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Abstract

A previously discovered but as yet undescribed mutation occurred in two separate spawnings of the same axolotl parents. The gene, designated p for premature death, is a simple Mendelian recessive lethal which in the homozygous condition, results in stasis of development at about stage 37, followed by disintegration of superficial tissue and eventual death of the affected embryos. Many of the abnormalities observed in sections of the stage 37 p/p embryo are related to the failure of this embryo to establish a functioning circulatory system, or to the resulting edema that distends the abdomen and flanks of the embryo. There are, however, several abnormalities which cannot be attributed to these conditions. All of these abnormalities can be indirectly related to the endoderm, particularly the anterior and dorsal endoderm of the developing p/p embryo.

Gynogenetic diploid axolotls were produced by activating eggs with ultraviolet-inactivated sperm, and then subjecting the activated eggs to heat shock. Optimal conditions for ultraviolet inactivation of the sperm, and for suppression of the second meiotic division by heat shock, were established. Gynogenetic diploids produced by these procedures included progeny homozygous

for recessive alleles carried by a heterozygous mother. Gynogenesis could, therefore, be used to uncover new mutations more rapidly than by conventional inbreeding techniques. However, some difficulty was encountered in recognizing mutant phenotypes because of the high incidence of abnormalities and deaths. Defective embryos probably resulted from a combination of heat-shock induced damage to the eggs and the expression of deleterious recessive alleles carried by the mother. Other potential uses for diploid gynogenesis, such as measuring gene-kinetochore distances, are discussed and examples provided.

RÉSUMÉ

Une mutation déjà découverte, mais pas encore décrite, s'est produite au cours de deux frais différents des mêmes parents d'axolotl. Le gène, p pour désigner la mort prématurée, est un caractère mendélien simple létal qui, dans la condition homozygote, résulte dans un arrêt du développement à peu près au stage 37, suivi par la désintégration des tissus superficiels et par la mort éventuelle des embryons affectés. Plusieurs des anormalités observées dans des sections d'embryon p/p au stage 37 sont reliées à l'impossibilité qu'a cet embryon à établir un système circulatoire fonctionnel, ou à l'oedème résultant qui distend l'abdomen et les flancs de l'embryon. Il y a , cependant, plusieurs anormalités qui ne peuvent pas être attribuées à ces conditions. Toutes ces anormalités peuvent être reliées à l'endoderme, particulièrement à l'endoderme antérieur et dorsal de l'embryon p/p en croissance.

Des axolotls diploïdes gynogénétiques furent produits en activant des oeufs avec du sperme inactivé avec la lumière ultra-violette, et ensuite en soumettant les oeufs activés au choc thermique. Les conditions optimales pour l'inactivation du sperme avec la lumière ultra-violette et pour la suppression de la deuxième division méiotique par le choc thermique ont été établies. Les diploïdes gynogénétiques produits avec ces procédures comprennent des descendants homozygotes pour les allèles récessifs de la mère hétérozygote. La gynogenèse peut donc être utilisée pour démasquer des nouvelles mutations plus rapidement qu'avec des techniques conventionnelles de croisements consanguins. Cependant, une certaine dif-

ficulté à été rencontrée pour reconnaître les phénotypes mutants à cause d'une forte incidence d'anormalités, et de décès. Les embryons défectueux résultent probablement d'une combinaison de dommages occasionnés aux oeufs par le choc thermique et par l'expression de gènes nuisible nécessifs de la mère. D'autres applications possibles de la gynogenèse diploïde, comme pour mesurer les distances gène-kinétochore, sont discutées et des exemples sont fournis.

