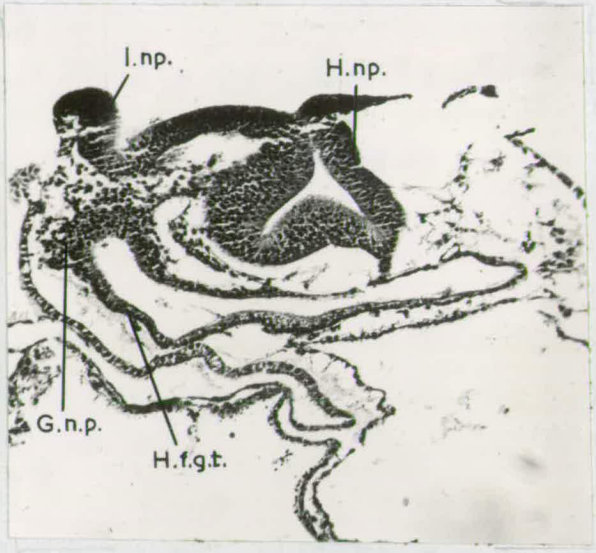

Fig. 3. HMa 30. Section through anterior region. (x 80).

HMa 30.

Length of the donor primitive streak 2 mm.

Length of the host primitive streak 1.5 mm.

Graft : To the left anterior region of the host.

In the section, (Fig. 3), induction into a secondary neural plate was seen. It was continouus with the host neural tube. The graft seems to have fused with the endoderm of the foregut/

blastoderm and the measurements were made with an ocular micrometer.

The position of the cuts may be ascertained by referring to the scale drawn map of the living embryo shown in Figure 2. The primitive streak with an average length of about 2 mm.was cut into units of 0.3 mm.; the original cut was made to include the node level of the primitive streak (a) and the subsequent cuts were made with it as an origin. The cuts containing the parts of the primitive streak at different levels were labelled as a, a^1, a^2, etc., successively hindwards. Those immediately next to them on the right and left at the corresponding levels as b, b^1, b^2, etc., and c, c^1, c^2 etc., respectively. The squares still further away from b, b^1, b^2 and c, c^1, c^2 were labelled as bb, b^1b^1, b^2b^2, etc., and cc, c^1c^1, c^2c^2 etc., respectively. The square 0.3 mm. anterior to the node was labelled as HMa; that on the right and left of it as HMb and HMc respectively.

Each square was then removed and grafted between the endoderm and the epiblast of the host embryo of the same age, (definite primitive streak) in a manner described by Waddington (1932).

foregut of the host to form a thick neural plate. A lot of mesenchyme is seen scattered round about the secondary neural plate and the graft. The fusion of the graft with the endoderm especially when the graft lies in the head, has been described previously by Abercrombie (1937).

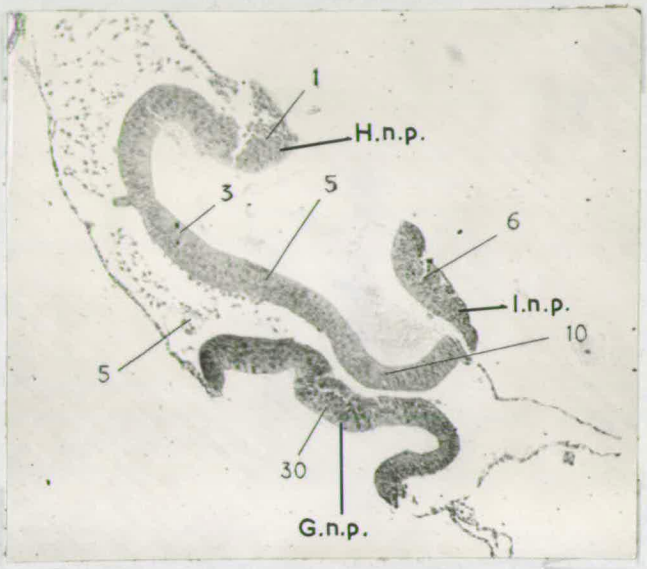

Fig. 4. HMa 28. Section. (x75).

HMa 28.

Length of the donor primitive streak 2 mm.

Length of the host primitive streak 1.8 mm.

The graft in this case was labelled with radioactive DL-methionine-S^{35}. From the section, (Fig. 4), the graft was seen to have differentiated into a big neural plate, probably representing the forebrain. It has induced the secondary neural plate which is confluent with that of the host. No mesenchyme is seen associated with the graft. This shows the capacity of the forebrain to differentiate independently of mesoderm as shown by Waddington (1932), Rudnick (1938b), and Spratt/

Spratt (1947c). The induction in this case is probably the homoiogenetic induction described in birds by Waddington (1933a), and in Amphibia by Mangold and Spemann (1927).

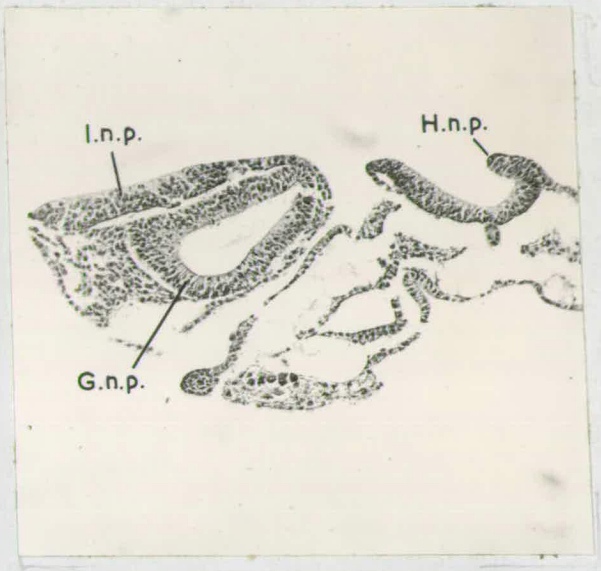

Fig. 5. HMa 26. Section. (x 70).

HMa 26.

The donor primitive streak 2 mm.

The host primitive streak 2 mm.

In the section, (Fig. 5), the graft was seen to have differentiated into a complete neural tube. The ectoderm of the host in contact with it shows a slight thickening. It may be called a weak induction. There is an attempt on the part of the graft to cause the host ectoderm to differentiate into neural tissue, but not into a definite neural plate.

Interpretation of the results.

The region HMa (a square of 0.3 mm. in front of the node level) has been described as forming forebrain by several investigators such as Wetzel (1929), Stein (1933), Spratt (1942), and others.

Thirty/

Thirty one grafts from this level were made. Two gave inductions and eleven differentiated into the neural tissue. The cases of induction are seen in Figs. 3 and 4. One instance of weak induction is seen in Fig. 5. Here there is an attempt to induce the ectoderm of the host into a neural plate. It is shown by the thickening of the host ectoderm though it does not form a neural plate. It seems probable that this region has an inducing capacity, though to a much lesser extent than that of the node level of the primitive streak. The self-differentiation of the graft into complete neural tube is seen in Fig. 5.

The graft HMa 28, (Fig. 4), which was labelled with radioactive methionine seems to show the capacity of this region for self-differentiation independently of mesoderm.

HMb and HMc Levels.

HMb and HMc levels are represented by the squares of 0.3 mm. on the right and left sides of the square HMa respectively. A few instances of each will be described.

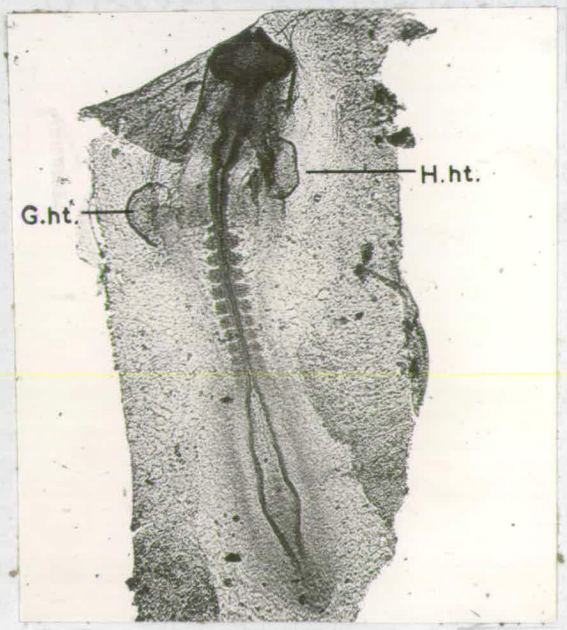

Fig. 6a. HMb1. Whole mount fixed after 24 hours

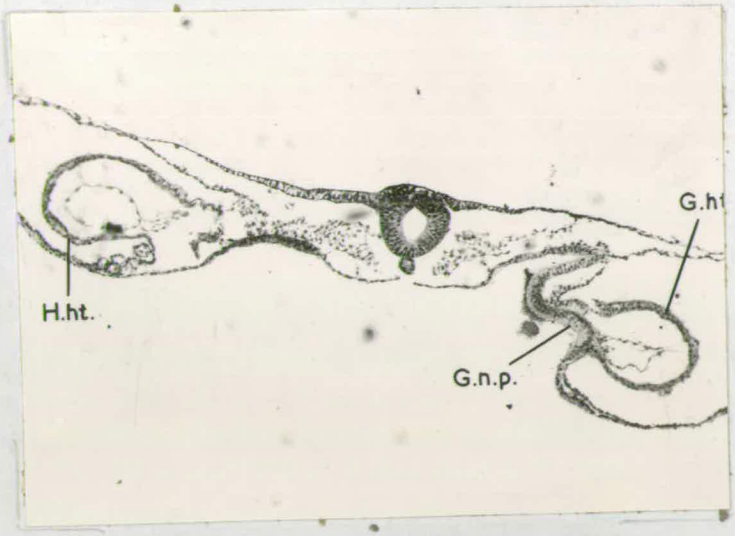

Fig. 6b. HMb1. Section of the same passing through two hearts. (x 70, sides reversed).

HMb1.

Length of the donor primitive streak 1.8 mm.

Length of the host primitive streak 1.5 mm.

Graft : In the left anterior region of the host.

A very beautiful heart, derived from the graft, was seen beating in the specimen (Fig. 6a) cultured for 24 hours. It seems to be of the same shape and size as that of the host. In the section (Fig. 6b), the graft neural plate is fused with the endoderm of the host.

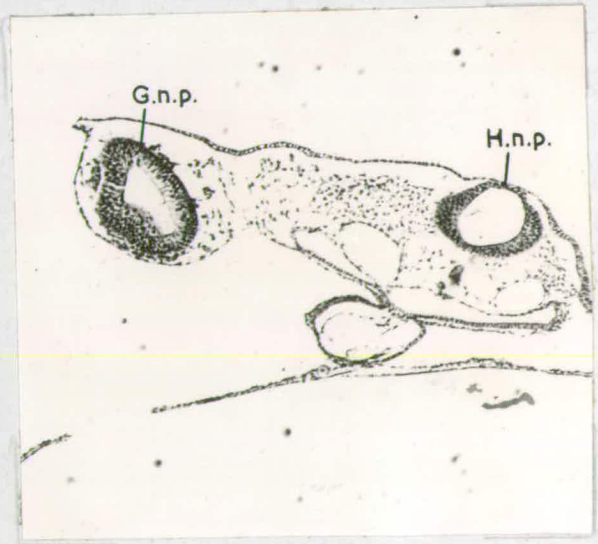

Fig. 7. HMb6. Section. (x 70, sides reversed).

HMb6/

HMb6

The donor primitive streak 2 mm.

The host primitive streak 1.9 mm.

Graft : In the right anterior region of the host.

The graft differentiated into a big neural tube (Fig. 7). Even though the neural tube derived from the graft is in contact with the host ectoderm, no induction is caused.

