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# Induction of Specific Differentiation by Samples of Proteins and Nucleoproteins in the Isolated Ectoderm of *Triturus*-gastrulae

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Transplantation and explantation experiments of HOLTFRETER, CHUANG, TOIVONEN, ROTMANN, FUJII, OKADA, HAMA, ENGLÄNDER, KAWAKAMI, VAHS and others have established that under the influence of many adult tissues of various vertebrates the ectoderm of amphibian embryos can differentiate in the definite direction characteristic for each adult tissue. Almost all principal organ rudiments belonging to the ectoderm and mesoderm are induced in the presumptive ectoderm of *Triturus*-gastrulae in this way. With few exceptions each adult tissue induces not a single kind but several kinds of structures. However, the array of structures produced under the influence of one of those adult tissues is usually not chaotic but has a distinct regional character. Thus several tissues, including the liver of the guinea pig, induce in the ectoderm archencephalic structures[1], other groups of tissues, including the kidney of the guinea pig, induce spino-caudal and deuterencephalic structures[2], and still other groups of tissues, including the bone marrow of the guinea pig, induce almost exclusively trunk-mesodermal structures[3]. This phenomenon, often referred to as the heterogeneous induction, deserves attention not only of embryologists but of all biologists who are interested in the mechanism of specific differentiation, because it presents a unique possibility of a chemical control of various specific differentiation processes. Although many papers[4] have been devoted to the study of the chemical aspect of the phenomenon, we are still far from understanding the chemical mechanism involved. In the present article, recent progress made in our laboratory in this field will be briefly reviewed.

The method of these experiments consists in fractionation of the extract from some adult tissues with a typical regional inductive effect, and in testing those fractions for their inductive effect on the isolated ectoderm of young gastrulae of the Japanese newt, *Triturus pyrrhogaster*. Chemical and physical characterization of the sample was tried so far as possible. The embryological test was made by implanting a small piece of the sample between two pieces of the isolated ectoderm. The ectodermal pieces fused together to form a vesicle enclosing the implant which was allowed to exert its morphogenetic effect on the internal surface of the ectoderm. The explants were cultured in Holtfreter solution adjusted to pH 7·2–7·4 for 9 to 14 days, exceptionally even for 39 days at 18°C. Differentiation of the explant was studied in microscopic sections. Without an implanted sample, the isolated ectoderm differentiated epidermis cells alone under the experimental conditions adopted.

## *Regional Inductive Effect of Pentose Nucleoprotein (PNP) from the Liver and Kidney*

It had been established by TOIVONEN[5] that ethanol-treated liver tissue of the guinea pig induces archencephalic structures which can be accompanied by deuterencephalic structures. Working in our laboratory, HAYASHI[6] found that 0·14 *M* NaCl extract of the tissue has the same effect, when tested as the ethanol-precipitate. The extract was fractionated by giving streptomycin sulfate. The precipitate obtained was washed repeatedly with a solution of streptomycin sulfate, suspended in a borate buffer and dialyzed. On chemical analysis the sample thus obtained was found to be pentose nucleoprotein (PNP)[7]. The electrophoretic pattern of the sample indicated a single boundary whose mobilities were found to be $-5{\cdot}1 \sim -5{\cdot}4$ $(cm^2/sV \times 10^{-5})$ at the descending limbs and $-6{\cdot}0 \sim -6{\cdot}3$ at the ascending limbs, when determined in 0·2 $\mu$ phosphate buffer at pH 7·5 and in 0·2 $\mu$ borate buffer at pH 8·5. The ethanol precipitate of the sample induced in the isolated ectoderm archencephalic

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[1] Eye, fore-brain, nose and lens are designated collectively as archencephalic structures.

[2] Spinal cord, tail-bud, tail-somites and tail-notochord are collectively called spino-caudal structures. Mid-, hind-brain and ear vesicle are called deuterencephalic structures.

[3] Trunk-mesodermal structures include trunk-notochord, trunk-somites, pronephros, blood cells, limb-bud and mesothelium.

[4] S. TOIVONEN and T. KUUSI, Ann. zool. Soc. 'Vanamo' *13*, 1 (1948). – S. TOIVONEN, Rev. suisse Zool. *57*, Suppl. 41 (1950). – T. KUUSI, Ann. zool. Soc. 'Vanamo' *14*, 1 (1951); Arch. Biol. *64*, 189 (1953).

[5] S. TOIVONEN, Ann. Acad. Sci. fenn. [A] *55*, 1 (1940); Exper. *8*, 120 (1952).

[6] Y. HAYASHI, Embryologia *3*, 57 (1956).

[7] Y. HAYASHI, Embryologia *3*, 57 (1956). – Y. HAYASHI and K. TAKATA (unpublished).

structures in a very high percentage of cases (Fig. 1). On the other hand, the non-precipitable part of the original extract was found to possess weak archencephalic effects. The regional effects of the sample of liver PNP varied according to that of its source material, the archencephalic tendency being combined with the deuterencephalic tendency in varying degree.

