The Two-gradient Hypothesis in Primary Induction. 
The Combined Effect of Two Types of Inductors 
Mixed in Different Ratios

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With one plate

On the basis of certain earlier suggestions made by Lehmann (1950) and Yamada (1950), together with our own experimental data, a modification of the two-gradient hypothesis of primary induction was presented by us some years ago (Toivonen & Saxén, 1955). Subsequently, this theory has often been referred to, accepted or criticized, and even misunderstood. There may thus be reasons for discussing it in the light of some recent experimental data.

At present there are limits to our opportunities of studying what is obviously the most important point in embryonic induction, the induction process itself. Simultaneously with such experiments on the induction process it is therefore necessary to continue research work on classical lines, and to obtain a further clarification of the causal relationships between the inductor and its morphogenetic action. A variety of qualitative investigations in this category have been made, but conceptions of the different quantities and the ratios of the active agents which participate in the primary induction are still based on indirect data. This is due to the lack of suitable methods for the testing of these compounds—which are still more or less hypothetical—in different quantities. A few attempts have been made in this direction, but the results are not quite conclusive (Shen, 1939; Niu, 1958): the complicated methods employed, the use of toxic materials, and the fractionation of inductively active samples may give rise to a variety of technical errors in experiments in which some chemical fractions are administered in different concentrations or amounts, either by adding them to the culture media or by incorporating them in inactive materials.

In the present experiments an attempt was made to approach the problem of the significance of the quantities of the inductors by employing the simplest possible method, i.e. by mixing two inductors with different inductive actions in different ratios, and then examining their combined action. The neatest way would have been to mix two chemical fractions of known inductive action, but the great number of unknown factors in such experiments (e.g. the degree of

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homogeneity of such mixtures, the release of the agents from certain materials used for their incorporation, &c.) made us decide to abandon this possible course of action. Instead, cells cultivated in vitro were used, both without pretreatment, and with a heat pretreatment known to inactivate the deuterencephalic and spinocaudal inductive action of the cells.

METHODS

HeLa-cells cultivated in vitro were chosen for the inductor material for two reasons. A homogeneous mixture of the cells could be expected according to preliminary results (Toivonen, Saxén, & Vainio, 1961) and according to certain control experiments (see below). Furthermore, the mixture could be used as such in implantation experiments without use being made of binding or incorporation material with an unknown effect on the process. Finally, the regulation of different quantities of the components in the experiments required no alterations in the size of the implant, which might otherwise have resulted in differences in the contact surface between the inductor and the reactive ectoderm. HeLa-cells had been used in a number of earlier experiments in our laboratory, and were known to be strong spinocaudal inductors (Saxén & Toivonen, 1957, 1958).

HeLa-cells were cultured in a standard culture medium containing 30 per cent. of human serum and 70 per cent. of Hanks's solution (see Saxén & Toivonen, 1958; E. Saxén & Penttininen, 1961). From our earlier experiments we knew that the spinocaudal inductive capacity of these cells was dependent on certain environmental factors, and especially on the pretreatment of the serum. In subsequent experiments we noted that during prolonged cultivation in the same, untreated, serum the inductive capacity of the cells changed (unpublished). Accordingly in the present experiments the culture medium was always changed 24 hours before using the cells, and only fresh, pooled human serum was used. In other respects the cultivation and the treatment of the cells did not differ from the methods employed in our earlier experiments (Saxén & Toivonen, 1958).

After 24 hours of cultivation in the fresh medium, cells from three Roux flasks were mechanically released, brought together, and centrifuged at 3,000 rev./min. The sediment was washed three times with Hanks's solution, and centrifuged after each rinse. Finally, the cells were suspended in 20 c.c. of saline, shaken to form a homogeneous suspension, and divided into two equal parts. The cells were counted from samples of these two halves. Subsequently, one-half of the cells was heated on a water-bath for 30 minutes at 70°, the other half being kept meanwhile in the culture incubator at 37°. These two cell suspensions were finally used for making the different mixtures of inductors.

