

FRONTISPIECE.



An adult frog (Xenopus laevis laevis) obtained from the
transplantation of a gut cell nucleus of a swimming
tadpole.

Circa natural size.

Photo: J. Haywood.

NUCLEAR TRANSPLANTATION IN XENOPUS.¹

A thesis submitted to the Board of the
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relationships during ~~differentiation~~ differentiation in Vertebrates.

NUCLEAR TRANSPLANTATION IN XENOPUS.

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NUCLEAR TRANSPLANTATION IN XENOPUS.

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- I. Introduction. The principle of nuclear transplantation. Nuclear transplantation in unicellular organisms. The conditions necessary for successful nuclear transplantation in Metazoa. Nuclear transplantation in Amphibia: previous records, and the type of information which it can provide. Difficulties in achieving the successful transplantation of nuclei in Xenopus, and a brief survey of the following chapters.
- II. A description of the technique used for nuclear transplantation in Xenopus, and information needed for its practical application.
- III. An analysis of the effects of ultraviolet irradiation on the uncleaved eggs of Xenopus, as required for nuclear transplantation. The effect on the following structures, of different doses of u.v. at different intervals after eggs have been laid: the egg membranes, the egg cytoplasm, and the egg nucleus. The survival of androgenetic haploids in Xenopus.
- IV. An analysis of the effects of each step in the technique on the development of transplant-embryos. The results of varying individually each part of the technical treatment of donor nuclei and recipient eggs. Variation in the innate quality of recipient eggs. The procedure for selecting and analysing experimental results, so that transplant-embryo survival can be directly related to the genetic qualities of transplanted donor nuclei.

- V. The results of transplanting nuclei from differentiating endoderm tissue. The latest developmental stage of donor embryo in which all nuclei are totipotent, and the latest in which some still remain totipotent. The proportion of normal and abnormal transplant-embryos, (also the proportion of regular and irregular cleavage), in relation to the developmental stage of the donor embryo. A comparison of the rate and time of onset of nuclear differentiation in Xenopus laevis and Rana pipiens; hence some comments on the significance of nuclear differentiation during embryonic development.
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preliminary experiments involving the serial transplantation of nuclei into and from the eggs of foreign species and subspecies of frogs.

ABSTRACT. An abstract giving an outline of the experiments carried out and the main results obtained.

APPENDIX. Copies of the following published papers.

Fischberg, M., Gurdon, J.B., and Elsdale, T.R., 1958. *Nature*, 181, 424.

Nuclear transplantation in Xenopus laevis.

Gurdon, J.B., Elsdale, T.T., and Fischberg, M., 1958. *Nature*, 182, 64-65.

Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei.

Fischberg, M., Gurdon, J.B., and Elsdale, T.R., 1958. *Exp. Cell Res.*, Suppl. 6,

161-178. Nuclear transfer in Amphibia and the problem of the potentialities of the nuclei of differentiating tissues.

INTRODUCTION

The time-scale of biological processes is an important factor in determining ⁱⁿ how they may best be investigated. There is a distinction between those which repeat themselves (or can be made to do so experimentally) many times within a human's working lifetime, such as embryonic development, and those continually changing processes such as evolution which rarely, if ever, repeat themselves exactly. Processes of the former kind have mainly been studied in two ways. The first concerns the analysis of a process as it normally occurs, and consists of following the fate of various structures or substances by means of direct microscopy, specific staining, isotopes, etc. Examples of such studies are the mapping of the presumptive regions of a cleaving Styela egg (Conklin, 1905) and of an Amphibian blastula (Vogt, 1929). The second method consists of interfering with a normal process and observing the result of the interference. This can either be done by creating an abnormal environment (e.g. metabolic poisons) or by experimentally placing structures concerned in the process in an abnormal relationship with each other. The method and result of the interference can then provide information about the part played by different factors in the normal process. An example of this kind of experimental investigation is the tissue grafting work of Spemann's school (review, 1938). Nuclear transplantation, which is the subject of the following thesis, is simply a technique for carrying out grafts at the subcellular level, instead of with whole cells or tissues.

The successful transplantation of a living nucleus was first achieved in *Amoeba* (Commandon & Fonbrune, 1939); it has subsequently been carried out with many other unicellular organisms, such as *Acetabularia* (Hämmerling, 1953), *Stentor* (Tartar, 1953), etc. However the transplantation technique is very different in Protozoa and Metazoa; also the conclusions derived from its application in unicellular organisms cannot necessarily be applied to multicellular organisms since the nucleo-cytoplasmic interactions which bring about differentiation are probably very different in organisms of the cellular as opposed to tissue grade of construction. For this reason further discussion of nuclear transplantation will be restricted to multicellular animals.

All attempts at nuclear transplantations in the metazoa have involved the transfer of an embryonic cell nucleus to an enucleated and unfertilised Amphibian egg-cell. An egg was chosen, since it is an exceptionally large cell which is likely to withstand experimental manipulation better than other cells. It is also the ideal kind of cell for ease of culturing and for testing the genetic qualities of a transplanted nucleus, since unlike other cells it should, with a normal nucleus, develop into a complete individual, during the life of which nearly all genes will act and probably reveal their presence. Amphibian eggs have soft penetrable membranes and contain a sufficient quantity of yolk to be large, but not so much as to prevent them being holoblastic; the resulting embryonic cells are large owing to their yolk content, and this facilitates the healing of the cell wall without damage to the nucleus.

In all grafting experiments it is desirable that the technique itself should be harmless, so that any abnormalities resulting from the

operation can be attributed to the altered relationship of the transplanted structures compared to normal. In nuclear transplantation it must therefore be shown that a normal nucleus can be transferred to an enucleated unfertilised egg which can then develop normally. The first real success with nuclear transplantation was achieved with frogs' eggs by Briggs and King (1952), who were able to obtain normal tadpoles in the majority of cases when they transferred blastula cell nuclei to eggs of Rana pipiens. In some instances abnormal development of embryos derived from transplanted nuclei may have been due to technical damage; however, the transplantation of nuclei into the eggs of different species, and that of nuclei from differentiating cells showed that the way in which transplant-embryos develop is, to a large extent, nucleus-specific and therefore depends mainly upon the specific qualities of the donor nuclei. Attempts have been made to transplant nuclei using newts' eggs (Waddington & Pantelouris, 1953†; Lehman, 1955) *, but none gave satisfactory results since in no case was normal development obtained from blastula nuclei which are believed to be undifferentiated. Thus it was not certain to what extent abnormal development was caused by imperfection in the transplantation technique.

Having established that an embryonic cell nucleus can be harmlessly transplanted to an enucleated egg, it is worth considering what kinds of problems this technique can be used to investigate. In effect it enables the zygote nucleus (of a fertilised egg) to be replaced by an embryonic cell nucleus. The development, adult characters, and reproductive capacity of the resulting individual can then be compared to that of an

* The injection of embryonic brei into unfertilised eggs (Rostand, 1943) is not regarded as a case of nuclear transplantation.

individual derived from a fertilised egg; the genetic qualities of the transplanted nucleus are thus directly compared with those of a zygote nucleus. One kind of experiment that can be done with this technique is to test the genetic qualities of somatic nuclei from embryonic cells. It is so far only possible to transplant nuclei from embryonic cells, because fully differentiated cells are very difficult to dissociate and their nuclei are protected by very little cytoplasm. The other kind of experiment for which this technique is suitable is to test the effect on nuclei of various natural and artificial environments; for instance nuclei may be transferred to the eggs of foreign species, and then transferred back to eggs of ^{their} own species, when the resulting transplant-embryos will show the effect of the foreign cytoplasm.

The success with nuclear transplantation enjoyed by **King** and **Briggs** (1956) has shown that their technique has a very wide application. However, Rana pipiens, with which they developed their technique, has a limited geographical distribution and cannot be at all easily kept or reared under laboratory conditions. It was therefore suggested by Dr. M. Fischberg that an attempt should be made to adapt Briggs and Kings' technique for use with the eggs of Xenopus. This is an extremely convenient laboratory animal, since it suffers little from disease, and can be easily reared in the laboratory to sexual maturity within one year. Another great advantage of this species is that eggs can be obtained from it at any time of year by the injection of commercial gonadotropic hormone. There are, however, considerable differences between the eggs of Rana and Xenopus which prevent the direct application of Briggs and King's technique to the latter species.

The most serious of these is the extreme elasticity of the vitelline membrane of Xenopus eggs, which prevents their penetration by a micro-pipette and their enucleation by the usual method of Porter (1939) with a needle. This difficulty was overcome by the fortunate discovery in collaboration with Dr. TR. Elsdale that ultraviolet irradiation weakens the vitelline membrane. It was later found that the same treatment with u.v. inactivates the egg pronucleus but does not damage the egg cytoplasm. After certain other difficulties had been overcome, nuclear transplantation in Xenopus gave results comparable to those obtained by Briggs and King with Rana.

The following brief survey is only intended to act as a guide to the ensuing chapters, showing how they are related to each other; it does not aim at giving a representative impression of the results obtained. Chapters II to IV are primarily concerned with the description and analysis of the transplantation technique, and chapters V to VIII with some results of its application.

Details of the technique and of rearing embryos are given in chapter II. Chapter III is an analysis of the effects of u.v. as used in nuclear transplantation experiments; particular attention is given to the fate of the irradiated egg pronucleus which dies in the egg cytoplasm, and might affect the further development of the egg and transplanted nucleus. Chapter IV is an analysis of the technique and shows to what extent abnormal

transplant-embryo development can be attributed to any part of the technical treatment of donor nuclei and recipient eggs, or to the poor quality of recipient eggs. As a result of this investigation, transplantation experiments with Xenopus can be analysed in such a way that the results only depend upon the innate qualities of the donor nuclei.

Chapters V and VI describe some experiments which show how somatic nuclei change as the endoderm cells from which they were taken ~~belong~~ become differentiated. Chapter V describes the embryonic development of transplant-embryos up to the swimming tadpole stage, and chapter VI describes the adult characters and reproductive capacity of the frogs to which these tadpoles gave rise. In this way endoderm nuclei have been tested not only for genes which affect embryonic differentiation but also for those which have their main effect later in life.

The changes which endoderm nuclei are found to undergo are presumably induced by the surrounding cytoplasm. Some experiments have been begun which aim at ^w demonstrating that a cytoplasmic environment can bring about genetic changes in nuclei. In these experiments nuclei have been transferred to eggs of different species or subspecies, and then, after they have synthesised from foreign cytoplasm, they have been transferred back to eggs of their own species. The results of these experiments are not yet conclusive, but are briefly described at the end of chapter IX, and in the appendix.

During these experiments some series of transplant-frogs have been obtained which are of interest apart from their connection with the main theme of nucleo-cytoplasmic interactions. For no obvious reason a

variable proportion of all transplanted nuclei become tetraploid before commencing development. Chapter VII describes the resulting tetraploid frogs and their gametogenesis, since very few tetraploid Anura have been previously recorded. Many genetically identical individuals can be obtained by nuclear transplantation in a species which only reproduces itself sexually. A series of such genetically identical frogs is described in Chapter VIII; these would be of value for any experiment in which it was important to exclude the effect of genetic variation from the results.

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CHAPTER II.

DESCRIPTION OF THE TECHNIQUE FOR NUCLEAR TRANSPLANTATION IN XENOPUS LAEVIS

Synopsis

- I. INTRODUCTION
- II. APPARATUS
- III. THE NUCLEAR MARKER
- IV. THE PREPARATION OF DONOR CELLS
- V. THE PREPARATION OF RECIPIENT EGGS
- VI. THE TRANSPLANTATION PROCEDURE
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- VIII. THE MAIN DIFFERENCES IN THE NUCLEAR TRANSPLANTATION TECHNIQUES
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- IX. SUMMARY

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References

DESCRIPTION OF THE TECHNIQUE FOR NUCLEAR
TRANSPLANTATION IN XENOPUS LAEVIS

by, T.R. Elsdale, J.B. Gurdon and M. Fischberg¹.

I. INTRODUCTION

A method by which nuclei can be successfully transplanted into Amphibian eggs was first worked out by Briggs and King (1952), for the eggs of Rana pipiens. Xenopus laevis is an atypical Anuran since its eggs can be obtained through^{aut} the year, and the resulting embryos can be reared to maturity within 12 months under laboratory conditions. Because of these advantages we have used Xenopus for nuclear transplantation experiments. Though the principle of Briggs and King's technique has been followed, differences between the eggs of Rana and Xenopus have made it necessary to modify their technique before it can be satisfactorily applied to the eggs of Xenopus. The purpose of this publication is first to give technical details of these modifications, and secondly to discuss the extent to which they might affect a direct comparison of the results of transplantations in Rana and Xenopus.

II. APPARATUS

A low power binocular microscope giving a magnification of between X 25 and X 50 has been found suitable for transplanting nuclei. A syringe capable of delivering small quantities of fluid is required. We have used an "Agla Micrometer Syringe Outfit" (Burroughs Wellcome & Co., London), in

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which a fairly strong spring was fitted to the shaft of the plunger, controlling the uptake of fluid. The needle of the syringe is connected to a micropipette by polythene tubing of about 1 mm bore. The micropipettes are constructed as follows. Lengths of thin-walled glass tubing are cleaned chemically and thoroughly washed out. They are then drawn out to an internal diameter of 20 to 50 μ as required. The syringe, tubing, and micropipette are all filled with liquid paraffin (B.D.H.); this has been found to give superior control to that given by air or water. It does not appear to have any harmful consequences on either the donor cell or recipient egg, with which it should not in any case come into contact. The pipette is held and controlled by a Singer "Microdissector" (Plate 1, fig. A). This instrument reduces all hand movements by a factor of about four times, and does not invert movements in any direction. Direct movement is particularly valuable in the vertical plane.

The source of ultraviolet irradiation which we have used for inactivating the egg pronucleus is a 100 Watt, medium pressure, mercury arc lamp, made by Hanovia Ltd., Slough, England. This source gives an appreciable emission at all wavelengths from 2,100Å to 3,200Å as well as visible light. Further details can be supplied by the manufacturers. The lamp is mounted horizontally giving a downwardly directed beam onto the eggs, which are placed 13 cm. below the bulb. A quartz condenser, 4 cm. above the eggs, focuses the beam onto an area about 2 cm. in diameter.

III. THE NUCLEAR MARKER

A nuclear marker (Elsdale, Fischberg, and Smith, 1958; Fischberg and Wallace, in press) has proved most helpful for our nuclear transplanta-

tion experiments. Various uses to which this marker has already been put have been described in recent publications from this laboratory (Fischberg, Gurdon, and Elsdale, 1958; Gurdon, 1960; Blackler, 1960). Diploid nuclei of wild-type Xenopus each have two nucleolar organisers, and usually have two nucleoli; however a variable proportion of such nuclei (average 30 - 50 %) have only one nucleolus resulting from fusion of the nucleolar material of the two organisers. The nuclear marker consists of a mutation causing inhibition or loss of the nucleolar organiser, of which there is one in every haploid set of chromosomes. Thus nuclei of diploid individuals heterozygous for this mutation have only one nucleolus, and may therefore be easily distinguished from nuclei of normal diploids, the majority of which have two nucleoli. In all other respects individuals heterozygous for this mutation are entirely normal. Animals homozygous for this mutation possess no proper nucleoli, but have small droplets of nucleolar substance which are of irregular size and number. However homozygotes are inviable, and die before feeding.

For nuclear transplantation experiments it has been customary to transplant marked donor nuclei into eggs of unmarked female frogs. Assuming that the ploidy and nucleolar-number of a transplant-embryo is known, the origin of its nuclei is immediately apparent, (see Table 1). As is described below (p. 8) the nucleolar marker can be rapidly identified under the phase-contrast microscope.

IV. THE PREPARATION OF DONOR CELLS

The developmental stages of donor embryos have been identified according to the Normal table for Xenopus laevis, by Nieuwkoop and Faber, (1956).

TABLE 1

The use of the *Xenopus* nuclear marker for determining the origin of nuclei in transplant-embryos. Donor nuclei from the 1-nucleolated strain are transplanted into recipient eggs from normal 2-nucleolated individuals.

FLOIDY	NUMBER OF NUCLEOLI		
	1	2	3
Haploid	Parthenogenetic development of haploid egg-pronucleus. Less than 1%.		
Diploid	Development of transplanted nucleus only. 60-100%.	Parthenogenetic development of diploid egg-nucleus. Less than 1%.	
Triploid		Haploid egg-nucleus combined with diploid transplanted nucleus. Less than 1%.	
Tetraploid		Doubled transplanted nucleus. Up to 40%. (Gurdon 1960 a).	Diploid egg-nucleus combined with transplanted nucleus. Less than 1%.

1956. The dissociation of donor cells has been carried out by the methods of King and Briggs (1955). We usually use Barth's saline solution "X" (Barth & Barth, 1959) instead of Niu and Twitty^{solution}, but the results are very little, if at all, affected by this change. Many tissues, especially those of younger embryos, can be dissected away from the rest of the embryo without the help of trypsin. The tissue is placed in Barth's solution "X", in which Calcium and Magnesium are replaced by 5×10^{-4} Versene. The time required in Versene to bring about dissociation increases the older the donor embryo. For early stages such as blastulae, 10 minutes of Versene is sufficient. Longer periods of 20 minutes or more are needed for later stages. Disaggregated cells can be kept without harm in normal strength Barth solution phosphate buffered to about pH 7.5, for over half an hour before being transplanted. When disaggregated, they are transferred to an agar-covered slide with at least 1 ml. of Barth solution on it. To prevent evaporation altering the strength of the saline solution, the donor cells should only be placed in the beam of the microscope lamp when drawing the cell into the pipette.

V. THE PREPARATION OF RECIPIENT EGGS.

Adult females of Xenopus can be induced to ovulate several times a year by injection of gonadotrop~~h~~ic hormone. We have used the commercial produce "Conan" (B.D.H.) or "Pregnyl" (Organon laboratories Ltd.), and find that a dose of about 300 units is suitable for females and one of 150 units for males (which should have black nuptial pads on the forearm before injection). The dose is varied according to the size of the animals and

their condition. The presence of a male is not necessary to induce a hormone injected female to lay eggs. It has been shown (Curdon, 1960 c) that eggs used as recipients for nuclear transplantation give very different results not only according to the frog used, but also from one ovulation to the next of the same frog. No way has yet been found of improving the quality of eggs, and we have no evidence that the eggs of frogs imported from Africa give consistently better results than the eggs of those reared in the laboratory.

Before transplanting a donor nucleus into an egg, it is necessary to remove or inactivate the egg-prenucleus. In frogs of the genus Rana and in newts this can be reliably done by lifting the egg-nucleus and a little surrounding cytoplasm out of the egg with a needle (Porter's technique, 1939). This method is used by Briggs and King for their nuclear transplantation experiments with Rana. The great strength and elasticity of the vitelline membrane of Xenopus eggs prevents this method being used both reliably and harmlessly for the eggs of this species. ^(Curdon, 1960 b) We have found that a short exposure (less than 1 minute) of the animal pole of an egg to ultraviolet irradiation inactivates the egg pronucleus but does not damage the egg in any other respect. Another very considerable advantage of this ultraviolet treatment is that it renders the vitelline membrane penetrable to a micropipette without destroying it. It may also sterilise the injection area. Unless weakened in this way, the vitelline membrane can not be penetrated by a pipette without damaging the egg. The effects of ultraviolet irradiation on Xenopus eggs forms the subject of another publication (Curdon, 1960 b); this describes the fate of the irradiated egg-nucleus, and considers the evidence that the ultraviolet does not damage the egg cytoplasm.

The main reason for this belief is that a considerable increase in u.v. dose does not increase the abnormalities of transplant-embryo development.

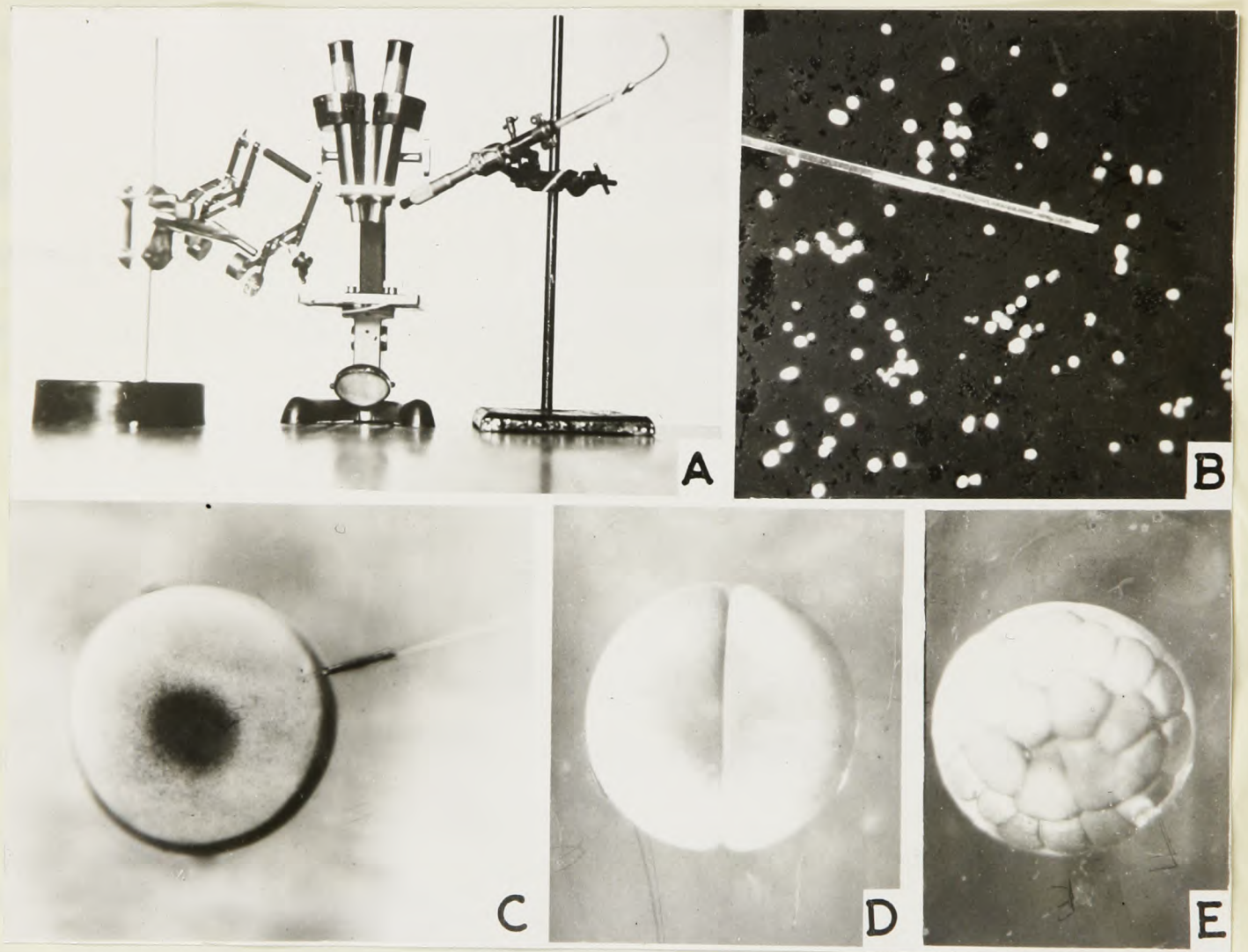
Best results are obtained if eggs are not used for transplantation immediately after laying, but are left in water for about 15 minutes (Gurdon, 1960 *c*). Eggs to be irradiated are placed dry, about six at a time, on a glass slide. Free water must be removed so that the egg remains with its animal pole facing upwards. The slide carrying the eggs is then placed under the ultraviolet beam for the appropriate amount of time (between 20 and 50 seconds according to the particular ovulation). The eggs are now ready for the injection of the donor nucleus.

VI. THE TRANSPLANTATION PROCEDURE

The disaggregated donor cells on an agar-covered slide, can be seen very easily if all light coming from below the slide is cut out, and strong illumination from above is used (Plate 1, B₁). There is some variation in the size of cells from any region of a donor embryo, and a cell is chosen which is just too big to fit into the end of the pipette, so that the cell-wall is broken when the cell is sucked up (Plate 1B). The cell cytoplasm should still completely surround the nucleus which must not be greatly distorted by this treatment. This part of the technique follows the technique of Briggs and King (1952).

The slide carrying the irradiated eggs (still not surrounded by fluid) and that carrying the donor cells can conveniently be placed on a larger glass slide. This enables the donor cells or recipient eggs to be quickly placed in the field of view as required. Before the donor cell is drawn into the pipette, a small air-bubble should be sucked into it, so as

PLATE 1, figures A - E.



Legends to figures.

PLATE 1.

- Fig. A. Photograph of the transplantation apparatus, showing a 'Singer' micromanipulator, a microscope, and an 'Aglar' syringe with polythene tubing. Illumination is provided by a small spot lamp (not shown).
- Fig. B. Disaggregated cells of a Xenopus laevis late blastula, one of which has just been sucked into the end of the micropipette; the air-bubble marker can be seen in the ~~end~~^{shaft} of the pipette. X 25.
- Fig. C. The micropipette is seen inserted into the recipient egg. The donor cell has been injected and the air-bubble marker (dark) is seen where the shaft of the pipette penetrates the egg. X 25.
- Fig. D. A 2-cell stage of a successfully transplanted egg. The penetration wound has completely healed and can no longer be seen. X 25.
- Fig. E. Another transplant-embryo at the morula stage. The small exovate from the injection (lower right of picture) has separated from the surface of the 'egg', and will not interfere with further development. X 25.

to separate the paraffin from the saline solution (Plate 1,B). This allows the passage of fluid out of the pipette to be accurately observed when the donor cell has passed out of sight, (Plate 1,C). The position of the air bubble must of course be adjusted each time a new donor cell is sucked up. While watching the air bubble the syringe is turned so that the donor cell and a small amount of saline solution is deposited near the centre of the egg. If this is done carefully and if the u.v. dose has been judged correctly, the egg will heal up very quickly after penetration (Plate 1,D,E). It is desirable to inject a small amount of saline solution with the donor cell since this does not affect development, and it helps to ensure that the cell is not damaged by the end of the pipette. During the operation the pipette has been controlled with the help of the microdissector; it is now withdrawn from the egg, and the same procedure repeated for the next egg. The average time required for the injection of one egg with a nucleus is about one minute. Thus if six eggs on one slide are irradiated and injected, the eggs will remain uncovered with fluid for about ten minutes. If the operation takes longer, fewer eggs should be done at one time. If eggs are left uncovered by water for too long, the jelly and vitelline membrane may dry onto the egg surface, causing abnormal development or preventing development altogether.

VII THE CULTURING AND SCORING OF TRANSPLANT-EMBRYOS

As soon as the eggs on one slide have all been injected with a nucleus, the slide is placed in a small petri dish containing full strength Niu and Tritty solution. The eggs are left in this solution for a few hours,

since this facilitates the healing of the injection wound. This solution is replaced by 1/10th strength Miu and Twitty solution some time during cleavage. Shortly before hatching the embryos are placed in tap-water, from which excess chlorine has been allowed to evaporate. When tadpoles are ready to feed they are placed in 50 ml. tubes and fed on a weak solution of powdered nettles (*Urtica* sp.). They are changed to a larger bottle (about 3 tadpoles per litre of water) as they grow, but are fed on nettle powder until metamorphosis which occurs about two months after laying. We experience very little mortality between the beginning of feeding and metamorphosis, and have not found it necessary to use antibiotics at any stage. After metamorphosis frogs are fed on tubifex, and later on liver.

Many transplant-embryos develop abnormally, and the following procedure is followed when it is clear that an embryo will not differentiate any further. It is drawn under a camera lucida; then a small part of it is lightly squashed on a slide under a cover slip. The number of nucleoli per nucleus can be quickly counted under the phase-contrast microscope (Elsdale, Fischberg & Smith, 1953). Chromosome numbers can be determined after ~~leaving the~~ leaving the tissue in aceto-orcein for about one minute before squashing. The rest of the embryo may then be fixed for sectioning. If an embryo is not required for sectioning, the whole of it may be squashed so that the ploidy and nucleolar number can be estimated in different regions. If the embryo is old enough to have a tail, part of this may be removed and stained in haemalum which shows the nucleoli and chromosomes clearly.

VIII THE MAIN DIFFERENCES IN THE NUCLEAR TRANSPLANTATION TECHNIQUES FOR XENOPUS AND RANA

The most obvious difference between Briggs and King's technique for Rana and our modifications of it for Xenopus concerns the method of recipient egg enucleation. With Rana the egg nucleus is removed from the egg, while in Xenopus it is irradiated with ultraviolet and then allowed to degenerate in the egg cytoplasm. The fate of the irradiated egg nucleus in Xenopus has been followed, and there is no reason to believe that this or the other consequences of the ultraviolet treatment affect the development of the transplanted nucleus in any way (Gurdon, 1960 b).

Another modification which we have introduced concerns the Xenopus nuclear marker. The presence of this marker is not associated with any abnormalities, but gives proof that the nuclei of transplant-embryos are derived from the injected nucleus alone.

Other differences also exist which are of considerable practical importance but which do not affect the results. For instance, Xenopus eggs are left uncovered by saline solution for about ten minutes during transplantation; this only causes damage if the eggs are allowed to become dry on the surface; but eggs in which this has happened can easily be recognised and thrown away.

When comparing nuclear transplantation experiments in Rana and Xenopus, variation in the quality of recipient eggs must be considered. We have found that nuclei from the same donor embryo, when transplanted into the eggs of different females, can give very different results. It seems that some of our abnormal transplant-embryos may be attributed to poor

quality of recipient eggs; this might be due to the fact that it is not possible to give Xenopus the same conditions in the laboratory as they enjoy in the wild.

One may conclude that, with the possible but unlikely exception of egg nucleus degeneration in Xenopus, none of the differences in technique between Rana and Xenopus influence the results of experiments with these species.

The results must therefore represent real differences between the species and not differences in technique. This conclusion only applies to the principle of the technique, and does not take account of practical variation due to inconsistency with which the technique is carried out on different occasions or by different workers. The effect of varying certain parts of the technique more than would be done under normal circumstances is described elsewhere (Gardon, 1960 c), thus estimating the consequences of practical inconsistencies. Since differences in technique between Rana and Xenopus probably do not affect the results, experiments on these two species may be directly compared.

IX. SUMMARY

1. Technical details are given of a method of transplanting embryonic nuclei in Xenopus laevis.
2. This technique is based on Briggs and King's method for Rana pipiens. Owing to certain differences between the eggs of Rana and Xenopus, their technique has been modified for Xenopus.

3. The main modification for Xenopus concerns the use of ultraviolet irradiation instead of enucleation by a needle.
4. Differences between transplantation techniques with Rana and Xenopus are considered unlikely to account for the differences in experimental results obtained with these species.

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THE EFFECTS OF ULTRAVIOLET IRRADIATION ON THE UNCLEAVED EGGS

OF XENOPUS LAEVIS.

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THE EFFECTS OF ULTRAVIOLET IRRADIATION ON UNFERTILIZED EGGS OF

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INTRODUCTION

Ultraviolet (u.v.) irradiation of newly laid Xenopus eggs has been found to kill the egg pronucleus without damaging the egg cytoplasm. This appears to be the most reliable and least damaging way of "enucleating" the eggs of this species. The enucleation of eggs is a technique required for many different kinds of experiment; these include: (a) the enucleation of fertilised eggs for the production of androgenetic haploids, hybrid merogons, etc., and (b) the enucleation of unfertilised eggs as in nuclear transplantation.

This paper describes the fate of the dying egg pronucleus after the u.v. treatment; this is of considerable importance in the interpretation of nuclear transplantation experiments which have been carried out with Xenopus (Fischberg, Gurdon, & Elsdale, 1958). The question of whether u.v. damage has been sustained by the egg cytoplasm has been investigated.

METHODS

U.V. Irradiation

The u.v. treatment of unfertilised Xenopus eggs has been worked out for the purposes of nuclear transplantation (Elsdale, Gurdon, & Fischberg, 1960). After the jelly has swollen by uptake of water, eggs are placed on a dry glass slide, their animal pole facing upwards; it is important to

remove free water from around them since their orientation may otherwise change. The eggs are now exposed to the u.v. beam for the appropriate time (see p. 6).

The u.v. source which has been used is a Henovia 100 Watt, medium pressure, mercury arc lamp; it was used without a filter but with a quartz ~~condenser~~ condenser. The eggs are placed about 13 cm from the source which is mounted so as to give a downwardly directed beam.

Enucleation of the egg by microdissection

Porter's (1939) method of enucleating eggs by lifting up the egg-spot with a needle cannot be reliably applied to the eggs of Xenopus, since the very elastic vitelline membrane impedes the formation of an exovate. The following method has therefore been developed. It enables eggs to be enucleated with considerable accuracy (97%), but ~~which~~ may disturb the organisation of the cytoplasm. A tungsten needle is pushed through the cortex of the egg so that its shaft lies just underneath the egg-spot. The needle is then lifted tangentially away from the egg, thus stretching the vitelline membrane, which carries the egg-spot and associated cytoplasm with it. Forceps are used to pinch off an exovate from the egg, and the needle disperses the exovate into the jelly, allowing the vitelline membrane to return to the surface of the egg. The use of forceps enables the size of the exovate carrying the egg nucleus to be easily controlled. Hamilton (1957) has also obtained androgenetic haploids in Xenopus by a modification of Porter's technique.

Criteria for determining the success of enucleation or of pronucleus inactivation

In order to determine the efficiency of these methods, fertilised eggs were used, and a resulting haploid individual was assumed to indicate a successful enucleation or irradiation. Though a haploid nucleus is known to occasionally become diploid, this occurs very seldom and the presence of a diploid was taken to indicate a failure of the operation. Chromosome numbers were estimated in different ways. The number of nucleoli per nucleus can be counted with the phase-contrast microscope from squash preparations of embryos. Nucleoli and chromosomes can also be counted after aceto-orcein or haemalum staining of embryos or tail-tips. Because the size of nuclei varies according to developmental stages as well as ploidy, and because the haploid syndrome of abnormalities is sometimes shown by diploids, these criteria were used as an indication rather than proof of haploidy. Only those embryos which passed the blastula stage are included in the results given below.

In the case of transplant-embryos (i.e. embryos resulting from an irradiated unfertilised egg and an injected nucleus) the successful inactivation of the egg nucleus was confirmed by the use of a nuclear marker (Elsdale, Fischberg & Smith, 1958; Fischberg & Wallace, in press). In transplantation experiments the donor nucleus possessed the nuclear marker, while the recipient egg did not. Analysis of the ploidy and nucleolar number of transplant-embryos shows clearly whether or not the egg-pronucleus has participated in development. This only happens rarely, and when it does the transplant-embryos concerned can be excluded from the results.

EXPERIMENTS AND CONCLUSIONS.

Evidence that u.v. irradiation kills the egg-nucleus and not the sperm nucleus.

In order to investigate which pronucleus is inactivated by the u.v., experiments have been made with the *Xenopus* strain possessing a nuclear marker (Elsdale, Fischberg, & Smith, 1958; Fischberg & Wallace, in press). The principle of the experiments has been to mate a normal frog, all of whose (haploid) gametes are uni-nucleolate, to a heterozygous mutant frog of which half the gametes possess no nucleolus at all (0-n), and half are uni-nucleolate (1-n). The eggs were irradiated soon after fertilisation, and all developed as haploids. One of two results are expected from such a cross, according to the way in which the cross is made. The haploid progeny may consist of both 0-n and 1-n individuals; the 0-n embryos could only have been derived from the mutant parent, while the 1-n embryos could have come from either parent. The gametes of a mutant parent are present in equal proportions of 0-n and 1-n (Elsdale, Fischberg, & Smith, 1958). Thus if both kinds of embryos are present in about equal numbers in the progeny of this cross, it is reasonable to assume that all the 1-n individuals have also come from the gametes of the mutant parent. It is only if the 0-n are significantly fewer than the 1-n that one must suspect that some of the 1-n were derived from the non-mutant parent. The other expected result is for all the progeny of the cross to be 1-n, with no 0-n present at all. The absence of 0-n means that none of the 0-n gametes from the mutant parent have escaped irradiation, but it is nevertheless possible that some 1-n gametes from the mutant parent have contributed to the progeny of the cross. The extent to which this may have occurred becomes smaller, the

greater are the number of embryos in the progeny, among which no 0-n's were found.

In the first experiment to be carried out, a normal female was mated to a mutant male. The progeny of this cross contained 61 1-n haploids and 56 0-n haploids. These figures do not differ significantly from equality. In this experiment the 0-n embryos were derived from the male gametes, and there is no reason to believe that any of the 1-n embryos were derived from the egg-pronucleus. This experiment was then repeated the other way round, by using a mutant female and normal male; the result was 117 1-n embryos and no 0-n's. This confirms the result of the previous experiment, since there is again no reason for believing that the pronucleus of any egg escaped u.v. inactivation.

Further confirmation of these results comes from nuclear transplantation experiments in which marked donor nuclei have been injected into unmarked recipient eggs. The nuclear marker shows that the great majority of the resulting embryos were derived from the injected nucleus alone. In less than 1 % of cases does the egg nucleus participate in development; the reason for the failure of inactivation on these occasions is not clear, but may be due to rotation of the egg during irradiation, or to the presence of foreign matter overlying the egg nucleus.

The results of these experiments show that u.v. irradiation of the animal hemisphere of the egg selectively kills the egg pronucleus, while this is situated near the surface of the egg. Haploids which develop after such irradiation of fertilised eggs are apparently always androgenetic. The u.v. treatment in these experiments does not seem to affect either the sperm nucleus or the injected nucleus.

The effect of u.v. on the jelly and vitelline membrane of the egg

One effect of u.v. irradiation on eggs is to "dissolve" the jelly and weaken the vitelline membrane. The latter effect of u.v. is very valuable for nuclear transplantation experiments, since the vitelline membrane cannot otherwise be penetrated by a micropipette without causing considerable damage to the egg. The author wishes to emphasise that this effect and its value for nuclear transplantation experiments was appreciated in collaboration with Dr. T.R. Eldale. If too great a dose is given, the vitelline membrane fails to heal after penetration, and a large exovate is formed; too large a dose may also lead to the vitelline membrane drying on to the cortex of the egg, so causing abnormal development. With a moderate dose of u.v. the vitelline membrane heals well, but later breaks open, liberating the embryo - unharmed - a few hours before the usual hatching time. Thus it is necessary to choose an optimum u.v. dose which is sufficient to enable the egg to be penetrated without greatly distorting it, but which is not so great as to prevent healing of the injection wound. The optimum dose largely depends upon the rate at which the jelly 'dissolves' and that at which the vitelline membrane becomes penetrable. The duration of the dose, which must be determined experimentally, varies from 15 to 50 seconds for the eggs of different frogs, though it is fairly constant for all eggs of any one ovulation. The reason for this variation is that eggs vary according to the ovulation in the amount of jelly they have, and in the extraneous matter attached to them. It seems to be necessary for the u.v. rays to break down the jelly before they can reach the egg nucleus, and it is probable that even in eggs which require very different optimum doses of u.v., the egg nucleus nevertheless receives the same dose.

The effect of the u.v. upon the egg nucleus

Variation of the u.v. dose.

The effect of u.v. upon the egg nucleus varies considerably according to the duration of the treatment (table 2), and also according to the length of time between fertilisation and irradiation (table 1). Table 1, which includes experiments done on the eggs of 3 different frogs, shows that the nucleus of eggs irradiated within 20 minutes (at 20° C) of fertilisation is almost always inactivated. Eggs irradiated more than 20 minutes after fertilisation are often diploid, since the egg pronucleus has sunk far enough below the egg surface to be protected from the u.v. If the egg is not fertilised, the egg nucleus does not sink below the surface at once. Thus there is no need to irradiate unfertilised eggs for subsequent transplantation immediately after laying.

Another important respect in which the u.v. dose can be varied concerns its duration. It was mentioned above that an optimum u.v. dose is selected for each set of eggs according to their penetrability by a micropipette. It is fortunate that this same optimum dose is sufficient in nearly all cases to kill the egg nucleus, thus producing androgenetic haploids from fertilised eggs. It is of interest to find out whether this dose is the minimum capable of killing the egg nucleus or whether a reduction in it would still provide a high proportion of haploids. Table 2 shows the effect of varying the dose above and below the optimum, which in this case was 50 seconds.

TABLE 1.

The effect of u.v. irradiation on eggs at different intervals
after fertilisation.

<u>Time between fertilisation</u> <u>and irradiation (mins)</u>	<u>Total embryos</u> <u>analysed</u>	<u>Haploid</u>	<u>Diploid</u>	<u>Mosaics</u>
5 - 11	31	29	2	0
12 - 20	53	53	0	0
21 - 29	23	11	11	1
30 - 39	23	0	23	0
40 - 50	18	0	18	0
CONTROLS (No u.v.)	187	0	187	0

TABLE 2.

The effect of different doses of u.v. on the ploidy and survival of embryos derived from irradiated fertilised eggs.