A parallel series of experiments was made earlier by YAMADA and TAKATA[8], using as the source material kidney tissue of the guinea pig, which had been shown by TOIVONEN[5] to be a good inducer of spino-caudal and deuterencephalic types. The sample of PNP from the kidney induced spino-caudal and deuterencephalic structures at a high frequency (Fig. 2). No archencephalic structure was recorded in this series. The results fit in with our earlier experiments[9], in which kidney tissue was fractionated into cell components according to the usual technique and these components were tested for their inductive effect. Among the cytoplasmic fractions, the 'small microsome' fraction showed the highest frequency of spino-caudal effect,

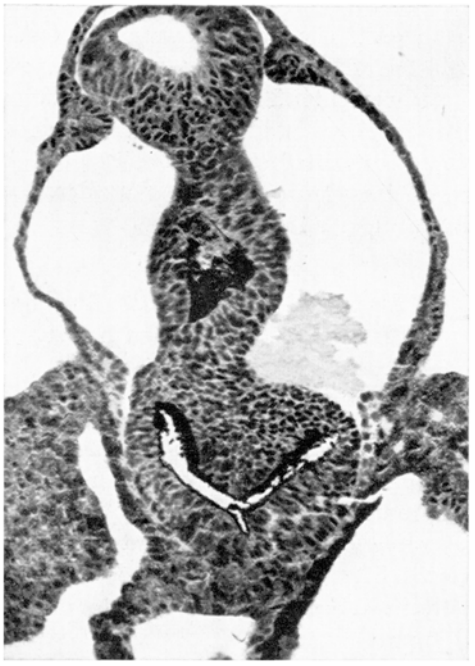


Fig. 1. — Archencephalic induction caused by liver PNP in the isolated ectoderm. Nose, fore-brain and eye-type structure with pigment.

while the 'mitochondria' and 'supernatant' fractions were distinctly weaker in the inductive effect. Very probably our sample of PNP prepared with the streptomycin technique is derived from the microsomes. This was particularly supported by the recent experiments of TAKATA and OSAWA[10] on the appendix of the rabbit, which demonstrated that streptomycin given to an extract of the microsome fraction precipitated the PNP component of the microsomes leaving their non-nucleoprotein component in solution.

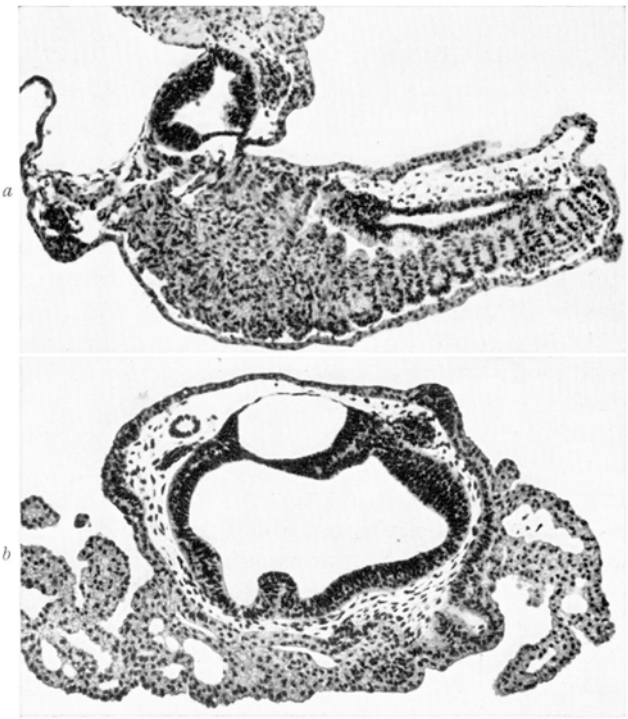



Fig. 2. — Effect of a sample of kidney PNP. *a* Somites and a spinal cord-type structure. *b* A deuterencephalon-type structure accompanied by mesenchyme, small ear vesicle-type and ganglion-type structures.

### *Effect of Liver PNP Samples Purified by Ultracentrifugation*

Although the sample of liver PNP obtained with the above method indicated a single boundary on electrophoresis, its sedimentation diagram suggested some heterogeneity. When the sample was fractionated into two or three subfractions by ultracentrifugation, and the PNA content of each subfraction was estimated, the sedimentable subfraction showed a very low value in contrast to a very high value obtained in the non-

*Table I.* The Pentose Nucleic Acid Content and Inductive Effect of Subfractions of a Pentose Nucleoprotein Sample Separated by Ultracentrifugation

| Subfractions | Precipitate of 100,000 × *g*, 1 h | Supernatant 100,000 × *g*, 1 h |
|---|---|---|
| PNA Content in μg PNA–P/mg Protein N . . | 10·2 | 92·7 |
| Number of Explants . . . | 53 | 59 |
| Total Neural Induction . . | 72% | 100% |
| Archencephalic Induction . | 55% | 34% |
| Deuterencephalic Induction | 9% | 71% |
| Non-specifiable Induction . | 38% | 8% |

[8] T. YAMADA and K. TAKATA, Embryologia *3*, 69 (1956).