Non-heated and heated cells were separately tested for their inductive effect. In addition, these two suspensions were mixed in the following ratios: 9 to 1, 7 to 3, 1 to 1, 3 to 7, and 1 to 9. The mixing of the cells in these experiments was performed as follows: both cell suspensions were shaken to a homogeneous suspension in 10 c.c. of saline. From these paired suspensions, 1 and 3 c.c. were
exchanged, the combined suspensions being repeatedly shaken and microscopically examined. In conclusion, each suspension was centrifuged for 10 minutes at 5,000 rev./min., the sediment being covered by cold, 70 per cent. alcohol and kept in a refrigerator for 4 to 8 hours at +4° C. After rinsing in Holtfreter's solution, small pieces were cut from the compact cell mass to serve as implantation material for 10 to 15 operations.

To check the degree of mixing of the cells, i.e. the homogeneity of the centrifuge sediment of a combined cell suspension, the following experiment was carried out. Before pretreatment, the culture media of HeLa-cells was transferred into Ringer's solution containing C-14 labelled algal protein hydrolysate (CFB-25, from the Radiochemical Centre, Amersham, England). After 6 hours of cultivation in this medium the cells were removed, suspended in 10 c.c. of saline, rinsed, and divided into two halves. One-half was again heated as described above, the other half being kept meanwhile in the incubator. Simultaneously, the same quantity of cells was cultivated in non-labelled Parker's solution 199, and after division into two halves, treated like the labelled ones. Heated and non-heated cells from these two cultures were combined 1 to 1. Consequently two combined inductors were again obtained:

(a) 1 part heated, C-14 labelled cells to 1 part non-heated, non-radioactive cells;
(b) 1 part heated, non-radioactive cells to 1 part non-heated, C-14 labelled cells.

After centrifugation and alcohol treatment these suspensions were used as implantation material. The implanted embryos were fixed after 24 hours of cultivation at a late neurula stage, sectioned serially and covered by a stripping film (Kodak AR 10). After 3 days of exposure the films were developed and the distribution of labelled and non-labelled cells examined.

Young gastrulae of *Triturus vulgaris* were used as host animals in all the experiments here described. After implantation of the combined inductors the embryos were cultivated for 11 days in an incubator at 18° C. Serial sections were made from the larvae and they were stained and examined under the microscope.

In the quantitative estimation of certain induced structures *camera lucida* drawings were used, made with a magnification of ×250. The drawings were made from complete series of 15 μ sections, 10 larvae in each series. To avoid the 'host influence' the larvae were selected according to the region of the secondary structures—all inductions here were located in the heart-liver region. Only the muscle tissue was drawn, and the total area of these drawings was measured by planimeter.

In each record of the secondary structures only the presence or absence of the structures was noted, and only in the case of muscle tissue was an attempt made to estimate the amount or volume of the tissues induced. In other words, the analysis of the series is qualitative in nature, and gives no information on the 'strength' of the induction.
RESULTS

Autoradiographic examination of the inductor

When a mixture of C-14 labelled and of non-radioactive HeLa-cells was used as inductor and the sections were covered by stripping film, 3 days' exposure was sufficient for demonstration of the labelled cells in an autoradiograph. Examination of the autoradiographs (Plate) strongly hinted at an almost homogeneous mixture of heated and non-heated cells in the inductor.
After the 24 hours' cultivation of the host, only a very slight release of the labelled compounds was noted by employment of the present methods, and the non-labelled, killed cells obviously did not incorporate these labelled compounds. Hence, the two cell types were distinguishable, and showed a homogeneous mixture.

**Text-Figs. 3 and 4.** Incidence of a secondary spinal cord and mesodermal structures in the experimental series.

**Distribution of secondary structures in the series**

In each experiment about 50 operations were performed. Some embryos died during the subsequent cultivation, and some were malformed and discarded. The final results are based on the following number of larvae:
In all series the total induction percentage was 100. The distribution of archencephalic, deuterencephalic, and spinal structures, together with the incidence of mesodermal formations in the different series, is shown in Text-figs. 1-4. In addition, the original records of one series (1:1) are given in Table 1. These results are analysed below.