	<u>DOSE OF U.V.</u>				<u>Controls</u>	
	<u>20-25 sec.</u>	<u>30-35 sec.</u>	<u>40-50 sec.</u>	<u>60-80 sec.</u>	<u>Microdis-</u> <u>section</u> <u>haploids</u>	<u>No</u> <u>treatment</u>
dy of yos: ploid ploid	9 0	17 0	10 0	10 0	169(97%) 6	0 34
ival of yos: tal numbers	8	20	15	12	169	127
rested te blastu- e:	12½%	10%	26½%	24½%	15%	0.75%
normal strulae	37½%	10%	---	---	20%	---
rested nural folds	---	5%	6½%	---	10%	0.75%
lightly unted post- nurulae	12½%	5%	---	8½%	20%	---
lorocephalic r oedematous haploid synd- rome)	37½%	70%	67%	67%	35%	---
ormal adpoles	---	---	---	---	---	98½

Table 2 and fig. 5 show that the survival of haploids from eggs irradiated for 30 to 80 seconds is very similar, but that many more abnormalities are found among eggs irradiated for 20 to 25 seconds. This almost certainly means that 20 to 25 seconds of u.v. only partially inactivates the egg nucleus, which can then interfere with development. Embryos in the 20 to 25 second group, though having only one nucleolus per nucleus, may well have been hyperhaploid, possessing extra chromosomes or chromosome fragments. The fact that the proportion of abnormalities found among the 3 groups of eggs irradiated for between 30 and 80 seconds is the same, indicates that 30 seconds or more of u.v. completely inactivates the egg nucleus, which does not then affect development. A similar effect was observed by Hertwig (1911) after X-ray treatment of amphibian eggs.

The observation that true haploids are obtained after little more than half the optimum u.v. dose, shows that this dose is more than sufficient for inactivation of the egg nucleus. It is, therefore, very unlikely that any chromosome fragments of the egg nucleus will escape complete inactivation, and then participate in development with the injected or sperm nucleus.

The fate of the irradiated egg nucleus.

This investigation is based upon the analysis of 30 irradiated unfertilised eggs into which blastula nuclei were transplanted, and in which the transplanted nucleus appeared cytologically to be developing normally. The majority of these eggs were fixed during the first mitosis of the

transplanted nucleus (50 to 80 minutes old), and the rest were fixed at various times between 5 and 50 minutes after transplantation. As eggs were fixed at intervals between transplantation and the two-cell stage, it is in most cases not possible to tell whether any one egg would have developed at all, if it had not been fixed. In the main series of eggs with transplanted nuclei used for this analysis, 16 of the 20 eggs fixed appeared to have normally developing transplanted nuclei. The remaining 4 eggs had abnormal transplanted nuclei and would probably have cleaved abnormally. Of 28 unfixed control transplantations done at the same time, 6 did not cleave, 4 cleaved abnormally and the remaining 18 cleaved normally and became normal tadpoles. This means that the conclusions reached should be typical for eggs with a normally developing transplanted nucleus and irradiated egg nucleus.

Eggs were fixed in Perenyi's fluid for about 6 hours, and washed out overnight in 70% alcohol. Before fixation most of the jelly was removed, and after fixation the vitelline membrane was removed from a small part of the vegetal pole, to allow free passage of fluids into the egg. As the egg is very fragile when fixed, it is desirable to leave the vitelline membrane to protect the rest of the egg. Serial sections at 10μ were cut parallel to the equator; they were then stained with Mayer's haemalum, and sometimes restained with Feulgen.

When analysing these sections the position of the nuclei distinguishes the developing nucleus from the egg pronucleus. However, the following criteria have been used to confirm their identification:

1, The egg pronucleus is never surrounded by a clear yolk-free area of cytoplasm, which is always associated with the transplanted nucleus (compare fig. 1, A-D with fig. 1, F,H; in fig. 1, E, G the egg nucleus is inside the clear area associated with the developing nucleus).

2. Nuclei of cells from the vegetal pole of the donor embryo have been used for transplantation; these nuclei have very large (vegetal) yolk platelets associated with them until their first mitosis after transplantation. These large yolk platelets are very conspicuous among the much smaller platelets of that part of the egg in which the transplanted nucleus lies (dyp in figs. 1, F and 2, A, B).

The events which take place after the successful irradiation of the egg nucleus are shown in figs. 1 and 2. At the time of irradiation the egg nucleus is at metaphase of the second maturation division; in spite of irradiation it completes that division, extruding the second polar body and forming an apparently normal pronucleus. The extrusion of the second polar body has only been observed in a single egg, and it is probable that in some irradiated eggs, the whole metaphase plate sinks into the egg as a diploid egg nucleus which then degenerates. The pronucleus now moves down towards the centre of the egg (fig. 1, A-D), just as it would if it had not been irradiated. 30 minutes after transplantation the irradiated pronucleus is still cytologically normal. After 40 minutes the transplanted nucleus has reached the position where pronuclei normally fuse - about one third of the way from the animal pole to the vegetal pole. The pronucleus joins the transplanted nucleus; by this time it has begun to degenerate, as is apparent from its crinkling nuclear membrane and granular nucleoplasm as opposed to the smooth membrane and more homogeneous nucleoplasm of the

PLATE 1.

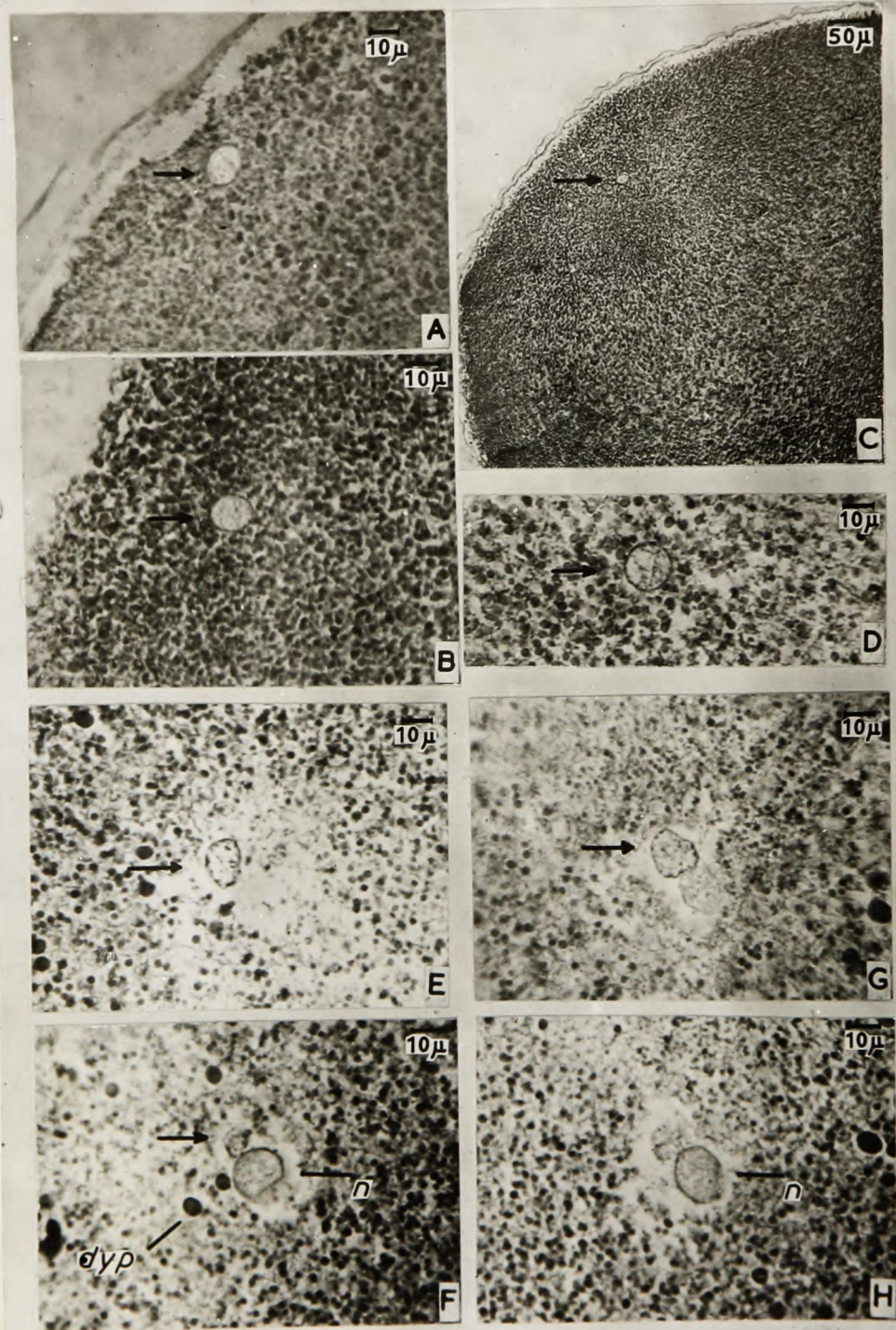


Fig. 1. (plate).

An arrow ($\rightarrow$) indicates the egg pronucleus.

- A, egg fixed 12 minutes after transplantation. The egg pronucleus has completed the second meiotic division.
- B, egg fixed 23 minutes after transplantation, showing the pronucleus moving towards the centre of the egg.
- C, egg fixed 25 minutes after transplantation, showing the egg pronucleus.
- D, egg fixed 30 minutes after transplantation. The pronucleus is still morphologically normal.
- E, egg fixed 40 minutes after transplantation. The dying pronucleus has a crinkled nuclear membrane and granular contents.
- F, the next section of the same egg as in E, showing the transplanted nucleus (n) and donor cell yolk platelets (dyp.). Part of the dying pronucleus (shown in E above) can be seen adjacent to the transplanted nucleus.
- G, another egg fixed 40 minutes after transplantation, showing dying pronucleus. Part of the transplanted nucleus can just be seen in this section below the pronucleus.
- H, the next section of the same egg as in G, showing the transplanted nucleus (n) and the dying pronucleus. The transplanted nucleus has a smooth nuclear membrane and a more homogeneous nucleoplasm.

PLATE 2.

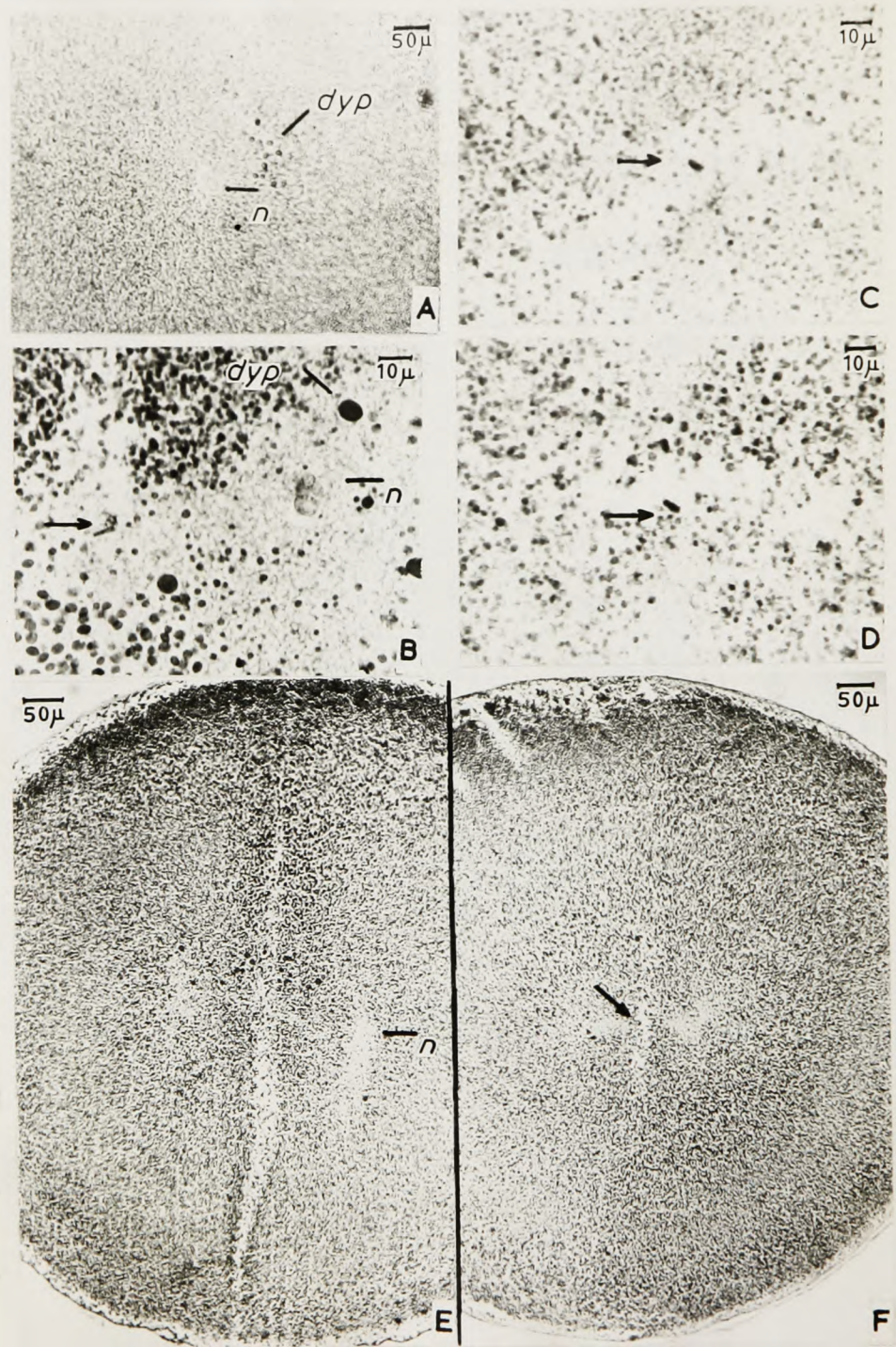


Figure 2 (plate). An arrow indicates the egg pronucleus.

- A. Section showing donor cell yolk platelets (dyp) and yolk-free region of cytoplasm around the developing transplanted nucleus. This is the same section as is shown in fig. 1H.
- B. Egg fixed 59 mins after transplantation, showing the pycnotic pronucleus and a daughter transplant nucleus (n); the latter, which is reforming after the first mitotic division, consists of small spheres at this stage.
- C. Egg fixed 61 mins after transplantation. The pycnotic pronucleus is seen in the centre of the future cleavage furrow.
- D. Another egg fixed 58 mins after transplantation, again showing the pycnotic pronucleus left between the two daughter transplant nuclei (not visible in this section).
- E. Egg fixed 76 mins after transplanation. One of the daughter transplant nuclei can be seen (n), having completed the first mitosis. The egg pronucleus has disappeared by this stage.
- F. Egg 58 mins old; this is the same section as is shown in fig. 2D above, containing the degenerated pronucleus. The transplanted nucleus is at anaphase of its first mitotic division (not visible in this section).

transplanted nucleus (In fig. 1, compare E with F, and G with H. E and F, and G and H show an almost identical situation in two different transplant-embryos.) The egg pronucleus, which remains adjacent to the transplanted nucleus but does not fuse with it, now becomes rapidly smaller and more condensed. About 60 minutes after injection the transplanted nucleus is at anaphase of its first mitosis. The pycnotic egg nucleus is now left between the two newly formed daughter nuclei in the equatorial plane of the spindle, which is also the plane of the future cleavage furrow (fig. 2, B, C, D, F). In fig. 2, B both the degenerating pronucleus and one of the daughter nuclei of the transplanted nucleus can be seen in the same section. When these sections were restained with Feulgen, no Feulgen-positive material could be seen in the cytoplasm, even in the precincts of the strongly staining pycnotic egg nucleus. This indicates that the egg nucleus does not give rise to chromosome fragments which then become incorporated into the transplanted nucleus. 70 to 80 minutes after transplantation, the daughter nuclei have reached the interphase stage (fig. 2, E); in 7 embryos of this stage, no trace could be seen of the egg nucleus, which must by now have completely disappeared.

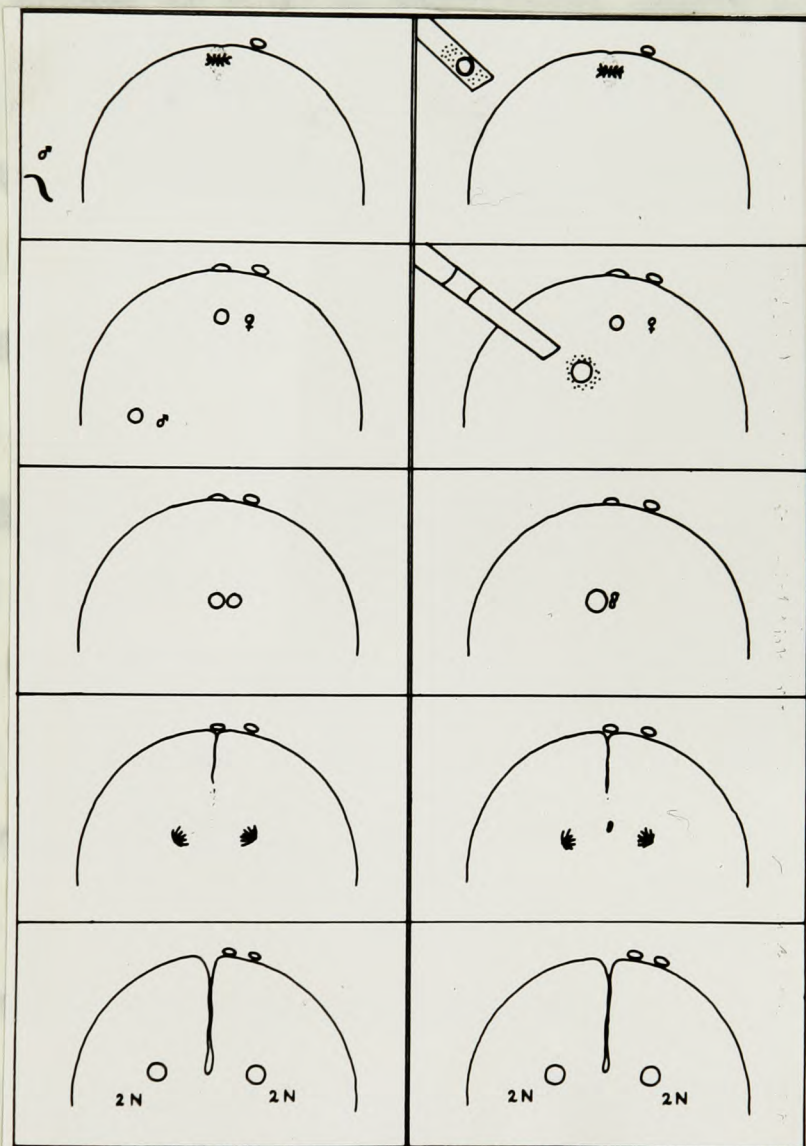
A few fertilised eggs were irradiated and fixed at various times between fertilisation and the 2-cell stage. Sections of these eggs showed that the irradiated egg nucleus behaves in the same way as in eggs with transplanted nuclei. The sequence of events taking place in non-irradiated fertilised eggs and in transplant-eggs with irradiated nuclei are shown diagrammatically in fig. 3.

Figure 3.

Diagram comparing the development of normal fertilized eggs

with irradiated eggs after nuclear transplantation.

Normal fertilized egg U.V.-irradiated egg with trans-planted nucleus.



The behaviour of the irradiated egg nucleus described above is very similar to what is already known to apply to the nuclei of tissues that have been heavily irradiated with X-rays or u.v. (Giese, 1950; Hollaender, 1955). The susceptibility of purines and pyrimidines to destruction by u.v. can account for the finding that the egg nucleus is killed long before the egg cytoplasm is damaged. Selman (1958) has described the effect of u.v. on fertilised newt's eggs (Triturus palmatus). He found that the same dose of u.v. could cause incomplete inactivation of the egg nucleus as well as damaging the cytoplasm. The reason for this rather marked difference in the results with Xenopus and Triturus is not clear, but might be connected with polyspermy.

The effects of u.v. irradiation on the egg cytoplasm

The optimum dose of u.v. irradiation does not seem to cause any damage to the egg cytoplasm. This cannot, however, be demonstrated clearly by any single experiment, so several different ones are described which all support the same conclusion.

The survival of androgenetic haploids.

The extent of u.v. damage can be estimated from the number of abnormalities among fertilised eggs after irradiation. However, eggs irradiated soon after fertilisation develop as haploids, and it is difficult to be certain whether the irradiation has caused any abnormalities, since haploids are inherently abnormal owing to their reduced chromosome number. Table 2 and figs. 5 and 6 show the frequency of abnormalities which have been found among embryos derived from u.v.-treated eggs, and also among haploids

PLATE 4.



Photograph of androgenetic u.v.-haploids (left)
and diploid controls of the same age (right).

The haploids were irradiated for 40 to 50 seconds. A similar amount of variation is found among haploid Xenopus obtained by other means.

obtained by the micro-dissection method described on page 2. In no case did an embryo differentiate further than the microcephalic and oedematous condition referred to as the haploid syndrome (fig. 4). This is generally true of Xenopus haploids obtained by other means (Rostand, 1951). Table 2 and fig. 6 show that 70% of all u.v. haploids irradiated for between 30 and 80 seconds reached the typical haploid syndrome. Since these embryos differentiated as far as is usually possible for haploids, this shows that 70% of eggs irradiated with the optimum u.v. dose do not suffer any cytoplasmic damage.

Figs. 5 and 6 show that the remaining 30% of u.v. haploids developed abnormally. These abnormalities may be due to cytoplasmic u.v. damage, or to considerable variation in the way in which haploids differentiate. Variation in the severity of haploid abnormalities would be expected in view of the fact that the haploid syndrome may be affected by exposed lethal genes (Subtelny, 1958). Owing to genetic variation these would be present in more lethal combinations in one spermatozoon than in another. Variation in the differentiation shown by haploids might also be expected to arise from poor quality eggs. Transplantation experiments (Gurdon, 1960) have shown that the quality of eggs varies considerably and poor eggs may result in abnormalities of development.

These considerations do not prove that u.v. has no harmful effect on the egg cytoplasm, but they constitute reasons for attributing abnormalities of development in fertilised irradiated eggs to other causes.

There is a tendency for eggs irradiated within five minutes of fertilisation to suffer from more abnormalities of development than those

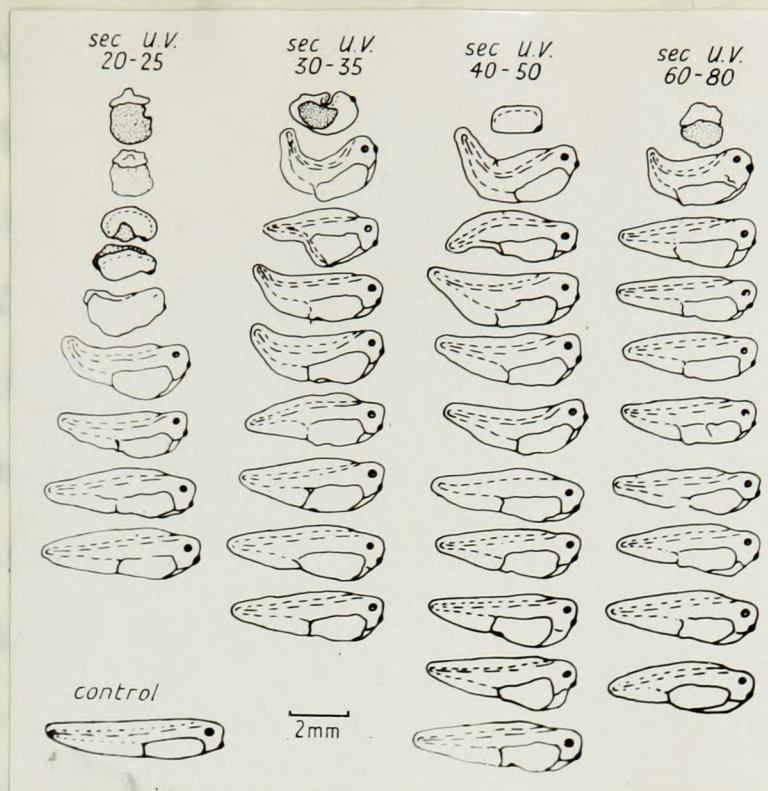


Figure 5. Camera lucida drawings of haploids resulting from different amounts of u.v. irradiation. Many abnormalities of early development can be seen in the 20 to 25 second group, while there is a much smaller proportion of early abnormalities in the next three groups.

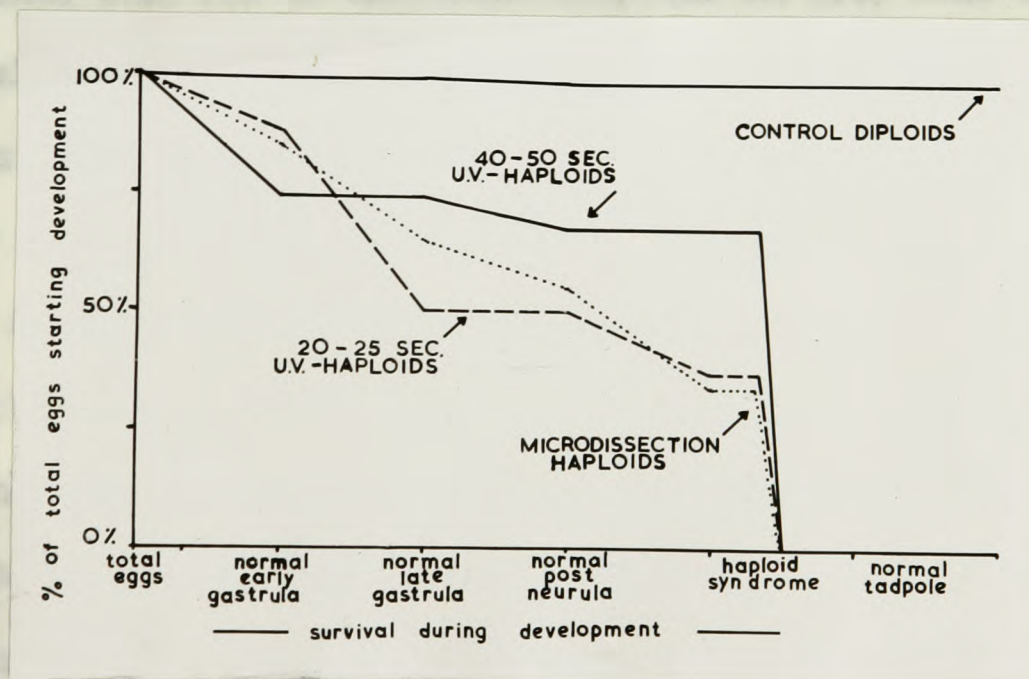


Figure 6. Survival curves for haploids and controls, based on figures given in table 2. U.V.-haploids of the 40 to 50 sec group have a very similar survival curve to those of the 30 to 35 sec and 60 to 80 sec groups, which are not shown. The method by which haploids are obtained causes different proportions of abnormalities in early development, but the furthest stage of differentiation reached is always the same.

irradiated after five minutes from fertilisation. These abnormalities may be due to the chance irradiation of sperm nuclei or to a greater susceptibility of eggs to u.v. at this time. However, this source of abnormality is not thought to affect unfertilised eggs used for transplantation, since these are usually irradiated at about 20 minutes after laying. By this time they have undergone a kind of "activation", which is also observed in fertilised eggs. This is recognised by rotation of the egg, and by a contraction of animal pole pigment; the egg cytoplasm also appears to become more solid in consistency.

The effect of increasing the u.v. dose.

Table 2 shows that there is no increase in the abnormalities shown by u.v.-haploids when the u.v. dose is increased from 30 to 80 seconds. The egg nucleus appears to have been completely inactivated by 30 seconds. The fact that this two to threefold increase in u.v. dose causes no increase in abnormalities is a very strong reason for believing that no cytoplasmic damage was incurred.

The survival of transplant-embryos.

The survival of transplant-embryos varies considerably according to the condition of recipient eggs. If an early donor stage with undifferentiated nuclei is used and the recipient eggs are of good quality, it may happen that all transplant-embryos which pass the late blastula stage become normal tadpoles. This shows that at least in these cases the u.v. treatment of the recipient eggs could not have caused any cytoplasmic damage which has any effect after the late blastula stage.

Irradiated fertilised eggs which become diploids.

If fertilised eggs are irradiated with u.v. more than 20 minutes after fertilisation, the pronucleus of most eggs has sunk below the surface and is not affected by the u.v. The resulting embryos are therefore diploid and these develop into normal tadpoles. This shows that in these embryos the u.v. causes no cytoplasmic damage.

Eggs which are irradiated for 10 to 20 minutes after fertilisation on the vegetal pole as opposed to animal pole, are diploid and suffer no externally visible abnormalities.

CONCLUSIONS OF IMPORTANCE FOR THE INTERPRETATION OF NUCLEAR TRANSPLANTATION

EXPERIMENTS

The main purpose of this investigation has been to analyse the effect of u.v. irradiation on unfertilised eggs which were subsequently used for nuclear transplantation. The following comments therefore draw attention to the conclusions from this work which are particularly relevant to the interpretation of nuclear transplantation experiments. An optimum u.v. dose is chosen for the eggs of each female frog, such that a pipette can be pushed into the egg without causing damage or preventing healing. This dose has been found to be nearly double the minimum required to inactivate the egg nucleus completely. This shows that the egg nucleus is very thoroughly inactivated before the egg is provided with a transplanted nucleus. In the case of unfertilised eggs there is no need to irradiate

them immediately after laying, since in contrast to fertilised eggs the egg nucleus remains on the surface. When the pronucleus degenerates it appears not to disintegrate into visible fragments, but to disappear gradually as a unit; this makes it unlikely that fragments from the pronucleus become attached to the developing transplanted nucleus and affect the future development of the egg.

Concerning u.v. damage to the egg cytoplasm it is worth pointing out that in fertilised eggs irradiated for between 40 and 80 seconds, only about 8% of eggs showed abnormalities after the late blastula stage (Table 2). This means that with the u.v. doses used for nuclear transplantation experiments, most of the damage which might be attributed to u.v. results in arrested late blastulae. For most of our transplantation experiments it is desirable to consider only those transplant-embryos which develop beyond late blastulae. Reasons have already been given (pp. 12-14) for believing that the egg cytoplasm suffers very little u.v. damage with the doses used; but in any case most of the developmental abnormalities which might be attributed to u.v. do not interfere with the interpretation of nuclear transplantation experiments.

SUMMARY

The effects are described of ultraviolet (u.v.) irradiation upon the eggs of Xenopus laevis. The results obtained apply to fertilised eggs and also to unfertilised eggs into which blastula nuclei had been transplanted. Eggs were irradiated up to 20 minutes after laying, for periods varying from 15 to 50 seconds.

The egg nucleus is completely inactivated by small doses of u.v. If fertilised eggs are used this gives rise to haploids, which the use of a nuclear marker has shown to be androgenetic. After irradiation the egg nucleus descends towards the centre of the egg, and comes to lie adjacent to the transplanted or sperm nucleus. At the first mitosis, however, it does not fuse but remains as a pycnotic clump in the centre of the spindle. Soon after this it disappears, without disintegrating into visible fragments. The transplanted or sperm nucleus appears to be unaffected by the irradiation and death of the egg nucleus.

The egg cytoplasm does not appear to be damaged, even after doses of u.v. which are considerably more than sufficient to kill the egg nucleus. The main reasons for this belief are that haploids obtained by other means develop no better than those obtained by u.v. An increase of u.v. treatment from 30 to 80 seconds results in no increase in abnormalities sustained.

The jelly is broken down and the vitelline membrane weakened. This enables the egg to be penetrated by a micropipette without causing damage or preventing healing.

This investigation was undertaken to facilitate the analysis of nuclear transplantation experiments in Xenopus. The increased penetrability of eggs is of technical value for this purpose. The interpretation of these experiments is greatly facilitated by the knowledge that the egg cytoplasm need not be damaged by the u.v. and that the egg nucleus is completely inactivated so as not to interfere with the development of the egg.

Acknowledgements

I wish to thank Professor Sir A.C. Hardy, F.R.S., for having enabled this work to be carried out in his Department. I am very much indebted to Dr. M. Fischberg for his many helpful suggestions throughout this work and for his attention to the manuscript. I am very pleased to acknowledge the contribution to this work of Dr. T.R. Elsdale, in collaboration with whom the usefulness of u.v. treatment and of other aspects of the technique was first appreciated. Miss A.G. Jewkes has provided most helpful technical assistance during this work.

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CHAPTER IV

FACTORS RESPONSIBLE FOR THE ABNORMAL DEVELOPMENT OF EMBRYOS

OBTAINED BY NUCLEAR TRANSPLANTATION IN XENOPUS LAEVIS.

Synopsis.

INTRODUCTION

DESCRIPTION OF THE TECHNIQUE

DONOR NUCLEI

The disaggregation and storage of donor cells.

The treatment of donor cells in the injection pipette.

RECIPIENT EGGS

Technical treatment of recipient eggs

Innate variation in the quality of recipient eggs

DISCUSSION AND CONCLUSIONS

SUMMARY

FACTORS RESPONSIBLE FOR THE ABNORMAL DEVELOPMENT OF EMBRYOS
OBTAINED BY NUCLEAR TRANSPLANTATION IN XENOPUS LAEVIS.

INTRODUCTION

In Xenopus, the embryos derived from nuclear transplantation often develop abnormally. These abnormalities must be due to the limited potentiality for development of either the donor nucleus or the egg-cytoplasm; this limited potentiality will in turn be due to technical damage during transplantation or to the innate condition of the nucleus or cytoplasm before the experiment. The extent to which these technical and innate factors are responsible for abnormalities of transplant-embryo development has been analysed by considering the effect of each factor in turn. Nuclei from early donor stages have been used, since these nuclei are believed to be undifferentiated (see p. 15) and therefore to have the innate capacity for entirely normal development. The effects of other factors have been investigated by experiments in which each factor is varied in different ways. Any correlation between variations in one factor and the resulting proportion of abnormal transplant-embryos is then recorded. In this way the effect of each factor on the development of transplant-embryos can be analysed.

These experiments show how nuclei can be transplanted with least damage, and whether small inconsistencies in carrying out the technique can bring about abnormalities of development. It is thus made clear to what extent abnormal transplant-embryos may be considered to represent innate qualities of donor nuclei.

TECHNIQUE

As the method of nuclear transplantation for Xenopus laevis has been described elsewhere (Elsdale, Gurdon, & Fischberg, in prepn.), it will be only very briefly outlined here. Cells are dissected as a tissue from the donor embryo, disaggregated chemically, and then kept in saline solution until required. Unfertilised recipient eggs are irradiated with ultraviolet to kill the egg-promucleus. A donor cell is sucked up into a pipette which is sufficiently narrow to disrupt the cell wall and leave the nucleus intact, protected by some cytoplasm. The nucleus with its cytoplasm is then injected into the recipient egg. This sequence of events justifies the distinction made below between the treatment of donor nuclei and that of recipient eggs.

DONOR NUCLEI.

The disaggregation and storage of donor cells

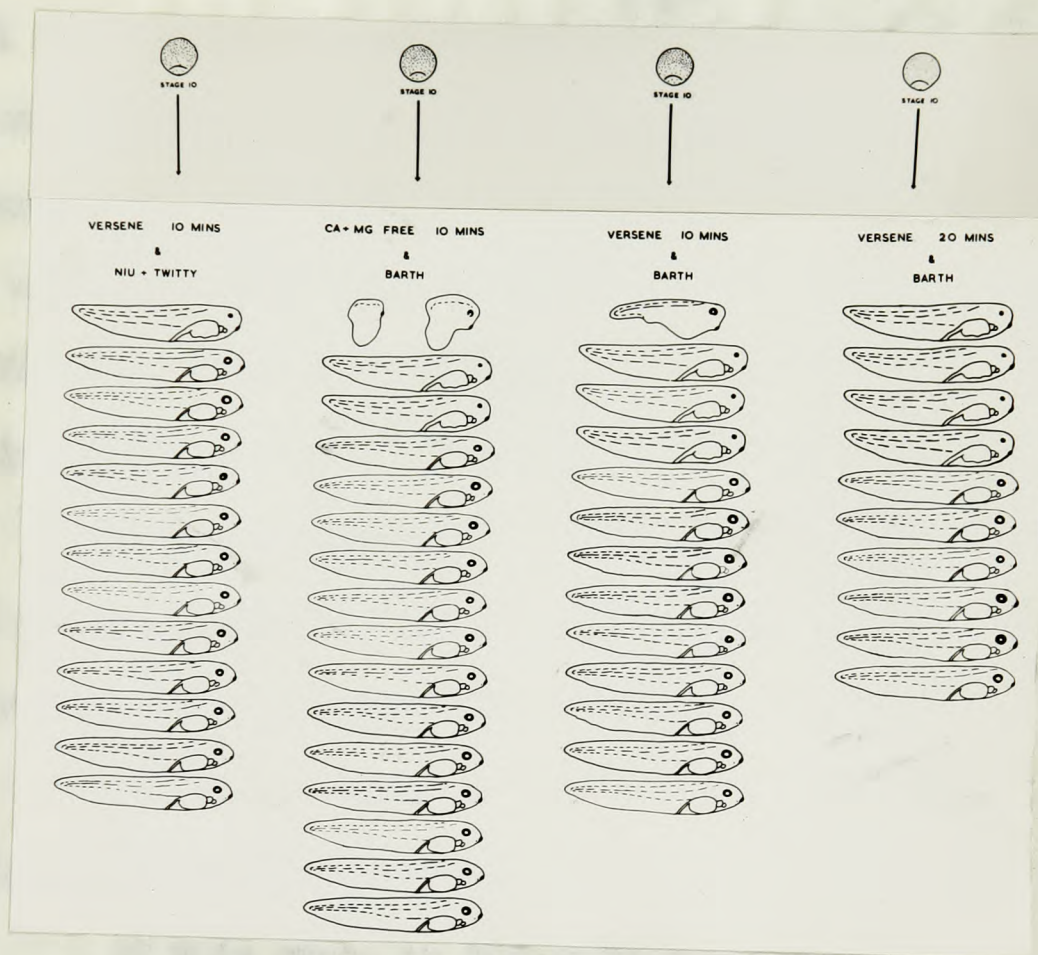
Donor cells are prepared by placing the isolated tissue (dissected from the embryo with needles), in a disaggregating solution for between 10 and 20 minutes. During this time many cells become free, and they are then placed in a full-strength saline solution until required for transplantation. The disaggregating solutions are made from standard saline solution either by omitting the Calcium and Magnesium salts, or by replacing these salts with 5×10^{-4} Molar Versene. Only the saline solutions of Niu & Twitty (Flickinger, 1949), and Barth (Barth & Barth, 1959) have been tested. Briggs and King (1953) have used Rana for nuclear transplantation and have found that Niu & Twitty saline causes fewer abnormalities of development than many organic

and inorganic solutions which they also tested. Since then Moor (unpublished), also using Rana, has found that fewer abnormalities resulted from the use of Barth's X-solution, which mainly differs from Niu and Twitty in containing one and a half times as much NaCl.

In text-figure 1, the three columns on the left compare the effects of different disaggregating solutions on the same kind of donor cells. In this case there is very little difference between Calcium- and Magnesium-free Barth, Barth-Versene, and Niu & Twitty-Versene; in other experiments there is usually no difference between Barth- and Niu & Twitty-Versene solutions, though more severe abnormalities are found after using Ca & Mg free Barth solution which has a weaker disaggregating effect than Versene. Thus inadequate disaggregation seems to result in nuclear damage. This may be due to the fact that cells insufficiently disaggregated by chemical means tend to be separated mechanically when being transferred to the standard saline solution; this may result in damage to the cell wall, which then fails to protect the nucleus from the saline solution. King & Briggs (1955) have found that donor cells are less damaged if disaggregated chemically than if dissected apart with needles. The conclusion from this experiment is that the nuclei of donor cells are more likely to be damaged after insufficient dissociation than by any direct effects of Versene.

To test whether disaggregation by even the best method was nevertheless harmful, donor cells were left for varying periods of time in Barth-Versene. Cells that were kept for longer times in Versene were left for correspondingly shorter times in the standard solution; thus the total time for which cells were in any saline solution was constant. The two columns

Text-figure 1.



Donor cells from early gastrulae were prepared in different ways and 36 transplantations made from each donor embryo. The disaggregating solution was the same as the solution in which cells were kept for transplantation, with either Ca and Mg salts omitted or replaced by 5×10^{-4} Versene.

on the right of text-figure 1 show that there is no pronounced difference in results if the donor cells are left for twice as long as is necessary in Versene solution. These tests have been carried out on blastula cells the nuclei of which are relatively well protected by cytoplasm. Endoderm ^{also} nuclei from hatched tadpoles have been transplanted after exposure to Versene solution for varying periods of time. Donor cells disaggregated for 20 minutes and 35 minutes gave equally good results, which were better than those obtained from cells disaggregated for only 10 minutes. It is apparent that Versene in the strength used does not damage nuclei of intact cells even if they are exposed to it for double the normal time.

These results mean that care should be taken to disaggregate donor cells thoroughly before transplanting their nuclei, but that exposure of cells to Versene for longer than the usual 10 - 15 minutes is not harmful.

The treatment of donor cells in the injection pipette

At this stage in the technique there are two ways in which the donor nuclei may be damaged. They may either be physically broken or distorted when being sucked up into the micropipette, or they may be chemically harmed by exposure to the saline solution. Each kind of damage is likely to be increased the more the donor cell is distorted when sucked into the pipette. Two kinds of experiment have been carried out to investigate this source of damage.