[9] T. YAMADA, K. TAKATA, and S. OSAWA, Zool. Mag., Tokyo *63*, 415 (1954). – T. YAMADA, Symp. Soc. cell. Chem. *4*, 1 (1956).

[10] K. TAKATA and S. OSAWA, Biochim. biophys. Acta *24*, 208 (1957).

sedimentable subfraction (Table I)[11]. This was interpreted to suggest that the original sample of PNP was contaminated with a protein which was sedimentable by the centrifugal force applied. When all those subfractions obtained by ultracentrifugation were studied for their morphogenetic effects on the isolated ectoderm the non-sedimentable subfractions showed a higher frequency of neural and deuterencephalic induction, and a lower frequency of regionally non-specifiable induction, compared with the sedimentable subfractions. The inductive effect of the original PNP sample was found to be closely comparable with that of the non-sedimentable subfraction which seems to represent a very pure sample of PNP, on account of its PNA content.

All these results lead to the conclusion that PNP of the liver itself has a strong inductive effect of archencephalic and deuterencephalic types. It may be added that the PNP sample prepared by the streptomycin technique does not lose its inductive effect after extraction with various organic solvents.

### *Significance of Nucleic Acid- and Protein-Components for the Induction caused by PNP*

The next question raised was the relative role of protein- and nucleic acid-components of PNP in the induction caused by the latter. In an attempt to answer this question ribonuclease, pepsin and trypsin were used to attack one of these components leaving the other more or less intact.

In the first experiment[11] of this group the sample of liver PNP was denatured with ethanol, suspended in a borate buffer of pH 7·6 and incubated with crystalline ribonuclease for 7 h at 28°C under continous dialysis. After the incubation, the sample contained 3·5 $\mu$g PNA-P per mg protein N in contrast to 86·6 $\mu$g PNA-P of the control sample. The both samples were tested for the regional inductive effect. The very high frequency of the over-all induction of the control sample remained unchanged in the experimental series, and small differences of frequency in regional types observed between the experimental and control series were found to be not significant.

In the second experiments[11] with ribonuclease, liver PNP purified by ultracentrifugation at 100,000 × $g$ for 1 h and not denatured with ethanol, was used as the substrate. Incubation with crystalline ribonuclease was made in a saline of pH 7·6 at 22°C for 3·5 h under constant dialysis. At the end of incubation, an equal volume of saturated ammonium sulfate was added to the mixture. The precipitate obtained was used as the sample for the induction experiment after a dialysis and ethanol-precipitation. Whereas the control sample contained a large amount of PNA, the treated sample showed only a trace of the latter (Table II). As indicated in the Table, the high over-all induction frequency of the control sample was unaffected by the treatment. The frequency of archencephalic, deuterencephalic and non-specifiable inductions also remained unchanged after the treatment.

*Table II.* Inductive Effect of PNP Purified by Ultracentrifugation and Treated with Ribonuclease

| | Control | Experimental |
|---|---|---|
| PNA Content in $\mu$g PNA–P per mg Protein N | 148·6 | 0·84 |
| Number of Explants | 49 | 52 |
| Total Neural Induction | 100% | 100% |
| Archencephalic Induction | 14% | 15% |
| Deuterencephalic Induction | 82% | 77% |
| Non-specifiable Induction | 18% | 17% |

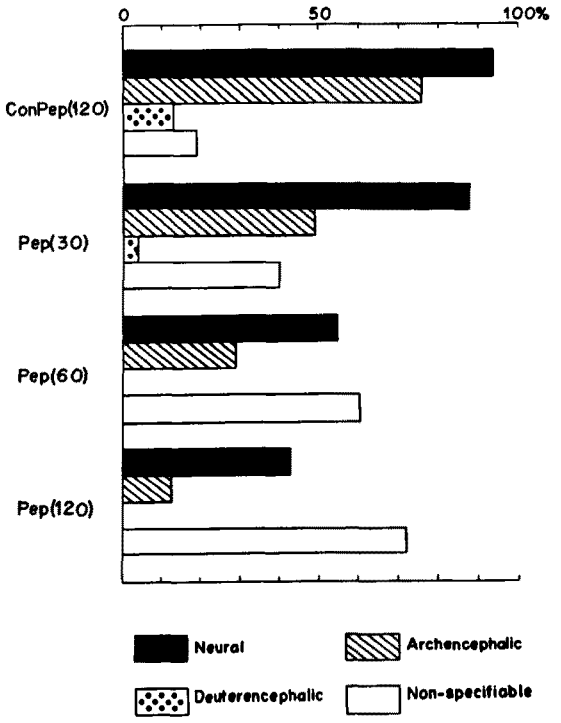



Fig. 3. – Diagrams showing progressive suppression of the inductive effect of a sample of liver PNP treated with pepsin for 0 min (ConPep120), 30 min (Pep30), 60 min (Pep60), and 120 min (Pep120). The inductive frequency of each type of induction is expressed by the percentage of the number of explants containing the type in question against the total number of explants in the series concerned (Hayashi).