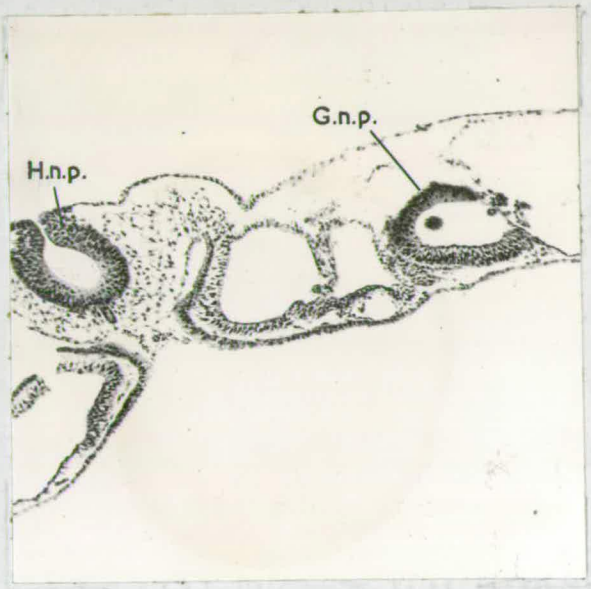

Fig. 8. HMc8. Section. (x 70).

HMc8.

Donor primitive streak 2mm.

Host primitive streak 2 mm.

The graft differentiated (Fig. 8) into almost a complete neural tube. No induction was caused. Close to the graft is seen the mesenchyme, probably derived from the graft.

Interpretation of the results.

Ten grafts were made from HMb level, out of which four differentiated into neural plates. Fourteen were made from/

from HMc level, five differentiated into neural plates. In some cases, for instance Fig. 6a, the heart differentiated. In both the levels no induction was caused. In some cases, Fig. 7, although the graft is close to the estoderm of the host, there is no indication of any induction.

Node level (a) of the primitive streak.

This is the square of 0.3 mm. passing through the node of the primitive streak.

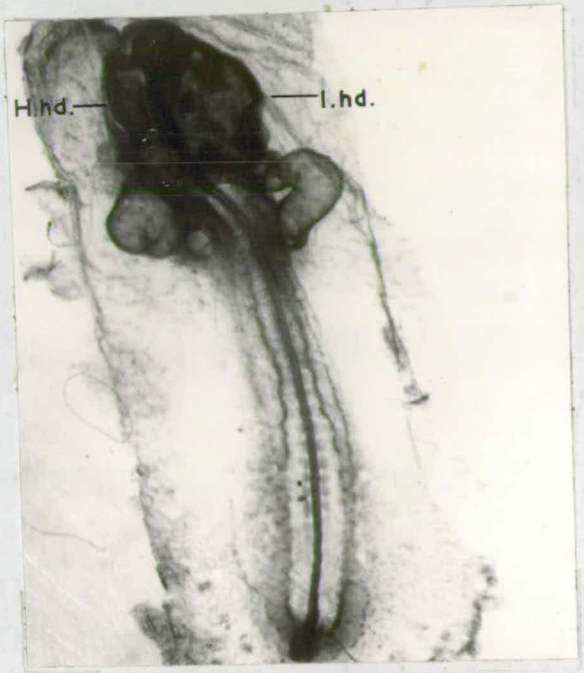

Fig. 9a, a2 Whole mount fixed after 46 hours of culturing (x 10).

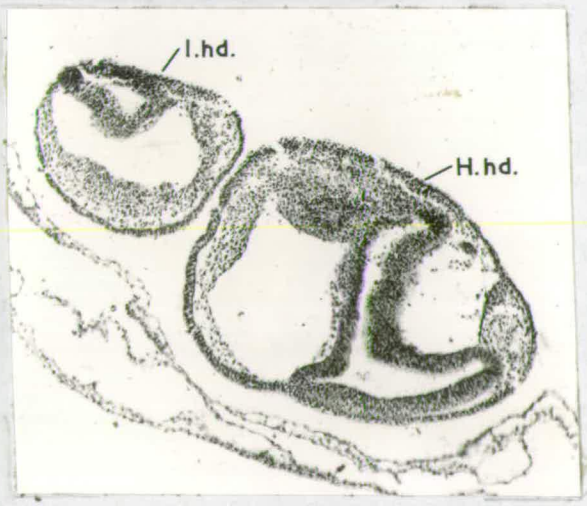

Fig. 9b. a2 Section through head (x 80, sides

a2

The whole mount (Fig. 9a) shows two heads and two hearts. The induced structures are on the right side. In the section (Fig. 9b) the two heads are clearly seen. The induced head is smaller than the head of the host. Both the hearts were beating at the time of fixation. The specimen was fixed after 46 hours of culturing.

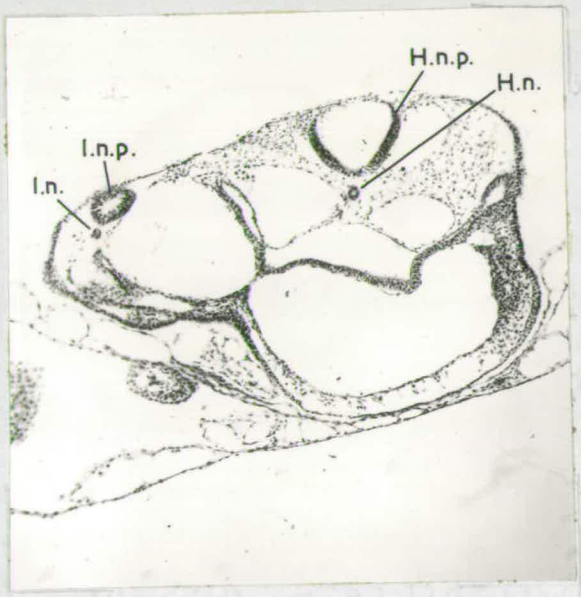

Fig. 10. all. Section showing the induction of a complete secondary axis. (x 70).

all

The graft appeared to have been incorporated into the host (Fig. 10) and had induced a complete secondary axis, with neural tube, notochord and foregut. The neural tube, notochord, foregut and a well developed heart of the host were clearly seen. The specimen was fixed after 46 hours of culturing.

In all 20 grafts were made from this region out of which 17 caused inductions. The capacity for induction seemed to be highest at this level of the primitive streak.

b and c levels.

b and c levels represent the squares of about 0.3 mm. on the right and left sides of the node level (a) of the primitive streak respectively. Some of the grafts are described from these levels.

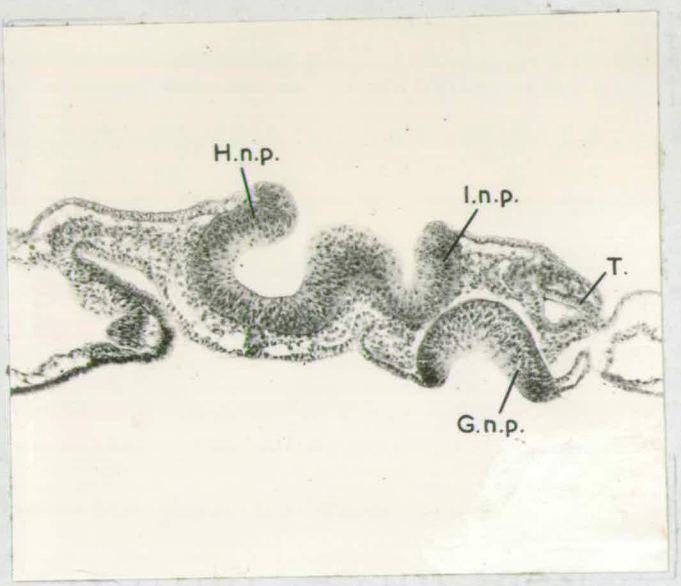

Fig. 11. b11. Section showing host, induced and graft neural plates. (x 70, sides reversed).

b11

Length of the donor primitive streak 2 mm.

Length of the host primitive streak 1.4 mm.

Graft: In the left anterior region of the host.

In the section, (Fig. 11), the induced neural tube is seen confluent with that of the host. The graft is differentiated into the neural tube though not completely, and there is a lot of mesenchyme. No notochord seems to be associated either with the induced neural tube or the graft.

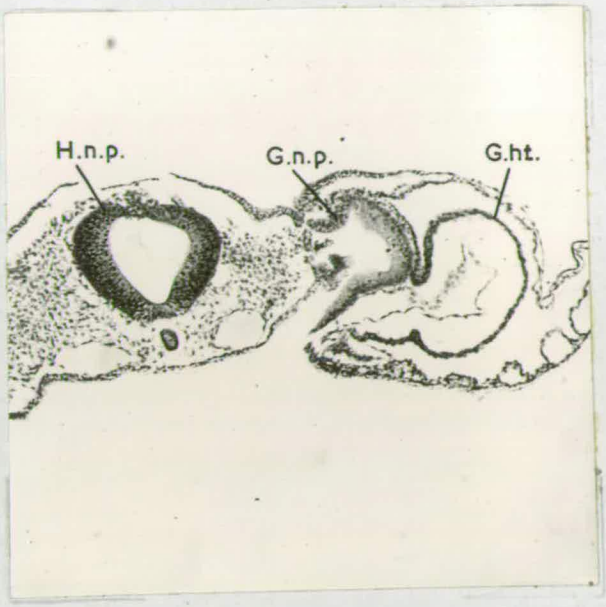

Fig. 12. b24. Section through the heart derived from the graft. (x 80, sides reversed).

b24.

Length of the donor primitive streak 1.8 mm.

Length of the host primitive streak 1.8 mm.

Graft: In the left anterior region of the host. The graft is differentiated into a neural plate and heart (Fig. 12). The heart derived from the graft appears as big as that of the host (not shown in photograph). At the time of fixation two hearts were seen beating.

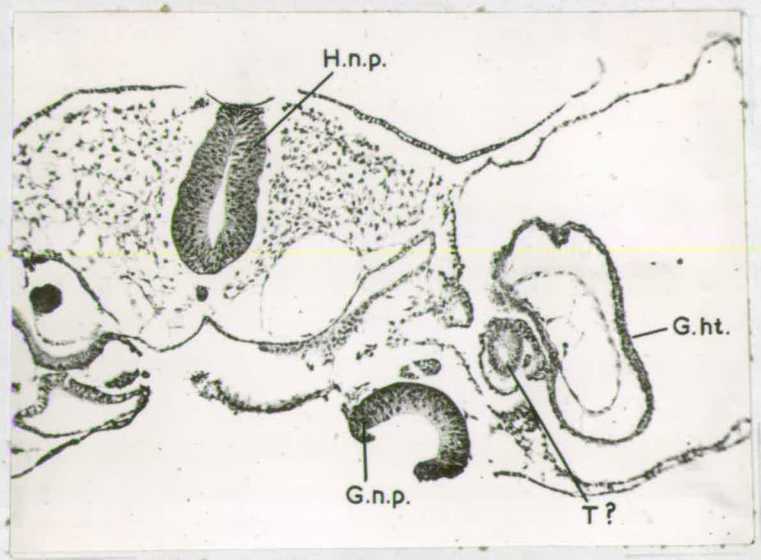

Fig. 13/

Fig. 13. b28. Section showing the structures of the host and those derived from the graft. (x 80).

b28.

Length of the donor primitive streak 1.8 mm.

Length of the host primitive streak 1.9 mm.

In the section, (Fig. 13), the graft seems to have fused with the endoderm of the host to form a neural tube, though not a complete one. Associated with the graft is mesenchyme as well as a well developed heart. On the inner side of the heart there is a tubule lined by endodermal cells.