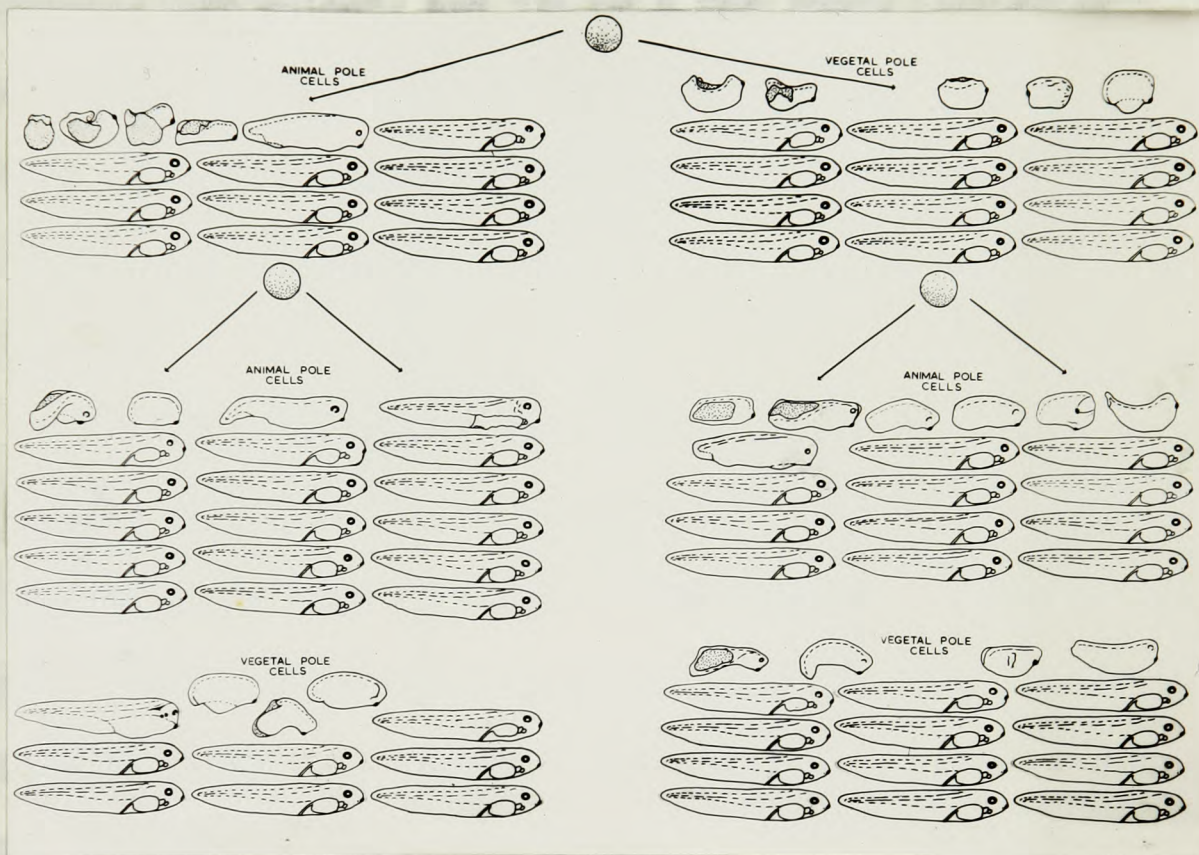
The first experiment involved transplanting animal and vegetal cells from blastula donor embryos. Cells from these regions differ considerably

in size, though their nuclei are of approximately the same volume. Both kinds of cells were distorted to the same extent, by sucking them into pipettes of different sizes; thus animal cell nuclei were protected by a thinner layer of cytoplasm, and so were more likely to be exposed to the saline solution; animal cell nuclei were also more pushed out of shape than the vegetal nuclei. If transplanted nuclei are affected by either of these factors, embryos derived from animal cell nuclei should develop more abnormally than those derived from vegetal nuclei.

In text-figure 2 this test has, in effect, been carried out three times; the fact that two of the blastula donor embryos happen to have been derived from the first by nuclear transplantation does not affect the present conclusions. Though some abnormal embryos are present in each transplantation group, it can be seen that there is no consistent difference in the proportion of abnormalities derived from animal or vegetal donor nuclei. This shows that the smaller amount of cytoplasmic protection possessed by animal hemisphere nuclei does not expose them to technical damage. The chief importance of this result concerns the interpretation of experiments in which nuclei are taken from much later donor stages. The endoderm cells of hatched prefeeding tadpoles, though slightly smaller than blastula ectoderm cells, have a similar ratio of nucleus to cell volume. This means that differences in the result of transplanting endoderm nuclei from early and late stages cannot be due to the smaller size of the cells rendering them more susceptible to technical damage.

In the second experiment different sized pipettes were used for donor cells of the same size and kind as each other; the donor cells were

Text-figure 2.



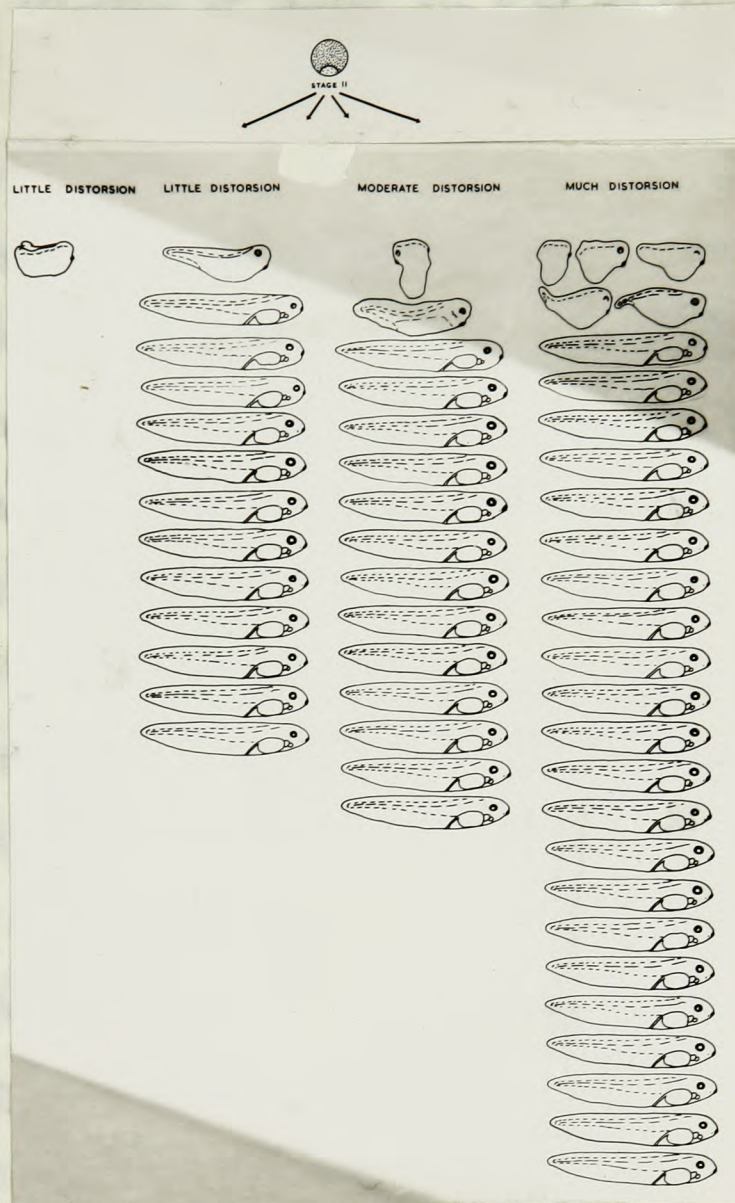
Transplantations were made from undifferentiated nuclei of small animal and large vegetal cells of a blastula donor. A blastula ~~from~~ from each of these groups served as a donor for two serial clones, in each of which transplantations from animal and vegetal cells are shown separately.

therefore distorted by the pipette to different degrees. Four categories of distortion have been recognised (text figure 3). The ratio between the diameter and length of the cell inside the pipette was about 2:3 for "very little distorted" cells, about 1:2 for "little distorted" cells, about 1:3 for "moderate distortion", and 1:4 or 5 for "much distortion". In cells which were very little distorted the cell wall was often not broken and no development took place; in much distorted cells the cytoplasm was much dispersed so that nuclei may have come into direct contact with the saline solution.

Text figure 3 shows two experiments of this kind. Within each, exactly the same number of transplantations were carried out for each group. These diagrams, however, show only those embryos that became regular late blastulas. Since the cause of abnormal cleavage after transplantation is very uncertain, eggs which cleaved irregularly have been omitted from the diagrams, but were in fact more frequent, the less the distortion of the donor cell. The most obvious result, equally clear in each experiment, is that the proportion of transplanted eggs which cleave normally varies directly according to the amount of distortion of the donor cell. The abnormalities in each group should be considered as a proportion of the number of embryos shown. Rather surprisingly embryos derived from "very little distorted" donor cells develop very abnormally. Among the other three groups the proportion of abnormal embryos and the severity of abnormalities slightly increases the greater the distortion.

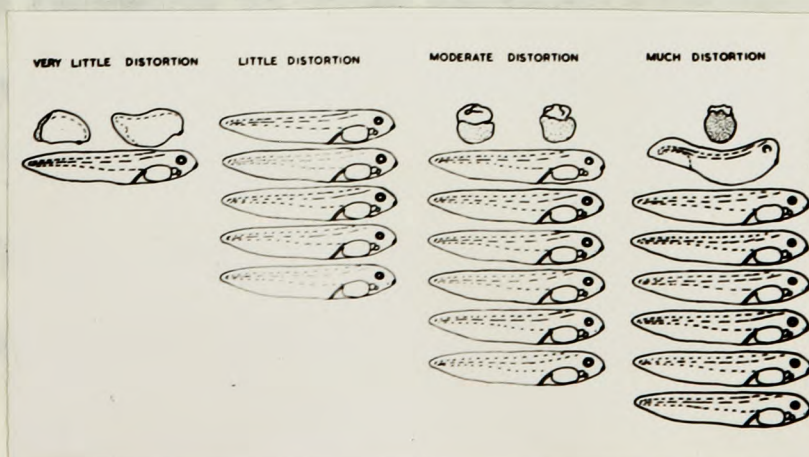
These experiments clearly show that to obtain the best results,

Text-figure 3a.



Cells from an early gastrula donor, disaggregated by Barth-versene, have been transplanted into recipient eggs of one frog. The distortion of the donor cells on being sucked into the ^{pipette} was varied, but in all other respects the technique and materials were the same for the 4 groups (36 transfers in each).

Text-figure 3b.



Similar to text-figure 3a in all respects, except that eggs were obtained from a different recipient frog, and that there were only 12 transfers per group.

donor cells should be distorted to a small or moderate degree forming in the pipette a cylinder of length 2 - 3 times its diameter. If cells are distorted too little, the cell-wall is often not broken properly and this results in much abnormal cleavage, though for no obvious reason the few regular late blastulae that occur also develop very abnormally. If donor cells are distorted too much, the abnormalities increase a little, presumably because the nuclei are exposed to the saline solution.

When interpreting nuclear transplantation experiments it is very important to remember that the amount of distortion of the donor cell is by no means the only factor that decides what proportion of transplanted eggs will cleave normally. In practice it is very difficult to distort each donor cell to exactly the same extent, so that up to 20% of variation in the percentage of normal cleavage will probably not convey information about the quality of donor nuclei. On the other hand, nuclei from late donor stages give conspicuously fewer normal late blastulae than those from early stages, even though both kinds of cells are distorted to a very similar extent (Gurdon, 1960 b).

RECIPIENT EGGS

Technical treatment of recipient eggs

The treatment to which recipient eggs are subjected during transplantation consists of:- Ultra-violet irradiation, penetration by a micropipette, the deposition near its centre of a nucleus, some donor cell cytoplasm, and a small amount of saline solution. In addition, the eggs remain unsupported by a fluid medium during these operations. These factors will

be discussed in turn.

The main purpose of Ultraviolet treatment is to kill the egg-nucleus, but it also renders the egg more easily penetrable to a micropipette by dissolving some of the jelly and weakening the vitelline membrane. The u.v. treatment of uncleaved Xenopus eggs is the subject of another publication (Gurdon, 1960 a). Several experiments are described none of which give any reason to believe that the u.v. irradiation of the animal hemisphere damages the egg cytoplasm. The clearest evidence was given by an experiment in which fertilised eggs were irradiated for different lengths of time. As the u.v. kills the egg pronucleus, fertilised irradiated eggs develop as androgenetic haploids which are never entirely normal. However, in this experiment no change in the proportion of abnormal embryos was obtained, even though the u.v. dose was considerably more than that normally used for transplantation experiments.

The donor cell cytoplasm, which is never more than one 30,000th of the egg by volume, has been found to have no effect on development when injected (without a nucleus) into a fertilised egg. The remaining three factors, (the penetration by a pipette, injection of saline solution, and lack of support by fluid) were first tested with fertilised eggs. Eggs were treated in three ways:- (a) u.v. irradiated only, (b) u.v. irradiated, and penetrated by a pipette, but with no fluid injected, and (c) u.v. irradiated, penetrated by a pipette, and a little Barth saline solution (but no nucleus) injected, as for transplantation experiments. In each case the eggs were left unsupported by fluid for the usual amount of time, i.e. 5-10 minutes.

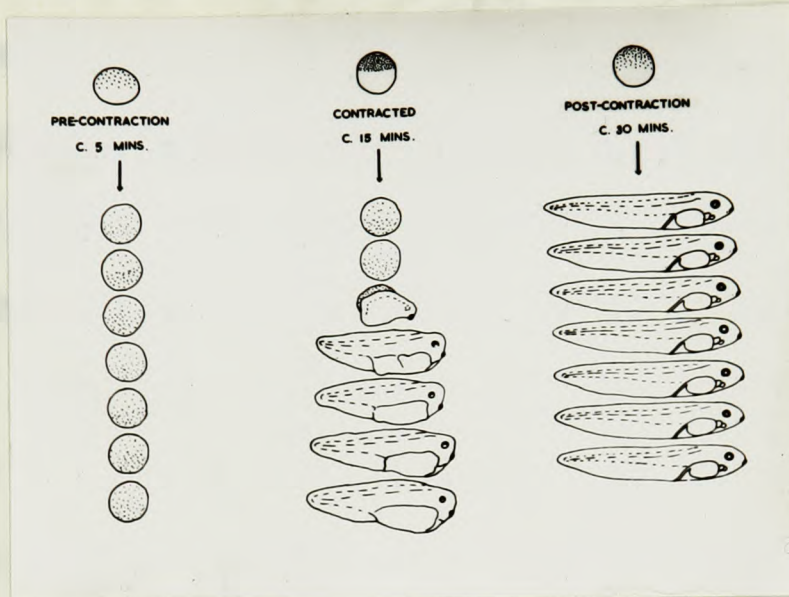
In this experiment again all embryos were haploid, and therefore none were entirely normal. The results varied considerably from the eggs of one frog to those of another; for this reason a typical experiment cannot be shown. There is a general tendency for most abnormalities to be derived from eggs which were irradiated and injected with saline solution (c), and for eggs which had been irradiated only (a) to develop most normally. These differences may be to some extent due to haploid embryos being able to recover less well from injection with solution than diploids. By whatever means haploid Xenopus are obtained, some develop more abnormally than others (Gurdon, 1960 a). It is unlikely that the poor developmental capacity of haploids can account for all the differences between eggs irradiated only (a), and eggs irradiated and injected (c). Since the difference between the three kinds of treatment is not apparent with some eggs but pronounced with others, it seems clear that the ability to withstand the transplantation treatment depends on egg quality. This matter is discussed further in a section below (p. 10). As it is unfortunately not possible to obtain diploid development from fertilised eggs treated for transplantation, further tests were carried out on unfertilised eggs with transplanted nuclei. The amount of saline solution injected into the eggs was varied from about the same volume as the donor cell to 2 or 3 times this amount. This had no effect on the proportion of abnormalities among the results. The time for which eggs remained unsupported by fluid could be increased to at least 20 minutes (which is double the usual time) without having any effects, so long as the surface of the eggs did not become dry.

The eggs of Xenopus often undergo a contraction of the area of

animal pole pigment and at the same time the consistency of the eggs seems to become stiffer (Gurdon, 1960 a). The duration and time of onset of this contracted state varies from the eggs of one ovulation to those of another; in each egg it usually starts about 10 - 20 minutes after laying and lasts for about ten minutes. Text-figure 4 shows the result of irradiating fertilised eggs and injecting them with saline solution (but no nucleus) at different times after laying. The eggs treated before contraction have all become arrested as late blastulae, but those in the contracted state have developed as well as haploids usually do. In those treated after the contracted state, the egg nucleus has had time to sink below the surface; these have therefore become diploid and all are normal. It seems that there is associated with this contraction an increased resistance to technical interference such as penetration by a pipette (text-fig. 4). It is not clear whether this contraction of Xenopus eggs is comparable to the activation of Rana pipiens eggs (Briggs & King, 1953). The present author has also found (Gurdon, 1960 a) that experimental manipulations, such as enucleation with a needle, result in developmental abnormalities if carried out on eggs immediately after they have been laid, but can be much less harmful if carried out about 15 minutes after laying. This applies to both fertilised and unfertilised eggs. For this reason recipient eggs are ~~used~~ for nuclear transplantation about 15 minutes after laying, when they are usually in the contracted state.

The above tests have shown that there is no reason to believe that the usual treatment of good recipient eggs, during transplantation, in any way reduces their capacity to develop normally. Though this seems to apply

Text-figure 4.



Fertilised eggs, u.v. irradiated at different intervals after laying, were all treated as for nuclear transplantation; they were penetrated by a pipette and injected with some saline solution but no nucleus.

to the eggs of most ovulations there may be some exceptions, since the quality of eggs varies (see next section).

Innate variation in the quality of recipient eggs.

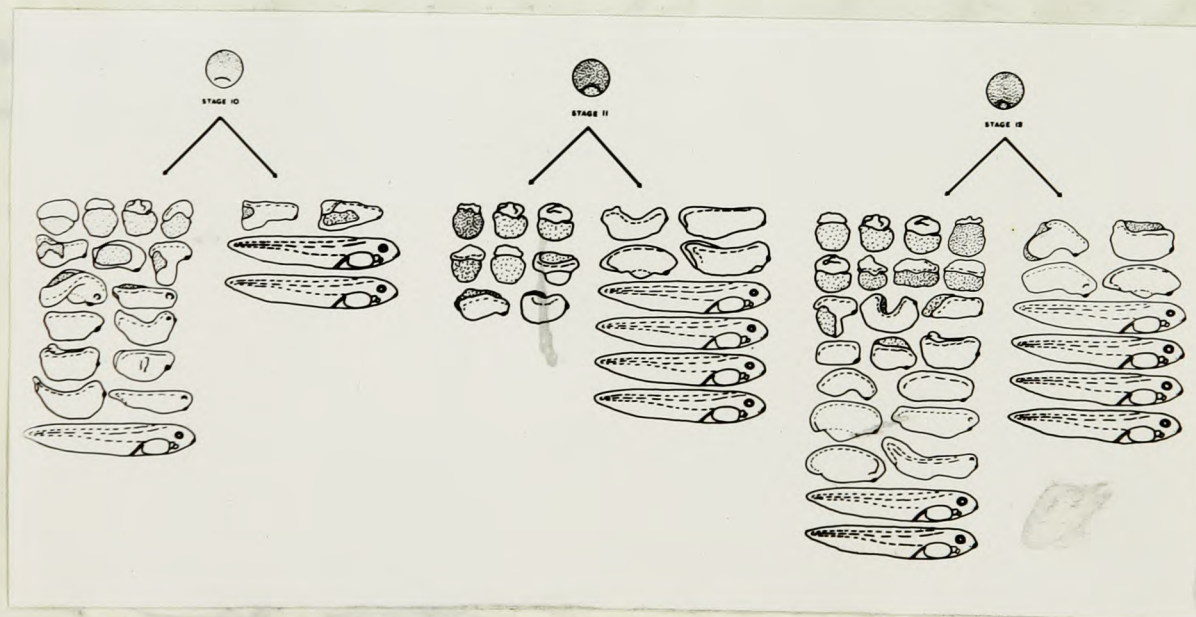
There seems to be a considerable discrepancy in the results obtained from recipient eggs of different female frogs, in spite of using the same donor cells. This was tested by transplanting nuclei from a single donor embryo into groups of eggs from two frogs laying at the same time. The results of these experiments are shown in text-figure 5. In these diagrams there are two groups of embryos under each donor. The left-hand group comes from the eggs of one frog, and the right hand group from those of another. In each case a very marked difference between the two groups of embryos can be seen, which must show a difference in egg quality since there was no other variable in these experiments. The poor eggs differ from the good ones not only in the greater proportion of developmental abnormalities, but also in the smaller number of regular late blastulae obtained (especially text-fig. 5c). This difference in the quality of eggs persists whatever donor stage is used (compare text-figures 5b and 5c). The eggs of one female are usually of the same quality throughout one ovulation though there is a tendency for the last eggs laid to give worse results, presumably because some eggs are immature. One female may lay good eggs at one ovulation but bad ones at the next, and it has not been found that certain females can be relied upon to give good or bad eggs every time they lay.

Many attempts have been made to improve the quality of eggs, as for instance by varying the diet, but so far none have been successful. Frogs recently imported from the wild do not always give much better eggs than ones

Text-figures 5 a, b, and c.

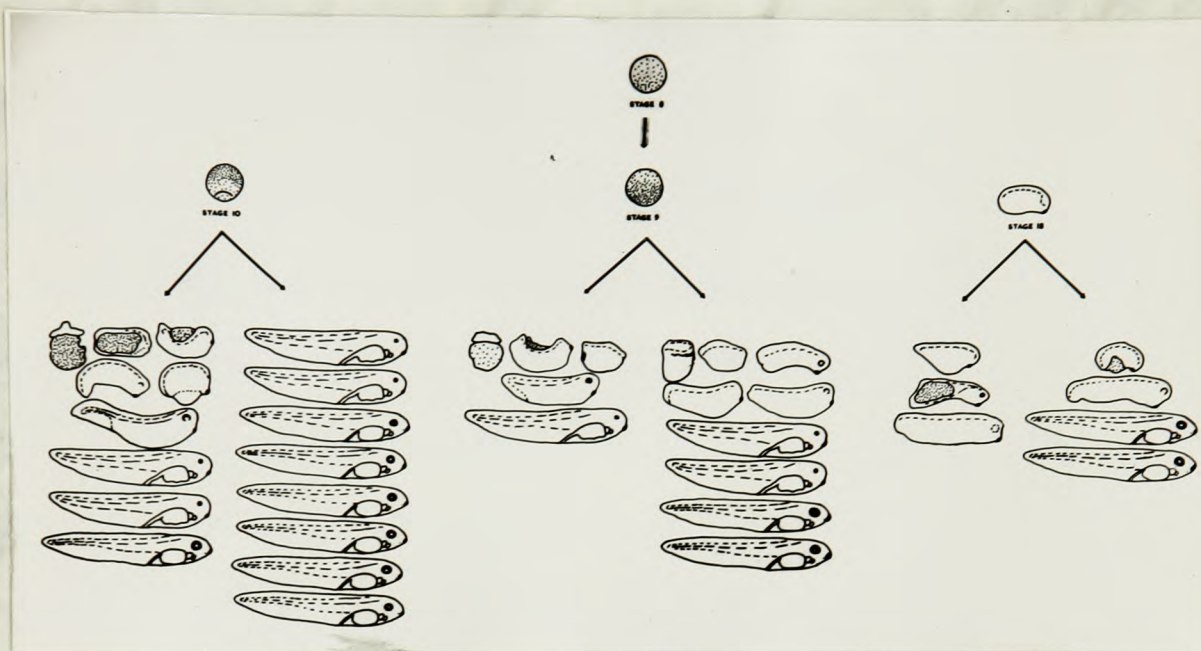
These experiments, in which there was no variation in technique, show how transplant-embryo development may vary according to the quality of recipient eggs. In each figure the eggs of two recipient frogs have been used, and nuclei from each donor embryo have been transplanted into the eggs of both frogs. The figures are set out according to the order in which transplantations were made, i.e. nuclei from the donor on the left were transplanted first and those from the donor on the right of each figure last.

Text-figure 5a.



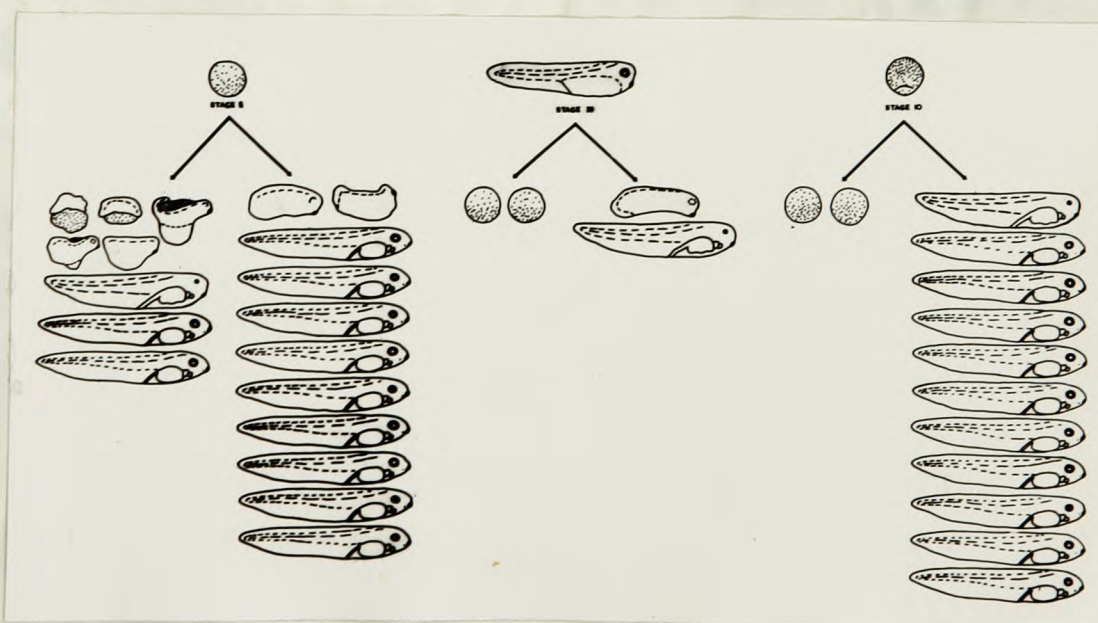
About 30 transplantations were made from early, middle, and late gastrula donors into the eggs of each frog. The eggs of both frogs gave abnormal transplant-embryos, but the ratio of normal to abnormal embryos was appreciably greater for the eggs of the female on the right of each series.

Text-figure 5b.



About 25 transplantations were made from a gastrula, serial blastula, and neural folds donor, to the eggs of each recipient frog. One female gave consistently worse results than the other.

Text-figure 5c.



About 30 transplantations were made from a blastula, hatched tadpole, and ~~neural folds~~ gastrula donor into the eggs of each frog. One female gave exceptionally good and consistent results while the eggs of the other gave very poor results and appear to have deteriorated in quality.

bred in our laboratory. Eggs are obtained from each female by injection of gonadotropic hormone at two to three monthly intervals.

This variation in the results obtained from the eggs of different females is rather surprising in view of the fact that naturally fertilised eggs from hormone injected frogs nearly always develop very well; usually over 90% of such fertilised eggs develop into normally feeding tadpoles. It is possible that the injection of a nucleus brings about development in some eggs which, owing to poor quality, would not have been fertilised, or have developed abnormally if they had been fertilised. It is found on the average that a higher percentage of eggs cleave regularly after transplantation than after fertilisation. On the other hand it may be that eggs can develop quite normally when fertilised, but are nevertheless very variable in their ability to withstand experimental manipulations. In either case the cause of variation is egg quality. This observation does not apply only to nuclear transplantation experiments since it is known that embryos derived from the eggs of different frogs are also very variable in their ability to heal up after tissue grafting.

No way has yet been found of distinguishing recently laid eggs which will develop well after nuclear transplantation from those which will not. It was mentioned on page 9 that recipient eggs are not usually used for transplantation until they have undergone certain changes recognised by contraction of the area of animal hemisphere pigment. While the selection of contracted eggs gives fewer abnormalities (text-figure 4), it is not found that all contracted eggs give equally good results. It is quite possible for the eggs of one frog to cleave well, ~~and even gastrulate well,~~

and even gastrulate well, but later to suffer from many more abnormalities than those of another frog. The quality of eggs is therefore judged entirely by the proportion of normal tadpoles obtained from them. This means it is necessary to start and end all experiments with a control series - that is with donor nuclei, the developmental potentialities of which are already known. Only if these control transplantations develop well can attention be paid to the results obtained from other donor nuclei.

DISCUSSION AND CONCLUSIONS

There are four main factors which determine the way in which embryos from transplanted nuclei develop; these are the technical treatment and innate qualities, of donor nuclei and recipient eggs. The majority of the experiments carried out have consisted of nuclear transplantations, since this is the most direct way of investigating these factors.

The technical treatment of donor nuclei can be carried out in such a way that it is harmless. To achieve this cells should be thoroughly disaggregated by exposure to versene for 10 - 20 minutes according to the stage of the donor embryo. When sucked up into a pipette donor cells should be distorted so that their length is 2 - 3 times their diameter. In practice, small variations within this range of cell distortion are unavoidable; these do not affect the further development of blastula transplant-embryos, but may account for up to 20% of variation in the number of total transplantations which form regular blastulae.

The effects of the technical treatment and innate qualities of recipient eggs will be discussed together since it is difficult to distinguish

between them. The innate quality of eggs is often responsible for variation in transplant-embryo development. It was shown that undifferentiated donor nuclei when transplanted under the same conditions, can give rise to very different results according to the female's eggs into which they were transplanted. These different results may either be due to variation in the innate developmental capacity of eggs, or to variation in their capacity to withstand, or recover from, the experimental manipulations to which they are subjected. In either case the cause of variable results is egg quality. A considerable reduction in abnormalities was obtained by using recipient eggs in the contracted state, but in spite of this the eggs of some frogs gave much better results than those of others. Unfortunately no way has yet been found of improving egg quality so as to eradicate this source of abnormalities, or of recognising the quality of eggs other than by the development to which they give rise. This makes it necessary to carry out a control series of transplantations using early stage donor nuclei, the developmental potentialities of which are already known. If the recipient eggs were of good quality these control transplantations will develop as expected, and attention can be paid to the results of the experiment; if the controls develop very abnormally, the whole experiment must be discarded. Thus technical treatment or innate quality of recipient eggs will not affect the conclusions if these are restricted to experiments in which egg quality proved to be good.

The innate qualities of donor nuclei were kept constant; this was done by using nuclei from early donor stages which are believed to be undifferentiated as shown in the experiment described below. One of the main uses of nuclear transplantation is to test the innate qualities of different kinds

of nuclei; experiments of this kind which are described elsewhere (Gurdon, 1960 b), can only be done when the effects of other factors have been excluded.

The effects of all four factors which influence transplant-embryo development have now been considered; a standard technique has been developed by which experiments can be carried out and analysed in such a way that the only variable factor is the innate quality of donor nuclei. This is demonstrated by the following experiment in which early gastrula endoderm nuclei were used as donors. Fourteen nuclei were transplanted; three did not cleave but the remaining eleven all cleaved normally. These eleven regular late blastulae all formed completely normal feeding tadpoles, which would almost certainly have metamorphosed as usual if they had not been fixed. These results give good reasons for believing that the endoderm cell nuclei of blastulae and early gastrulae are all totipotent, or capable of giving normal transplant-embryo development. It shows that if all conditions are perfect (most especially the quality of recipient eggs), every undifferentiated nucleus which initiates cleavage will bring about normal development. This experiment is atypical, since in most cases a few abnormal embryos occur in series which otherwise consist of normal tadpoles. However, its importance is not decreased by its uniqueness, since the failure of other experiments to give such clear results seems to be due to the quality of recipient eggs.

The author has pleasure in thanking Professor Sir A. H. H. for having provided facilities for this work in his department. The author

SUMMARY

1. An analysis has been made of factors which can cause abnormal development of embryos derived from transplanted nuclei in Xenopus laevis. A distinction is made between innate and technical factors, and between those which affect donor nuclei and recipient eggs.
2. The innate developmental potentialities of endoderm donor nuclei do not appear to be limited, at least up to the early gastrula stage. Abnormalities known to be due to differentiated donor nuclei from later stages are described elsewhere. The technical treatment of donor nuclei can be carried out so as not to damage them, even if this is purposely made more severe than usual.
3. The innate capacity for development of recipient eggs was found to vary considerably from one spawning to another; this variation seems to be due to an inability of the eggs of one frog to withstand technical treatment which was shown to be harmless to the eggs of other frogs.
4. As the quality of recipient eggs can only be judged from results, experiments using Xenopus eggs should be accompanied by control transplantations, using donor nuclei the developmental capacity of which is already known. With these control transplantations for comparison, the technique is suitable for testing unknown developmental potentialities of different kinds of donor nuclei or recipient eggs.

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CHAPTER V

THE DEVELOPMENTAL CAPACITY OF NUCLEI TAKEN FROM DIFFERENTIATING ENDODERM CELLS OF XENOPUS LAEVIS.

Synopsis.

I. INTRODUCTION

II. THE TECHNIQUE, DONOR NUCLEI, AND RECIPIENT EGGS

III. FACTORS AFFECTING TRANSPLANT-EMBRYO DEVELOPMENT

IV. THE RESULTS OF EXPERIMENTS

A. First Transfer Experiments

B. Serial Transfer Experiments

C. Conclusions concerning the Potentialities of Endoderm Nuclei in Xenopus

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The part of a nucleus in which differentiation takes place.

The significance of nuclear changes.

THE DEVELOPMENTAL CAPACITY OF NUCLEAR TRANSPLANTS

V. COMPARISON OF NUCLEAR DIFFERENTIATION IN XENOPUS LAEVIS AND RANA PIPIENS

SUMMARY

Acknowledgements

References

... of nuclear differentiation in different parts of the embryo ... differ genetically from each other during development. It was ... possible to investigate this matter using a single ... that nuclear transplantation is a satisfactory technique for ... developmental potentialities of embryonic nuclei. These ... used ... for transplantation experiments with ... and have found that these nuclei were progressively ... developmental capacity after the late blastula stage.

This paper describes some similar experiments carried out with ... of *Xenopus laevis*. The general conclusion was that ... change in development proceeds in a similar way ... differences between *Rana* and *Xenopus* in the rate and ... of nuclear changes. These differences also ... the significance of nuclear differentiation during embryonic development.

1. THE EXPERIMENTAL METHOD

The technique used in these experiments with *Xenopus* has been modified from that of Briggs and King (1938), and is described elsewhere together with the method of culturing transplanted embryos (Mintala, Gordon and Fischberg, in press).

THE DEVELOPMENTAL CAPACITY OF NUCLEI TAKEN FROM DIFFERENTIATING
ENDODERM CELLS OF XENOPUS LAEVIS

I. INTRODUCTION

An important question concerning embryonic differentiation is whether the nuclei of somatic cells in different parts of an embryo come to differ genetically from each other during development. It has become possible to investigate this matter since King & Briggs (1955) have shown that nuclear transplantation is a satisfactory technique for testing the developmental potentialities of embryonic nuclei. These authors (1957) have used Rana pipiens for transplantation experiments with endoderm nuclei, and have found that these nuclei become progressively limited in their developmental capacity after the late blastula stage.

This paper describes some similar experiments carried out with endoderm nuclei of Xenopus laevis. The general conclusion that nuclei change as development proceeds is confirmed; there are however considerable differences between Rana and Xenopus in the rate and time of onset of nuclear changes. These differences make it easier to understand the significance of nuclear differentiation during embryonic development.

II. THE TECHNIQUE, DONOR NUCLEI, AND RECIPIENT EGGS

The technique used in these experiments with Xenopus has been modified from that of Briggs and King (1953), and is described elsewhere together with the method of culturing transplant-embryos (Elsdale, Gurdon and Fischberg, in prepn).

Donor cells have been prepared by dissecting out part of the endoderm with the help of needles. Cells are disaggregated by placing the isolated tissue in Barth-Versene solution for between 10 and 20 minutes (Gurdon 1960 c); they are then transferred to standard Barth solution (Barth & Barth, 1959) and kept until required for transplantation.

The developmental stages of donor embryos have been identified according to Nieuwkoop and Faber's (1956) normal table for Xenopus laevis. All the experiments described below have been carried out with vegetal or endoderm donor cells. In blastulae and gastrulae donor cells were taken from the centre of the vegetal cell mass, and in later stages from the floor of the gut lumen. In hatched tadpoles, cells were taken from the floor of the anterior part of the gut; these cells are rather larger and more easily disaggregated than those forming the outer wall of the gut. In hatched tadpoles the presumptive germ cells do not lie in that part of the gut from which donor cells were taken.

Proof that the nuclei of a transplant-embryo have all been derived from the injected nucleus alone is provided by the use of marked donor nuclei. These are diploid and were obtained from the one-nucleolated strain of Xenopus (Elsdale, Fischberg, & Smith, 1958). The presence of the nuclear marker can be recognised in a squash preparation of part of the donor embryo, since the number of nucleoli per nucleus can be quickly counted under the phase-contrast microscope (Elsdale, Gurdon, & Fischberg, in prepn.). Since marked donor nuclei have been injected into unmarked and enucleated recipient eggs, all transplant-embryos should have marked nuclei (with one nucleolus). In some cases transplant-embryos are obtained

which have two or more nucleoli in each nucleus. If the embryo's ploidy and nucleolar number is known, the origin of its nuclei is at once evident (Elsdale, Gurdon & Fischberg, in prep.). Tetraploids resulting from the doubling of the injected nucleus have been obtained quite frequently, but are not apparently any more abnormal than comparable diploid embryos (Gurdon, 1960 a). However, because it is often troublesome to distinguish triploid from tetraploid embryos, only one-nucleolated diploids have been counted in the results below.

Recipient eggs have been obtained by injecting frogs with gonadotropic hormone. Normal diploid frogs were used which did not therefore carry the nuclear marker. These frogs were either those which have been kept in the laboratory since they were imported, or else those which have been reared in the laboratory from imported frogs. The quality of eggs laid by recently imported frogs does not seem to differ consistently from that of frogs reared in the laboratory.

III. FACTORS AFFECTING TRANSPLANT-EMBRYO DEVELOPMENT

The way in which transplant-embryos develop is determined by many different factors, which fall into the four categories below. The effects of these must be known before it is possible to relate transplant-embryo development to the first of these factors: (a) Innate qualities of donor nuclei; (b) Technical damage to donor nuclei; (c) Innate qualities of recipient eggs; (d) Technical damage to recipient eggs. Study of variation in the last three of these has shown that transplant-embryo

development may be affected in only two ways other than by innate donor qualities (Gurdon, 1960 c). The first concerns variation in the degree of donor cell distortion, and the second concerns variation in egg quality.

When donor cells of a similar volume are transplanted with different sized pipettes, this varies the extent to which the donor cell wall is disrupted. It is found that the degree of donor cell distortion is directly related to the proportion of total transplantations which form regular late blastulae (Gurdon 1960 c). It seems that, if the donor cell wall is insufficiently broken, the injected nucleus is unable to combine satisfactorily with the recipient egg cytoplasm. Though the degree of donor cell distortion has been kept constant as far as possible, small variations in this respect are unavoidable and can cause a variation of up to 20% in the proportion of late blastulae formed. For this reason it is best to base the main conclusions from transplantation experiments on the development of transplant-embryos expressed as a proportion of regular late blastulae, and not as a proportion of total transplantations.

When nuclei from the same donor embryo are transplanted (with no variation in technique) into eggs of different frogs, it is found that the development of transplant-embryos is appreciably more normal with the recipient eggs of some frogs than with those of others (Gurdon, 1960 b). A test (ibid) in which fertilised eggs were treated just as for nuclear transplantation except that no nucleus was injected, showed that the standard transplantation treatment may result in abnormal development with the eggs of some frogs, but may cause no harm to those of others. This demonstrates that the eggs of different frogs vary considerably in their ability to withstand the experimental manipulations to which they are

subjected. It is unfortunately not possible to recognise the quality of recipient eggs other than by the development to which they give rise. This makes it necessary to do control experiments using donor nuclei of known developmental potentialities, such as undifferentiated blastula nuclei. Control transplantations show in which experiments egg quality was good; only these "selected" experiments can be regarded as giving a clear indication of innate nuclear qualities. The quality of recipient eggs is usually consistent throughout an ovulation, but it sometimes happens that eggs deteriorate in quality when a frog has already been laying for several hours. For this reason control transplantations are if possible carried out at the beginning and end of each experiment.

Experiments published elsewhere (Gurdon 1960 c) have shown that abnormal development of transplant-embryos beyond the blastula stage cannot be caused by any part of the standard technical treatment of donor nuclei. If donor nuclei from several normal blastulae are transplanted into the eggs of one frog, they give very similar results (fig. 4 b). This demonstrates that blastula nuclei do not differ in their capacity to give normal transplant-embryo development, and are therefore suitable for controls with which to test the quality of recipient eggs. It is now evident that transplantation experiments can be analysed in such a way that the only variable affecting transplant-embryo development is the innate quality of donor nuclei.