In the further series of experiments[11], the effect of crystalline pepsin on the inducing ability of the sample of liver PNP was studied by incubating the sample with the enzyme for 30 min, 60 min, and 120 min at pH 4·0. At the end of the incubation period, the pH was adjusted to 7·5 and ethanol was added to the reaction mixture to precipitate the sample to be tested. As is clear from Figure 3, a progressive decrease of archencephalic and deuterencephalic induction and

[11] Y. Hayashi (unpublished).

concomitant increase in the non-specifiable induction were registered. This can be interpreted to indicate that under the action of the enzyme the formation of essential structures of the central nervous system was suppressed, while the differentiation of non-axial structures derived from the neural fold or of unorganized neural tissue was enhanced.

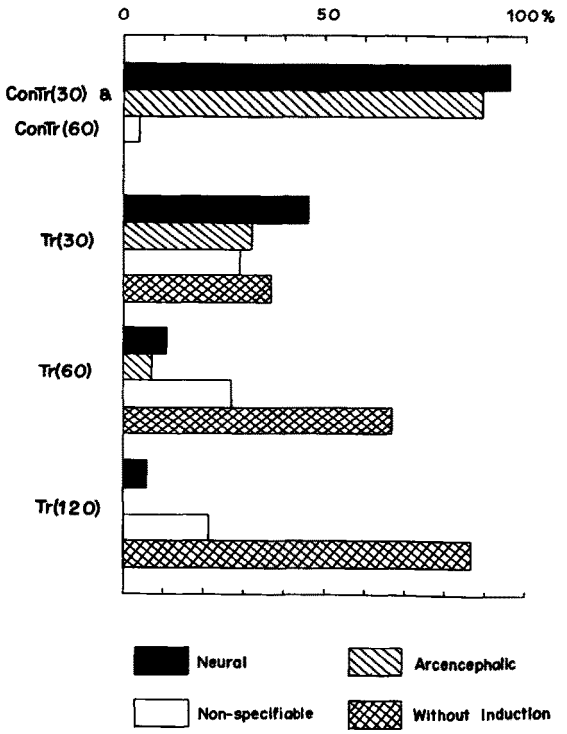



Fig. 4. — Diagrams showing progressive suppression of the inductive effect of a sample of liver PNP by a treatment with trypsin for 10 min (ConTr30, 60), 30 min (Tr30), 60 min (Tr60), and 120 min (Tr120). (HAYASHI).

In a comparable series of experiments[11], the effect of crystalline trypsin was studied after the same time-intervals. After each incubation period, the reaction mixture was heated at 85°C in 95% ethanol in order to inactivate the enzyme. The result demonstrated a progressive suppression of archencephalic induction, leading to a complete disappearance of the latter at the end of 120 min-treatment (Fig. 4). The non-specifiable induction increased weakly up to 60 min, while the number of explants without any induction increased steadily during the whole incubation period to attain the majority of cases at the end of 120 min-treatment. The effect of the heat-treatment was carefully controlled with a special series of experiments, which demonstrated that the treatment does not change the over-all induction frequency but changes the regional effect so that the archencephalic type alone is induced after the treatment.

Taken altogether, the results of the experiments with enzymes harmonize with each other in indicating that in the induction caused by liver PNP the main role is played by the protein-component and not by the nucleic acid-component. This conclusion is compatible with the observations[12] that PNAs from various sources give only weak inductions of the non-specifiable or of archencephalic type in the competent ectoderm. There is also an increasing body of evidence[13] suggesting the inability of ribonuclease to suppress the inducing power of tissues and extracts.

### *The Chemical Agent Responsible for the Mesodermal Induction by the Bone Marrow*

Although in the liver and kidney of the guinea pig, the PNP fraction carries the inductive ability characteristic for each tissue, this is not the case for the bone marrow, the potent inducer of mesodermal structures discovered by TOIVONEN[14]. This was demonstrated in the following two series of experiments[15].

As the starting sample, 0·14 *M* NaCl extract of bone marrow tissue of the guinea pig was precipitated with ethanol and tested for the regional inductive effect as usual. It induced trunk-notochord (50%), trunk-somites (45%), pronephros (45%), blood island (55%) and other mesodermal structures. Neural structures were obtained only very infrequently and in small amounts, always accompanying mesodermal structures.