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Introduction

The amphibian embryo has long been a favorite organism for the study of embryology and genetics. The availability of various species, and the ease with which they may be bred and raised in the laboratory have contributed to their popularity in these fields. Amphibian eggs are relatively large and develop externally, facilitating observation during morphogenesis, and enabling operative procedures such as microsurgery and microinjection to be carried out with relative ease. Since the females typically lay large numbers of eggs, abundant material is present for biochemical and genetic studies. Since they are poikilothermic, the rate of development of amphibian embryos can be controlled within wide limits, and any desired stage from oogenesis to metamorphosis can be obtained (De Berardino, 1966). Malacinski and Brothers (1975) have cited a variety of genetic manipulations that can be carried out in the amphibian embryo. These include the production of haploid or polyploid individuals (Fankhauser and Humphrey, 1942; Sladecek and Lanzova, 1959), and production of gynogenetic or parthenogenetic individuals (Nace, Richards and Asher, 1970; Jones, Jackson and Whiting, 1975). Nuclear transplantation can be used to produce isogenic strains of amphibians (Gurdon, 1964).

The axolotl, Ambystoma mexicanum, is a neotonous salamander and is normally aquatic for its entire life cycle (Brunst, 1955). Mature females lay several hundred eggs, yielding statistically significant ratios for genetic studies in a single spawning. The generation time of the axolotl is about one year, and mature females can be hormonally induced to spawn every three months (Ketterer and Forbes, 1972). Each of the stages in the life cycle can be readily observed, and because of their large size (2mm), the egg and embryo are easily surgically manipulated. Perhaps the greatest single advantage of the axolotl is that the largest array of mutant genes in any single species is available in this animal (Humphrey, 1975). To date, some thirty-three genes are known that affect oogenesis and embryonic or larval development of the axolotl. The discovery of most of these genes is a result of thirty years of inbreeding and analysis by R.R. Humphrey, currently at Indiana University. These genes have been placed into five groups according to their effect on development (Briggs, 1973; Malacinski and Brothers 1975). These groups are; the maternal effect genes; those genes whose effect is on specific organs or tissues; those that affect the nucleolus; those that affect pigment cell differentiation and/or patterns formed by pigment cells; and finally, those genes that affect cell and tissue function.

In attempts to characterize phenotypic changes that result from genetic mutations, the developmental geneticist has two powerful tools with which to work. These are reciprocal grafting of various organ primordia between mutant and normal embryos, and parabiosis. Grafting experiments have long been used in genetics to determine the competence of mutant tissue to differentiate into normal primordia, or to test the ability of mutant hosts to induce a normal graft to differentiate into normal primordia. Such experiments are of particular value when the mutation affects a single organ or tissue, and genes of this group (those that do affect a single organ or tissue) are important for their potential usefulness in studies of embryonic induction (Briggs, 1973). Reciprocal transplantations are less useful when the gene in question is an autonomous lethal. Autonomous cell lethals are defined as genes which exert lethal effects which cannot be corrected by parabiosis of mutant embryos with normal ones, nor by grafting of organ primordia to normal hosts. The effects are thus autonomous in the sense that they cannot be corrected by substances capable of diffusing across cell membranes from the normal co-twin or host (Chung and Briggs), 1975. The best way to determine whether a mutation is an autonomous lethal is to parabiose mutant and normal embryos. From such experiments three results are possible. First, the mutant can be completely corrected by parabiosis. This result would indicate that the mutation can be corrected by a humoral agent.

transmitted from normal to mutant either by common circulation or by diffusion, and thus demonstrate that the mutation in question is not an autonomous lethal. The second possibility is that the defect can remain uncorrected in the mutant, but the normal partner is able to supply the necessary environment for the mutant to develop normally in all aspects except that organ or tissue which is affected by the mutation. The third type of result is that the mutant does not derive any benefit from its normal partner. They may survive longer than they would by themselves, but are otherwise unimproved (Chung and Briggs, 1975). This result indicates that the mutation is such that it cannot be corrected by any diffusible substances from the normal partner. The fact that all parts of these embryos eventually die indicates that the gene in question affects all of the organs and cell types. By definition, mutations such as these are autonomous lethals.