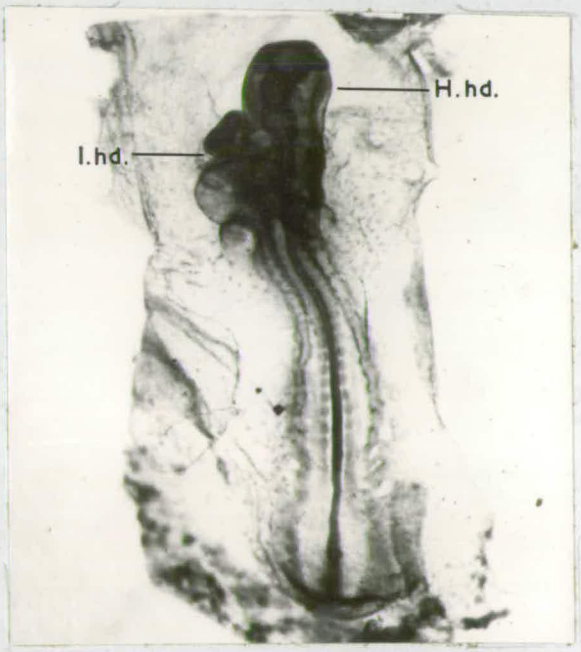

Fig. 14. c1. Whole mount showing two heads. (x 10).

c1.

Graft in the left anterior region of the host.

In the entire stained and cleared specimen, (Fig. 14), a beautiful induced head is seen lying on the left of the host. In the section the induced neural tube was clearly seen, but unfortunately the sections were lost in procedure.

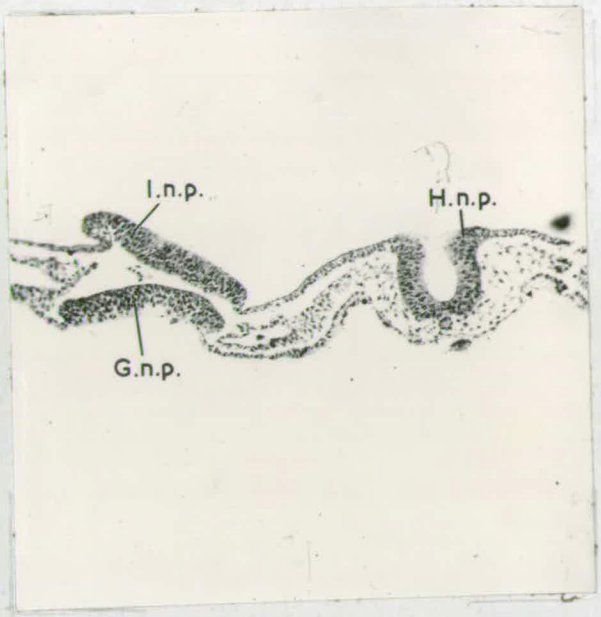

Fig. 15. c6. Section (x 75).

c6.

Graft in the left anterior region of the host.

In section, (Fig. 15), the graft itself seems to have differentiated into a neural plate, and to have induced a neural plate above it.

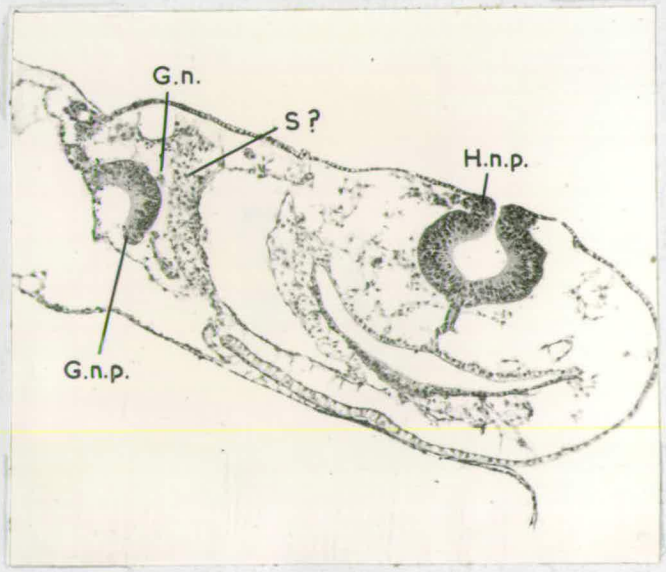

Fig. 16. c28. Section. (x 70 sides reversed).

c28/

c28.

Length of the donor primitive streak 1.9 mm.

Length of the host primitive streak 1.5 mm.

Graft : In the right anterior region of the host.

The graft is differentiated (Fig. 16) into a neural plate, notochord, and a group of mesenchyme on either side of it, probably forming a somite. This was the only instance where the notochord was seen to have differentiated. This is probably the region where presumptive notochord lies; and as the graft is from the left side, it perhaps shows the lateral extent of the notochord. The section is an interesting instance showing the inter-relation between the formation of somites and the notochord, which will be considered in the discussion.

In this case, although the graft is self-differentiated into neural tube and notochord, and lies close to the host ectoderm, it has not produced induction.

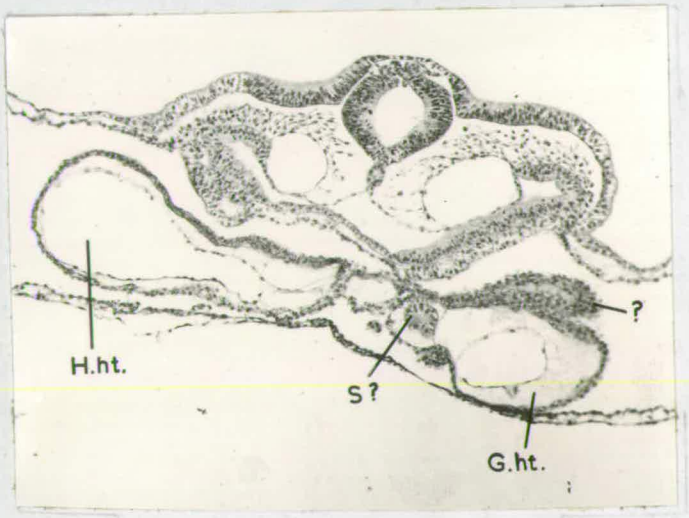

Fig. 17. c27. Section through heart (x 80, sides reversed).

c27.

The donor primitive streak 2 mm.

The host primitive streak 1.5 mm.

Graft : In the left anterior region of the host.

In the section (Fig. 17), the graft is seen to have differentiated into a well developed heart, some mesenchyme and a round mass of cells, probably the somite. At the time of fixation two hearts were seen beating. The heart of the host is bigger in lateral extent than that of the graft, and lies opposite to it.

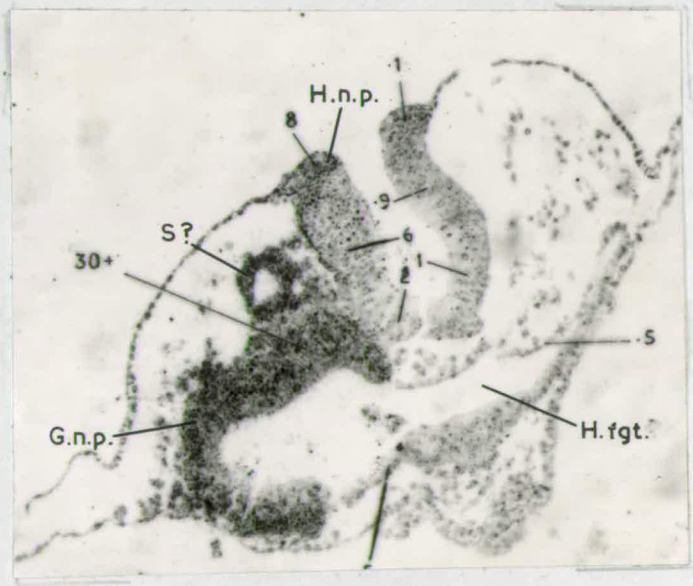

Fig. 18. c39. Section through anterior region (x 150).

c39.

The graft was labelled with radioactive DL-methionine-S^{35}. It differentiated into a neural plate, mesenchyme and somite? (Fig. 18). The neural plate of the graft is fused with/

with the endoderm of the foregut of the host. As described before, this is a good instance showing the fusion of the graft with the foregut endoderm of the host. No induction is caused although the neural tube and the mesoderm derived from the graft are in contact with the ectoderm of the host.

Interpretation of the results.

Thirty grafts of level b were made, out of which two caused inductions, and eight differentiated into neural tissue, four differentiated only into mesenchyme. Thirty nine grafts were made from level c, out of which 3 gave inductions and 14 differentiated into neural tissue, 4 into mesenchyme alone. It seems then that these levels also have a capacity for induction. Good differentiation of the heart was seen from both the levels in some of the grafts. In one instance only, c28 (Fig. 16), the notochord was differentiated from the graft. This is perhaps the region in which the presumptive notochord lies; and in some cases, (Figs. 16, 17), a tendency of the mesenchyme to form somites is seen. This may indicate the inter-relationship between the somites and the notochord which will be considered in the discussion. Although some of the grafts are differentiated into neural plate and are in contact with the host ectoderm, they have not caused any induction (Figs. 16, 18).

Second level of the primitive streak (a^1).

This is a square of about 0.3 mm. behind the node level. In all 16 grafts were made from this region, 9 of which/

which caused inductions.

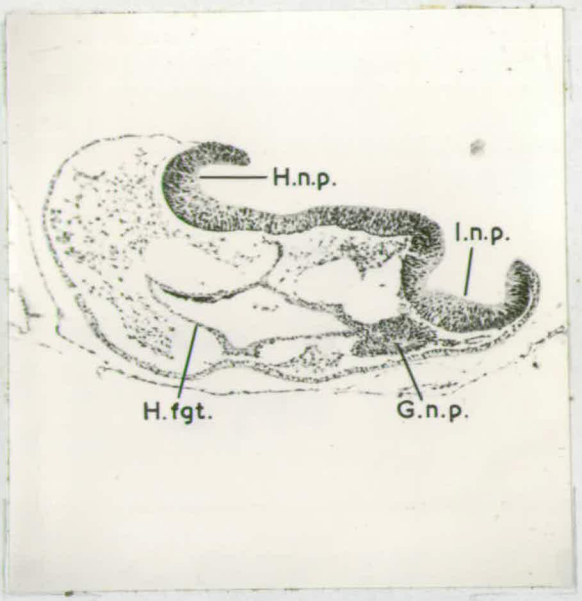

Fig. 19. $a^1 6$. Section (x 75, sides reversed).

$a^1 6$.

In the section, (fig. 19), the graft is represented by a mass of mesoderm? and above it is seen the induced neural plate continuous with the host neural plate.

b^1 and c^1 levels.

These levels represent the region 0.3 mm. to the right and left of the second anterior level of the primitive streak (a^1). The description of some of the grafts of each level is as follows:

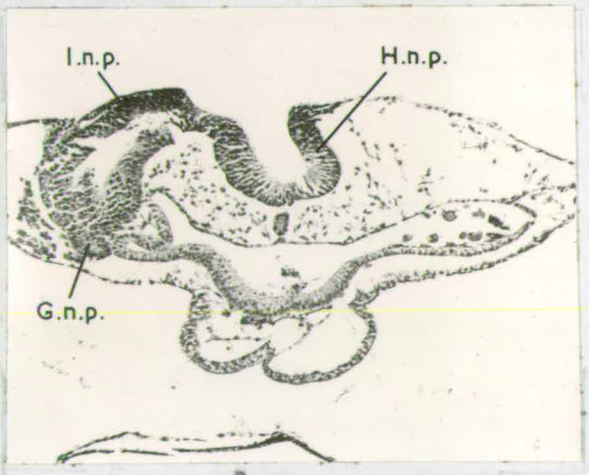

Fig. 20a. $b^1 25$. Section through anterior region. (x 80).