IV. THE RESULTS OF EXPERIMENTS

Table 1 is a summary of all experiments with endoderm nuclei carried out since the technique became standardised; it includes only

TABLE 1.

DONOR EMBRYO AGE GROUP And normal stage numbers of Nieuwkoop (1956).	TOTAL TRANSPLANTATIONS	STAGES OF TRANSPLANT-EMBRYO DEVELOPMENT				
		Late Blastula	Early Gastrula	Late Gastrula & Neural Folds	Swimming Tadpole	Normal Feeding Tadpole
Blastulae (7-9)	533 100%	172 32%	163 31%	147 28%	116 22%	109 21%
Gastrulae (10-13)	1014 100%	452 45%	421 42%	354 35%	242 24%	229 23%
Neurulae (14-22)	469 100%	182 39%	135 29%	99 21%	58 13%	51 12%
Muscular Response Post-neurulae (23-26)	236 100%	77 34%	65 29%	47 21%	29 13%	26 12%
Heart-beat Tadpoles (29-34)	282 100%	72 25%	54 19%	34 12%	15 5%	12 4%
Hatching Tadpoles (35-37)	471 100%	95 20%	70 15%	44 10%	20 4%	13 3%
Swimming Tadpoles (39-41)	681 100%	77 11.5%	54 8%	27 4%	20 3%	13 2%

TABLE 1. Total results with endoderm nuclei. The percentages represent transplant-embryo survival in terms of total transplantations for each donor.

TABLE 2.

DONOR EMBRYO AGE GROUP	TOTAL TRANSPLANTATIONS	STAGES OF TRANSPLANT-EMBRYO DEVELOPMENT				
		Late Blastula	Early Gastrula	Late Gastrulae & Neural Folds	Swimming Tadpole	Normal Feeding Tadpole
And normal stage numbers of Nieuwkoop (1956)						
Blastulae (7-9)	327 100%	113 35% 100%	110 34% 98%	106 33% 94%	96 30% 85%	91 28% 81%
Gastrulae (10-13)	502 100%	221 44% 100%	220 44% 100%	216 43% 98%	175 35% 79%	169 34% 77%
Neurulae (14-22)	140 100%	50 36% 100%	48 35% 96%	40 29% 79%	27 19% 53%	26 18% 52%
Muscular Response Post-neurulae (23-26)	152 100%	53 34% 100%	50 32% 94%	40 26% 75%	25 17% 47%	22 15% 41%
Heart-beat and hatching tadpoles (29-34)	174 100%	47 27% 100%	36 21% 76%	28 16% 59%	17 10% 36%	13 6% 27%
Swimming Tadpoles (39-41)	436 100%	89 21% 100%	71 16% 80%	48 11% 54%	17 4% 19%	13 3% 15%

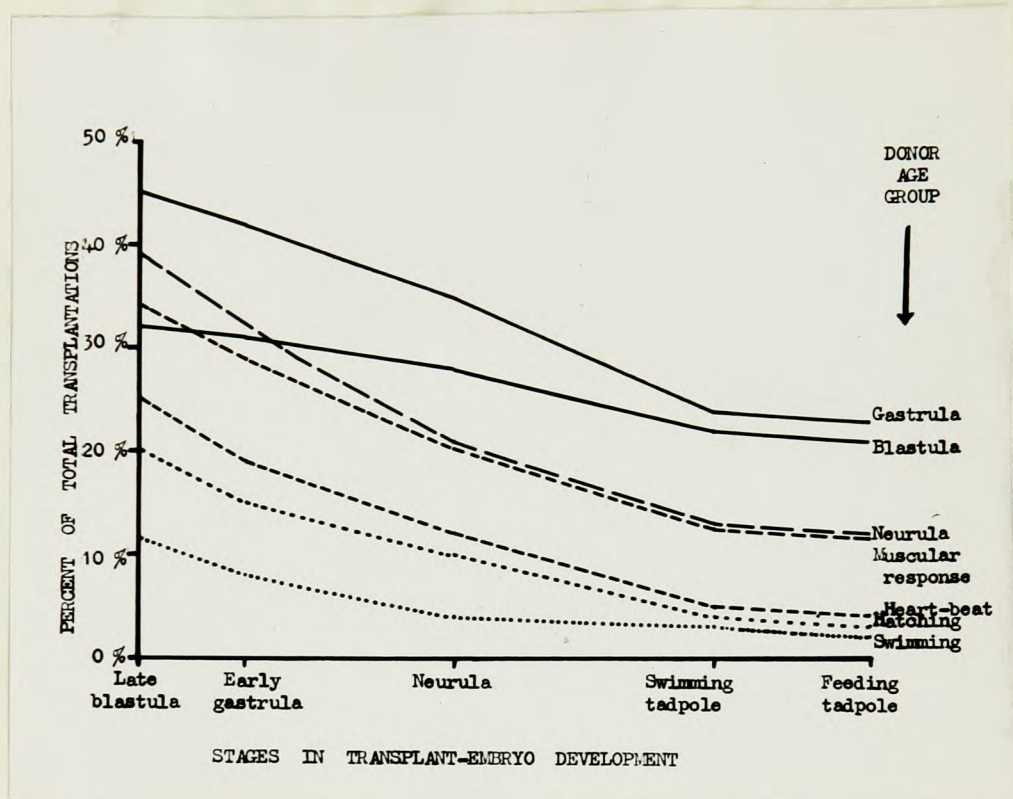
TABLE 2. Selected experiments only. The percentages in the middle of each square represent transplant-embryo survival in terms of total transplantations; the percentages at the bottom of each square indicate transplant-embryo survival in terms of the number of late blastulae for that donor stage.

diploid transplant-embryos from first transfer experiments and does not contain the results of serial transfers. In the survival curves of fig. 1, there is an appreciable mortality among transplant-embryos from all donors; it happens that a higher proportion of late blastulae were obtained from gastrula and neurula donors than from blastula donors, but this difference is not significant. Apart from this it can be seen that the older the donor embryos, the smaller is the proportion of late blastulae and normal tadpoles obtained from their nuclei. It is not profitable to examine these general trends in any detail, since they include experiments done on bad recipient eggs, many of which developed abnormally irrespective of the type of injected nuclei.

Table 2 contains only the results of "selected" experiments in which the recipient eggs were of good quality. There is no clear-cut way of classifying recipient eggs as good or bad, since all degrees of egg quality are found. The best that can be done is to group together all those experiments in which control blastula nuclei have given predominantly normal development. These experiments should provide a clearer indication of trends that have already been seen in fig. 1, but which were then obscured by poor egg quality. In fig. 2, the point at which each survival curve starts shows the percentage of total transplantations which became regular late blastulae.

In fig 3 transplant-embryo survival is shown as a proportion of late blastula transplant-embryos and not as a proportion of total transplantations. This method of presentation avoids the consequences of

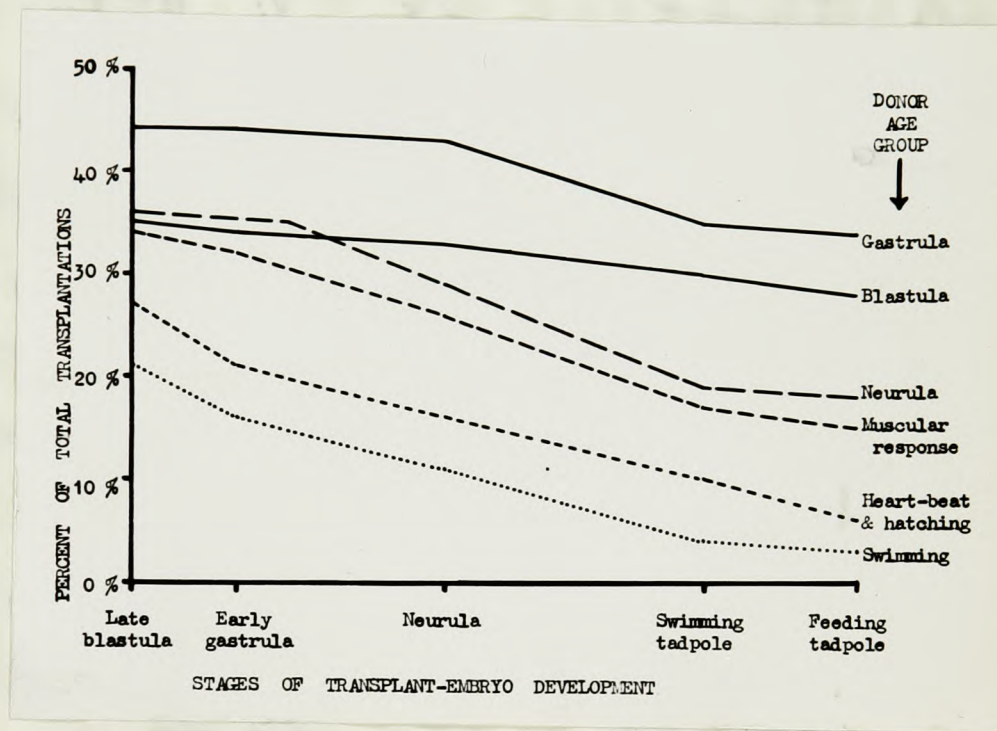
Figure 1.



Survival curves for all experiments (figures from Table 1).

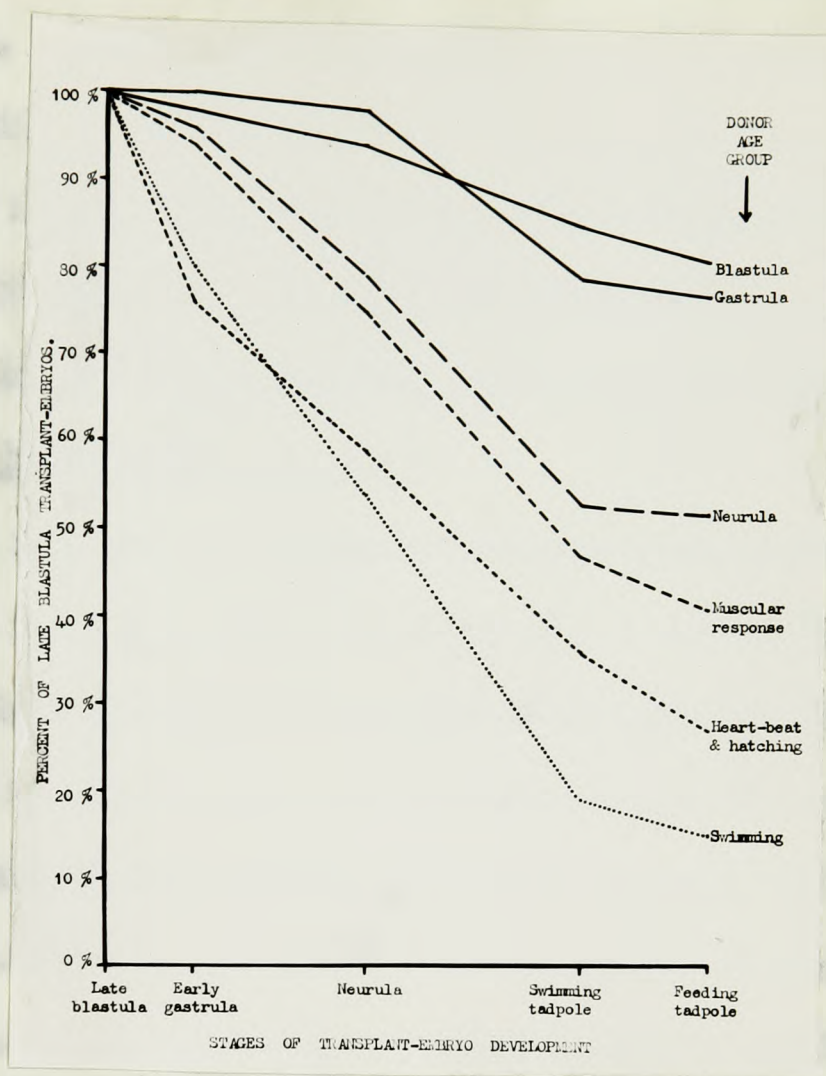
Each curve shows the survival of transplant-embryos for one donor age group. Within each donor group, survival is expressed as a percentage of total transplantations.

Figure 2.



Survival curves for selected experiments only (figures from Table 2). Transplant-embryo survival is expressed as a percentage of total transplantations for each donor age group.

Figure 3.



Survival curves for selected experiments only (figures from Table 2). Transplant-embryo survival is expressed as a percentage of the number of regular blastula transplant-embryos obtained from each donor age group.

The present study for organ development. The present transplant-embryo experiments are therefore believed to be entirely consistent with the view that blastula nuclei are undifferentiated. The survival curve for blastula nuclei in Fig. 3 will be used for comparison with the other survival curves for nuclei of later donor stages.

Gastrula nuclei have given the results shown in Figs. 4, 5, 6,

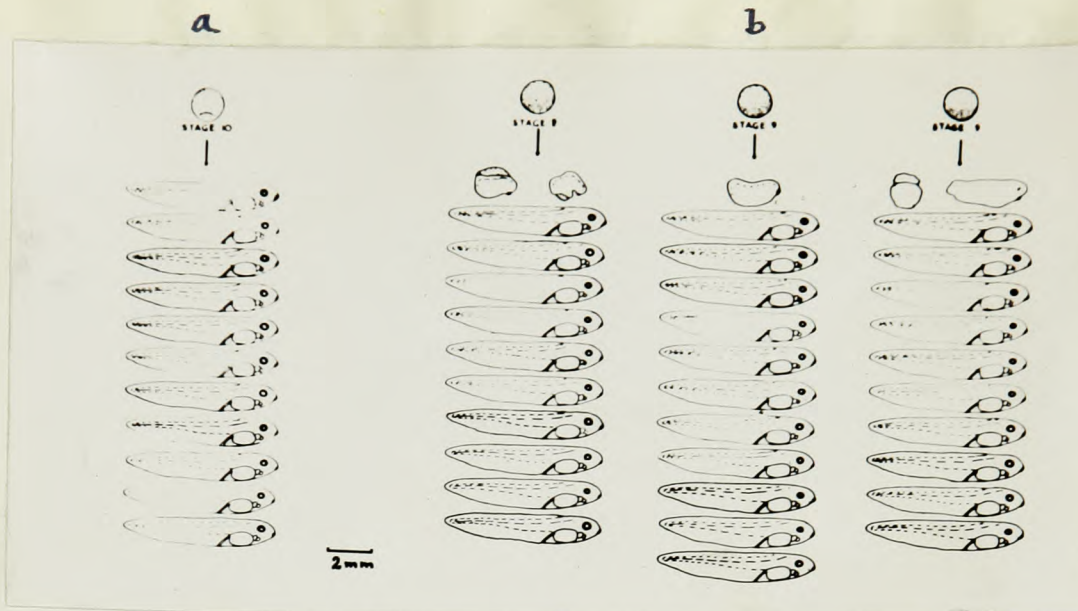
technical variation in the degree of donor cell distortion, but only shows transplant-embryo survival beyond the late blastula stage. The survival curves in fig. 3 are therefore believed to be a good representation of the innate qualities of different donor nuclei, since the effects of other factors which might affect these curves have been removed. Some of the "selected" experiments concerned have been shown in diagrammatic form (figs. 4-9), and will be discussed below with the survival curves.

A. First Transfer Experiments

The most normal transplant-embryo development that has been obtained from blastula nuclei is shown in figs. 4b, 6 and 9. In these experiments nuclei from eight different blastulas have been tested. In the second series of fig. 6 all transplant-embryos became normal tadpoles; in the other seven series most embryos were normal, but in each case a few developed abnormally. The cause of these abnormalities is not clear. It may be that the quality of all eggs is rarely good enough to withstand transplantation; it is often found in this laboratory that up to 10% of naturally fertilised eggs, obtained after hormone injection, develop abnormally; these abnormalities must presumably be attributed to poor egg quality. The small percentage of abnormal development obtained from blastula nuclei certainly does not justify the conclusion that some of these nuclei are differentiated, or lack the potentiality for normal development. The present transplantation experiments are therefore believed to be entirely consistent with the view that blastula nuclei are undifferentiated. The survival curve for blastula nuclei in fig. 3 will be used for comparison with the other survival curves for nuclei of later donor stages.

Gastrula nuclei have given the results shown in figs. 4a, 5, 6,

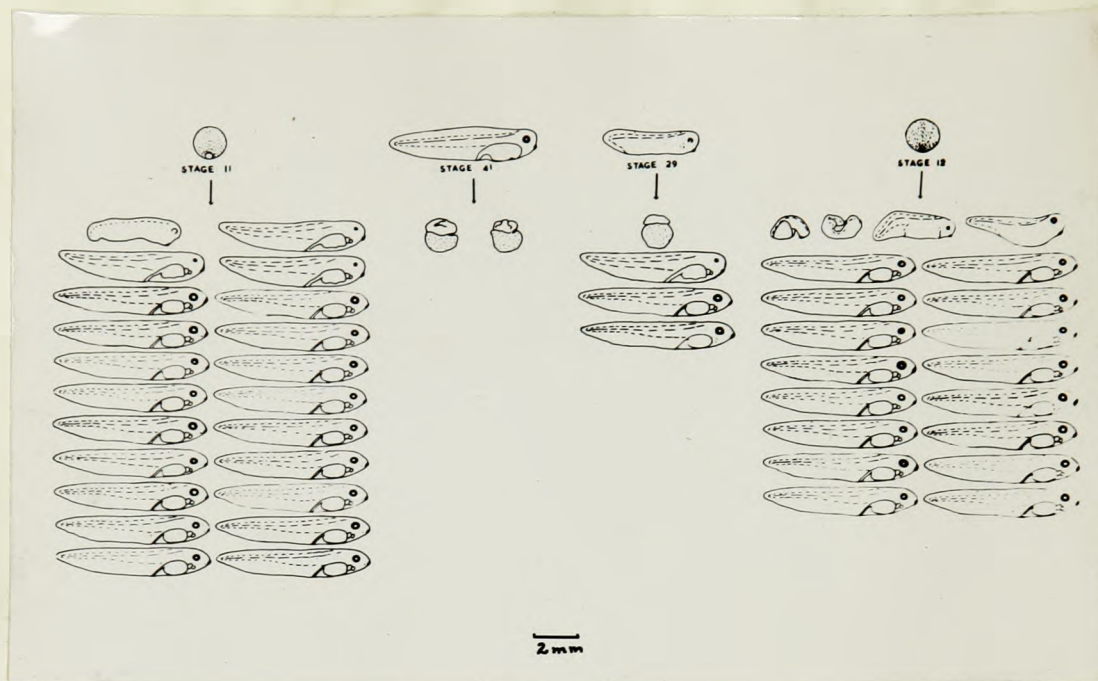
Figure 4.



The development of transplant-embryos derived from the endoderm nuclei of an early gastrula (fig. 4 a), and from the nuclei of three late blastulae (fig. 4 b). Recipient eggs from the same frog were used for all three series in fig. 4 b, which have given very similar results. The total number of transplantations carried out for **fig. 4a** was 14, and for each series of fig. 4 b, about 30.

In this and all following diagrams only those transplant-embryos which survived as regular late blastulae are shown; transplanted eggs which cleaved irregularly have therefore been omitted. Also in this and following diagrams, the series are arranged according to the order in which they were done; that is, the series on the left was made first, and that on the right last.

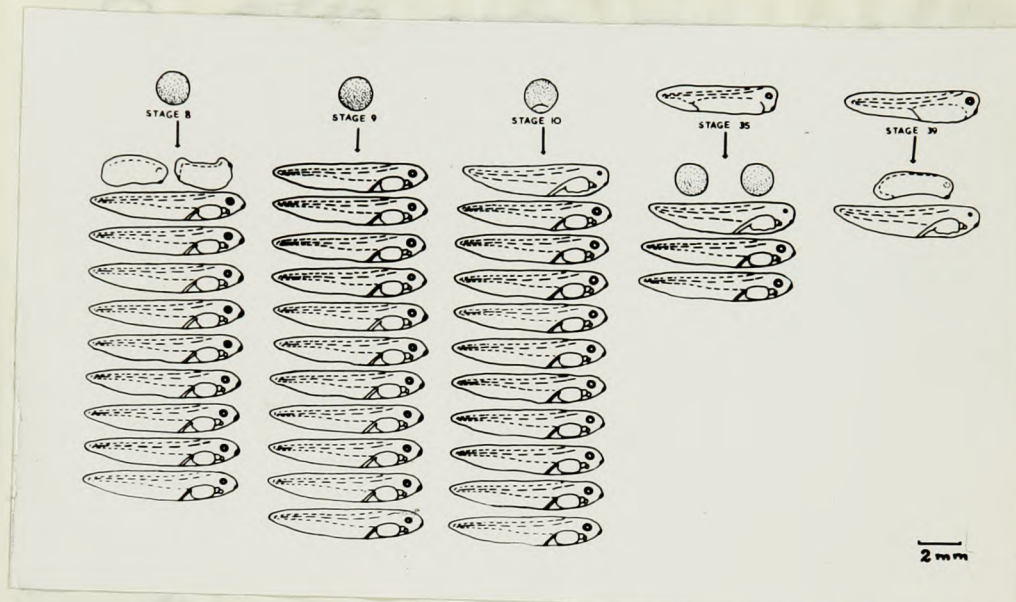
Figure 5.



Transplant-embryos derived from nuclei of a mid-gastrula, a swimming tadpole, a heart-beat tadpole, and a late gastrula. About 40 transplantations were made for each series, and recipient eggs from the same frog were used throughout.

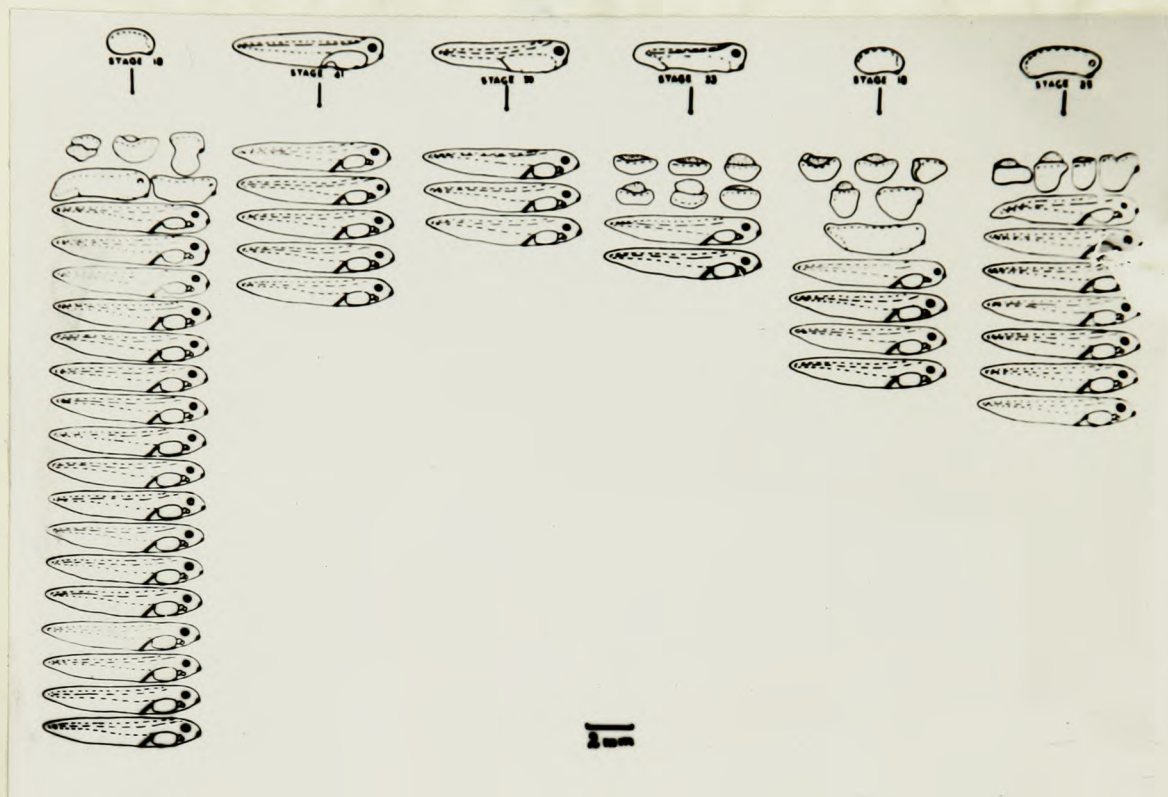
In this and all following diagrams, microcephalic and oedematous tadpoles are represented diagrammatically with a small eye and heart oedema in contrast to normal tadpoles (with a large eye). One microcephalic and two normal tadpoles are shown under the stage 29 donor.

Figure 6.



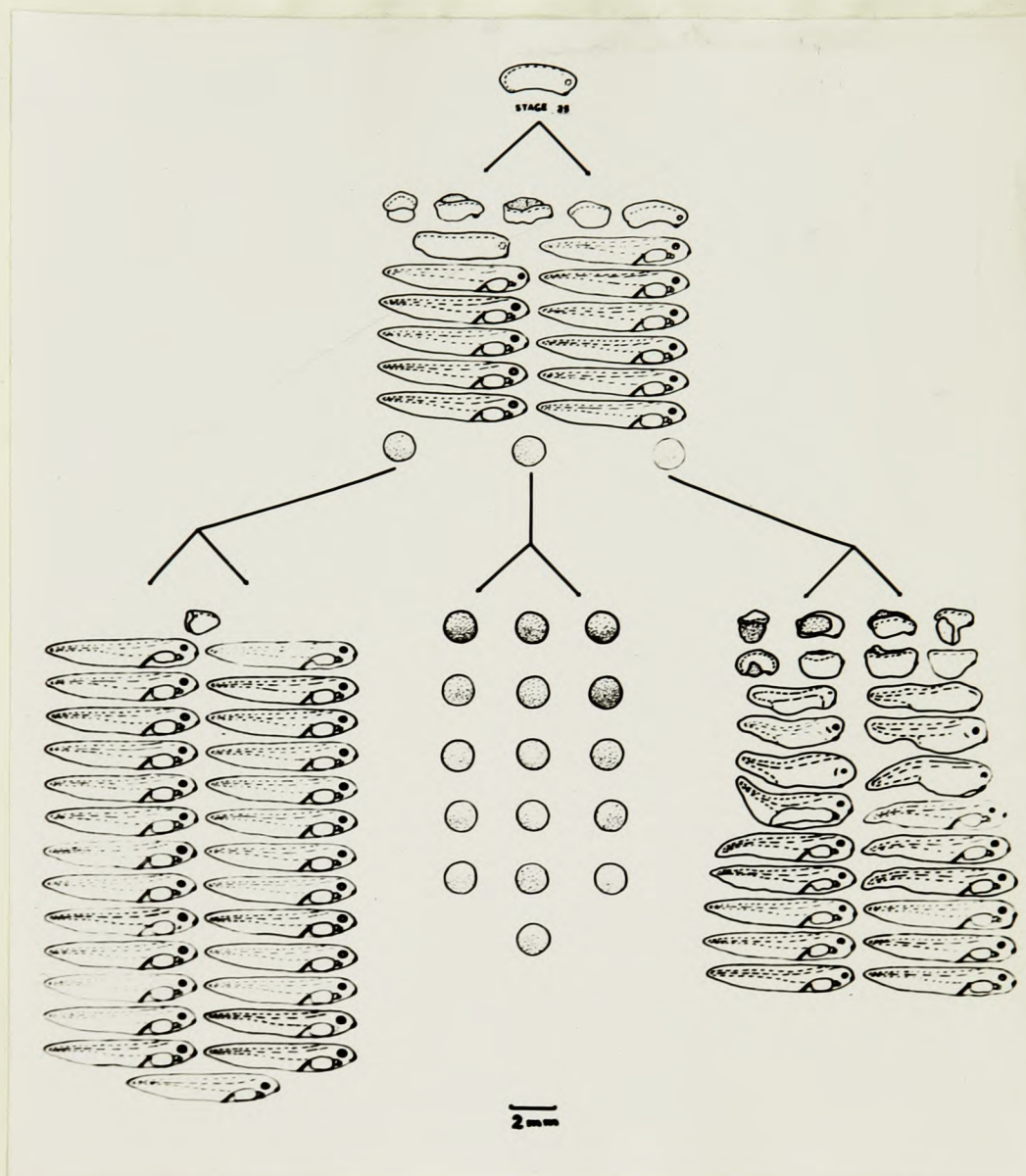
Transplant-embryos from a mid-late blastula, late blastula, an early gastrula, a hatching tadpole, and a swimming tadpole, all from recipient eggs of the same frog. c. 30 transplantations per series.

Figure 7.



Transplant-embryos from a neural-fold stage, a stage 41 swimming tadpole, a stage 39 swimming tadpole, a heart-beat tadpole, another neural-fold stage, and a muscular-response stage; all into the eggs of one frog, and about 35 transplantations per series.

Figure 8.



20 Transplant-embryos were obtained from endoderm nuclei of an original muscular-response stage tadpole. When these embryos had become late blastulae, three were used to provide nuclei for serial transplantations. The three resulting clones differ strongly from each other, but the transplant-embryos within each clone are very similar. About 50 transplantations were made for each of the four series. The serial clones (but not the first transfers) were made with the eggs of one frog.

Figure 9.

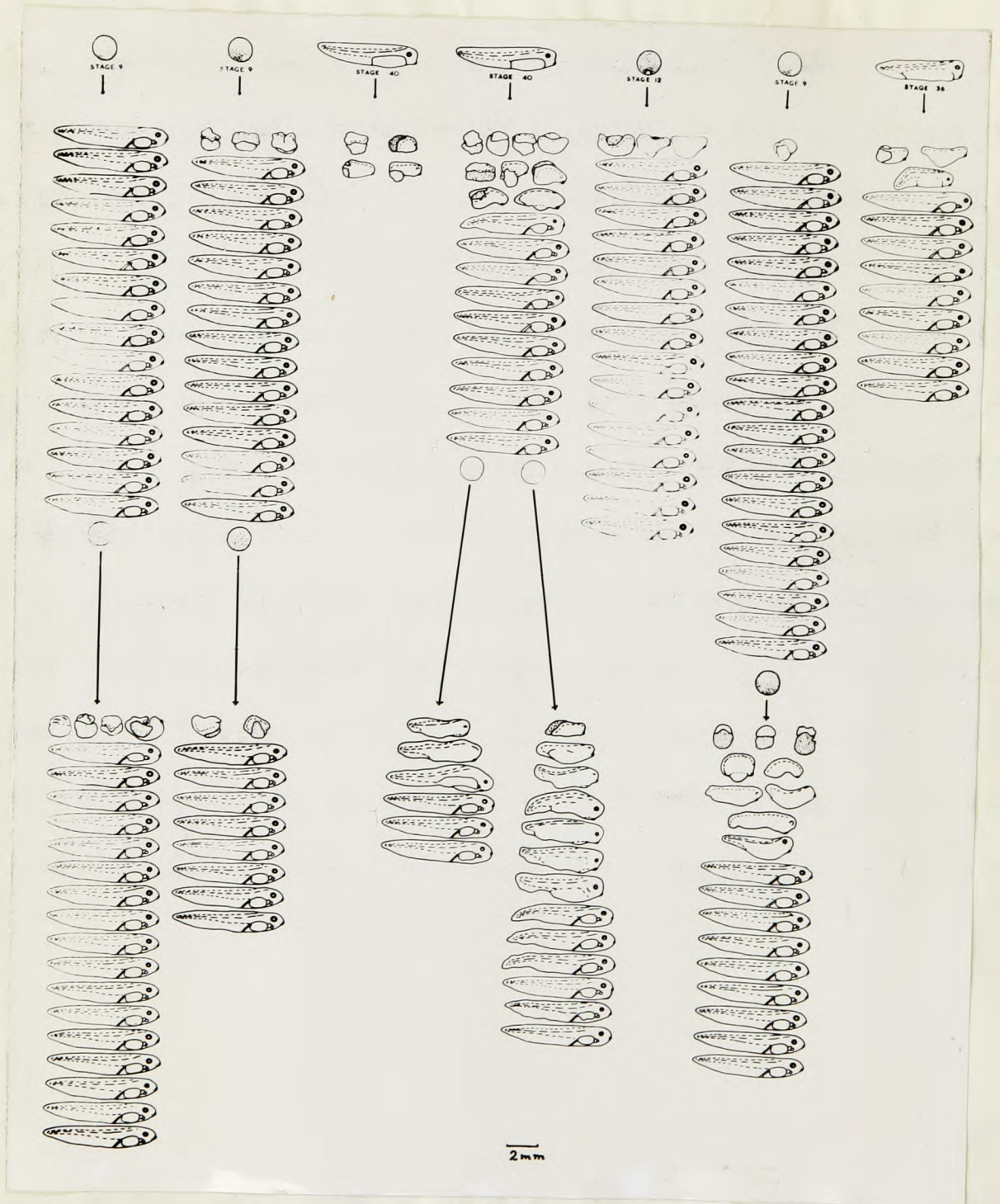


Figure 2.

First transfers were made from two late blastulae, two swimming tadpoles, a late gastrula, a late blastula and a hatching tadpole. There is a marked difference in the results from the two prefeeding tadpoles, in spite of consistently good quality recipient eggs (all from one frog) as shown by the three late blastula series. Five serial clones were made into the eggs of a second frog, using late blastula transplant-embryos as donors. It appears that the eggs of this second frog deteriorated towards the end of the experiment, since the proportion of abnormal transplant-embryos increases irrespective of donor age. About 50 transplantations were made for each series, both among the first transfers as well as in serial clones. The serial transplantations like the first transfers are arranged according to the order in which they were done, though the serial clones were of course not commenced until all first transfers had been completed.

and 9. In the experiment depicted in fig. 4 a, 14 nuclei were transplanted: 3 eggs failed to cleave at all, but the remaining 11 cleaved regularly and all formed normal tadpoles, which, had the experiment been continued, would almost certainly have metamorphosed. Since the results of this experiment did not contain any abnormally cleaved eggs it constitutes particularly clear evidence that the endoderm nuclei from early gastrulae are totipotent and undifferentiated. In the other series with gastrula nuclei a few transplant-embryos are abnormal, though no more than from blastula nuclei. The survival curve for gastrula nuclei in fig. 3 is within 5% of that for blastula donors at each point, a difference which is certainly not significant. There is no indication of any decrease in nuclear potentialities during gastrulation, since late blastulae and late gastrulae give similar results (of the second and fifth series of fig. 9).

It is worth drawing attention here to the consistent results that have been obtained when nuclei from several different blastula and gastrula donors are transplanted into the recipient eggs of one frog, as in figures 4b, 5, 6, and 9. The proportion of total transplantations which become late blastulae is very similar in these experiments, as shown by the number of embryos under each donor; also the proportion of late blastulae which become normal tadpoles is about the same in each case. The similarity in results is most marked among series in which recipient eggs of the same frog were used, that is among all those shown in one figure. These consistent results would be expected from totipotent, undifferentiated nuclei.

Nuclei from older donor embryos appear to become progressively limited in their developmental potentiality. This differentiation is reflected not only in the proportion of total transplantations which form

regular late blastulae, but also in the proportion of late blastulae which develop normally. The point at which survival curves commence in fig. 2 is lower, the older the donor stage; this illustrates the decline in the proportion of late blastulae formed from older donor nuclei. It was pointed out above (p. 4) that the technique may cause up to 20% of variation in the proportion of blastula transplant-embryos. Such variation in technique is probably responsible for the fact that a greater proportion of blastula transplant-embryos was obtained from neurula and gastrula donors than from earlier stages. In donors later than the heart-beat stage there is a pronounced reduction in the numbers of blastula transplant-embryos. This difference is too large to be accounted for by technical variation, and must therefore represent a real difference in nuclear potentialities. In most experiments the proportion of blastulae formed decreases progressively from the heart-beat stage, the older the donor; this typical condition is seen in figs. 6, 7 and particularly clearly in fig. 5. However, this trend is not always consistent. It may happen that nuclei from two donor embryos of exactly the same age, when transplanted into eggs of the same frog, will give entirely different results; this can be seen by comparing the 1st and 5th series of fig. 7, and particularly clearly from a comparison of the 3rd and 4th series of fig. 9. One may conclude from these results that the ability of endoderm nuclei to form regular blastulae after transplantation becomes progressively reduced the older are the donor embryos beyond the heart-beat stage; on the other hand, the rate at which this capacity is lost is not always closely correlated with the developmental stage of the donor embryo.

The second respect in which endoderm nuclei appear to become

differentiated concerns their decreasing capacity for giving normal transplant-embryo development. Blastula transplant-embryos derived from older donors are much less likely to become late gastrulae and normal tadpoles than transplant-embryos from younger donors (fig. 3a). This loss of capacity begins in nuclei from neurula donors. From this stage onwards the developmental potentiality of nuclei becomes increasingly restricted right up to the swimming tadpole stage, which is the latest from which nuclei have been transplanted. Even at this advanced stage, transplant-embryos from some nuclei have developed into normal tadpoles, though the proportion of such undifferentiated nuclei is very small.

As before, there is great variation in the extent to which this loss of capacity has proceeded in donor embryos of the same age. In fig. 5, nuclei from a swimming tadpole have given wholly abnormal development, but in fig. 7, two swimming tadpoles have given mostly normal development; the proportion of abnormalities is markedly different in the two swimming tadpoles of fig. 9. Thus, though there is a clear tendency for more abnormal transplant-embryo development to be obtained from the nuclei of later donor stages, there is no exact correlation between the age of the donor embryo and the developmental potentialities of its endoderm nuclei.

B. Serial Transfer Experiments

Serial nuclear transfers involve exactly the same technique as first-transfer experiments. The only difference is that the donor embryo is not derived from a normally fertilised egg, but is itself a transplant-embryo obtained by nuclear transplantation (King, & Briggs, 1956). Reasons have been given above for believing that Xenopus endoderm nuclei do not

differentiate until after the late gastrula stage; thus if a transplant-embryo is used for serial transplantation before it has developed beyond a blastula or gastrula, all its endoderm nuclei should have the same potentiality as each other. If a blastula transplant-embryo derived from a normal nucleus is used as a donor, serial transplantation of its nuclei should yield a high proportion of normal development. On the other hand, if the original nucleus was differentiated so that it would have developed abnormally, then serial transplantation from it should (if the abnormality is inherited) provide many embryos (a clone) all suffering from the same kind of abnormality.

Two points can be investigated by serial transplantation of nuclei from transplant-embryos which have not developed beyond the blastula or gastrula stage. First, such experiments show whether the lack of potentiality of differentiated nuclei is inherited or not, and secondly whether the differentiation of nuclei is reversible.

The extent to which nuclear changes are inherited is apparent from a comparison of one clone with another. If they are consistently different from each other this can only be attributed to heritable differences between the original donor nuclei. In fig. 8, nuclei were taken from an original muscular response tadpole, and three transplant-embryos were used at the blastula stage to provide donor nuclei for serial clones. In the left hand clone the great majority of embryos were normal; the proportion of abnormal embryos is very small and not more than is obtained with first-transfer blastula nuclei. This clone therefore shows that the original donor

nucleus was normal. In the middle clone every transplant-embryo was arrested as a late blastula. The third clone on the right contains a range of abnormalities, but none of the tadpoles shown were quite normal, all being microcephalic and oedematous. The variation within this clone is discussed below. The three clones in this experiment are consistently different from each other, and so demonstrate clearly that the abnormalities in the 1st transfer series are heritable.

In this experiment, three blastula transplant-embryos were used to provide serial donor nuclei. It was not of course known how these three blastulae would have developed if they had been allowed to differentiate further. However, an indication of the way they might have developed can be derived from the development of the other 17 1st transfer embryos. The three serial donor embryos were chosen at random from 20 blastula transplant-embryos, and would therefore be expected to have developed in the same kind of way as did the 17 which were left. The potentialities of the 3 serial donors turned out to be consistent with the 17 1st transfer embryos. This result suggests that all the abnormalities present in the first transfer group are heritable in the way that the abnormalities of the three embryos used for serial transplantation have been found to be.

The second kind of information which serial transplantation can give concerns the reversibility of nuclear differentiation. This is shown by the variation within a clone as opposed to the variation between clones. Clones from original blastula donors are shown in fig. 9. Since the great majority of embryos in the first transfer series were normal, nuclei from embryos selected at random for serial donors would also be expected to be

totipotent in the majority of instances. The first and second clones of fig. 9 are predominantly normal. the clone from the sixth series contains more abnormal embryos than would be expected, but this series was carried out on the last eggs laid in one frog's ovulation, and some of these may have been immature. The first two clones of fig. 9 and the first of fig. 8 show that totipotent nuclei have no obvious tendency to age or become abnormal after continued transplantation. These series also confirms that the technique and egg quality cannot explain the abnormalities found in other clones. There is very little variation in clones from normal nuclei, and no variation in ones with very abnormal nuclei (2nd clone of fig. 8); *however, clones in which no embryos are quite normal though some are nearly so, are very variable.* This is seen in the third clone of fig. 8, and in the two clones from the fourth series of fig. 9; in both cases the original donor was advanced and would therefore have contained differentiated nuclei. This variation could be explained in two ways. It might be that the potentiality of the original donor was like that of the worst or best embryo in the clone derived from it, and that after serial transplantation some nuclei increase or decrease their potentialities. Or it might be that embryos obtained from abnormal nuclei are much more sensitive to technique etc. than embryos with normal nuclei. In connection with the latter, haploid embryos, which have abnormal nuclei, differentiate very variably (Gurdon 1960 a); though this variation might be due to genetic differences from one haploid nucleus to another, it might also be due to the effect of various environmental factors on nuclei suffering from the same abnormality (haploidy). Sufficient experiments have not yet been carried out

to show whether variation in abnormal transplant-embryo clones represents a change in nuclear potentiality or not. Until other possibilities have been excluded, this limited variation cannot be regarded as evidence that nuclear differentiation is reversible.