Quantitative estimation of the secondary muscle tissue

As already mentioned, the above results show only the qualitative analysis of the distribution of the secondary structures, as in most of the previous studies. Earlier, some authors have tried to estimate the volume of induced structures (Chuang, 1955; Nieuwkoop, 1958), and a similar approach was made in the present study. The different regions of the central nervous system can hardly be used in such estimations because one cannot distinguish their borders in induced and often less organized formations. The mesenchyme also is not suitable for such calculations, and only occasionally was notochord induced in our series.
The original records of the microscopical examination of 47 larvae induced by non-heated and heated cells mixed in ratio 1 to 1

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Consequently, muscle tissue seemed to offer the only possibility for quantitative studies. The results are shown in Table 2 and Text-fig. 5. The variation in size of the secondary muscle formations was very great. In all series in which muscle
tissue was induced, there was observed a wide range from very small 'muscle fibres' to large masses of $50 \times 10^{-3}$ mm. Simultaneously, with an increasing rate of muscle induction (Text-fig. 4), there seems to be a slight increase in the volume of this tissue. This can be seen as a decrease in the number of very small inductions, and as a slight increase in the mean volume.

**Table 2**

*The total volume of the secondary muscle tissue in 10 larvae of the series 3/7, 5/5, 7/3, 9/1 and non-heated*

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**Mean:** 17.6 30.8 21.1 27.2 $47.3 \times 10^{-3}$ cmm.

Thus the results of quantitative estimation of the 'muscle induction' may be summarized as follows: with an increasing rate of muscle induction the appearance of very small inductions seems to diminish, and simultaneously very large masses become rather more frequent. However, the differences are small, and in all the experiments complete series were noted, ranging from very small to relatively massive muscle inductions.

**Discussion**

The first question for discussion is the homogeneity of the implanted material. Despite the fact that HeLa-cells, with and without pretreatment, were chosen as a suitable material in the 'mixing' of two inductors, we could not be sure that the two components of the final implantation material were homogeneously mixed to form one 'inductor' with evenly distributed active factors. If this was not the case, then the experiments would be comparable to our earlier implantation with two different inductors implanted simultaneously, but not mixed (Toivonen & Saxén, 1955). As stated above, every precaution was taken to maintain the homogeneity of the cell suspension during the various treatment stages, but there still existed the possibility that the heat pretreatment had changed the physical properties of the cells (e.g. their sedimentation rate, aggregation potentialities, &c.), and consequently that during centrifugation these cells would be separated from the non-heated ones.

As stated above, the control experiments with C-14 labelled cells suggested
that an almost complete mixing of the cells was obtained (Plate). The same conclusion can be drawn from the results. One complete experiment has been demonstrated in Table 1, and two points need stressing. First of all, no ‘grouping’ of certain types of induction can be noted despite the fact that on each occasion a new piece of the precipitated cell suspension was taken after about 10 implantations. If a fractionation by centrifuging had occurred, due to different physical properties of the cells, there would certainly have been layers yielding different inductive actions. Secondly, in all those cases showing archencephalic inductive action, i.e. the pure action of one of the mixed components, both deuterencephalic and spinocaudal formations were induced, which demonstrates the presence of the other component. From this it may be concluded that in different experimental series our implantation material represents a quite homogeneous mixture of the two inductors tested.

Nevertheless, the conclusion that we are obviously dealing with an almost homogeneous mixture of cells with different inductive actions does not mean that the implantation material can be considered as a chemical mixture of two or more inductively active agents. The differences are restricted to a ‘cellular level’, and in considering the suggestion that the inductive stimulus is mainly transmitted through the surface of contact between the inductor and the reactive ectoderm, it should be remembered that this surface of the implant is actually like a mosaic, the two cell types being obviously represented in a ratio which corresponds to the ratio on mixing. We return to this point below.

As mentioned in the introduction, the experiments were carried out in order to test our two-gradient hypothesis which suggests a neuralizing and a mesodermalizing principle. Before we embark further on these still hypothetical considerations, we have to repeat what can actually be read directly from the present results, and we shall only subsequently discuss them in the light of our theory.

The regional type of induction is usually divided into archencephalic, deuterencephalic, and spinocaudal, and some authors still suggest that these regions have their specific regional inductors (Tiedemann, 1959). In the present experiments we confirmed our earlier observations of the inductive action of HeLa-cells grown in fresh human serum (Saxen & Toivonen, 1958). They yielded a strong spinocaudal effect, and had a relatively weak deuterencephalic capacity. Furthermore, the well-known effect of short-term heat treatment was demonstrated. Following this treatment, the HeLa-cells induced purely archencephalic structures. In both series the induction rate was 100 per cent.