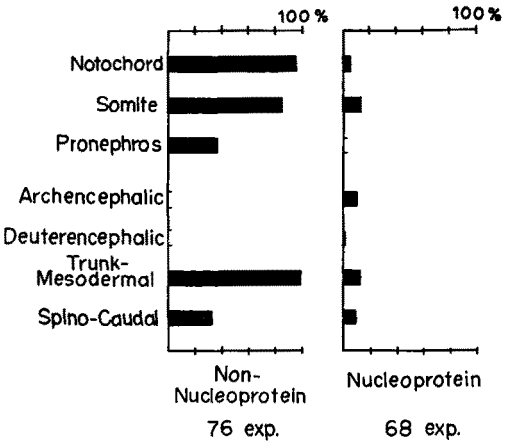



Fig. 5. Diagrams showing the inductive effect of nucleoprotein fraction and non-nucleoprotein fraction from the bone marrow extract.

The inductive effect of the extract was thus closely comparable with that of the original tissue. The ex-

[12] T. KUUSI, Arch. Biol. *64*, 189 (1953). – T. YAMADA, K. TAKATA, and S. OSAWA, Embryologia *2*, 123 (1954). – H. TIEDEMANN and H. TIEDEMANN; Hoppe-Seiler's Z., *306*, 132 (1957).

[13] T. KUUSI, Ann. zool. Soc. 'Vanamo' *14*, 1 (1951); Arch. Biol. *64*, 189 (1953). – H. ENGLÄNDER, A. G. JOHNEN, and W. VAHS, Exper. *9*, 100 (1953). – Y. HAYASHI, Embryologia *2*, 145 (1955). – H. ENGLÄNDER and A. G. JOHNEN, J. Embryol. exp. Morphol. *5*, 1 (1957). – W. VAHS, Roux' Arch. *149*, 339 (1957).

[14] S. TOIVONEN, Arch. Soc. 'Vanamo' *7*, 113 (1953); J. Embryol. exp. Morphol. *1*, 97 (1953); *2*, 239 (1954); Ann. Acad. Sci. fennicae [A IV] *22*, 1 (1954).

[15] T. YAMADA, Y. HAYASHI, K. TAKATA, and S. OSAWA (unpublished).

tract was fractionated into two fractions by the use of streptomycin sulfate. The nucleoprotein fraction which contained the bulk of PNA present in the original extract showed very weak inductive effect of neural and mesodermal nature. A large number of explants failed to show any sign of induction at all (Fig. 5). On the other hand, the non-precipitable fraction which contained only a very small amount of PNA proved to be a potent inducer of trunk-mesodermal structures. The effect of the fraction was characterized by very frequent occurrence and good differentiation of the notochord and somites (Fig. 6). Spino-caudal structures including the spinal cord and tail-bud often accompanied the trunk-mesodermal structures.

In the other series of experiments, the same saline extract was fractionated into four fractions according to the scheme shown in Figure 7. Those fractions were tested for their regional effects by the usual technique. As shown in Figure 8 the microsome fraction and final supernatant fraction were very poor in inductive activity, while both acid-precipitable fractions induced typical trunk-mesodermal structures in all explants. In most cases, the whole mass of the explant was converted into mesodermal structures, except the thin layer of the epidermis covering the surface. Very high frequency of notochord and somites was characteristic for both series. Judged from the mode of separation, both the acid-precipitable fractions may be chemically not much different. An electrophoretic study of the acid-precipitable fraction showed at least two boundaries. The fast moving component which represented only a small fraction of the material showed a mobility comparable with that of PNP of liver and kidney tissues and was assumed to be PNP of the supernatant fraction. The other boundary representing the bulk of the fraction was very slow in movement and assumed to be a protein free of nucleic acid.

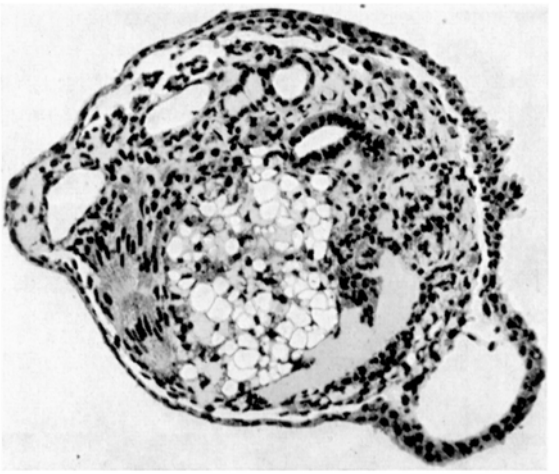


Fig. 6. – Notochord, somites and pronephros induced by the non-nucleoprotein fraction of the bone marrow in the isolated ectoderm.