$b^1 25$/

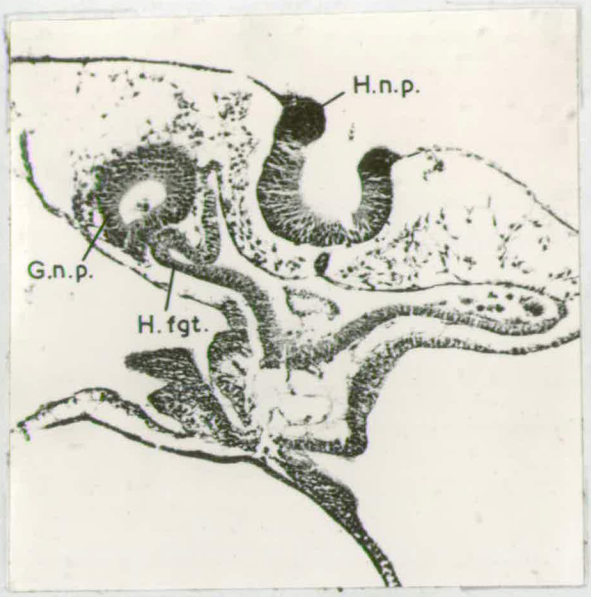

Fig. 20b. $b^{1}25$. Section of the same, slightly through posterior region. (x80).

$\underline{b^{1}25}$.

Length of the donor primitive streak 2 mm.

Length of the host primitive streak 1.7 mm.

Graft : In the anterior region of the host.

Two sections in the same slide may be studied. In the anterior one, (Fig. 20a), the graft is not seen to differentiate into a complete neural tube. The ectoderm of the host appears to form a slight thickening, suggesting a weak induction. In the slightly posterior section (Fig. 20b) the graft forms a complete neural tube; due to the presence of the graft the diverticulum of the foregut on that side is withdrawn and is divided into two short diverticula.

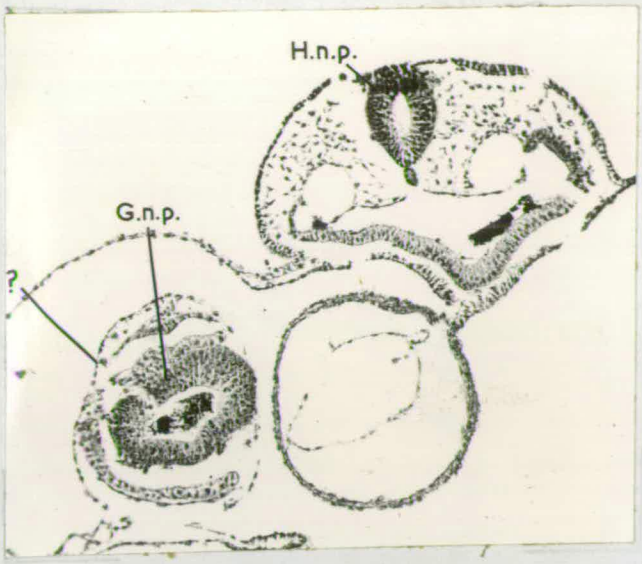

Fig. 21. $b^1 22$. Section. (x 80).

$\underline{b^1 22}$.

Donor primitive streak 2 mm.

Host primitive streak 1.8 mm.

Graft : To the left anterior region of the host.

The graft is differentiated into a complete neural tube (Fig. 21). A double layer of cells, separated by a narrow streak, is seen enclosing almost half of the graft. The nature of this structure cannot be defined.

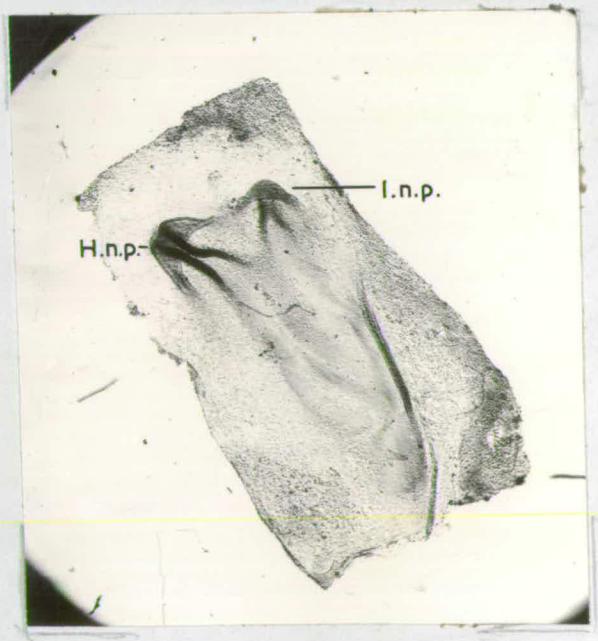

Fig. 22a. $c^1 15$. Whole mount showing two embryonic axes. (x 10 , sides reversed).

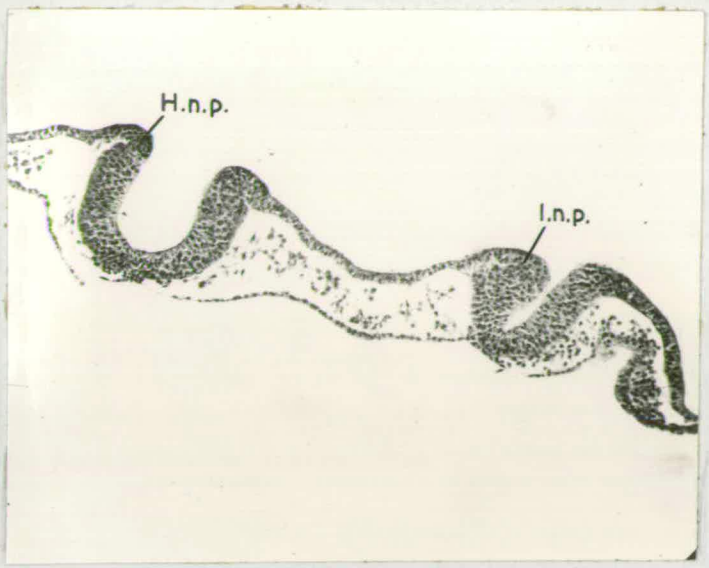

Fig. 22b. $c^1$15. Section of the same through neural plates. (x 70, sides reversed).

$c^1$15.

Donor primitive streak 1.8 mm.

Host primitive streak 1.2 mm.

Graft : In the left anterior region of the host.

A well induced neural plate is seen in the entire specimen, (Fig. 22a). In the section, (Fig. 22b), the induced neural plate is seen far apart from that of the host. No trace of graft is seen except a mass of mesoderm.

Interpretation of the results.

Out of twenty five grafts made from b^1 region, only one, (Fig. 20a), caused a weak induction and five differentiated into neural tissue. Thirty three grafts were from level c^1, out of which five caused inductions and eight differentiated into neural tissue. It seems probable that these levels have an inducing capacity, as has the node and the/

the anterior third of the primitive streak. Moreover, it seems that the inducing capacity at these levels is less on the right side than that on the left. Only one instance of weak induction is seen in b^1 graft in which the ectoderm of the host shows a slight thickening, but not formation of a neural plate. On the contrary, on the left side five inductions are caused. The frequency of differentiation and induction of the grafts on the right side, as shown in the table, seems to be less than that on the left. The superior developmental capacity of the left grafts over the right has been described by Rawles (1936, 1943). The asymmetry shown in the early stages of development may be the manifestation of the asymmetry of structures seen in the adult bird.

Third level of the primitive streak (a^2).

This region represents a square of about 0.3 mm. posterior to the second level of the primitive streak (a^1). Eight grafts were made, out of which three gave inductions.

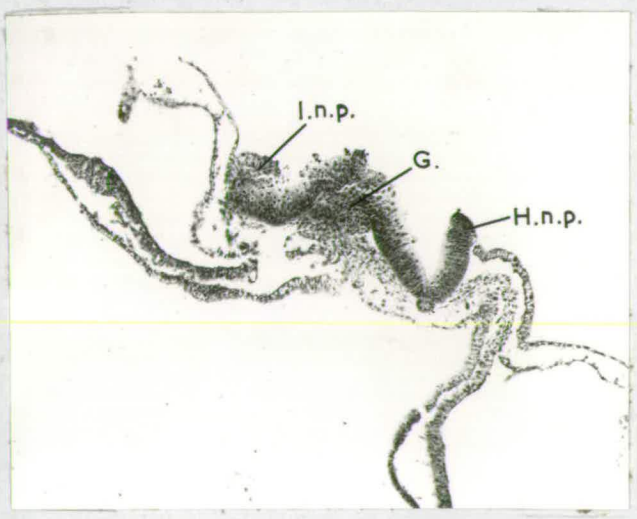

Fig. 23. a^2_3 section. (x 75).

a^2_4

a^2_3 Unfortunately, the section was damaged but by the side of the host was seen the induced neural plate (Fig. 23). Between the host and the induced neural plate is a group of mesodermal cells, probably representing the graft.

b^2 and c^2 levels.

These levels correspond to the squares about 0.3 mm. to the right and left of the anterior third level (a^2) of the primitive streak.

b^2 grafts.

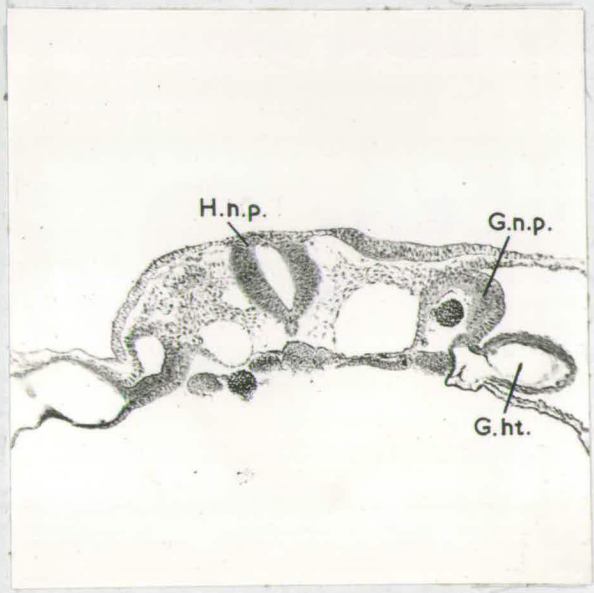

Fig. 24. $b^2 14$. Section. (x 70).

$b^2 14$.

In section (Fig. 24) the graft seems to have differentiated into a neural plate and heart. The ectodermal thickening between the host and graft neural plate may be the auditory thickening.

e^2 grafts.

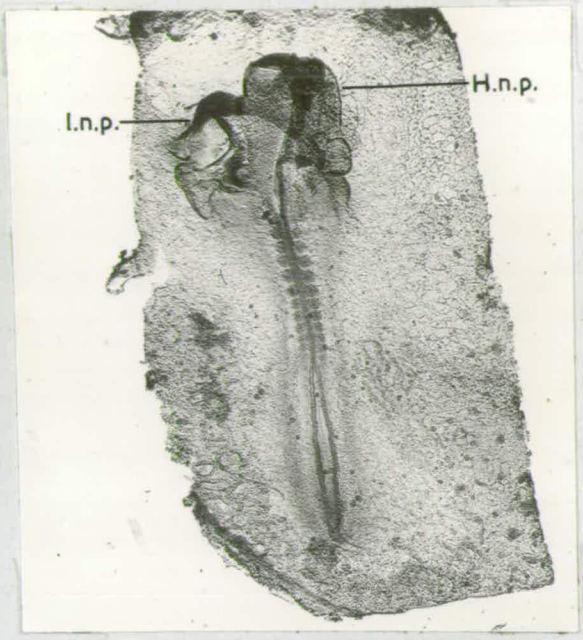

Fig. 25a. $e^2 5$ whole mount. (x 10).