In mixing these two inductors with different regional inductive actions, a progressive shift of the regional character of the inductions was observed (Text-fig. 6)—obviously as a result of the combined action of the components. Starting from the purely archencephalic-inducing heated cells, even an addition of 10 per cent. of non-heated cells resulted in an induction of hindbrain structures to the extent of almost 100 per cent.; but it was hardly possible to detect the original action of the non-heated cells: renal tubules were frequently noted, but both
mesenchyme and myotomes were almost entirely absent, and spinal cord was never obtained. Thus the inductive action of this mixture is definitely a new one, and does not represent the regional inductive types of either of the two components of the mixture. This combined action is seen even more markedly in the next step, in which 30 per cent. of non-heated cells were added: all regional types are now represented in the inductions, but the dominating activity is a deuterencephalic inductive action. When heated and non-heated cells were mixed 1:1, this activity was more inclined towards a spinocaudal type, and when non-heated cells were added in increasing amounts the type of inductive action was progressively shifted towards the pure action of non-heated cells. This change is noted as a slight decrease in archencephalic and deuterencephalic inductions, accompanied by a slight increase in spinocaudal structures. In this connexion the appearance of notochord can be noted (Text-fig. 4).

Some of the above-mentioned results are, of course, quite as expected, and they are easily explained as a competition of the reactive material between the two inductively active components. For instance the progressive changes noted when the relative amount of spinocaudal inducing, non-heated cells was raised from 50 per cent. to 100 per cent., can thus simply be referred to as a 'masking' of the archencephalic action. However, this is not true as regards the deuterencephalic action. As emphasized above, the heated cells do not possess the capacity to induce hindbrain structures, and when non-heated cells were tested alone, deuterencephalic inductions were noted only in about one-third of the cases. Thus, the induction of a high percentage of deuterencephalic structures in series 1/9 and 3/7 must be due to a combined effect of archencephalic and spinocaudal actions. It may therefore be stated that in this instance the
The deuterencephalic inductor was experimentally built up—an observation which has to be discussed in the light of certain earlier data.

Yamada (1958, 1959) demonstrated a progressive shift in the inductive action of an originally purely mesodermalizing inductor when exposed to steam for various lengths of time. In this series there appeared a transitional deuterencephalic inductive action which disappeared after a prolonged heat treatment, to become a purely archencephalic action. The author was inclined to explain this as a progressive change in the protein molecule responsible for the inductive action. The present results, indicating that the same effect was obtained after an experimental combination of two killed inductor tissues, does not seem to support this suggestion. This might also be true of the earlier ideas of a specific inductor with deuterencephalic inductive action. Tiedemann (1959) isolated ribonucleoprotein samples yielding an almost pure deuterencephalic action, and seemed to believe in the possibility of the existence of a regionally specific deuterencephalic inductor. The present results may not definitely exclude such a possibility, but they do show that a similar effect can be obtained by mixing two inductors with different actions. Nevertheless, we are fully aware of one weak point in this argument; the deuterencephalic action is not completely lacking in the other component used in the mixtures, and consequently the definite rise of deuterencephalic structures might be explained as some kind of ‘unmasking’ of this action as a consequence of the increasing amounts of the heated cells, and not of a combined action. This possibility is dealt with below.