C. Conclusions concerning the Potentialities of Endoderm Nuclei in *Xenopus*.

Nuclear differentiation in relation to the developmental stages of donor embryos.

The nuclei of differentiating endoderm cells become progressively limited in their developmental capacity. This differentiation is evident not only from the proportion of transplantations which become late blastulae, but also from the proportion of blastula transplant-embryos which develop normally. The proportion of late blastulae obtained from the nuclei of embryos up to the muscular-response stage does not vary, but becomes progressively reduced from nuclei of heart-beat and later stages. Although variation in technique can effect the proportion of late blastulae, it cannot account for the great difference in this respect between nuclei from young and advanced donor embryos. The majority of transplant-embryos derived from blastula and gastrula nuclei develop normally as far as swimming tadpoles; however, the more advanced the donor, the more transplant-embryos develop abnormally, and the more severe are the abnormalities from which they suffer.

These results indicate that the endoderm nuclei of *Xenopus* only begin to become differentiated in embryos older than late gastrulae. From the neurula stage onwards there is a progressive increase in the proportion of differentiated nuclei, but even in swimming tadpoles there remain a few

endoderm nuclei with entirely unrestricted developmental potentialities.

These conclusions have been based on the results of selected experiments. It was, however, pointed out (p. 6) that the same conclusions can be drawn, though less obviously, from all experiments. Fischberg, Gurdon, & Elsdale (1958) reached similar general conclusions from some earlier experiments with Xenopus.

Limited application of results.

Attention must now be drawn to certain limitations which apply to these results. Only endoderm nuclei of Xenopus have been investigated, but comparable changes will probably be found to take place in the nuclei of other differentiating tissues. Briggs & King (1957) have described specific abnormalities associated with nuclei from different embryonic tissues of Rana pipiens.

The potentialities of nuclei have not been tested for all developmental stages up to that in which the endoderm is differentiated. No transplantations have been made from nuclei of earlier stages than a mid-blastula, but there is no reason to believe that such nuclei would give more normal development than was obtained from late blastula and gastrula nuclei. The latest donor stage from which nuclei were transplanted was a swimming tadpole in which the gut had just formed two right-angled bends. In order to transplant from later stages, other substances than Versene would be required for cell disaggregation, and it is not certain that these would be harmless. The potentialities of endoderm nuclei have therefore only been tested between Nieuwkoop's stages 8 and 41, but some gut cells remain undifferentiated until stage 46, several hours later.

The development of transplant-embryos has only been followed up to the formation of normal tadpoles. Transplanted nuclei have therefore only been tested for their ability to bring about embryonic development up to the tadpole stage. Transplant-embryos which become normal tadpoles can be reared into sexually mature frogs (Gurdon, Elsdale, & Fischberg, 1958); the study of these frogs gives additional information about the nuclei from which they were derived, and will be dealt with elsewhere.

The part of a nucleus in which differentiation takes place.

The changes undergone by differentiating endoderm nuclei were described above as "innate nuclear qualities". This term refers to the conclusions based on transplant-embryo development when all effects of the transplantation technique and egg-quality have been removed from the results. Serial transfer experiments have shown that these innate nuclear changes are hereditary and, to a large extent at least, irreversible. These two characteristics are associated with gene controlled factors. Moreover, it is shown below that there is no correlation between these nuclear changes and certain general, reversible nuclear qualities, such as size and stage in mitosis. Most nuclear components other than the chromosomes are changed at each mitosis; nuclear changes, which are heritable, cannot therefore be associated with the nuclear membrane, nuclear sap, or nucleolus. Thus there are strong reasons for believing, as in Rana (Briggs & King, 1956), that the changes in endoderm nuclei have taken place in the chromosomes.

The smallest endoderm nuclei to have been transplanted (stage 41) are at least as large and as well protected by cytoplasm as nuclei from the animal hemisphere of a blastula; but, as animal and vegetal blastula nuclei

give equally good results, nuclear changes cannot be associated with the size of nuclei (Gurdon, 1960 c). The proportion of endoderm nuclei "in mitosis" (nuclear membrane disappeared) have been counted in embryos at different stages of development, reared at 21° C. 100 endoderm nuclei of the kind used as donors for nuclear transplantation were counted for each donor stage. The proportion of nuclei in mitosis was 17% in a blastula, 7% in a late gastrula, 5% in a neural-folds embryo, 2% in a heart-beat embryo, and 1% in a stage 41 swimming tadpole. These figures give only a rough indication of the frequency with which nuclei in mitosis were used for transplantation; *they show, however, that the transplantation* of mitotic nuclei, whatever effect this may have, is too infrequent to effect the evidence for nuclear differentiation.

The significance of nuclear changes.

There is no obvious correlation between nuclear differentiation and morphogenetic development of tissues. This is shown below (p. 18) by comparison of nuclear differentiation in Rana and Xenopus. Very little seems to be known about the state of determination of endoderm tissue in Xenopus. Though the endoderm of Amphibia is described as "regionally determined histologically sometime before gastrulation" (Kemp, 1951), this generalisation may not be true of Xenopus. As it is not known what tissues endoderm can form when grafted into various regions of an embryo, it does not seem possible to compare tissue determination and nuclear differentiation. It seems possible however that nuclear differentiation may be connected with cell differentiation. In Xenopus the nuclei used for transplantation have been taken from the most undifferentiated endoderm cells of each donor stage. As the gut becomes formed, many of the peripheral endoderm cells become differentiated - that, they lose their more or less spherical shape, and their

nuclei often become elongated; these cells also become very strongly connected to each other, and it is for this reason that nuclei have not been transplanted from fully differentiated cells. What has in effect been done in these experiments is to transplant nuclei from undifferentiated endoderm cells, and show that the nearer the time of cellular differentiation, the more nuclei become differentiated. These results are consistent with the considerable variation that has been found in the degree of differentiation of nuclei from late donor stages (p. 10). Some donor cells were probably nearer this differentiated state than others. It is therefore suggested that nuclear differentiation may play a significant part in the processes which take place when individual cells attain their final functional state. This hypothesis can only be tested when a method is found by which differentiated cells can be harmlessly dissociated.

V. COMPARISON OF NUCLEAR DIFFERENTIATION IN XENOPUS LAEVIS AND RANA PIPIENS

Briggs & King (1957), using Rana pipiens, have transplanted endoderm nuclei from donors of various ages between late blastulae and tail-bud embryos. The survival of transplant-embryos derived from Rana pipiens nuclei is shown in Table 3 and has been expressed as a percentage of late blastula transplant-embryos for each donor stage; these figures are therefore comparable to those taken from Table 2 for Xenopus. The survival of transplant-embryos in Rana and Xenopus is compared in Table 3 and fig. 10.

Transplant-embryos derived from blastula nuclei of Rana are mostly normal; there is however a sharp decline in the developmental potentialities of endoderm nuclei from gastrulae and later stages. Thus in Rana the differentiation of endoderm nuclei starts at the beginning of gastrulation

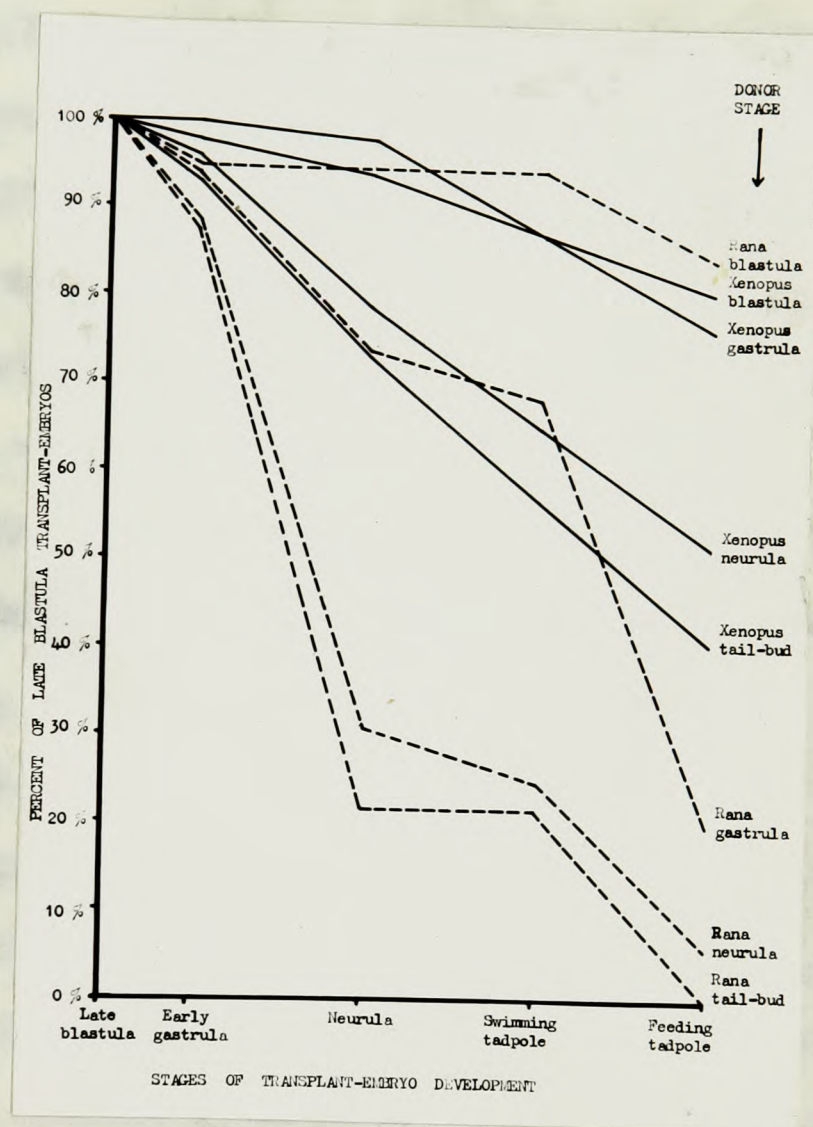
TABLE 3

DONOR STAGE	SPECIES	TOTAL TRANSPLANTATIONS	'STAGES IN TRANSPLANT-EMBRYO DEVELOPMENT				
			Late Blastulae	Early Gastrulae	Neurulae	Tail-buds	Normal Tadpoles
late blastula or early gastrula animal cells, undifferentiated nuclei).	<u>Rana</u>	92	39 100%	37 95%	37 95%	37 95%	33 85%
	<u>Xenopus</u>	327	100%	98%	94%		81%
late gastrula endoderm	<u>Rana</u>	155	79 100%	75 95%	58 74%	54 69%	16 20%
	<u>Xenopus</u>	502	100%	100%	98%		77%
neurula endoderm	<u>Rana</u>	98	16 100%	14 88%	5 31%	4 25%	1 6%
	<u>Xenopus</u>	140	100%	96%	79%		52%
tail-bud endoderm	<u>Rana</u>	130	9 100%	8 89%	2 22%	2 22%	0 0%
	<u>Xenopus</u>	152	100%	94%	75%		41%

TABLE 3. A comparison of nuclear differentiation in Rana and Xenopus. The percentages are expressed in terms of the number of late blastula transplant-embryos for each donor stage. The figures for Rana have been taken from Briggs & King (1957), and for Xenopus from Table 2 above. (solid lines): the numbers have been taken from Table 2. Each

curve shows the survival in terms of the late blastula transplant-embryos of one donor stage.

Figure 10.



Survival curves for endoderm nuclei of Rana (dotted lines) and Xenopus (solid lines); the numbers have been taken from Table 3. Each curve shows the survival in terms of the late blastula transplant-embryos of one donor stage.

and continues from that stage onwards; by the tail-bud stage all endoderm nuclei are differentiated, since none have given rise to normal transplant-embryo development, (Briggs & King, 1957).

In Xenopus, on the other hand, the differentiation of endoderm nuclei does not begin until the neurula stage, and then only takes place very gradually from that stage onwards; even in swimming tadpoles some endoderm nuclei remain undifferentiated. In both Rana and Xenopus a similar proportion of normal transplant-embryos are obtained from undifferentiated blastula nuclei, but in other respects the rate and time of onset of endodermal nuclear differentiation is very different for the two species.

The possibility that transplant-embryo survival is affected by differences in the technique for the two species seems improbable (Elsdale, Gurdon & Fischberg, in prepn.). The only significant difference in technique concerns the method of enucleation of recipient eggs. In Rana the egg nucleus is removed, while in Xenopus it is inactivated by irradiation and allowed to degenerate in the egg cytoplasm (Gurdon 1960 b). In Xenopus, there is no reason to suppose that the dying egg nucleus has any effect on either the injected nucleus or the egg cytoplasm, but this question cannot be finally settled until it is possible to enucleate the eggs of both species by the same method.

The Xenopus transplantations used for fig. 10 are selected results, while the results for Rana are the average of all experiments with endoderm nuclei. The use of selected results for Xenopus does not however affect the conclusions, since a similar comparison with Rana is obtained if the total results for Xenopus (Table 1) are used instead of selected results. It appears that Briggs & King have not found much variation in their results with

Rana corresponding to the eggs of individual frogs. They have used frogs which have been taken from their natural conditions, and this might account for the eggs of Rana being more consistent in quality than those of Xenopus. It could, on the other hand, be that the much greater size of Rana eggs renders them less susceptible to damage by experimental manipulation, so that all Rana eggs are resistant to the technique while this is only true of some eggs in the case of Xenopus. Whichever of these possibilities is correct does not invalidate the very marked difference in nuclear differentiation between the two species.

Both Rana and Xenopus pass through very similar stages of morphological differentiation. Cleavage, gastrulation, and elongation of the embryo are proportionately faster in Xenopus, but this is largely due to the higher temperature at which Xenopus develop. The rate of embryonic development does not appear to affect the rate of nuclear differentiation, since nuclei from Xenopus embryos which were reared at 18° C and 26° C gave a similar proportion of normal and abnormal transplant-embryos. These experiments lead to the conclusion that no consistent relationship exists between the rate of nuclear differentiation and that of morphological tissue differentiation. This confirms the conclusion (p. 17) that there is no causal connection between the differentiation of a tissue and that of its cell nuclei.

SUMMARY

1. The developmental potentiality of embryonic endoderm nuclei in Xenopus laevis is shown to change as the tissue to which they belong becomes differentiated. This change has been demonstrated by taking nuclei from endoderm tissue in different stages of differentiation, and

transplanting them into enucleated unfertilised eggs; the development of the resulting transplant-embryos indicates the developmental capacity of their nuclei.

2. Since the proportion of total transplantations which become late blastulae is affected by the technique, the main conclusions have been drawn from the further development of late blastula transplant-embryos. The quality of recipient eggs, which is always variable, may also affect transplant-embryo development, and conclusions have therefore been mainly derived from selected experiments in which control donor nuclei have shown that egg quality was good. Reasons have been given for believing that non-hereditary nuclear qualities, such as their size and stage in mitosis, do not affect the conclusions drawn from transplant-embryo development; their development is therefore solely dependent on the specific genetic qualities of the donor nuclei used.

3. No decline in the developmental potentiality of endoderm nuclei was found in blastulae and gastrulae. However, the capacity of nuclei to form normal tadpoles decreased progressively from after gastrulation until the beginning of torsion in the gut of swimming tadpoles; at this late stage there was still a small proportion of undifferentiated nuclei from which normal tadpoles have been obtained. Thus nuclear differentiation affects an increasing proportion of nuclei to an increasing extent, as the endoderm becomes differentiated. Serial transplantation has shown that the changes involved in nuclear differentiation are heritable and, at least to a large extent, irreversible.

4. These results are compared with those of Briggs and King who transplanted nuclei from endoderm cells of Rana pipiens. In both Xenopus and Rana, nuclei are undifferentiated at the late blastula stage, but after this the rate and time of onset of nuclear differentiation is very different for the endoderm of the two species. These differences show that there is no causal connection between nuclear differentiation and tissue differentiation. It is suggested that nuclear differentiation may be concerned in the processes which take place when individual cells become differentiated into their final functional stage.

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THE REPRODUCTIVE CAPACITY AND OTHER CHARACTERS OF ADULT XENOPUS

LAEVIS DERIVED FROM THE TRANSPLANTATION OF EMBRYONIC ENDODERM

NUCLEI

Synopsis.

I. INTRODUCTION

II. THE NUCLEAR ORIGIN OF FROGS; REARING METHODS AND CONTROLS

III. DESCRIPTION OF ADULT TRANSPLANT-FROGS, THEIR REPRODUCTIVE CAPACITY
AND OFFSPRING

- A. Weight
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THE REPRODUCTIVE CAPACITY AND OTHER CHARACTERS OF ADULT XENOPUS
LAEVIS DERIVED FROM THE TRANSPLANTATION OF EMBRYONIC ENDODERM
NUCLEI.

I. INTRODUCTION

When an embryonic cell nucleus is transplanted into an unfertilised and enucleated egg, many of the resulting transplant-embryos develop into tadpoles and frogs. The development of these embryos and the characters of the adult frogs indicate the genetic qualities of the transplanted nucleus. Nuclear transplantation is, in effect, a means of replacing the zygote nucleus of a fertilised egg by an embryonic cell nucleus. If embryos and frogs derived from transplanted nuclei are compared with normal embryos and frogs from fertilised eggs, it can be seen how the transplanted nuclei differ genetically from normal zygote nuclei.

The developmental potentialities of endoderm nuclei have recently been investigated in Xenopus laevis (Gurdon, d). Conclusions were based on the development of transplant-embryos up to the stage when tadpoles begin feeding. Many of these transplant-embryos have been reared into frogs which can reproduce themselves (Gurdon, Elsdale, & Fischberg, 1958); these frogs, which are described below, provide further information about the genetic qualities of endoderm nuclei.

II. THE NUCLEAR ORIGIN OF FROGS; REARING METHODS AND CONTROLS

The frogs described below have been reared from the same transplant-embryos as were used in an analysis of the developmental potentialities of

endoderm nuclei (Gurdon, d). They were obtained by the standard nuclear transplantation technique, and carry the Xenopus nuclear marker, which proves that their nuclei are derived from the transplanted nucleus (Elsdale, Gurdon, & Fischberg, in prepn.). These frogs are derived from endoderm nuclei of donor embryos ranging from blastulae to swimming tadpoles.

The method of rearing transplant-embryos into adult frogs has been described elsewhere (Elsdale, Gurdon, & Fischberg, in prepn.). Under these conditions sexually mature frogs are obtained from fertilised eggs within one year. It is probable that the rearing conditions and diet are not ideal, and that deficiencies of some kind are responsible for a lower average fertility than would be expected from animals living in wild conditions. For this reason transplant-frogs are compared to control frogs which have been reared from fertilised eggs under the same laboratory conditions, and not to frogs recently imported from the wild. It is, however, worth pointing out that there are only small differences (even in fertility) between laboratory-reared frogs and those that have grown up in a natural, wild environment.

III. DESCRIPTION OF ADULT TRANSPLANT-FROGS, THEIR REPRODUCTIVE CAPACITY AND OFFSPRING

The general appearance of transplant-frogs is very similar to that of controls. Gross abnormalities, such as limb or organ deficiencies occur very rarely, and are found as frequently (less than 0.5%) in control frogs. There are also no appreciable differences between transplant-frogs

and controls in body-proportions, activity, and behaviour; these characters have not therefore been described. Records and measurements of other characters of these frogs will be found in Table 1, which includes all adult diploid frogs that have so far been obtained from the transplantation of endoderm nuclei. Tetraploid frogs have been ~~ex~~cluded from the results, since their gametogenesis is characteristically abnormal owing to their polyploid conditions; in other respects they are very similar to their diploid brothers and sisters (Gurdon, 1960 a).

Since all cell nuclei of one embryo are genetically related if not identical, it is not surprising that transplant-frogs derived from one donor should show many similarities not shared with frogs from other donors. If all transplant-frogs from one donor embryo (bracketed together in Table 1) suffer from the same kind of abnormality, this indicates that it is due to a genetic abnormality in the zygote nucleus from which the donor embryo was derived. On the other hand if some transplant-frogs from a donor show an abnormality that is not present among others from the same donor, this may be due to either (i) an abnormality in the particular eggs from which the abnormal frogs developed (see p. 4), or (ii) to some environmental factor affecting the frog's growth, or (iii) to nuclear differentiation in the endoderm cells from which the frogs were derived. No obvious similarities have yet been observed among transplant-frogs derived from the recipient eggs of any one frog, whether a comparison is made of the results from eggs laid on different occasions or only of those from eggs laid during one ovulation.

A. Weight.

In Xenopus laevis there is a considerable variation in adult

TABLE 1. The gametogenesis and other characters of adult transplant-frogs derived from embryonic endoderm nuclei.

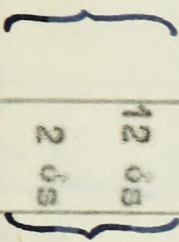
ORIGIN OF DONOR NUCLEI	DEVELOPMENTAL STAGE (Neurotop & Faber, 1956)	Donor Origin of nuclei.	SEX	Origin of recipient eggs	WEIGHT WHEN ADULT in grams.	FERTILITY (% of all eggs which begin develop- ment).	DEVELOPMENT OF OFFSPRING (% of blast- ulae which became nor- mal tadpoles)	NOTES & reference numbers.
IMPORTED FROGS	Reared under natural conditions	24 ♀s	—	Range: 70-104.	5% - 95%	50% - 90%.		
CONTROLS (from fertil- ized eggs)	Of same parentage	5 ♀s	—	71-104 (average: 88).	5% - 70%	50% - 90%.	2N series ♀s Nos. 1, 4, 5, 6, 7.	
		1 ♀	—	136.	0% - 60%	50% - 90%	2N, No. 77.	
		6 ♂s	—	35-56 (average, 52.).	5% - 90%	50% - 90%	17.20 ♂s.	
BLASTULA (animal hem- isphere cells).	8	1 ♀		85	20 %	30 %	Tr. End. 1, (old ser.).	
		 12 ♂s 2 ♂s	30x, 300x, 16, 24, 26L.	40-64 (average, 52.).	5% - 90% 0% - 3%	50% - 80%	Tr. Pot. ser. ♂s.	

TABLE 1, Continued.

ORIGIN OF DONOR NUCLEI	DEVELOP- MENTAL STAGE (Neuwkoop & Faber, 1956).	Origin of donor nuclei	SEX	Origin of recipient eggs & date	WEIGHT WHEN ADULT IN GRAMS	FERTILITY (% of all eggs which begin development).	DEVELOPMENT OF OFFSPRING (% of blast- ulae which became nor- mal tadpoles).	NOTES, & reference numbers.
BLASTULA (vegetal hemi- sphere cells).	8	{	♀	1x:57.	95	—	—	Tr. End. 5.
	8	{	♀	12E	73	—	—	Tr. End. 37.
	8	{	♀	1x:57.	74	—	—	Tr. End. 40.
	8	{	♀	12E	24	—	—	Normal looking but entirely infertile eggs. Tr. End. Nos. 31, 32, 33, 34, 35, 36.
	8	{	♀	1x:57.	45	—	—	
	8	{	♀	1x:57.	110	0%	—	
	8	{	♀	1x:57.	116	0%	—	
	8	{	♀	1x:57.	116	0% (twice)	—	
	8	{	♀	1x:57.	127	0% (twice)	—	
	8	{	♀	1x:57.	120	5% - 30%	30% - 70%	Tr. End. 17.
	8	{	♀	1x:57.	120	5% - 30%	30% - 70%	Tr. End. 17.
	8	{	♀	1x:57.	120	5% - 30%	30% - 70%	Tr. End. 17.
Transplant-frog offspring	9	{	♀	1x:57.	71-108	30%	90%	17.20 F ₁ .
	9	{	♀	1x:57.	35-47	30%	90%	Tr. End. 20.
	9	{	♀	1x:57.	49	10% - 90%	30% - 70%	Tr. End. 20.
	9	{	♀	1x:57.	49	—	—	Sex-reversed, but now mnl. male. Tr. End. 16.

TABLE 1. Continued.

Headings to columns as on previous page.

GASTRULA (endoderm cells).							
12	{	♀	20ox x1:57.	72	—	—	Tr. End. 26.
13	{	♀	31E x11:57.	89 106 120	— 10% 5%—30%	70%	Tr. End. 41. 42. 43.
12	{ 12 {	♀ ♀ ♀ ♀	24E x1:57.	51 56 69 39 39	5%—10%	c. 60%	Tr. End. 51. 52. 53. 47. 50.
13	{	♂	43E v1:58.	28	—	—	Tr. End. 67.
12	{	♂	12E x1:57.	45	—	—	Tr. End. 44.
10	{	♂ ♂ ♂ ♂	16E x:57.	36 37 39 44	10%—30%	—	Tr. End. 21. " 22. " 23. " 24.
NEURULA AND POST-NEURULA (endoderm cells).							
21E	{	♀	27L x1:57.	85	—	—	Tr. End. 30.
22	{	♀	20ox x:57.	75	—	—	Tr. End. 27.
22	{	♀	24E x1:57.	99	10%—70%	50%—90%	Tr. End. 57.
18	{	♀		146	5%—20%	c. 70%	Tr. End. 54.
19	{	♂ ♂ ♂	31E x11:57.	56 33 38 46	5%—60% 45%—55% —	30%—80%	Tr. End. 55. " 56. " 60. " 61.

TABLE 1, Continued.

Headings to columns as on previous page.

MUSCULAR RESPONSE (endoderm cells)	24	{	♀	24E x1:57.	100	5%	c. 50%	Tr. End. 65.
	26	{	♀	31E x11:57.	130 131	20%-95% 70%-80%	70%-90% 80%-95%	Tr. End. 65. " 66.
	26	{	6♂s 19♂s	700x 35L v1:59.	10-32 8-32, + 2 dwarfs	—	—	041.6-9 041.19-21.
HEART-BEAT TADPOLES (endoderm cells).	33	{	♀ ♀ ♀	12E v1:58.	94 106 106	No eggs. 0% (c. 500 eggs only) 0%-5% (800 eggs only).	35%	Tr. End. 70. " 71. " 72.
	34	{	♀ ♂	13E v1:58.	95 52	No eggs 3%	c. 30%	Tr. End. 73. Tr. End. 74.
HATCHING TADPOLES (gut cells)	37	{	8 ♀	16E v11:58.	89	30%	c. 70%	Tr. End. 77.
	37	{	♀	24E v1:58.	80	5%	c. 50%	Tr. End. 75.
SWIMMING TADPOLES (endoderm)	40	{	♀	17E 1v:59.	71	0%	—	Tr. End. 1, new ser.
	40½	{	♀	34L v1:59.	Not yet mature.			Tr. End. 2.
	40	{	♂ ♂	13E v1:59.				Tr. End. 3. " 4.

size. Since the relative proportions of the body are very similar in transplant-frogs and controls, body size is satisfactorily indicated by weight. Though frogs imported from Africa are by no means a representative sample, some of these are more than twice the weight of others of the same sex. This shows that in this species adult size is a variable characteristic. At least 90% of frogs reared in the laboratory from fertilised eggs fall within the range of 70-135 gms for females, and 35-55 gms for males. The remainder are exceptionally small, sometimes weighing less than 20 gms, and do not show secondary sex characters. Laboratory frogs differ from imported "wild" frogs in having a smaller average size, and possibly by including a greater number of dwarf individuals, though these might fail to survive in natural conditions and so be overlooked. There are two possible reasons for this difference between wild and laboratory reared frogs. One is the laboratory diet which is probably deficient in some unidentified respect; this could affect adult size directly as well as by its effect on the quality of eggs from which transplant-frogs are derived. The injection of hormone to induce ovulation probably results in immature and overmature eggs being laid as well as normal ones. These two factors are apparently responsible for the very variable quality of recipient eggs used for nuclear transplantation (Gurdon, c), and they may give rise to abnormal adult frogs as well as to abnormal transplant-embryos.

The majority of transplant-frogs in table 1 are within the normal range of weight for controls. The exceptions to this are the five females from a gastrula donor, the male from a stage 13 gastrula, two of the six females from blastula vegetal nuclei, and the 25 males derived directly and indirectly from a stage 26 these embryo (two of these being very much

smaller than the others); all these frogs are below the normal range of weight for laboratory reared controls (down to 70 gms for females and 35 gms for males). Three of the donor embryos from which these frogs ^{have been} ~~were~~ derived (stages 12, 13, and 26) gave rise to no normally sized frogs, and it is therefore probable that the zygote nucleus from which these donor embryos developed carried a genetic factor responsible for small size, such that all transplant-frogs derived from them would also be small. On the other hand the blastula donor and the serial stage 26 donor each gave rise to two exceptionally small individuals as well as to other normally sized ones. These dwarfs must therefore have arisen from any one of the causes suggested on p. 3, but most likely from variable egg-quality (see discussion, p. 15). Owing to the great variation in weight among control frogs, and to the relatively small number of transplant-frogs so far reared to maturity, it is not yet clear whether these dwarf transplant-frogs have occurred with a higher frequency than would be expected among controls, and thus whether or not they are really "abnormalities".

Two conclusions can be drawn from this study of adult size in transplant-frogs. One concerns the 19 serial transplant-frogs derived from a stage 26 donor embryo, which should all be genetically identical (see chapter IX). These "genetic twins" resemble each other inasmuch as all are abnormally small, but otherwise there is considerable variation in size among them. This indicates that the average size of a series of frogs is genetically controlled, but that there is considerable variation around the average, caused by difference in environmental factors or egg quality.

Two frogs from nuclei of a stage 24 tadpole. One of these frogs is a typical

The other conclusion that can be drawn from the weight of these frogs is that there is no obvious correlation between decrease in size and increase in the donor stage from which they were derived. This shows that as endoderm nuclei approach the differentiated stage, they do not undergo any changes which affect the size of the individual to which they can give rise.

B. Colour pattern.

There is considerable variation in the pattern on the dorsal surface of Xenopus laevis. The type of pattern appears to be genetically inherited, and there is a very noticeable similarity in the pattern of all transplant-frogs derived from the same donor embryo. No method has yet been devised for the numerical classification of Xenopus colour patterns.

C. Sex differentiation

The majority of transplant-frogs have undergone normal sex-differentiation, and secondary sex-characters are generally well developed. However, one frog from a blastula vegetal nucleus at first had the external appearance of a female, but has since become a normal male; a case of sex-reversal in this direction was also observed in a female frog imported from the wild. In frogs which are abnormally small (described above), secondary sex characters are often poorly developed, but are sufficiently clear to indicate the sex of the frogs.

As can be seen from Table 1, frogs from the same donor have nearly all become the same sex. This generalisation has been clearly demonstrated by 14 frogs from blastula animal cells, and by the 25 frogs from stage 26 embryo, which developed into males. The only exceptions are two frogs from nuclei of a stage 34 tadpole. One of these frogs is a typical

male and the other a sterile female; since both were derived from the same donor, sex-reversal has presumably taken place in one of them. The fact that the reversal from male to female has never been observed in this laboratory, and that in this case the female is entirely sterile while the male is fertile, indicates that it is the male that is sex-reversed from a sterile female, rather than the other way round. If these frogs had both been genetically male, there is no reason why one should have changed into a female; but if both had been sterile females, it is not unlikely that one should reverse its sex, since genetic females of Xenopus in which ovarian development is suppressed will often develop into functionally normal males (Chang and Witschi, 1956).

Frogs reared in the laboratory from fertilised eggs are about 50% males (98 females: 113 males), showing that the laboratory conditions do not favour one sex much more than the other. However, the sex-ratio among transplant-frogs is not as near equality as would be expected. Transplant-frogs from one donor (with the single exception above) are of the same sex, clearly indicating the genetic sex of the donor embryo; thus the ratio of male-producing to female-producing donor embryos should be nearly equal. The transplantation of endoderm nuclei has produced 39 females frogs from 23 different donors, and 14 males from 8 donor embryos. This is in contrast to frogs derived from the ectoderm nuclei of blastulae and gastrulae, which consisted of 30 males from 14 different donors but only one female. Thus the ratio of female donors to male donors was 23:8 for endoderm nuclei, 1:14 for ectoderm nuclei.

It seemed worth testing the possibility that ectoderm nuclei become differentiated, so that ovarian development is suppressed in the resulting transplant-frogs, which then become male. This was done by transplanting endoderm and ectoderm nuclei from three gastrula donors. Frogs derived from both endoderm and ectoderm nuclei of two of these donors were all females. From the third donor all "endoderm" transplant-frogs were female, and one frog from an ectoderm nucleus, which was killed soon after metamorphosis, had under-developed ovaries; however, two other frogs derived from ectoderm nuclei of this donor both became males. Thus sex-reversal must have taken place in one direction; it seems more likely for the reasons given above that all three donors were by chance genetically female, and that the ectoderm nuclei of one gave rise to sex-reversed males, rather than the alternative explanation that all donors were genetically male, and that all endoderm and most ectoderm nuclei gave rise to completely sex-reversed females. While it is not true that differentiating ectoderm nuclei always give rise to male transplant-frogs, there seems to be a tendency in this direction which needs further investigation with frogs derived from ectoderm nuclei of older embryos.

D. Gametogenesis.

The reproductive capacity of transplant-frogs has been judged from their fertility and the development of their offspring. The percentages for fertility given in Table 1 show the proportion of all eggs laid which began regular cleavage irrespective of their later development, and hence show the proportion of eggs fertilised. In control frogs there is great variation (5% - 90%) not only in the fertility of different frogs, but also in the fertility of the same frogs in consecutive matings. Thus

the fertility of transplant-frogs can only be considered abnormal, either when a lack or deficiency of gametes is produced in several matings, or when a certain frog consistently fails to give rise to any fertilised eggs.

The majority of transplant-frogs have laid as many eggs, and proved as fertile, as controls. Nuclei from four donor embryos have however given rise to sterile or nearly sterile frogs. Six female frogs derived from one blastula donor are entirely sterile in a peculiar way. Four of these frogs, which are of normal adult size, responded to hormone as usual and laid large numbers of apparently normal eggs. Although these frogs were mated on six occasions to different males which were known to be fertile, not a single egg showed any signs of cleavage. It was also found that 50 unfertilised eggs showed no sign of development after blastula nuclei had been transplanted into them. A complete lack of cleavage of this kind has never before been found after nuclear transplantation. These eggs were normal in external appearance (including pigmentation, etc.), and sections of fixed eggs showed that their yolk-gradient and structure was normal. Hence this peculiar abnormality (clearly associated with the original donor embryo) is probably due to a relatively small genetic defect, concerning only a few genes, since a large part of the genome of these frogs, controlling development, growth, and most adult characters, must be normal. This case is a good example of the way transplant-frogs can demonstrate small genetic abnormalities. Since this abnormality affects all transplant-frogs tested from the original donor, it probably has nothing to do with nuclear differentiation during development which would be expected to affect some

nuclei more than others; it is probably due to a defect in the zygote nucleus from which the donor embryo developed (see p.3).

Some female frogs derived from endoderm nuclei of one swimming and two heart-beat tadpoles suffer from a reproductive abnormality which appears to be due to deficient rather than abnormal gametogenesis. The three females derived from a stage 33 tadpole all have very poorly developed sex-characters; they are thin and have the external shape of large males. In one of them the cloaca is very small and did not respond to hormone in the usual way; this female appears to be entirely sterile since it laid no eggs at all. The other two females both have reduced cloacae, and laid appreciably smaller numbers of eggs than usual. One of these females gave rise to offspring of which 60% were abnormal; these suffered from a wide range of abnormalities none of which were more frequent than others. ¶

The other heart-beat donor (stage 34) gave rise to a completely sterile female, and also to a functional male which is thought to be sex-reversed from an entirely sterile female (as discussed above).

The only adult transplant-frog derived from a swimming tadpole is completely sterile; this frog (frontispiece) has the external appearance of a female, and the cloaca which is smaller than usual responded to hormone injection in the usual way, but not a single egg was laid.

These frogs from the heart-beat and swimming tadpole donor embryos were largely or completely unable to produce gametes, but the gametes that were produced were as normal as those of controls. These frogs therefore seem to suffer from a deficiency and not an abnormality of germ-cells. The fact that the transplant-frogs derived from each of the heart-beat donors

differ among themselves, suggests that their germ-cell deficiency is not due to an innate character of the donor embryo. It seems more likely that the endoderm nuclei from which they were derived had lost their totipotency needed for germ-cell formation. This suggestion is supported by the fact that germ-cell deficiency has only been found among frogs from rather late donor stages. In Xenopus, the germ-plasm is continually in contact with the prospective germ-cell nuclei from the late blastula stage (Blackler, 1958). This relationship is the kind that might be responsible for initiating the germ-line by preventing those nuclei which remain in contact with this special cytoplasm from becoming differentiated (as in Ascaris, Sciara, etc.). However, nuclei of endoderm cells are entirely separated from the germ-plasm from the blastula stage onwards. The fact that some endoderm nuclei from hatching tadpoles can give rise to transplant-frogs capable of producing normal germ-cells, shows that some endoderm nuclei may remain undifferentiated though separated from the germ-plasm for a long period of development. On the other hand there is some evidence that endoderm nuclei may lose their totipotent stage so that they can give rise to normal frogs but not to normal germ-cells. It would perhaps be expected that differentiating nuclei would first lose their ability to form germ-cells since the latter require a complete complement of genes for their formation, while other cell types may not.

E. The Development of offspring from transplant-frogs.

Fischberg (personal communication) has pointed out that information on the genetic constitution of transplant-frogs can be obtained from a breeding analysis of them and their offspring. It is known that

endoderm nuclei of both Rana and Xenopus become differentiated by losing certain developmental potentialities (Briggs and King 1957 $\frac{1}{2}$; Gurdon, d). Though the exact nature of these changes is not known, they are probably alterations or deletions of genetic material (Briggs and King, 1956) which, like mutations, should be recognisable by appropriate breeding experiments. If the nuclei of a transplant-frog were to contain one or more abnormal genes the effects of which are dominant to the wild-type allele, this should be evident during the life of the frog itself or, if only its gametes are affected, from some of its offspring. On the other hand, if the nuclei of a transplant-frog were to suffer~~from~~^{not} a recessive change, this would be shown by the transplant-frog or by any of the F_1 progeny of this frog mated to a wild-type individual. The purpose of the breeding analysis is to show the existence and inheritance of any genetic abnormalities which behave like recessive mutations. If such an analysis is to be made of a transplant-frog which carries a recessive mutation, it is first mated to a wild-type individual; half its F_1 offspring will be heterozygous for the mutation and the other half entirely normal. These F_1 offspring are then mated to each other; when a cross is made between two of these individuals both of which happen to be heterozygous for the mutation, a quarter of their offspring will be homozygous for, and so will suffer from the effects of, this mutation. Since the F_1 progeny heterozygous for this recessive mutation, cannot be distinguished from their brothers and sisters which do not carry the mutation, only one in four such F_1 crosses will by chance involve two heterozygous individuals and so produce recessive homozygotes showing the 'mutation'. The discovery of such 'recessive mutations' will be made quicker and more frequent (i) by mating transplant-frogs together to provide the F_1 generation, when there

is a remote chance that both will carry the same recessive mutation, (ii) by using transplant-frogs from the nuclei of the latest donor stages, which, by analogy with developmental abnormalities of transplant-embryos, should have the highest proportion of differentiated gene loci, and (iii) by studying the development of transplant-embryos, and the characters of adult transplant-frogs, in great detail.

As shown in Table 1, transplant-frogs from many different donor stages have been mated to wild-type individuals. Some of these frogs, such as those derived from heart-beat and hatching tadpole nuclei, have, when mated to wild-type individuals, produced offspring of which only 30% have developed into normal tadpoles. The remaining embryos suffered from a wide range of different abnormalities, and there was no indication that half of the offspring owed their abnormality to the same abnormal gene. Thus all that has so far been shown is that the nuclei of these frogs did not contain any dominant genetic abnormalities. The F_1 progeny of these frogs are not yet old enough to be mated to each other so as to provide a test for recessive genetic abnormalities, though this has been done for two blastula transplant-frogs (table 1).

IV. DISCUSSION AND CONCLUSIONS

In chapter IV an analysis was made of the factors responsible for the abnormal development of transplant-embryos, but the results of ^{this} ~~the present~~ analysis cannot be directly applied to abnormalities of adult transplant-frogs. In a similar way, the rate and time of onset of the nuclear differentiation which affects development (chapter V) may be quite different

from that of the nuclear differentiation which affects adult characters. It is quite possible for instance that somatic nuclei may lose their potentiality for giving rise after transplantation to normally reproducing adult frogs before or after they lose their potentiality for giving rise to normal embryonic development.