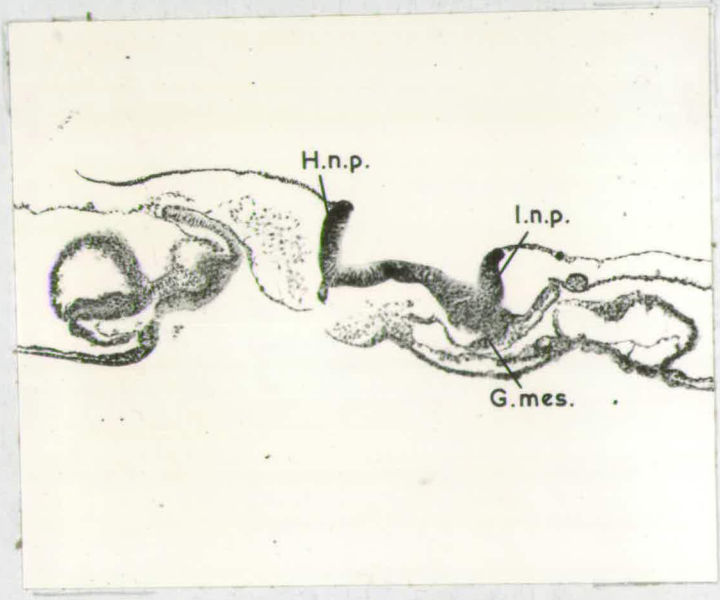

Fig. 25b. $e^2 5$ section of the same through the heart region. (x 70, sides reversed).

$e^2 5$.

Donor primitive streak 1.5 mm.

Host primitive streak 1.8 mm.

Graft : In the left anterior region of the host.

In/

In the entire specimen, (Fig. 25a), as well as in the section, (Fig. 25b), an induced neural plate can be seen attached to that of the host in the hindbrain region. No neural tissue has developed in the graft, but it is seen in section as a strand of mesoderm beneath the induced neural plate. It seems that the presence of the graft in the heart region has upset the formation and extent of that organ in the host.

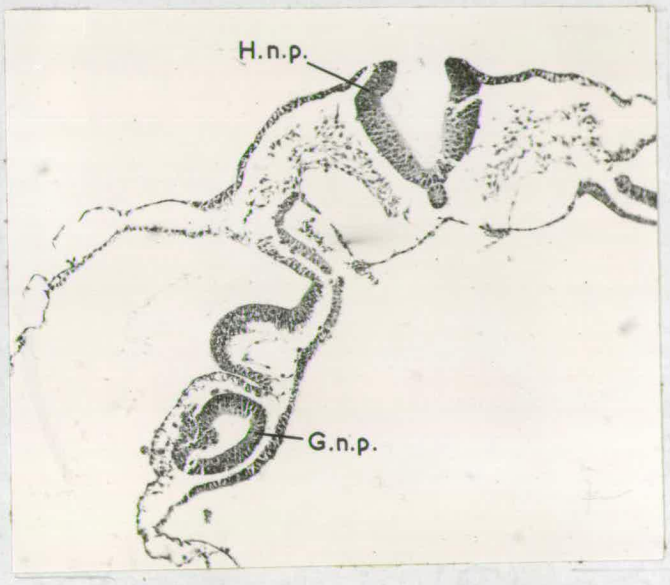

Fig. 26. $C^2$11. Section. (x 70).

$\underline{C^2 11}$.

Donor Primitive streak 2 mm.

Host primitive streak 1.9 mm.

Graft : In the left anterior region of the host.

In the section, (Fig. 26), the graft is seen to have differentiated into an almost complete neural tube. Round about are a few scattered mesenchymal cells. This is a good instance which shows that the neural tissue can develop about 0.6 - 0.9 mm. behind the node. The posterior extension of the neural tissue is considered in detail in the discussion.

TABLE NO. 1.

Table showing the differentiation of the axial and lateral grafts and their capacity for induction.

Level of the graft	No. of cases grafted	No. of inductions	No. of grafts differentiated	Differentiation of graft				Structure of the induced embryo when present	
				Neural plate	Noto-chord	Meso-derm	Heart	Neural Plate	Noto-chord
HMa	31	2	16	11	-	+	8	+	-
HMb	10	-	6	4	-	+	3	-	-
HMc	14	-	7	5	-	+	1	-	-
Node level a	20	17	13	10	+ in some	+ in some	+ in some	+	+ in some
b	30	2	12	8	-	+ in some	3	+	-
c	39	3	18	14	1	+ in some	3	+	-
a^1 Second level of the primitive streak	16	9	7	6	-	+	-	+	
b^1	25	One instance of weak induction	9	5	-	4	3	-	-
c^1	33	5	12	8	-	+	2	+	-
a^2 Third level of the primitive streak	8	3	3	1	-	+	-	+	-
b^2	16	-	3	2	-	+	-	-	-
c^2	13	1	4	3	-	?	-	+	-

\+ Positive
\- Negative

Interpretation of the results.

Out of sixteen grafts made from b^2 level none caused induction and only two differentiated into neural tissue. Thirteen grafts were made from c^2 level out of which one caused induction and three differentiated into neural tissue. These levels also seem to possess an inducing capacity. In one case a perfect neural tube seems to have differentiated from these levels (0.6 - 0.9 mm. behind the node) as seen in Fig. 26. Perhaps it might have been formed by regulation of the graft. The point about the posterior extension of the neural tissue will be considered in the discussion. Here again the frequency of differentiation of neural tissue and of induction in the right grafts seems to be less than that in the left grafts.

The results of the differentiation of grafts at various median and lateral levels and their capacity for induction are given in Table 1.

Peripheral grafts.

bb and cc levels.

These grafts were taken 0.4 - 0.6 laterally from the first level (node level a) of the primitive streak on right and left respectively. Out of eight grafts of bb level, one differentiated into mesenchyme. Fourteen grafts of cc level were made, out of which three differentiated either into mesenchyme or myoepicardium. One of each is described below.

Fig. 27. bb3. Section through the graft.
(x 320).

bb3.

Donor primitive streak 2 mm.

Host, early head process stage.

Graft : In the right anterior region of the host.

In the section, (Fig. 27), the graft differentiated into a mass of mesenchyme arranged round the circular cavities.

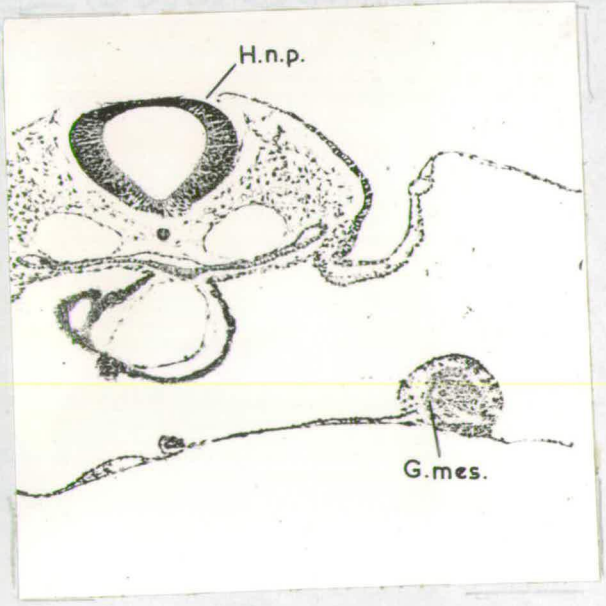

Fig. 28. cc14. Section (x 80, sides reversed).

cc14/

cc1/4.

Donor primitive streak 2 mm.

Host primitive streak 1.9 mm.

Graft : In the left anterior region of the host.

Here also in the section, (Fig. 28), the graft differentiated into a small round mass of mesenchyme.

b^1b^1 and c^1c^1 levels.

These regions are 0.4 - 0.6 mm. on the right and left sides of the second level of the primitive streak respectively. Seven grafts of b^1b^1 level were made, out of which two differentiated into myoepicardium. Out of nine grafts from c^1c^1 level, two differentiated into myoepicardium and mesenchyme.

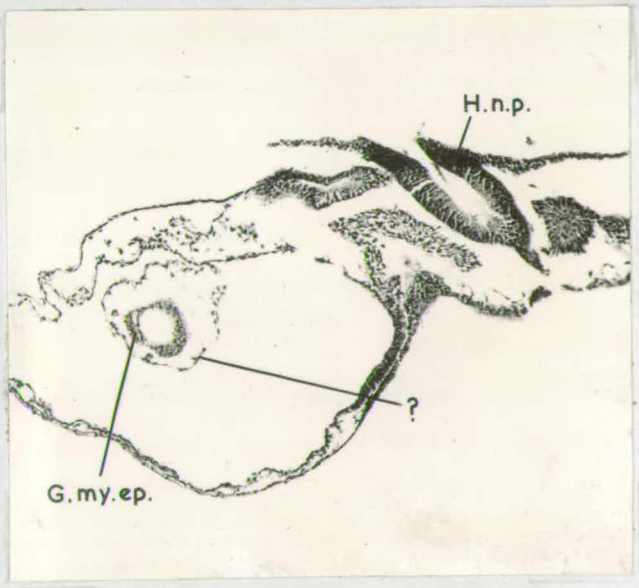

Fig. 29. $b^1b^1$1. Section (x 80).

$b^1b^1$1.

Donor primitive streak 2 mm.

Host primitive streak 1.7 mm.

Graft : In the left anterior region of the host.

In/

In the section, (Fig. 29), the graft differentiated into a round thin-walled structure surrounded by a thin layer of mesenchyme. It was beating at the time of fixation, thus it may perhaps be the myoepicardium. The surrounding layer looks like a layer of somatic mesoderm, the nature of which cannot be said definitely.

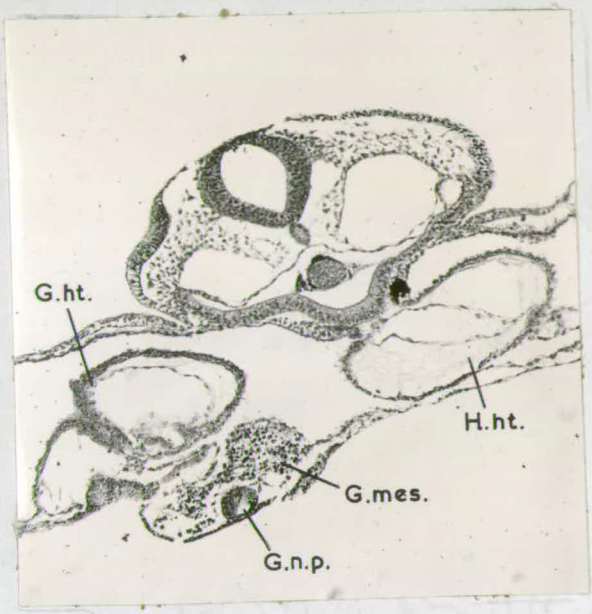

Fig. 30. $c^1c^1_4$. Section (x 80).

$c^1c^1_4$.

Donor primitive streak 2 mm.

Host primitive streak 1.9 mm.

Graft : In the left anterior region.

The graft, (Fig. 30) seemed to have differentiated into a well developed heart, (endocardium and myoepicardium), and a group of mesenchyme. Beneath the mesenchyme is seen a small, yet complete, neural tube. It appears to have been formed by regulation. The heart derived from the graft is almost as big as the heart of the host.

b^2b^2 and c^2c^2 levels

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b^2b^2 and c^2c^2 levels.

These levels are 0.4 - 0.6 mm. on the right and left respectively of the third level of the primitive streak (a^2). Six grafts of b^2b^2 level were made, out of which two differentiated into mesenchyme or myoepicardium. Out of eleven grafts of c^2c^2 level, three developed into myoepicardium or mesenchyme.