The two-gradient hypothesis

According to this hypothesis, not only is the deuterencephalic action discussed above a combined action of two active principles, but so also is the spino-caudal effect. We have termed these the neuralizing and the mesodermalizing principle; the former corresponds to the classical archencephalic inductors. Thus the effect of this principle is an induction of forebrain structures and the corresponding sense organs and placodes. The mesodermalizing action has never been demonstrated in a completely pure form, but certain heterogeneous inductors (such as guinea-pig bone-marrow, frog ventral skin) and protein fractions show a predominantly mesodermalizing action, accompanied only occasionally by the induction of small neural structures (Okada, 1948, Toivonen, 1953, 1954; Yamada, 1958, 1959; Tiedemann, 1959). In contrast, the neuralizing action can be obtained in pure form, e.g. after heat treatment of combined inductors (Toivonen & Kuusi, 1948). The combined action of these two factors has earlier been experimentally produced by us by the simultaneous implantation of tissues with neuralizing and almost purely mesodermalizing actions (guinea-pig liver + bone-marrow). As a result of this simultaneous implantation, an induction of spinal cord was noted in about 90 per cent. of cases as opposed to the series with pure bone-marrow in which the corresponding percentage was less than ten. At the same time the occurrence of hindbrain structures was
definitely increased compared with the action of liver and bone-marrow alone. If we now combine these series with the present observations with heated and non-heated HeLa-cells, there appears to be a continuous series from an almost pure mesodermal inductive action (bone-marrow) through spinocaudal (non-heated HeLa-cells and liver-bone-marrow combination) and deuterencephalic (series 3/7 and 1/9) to a pure neuralizing action (heated HeLa-cells). Very similar series were obtained by Engländer & Johnen (1957) after prolonged alcohol-treatment of a kidney tissue which originally induced spinocaudal structures, and by Yamada (1958, 1959) by short-term exposure to steam. Furthermore, our earlier series, in which HeLa-cells were cultivated in heat-inactivated serum for different periods of time, showed similar progressive

changes in the inductive capacity (Saxén & Toivonen, 1958). We were inclined to explain this as a progressive inactivation of the mesodermalizing principle resulting in an 'unmasking' of the neuralizing principle—first combined with a weakened mesodermalizing action, and, finally, in a pure neuralizing action. If the present results are read 'from left to right', i.e. by starting from the neuralizing, heated cells, the result can consequently be explained as a progressive increase of the mesodermalizing principle (Text-fig. 7). Several earlier experiments with heat treatment suggest that the neuralizing principle is not affected by the 30-minute treatment at 70° (Toivonen & Kuusi, 1948; Kuusi, 1951). This idea apparently finds confirmation in the induction rate of 100 per cent. obtained in the present series with heated cells. Thus, when one takes into account that in all the present series the amount of HeLa-cells was roughly the same, it follows that the neuralizing principle was constant. Accordingly, the changes in the regional induction character must be due to changes in the labile mesodermalizing component. As a

Text-Fig. 7. The incidence of archencephalic, deuterencephalic and mesodermal structures in the present experimental series.
consequence, it may be possible to state that the regional inductive action of certain implanted tissues can be progressively altered by inactivation of the mesodermalizing principle in an originally combined inductor, but, vice versa, if a start is made from a purely neuralizing inductor the change can be obtained in the opposite direction by adding increasing amounts of this mesodermalizing principle to the inductor. We are therefore inclined to feel that the present results, combined with earlier observations, give us strong evidence in favour of the two-gradient hypothesis.

As stated earlier, the hypothesis discussed here is a simplified model, and might even be viewed as an over-simplification (Dalcq, 1960). Actually, it is an attempt to explain the relation between the more or less hypothetical agents used in induction experiments and their inductive action. So far, it seems to explain such experimental data satisfactorily, but apart from the actual mode of their action, two more questions await an answer. The experiments were made under controlled conditions, using the same scheme of operations and the same material, and the role of the time factor and the competence of the reactive material may thus be excluded. Certain comments must be made, however. As shown by Johnen (1956) and Toivonen (1958), the induction process requires a definite time to occur, and according to the latter investigation it seems that the minimum time required is the same for both types of induction when killed tissues are used as inductors. However, Nieuwkoop has presented a hypothesis of the time factor in the induction process with which we are not in full agreement (Nieuwkoop et al. 1952; Nieuwkoop & Nigtevecht, 1954; Nieuwkoop, 1958). In this concept the differentiative stimulus starts with a general 'activation' which results in the formation of forebrain structures if no subsequent stimuli are present. This subsequent stimulation or 'transformation' leads to a regional segregation of the central nervous system and the induction of spinocaudal structures. If Nieuwkoop's 'activation' is termed 'neuralizing action', and his transformation 'mesodermalization', we are very close to our theory. The main difference, apart from the terminology, seems to be the time sequence of these two processes. According to Nieuwkoop, the preceding activation is a prerequisite for the subsequent transformation, and the present results might not exclude this possibility. However, one of the present authors has recently tested this hypothesis by employment of a somewhat different technique (Toivonen, 1961), and he has discovered that this sequence can be changed experimentally: the combined action of inductors was again tested. Heated, purely neuralizing HeLa-cells and mesodermalizing guinea-pig bone-marrow were used, not simultaneously as in the present series, but one after the other. The competent ectoderm was first induced by the bone-marrow for 3 hours, following which this inductor was replaced by the neuralizing heated HeLa-cells. In comparison with the effect of bone-marrow alone for 3 hours, and with the effect of the heated HeLa-cells, a combined action was again obtained. In other words, the ectoderm was first ‘activated’ into a mesodermal direction, and subsequently partly ‘transformed’
into a neural direction. The observation does not seem to be in accordance with Nieuwkoop's idea of the temporal relationships of the induction process.