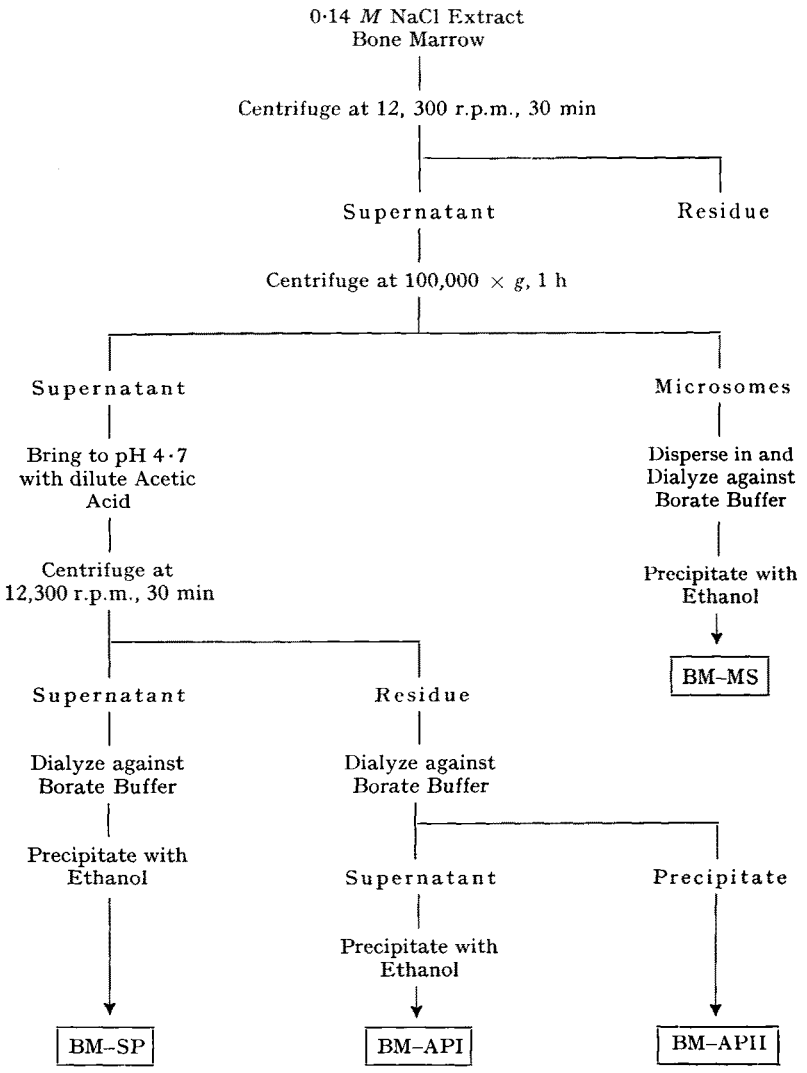



Fig. 7. – The scheme of fractionation of the bone marrow.

Thus intercellular localization of the inductive ability of the bone marrow is quite different from that of the liver and kidney where, as stated, the microsomes appear to be the most important site of localization in the cytoplasm.

The data reviewed above, together with contributions of KUUSI[16] and of TIEDEMANN and TIEDEMANN[17],

[16] T. KUUSI, Ann. zool. Soc. 'Vanamo' *14*, 1 (1951); Arch. Biol. *64*, 189 (1953).

[17] H. TIEDEMANN and H. TIEDEMANN, Hoppe-Seyl. Z. *306*, 7 (1957).

demonstrate that in the differentiated tissue cells a number of proteins or proteids are present which give different regional effects in the same reacting system under identical experimental conditions. They may be called the specific inducing substances. However there

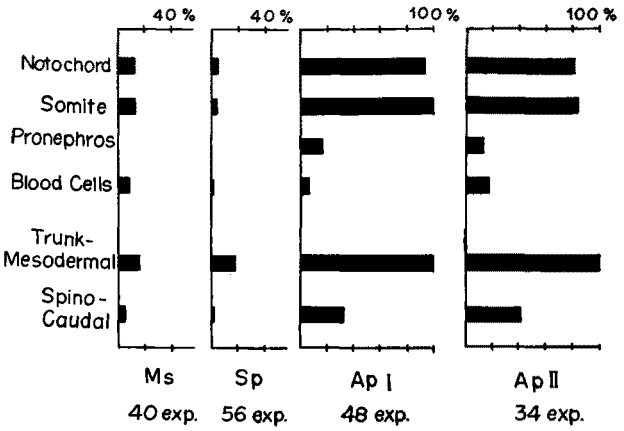



Fig. 8. – Diagrams showing the inductive effect of microsome fraction (Ms), final supernatant (Sp), acid-precipitable fraction of the supernatant I and II (Ap I, Ap II) of the bone marrow (see Fig. 7).

are grounds to assume that their regional specificity is not absolute. For instance, the regional effect of many samples can be shifted or altered by treating them with different physical or chemical agents. Further, by combining one of the samples with another the regional effect can be obtained, which is not produced by either

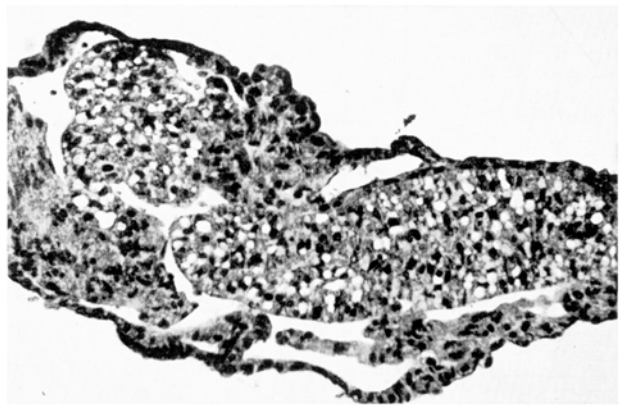


Fig. 9. – Notochord and somites induced by the acid-precipitable fraction II of the bone marrow.