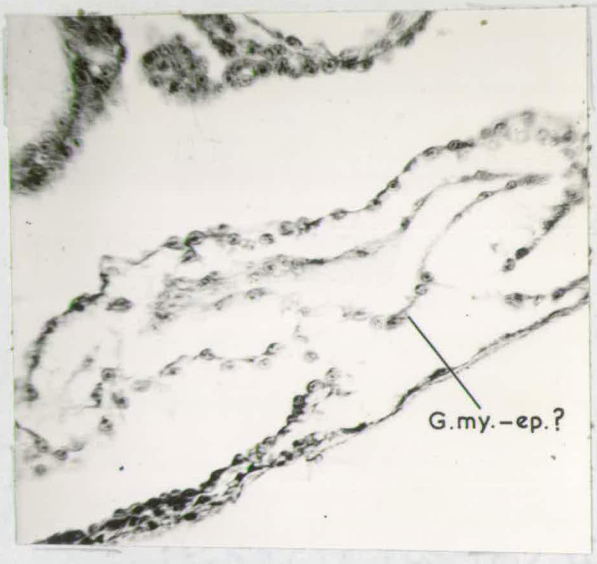

Fig. 31. $b^2b^2$3. Section through the graft. (x 270).

$b^2b^2$3.

A very small graft was seen to have arranged itself into a thin layer surrounding a cavity, (Fig. 31). The thin layer probably represented the myoepicardium.

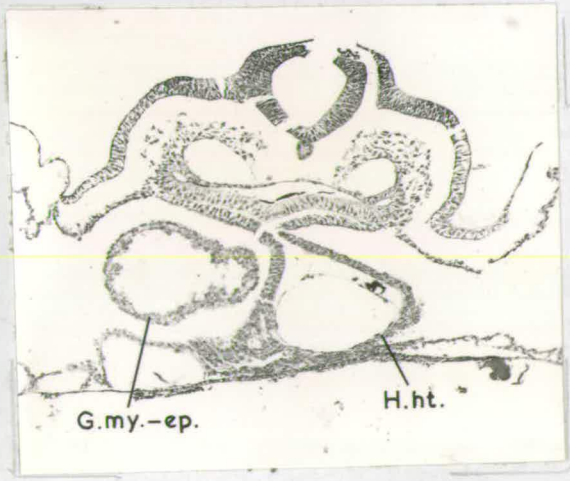

Fig. 32. $c^2c^2$8. Section. (x80).

$c^2c^2$8/

TABLE NO. 2.

Table showing the differentiation of the peripheral grafts and their capacity for induction.

				Differentiation of graft		
Level of graft	No. of cases grafted	No. of inductions	No. of grafts differentiated	Neural tissue	Mesenchyme	Myoepicardium
bb	8	-	1	-	+	
cc	14	-	3	-	++	+
b^1b^1	7	-	2	-	+	+
c^1c^1	9	-	2	+ in one	++	+
b^2b^2	6	-	2	-	+	+
c^2c^2	11	-	3	-	+	+

\+ Positive

\- Negative

$c^2c^2_8$.

The graft in the anterior sections seemed to have differentiated into a mass of mesenchyme. In a few sections behind the anterior ones, the mass arranged itself into myoepicardium surrounding an oval cavity full of clot, (Fig. 32). The endocardium (primordium of the heart) is not found.

Interpretation of the results.

The peripheral grafts are difficult to distinguish from the tissues of the host. The summary of the results is given in Table No. 2. It seems from the Table given that none of the peripheral grafts have a capacity for induction. None have formed neural tissue, except in one instance $c^1c^1_4$, (Fig. 30), where a small, though complete, neural tube is seen, probably formed by regulation.

It appears that the peripheral grafts differentiate mostly into myoepicardium or mesenchyme, (Figs. 27, 28, 29 and 30).

The formation of heart at the definite streak stage blastoderm has been studied by Hunt (1932) using chorio-annantoic method. Rawles (1936, 1943), using the same method, mainly considered the differentiation of heart in the head process stage blastoderm; while Ebert (1953) using immuno-chemical technique, detected the presence of heart protein (cardiac myosin) in the various parts of the primitive streak stage blastoderm. The details regarding the formation of heart in the definite streak stage blastoderm will be considered in the discussion. But it seems probable that the area of heart formation in the primitive streak stage is either widely extended, or that in the various peripheral grafts/

grafts where the heart is differentiated, it is formed by the processes of regulation.

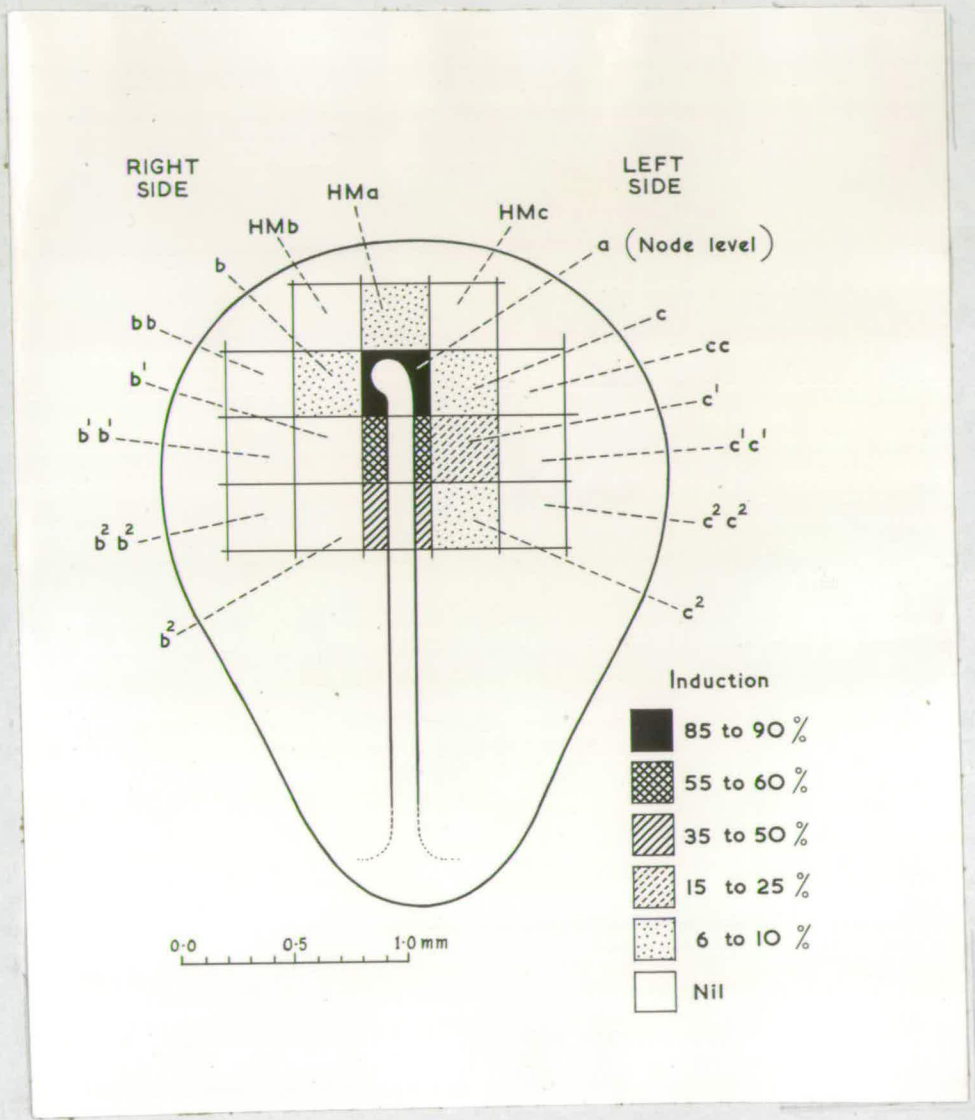

Fig. 33. A map showing an extent of the organiser in a blastoderm of a definite streak stage. (Ventral view).

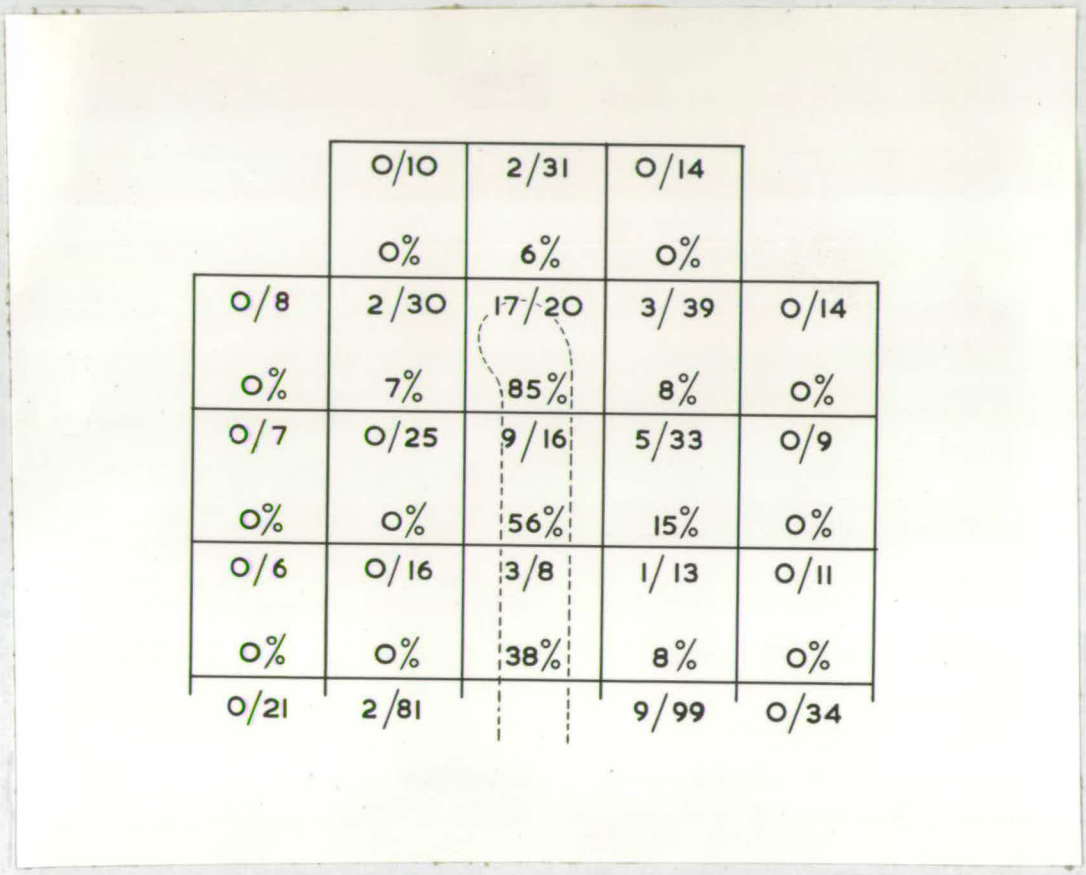

Fig. 34. Graphic representation of Fig. 33.

List of abbreviations.

c cavity

fgt. foregut

hd. head

ht. heart

mes. mesenchyme

my.ep. myoepicardium

n notochord

n.p. neural plate

s somite

T tubule lined by endoderm

The prefixed letters H., G., and I denote the structures of the host, graft and of the induced axis respectively.

Discussion.

From the results of the present work it seems probable that besides the anterior thirds of the primitive streak, the regions at least about 0.3 mm. in front and to the side of it have an inducing capacity, though to a much lesser extent. This does not seem unlikely from consideration of the results obtained by Abercrombie (1950), who found that after reversal of a variety of different parts of the streak many cases of regulative development were obtained, showing that the primitive streak is subject to control by the surrounding blastoderm. Bautzmann (1926) also found that in Amphibia the organising capacity is not restricted to the blastopore along, but is spread over a quadrant of the egg.

On the basis of the results obtained in the present work, a map showing the extent of organiser in the chick blatoderm at the definitive streak stage may be presented. It (Figures 33 and 34) shows that the frequency of induction is highest at the node of the primitive streak, and falls off along the axis as well as laterally. It seems that there is an antero posterior and a medio-lateral gradient of inducing activity. The gradient theory was applied in connection with the organiser by Child (1915). Child proposed the metabolic gradients, especially the respiratory gradients to explain the inducing capacity of the organiser in particular. Buchanan (1926), Hyman (1927), Hinrichs (1927), Rulon (1935), Moog (1943a, b) all supported him. Besides the metabolic gradients, gradients in different chemical substances have been pointed out by Jacobson (1938b), Brachet (1940, 1944), Spratt (1947b), and others. From the present work nothing can really be said about the nature of the gradients. It can only be said that the regions (b, c, c^1) near the

notochord seem to have a greater inducing capacity than the parts (bb, b^1b^1, cc, c^1c^1, etc.) away from it.