As regards the third component in the inductor–time–competence chain, the competence, Kuusi (1961) has recently presented an interesting idea. She points to the possibility that heat treatment known to 'inactivate' the mesodermalizing component can act in an indirect way by altering the physical properties of the inductor tissue. If the release of inductively active agents from the implant is delayed, the competence of the reacting tissue will change before these active compounds reach it. If the mesodermal competence is lost first, then the 'heat-inactivation' of the mesodermalizing principle could be explained in this way. However, certain earlier observations suggest that during normal gastrulation the neural competence is lost first (Holtfreter, 1938; Gallera, 1952; Nieuwkoop, 1958). As a result, much more experimental data are needed before this tempting hypothesis can be taken as proved. However, these two examples, the time factor theory of Nieuwkoop and the recent hypothesis of Kuusi, show us how complicated the study of the dynamic process of induction is.

Primary stimulus and subsequent interactions

At present our histochemical and submicroscopical methods do not allow us to follow the process of differentiation into neural versus mesodermal directions from the very beginning. Consequently, in all experiments which are concerned with the primary induction, the result of an inductive stimulus is not obtained until 7–12 days after the actual process has occurred. We know from the earlier experiments that a 3-hour contact with an inductor results in an inductive stimulus (Toivonen, 1958). From this it follows that the end-result after 11 days of cultivation, as, for instance, in the present series, must be a result of the action of the primary stimulus followed by a number of subsequent environmental actions. Thus our model of the effect of different ratios of mesodermalizing/neuralizing principle does not entail that the ectodermal cells, when stimulated by a definite 'mixture' of these agents, will be directly transformed into hind-brain or spinal cord cells, for instance, but that the relative amounts of the primarily mesodermalized and neuralized areas respectively of the ectoderm are dependent on the M/N-ratio. Their fate is determined on the basis of the relative amounts of these primarily determined cells. This seems to be dependent not only on the relative amount of these cells but also on their absolute amount.

These ideas are corroborated by a variety of experimental results. As regards the total amount of determined tissue, and its relation to the future fate of its cells, the results of Lopashov (1935) should be mentioned. He explanted fragments of presumptive head mesoderm of Triturus and obtained muscle differentiation only when the fragment was small, but notochord and brain vesicles were formed when several fragments were fused. Working with presumptive neural tissue of the mouse and chicken embryos Grobstein obtained a progressive decrease in the differentiation of the central nervous system when the
fragments were decreased from halves to one-sixteenths (Grobstein, 1952, 1955; Grobstein & Zwilling, 1953). From these results, Grobstein (1955) concluded that there 'appears to be a limiting minimum mass essential to continued morphogenesis and differentiation'. Muchmore (1957) used *Amblystoma* tissues in similar experiments. He explanted pieces of presumptive somite tissue from young neurulae, the smallest explanted piece corresponding to five presumptive somites. These small fragments differentiated only occasionally into muscle tissue, but when several such fragments were aggregated and explanted, muscle differentiation occurred in increased amounts. In addition, nephric tubules were differentiated more often, and certain other structures were also developed (neural formations, notochord, limb-buds). More recently Wilde (1961) demonstrated, in microdrop cultures of *Amblystoma* neuroepithelial cells, that a single isolated cell never differentiated, but that when two of these cells were cultivated in the same drop one of them would differentiate. Thus all these results demonstrate the "mass effect" under experimental conditions. It is tempting to suggest that similar masses are required for a manifestation of inductive stimuli in our experiments, i.e. that there is a *limiting minimum mass to be originally induced* for the manifestation of the differentiative stimulus. Thus a small amount of neuralizing principle cannot manifest itself when most of the cells are induced by the strong mesodermalizing principle present, as obviously in the experiments of Yamada (1959). Furthermore, the results of Muchmore (1957) might explain the old concepts of 'weak' and 'strong' mesodermalizing induction where the former leads to the formation of mesenchyme and nephric tubules, the latter to somites and notochord. This is also seen in our present results: nephric tubules are induced if 10 per cent. of 'mesodermalizing' cells are added, whereas myotomes need about 30 per cent. and notochord not less than 90 per cent. of these cells. In the light of the observations of Muchmore (1957), this could be explained as an induction of increasing amounts of ectodermal cells into a mesodermal direction. The same phenomenon might explain the somewhat unexpected results obtained in the quantitative estimations of the muscle tissue: if a comparison is made of Text-figs. 4 and 5, it may be noted that where an increase of the volume of secondary muscle tissue might be expected (series 7/3 to non-heated), this was hardly to be seen, but that simultaneously the incidence of notochord was increasing. Thus, a part of the originally mesodermalized ectoderm, instead of being differentiated into muscle tissue, was in fact differentiated into notochord, representing the 'strongest' mesodermalization.