Insufficient transplant-frogs have so far become adult as well as abnormal (in some respect) to identify the cause of their abnormalities at all precisely ^(see Table 2) A. Harmful effects of the transplantation technique should result in abnormalities which are not correlated with any one donor embryo or with the recipient eggs from any one female frog. Damage of the recipient eggs would in any case be likely to lead to relatively severe abnormalities, affecting development before the adult stage. It is quite possible that ~~versene~~ ^{versene} treatment could lead to random chromosomal damage which might be expressed as small abnormalities of adult transplant-frogs, but which is most unlikely to affect all donor cells from one embryo in exactly the same way. Any genetic abnormality carried by all nuclei of a donor embryo and therefore probably present on the zygote nucleus from which this donor embryo developed, should be shown in exactly the same way by all transplant-frogs derived from this donor. This in fact happened in the transplant-frogs all of which suffered from the unusual abnormality of their eggs. Abnormalities due to the quality of recipient eggs should be shown by all transplant-frogs from the eggs of one female if the abnormality is genetic and present in the parent frog; no example of this kind has been observed in the transplant-frogs described here. If the abnormality is environmental such as the state of maturity of eggs, then this should effect some eggs from one

TABLE 2. Abnormalities of transplant-frogs derived from endoderm nuclei.
This table includes all abnormal transplant-frogs listed in yable 1.

DONOR EMBRYO	TOTAL NO. TRANSPLANT-FROGS FROM THIS DONOR	WEIGHT	SEX-DIFFERENTIATION	FERTILITY
Stage 8, veg. cells.	6 ♀s	2 out of 6 abnormally small.	_____	4 out of 6 gave abnormal eggs only.
Stage 12.	5 ♀s (2 serial)	All smaller than usual.	Probably normal.	
Stage 13.	1 ♂	Abnormally small.	_____	_____
Stage 26.	25 ♂s (19 serial)	All smaller than usual; 2 abnormally small.	Probably normal.	
Stage 33.	3 ♀s	Normal.	Secondary sex-characters very poorly developed.	1 ♀ --- no eggs. Other 2 ♀s --- reduced number of eggs.
Stage 34	1 ♀ 1 ♂	♀ with very poorly developed sex-characters. ♂ normal.	♀ gave no eggs.	

NOTES: (i). The stage 9 ♀ reversed to ♂ (table 1) is not regarded as abnormal since a similar case was observed among some imported frogs.

(ii). Frogs which are abnormally small cannot be scored for sex-differentiation or fertility, since they usually fail to show secondary sex-characters.

female more than others. In Xenopus, eggs are only ovulated when about to be laid (in contrast to the uterine storage of ovulated eggs as in Rana), but nevertheless vary considerably in quality (Chapter IV). Over maturity of eggs can bring about sterility in the resulting frogs (Witschi, 1952), and it may be that immaturity and overmaturity also have other affects on adult frogs. Abnormalities due to nuclear differentiation, if comparable to the effects of nuclear differentiation on development, should affect the endoderm nuclei from one donor embryo in different ways; in other words such abnormalities should not be strongly donor specific. The only completely certain conclusion that can be drawn from this study is that nuclei which give rise to normal adult-frogs with normal gametogenesis and offspring must have been totipotent.

The main result of this investigation is that some endoderm nuclei may remain completely undifferentiated at least ^{as far} ~~as~~ as the hatching tadpole stage. This finding is of particular relevance in connection with germ-cells, since these may remain unchanged even though not in contact with the germ-plasm in contrast to cells of the germ-line which usually are. The fact that there is very little mortality between the earliest feeding tadpole stage and the adult frog, shows that there is no marked tendency for endoderm nuclei which are normal for genes required ^{for} development to become differentiated in respect of genes required for adult characters and reproduction. It is not of course possible to say whether the latter kind of genes become lost or changed later than those required for development, since transplant-embryos which are abnormal for developmental genes will not become adult. It is most likely that genes required for adult life will have other functions connected with development; this is consistent ~~that comes~~ with the conclusion reached above that none of the abnormalities of adult frogs can be attributed to nuclear differentiation with any certainty.

The frogs derived from the serial transplantation of nuclei of an original stage 26 donor (Table 1) show that there are no effects on adult characters caused purely by the continued transplantation of nuclei. The serial transfer frogs are no smaller (on average) or more abnormal than the first transfer frogs from the same donor. Since these frogs are not affected by the technique or by any ageing process of the somatic nuclei, this means that nuclei can be serially transplanted for several generations between the eggs of two or more different species, and the characters of the resulting frogs directly compared.

V. SUMMARY

1. Adult frogs, derived from transplanted nuclei of ^{endoderm} differentiating / cells of Xenopus laevis, have been described with special reference to their size, sex differentiation, and reproductive capacity. The study of these frogs shows to what extent the donor nuclei from which they were obtained were normal in respect of genes affecting adult life and reproduction; all donor nuclei which give rise to frogs must have been normal for genes concerned with development, since adult frogs are only obtained from embryos which develop normally.
2. Transplant-frogs which are normal in every respect have been obtained from endoderm nuclei ^{of} embryos ranging from late blastulae to hatching tadpoles. At least some endoderm nuclei therefore remain undifferentiated up to this late developmental stage. Since these frogs can give rise to normal offspring this demonstrates that endoderm nuclei can give rise to normal germ-cells, even though they have been separated from the special germ-plasm since the late blastula stage.

3. A few transplant-frogs have been obtained which are abnormal in respect of size or gametogenesis; some of these abnormalities are apparently due to a mutation in the zygote nucleus from which the donor embryo developed; the abnormality consisting of a lack or deficiency of germ-cells affected some frogs from the most advanced donor stages, and may be due to nuclear differentiation or to over-maturity of the recipient eggs.

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TETRAPLOID FROGS.

I. INTRODUCTION

CHAPTER VII.

Polyplaid animals have been obtained in various anuran groups, but so far **TETRAPLOID FROGS.** This paper describes some tetraploid individuals of *Xenopus laevis*, together with previous records. **Synopsis.**

I. INTRODUCTION

II. METHODS BY WHICH TETRAPLOID ANURA HAVE BEEN OBTAINED

- A. Spontaneous tetraploids.
- B. Progeny of diploids mated to triploids.
- C. Temperature shock treatment of eggs soon after fertilisation.
- D. Parthenogenetic development.
- E. Colchicine and sulphanilamide treatment.
- F. Nuclear transplantation.

III. DESCRIPTION OF TETRAPLOID FROGS.

- A. Evidence of tetraploidy.
- B. Morphology.
- C. Rate of growth and adult size.

IV. REPRODUCTIVE CAPACITY.

- Males
- Females

V. TETRAPLOID XENOPUS COMPARED WITH OTHER TETRAPLOID ANURA.

VI. TETRAPLOID AND TRIPLOID XENOPUS COMPARED.

SUMMARY.

Acknowledgements

References

TETRAPLOID FROGS.

I. INTRODUCTION

Polyploid animals have been obtained in various vertebrate groups, but so far very few cases have been recorded of tetraploid Anura. This paper describes some tetraploid individuals of Xenopus laevis. These, together with previous records of tetraploid Anura, are compared with:-

- (i) Tetraploid Urodeles, which have been obtained in several species.
- (ii) Haploid, diploid, and triploid individuals of Xenopus laevis.

This last comparison serves to distinguish the effects of an increase in cell size (seen most clearly in tetraploids) from the effects of an uneven number of chromosome sets per nucleus (as in triploids).

Xenopus is particularly suitable for studying the effects of poly-ploidy in Anura, since it is one of the few species which can be conveniently raised to maturity in captivity.

II. METHODS BY WHICH TETRAPLOID ANURA HAVE BEEN OBTAINED (Table 1)

A. Spontaneous tetraploidy.

Tetraploids occur spontaneously from the offspring of diploid parents with a very low frequency. There seem to be no published records for the frequency of spontaneous tetraploidy in Anura, but the following unpublished figures apply to Xenopus laevis. From 992 analysed tadpoles, one was tetraploid and two were mosaics being partially tetraploid only (Fischberg, Elsdale & Smith, unpublished). In another series no tetraploids but two diplotetraploids were found among 135 analysed tadpoles (S. Smith, unpublished). Thus from 1127 embryos, just under 0.1% were pure tetraploids. This figure is very similar to the frequency of spontaneous tetraploidy in

TABLE 1.

RECORDS OF TETRAPLOIDY IN ANURA.

Method of production	Species	Frequency of tetraploids	Survival and abnormalities	References
Spontaneous	<u>X. laevis</u>	0.1%	Not known	Fischberg, Elsdale, and Smith, unpublished.
Progeny of diploid-triploid crosses	<u>X. laevis</u>	1.6%	Died as tadpoles or after metamorphosis	Smith, unpublished.
Temperature treatment of eggs:				
Hot shock	<u>X. laevis</u>	---	Adult frog.	
Cold shock	<u>X. laevis</u>	0.5%	Died as tadpoles or soon after metamorphosis	Smith, unpublished.
" "	<u>Rana limnocharis</u>	---	Became oedematous soon after metamorphosis	Kawamura and Moriwaki, 1953.
Parthenogenesis by needle dipped in blood cells	<u>Rana nigromaculata</u>	---	Died as tadpoles	Kawamura, 1939.
Colchicine and sulphanilamide treatment	<u>Rana temporaria</u>	---	Some became adult ⁺	Jahn, 1952.
Nuclear transplantation	<u>X. laevis</u>	5 - 13 % according to donor stage	Abnormalities not associated with tetraploidy; some became adult.	See Table 2, below.
	<u>Rana pipiens</u>	20 - 65% according to donor stage.		Briggs and King, 1957.

These individuals may have been aneuploids or mosaics as opposed to true tetraploids.

Urodeles, which varies from 0.04 to 0.4% according to the species (Fankhauser, 1945).

B. Progeny of diploids mated to triploids.

A small proportion of tetraploids or near-tetraploids result from crossing a triploid female with a diploid male, or vice versa. The following information applies to Xenopus laevis, and has been provided by S. Smith, unpublished. The combined results of three crosses involving a triploid female and a diploid male were that two tetraploids were found among 69 analysed tadpoles (i.e. 3%); on the other hand, after mating a diploid female with a triploid male, 2 out of 170 tadpoles from three separate crosses were tetraploids (1.2%). Owing to the difficulty of making exact counts among so many chromosomes, these 'tetraploids' may have been aneuploids differing by a few chromosomes from the tetraploid number (72). All of these tetraploids died before becoming adult.

C. Temperature shock treatment of eggs soon after fertilisation.

Hot shock treatment of newts' eggs a few hours after fertilisation has given up to 93% tetraploids among those which developed furthest (Fischberg, 1958). However hot and cold shock treatment designed to produce tetraploids by a similar means have proved unsuccessful in Xenopus, though hot and cold treatment designed to produce triploids (shock treatment a few minutes after fertilisation) has produced 6 tetraploids out of 1183 analysed tadpoles, i.e. 0.5% (Smith, unpublished). Of these only the tetraploid derived from the cold treatment became adult, the others having died.

Kawamura and Moriwaki (1953) found one tetraploid Rana limnocharis among 231 triploids obtained by cold shock treatment; this individual became abnormal after metamorphosis.

D. Parthenogenetic development.

A very few parthenogenetic tetraploid tadpoles, none of which metamorphosed, were obtained by Kawamura (1939) in Rana nigromaculata. Parthenogenetic development was induced by pricking unfertilised eggs with a needle which had previously been dipped in blood cells.

E. Colchicine and sulphanilamide treatment.

Various degrees of polyploidy have been obtained in Rana temporaria by colchicine and sulphanilamide treatment of early embryos (Jahn, 1952). Many 'tetraploid' frogs resulting from such treatment seem to have been mosaics containing diploid as well as tetraploid nuclei.

F. Nuclear Transplantation.

Nuclear transplantation is a technique in which a nucleus from an embryonic cell is transferred to an enucleated egg. It seems to be usual for a certain proportion of the embryos resulting from this kind of experiment to be tetraploid, instead of diploid like the injected nucleus (Briggs & King, 1953; Fischberg, Gurdon & Elsdale, 1958 a.).

There are many possible reasons for this occurrence of tetraploidy after nuclear transplantation, but one or both of the following causes are

possibly responsible. Tetraploidy might occur when nuclei to be transplanted are taken at a particular stage in mitosis. If this were the case, the proportion of tetraploids obtained would be expected to decrease as later donor stages are used, since later stages have fewer nuclei undergoing mitosis at any one time. On the other hand tetraploidy might be due to the small size of the donor nuclei; because of their small volume compared to that of the zygote nucleus, these might be unable to bring about the first cleavage in a large egg. The products of the first mitotic division might fuse, resulting in a considerably larger tetraploid nucleus. Since donor nuclei become smaller as embryonic development proceeds, the proportion of tetraploids should increase with later donor stages if this explanation is correct.

Table 2 shows that in Briggs & King's experiments (1957) tetraploids are obtained much more frequently from later donor stages. But in the present author's experiments with Xenopus the highest frequency of tetraploids is from earliest and latest donor stages (blastula and tadpole), while middle aged donors (neural folds) gave the minimum proportion of tetraploids.

These two results are consistent with the possible causes of tetraploidy described above; in both Xenopus and Rana there is an increased proportion of tetraploids associated with the latest donor stages used for transplantation, which could be explained by the small size of the late stage donor nuclei in relation to the large volume of the cytoplasm of an egg. In Xenopus there is also an increase in the proportion of tetraploids derived from earliest donor stages, but this tendency is not apparent in Rana pipiens. The rate of development in Xenopus, however, is very much more rapid than that of Rana pipiens, and this should result in a greater

TABLE 2.

THE FREQUENCY OF TETRAPLOIDS OBTAINED FROM NUCLEAR TRANSPLANTATION.

Species	Donor stage	Numbers of individuals		Percentage of tetraploids
		Diploids	Tetraploids	
<u>Rana pipiens</u>	Late blastula	---	---	17 %
	Tail-bud	---	---	67 %
<u>Xenopus laevis</u>	Late blastula	67	9	12 %
	Gastrula	52	5	9 %
	Neurula	22	1	4½ %
	Tail-bud	16	1	6½ %
	Pre-hatching	47	4	8½ %
	Post-hatching	25	3	13 %

ploids begin their first cleavage when tetraploids are already well advanced (Briggs & King, 1937). Fawcett's (1929) evidence concerning the origin of tetraploidy in mouse after hot point treatment has shown that in this case the doubling of the chromosome number occurs after the first cleavage division.

III. INDICATIONS OF SPONTANEOUSNESS.A. Evidence of spontaneousness.

Tetraploid embryos have been recognized by sectioning well-laid preparations in which the number of nuclei per nucleus, the number of nuclei per area of epiblasts, and other characteristic structures were

proportion of nuclei being in mitosis. One may conclude that the stage in mitosis reached by a nucleus at the time of transplantation, as well as other characteristics such as size, etc. may be to some extent responsible for tetraploidy.

Nuclear transplantation can provide some information about the origin of tetraploid nuclei. In Xenopus a nuclear marker is known (Elsdale, Fischberg and Smith, 1958) in which all diploid nuclei have only one nucleolus per nucleus instead of some having two. For most transplantation experiments with Xenopus, this marker has been present in donor nuclei, and resulting tetraploids possess two nucleoli per nucleus. If normal, unmarked donor nuclei are used, tetraploids have four nucleoli per nucleus. These points show that the tetraploid condition has arisen as a result of the doubling of the injected nucleus, and is not derived from the egg-pronucleus. The same process probably occurs in Rana pipiens, in which species tetraploids begin their first cleavage when diploids are starting their second (Briggs & King, 1957). Fischberg's (1958) evidence concerning the origin of tetraploidy in newts after hot shock treatment has shown that in this case the doubling of the chromosome number occurs after the first mitotic division.

III. DESCRIPTION OF TETRAPLOID FROGS.

A. Evidence of tetraploidy.

Tetraploid tadpoles have been recognised by making tail-tip preparations in which the number of nucleoli per nucleus, the number of nuclei per area of epidermis, and also approximate chromosome numbers, were

counted. In the case of frogs derived from nuclear transplantation, tetraploids probably had the exact tetraploid chromosome number since it is not likely that a hyper- or hypo-tetraploid condition could have arisen as a result of transplantation. In order to exclude the possibility of mosaics, estimations of nuclear volume were made for several different tissues. Epidermal nuclei are very flattened and as the difference in thickness of these nuclei in diploids and tetraploids is very small, if it exists, it has been ignored; thus the volume of epidermal nuclei has been compared by multiplying together the shortest and longest diameters. The nuclei of internal organs have been regarded as rotational ellipsoids. As it is very difficult to measure the depth of these nuclei in sections, it has been assumed that the depth is equal to their shortest diameter. The volume of a rotational ellipsoid is calculated from the formula $\frac{4}{3}\pi r^3$; as $\frac{4}{3}\pi$ does not vary according to the size of an ellipsoid, the volume of ellipsoids can be compared by r^3 , which, in this case is calculated by multiplying the largest diameter by the square of the shortest diameter. In each case ten nuclei were taken at random for measurement. Table 3 shows that the average volume of tetraploid nuclei was twice that of diploids. It also shows that the epidermal cells of tetraploid tadpoles increase in size in the same way as tetraploid nuclei. The units of measurement are unimportant for the purposes of comparison, and are not stated.

B. Morphology.

Though many tetraploid Xenopus develop normally, those which do not have suffered from the same kinds of abnormalities as diploids also obtained

TABLE 3.

COMPARISON OF THE VOLUME OF DIPLOID AND TETRAPLOID NUCLEI.

Tissue	Total volume of 10 nuclei		Diploid:tetraploid volume ratio.
	Diploid	Tetraploid	
Kidney, ♂	7,822	15,735	1 : 2.011
Kidney, ♀	8,153	16,793	1 : 2.060
Liver, ♂	5,621	11,749	1 : 2.090
Liver, ♀	7,072	12,825	1 : 1.813
Oviduct	6,916	13,981	1 : 2.021
Epidermis (adult)	11,008	20,685	1 : 1.879
Number of cells per area			
	Diploid	Tetraploid	
Epidermis (Tadpole tail-tip)	683	326	1 : 2.095
Average of ratios for all tissues: 1 : 1.996			

by nuclear transplantation; thus these experiments give no indication of any specific abnormalities associated with tetraploidy. This conclusion was also reached By Briggs & King (1957) from their tetraploids^{tadpoles} in Rana pipiens from nuclear transplantation. Tetraploids~~from~~ obtained by other means have mostly died as tadpoles or become oedematous as young frogs. It is not certain to what extent this mortality is associated with tetraploidy, since some methods of obtaining tetraploids are liable to give aneuploids and mosaics in addition to tetraploids. Although normal diploid frogs of any other genus than Xenopus are very difficult to rear to maturity, and may become abnormal, tetraploid frogs other than Xenopus have often suffered mortality or abnormalities to an extent that would not be expected of diploids. It is possible that tetraploids in Xenopus are more viable than tetraploids in phylogenetically less 'primitive' families of Anura.

^{Eight}~~Eleven~~ tetraploid frogs derived from transplanted nuclei have become adult. These appear entirely normal in every way except as regards gametogenesis and certain secondary sex characters.

C. Rate of growth and adult size.

Nuclear transplantation is a very suitable technique for obtaining almost perfect controls. The nuclei from a presumptive organ of a single embryo should be genetically identical unless some of the nuclei have undergone natural differentiation (Fischberg, Gurdon & Elsdale 1958; Briggs & King, 1957). As young donor embryos were used for obtaining tetraploids, such differences between the nuclei of the original donor would be expected to be very small, if existing at all. Natural differentiation is not

believed to commence until after the blastula stage; thus all transplant-embryos derived from the undifferentiated nuclei of a single embryo should be genetically identical, or what might be called nuclear twins. Differences between such nuclear twins could of course be due to differences in the cytoplasm of the recipient eggs, though as these are taken from the same female frog, such differences would be small.

The growth rate of tetraploid males was compared to that of diploids by keeping under identical conditions tetraploid and diploid nuclear twins, both having been derived by nuclear transplantation from the same donor embryo. Measurements on four such pairs of tadpoles were made every few days from head to anus, between the eyes, and of total length. After metamorphosis measurements were made by weight at two monthly intervals. Though tetraploids were usually slightly smaller than diploid controls, the difference was usually less than 20%. Among the progeny of one original donor, three tetraploid males weighed 53 gms., 50 gms., and 47 gms. (average 50 gms.). Nuclear twin diploids, which were therefore also males, weighed 66 gms. 59 gms. and 57 gms. (average 61 gms.). The average weight of diploid females derived from nuclear transplantation varied between 70 gms. and 140 gms: the weight of four tetraploid females varied between 50 gms. and 85 gms. Most of this difference is due to the lack of eggs in tetraploid females; thus tetraploids weigh less than diploids but with the small numbers at present available, the difference is not yet significant.

The size of organs as well as adult size is similar in tetraploid and diploid Xenopus, but the size of tetraploid nuclei, and apparently also

of cells, is twice that of diploids. Thus, as in all other Amphibia, tetraploid frogs must have half the number of cells as are present in diploids. One may conclude that in Xenopus there is no evidence of a significant difference in growth rate, though diploids attain a slightly but not yet significantly, greater adult size.

IV. REPRODUCTIVE CAPACITY.

Eight tetraploid Xenopus have so far become adult; four of these are indistinguishable in external appearance from normal males; the other four appear to be genetically female.

Males.

In tetraploid males the black nuptial pad on the fore-arms (Text fig. 1), and the condition of the cloaca are identical to those of diploids. When tetraploid males and control diploids of the same age have been given a gonadotrophic hormone injection to stimulate mating, the tetraploids croak much less; on only two occasions out of nine have tetraploid males clasped a female which was about to lay, even though control diploids have immediately clasped the same females. On each occasion when a tetraploid male clasped a female which was laying apparently good eggs, none of the eggs developed. Under similar conditions, 30-70% of eggs were fertilised by control diploid males and developed normally.

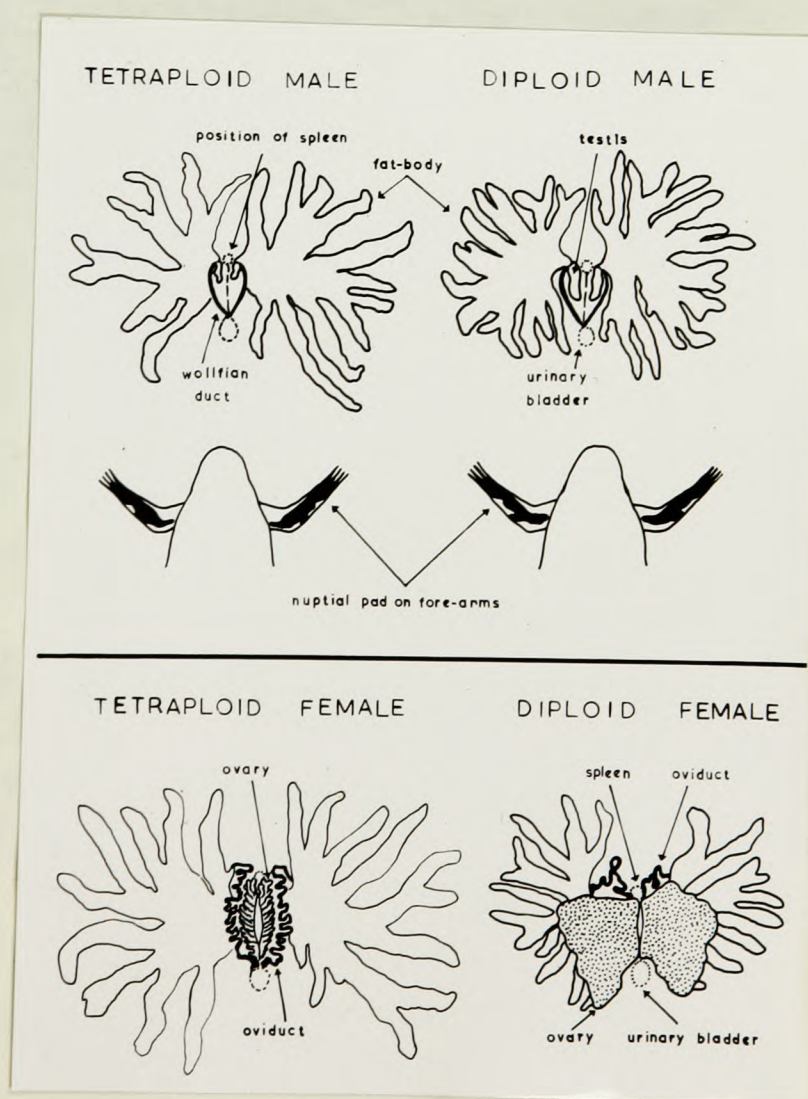
Two tetraploid males have been dissected. In each tetraploid it was found that the testes were noticeably smaller, and the fat-body ~~was~~ less lobed and greater in relation to body weight, than in control diploids (see Table 4 and Text fig. 1).

The tetraploid testes were sectioned and compared to diploid testes.

TABLE 4.

COMPARISON OF FAT-BODY IN TETRAPLOID AND DIPLOID XENOPUS.

	Total body weight	Weight of fat-body	No. of lobes of fat-body	Fat-body as % of total weight.
Tetraploid ♀	85 grams	8.9 grams	17	10.5 %
Diploid ♀	124 grams	4.0 grams	18	3.5 %
Tetraploid ♂	35 grams	4.4 grams	18	12.5%
Diploid ♂	47 grams	3.75 grams	28	8.0 %



Text-figure 1.

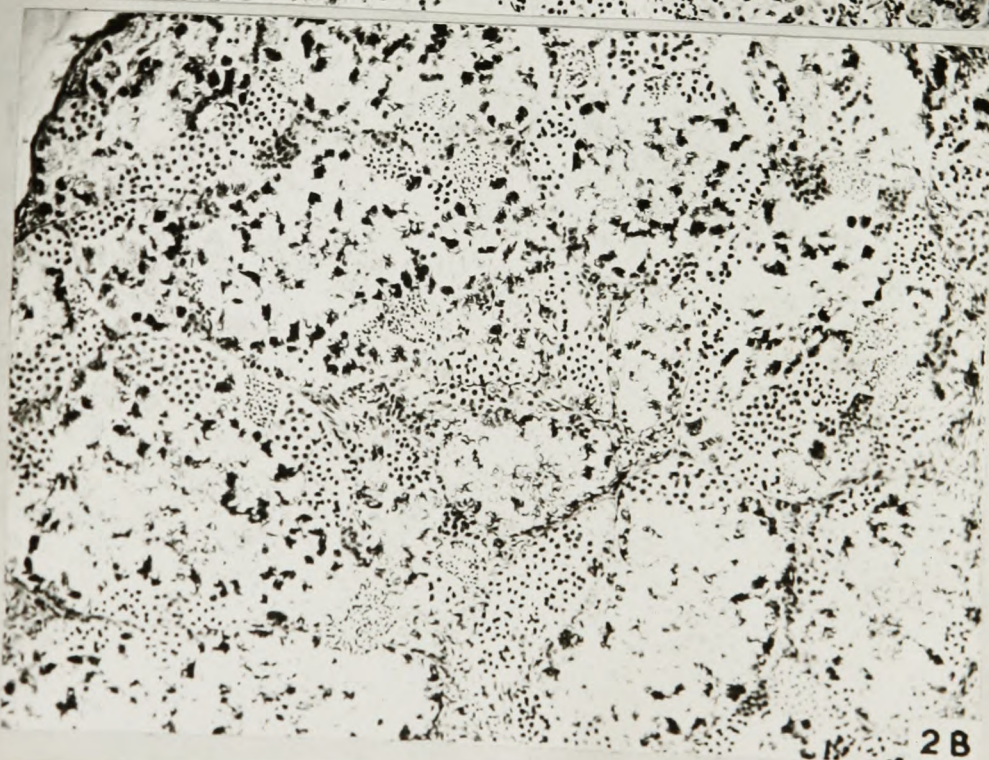
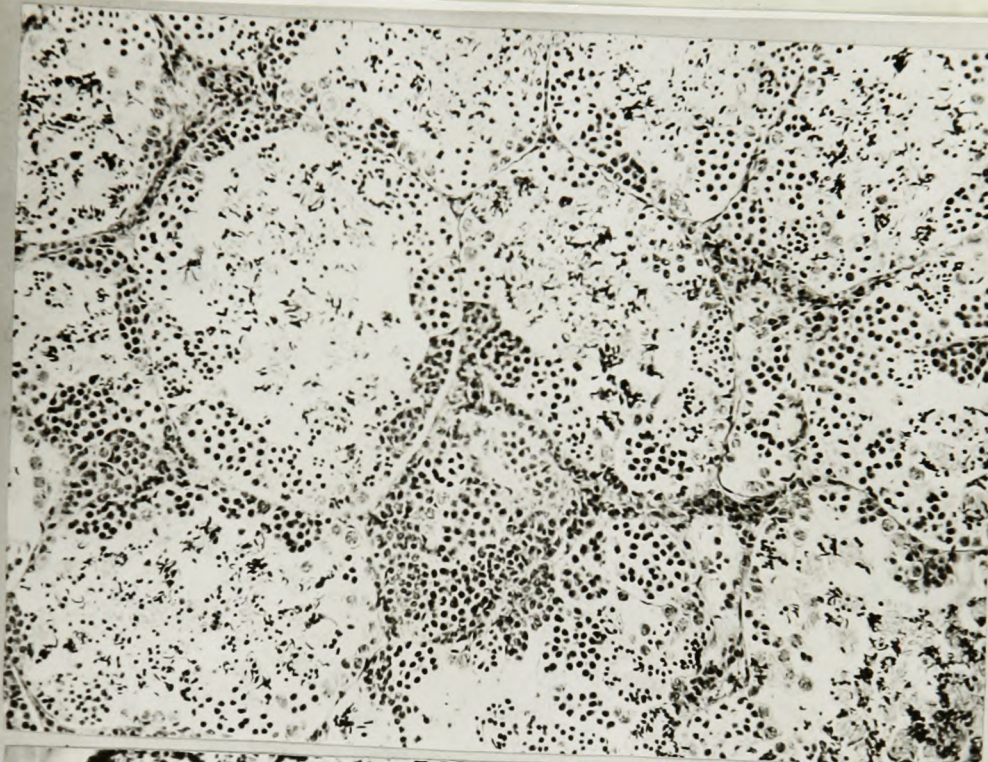
Diagrams of reproductive organs of diploid and tetraploid *Xenopus*; also of nuptial pads on fore-arms of males.

Text-Figure 1. Diagram of representative regions of diploid and tetraploid testes showing relative abundance of various parts of spermatogenesis.

Plate 1, fig. 2A. Section of tetraploid testis, showing abundance of nests of premeiotic germ-cells (large nuclei) and noticeable lack of sperm, X 133.

Plate 1, fig. 2B. Section of diploid testis, showing abundance of sperm (irregular dark blotches). X 133.

In the tetraploid testis the ... were present (fig. 2a). In ... were ... in preparation. **PLATE 1, figs. 2A and 2B.**



2B

In the tetraploid testis the typical nests of spermatogonia were present (fig. 2a). In interphase before meiosis (fig. 3a), and when more condensed in preparation for the first meiotic division (fig. 4a), tetraploid nuclei appear quite healthy. However after the first meiotic division tetraploid spermatocytes are variable in size and shape, while diploid nuclei are very similar to others in the same nest at this stage (cf. figs. 5a and b). After the second meiotic division nearly all diploid spermatids become normal spermatozoa, while tetraploid spermatids are at this stage mostly arrested as pycnotic nuclei, the remainder forming spermatozoa of very variable size and shape (fig. 6a). These findings indicate that tetraploid spermatocytes behave normally up to the first meiotic division. During meiosis in tetraploid cells, chromosomes are not distributed equally, such that most daughter nuclei receive too many or too few chromosomes and only by chance do occasional nuclei receive exactly the diploid number. This interpretation is suggested by the variation in size of spermatocytes after each meiotic division, and could be brought about by abnormal chromosome pairing. It is apparent that even after two irregular meiotic divisions, some spermatozoa are formed, (fig. 6a). It seemed worth testing the ability of these "diploid" spermatozoa to bring about development with normal unfertilised eggs. This was investigated by artificially fertilising the eggs of a diploid female with a sperm suspension from the testes of a diploid and tetraploid male. Of 22 eggs treated with sperm from the diploid male 13 developed normally. But of 18 eggs treated with the sperm from the tetraploid male, none developed at all. The probability of obtaining this difference by chance is 1% and the difference may therefore be considered significant. It is not possible with these small numbers to conclude that no functional sperm is produced by

Plate 2, fig. 3. Section of testis, showing spermatocytes before entering meiosis.

Fig. 3A, Tetraploid. Fig. 3B, Diploid. X 400.

" fig. 4. Section of testis, showing spermatocytes beginning to condense in preparation for the first meiotic division.

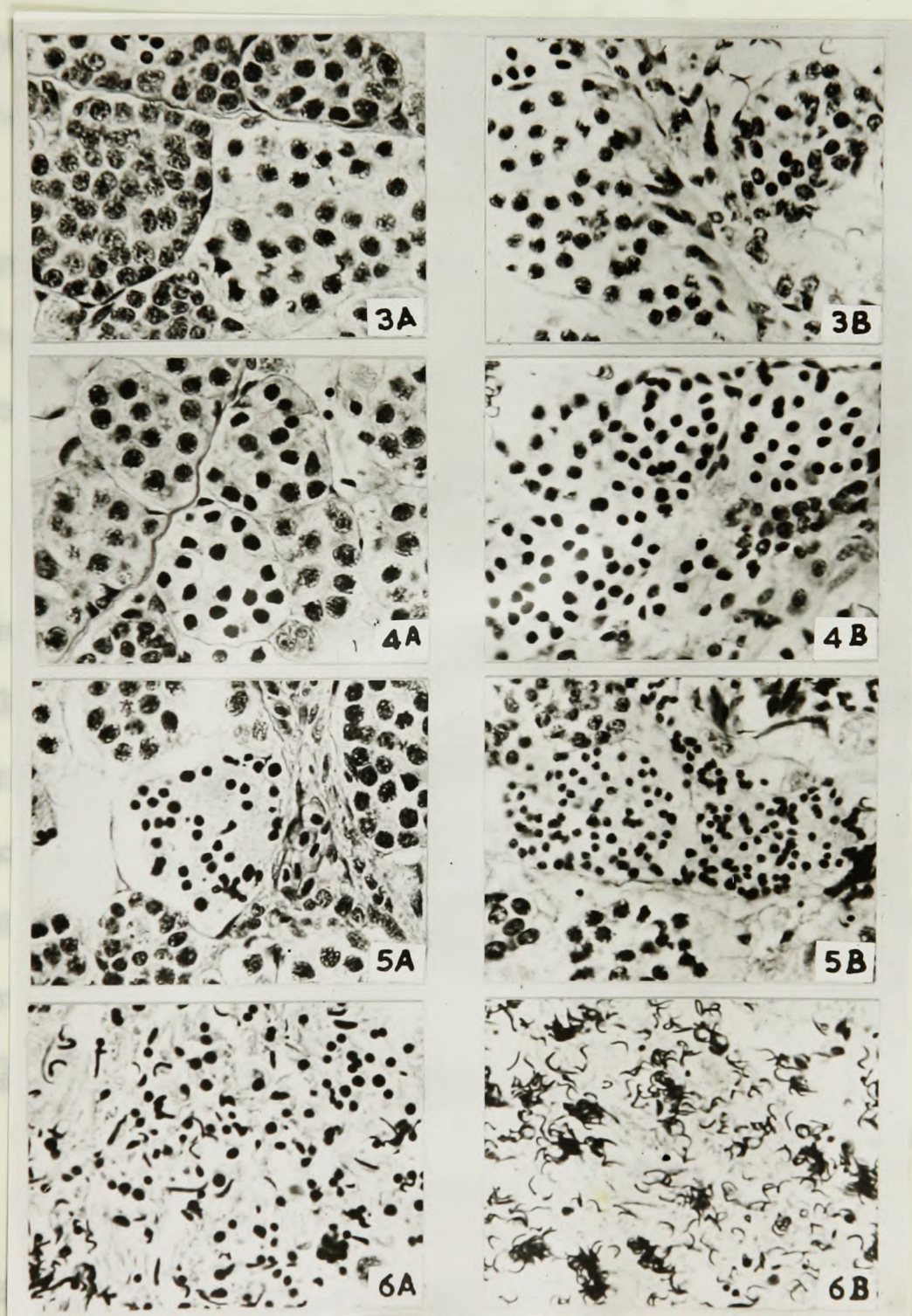
Fig. 4A, Tetraploid. Fig. 4B, Diploid. X 400.

" fig. 5. Section of testis, showing spermatocytes after first meiotic division.

Fig. 5A, Tetraploid. Fig. 5B, Diploid. Note irregular size of these nuclei in the tetraploid testis compared to the uniform appearance of these in the diploid testis. X 400.

" fig. 6. Section of testis, showing spermatozoa. Fig. 6A Tetraploid. Fig. 6B, Diploid. The diploid sperm is almost entirely normal, while that of the tetraploid consists mostly of pycnotic post-meiotic nuclei, with a few sperm of irregular size and shape. X 400.

PLATE 2, figs.3 - 6.



a tetraploid male; but it is apparent that very little sperm is produced, and that most of that which is, cannot bring about development with normal eggs. This experiment with artificial fertilization shows that the infertility of tetraploid males is not wholly due to their ineffective mating behaviour. It also ~~excludes~~ the possibility that although a few functional spermatozoa may be produced, these are never actually released by tetraploids which show some reluctance to clasp.

The differences in the reproductive capacity between diploid and tetraploid males may be summarised as follows. In external morphology tetraploids have well developed secondary sexual characters, and are indistinguishable from diploids. However the mating behaviour of tetraploids is inferior to that of diploids. Spermatogenesis in tetraploids is very abnormal; it appears that the pairing of chromosomes in tetraploid nuclei is irregular. This seems to result in an almost random distribution of chromosomes such that only occasionally is exactly the diploid number of chromosomes given to any nucleus. Most products of meiosis in tetraploid males result in pycnotic nuclei, but a few spermatozoa are formed. These are variable in size and shape, and are mostly incapable of bringing about any development with normal eggs.

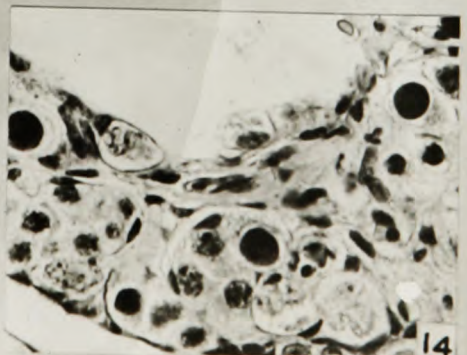
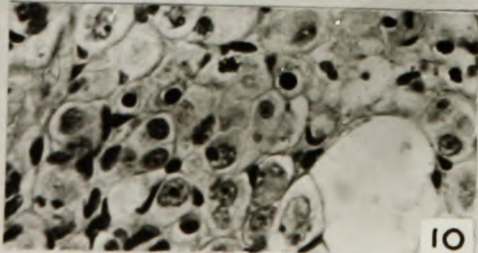
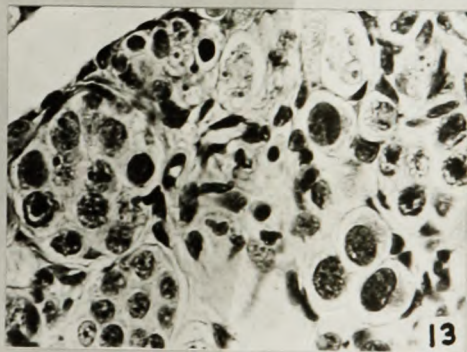
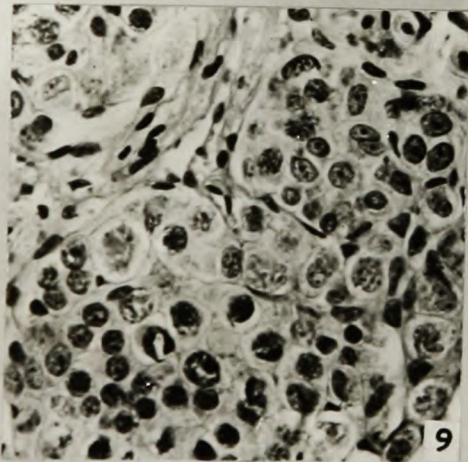
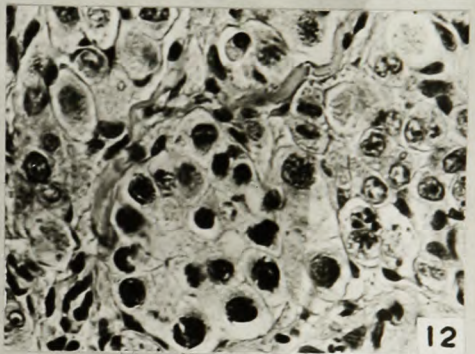
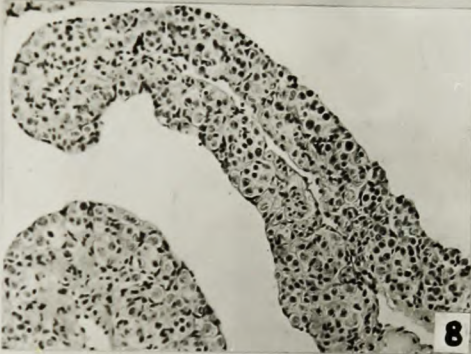
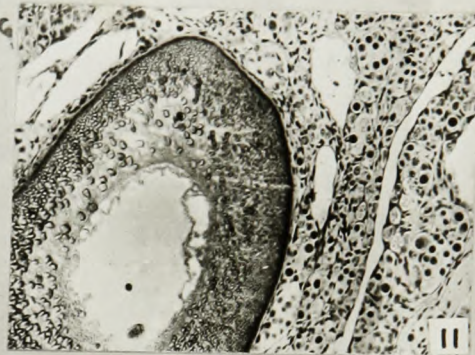
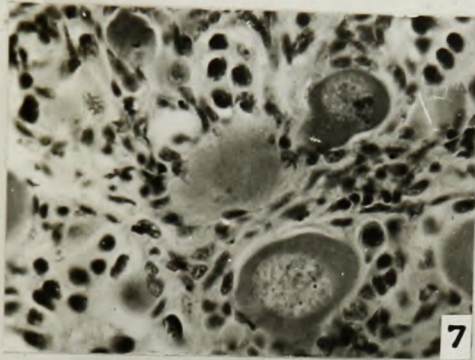
Females

Four adult tetraploid frogs are similar to females. There is no nuptial pad on their fore-arms; but ^{the} cloaca is smaller in size than that of a normal female, and is similar to that of immature frogs.