of the two samples[18]. The former type of evidence[19] was first demonstrated in the experiments using the tissue as the inducer, and was later extended to extracts and fractions or even to samples of considerable purity. They are of special interest for embryologists, because they suggest the interrelationship of various regional differentiations and eventually the physico-chemical basis of regionality. The following data indicate that also in the PNP samples alteration of the regional inductive effect does occur: a liver PNP sample with a considerable deuterencephalic tendency induced only archencephalic structures after a short heat-treatment. Further, a kidney PNP sample possessing spino-caudal and deuterencephalic effects in the fresh condition induced archencephalic structures after a longer standing in the solution. Especially suggestive results were obtained by HAYASHI[20] when he studied the effect of pepsin on the sample of kidney PNP. Addition of the enzyme to the sample at a sub-optimum condition resulted in appearance of archencephalic effects and suppression of the spino-caudal tendency. A similar effect of pepsin has been reported by TIEDEMANN and TIEDEMANN[17] on a sample of protein from the extract of the chick embryo. As to the bone marrow and its fractions, there have been no data in favour of a change in regionality. According to TOIVONEN[21], a short heat-treatment of the tissue leads to an almost complete loss of inductive ability. However, for the frog skin, the other inducer of mesodermal structures, OKADA[22] demonstrated the capacity of neural induction after heat-treatment. In the following experiments, a clear shift in regional effects was demonstrated for the bone marrow by a progressive heat-treatment of short duration[23].

### *Progressive Shift of the Regional Effect by a Heat-Treatment of the Bone Marrow*

Small pieces of frozen tissue were spread on a glass plate in a thin layer and exposed to steam in a steam-sterilizer for 25 s, 40 s, 60 s, and 150 s, and then quickly dipped in cold 90% ethanol. The morphogenetic effects of these samples were tested as

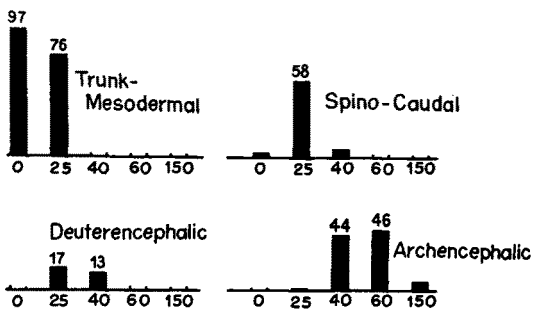



Fig. 10. – Diagrams showing the frequency of various regional types of induction caused by the bone marrow treated with steam for 0 s (0), 25 s (25), 40 s (40), 60 s (60), and 150 s (150).

[18] S. TOIVONEN and E. SAXÉN, Exp. Cell. Res. Suppl. *3*, 346 (1955). – K. YAMANA and I. KAWAKAMI, Zool. Mag., Tokyo *66*, 58 (1957). – T. YAMADA, Zool. Mag., Tokyo *66*, 60 (1957).

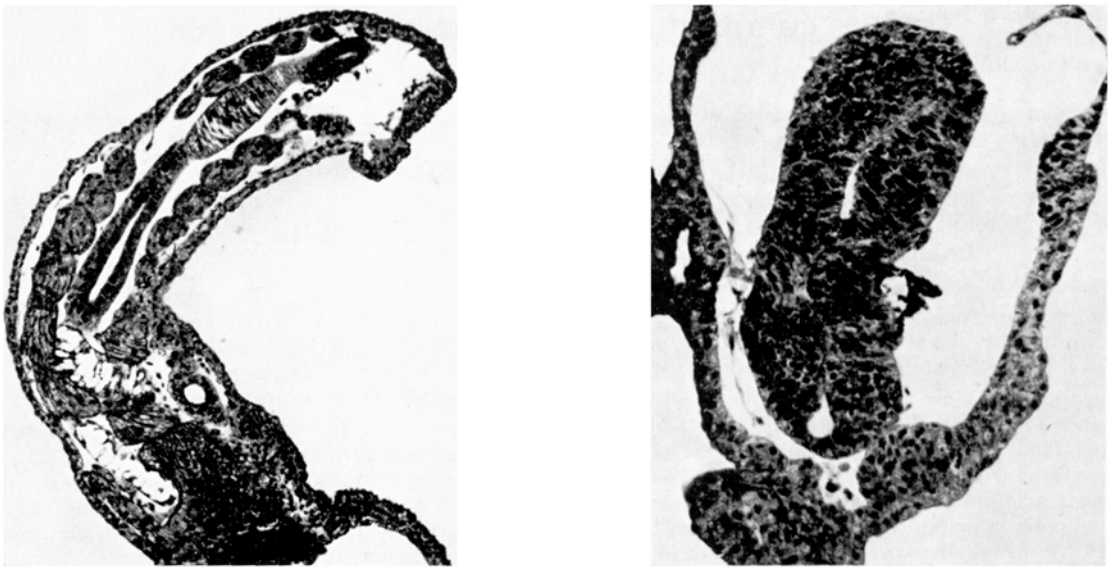
[19] H. H. CHUANG, Roux'Arch. *139*, 556 (1939); *140*, 25 (1940). – H. ENGLÄNDER, A. G. JOHNEN, and W. VAHS, Exper. *9*, 100 (1953). – T. YAMADA and K. TAKATA, J. exp. Zool. *128*, 291 (1955). – W. VAHS, Z. Naturf. *10b*, 412 (1955). – I. KAWAKAMI and S. MIFUNE, Mem. Fac. Sci. Kyushu Univ. [E] *2*, 141 (1957). – H. TIEDEMANN and H. TIEDEMANN, Hoppe-Seyl. Z. *306*, 7 (1957).