We thus start from the concept that the first inductive stimulus will determine only the first step of differentiation of the ectodermal cells, i.e. the differentiation into either the neural or the mesodermal direction and probably also into the entodermal (Takata & Yamada, 1960). Due to the obvious mosaic-like distribution of the neuralizing and mesodermalizing 'units' on the contact surface of the implant, the overlying ectoderm will consequently be induced into intermixed islands of neural and mesodermal cells. The fate of these areas is then
determined by subsequent conditions. One of those essential factors which conducts the future development of these primarily induced cells might be the 'mass effect' mentioned above, leading to the lack of manifestation of the differentiative stimulus of very small areas. In addition, there are experimental data which demonstrate different 'inductive interactions' during the period of development following primary induction. For instance, the development of limb-buds might thus be correlated with the formation of nephric tubules (Muchmore, 1957), the differentiation of muscle tissue seems to be dependent on the presence of notochord (Yamada, 1940) and its growth enhanced by spinal cord (Holtzer, Lash, & Holtzer, 1956; Avery & Holtzer, 1958). In connexion with our present findings, the convincing experiments of Takaya are of special interest (Takaya, 1955, 1956#, 19566). When explantation was made of the branchial part of the neural plate, the type of neural differentiation seemed to be dependent on the amount and the contact of mesenchymal and muscle tissue. In the total absence of these mesodermal components, the neural tissue developed into a vesicle resembling forebrain. When a larger amount of muscle tissue was in contact with the neural structures, these often resembled hindbrain structures, and ear vesicles were observed. In the presence of large amounts of muscle tissue, this often encircled the elongated neural tissue, which morphologically closely resembled the spinal cord. The author thus concluded that the regional character of the neural formations is not determined during the first step of induction, and that this is due to the neighbouring mesodermal structures. The results and ideas of Takaya thus seem to be fully in agreement with the two-gradient theory: a strong mesodermalizing action \( M \) combined with neuralizing action \( N \) was suggested as leading to differentiation of spinocaudal structures, and this seems to be the consequence of an induction of a large amount of mesenchymal tissue which determines the eventful fate of the simultaneously induced neural structures.

In the light of the ideas mentioned above, it might be interesting to compare the results of our earlier experiments (Toivonen & Saxén, 1955) with those of the present series. In the earlier series, the two different inductors were implanted simultaneously, but not intermixed, and thus the 'fields' of their inductive effects overlapped each other only partly, whereas in the present series no such individual fields could be expected. Consequently, in the liver–bone-marrow series we frequently obtained very complete inductions, closely resembling the dorsal side of the normal embryo. In these inductions, all the regional types of induction were represented, and usually in the normal sequence from pure archencephalic induction (liver effect) through deuterencephalic and spinocaudal structures (combined effect) to pure mesodermal masses (bone-marrow effect). In the present series, such complete inductions were seen only occasionally, and when archencephalic structures dominated, spinocaudal structures were usually absent.

To summarize the concepts embodied in this extensive and partly hypothetical discussion, it may be stated that the present experimental results, as well as a
variety of data obtained by other authors during the last five years, appear to corroborate our two gradient hypothesis (Toivonen & Saxén, 1955). Accordingly, these results can be explained by a primary neuralization versus mesodermalization by two different inductive principles, followed by a variety of interactions between these two tissue components, and leading to the regional type of induction noted under experimental conditions.