When injected with gonadotropic hormone in the way that causes normal females to lay, these tetraploid females were not clasped by males. Thus these frogs lacked some physiological factor or behaviour pattern which

- Plate 3, fig. 7. Section of ovary of normal diploid frog, a few months after metamorphosis, showing premeiotic oocytes and young auxocytes. X 340.
- " fig. 8. Section of ovary of first tetraploid female. X 85.
- " fig. 9. Section of ovary of same female tetraploid. Nests of nuclei at the same stage can be seen, suggesting incipient sex-reversal. X 340.
- " fig. 10. Section of ovary of same female tetraploid, showing dying auxocytes and pyknotic nuclei. X 340.
- " fig. 11. Section of ovary of second tetraploid female. A single large oocyte is seen. The almost clear spaces are probably the result of decayed oocytes. X 85.
- " fig. 12. Section of ovary of same tetraploid female, showing a group of normal premeiotic nuclei. X 340.
- " fig. 13. Section of ovary of same tetraploid female, showing four young growing auxocytes, which appear quite normal. Very few auxocytes develop beyond this stage. X 340.
- " fig. 14. Section of ovary of same tetraploid female, showing auxocytes beginning to die, with pyknotic nuclei. X 340.

PLATE 3, figs. 7 - 14.



induces males to clasp.

Two females have been dissected. In each case the fat-body was considerably more voluminous than in normal females (see Table 4); this is known to be associated with sterility (Gallien, 1956). In one female (18 months old) the ovaries were represented only by small folded ridges such as are found in immature females a few months after metamorphosis (text fig. 1.). Sections of the ovaries of this female showed that only very young auxocytes were present. Apart from the usual premeiotic nuclei of very early oocytes only dying oocytes could be seen (figs. 8-10). The glandular part of the oviduct wall of this female was poorly developed, though that of the other tetraploid female to be described was normal.

A second tetraploid female was killed after 14 months; it failed to lay any eggs after the usual injection of hormone. The ovaries of this frog were similar in general appearance to those of the previous frog (text. fig 1), but sections showed that a few growing oocytes were present. The majority of oocytes had died or were dying at an early stage (fig. 14). It seemed that oocyte development had proceeded a little further in this female than in the previous frog. The oviduct of this female was like that of a normal diploid.

Certain generalisations can be made about the capacity for oogenesis in tetraploid females. In the ovaries of both females referred to, groups of condensed premeiotic nuclei can be seen (fig. 12). At this stage the tetraploid germ-cells seem normal, and are very similar to comparable cells seen in the ovary of a control diploid female killed a few months after metamorphosis (fig. 7). In both tetraploids the oocytes began growth, and fig. 13

shows a group of three auxocytes which still appear quite healthy. However at about this stage almost all auxocytes from tetraploid ovaries seem to die, their nuclei becoming pycnotic (figs. 10 and 14). The ovaries of both females contained large non-cellular vacuoles (figs. 10 and 11) which probably result from the contents of one or more decayed oocytes. In one female a very few, exceptional, auxocytes have survived the critical stage in their early growth when most die; they have then grown into large oocytes with normal yolk formation (fig. 11). In the other female no auxocytes seem to have survived the critical stage, and there is a slight indication of sex-reversal towards the male condition. This is suggested by large nests of cells the nuclei of which are all at the same stage (fig. 9). It is possible that this merely represents delayed or arrested gonad differentiation, but on the other hand Chang (1953) from his work on hormone induced sex-reversal regards such nests as the first step towards reversal. In support of this suggestion it is known that testicular differentiation of a gonad often tends to increase at the expense of ovarian differentiation, as for instance when a gonad has suffered a general inhibition (Witschi, 1956). Humphrey, Briggs, & Fankhauser (1950) have found that triploid females of Rana pipiens may undergo reversal to males as a result of triploidy. It is possible that tetraploid Xenopus females will show a more clear tendency towards male differentiation as they become older.

The only two tetraploid females which have been dissected so far have shown a considerable difference in their powers of gametogenesis; one was completely sterile, while the other produced a very few nearly mature and possibly normal eggs. The reason for this difference is not clear, and

it will be necessary to wait until more tetraploid females of different ages have been sacrificed. A greater variation was found among triploid females in Xenopus (Smith, unpublished, see Section VI below).

The following conclusions can be made concerning oogenesis in tetraploid Xenopus. The great majority of oocytes become arrested or die at an early stage in oogenesis. Thus gametogenesis is mostly arrested at an earlier stage in females than in males; in the latter, gametogenesis first becomes abnormal during the first meiotic division. So far too few tetraploid females have been examined to comment on the fact that one female could produce a few almost normal eggs, while another was completely sterile.

V. TETRAPLOID XENOPUS COMPARED WITH OTHER TETRAPLOID ANURA AND URODELES

There are very few previous accounts of tetraploid frogs. Kawamura (1939) has described four tetraploid tadpoles of Rana nigromaculata, the most normal of which died before metamorphosis, but lived longer than is usual for haploids and mosaics, though shorter than triploids. It is probable that the parthenogenetic origin of these frogs (with partial homozygosity) would account for some of the abnormalities, which may not have been wholly due to the tetraploid condition of their nuclei.

Kawamura and Moriwaki (1953) have described a tetraploid Rana limnocharis which as a tadpole grew about 10% faster than comparable diploids. This individual died two months after metamorphosis; its tetraploid ovaries, though more normal than triploid ovaries, were inferior to those of diploids. This frog did not seem likely to reach sexual maturity, had ^{it} not died.

Kawamura and Miyada (1951) described mosaic diplo-tetraploid Rana nigromaculata; the gametogenesis of these is more normal than that of pure

tetraploids, showing that an environment of diploid cells can improve the production of tetraploid gametes.

Jahn (1952) has described tetraploid Rana temporaria though it appears from chromosome numbers given in that paper that these tetraploids were in fact diplo-tetraploid mosaics. These tetraploid frogs are said to have grown faster and to have reached a greater adult size than their diploid controls; it is however stated that the controls produced inferior development to typical diploids. The performance of these tetraploids is different from all others that have been described.

In Urodeles, tetraploids are in general less viable than triploids. Sanada (1956) has described nearly normal gonads in a tetraploid Triturus pyrrhogaster, though this is apparently exceptional, (Kawanura and Utsonomiya, 1957). Tetraploid axolotls are apparently much more viable than any other tetraploid urodeles (Humphrey and Fankhauser, 1956). Kawanura and Utsonomiya attribute this better performance of tetraploid axolotls compared to other tetraploid urodeles to neoteny in this species. Tetraploid axolotls and tetraploid Xenopus are more viable than any other Amphibia; this is probably due to the fact that these species are unusual in leading an aquatic life as adults, and can thus be kept satisfactorily under laboratory conditions.

The viability of tetraploid Anura seems in general to be inferior to that of diploids, especially after metamorphosis; the tetraploid individuals of Xenopus laevis seem to be exceptional in this respect. As regards gametogenesis Xenopus resembles other tetraploid Anura in being very inferior to diploids, as they are often sterile. With the exception of the Axolotl, tetraploid urodeles seem to suffer from the same defective gametogenesis as tetraploid Anura.

Tetraploid species are distinctly uncommon in Vertebrates if they exist at all and are unknown in Anura, whereas tetraploid species are known to exist in many plants and in some invertebrate animals. As has been shown the viability of artificially induced tetraploid vertebrates need not be less than that of diploids; the reason for the scarcity of tetraploid species in vertebrates is most probably associated with their very abnormal gametogenesis. The distribution and behaviour of chromosomes during meiosis in tetraploid Xenopus is very irregular, while tetraploid plants can give a high proportion of viable gametes. The reason for this difference is not clear, but may be connected with the pairing of homologous chromosomes during meiosis.

VI. TETRAPLOID AND TRIPLOID XENOPUS COMPARED.

The following information concerning triploid Xenopus has been very kindly provided by S. Smith (unpublished), and the author is grateful for being allowed to quote the results of this work before its publication.

The rate of growth of triploid tadpoles and frogs in Xenopus is indistinguishable from that of diploids. Adult size is more variable and slightly greater in triploids than in diploids, but this difference which is by no means significant, seems to be related to degree of sterility.

As regards gametogenesis, triploid females are of two kinds; they are either entirely sterile with no growing oocytes (ca. 80% of females are of this kind), or they are capable of producing a few apparently normal eggs. After fertilisation by a normal diploid male, most eggs from a triploid female which begin development give abnormal embryos, but a few have developed into frogs. Most triploid males are quite unable to fertilise normal eggs;

some eggs fertilised by a triploid male did however start development, but nearly all died before metamorphosis. Thus the gametogenesis of triploid males and females is much inferior to that of diploids. It is probable that the meiotic distribution of chromosomes is more or less random, so that only occasionally will a gamete arise with exactly the diploid or haploid chromosome number.

One may conclude that the morphology and viability of tetraploid, triploid and diploid Xenopus is similar; this is in contrast to haploid Xenopus, which only very rarely reach normal tadpoles, and are usually strongly oedematous, dying soon after hatching (Gurdon, unpublished). The physiology of tetraploids has not yet been investigated. Their reproductive capacity is variable and is inferior to that of triploids. It is hoped to investigate this further when more young tetraploids become adult.

This comparison of Xenopus polyploids shows that the main factor causing sterility in polyploid Xenopus is independent of the chromosomal sex-determining mechanism, but is due to the general increase in chromosome number. This is shown by the fact that tetraploids with the normal ratio of sex-chromosomes are less efficient at gametogenesis than triploids, which in the heterogametic sex, have an abnormal ratio of sex-chromosomes, (both of X:Y, and of X:autosomes).

SUMMARY.

1. A description is given of some tetraploid individuals of Xenopus laevis, which were obtained by nuclear transplantation. In external morphology they are almost identical to normal diploids. Their size is slightly smaller than that of diploids, but their rate of growth does

not differ significantly.

2. Gametogenesis is very abnormal in tetraploid Xenopus, which have so far been entirely sterile. Spermatogenesis becomes more abnormal at each meiotic division, probably due to a random distribution of chromosomes to daughter nuclei. Oogenesis becomes abnormal when the great majority of oocytes die at an early stage of their development. Occasionally a very few oocytes develop beyond this stage into almost normal eggs.
3. The results of different methods used to obtain tetraploid frogs are described. All previously recorded tetraploid frogs are compared with tetraploid Xenopus, which appear to be more normal than the former.
4. Tetraploid Xenopus are compared with haploid and triploid frogs of the same species. Haploids nearly always die as abnormal tadpoles before feeding, while triploids and tetraploids mainly differ from diploids in their reproductive capacity; secondary sex characters are often well developed in these polyploids. The gametogenesis of tetraploid Xenopus is inferior to that of triploids. This fact shows that the infertility of polyploids is due to a general increase in chromosome number and not to the ratio of sex-chromosomes.
5. In contrast to plants, natural tetraploid species are unknown in the Anura, and probably do not exist in other vertebrates. This is possibly due to the behaviour of chromosomes during gametogenesis, (either X:Y, or X:autosomes).

Acknowledgements.

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CHAPTER VIII.

A METHOD OF OBTAINING MANY GENETICALLY IDENTICAL FROGS.

In biological experiments designed to analyse the effect on a process of a certain factor, it is often desirable to reduce to a minimum the variation in results due to other factors. One way of doing this is to use genetically identical individuals. It is not difficult to obtain these in organisms which can be reproduced vegetatively, or mitotically, like most plants and micro-organisms, and many invertebrate animals. Vertebrates, however, only reproduce by sexual means, and so nearly always give rise to offspring which differ from each other in some respects at least. Identical twins arise naturally and can be reproduced experimentally in Vertebrates by division of an egg or embryo, ^{but} ~~though~~ it is very unusual for more than two such individuals to be obtained, though as many as eight are known. Continued inbreeding can give rise to a high degree of genetic homozygosity, but this is usually associated with weak, abnormal, or even sterile individuals. It is therefore very desirable to be able to obtain a stock of genetically identical individuals among the Vertebrates, without the time, labour, and loss of vigour associated with inbreeding.

In Amphibia, such individuals can be obtained by nuclear transplantation, which is in effect a means of replacing the zygote nucleus of a fertilised egg by an embryonic cell nucleus. If a normal (undifferentiated) nucleus is transplanted to an unfertilised and enucleated egg, a normal transplant-embryo will often result (King and Briggs, 1955). ^{Gurdon 1961} In the case of Xenopus such transplant-embryos have been reared into adult sexually mature frogs (Gurdon, Elsdale, and Fischberg, 1958). There are good reasons for believing that endoderm cell nuclei in Xenopus do not begin to differ from each other until after gastrulation has ended (Gurdon, a). Thus all

transplant-embryos or -frogs derived by nuclear transplantation from the endoderm nuclei of a single blastula or gastrula Xenopus embryo should be identical. They are, as it were, the products of vegetative propagation. It does not alter the result if the original embryo used to provide donor nuclei was itself the product of a transplanted nucleus, as in serial nuclear transplantation (Gurdon,a).

In one experiment with Xenopus, the transplantation of blastula donor nuclei gave rise to 20 regularly cleaving transplant-embryos, of which 19 became normal feeding tadpoles and subsequently metamorphosed. These 19 frogs were reared under good laboratory conditions with maximal feeding and no overcrowding, but in spite of this there is a considerable variation in size, of which weight is a good indication. Ten frogs weighed between 26 and 52 gms, seven between 10 and 23 gms, and there were two dwarfs which weighed only $3\frac{1}{2}$ gms each. The average weight of these frogs, excluding the two dwarfs, was 23 gms, which is much lower than the average of 52 gms for controls. However, among controls there is also a considerable variation in size, and a few dwarf individuals occur (Gurdon,b). Results described elsewhere (Gurdon,b) have shown that the average size of transplant-frogs is often donor specific, i.e. genetically controlled, while individual variation within the progeny of a single donor is affected by environmental factors. The present series of genetic twins confirms the evidence that the variation in size of laboratory reared Xenopus is not only due to individual genetic differences. There are several possible causes of this range in adult size; perhaps the most likely of these is the variation in egg quality (including immaturity and overmaturity) which seems to be inevitably associated with unidentified deficiencies of a laboratory diet (Gurdon,b). It must be born in mind that some of the size variation in

genetic twins could be due to gene induced differences in the eggs from which they were derived; however, since genetic twins are usually all derived from the eggs of one frog, the differences between egg nuclei will be small and are unlikely to have any effect on the quality of eggs. In other respects than size these genetic twins are very similar to each other. They are all males except the two dwarf individuals, which have not yet shown secondary sex characters. The pattern on the body, which is variable and under genetic control, is essentially the same in all individuals. In due course it is hoped to actually test their identity by experimental means.

These genetic twin frogs have been described merely to illustrate the principle of the means by which they were obtained. In most series or clones of genetic twins one would expect the average size to be normal. A completely pure breeding stock (apart from non-lethal mutations) could be obtained by sex reversing some of the tadpoles in a clone. This could be done in either direction by hormone treatment of tadpoles at the appropriate stage in their growth when sex determination becomes fixed, i.e. in Xenopus stage 52 of Nieuwkoop and Faber, 1956 (Gallien, 1956; Chang and Witschi, 1956). It would in fact be necessary to treat separate groups of tadpoles for sex reversal into both males and females, since their genetic sex would not be known at the time of treatment.

I am very grateful to Dr. M. Fischberg for having pointed out the possible use of multiple genetic twins.

For references, see next page.

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DISCUSSION OF CERTAIN POINTS ARISING FROM CHAPTERS II TO VIII.

CHAPTER IX.

DISCUSSION OF CERTAIN POINTS ARISING FROM CHAPTERS II TO VIII,

in connection with:

The technique,

The specificity of nuclear changes, and

Heterospecific nuclear transfers.

DISCUSSION OF CERTAIN POINTS ARISING FROM CHAPTERS II TO VIII.

The direct conclusions have been discussed at the end of each chapter, but some further comments are required on three points not adequately dealt with so far: the technique, the specificity of nuclear changes, and the effects on nuclei of foreign cytoplasmic environments.

Haploid, diploid, triploid, and tetraploid Xenopus have been described in chapters II and VII. These embryos and frogs demonstrate, within the same species, the effects of different numbers of chromosome sets per nucleus; this subject has already been briefly discussed at the end of chapter VII, and does not require further comment.

The technique.

The technique and the results of its application have been described (chapters II to IV); it now remains to consider whether these results are consistent with the belief that the technique does not cause any abnormal ~~transplant-embryo~~ ^{transplant-embryo} development. Though blastula nuclei are believed to be undifferentiated, they nearly always seem to give rise to a few transplant-embryos which develop abnormally; similarly it is unusual for more than 80% of all transplantations from these nuclei to cleave regularly. It is necessary to account for these abnormalities, which are not due to the quality of donor nuclei. The technical handling of donor cells may be responsible for up to 20% of variation in the proportion of transplantations which cleave regularly, but in other respects the technical treatment of donor nuclei and recipient eggs has not been found to affect transplant-embryo development in any way (chapter IV). It was however found that the quality of recipient eggs could account for some abnormalities; as this is the only variable that was found to be correlated with abnormal transplant-embryo development, it is probably responsible for all abnormal development

from blastula nuclei. It was pointed out in chapter V that sometimes well over half of all transplantations from blastula nuclei may develop abnormally, but that it is very rare for more than 20% of fertilised eggs to do so. One would therefore expect, as is indeed found in practice, that the proportion of transplantations which commence development is higher than the proportion of fertilised eggs which do so. Many of the eggs laid by a laboratory female with a clasping male fail to cleave. This is also true of artificially inseminated eggs, so is probably not due to deficient or abnormal sperm. Thus laboratory females seem to lay a variable proportion of abnormal eggs which are incapable of natural fertilisation, but which commence development when injected with a nucleus (though fail to develop normally thereafter). The respect in which these eggs are abnormal is not known, but it is almost certainly due to the frogs' laboratory diet which must be deficient in some ways. This variation in quality would not be expected in the eggs of animals taken from their natural environment, and often 90% or more of eggs laid by Rana pipiens are fertilised and develop normally. This accounts for the smaller variation in Briggs and King's results with the latter species than has been obtained with Xenopus.

In chapter II considerable attention was paid to the fate of the irradiated egg nucleus, in order to find out whether it can affect transplant-embryo development. Any effect it might have must be by fragments of the irradiated nucleus becoming incorporated into the developing nucleus. If these fragments are unspecific (? small molecules), they would not be expected to have any effect. But if they are specific (? large molecules or chromosome fragments) they would be expected to have a disturbing effect on normal development; this is in fact what happened

after very short doses of u.v., which presumably resulted in partially irradiated chromosomes becoming incorporated into the developing nuclei (chapter III). With the usual u.v. dose, transplant-embryo development is more normal, and a further increase in the dose has no effect on the frequency of abnormalities. Thus there are theoretical reasons (see also chapter III) for believing the irradiated egg nucleus has no effect on transplant-embryo development. However, this is only negative evidence and the question must be finally settled by comparing the results of nuclear transplantation into the eggs of the same species some of which have been 'enucleated' by u.v., and others by microdissection. Since Xenopus eggs enucleated by u.v. give better transplant-embryo survival than Rana pipiens eggs enucleated by microdissection, it appears that any effect of the irradiated nucleus would be to improve transplant-embryo survival. Since u.v. ^{probably} ~~also~~ causes less damage than microdissection, this problem should be investigated by finding out whether Rana pipiens transplantations survive better when enucleated by u.v. than by microdissection as used by Briggs and King.

Lastly it is worth drawing attention to the limitations of this technique. Though it appears to be satisfactory for the transplantation of living embryonic nuclei, it cannot be applied without further modifications and tests to the nuclei of differentiated cells.

The specificity of nuclear changes.

In chapters V and VI the main attention has been devoted to the frequency of normal and abnormal transplant-embryos and -frogs. Very little has been said about the specificity of abnormalities in relation to the kind

of nuclei from which they are derived. Briggs and King (1957) have described certain abnormalities as being characteristic for endoderm nuclei and others for ectoderm or mesoderm nuclei. It appears however that it is not so much the nature of the abnormality as its frequency which is characteristic. For instance the most easily defined syndrome of abnormalities is that associated with embryos derived from endoderm nuclei (ectodermal derivatives deficient or abnormal, and mesodermal derivatives often abnormal). This syndrome is found in a majority of endoderm transplant-embryos, but is also found in some embryos derived from mesoderm and ectoderm (Briggs and King, 1957). In Xenopus no single type of abnormality has been found more often among endoderm transplant-embryos than among embryos in which abnormalities are due to other causes, e.g. haploidy. A few unpublished experiments with gastrula ectoderm nuclei indicate that similar abnormalities ^{occur} among ectoderm transplant-embryos. In Xenopus it seems that all developmental abnormalities appearing between the blastula and neurula stages depend upon the extent to which the yolk is invaginated during gastrulation. The differentiation of the head and central nervous system can be related to the degree of yolk cell invagination, which can also be influenced by purely environmental conditions such as the tonicity of the solution. For this reason it seems to the writer that several embryos which are genetically abnormal in exactly the same way (i.e., in respect of the same genes) may nevertheless show a wide range of developmental abnormalities (cf. chapter V). This is the same as saying that normal development is canalised so that all genetically normal embryos develop almost identically, while canalisation would not be expected to take place in abnormal development. These considerations have not been brought forward as evidence that nuclear changes are unspecific

for the type of donor nucleus, but merely to draw attention to the difficulty of demonstrating the specificity of changes by observing the type and frequency of abnormal transplant-embryos.

Specific abnormalities are much less likely to be found in adult transplant-frogs which, because they have reached the adult stage, must be normal for the greater part of their genotype. As was pointed out in chapter VI, the abnormalities which occur in adult frogs are presumably due to relatively small abnormalities of the genotype; the expression of these will not be modified by lack of developmental canalisation and should therefore be much more constant. No clear examples of endoderm specific abnormalities have yet been found in Xenopus transplant-frogs, since the donor specific abnormalities described in chapter VI may not be related to the differentiation of endoderm nuclei.

The significance of nuclear changes is still a matter for conjecture, and has been discussed towards the end of chapter V. Nuclear changes are presumably caused by the particular cytoplasmic environment in which different somatic nuclei come to lie. Examples of cytoplasmic effects on nuclei are quoted by Fischberg, Gurdon, & Elsdale, (1958, p.176), where it is suggested that nuclear changes are brought about by the inability of nuclei to synthesise copies of all their gene loci, owing to the lack of the necessary substances in different cytoplasms. This particular point has been investigated further by the experiments described immediately below.

Heterospecific nuclear changes, transfers.

The principle of these experiments is to transplant an undifferentiated nucleus into the egg of a different species of frog; when the resulting transplant-embryo has become a blastula, some of its nuclei are transplanted

back into eggs of its own original species. The development of these transplant-embryos, whose nuclei have replicated and synthesised in foreign cytoplasm, is then compared with that of controls whose nuclei have only done so in the cytoplasm of their own species. This experimental design can be modified in various ways, by serially transplanting nuclei into the eggs of both species for different numbers of times. For instance, nuclei may be transferred to the eggs of a foreign species one, two, three, etc. times before being transferred back to the eggs of their original species, as a nucleus divides nearly fifteen times between its first division and the late blastula stage. This experiment would show the effect on these nuclei of approximately 15, 30 45 etc. syntheses from foreign cytoplasm.

The first series of experiments were made with Xenopus laevis and Rana temporaria, and are described in Fischberg, Gurdon, & Elsdale, 1958, pp. 170-175 (see appendix). These experiments are not very conclusive, since the nucleus of one species, having synthesised in the cytoplasm of the other, becomes changed and cannot after back transfer to its own species develop beyond a late gastrula. This means that the main criteria of the effect of foreign cytoplasm is the degree of gastrulation, which as pointed out above may be influenced by environmental factors. Because these species are too distantly related (that is, the nucleocytoplasmic incompatibility is too great) for species characters to be developed in heterospecific transplant-embryos, it was decided to try and repeat these experiments using more closely related species or subspecies.

4 Experiments have therefore been started making heterospecific nuclear transfers between Xenopus laevis laevis and Xenopus laevis victorianus. These two subspecies differ in a number of small but consistent characters

such as adult size, colour pattern, length of arms, etc. There appears to be no nucleocytoplasmic incompatibility between these species, and a nucleus from one species with the egg from the other will form adult individuals, in which specific characters can be clearly seen. These hetero-specific transplant-frogs are not yet sexually mature, but all characters that have so far become apparent appear to be those of the species from which the nucleus, and not the egg, is derived. Thus individuals derived from a Xenopus laevis victorianus nucleus and Xenopus laevis laevis egg are not yet distinguishable from normal Xenopus laevis victorianus, showing that the nucleus of one subspecies is very little if at all affected by having to synthesise from the cytoplasm of the other.

These experiments and conclusions are very incomplete at the present. They show so far that nuclei are able to synthesise exact copies of themselves from the egg-cytoplasm of a genetically similar kind to their own, but cannot do so from the cytoplasm of a genetically very different species. This is probably a reflection on the specificity of the precursors in an egg required for chromosome synthesis; these are believed to be polynucleotides which, it seems, do not differ qualitatively in Xenopus and laevis laevis and Xenopus laevis victorianus but do so in Xenopus laevis and Rana temporaria.

(A summary of the main results and conclusions will be found at the end of chapters II to VII inclusive).

1. In 1952 Briggs and King devised a method of transplanting a living embryonic nucleus into an enucleated egg; the development of the resulting embryo provides information about the developmental potentialities of the transplanted nucleus. These authors used Rana pipiens for their experiments. The present thesis describes how this technique has been modified for use with Xenopus laevis which is in some respects more suitable than Rana pipiens for the kind of problems which can be investigated by this technique. The description and analysis of the technique is summarised in section 2 below. Section 3 describes the results of transplanting nuclei from differentiating endoderm cells; it also summarises the development of embryos, as well as the adult characters and reproductive capacity of frogs, derived from these nuclei. Section 4 concerns an incomplete series of experiments in which nuclei are transferred into eggs of different species and subspecies of frogs.
2. Nuclear transplantation in Xenopus laevis is carried out by a similar method to that of Briggs and King for Rana pipiens. When embryonic cells have been disaggregated chemically, each is sucked up into a pipette just narrow enough to break the cell wall and is then injected into an enucleated egg. The main differences in the technique for the two species are: (1) a nuclear marker is used

which provides proof that the nuclei of the resulting transplant-embryos and frogs are derived from the injected nucleus and not the egg nucleus. (ii) the recipient eggs are injected while not immersed in a fluid medium; this greatly facilitates the operation, and is harmless to the eggs which are not exposed to the air long enough for their surface to become dry. (iii) the unfertilised recipient eggs are "enucleated" by a short exposure of their animal hemisphere to ultraviolet irradiation. There are many reasons for believing that this irradiation treatment has no effect on the egg cytoplasm. It has a most useful result of weakening the vitelline membrane sufficiently to enable the egg to be penetrated by a micro-pipette. Lastly it inactivates the egg nucleus which does not then participate in the subsequent development of the egg. The fate of the egg nucleus has been followed. It sinks towards the centre of the egg and comes to lie adjacent to the developing nucleus; it does not fuse with it but eventually disappears as a pyknotic clump in the centre of the spindle formed for the first mitotic division of the developing nucleus. There is no reason to believe that the degenerating egg nucleus has any effect on the subsequent development of the egg. When eggs are u.v. irradiated soon after fertilisation, they develop as androgenetic haploids, none of which survive beyond typically microcephalic and oedematous tadpoles.

Each part of the technique has been analysed in order to find out whether ~~it~~^c can account for any developmental abnormalities of transplant-embryos. This was done by taking each step in the technique in turn, varying the way it is carried out, and then looking for any

correlation with developmental abnormalities of transplant-embryos. Only one part of the technical treatment of donor nuclei and recipient eggs was related to abnormal transplant-embryo development. A direct correlation was found to exist between the degree of donor cell distortion in the pipette and the proportion of total transplantations which became regular late blastulae. As the degree of cell distortion cannot be kept entirely constant, a variation of up to 20% in the proportion of late blastulae formed may be due to unavoidable inconsistency in the technique. This effect can be excluded from the results by drawing conclusions from the further development of blastula transplant-embryos, rather than from the development of all transplantations.

Eggs laid by laboratory frogs vary greatly in their ability to withstand the treatment to which they are subjected during transplantation; thus poor egg quality is indirectly responsible for abnormal transplant-embryo development. Egg quality can only be recognised by the frequency of abnormalities among the resulting embryos. As the quality of all eggs laid during one ovulation of a frog is about the same, control transplantations using undifferentiated donor nuclei show whether egg quality was good in each series of experiments. The clearest results can therefore be obtained by considering only those "selected" experiments in which egg quality was subsequently found to have been good.

This analysis of the technique has subsequently shown that, if attention is restricted to selected experiments, then the way in which

blastula transplant-embryos develop gives a good indication of the developmental potentialities of the transplanted nuclei.

3. A survey has been made of the developmental potentialities of nuclei from differentiating endoderm or vegetal cells; donor nuclei were taken from this region of a series of embryos ranging from late blastulae to swimming tadpoles. All endoderm nuclei appear to remain totipotent until the early neurula stage and there are still a few nuclei in the mid-gut region of swimming tadpoles which have given rise to normal tadpoles and frogs. However endoderm nuclei tend to become changed genetically as the tissue from which they are taken becomes differentiated. This is shown by a progressive increase in the frequency and severity of developmental abnormalities derived from transplanted nuclei, the older the stage from which they are taken. The capacity of endoderm nuclei for normal cleavage (i.e. the proportion of total transplantations which become regular late blastulae) first shows a decline in muscular response embryos, and decreases progressively from this stage onwards. On the other hand blastula transplant-embryos develop increasingly abnormally from the early neurula stage onwards. There is no clear indication that the kind of abnormalities from which these transplant-embryos suffer are specific for the endodermal origin of their nuclei. Serial transplantation experiments have shown that nuclear differentiation is heritable, and at least to a large extent, irreversible. These results are compared with those of Briggs and King who transplanted endoderm nuclei in Rana pipiens. There is a

considerable difference in the rate and time of onset of endodermal nuclear differentiation in the two species. This difference shows that nuclear differentiation is not directly related to the morphological differentiation of endoderm tissue which is similar in the two species. It is suggested that nuclear differentiation is related to the fully differentiated state of individual cells.

Normal transplant-embryos derived from endoderm nuclei have been reared into adult frogs; the study of these frogs and their reproductive capacity gives further information about the genetic qualities of the nuclei from which they are derived. Most of these frogs are normal and give rise to normal offspring, but a few are abnormal in some way, and in these cases it is found that all frogs derived from the nuclei of one embryo suffer from the same type of abnormality. There is an indication that endoderm nuclei from advanced embryos are deficient for genes concerned in oogenesis, so that some of the resulting frogs are either sterile or sex-reversed into males.

All transplant-frogs derived from the single or serial transplantation of undifferentiated (e.g. blastula) nuclei should be genetically identical. A series of 19 such frogs have been reared to sexual maturity; they differ to some extent in size (which is not only controlled by the genetic quality of their nuclei), but in other respects appear to be identical. This is the only way of obtaining several identical "genetic twins" in animals which can only reproduce themselves sexually; they would be of value in any experiment in which the effect of genetic differences between individuals needs to be excluded.

In a variable proportion of transplantations, the transplanted nucleus doubles itself before undergoing its first mitotic division, and so gives rise to a tetraploid individual. As tetraploid *Anura* have been very seldom recorded, these tetraploid transplant-embryos have been reared into adult frogs which are normal except that they are sterile. During gametogenesis the pairing of chromosomes is more or less random, giving rise to aneuploid gametes. As would therefore be expected tetraploid Xenopus are more sterile than triploids which can sometimes produce a few normal gametes. Haploid Xenopus have been described in connection with the u.v. irradiation of eggs; thus the effect of one, two, three, and four chromosome sets per nucleus have been demonstrated within one species.

4. Some experiments have been started which are designed to show the effect of making nuclei synthesise and replicate in foreign cytoplasm. This has been done by serially transplanting nuclei into the eggs of different species or subspecies of frogs, and then back into the eggs of their own species. In one series of experiments nuclei were transplanted between Rana temporaria and Xenopus laevis. The results are inconclusive but it appears that nuclei are cumulatively and reversibly changed by the foreign cytoplasm. In another series of experiments, nuclei were transplanted between two subspecies of Xenopus laevis, but in these the nuclei appeared to be unaffected by the foreign cytoplasm. Thus qualitative differences in the substrates required for chromosome synthesis do not exist between the two subspecies, but do so between the two species.

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APPENDIX.

Sexually Mature Individuals of *Xenopus laevis* from the Transplantation of Single Somatic Nuclei

A METHOD of testing the potentialities of nuclei from embryonic cells has been described by Briggs and King¹. The method consists of transferring a nucleus from an embryonic cell into an enucleated and unfertilized egg of the same species. King and Briggs², who have performed their experiments on *Rana pipiens*, found that normal tadpoles resulted from eggs with transplanted nuclei in about 35 per cent of cases in which the nuclei were taken from blastulæ, but in only about 6 per cent of cases in which the nuclei were taken from late gastrula endoderm; they have not reported normal development from nuclei of post-neurulæ or later stages.

We have performed similar experiments on *Xenopus laevis*. In this species we do not enucleate the eggs, because the female pronucleus participates in the development of only a very few of the transplanted eggs; these cases can be recognized by the use of a nuclear marker³, and are excluded from our results. The marker is introduced by taking donor nuclei from a stock of mutant individuals having only one nucleolus in all cells, as opposed to wild-type individuals in which all cells contain potentially two nucleoli. Large series of observations and measurements show no differences in the embryonic development, viability, growth-rate, and fertility between mutant individuals and their wild-type full sibs. The host eggs are taken from the wild-type stock and the female pronucleus introduces an additional nucleolar organizer when it participates in development. The origin of the nuclei in transplant-tadpoles can be interpreted with certainty by knowing the ploidy and number of nucleoli.

We have found that the great majority of normal tadpoles resulting from transferred nuclei pass through metamorphosis normally. The older frogs appear to be sexually mature males and females; the rate of growth and sexual differentiation is similar in transplant-embryos and controls. In order to test the capacity of gametogenesis in transplant-individuals, we hope shortly to breed from these. Immature oocytes were found in one transplant-frog which died two months after metamorphosis.

As with *Rana pipiens*, the proportion of normal development obtainable from transferred nuclei

Table 1. METAMORPHOSED FROGS WHICH RESULTED FROM TRANS-PLANTED NUCLEI

Donors			Transplants		
Stage	Germ-layer	Total transfers	Metamorphosed frogs		
			No.	Percent-age of total	Percent-age of normal late blastulae
Mid and late blastula	Ectoderm	565	17	3.0	12
	Endoderm	159	12	7.5	16
Early gastrula	Ectoderm	94	7	7.5	40
	Endoderm	287	12	4.2	15
Late gastrula	Ectoderm	26	2	7.7	33
	Endoderm	185	11	5.9	16.5
Neural folds	Ectoderm	9	0	0	0 out of 1
	Mesoderm	325	4	1.2	6
Tail bud	Endoderm	137	7	5.1	14
	Endoderm	163	4	2.5	9
Muscular response	Endoderm	287	1	0.35	3
Pre-hatching tadpoles	Endoderm	357	0	0	0 out of 70
Total		2,594	77		

decreases with increasing age of the donor embryo. *Xenopus laevis* gives a smaller proportion of normal development from blastula nuclei than *Rana pipiens*, but on the other hand normal development can be obtained from much later donor stages; a pre-hatching tadpole (Nieuwkoop⁴ stage 32, endoderm) was the most advanced stage of donor from which a normal individual was obtained, but this was accidentally killed shortly before metamorphosis. We have also a few frogs from nuclei which have been transferred twice (serial transfers).

Table 1 gives the numbers of metamorphosed frogs that have been obtained from different germ-layers and developmental stages of donor embryos. When transplanting endoderm nuclei from post-neurula stages care has been taken to avoid using nuclei from the region of the presumptive germ-cells.

When considering our results it is important to appreciate that the normal development of eggs with transferred nuclei may mean either that the nuclei were undifferentiated and totipotent, or that they were differentiated, but were able to return to a totipotent state as a result of developing again in the cytoplasm of an uncleaved egg.

Our results may be summarized as follows: (1) It is possible to transplant nuclei without impairing their ability to bring about normal development. (2) Some nuclei are capable of giving normal development very shortly before the organ of which they are part becomes functional; normal development was

obtained from presumptive somite nuclei nine hours before the first muscular responses. (3) Normal development results from nuclei of more advanced donor stages in *Xenopus* than in *Rana pipiens*. (4) A number of monozygotic frogs have been obtained from single donors.

It may now become possible to breed from our sexually mature individuals which have developed from single somatic nuclei, and to test their genetic qualities.

We gratefully acknowledge the technical assistance of Miss A. Jewkes. This work has been made possible by contributions from the British Empire Cancer Campaign (M. F. and T. R. E.) and the Medical Research Council (J. B. G.).

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¹ Briggs, R., and King, T. J., *J. Exp. Zool.*, **122**, 485 (1953).

² King, T. J., and Briggs, R., Cold Spring Harbor Symp., **21**, 271 (1956).

³ Elsdale, T. R., Fischberg, M., and Smith, S., *Exp. Cell Res.* (in the press).

⁴ Nieuwkoop, P. D., and Faber, J., "Normal Table of *Xenopus laevis*" (1956).

Table 2

	No.	Per cent
Total transfers	905	100
Uncleaved	387	43
Cleaved normal and abnormal	518	57
Normal gastrulae	193	21
Normal at hatching	89	10
Normal feeding tadpoles	61	7

small to relate the abnormalities of transplanted embryos to the origin of the donor nucleus.

We have obtained normal tadpoles from eggs injected with ectoderm and endoderm nuclei from late gastrulae. King and Briggs¹ obtained 10-12 mm. tadpoles from nuclei of ectoderm, chorda-mesoderm, and endoderm from late gastrulae of *Rana pipiens*; but the majority of transplants from chorda-mesoderm and endoderm gave embryos developing syndromes of abnormalities specific for the origin of the donor nucleus.

We are continuing the work along two main lines: (a) to find the latest stages that provide donor nuclei which give normal development when transplanted into unfertilized eggs; (b) to see if embryos develop syndromes of abnormalities specific for the origin of the donor nucleus.

This work has been made possible by contributions from the British Empire Cancer Campaign (M. F. and T. E.) and the Medical Research Council (J. B. G.).

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² King, T. J., and Briggs, R., *Proc. Nat. Acad. Sci.*, **41**, 321 (1955).

Nuclear Transplantation in *Xenopus laevis*

Briggs and King¹ have devised a technique with which they were able to transplant nuclei from cells of early embryos into unfertilized eggs of *Rana pipiens* from which the female pronucleus had been removed. In this way they could test the extent to which a nucleus from embryos up to neurula stages could produce normal development when transplanted into an enucleated egg².

We have modified this technique to start similar experiments on *Xenopus laevis*. We have not so far removed the female pronucleus because it does not usually participate in development. This useful fact was discovered through the use of a nuclear marker, visible in interphase nuclei (Fischberg, M., Smith, S., and Elsdale, T., unpublished).

A survey of the number of chromosome sets present in 70 normal and abnormal tadpoles gave the results shown in Table 1. The tetraploid embryos are usually due to the doubling of the diploid chromosome number of the injected nucleus, and not to the participation in development of the female pronucleus¹.

Table 1

	No.	Per cent
Diploids	55	79
Tetraploids	12	17
Mosaics, etc.	3	4
Total analysed	70	100

Table 2 consists of the combined results from six of our more successful experiments, and shows the number of embryos that appear normal at various stages of development up to the feeding tadpole. The donor nuclei came from embryos between late blastula and late gastrula stages, and between these limits the proportions of normal host embryos and the types of host abnormalities at different stages are similar; these appear not to be dependent upon the developmental stage of the donor nucleus and the germ layer from which it derives. Table 2 is heterogeneous, because it consists of the results from twelve different categories of donor nuclei (four temporal stages and three germ layers); we find that the numbers in the individual categories are too

NUCLEAR TRANSFER IN AMPHIBIA AND THE PROBLEM OF THE POTENTIALITIES OF THE NUCLEI OF DIFFERENTIATING TISSUES¹

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This paper deals with the question of whether nuclear changes occur during the embryonic development of *Amphibia*. The stability and probable nature of those changes which have been observed after nuclear transfer will be discussed.