It is interesting to note that the frequency of induction as well as of differentiation, as shown in Table (1), in the grafts on the right b^1, b^2 etc., is less than that at the corresponding levels on the left (c^1, c^2 etc.). The grafts on the right at these levels,(with a single instance in each at b^1 and b^2 showing weak inductions, Figs. 20a and 24)), have not caused any induction. It is probable that the asymmetry shown in the adult bird is seen even at such an early stage of development. Reference to the asymmetry of the node at the primitive streak stage, has been made by Jacobson (1938b). The superior developmental capacity of the grafts from the left over the right, of the head process blastoderm has been shown by Rawles (1936, 1943), and Rudnick (1932), with chorio allantoic technique.

A correlation between the induction and the self-differentiation of graft into neural tissue is presumed by Waddington (1952a). The present results agree in some respects with this suggestion. The more peripheral grafts, bb, b^1b^1, b^2b^2, and cc, c^1c^1, c^2c^2 etc., (Figs. 27, 28, 29, etc.) which have never differentiated into neural tissue have not caused any induction, while some grafts b, c, c^1 etc., close to the anterior levels of the primitive streak have done so. (Figs. 11, 15, 22a). At the same time it must be noted that some grafts (Figs. 7, 16, 18, etc.) which have differentiated well have not produced inductions. It is further suggested by Waddington (1932, 1952a), and many others/

others (Hoadley 1927, Gräper 1929, Wetzel 1929, Umanski 1931, Dalton 1935) that the induction is primarily due to the axial mesoderm. It is thus worthwhile discussing the position and extent of the various presumptive tissues with the help of the results obtained in the present work. But it must be remembered that in any isolation experiments (chorio-allantoic, in vitro, intrablastodermal grafts) there may be regulation of the isolate, and that organs may appear from regions which are not their presumptive origin. With this caution in mind, an attempt will be made to discuss the position and extent of the various presumptive tissues.

The localisation of the presumptive tissues in the early and definite streak stages has been mapped out by several investigators, using different methods. Gräper (1929), Wetzel (1929) and Pasteels (1937) used the vital staining technique. Waddington (1932) on the basis of the results of his experiments, isolating the different pieces and culturing them in vitro, combined the maps of Wetzel and Gräper and gave sketches for the distribution of the presumptive areas. Rudnick (1938 a, b, c) used the in vitro technique to obtain her own maps. Spratt (1942, 1947 a, b, 1952, 1955) using several methods such as of carbon marking, vital staining etc., published his maps for these areas. Very recently Waddington (1952a), in view of the results of the recent researches, has proposed new maps. Besides these Kopsch (1927, 1934), Jacobson (1938b), and Rawles (1936, 1943), have also contributed to the mapping of the presumptive areas in the chick blastoderm. It is beyond the scope of the present work to discuss all the maps; only some of the important/

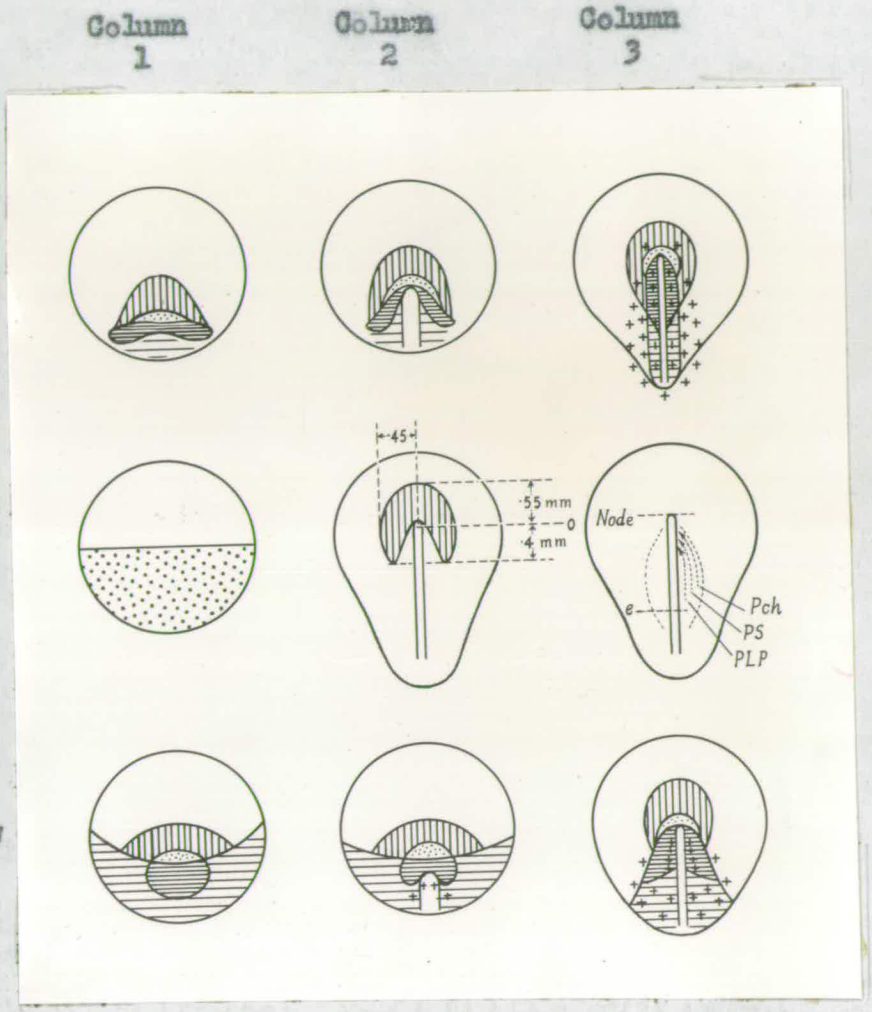

Fig. 35. Maps of the presumptive areas in the chick.

Top Row. After Pasteels (1937).

Middle Row. After Spratt (1942, 1947b, 1952, 1955).

Bottom Row. After Waddington (1952a).

Column 1. Pre-streak stage.

Column 2. Early streak stage (except in middle row, column 2).

Column 3. Long streak stage. (also in column 2, middle row).

White represents presumptive epidermis: vertical lines, neural tissue: dots (except in middle row, column 1) notochord: close horizontal lines, axial mesoderm.

important ones will be discussed.

The maps given by Pasteels (1937), Spratt (1947 a, b, 1952, 1955) and by Waddington (1952a) are shown in the figure (Fig. 35). The map of the definite streak stage can best be understood by studying the early stages. It appears from the figures that they differ essentially in the shape and extent of the presumptive areas, and in the interpretation of the invagination movement.

Presumptive neural tissue.

In Pasteels's and Waddington's maps the presumptive neural tissue seems to occupy only a small portion in the centre of the early blastoderm. On the other hand, Spratt (1942) obtained neural tissue from the anterior half of the prestreak blastoderm, even when the cut was 0.88 mm. away from the posterior border of the area pellucida.

In the definite streak stage, according to Pasteels, the presumptive neural tissue is crescentic in shape, the two arms tapering off and extending towards the middle of the streak. In Spratt's map, the neural plate is "oval in outline (the node lying in the centre), extends 0.25 ± anterior 0.4 ± posterior and 0.45 ± lateral to the primitive pit" (Spratt 1947b, 1952). Moreover, Spratt has claimed to have seen the presumptive neural plate in the definite streak blastoderm stained with haematoxylin, as a more darkly staining region occupying the same position and extent. It was pointed out by Jacobson (1938b) that, due to the higher lipoid contents, the area of the presumptive neural plate could be demonstrated with histochemical stains. In Waddington's maps the presumptive neural area is in a shape of/

of a horseshoe, the two arms extending across about one third of the streak.

No measurements are given either by Pasteels or by Waddington, and nothing could really be said regarding the extent of this area.

In the present work the neural differentiation is obtained in the region HMa, (Figures 4 and 5), (0.3 mm. in front of the node level), HMb and HMc (Figures 7 and 8), (0.3 mm. adjacent to HMa) and up to 0.6 - 0.9 mm. (Figure 26) behind the node level of the primitive streak. The posterior extension of the neural tissue in the definite streak stage has been studied by several investigators. Wetzel (1924) at first failed to get the neural tissue from the posterior pieces behind the node, though later on (1926) he could get it in the more posterior pieces. Hunt (1932) using the chorio-allantoic technique of culture could not obtain neural differentiation beyond the node level, though Dalton (1935) using the same technique succeeded in getting neural differentiation about 0.5 mm. beyond the node. Waddington (1935) obtained the neural tissue <u>in vitro</u> as far back as 0.7 mm. posterior to the pit. However, Rudnick (1938b) and Spratt (1952) using the <u>in vitro</u> technique found that neural plate never developed in posterior pieces cut about 0.4 mm. behind the pit. As mentioned above, the region 0.6 - 0.9 mm. behind the node has formed perfect neural tube (Figure 26), and not only that but one of the grafts at the same level has caused induction as well (Figure 25a). Waddington (1952a) has presumed the boundary between the inducing and non-inducing region to be shown by the limit at which the neural tissue

can/

can develop in vitro; however, Abercrombie and Bellairs (1954), and ul Islam A. (1953), have reported inductions through the posterior third of the primitive streak, which is the site of presumptive lateral plate mesoderm and which itself has not been described as forming neural tissue so far. Induction through the side plate mesoderm has also been demonstrated in Amphibia by Holtfreter (1933) and by Waddington (1936).

The capacity for self-differentiation independently of the mesoderm has been shown only in the case of forebrain by Waddington (1932), Rudnick (1938b) and Spratt (1942, 1947c). In the present work, the graft HMa (Figure 4), (0.3 mm. in front of the node) which was labelled with radio-active methionine clearly shows the self-differentiation of the graft (forebrain) independently of the mesoderm. However, besides the forebrain no other part of the central nervous system is described so far to possess this capacity and thus the differentiation of neural tissue at the posterior levels, as suggested by Waddington, may be due to its inductions from the underlying mesoderm. Similar results have been reported in Amphibia by Holtfreter (1948). Thus the definite limit of the presumptive neural area cannot be stated with accuracy from the techniques available at hand at the present moment.

Presumptive axial mesoderm.
(somites and notochord).

In the early stages, the area of presumptive mesoderm shown by Pasteels as well as by Wetzel (not shown in the figure) is very small compared to that shown by Waddington/

Waddington or Spratt. According to Spratt (1946), the entire posterior half of the pellucid area at the pre-streak stage is represented as prospective mesoderm. These differences are due to the different interpretations of the process of invagination which is beyond the concern of the present discussion. In the definite streak stage, the presumptive axial mesoderm in Pasteels's map is seen as a narrow band occupying more than half the length of the streak. This corresponds more or less with the position and extent of presumptive axial mesoderm shown by both Gräper (1929) and Wetzel (1929). In Spratt's map (1955) the prospective axial mesoderm area seems to extend across the posterior two-thirds of the primitive streak as Wetzel (1929) and Pasteels (1937) found. Spratt (1955) makes a further distinction between the somite centre, restricted to about 0.1 - 0.2 mm. posterior to the primitive pit and the prospective somite cells found as far as 0.6 - 0.8 mm. behind the node. In Waddington's map the presumptive axial mesoderm appears to be in the form of a triangle, (the base of the triangle is situated about the middle of the streak), and seems wider in lateral extent than in Pasteels's map. Though at first sight there appears to be a difference between the maps of Pasteels and Waddington in the position and extent of the presumptive axial mesoderm, if we take into consideration the stages of the primitive streak in the two maps, we find that in Waddington's sketches, the primitive streak appears to be in an earlier stage of development than that shown in Pasteels's. By the time it reaches the length shown in Pasteels's figures, the position and/

and extent of the axial mesoderm shown by Waddington will be the same as shown by Pasteels.