SUMMARY

The authors earlier presented a modified two-gradient hypothesis of primary induction, suggesting two inductively active principles. The regional type of induction would, according to this theory, be determined by the ratio of a neuralizing principle and a mesodermalizing principle. The present experiments were made in order to test this idea by the use of more quantitative methods.

HeLa-cells were used to produce, under experimental conditions, different ratios of the two principles in the implants. From earlier experiments it was known that these cells were, when cultivated in fresh human serum, strong inducers of spinocaudal structures, but after a short heat treatment, purely archencephalic inductors. Thus, cells with and without heat pretreatment were mixed in different ratios (1/9, 3/7, 1/1, 7/3, and 9/1). The inductive action of these cell mixtures was tested in the usual way in implantation experiments.

The results show that when non-heated cells are added to heated cells in increasing amounts, the regional type of induction produced by this mixture is progressively shifted from an archencephalic induction, through a deuterencephalic type, to a principally spinocaudal inductive action. Thus, the progressive shift from spinocaudal to archencephalic action earlier obtained by physical and chemical treatment of spinocaudal inductors, could be 'rebuilt' in the opposite direction.

The results are interpreted as corroborating the two-gradient theory. It is concluded that the primary inductive stimulus in these series led to determination of different amounts of neural and mesodermal cells, the final regional results being brought about by subsequent interactions of these two components. Certain recent findings which demonstrate a 'minimum mass effect' and late interactions between tissues are discussed.

RÉSUMÉ

L'hypothèse des deux gradients dans l'induction primaire. L'action combinée de deux types d'inducteurs mélangés selon des proportions différentes

Les auteurs ont présenté auparavant une hypothèse modifiée concernant l'existence de deux gradients dans l'induction primaire, suggérant la présence de deux principes actifs. Selon cette théorie, le type régional d'induction serait déterminé par le rapport entre un principe neuralisant et un principe mésoder-
Les expériences rapportées ici ont été faites pour vérifier cette hypothèse au moyen de méthodes plus quantitatives.

On a utilisé des cellules HeLa pour produire dans les implants, dans des conditions expérimentales, de telles différences de rapport entre les deux principes. A partir d’expériences antérieures, on savait que ces cellules, quand on les cultive dans du sérum humain frais, sont de puissants inducteurs de structures spinocaudales mais, après un court traitement par la chaleur, sont des inducateurs purement archencéphaliques. Ainsi, des cellules après et sans prétraitement par la chaleur ont été mélangées selon diverses proportions (1/9, 3/7, 1/1, 7/3 et 9/1).

L’action inductrice de ces mélanges cellulaires a été testée de la manière habituelle par des expériences d’implantation.

Les résultats montrent que lorsqu’on ajoute en proportion croissante des cellules non-chauffées à des cellules chauffées, le type régional d’induction produit par le mélange passe progressivement d’une action inductrice archencéphalique à une action principalement spinocaudale, par l’intermédiaire d’un type deutérencéphalique. Ainsi, le passage progressif de l’action spinocaudale à l’action archencéphalique, obtenu antérieurement par traitement physique et chimique d’inducteurs spinocaudaux, a pu être reconstitué en direction opposée.

Ces résultats sont interprétés comme corroborant la théorie des deux gradients. On conclut que le stimulus inducteur primaire dans ces séries a conduit à la détermination de quantités différentes de cellules neurales et mésodermiques, les résultats régionaux étant finalement provoqués par des interactions ultérieures entre ces deux composants.

On discute certains résultats récents qui démontrent l’existence d’un ‘effet de masse minimum’ et d’interactions ultérieures entre les tissus.

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EXPLANATION OF PLATE

Micrographs and autoradiographs of HeLa-cell mixtures after implantation and a subsequent cultivation of 24 hours. Stained with haematoxylin and eosin, autoradiographs by Kodak-AT-10 stripping film.

FIGS. A and B. 1 part heated, C–14 labelled cells to 1 part non-heated, non-radioactive cells.

FIGS. C and D. 1 part heated, non-radioactive cells to 1 part non-heated, C–14 labelled cells.

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