SINGLE, AS OPPOSED TO SERIAL, TRANSFERS OF EMBRYONIC NUCLEI

King and Briggs [6] transferred single embryonic nuclei into unfertilized eggs of *Rana pipiens* that had previously been activated and enucleated. They concluded from their brilliant experiments that many of the nuclei of late gastrulae had undergone some kind of differentiation which restricted their capacity to promote normal development. Post-neurula embryos derived from nuclei of different germ-layers showed different syndromes of abnormalities [1, 6]. The frequency and severity of these abnormalities increased with increasing developmental stage of the donors [1, 5].

We have adapted Briggs and King's nuclear transfer technique, introducing the following modifications: (a) The eggs of *Xenopus laevis* were used instead of those of *R. pipiens*; (b) the eggs were not activated previous to the injection of the donor nucleus; (c) the pro-nucleus of the recipient egg was not removed.

This last deviation from King and Briggs' method needs some qualification. A mutation that reduces the number of nucleoli in *X. laevis* has been discovered in our laboratory [3]. Heterozygotes for this mutation only differ from normal controls by containing one nucleolus in each nucleus instead of possessing potentially two nucleoli in all cells. This mutation therefore supplies an ideal nuclear marker visible in interphase nuclei. If unfertilized eggs from the two-nucleolus strain are injected with single diploid nuclei possessing one nucleolus, the origin of the nuclei of the developing embryos can, even in the case of fusion of egg pronucleus with the transferred nucleus, be determined with

¹ We wish to thank the British Empire Cancer Campaign (M. F. and T. R. E.), and the Medical Research Council for financial help (J. B. G.).

certainty. In our experiments with *X. laevis* we found that the egg pronucleus only participates in development in about 1 per cent of the transfers. The other 99 per cent of the transfer embryos possess nuclei derived from the injected donor nucleus alone.

In Fig. 1 (*a, b*) an attempt has been made to summarize the results obtained by Briggs and King [5, 6] from single transfers of endoderm nuclei in *R. pipiens* and to compare them with our experiments on endoderm nuclei of *X. laevis*. The curves representing King and Briggs' material have been constructed as exactly as possible with the help of their available published data, and we wish to apologise for any minor inaccuracies.

Fig. 1 *a* demonstrates how the proportion of recipient *R. pipiens* eggs, which develop into blastulae, decreases as the developmental stage of the donor embryo increases. The curve for recipient eggs which develop into apparently normal larvae falls more or less parallel to the curve for the blastulae. No metamorphosed frogs developed from these larvae. This is probably due to the difficulties in raising *R. pipiens* to the adult stage.

By comparing our results with those of Briggs and King it may be seen (Fig. 1 *a, b*) that fewer of the nuclei of early donor stages of *Xenopus* lead to the development of apparently normal blastulae, neurulae and larvae, but more of the nuclei of later donor stages lead to blastulae, neurulae, larvae and even metamorphosed adult frogs than is the case for *R. pipiens*.

Donor nuclei from post-blastula stages of *R. pipiens* show a progressive decline in their ability to promote normal development. A similar decline in *Xenopus* only becomes apparent from the transfer of nuclei from post-neurula embryos and later stages.

These differences in the results are probably due to two factors, the first technical, the second of great importance to the question of nuclear differentiation. First, many of our *Xenopus* eggs are of bad quality and do not develop normally after fertilization. This is probably due to the keeping and rearing of the frogs in laboratory conditions that can never quite replace the normal environment. At the time the eggs are used as recipients, not all the bad ones can be distinguished from the good ones.

Second, *Xenopus* develops three times as fast as *R. pipiens* at the temperatures used, and because nuclei in mitosis can probably not be transplanted successfully, it is likely that more transfers will fail for this reason in our experiments than in those of Briggs and King. Here are two situations leading to abortive transfers; both are likely to be more frequent in experiments on *Xenopus* than in those on *R. pipiens*, and must be borne in mind when results from the two species are compared. The low proportion of transplant late

blastulae of *Xenopus* that attain later embryonic stages is due, at least in part, to a tendency of the surface coat to disintegrate, causing the death of the late blastula or the early gastrula. Immature eggs are particularly prone to this disturbance, and different batches of eggs are exceedingly variable with respect to it.

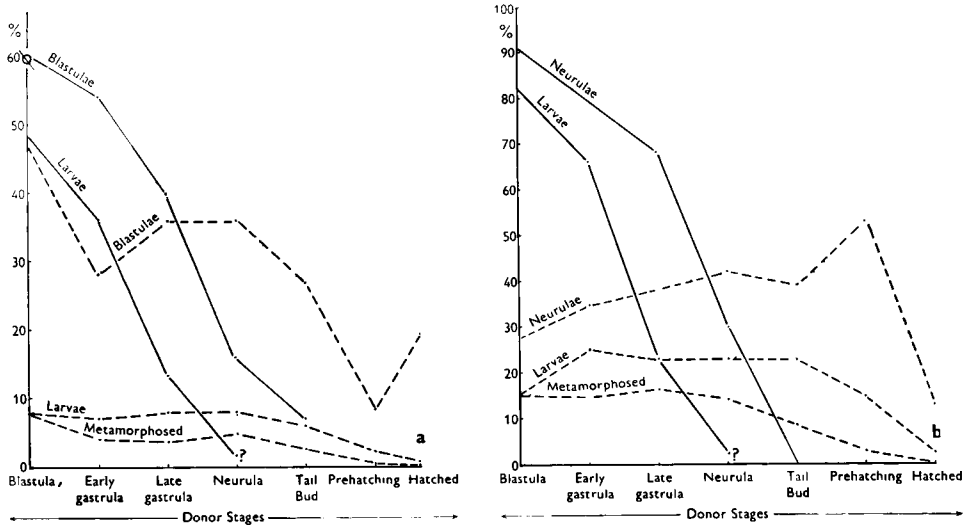


Fig. 1.—Development of transplant-embryos of *Xenopus laevis* (dotted lines) and *Rana pipiens* (continuous lines), from different donor stages. a, Percentages in terms of total transfers made; b, Percentages in terms of all transplant-embryos which reached normal late blastulae.

These reasons can explain the differences between the curves for *Xenopus* and *Rana* up to the point where the curves cross. The much better development of our recipient eggs which received a nucleus from late donor stages can be interpreted on the assumption that nuclear differentiation in *Xenopus* progresses less rapidly than in *R. pipiens*.

The data on the survival of transplant embryos of *Xenopus* show the same fundamental trends as those for *R. pipiens* and can be interpreted according to Briggs and King's hypothesis of nuclear differentiation. At present we have no adequate observations to confirm Briggs and King's finding that the abnormalities of transplant embryos are as specific to the origin of the donor nucleus as these authors claim.

SERIAL TRANSFER OF EMBRYONIC NUCLEI

Embryonic nuclei from the same region of a single donor embryo, when transplanted into recipient eggs, give rise to embryos exhibiting a considerable

variation of morphological abnormalities. This variation is rather difficult to interpret in the material so far demonstrated. It is desirable to find out if this variation is the result of different degrees of injury to the nuclei during transfer, or if it is the expression of natural heritable differences between the

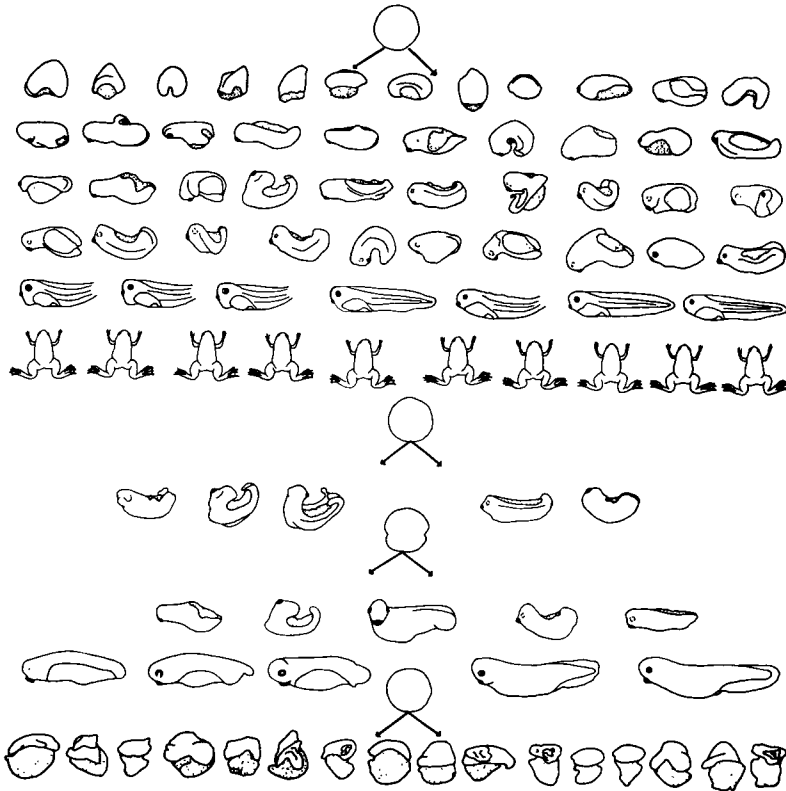


Fig. 2.—Serial transplantation of endoderm nuclei in *Xenopus laevis*; 1st (original), 2nd, and 4th donors: late blastulae. 3rd donor: incipient exogastrula. Endoderm nuclei were used for all transfers. Dotted areas in Figs. 2–6 represent exposed endoderm.

nuclei of the same donor region. King and Briggs [5] tried to solve this question by transferring nuclei serially. They first transplanted endoderm nuclei of a certain donor stage of *R. pipiens* into recipient eggs. When these reached the blastula stage they transferred the nuclei from the animal pole region of one of these embryos into other recipient eggs. They were able to produce in this way a clone of embryos containing nuclei derived from one original nucleus of the initial donor. This is the meaning of the term “clone” henceforth.

This procedure can be continued at will to produce third and fourth transfer generations of the clone derived from a single nucleus of the original donor.

Briggs and King found that members of a clone were strikingly similar, and that clones differed markedly from one another in the developmental

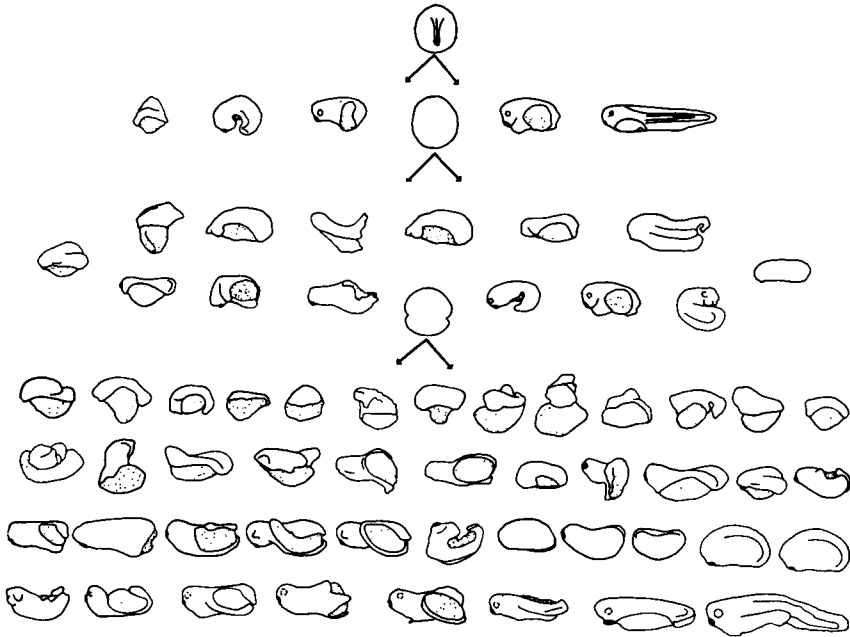


Fig. 3.—Serial transplantation of endoderm nuclei in *Xenopus laevis*. 1st donor, incipient neurula; 2nd donor, late blastula; 3rd donor, incipient exogastrula. Endoderm nuclei were used for all transfers.

stages reached by their members. The particular characteristics of a clone were maintained through a third and fourth transfer. Briggs and King concluded from the results of their single and serial transfers that blastula nuclei are totipotent and that post-gastrula nuclei had undergone highly stabilized heritable changes.

The possibility that irreversible nuclear changes occur during development being one of such outstanding interest, it was decided to perform similar experiments on *Xenopus*.

The experiments are of two types, first, the production of parallel clones (see Figs. 5 and 6). Two or more blastulae from the first transfer generation are used to produce separate clones; the nuclei within each clone stem from a single nucleus of the original donor. Second, serial transfer through several

generations as described above (see Figs. 2, 3, and 4). Only those embryos that developed beyond the late blastula stage are represented in the figures, except for Fig. 6, which includes arrested late blastulae.

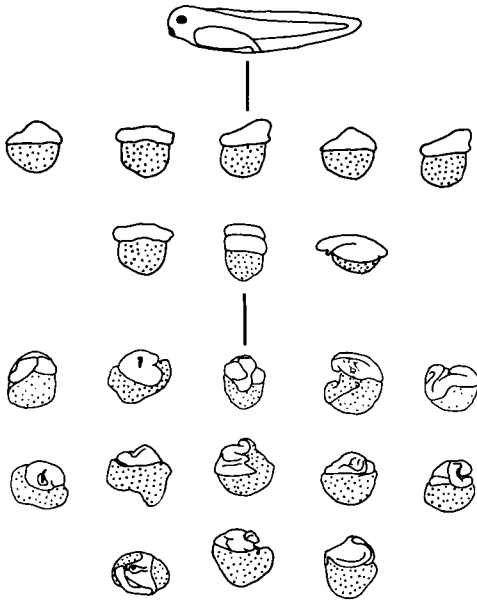


Fig. 4.—Serial transplantation of endoderm nuclei in *Xenopus laevis*; 1st donor, hatched tadpole; 2nd donor, exogastrula. Endoderm nuclei were used for all transfers.

Serial Transfer of Endoderm Nuclei

The initial donor nuclei of the first experiment were derived from endoderm of a late blastula; the result is shown in the upper part of Fig. 2. This result is typical for endoderm from early embryonic stages. The second and fourth generation donor nuclei were also taken from endoderm of a late blastula, the third generation from endoderm of an incipient exogastrula.

The variation in the second clone generation seems greater than the first or third generations. The third generation shows a deterioration, possible explanations for which will be dealt with later (p. 169).

Another series began with endoderm nuclei from a late gastrula. Three parallel clones were made from members of the first transfer generation; two of those donors were entering the neural plate stage, the third was an exogastrula. One of the clones from a neural plate stage was similar to the first transfer generation which ranged from exogastrulae to metamorphosed frogs. The other two clones showed a marked deterioration compared with the first transfer generation; the most advanced embryos in the clones were, in the case

of the early neural plate donor, an abnormal young larva, and in the case of the exogastrula, early postneural stages.

The third series is shown in Fig. 3. The initial donor was an embryo entering

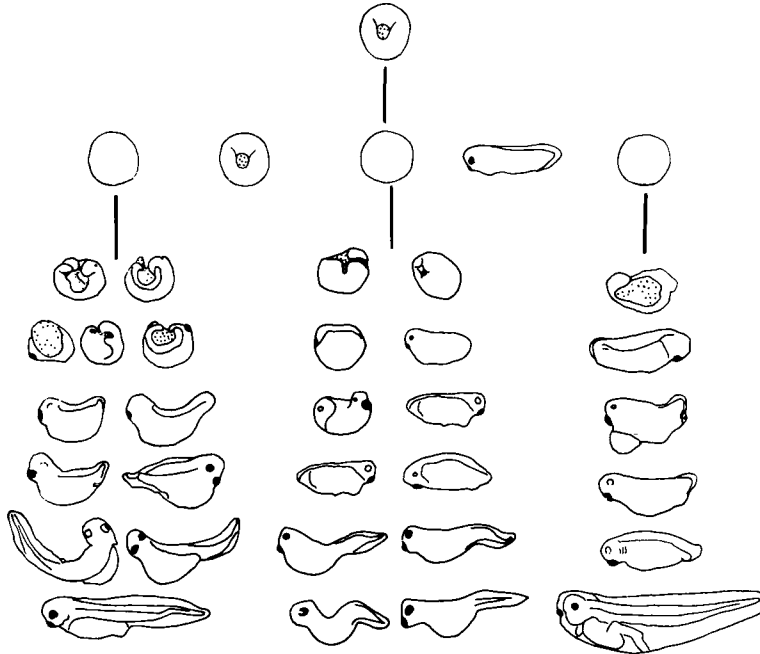


Fig. 5.—Serial and parallel transplantation of mesoderm nuclei in *Xenopus laevis*. 1st donor, late gastrula (mesoderm nuclei). 2nd donors for parallel series, 3 late blastulae (animal hemisphere nuclei).

the neural plate stage. The results of this transfer range from an ~~exogastrula~~ to a nearly normal tadpole. The clone derived from the endoderm of a late blastula is less variable and centres around abnormal gastrulae, some of which have developed appreciable inductions. A further generation of this clone derived from an incipient exogastrula is similar, but the range is greater.

The fourth series shown in Fig. 4 began with endoderm nuclei from a young tadpole. The first generation consisted of exogastrulae; one of these was used to produce a clone, the members of which exhibited an even more extreme exogastrulation than the first transfer generation.

Serial Transfer of Chordamesoderm Nuclei

The initial donor of the first series shown in Fig. 5 was a late gastrula. Parallel clones were derived from the animal hemisphere nuclei of three

late blastulae from the first transfer generation. The three clones are similar one to another, but their similarity to the first transfer generation is uncertain, due to the small numbers in the latter.

The second series (see Fig. 6) derives initially from somite mesoderm nuclei from a neural fold embryo due to give muscular responses within nine hours. The first generation shows the greatest possible variation, ranging from arrested late blastulae to metamorphosed frogs. Parallel clones from animal hemisphere nuclei from two late blastulae were produced. The right-hand clone in Fig. 6 exhibits the same range as the first transfer generation, but it is to be noted that, with the exception of one, all the embryos fall into two classes: those arrested before or entering gastrulation, and those developing into normal or abnormal larvae. The second clone showed almost no ability to enter gastrulation. Judged on the number of primary axes produced, these two clones are significantly different from one another at the 1 per cent level.

Discussion

Some general comments on the results of serial transfer of endoderm and mesoderm nuclei can be made.

The donor embryos from which the clones derived can be considered as a random sample of their own transfer-generation that passed through the blastula stage. The variation within a clone is usually less than that within the initial transfer generation, and is limited by the range between the arrested late blastula and the most advanced embryos. For this reason transfer generations are most readily compared by their most advanced embryos, and on this basis there are obvious, marked differences between the clones produced by serial transplantation. Similar large differences between clones are found in Briggs and King's data, but here the variation within clones seems to be much less than in *Xenopus*. In many cases their clones form a homogeneous group of embryos, narrowly centred about a particular developmental stage, the spectrum of less well-developed embryos of preceding stages being sometimes absent and often fragmentary.

Part of the heterogeneity within a transfer generation, which distinguishes the *Xenopus* results from those on *R. pipiens*, may be due to the variable quality of the eggs. It has already been stated that some eggs from our frogs fail to develop normally after fertilization. Gastrulation seems to be an especially critical stage for these embryos and most die at this time. The most significant effect of this on our experiments is that a variable proportion of transfers will fail to complete gastrulation normally, regardless of the nucleus introduced,

due to inherent deficiencies in some of the recipient eggs. This will account for at least a part of the greater variability within *Xenopus* clones compared to *Rana*, but it would be quite unsound to account for all the transfers that fail to gastrulate normally by this cause. Consider for example Fig. 6 where

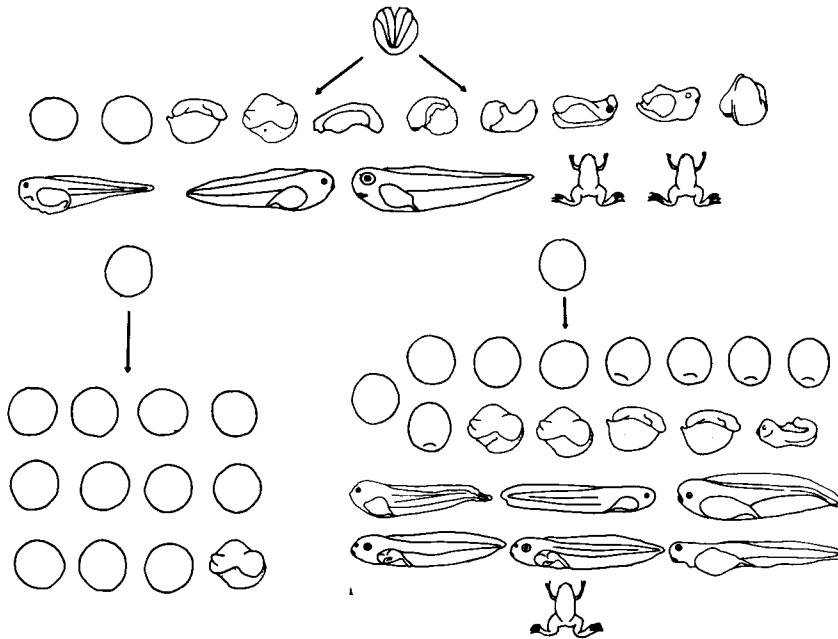


Fig. 6.—Serial and parallel transplantation of mesoderm nuclei in *Xenopus laevis*. 1st donor, neural fold stage (mesoderm nuclei). 2nd donors for parallel series, 2 late blastulae (animal hemisphere nuclei).

the two parallel clones represent a random sample of recipient eggs from the same ovulation.

The decline in the ability of transferred nuclei from progressively older embryos to promote normal development could be due to damage during transfer. Briggs and King have argued that the reduced variability within a clone and the marked differences between clones and their stability on repeated transfer renders this possibility unlikely. If damage were invoked to explain the results of serial transfer experiments, certainly rather special additional assumptions would be necessary. This is not to say, however, that nuclei are not susceptible to injury during transplantation. It is notable from Briggs and King's material that clones never improved on repeated transfer and sometimes deteriorated, and this might be a measure of the incidence of damage.

King and Briggs [5] claim that embryos derived from nuclei of the different germ layers develop somewhat differently and display different syndromes of abnormalities. A close examination of *Xenopus* transfers for such differences has not been made but, if Briggs and King's claim is correct, further additional assumptions would be necessary before a hypothesis of nuclear damage could be accepted. Briggs and King have injected cytoplasm from embryonic nuclei into fertilized eggs, and found that it has no effect on development. They have rejected on theoretical grounds the possibility that a portion of the cytoplasm adhering closely to the transferred nucleus might be responsible for their results. The assumption that the treatment to which the nuclei are subjected during transplantation causes heritable changes requires to be proven as vigorously as any other hypothesis. It is a limitation of the nuclear transplantation experiments so far performed that, although they demonstrate differences in the potentialities of embryonic nuclei, they can shed no direct light on how these differences originate. Considering all the evidence, it is our opinion at the present time that the most plausible origin of these differences is King and Briggs' hypothesis of a natural, progressive, nuclear differentiation during embryonic development, related in some way to the cytoplasmic environment of the nuclei. This conclusion could also explain part of the variability of members of those clones in *Xenopus* which derived from second and third donors which had passed the blastula stage. In these donors additional nuclear differentiation may have occurred. This hypothesis is supported by the experiments of one of us (J. B. G.), described in the next section, demonstrating that specific cytoplasms can induce changes in the potentialities of nuclei.

NUCLEAR TRANSFER BETWEEN SPECIES

Introduction

Homospecific transfers (transfers within a species) have shown that nuclei become differentiated. This is probably due to an effect of the different kinds of cytoplasm in which the nuclei lie. It seemed that the fact that cytoplasm can change nuclei could be most clearly demonstrated by heterospecific transfers (transfers between species); this would increase the contrast between nucleus and cytoplasm and so make any effect more easily seen.

Xenopus laevis and *Rana temporaria* proved to be suitable animals for this work; as has been said above enucleation is not necessary in *Xenopus*, but it is required in *Rana* and has been carried out by a modification of Porter's technique. The technique has been found to be 96 per cent successful. The

few cases when the female pronucleus, through not having been removed, has participated in development, can be recognised by the approximate chromosome number of such embryos; the female pronucleus appears not to double itself from the gynogenetic haploid state, and only diploid transplant-

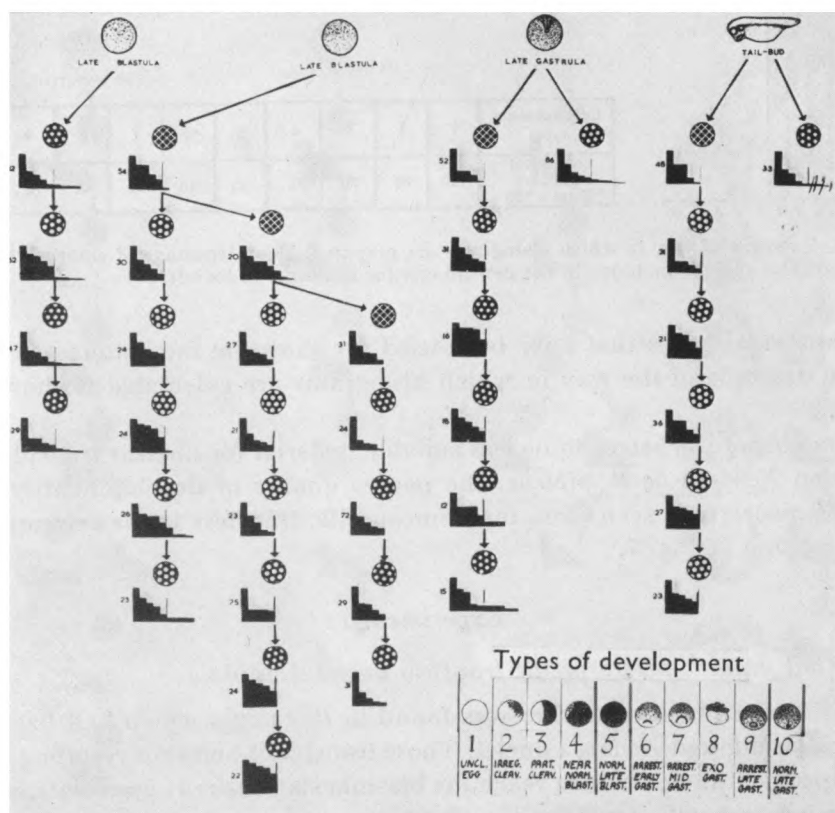


Fig. 7.—The serial transplantation of nuclei between *Rana temporaria* and *Xenopus laevis*. Experiments using three different donor stages of *Rana temporaria* as original donors. From each donor both homospecific and heterospecific transfer series were made. Three heterospecific series were derived from the blastula donor, when *Rana* nuclei were left in *Xenopus* cytoplasm for 1, 2, and 3 generations before "back-transfer" to *Xenopus*. See also legend to Fig. 9 for explanation of diagrams.

embryos have been used as donors for serial transfers. When making serial transfers only late blastulae were used as donors, and all the remaining blastulae of each group have been allowed to develop as far as they were able.

The results of these experiments have been expressed by using a histogram

to represent the proportions of each kind of abnormality present in the direct product of each single donor embryo. The numbers of each developmental type are expressed as percentages of the total transfers done per histogram; this total is represented by the extreme left block of each histogram. The

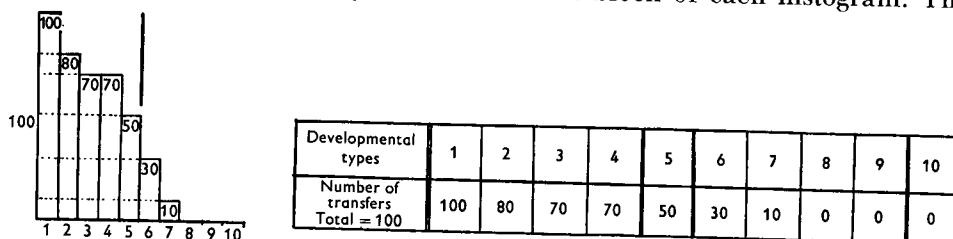


Fig. 8.—Example of way in which histograms are prepared. The percentage of embryos of each developmental stage is included in the percentages for all stages to its left.

developmental types that have been used are shown at the bottom of Fig. 7, and an example of the way in which histograms are calculated is shown in Fig. 8.

Rana temporaria seems to be less suitable material for nuclear transplantation than *Xenopus* or *R. pipiens*; the poorer quality of development typical for *R. temporaria* is seen from the homospecific transfers in the extreme left-hand column of Fig. 7.

Experiments

Proof that genuine heterospecific transfers have taken place

The *Xenopus* nuclear marker was found in *Rana* eggs which had been injected with marked *Xenopus* nuclei. Those transplant-embryos resulting from homospecific transfers which reach the blastula stage usually gastrulate, while similar heterospecific transfers usually do not.

Effect of foreign cytoplasm upon nuclei

Rana nuclei transferred to *Xenopus* cytoplasm and then back to *Rana* cytoplasm (Fig. 7, 2nd column, 2nd row) do not gastrulate—shown by the fact that there is no black beyond the vertical line in the histogram; this line at Stage 5 (see developmental types, Fig. 7) represents late blastulae; but *Rana* nuclei transferred to *Rana* cytoplasm (Fig. 7, 1st column, 1st row) do show some gastrulation, since there is some black on the right-hand side of the vertical black line.

This experiment shows that nuclei have been in some way affected by the

foreign egg-cytoplasm. This effect has already been reported by J. A. Moore (unpublished) from work on heterospecific transfers between *R. pipiens* and *R. sylvatica*. This effect has been confirmed cytologically in *Rana-Xenopus* transfers, since the nucleoli of heterospecific transfers in both directions are

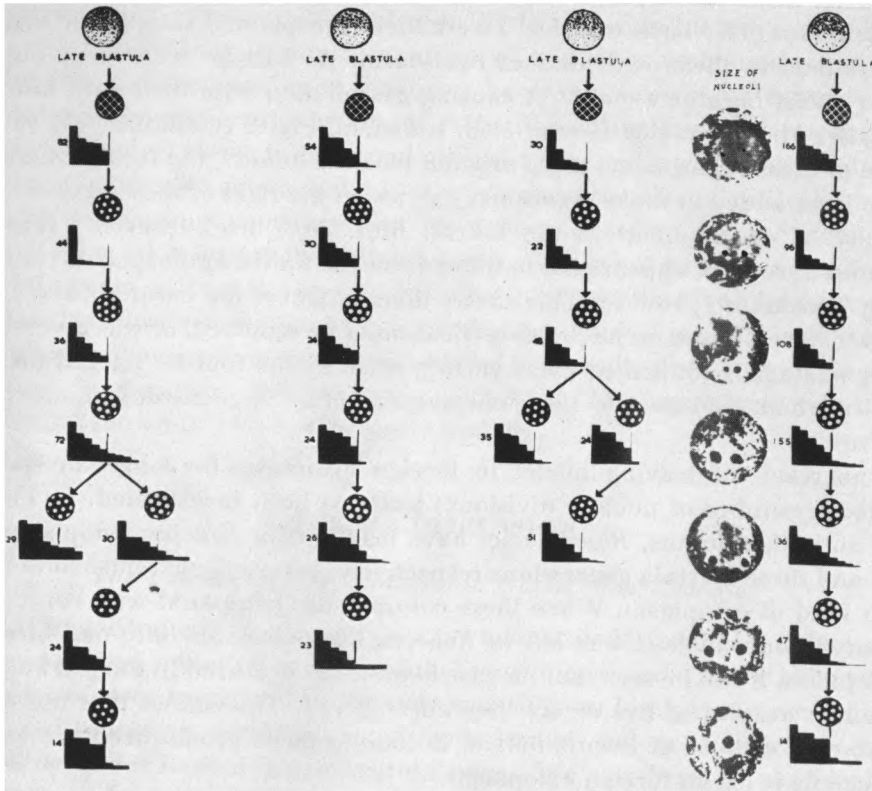


Fig. 9.—The serial transplantation of nuclei between *Rana temporaria* and *Xenopus laevis*. Three experiments using three different late blastulae of *Rana temporaria* as original donors. On the right are shown the combined results of these three series with photographs representing typical nucleoli for each transfer generation. Arrows indicate new transfer generations. Black discs with white cross-markings = recipient eggs of *Xenopus*. Black discs with white spots = recipient eggs of *Rana temporaria*. Histograms represent the quality of development of the recipient eggs directly above them.

very small (Fig. 9, upper two photographs of nucleoli) while nucleoli in homospecific transfers are of the usual large size seen in normal late blastulae (comparable to bottom nucleolus photograph, Fig. 9). As nucleoli are considered to be concerned with RNA and protein synthesis, and as some protein

synthesis seems necessary for gastrulation but not cleavage, this diminished nucleolar size is correlated with failure to gastrulate.

An analysis of the nature of this cytoplasmic effect upon foreign nuclei

Nuclei which had been transplanted into foreign cytoplasm and then back to their own (1st "back-transfer") were then transplanted serially for several generations in their own kind of cytoplasm. As can be seen from Fig. 9, these nuclei became capable of causing gastrulation with their own kind of egg after about three or four of such transfers. Fig. 9 (columns 1-3) shows three of these experiments using original blastula donors, the results of which have been added to make a summary series on the right of these figures. The quality of development in the fourth, fifth, etc. "back-transfer" (Fig. 7, column 2, row 5), appears not to differ from the fourth homospecific transfer (Fig. 7, column 1, row 4). This shows that, whatever the nature of the effect of foreign cytoplasm on nuclei, this effect could be removed, or was reversible. This was again confirmed cytologically, since by the fourth "back-transfer" nucleoli had increased to their normal size (Fig. 9, nucleolus-photograph, 4th row).

The result of leaving nuclei in foreign cytoplasm for longer or shorter periods (number of nuclear divisions) has also been investigated. In Fig. 7, 3rd and 4th columns, *Rana* nuclei have been left in *Xenopus* cytoplasm for two and three blastula generations respectively, before being returned to their own kind of cytoplasm. When these columns are compared with column 2, when a *Rana* nucleus was left in *Xenopus* cytoplasm for only one blastula generation, it can be seen that no gastrulation has occurred in the 3rd and 4th columns even after five or six "back-transfers". This shows that the effect of foreign cytoplasm is cumulative, becoming more pronounced the longer a nucleus is left in foreign cytoplasm.

Lastly, the result of using different donor stages for these experiments has been investigated. When nuclei from tail bud *Rana* embryos are transplanted homospecifically, the majority of the resulting blastulae do not gastrulate and, as might then be expected, no gastrulation took place after 4, 5 or 6 "back-transfers" in the heterospecific series (Fig. 7, 7th and 8th columns). Similarly, late gastrula nuclei usually do gastrulate in homospecific transfers, and also do so in heterospecific transfers again after about four "back-transfers" (Fig. 7, 5th and 6th columns). These experiments confirm the results so far obtained and give additional proof that the improvements of the fourth "back-transfers" are not simply a result of the participation of the female pronucleus.

Conclusions

In these experiments the possibility that technical damage can influence the results can be excluded. This is because the technique has been the same for all series (including the constant use of the same donor stage) so that any change which occurred would apply equally to all series; but the conclusions have only been drawn from a comparison of one series with another.

The only definite conclusions that can be drawn from these experiments are that nuclei are affected when made to divide and synthesis in foreign cytoplasm. This effect seems to be cumulative and eventually reversible, and has also been confirmed cytologically by variations in the size of nucleoli.

There are various considerations from these experiments and from the work of others that lead to the belief that this effect is not a loss but a replacement of certain parts of the nucleus by other parts specific to the foreign cytoplasm. This effect would then be comparable to a kind of genetic transformation. This replacement-change is considered to be quite distinct from change resulting from natural differentiation; both kinds of change have occurred together as shown in Fig. 7, columns 7 and 8.

GENERAL DISCUSSION

The Potentialities of Embryonic Nuclei after Transfer

A transferred nucleus giving rise to a normal fertile individual is totipotent. Between 80 and 100 adult or near adult *Xenopus laevis* have so far been produced by nuclear transfer. Their donors ranged from blastula to prehatching stages [4], although ectodermal and mesodermal donors leading to adult frogs were so far limited to pre-neurula stages. We can therefore confidently claim that at least some of the somatic nuclei of these donor stages were still totipotent in respect of somatic differentiation. So far only one male deriving from late blastula endoderm nucleus has been tested for its fertility. It gave rise to normal offspring when mated to a control female.

The results of single and serial transfer of nuclei of *R. pipiens* [5] and *Xenopus* show clearly that many of the nuclei are, however, not able to give rise to normal development after transfer. In *R. pipiens* the majority, if not all, of the nuclei of a blastula are totipotent but their potentiality declines drastically in later stages. The experiments in *Xenopus* are not conclusive as to the totipotency of all the blastula nuclei. Their potentiality after transfer only falls significantly in post-neurula stages.

The serial transfers demonstrate clearly the heterogeneity of a nuclear population from a particular region of a donor embryo in respect of developmental ability.

The Probable Nature of the Loss in Potentiality of Nuclei after Transfer

Damage to some nuclei during transplantation cannot be denied, but it is certain that not all the nuclei suffered from it. Reasons have been given (pp. 169 and 175) why all the cases of abnormal development should not be interpreted in terms of damage. Our main conclusions are not invalidated by the fact that damage may have occurred in some cases. It can therefore be stated with confidence that natural progressive nuclear changes occur during differentiation. These are heritable and relatively stable.

Our experiments are unable by their nature to locate the site within the nucleus where these changes are produced. The heritability and relative stability of this nuclear differentiation suggest, of course, the chromosomes as the site. The chromosomes are, as far as we know, the only nuclear structures showing continuity through mitosis, all other parts of the nucleus being neogenic. It is therefore most likely that the nuclear changes occur in the chromosomes and, probably, at the level of genes.

More and more cases of irreversible nuclear changes induced by cytoplasmic factors have been brought to our knowledge. They include examples from Protozoa [2, 7], *Ascaris* and Gallmidges [8]. Moore demonstrated that the cytoplasm of *R. pipiens* altered the nuclei of *R. sylvatica* and our paper deals with the influence of *Xenopus* cytoplasm on *R. temporaria* nuclei (p. 172). There is therefore no doubt that cytoplasm can change nuclear qualities.

The nuclei of an amphibian embryo are derived by equational mitosis from the original zygote nucleus and are, as demonstrated by Spemann and also by King and Briggs [5] identical in early developmental stages. At later stages they are no longer identical. This can only be a result of the regionally-unequal distribution of the cytoplasmic qualities.

It is possible to think that the observed nuclear changes were brought about in the following way: initially identical nuclei reduplicate in cytoplasms of different composition. They have identical requirements for this synthesis but possibly not an identical supply of precursors. This might then lead to some of the genes not being able to reduplicate at all, by being starved of one or more necessary precursors. Other genes might synthesize a modified structure by the incorporation of substances of a similar, but not identical, kind. These genes will then be inactive or changed in their function.

The results so far obtained from homospecific transfers, that is, transfers

within a species, provide no evidence of improvement in these developmental potentialities after repeated transfer into the cytoplasm of uncleaved eggs. This rather points to an irreversible loss of capacity, perhaps due to gene starvation. This evidence is, however, weak as not enough generations of transfers have been carried out.

The results obtained from heterospecific transfers between *Rana* and *Xenopus* show, however, clear evidence of an eventual reversal of the nuclear change acquired while in the foreign cytoplasm. This phenomenon could be interpreted as genetic transformation, similar to bacterial transformation, and an eventual reversal of it.

Results from nuclear transfers indicate that relatively stable, nuclear changes occur; these changes are reversible in the case of heterospecific transfers. It cannot, however, be decided whether, during natural differentiation, gene starvation or gene transformation or both of these types of change can take place.

SUMMARY

1. The possibility that nuclear changes occur during embryonic development of amphibia has been investigated, using the technique of nuclear transplantation.

2. Nuclei taken from progressively older embryos, show a decline in their ability to promote normal development when transplanted into unfertilised eggs. The results from *Xenopus* transfers are compared with those of Briggs and King, using *Rana pipiens*.

3. Clones derived initially from a single embryonic nucleus have been produced by serial transplantation. The variation within a clone is limited. Although the characteristics of a clone are relatively stable over several transfer generations, clones can differ strikingly one from another.

4. Serial transfers between *Rana temporaria* and *Xenopus laevis* have shown that *Rana* nuclei are affected by *Xenopus* cytoplasm. This effect was shown to be cumulative but eventually reversible. The effect is believed to represent a genetic transformation.

5. The results of these experiments demonstrating differences in the potentialities of embryonic nuclei are discussed in relation to the technique.

6. It is considered at present that these differences can be most plausibly attributed to a natural, progressive differentiation of embryonic nuclei in response to regional differences in the cytoplasmic environment.

7. The possible nature of these nuclear cytoplasmic interactions is discussed.

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