In the present study, it is observed that the differentiation of somites and notochord, (to be described separately) typical structures of the axial mesoderm are rarely seen except in a few grafts at level c (Figures 16 and 17). The mesenchyme in these grafts tends to arrange itself into round masses appearing like somites. Apart from these the mesoderm in the rest of the grafts is seen to be scattered in the form of mesenchyme.

The tendency of the mesoderm to form the somites in some grafts at level c may be due to the fact that these grafts lie adjacent to the node level (a) of the primitive streak, which is perhaps the region of the notochord.

It has been pointed out by Spratt (1942) and Ul Islam A. (1953) that the somites are formed only in the region in which the notochord lies; outside the region of the notochord the mesoderm is represented by the mesenchyme. Such inter-relation between the chorda and somites has been suggested in Amphibia by Yamada (1940), although Abercrombie and Bellairs (1954), on the basis of their experiments in the chick, do not think that the differentiation of notochord is necessary for the initial formation of the parachordal mesoderm (the somites and head mesenchyme). However, the failure to obtain somites in the grafts at levels b^1, b^2, c^1, c^2 etc., does not mean the absence of axial mesoderm in these regions. Very recently it was suggested by Spratt (1955) that, although the prospective somite cells extend as far back as 0.6 - 0.8 mm. behind the node, the formation of these cells/

cells into somites depends on the presence of the somite centre which is localised 0.1 - 0.2 mm. behind the primitive pit.

The lateral extension of the axial mesoderm seems to correspond to a narrow band as shown in Pasteel's mapping scheme. The more peripheral grafts, bb, b^1b^1, b^2b^2, cc, c^1c^1, etc., in the present study have shown poor differentiation except the myoepicardium and, in some cases, endocardium as well. Regarding the notochord, in the early stages both Pasteels and Waddington have shown notochord in the posterior third quarter, between the centre and posterior edge of the area pellucida. Spratt has demonstrated the differentiation of notochord in early stages in a region of area pellucida lying between 0.88 mm. and 0.44 mm. Essentially it seems that they all agree that in the early stages the notochord is situated at the anterior end of the developing streak.

In the definite streak stage, the posterior extent of the notochord in Pasteel's map appears to be extended much behind that shown in Waddington's scheme, but here again the apparent disagreement is only because of the age differences. Moreover, Waddington (1932) has shown the presumptive notochord extending posterior to the primitive pit. According to Spratt (1947a) the median chorda material lies approximately 0.03 mm. behind the pit to 0.2 mm. anterior to it; and the chorda somite mesoderm extends from 0.13 - 0.3 mm. behind the pit and extends 0.3 mm. laterally to the streak. Recently Spratt (1955) has distinguished a chorda forming centre from the prospective notochord cells. The chorda forming centre lies beneath the primitive pit and extends anteriorly to the edge/

edge of the node. Rudnick (1944) also places the chorda material only at the node level of the definite streak.

In the present study, the notochord is not seen to differentiate in any region except one single instance, (Figure 16), in which it is formed from the region 0.3 mm. adjacent to the node level on the left (c). It is probably because the notochord lies in that region. Moreover, the graft was removed from the left side of the node level of the streak, it may point out the lateral position of the notochord.

It is also seen that the inductions (when caused with lateral grafts) in the present work were mostly neural inductions with little or no mesenchyme. The induced neural plate was not associated with notochord in any case, (Figures 15, 22b, 25b). It may be possible that the graft at these various lateral levels did not contain the region of the presumptive notochord and hence the absence of notochord in the induced neural plate is to be accounted for. It will then lead to the question, are the notochordal inductions specific? Or, in other words, are there different substances for the neural and mesodermal inductions? This question has aroused the interest of the investigators since Spemann (1931) found in Amphibia that the organiser shows regional differentiation, on the basis of which he classified it into head or trunk organisers. On the basis of specific effects caused by treating Triton's eggs with lithium at different stages Lehmann (1936, 1938) supported him. Waddington (1938, 1940) seemed to suggest among many possibilities a different concentration of the same chemical substance in the different parts of the organiser/

organiser, or in each organ a presence of a basic evocator and a modulator, the latter acting as specific substance. However, Shen (1939) using different concentrations of the same substance (the sodium endosuccinate salt of 1:2:5:6 - dibenzanthracene) did not get any regional differentiation. On the contrary, Lopachov (1935) obtained inductions of eye in the entire young gastrula, or in the explants through the grafting of a dead eye. Chuang (1938) reported specific inductions both in the ectodermal explants and the entire gastrulae of Amphibia by implanting pieces of Triton liver and mouse kidney. In the entire gastrulae the specificity was not marked due to the influence of host. Chuang (1940), further studying the effect of heat on the mouse kidney as well as on Triton liver, found that different periods of heating affect the nature of the induced structures. He thus maintained that there are specific substances for the neural and mesodermal inductions. The experiments of Barth and Graff (1943), and Toivonen (1953a, b), also suggest the specificity of inductions. A detailed account of work regarding the specificity of induction is given in the introduction. But in spite of several researches the question of specificity of induction yet remains unanswered.

Heart forming areas.

The maps of presumptive areas given by Wetzel or by Pasteels give us no indication about the presumptive heart areas. In the unincubated blastoderm, Olivo (1928), obtained differentiation of heart muscle in tissue culture from the peripheral regions. On the other hand, Butler (1935) using the same stage with chorio-allantoic technique found that the posterior/

posterior as well as anterior grafts could also differentiate into heart.

In vitro culturing experiments of Rudnick (1938c) and Spratt (1942) on the short streak blastoderm seemed to indicate that the heart forming area lies somewhere in the posterior half of the blastoderm.

In the definite streak stage, Hunt (1932) while studying the potencies of transverse levels of the chick embryo on chorio-allantois, found that the frequency of heart formation was greatest in the pieces containing the posterior node level that is about 0.3 mm. behind the primitive pit, and that the heart normally develops from that level while, in other levels, it arises by regulation. Rudnick (1938b) with the same stage of development, with in vitro culturing technique, reported heart differentiation as far back as .59 - 1.00 mm. behind the pit. The lateral extent of the heart in these experiments was not known as longitudinal cuts were not made. Later on, however, Rudnick (1948) in her maps has shown the heart area on either side of the node. No measurements are given, so nothing could be said regarding the lateral extent of the heart from that map. Rawles (1936, 1943), using chorio-allantoic technique, studied extensively the organ forming areas in the head process stage blastoderm. In that she has mentioned the mediolateral extent of heart in a few cases from definite streak blastoderm. According to her, heart differentiation can be found laterally as far away from the pit as 0.6 mm. The antero-posterior extent of the heart seems to be 0.2 mm. in frontand 0.4 mm. behind the pit. The medio-lateral and antero-posterior extent/

extent of the heart in the definite primitive streak stage corresponds more or less to that in the head process blastoderm. She has also pointed out the bilateral nature of the heart areas in consequence of the blood vascular system. Recently Ebert (1953) using immunochemical technique has detected the presence of cardiac myosin in various stages of chick embryo. He found that in earlier stages the cardiac myosin is of wide spread occurrence, though more concentrated in mid and posterior regions than the anterior ones. At the head process stage it is found in both the central and lateral pieces, though more concentrated in the latter than that in the former. In still later stages it is detected only in lateral regions showing the bilateral nature of the heart areas.

In the present study, differentiation of heart has come across in the grafts of various levels. To give a few instances, the graft HMb, (0.3 - 0.6 mm. lateral to HMa), (Figure 6a), has formed a beautifully developed heart beating at the time of fixation. It appears to be formed at a lower level than that of the host embryo; yet it seems to be of the same shape and size as that of the host. Hearts were seen to differentiate from the grafts at levels b, c, (Figures 12, 13 and 17), and from a number of peripherial grafts, b^1b^1, c^1c^1, c^2c^2, as shown in Figures 29, 30, 32. The peripheral grafts c^1c^1, c^2c^2, in which the heart muscle seems to develop are 0.6 - 0.9 mm. lateral to the various levels of the primitive streak, and some (c^2c^2, Figure 32) were 0.6 - 0.9 mm. behind the node. It appears from this that

the heart areas are probably widely extended at the definite streak stage or, in some of the grafts, heart might have been formed by regulation.

However, as pointed out by Waddington (1952a), "Our knowledge of the presumptive map is not accurate enough to enable certain conclusions to be drawn", and as such the interpretation of the results remains highly speculative.

Summary.

1. In order to test the extent of organiser in the chick embryo, blastoderm of the definitive primitive streak stage was cut into as many as 18 squares of about 0.3 mm., 4 median and 14 lateral, with Hensen's node as an origin. The inducing capacity of each small piece was tested by transplanting it into the area pellucida of the host blastoderm, (in the definitive streak stage), cultured by an *in vitro* technique.

In all 303 grafts were made and 270 histologically examined.

2. From the results obtained it seemed probable that the grafts at the level immediately in front of the node level, (0.3 mm - 0.6 mm), and (0.3 mm - 0.6 mm), laterally adjacent to the node and the anterior thirds of the primitive streak, had an inducing capacity though to a much lesser extent than the node level.

3. The results of the present work made it probable that the inducing capacity falls off not only along the long axis of the blastoderm but also medio-laterally. It appeared as if there were gradient in inducing capacity along the longitudinal and transverse axes of the blastoderm.

4. A map showing an extent of the organiser in a blastoderm of a definite streak stage is given.

5. The grafts at various levels causing induction or differentiation are described.

6. The frequency of induction and differentiation seemed to/

to be higher in the grafts on the left side of the blastoderm than that on the right. It may perhaps be the manifestation of the asymmetry shown in the adult bird.

7. Peripheral grafts, (bb, b^1b^1, b^2b^2, cc, c^1c^1, c^2c^2 in text) appeared to differentiate only into myoepicardium and endocardium.

8. The problem of determination in the chick blastoderm is discussed with reference to the results obtained.

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ABSTRACT OF THESIS

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Degree Ph.D. *Date* 1st November, 1956.

Title of Thesis Studies in the processes of determination in the Chick Embryo.

The discovery of organiser and organiser centre of Amphibia by Spemann (1918) opened a new field in the working of experimental embryology; but there were several technical difficulties to be overcome in the application of the conception of organiser (Spemann) to avian embryology.

Tissue culture technique has several advantages over the earlier methods of in situ sectioning or chorio-allantoic techniques; and much work has been done on the development of the chick using the same method.

It has already been proved that, in the chick, at least the anterior third of the primitive streak has an organising function, but regions outside the primitive streak have not been systematically tested for the inducing capacity. In the present studies, an attempt is made to study the extent of organiser in the chick embryo at the definitive primitive streak stage.

A blastoderm of the definitive streak stage was cut into a certain number of median and lateral squares, each of about 0.3 mm. area. The inducing capacity of each small piece was tested by transplanting it into the area pellucida of the host blastoderm of the same age, cultured by an in vitro technique.

From the results obtained, it seemed probable that the grafts at the level immediately in front of the node level, (0.3 mm. - 0.6 mm.), and (0.3 mm. - 0.6 mm.), laterally adjacent to the node and the anterior thirds of the primitive streak, had an inducing capacity, though to a much lesser extent than the node level. Moreover, it appeared as if there were gradient in inducing capacity along the longitudinal and transverse axes of the blastoderm. The frequency of induction and differentiation seemed to be higher in the grafts on the left side of the blastoderm than that on the right. It may perhaps be the manifestation of the asymmetry shown in the adult bird. Peripheral grafts appeared to differentiate only into myoepicardium and endocardium.

The problem of determination in the chick blastoderm is discussed with reference to the results obtained.

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