[20] Y. HAYASHI (unpublished).

[21] S. TOIVONEN, Ann. Acad. Sci. fennicae [A IV] *22*, 1 (1954).

[22] Y. K. OKADA, Proc. imp. Acad. Japan *24*, 22 (1948).

[23] T. YAMADA, Zool. Mag., Tokyo *66*, 60 (1957).



*A* *B*

Fig. 11.—(*A*) Typical spino-caudal induction caused by 25 s-steamed bone marrow tissue. Spinal cord, notochord, somites and ear vesicle are visible. (*B*) An archencephalic brain induced by 40 s-steamed bone marrow tissue.

usual and compared with those of the untreated tissue fixed with cold 90% ethanol. Expressed in terms of regional types, a progressive shift in the quality of induction during the whole period was demonstrated (Fig. 10): The trunk-mesodermal type of induction diminished slightly within 25 s and then suddenly disappeared. The spino-caudal type was very infrequent at first, became frequent in 25 s, but perfectly vanished subsequently. The deuterencephalic type was represented only weakly in 25 s and 40 s. It might have a peak between two series. The archencephalic type was completely absent in the control, appeared later to increase remarkably during 40 to 60 s steaming and began to decrease after 150 s steaming. Thus the following sequence of regional types occurred during steaming:

trunk-mesodermal→spino-caudal→deuterencephalic →archencephalic

Inspection of earlier data suggests that this sequence can also be applied to other types of inducer: spino-caudal, deuterencephalic and archencephalic inducers. It must be pointed out, however, that concomitant with this change in regionality a gradual suppression of the total induction frequency may occur. We need further analysis to understand the physical basis of the change in inductive quality. It appears not improbable that the protein molecule responsible for the regional induction undergoes a progressive change in configuration under diverse experimental conditions, and this change is reflected in the progressive change in its regional effect. Such a possibility must have an important implication for the problem of embryonic organization, because the above sequence of regionality happens to coincide with the sequence of distribution of corresponding presumptive areas in the embryo[24].

*Zusammenfassung*

Ein Präparat von Ribonukleoproteiden der Meerschweinchenleber induziert im isolierten Ektoderm der jungen Gastrula von *Triturus pyrrhogaster* archenzephale und deuterenzephale Strukturen. Die Induktionsfähigkeit des Präparates bleibt unverändert nach der Reinigung durch Ultrazentrifugierung oder nach der Entfernung von Ribonukleinsäure mit Ribonuklease. Anderseits kann die Behandlung des Präparates mit Pepsin und Trypsin die Induktionsfähigkeit progressiv und weitgehend inaktivieren. Mit Präparaten von Ribonukleoproteiden der Meerschweinchenniere werden spino-caudale oder deuterenzephale, aber keine archenzephalen Differenzierungen im Ektoderm ausgelöst. Die Induktion der rumpfmesodermalen Strukturen im Ektoderm durch das Meerschweinchenknochenmark ist mit dem bei pH 4,7 ausfällbaren Anteil der überstehenden Flüssigkeit nach der Ultrazentrifugierung des Extraktes verbunden, und nicht mit der Mikrosomenfraktion, wie das in der Leber und Niere der Fall ist. Die qualitative Veränderung der induktiven Fähigkeiten, die das Knochenmark während der progressiven Hitzebehandlung erfährt, wird analysiert und ihre theoretische Bedeutung kurz diskutiert.

[24] For the further discussion cf. F. E. Lehmann, Rev. Suisse Zool, *57*, 141 (1950). – T. Yamada, Embryologia *1*, 1 (1950). – T. Yamada and K. Takata, J. exp. Zool. *128*, 291 (1955); Exp. Cell Res. Suppl. *3*, 402 (1955). – S. Toivonen and L. Saxén, Ann. Acad. Sci. fenn. [A] *30*, 3 (1955).