Two groups of genes in the axolotl are of particular interest. Genes which exert effects on the egg cytoplasm, the maternal effect genes, are of special embryological interest since they may provide new means of attacking the problem of how the egg cytoplasm controls early embryonic development (Briggs, 1973). The recessive gene f, characterized by Humphrey (1960), is such a gene. The gene f modifies the egg cytoplasm in such a way as to lead, during cleavage, to an excessive accumulation of fluid in the blastocoel. In F/f embryos the excess fluid is eliminated during gastrulation, but in f/f embryos from

f/f mothers the accumulation persists and all the embryos die. Embryos homozygous for f from F/f mothers are usually evident in early tailbud stages; excess fluid then usually produces a detectable enlargement of the head. This may be followed by the formation of blister like elevations dorsal to the branchial arch region. It is believed that the excess fluid is primarily a product of the endoderm, since the trunk also becomes distended. The excess fluid usually disappears as the circulation develops, and the embryos develop at the same rate as their normal sibs. Embryos that are homozygous f/f from f/f mothers never survive to hatching. Development of the head is retarded and fluid appears in the suprabranchial region, although there is a marked accumulation in the trunk. The foregut region shows no lumen. Development of the gills is either greatly delayed and reduced, or completely suppressed, with the head remaining subnormal in size, conditions which probably relate to the complete absence of circulation. F/f embryos are generally wider and show a dorso-ventral increase in measurement in the liver region. The head is noticeably subnormal in size, a feature probably resulting from the neural plate suffering some retardation of growth during the earlier period of marked distension. Since the accumulation and elimination of excess fluid involves a variable amount of damage to the midpart of the belly, in which region the blood island is situated, it may sometimes result in the failure of circulation to become established.

The second group of genes of exceptional embryological interest are those with specific effects on single organs or related groups of organs. These genes may provide a basis for examination of inductive relationships during development, and may provide a system whereby the molecular basis of specific inductive processes may be examined (Malacinski and Brothers, 1975; Briggs, 1973). Embryos that are homozygous for the gene c do not develop a functional heart. The initial heart development is normal, but c/c embryos fail to establish a heartbeat. Subsequently, the heart becomes distended and remains thin walled, and the animals become edematous. The affected animals survive for about twenty days after the time when the heart would normally have begun to beat. Mutants are capable of normal swimming movements, suggesting that the cardiac lethal mutation does not affect skeletal muscle (Lemanski, 1973). Because of the absence of circulation, the gills remain small, each showing only a few filaments. Since the intermedin produced by the hypophysis is not distributed to the more distal parts of the body, the melanophores of the tail and caudal half are contracted. Diffusion of this hormone through the tissue causes expansion of these pigment cells over the head and cranial portions of body. The larvae of c/c genotypes begin swimming much as do the normals, move in response to stimuli, and right themselves if inverted. They do not eat, and since there is no circulation, growth in any part ceases when its own yolk stores are

exhausted. Renal function is lacking, and a gradual accumulation of fluid converts the body to the pear shaped form that first attracted attention to this mutant type (Humphrey, 1972). Through parabiosis and reciprocal grafting Humphrey further demonstrated that the gene c specifically affects the heart, and that this gene affects not the heart mesoderm itself, but rather the endoderm, which provides the specific inductive influence for heart development (Jacobson, 1960). Fine structure studies have demonstrated that mutant myocardial cells fail to differentiate organized, functional myofibrils (Lemanski, 1973).

A third group of genes are those that affect the nucleolus. All three genes of this group act to reduce the size of the nucleolus. The molecular basis for the reduction in size is unknown, but these mutants should prove to be beneficial to studies of nucleolar function (Carroll, 1972).

The fourth group of mutant genes are those which affect pigment cell differentiation and/or patterns formed by pigment cells. The wild type axolotl has three types of pigment cells in the dermis. These types are: melanophores, which produce the black or brown pigment of melanoproteins; xanthophores, which produce yellow pigment derived from pteridines; and iridophores, which produce iridescent white pigment from purines. In young wild type

larva approximately equal numbers of melanophores and xanthophores give the dorsal surface a mottled dark green appearance. Iridophores are present on the ventral surface, on the gills, and in the iris of the eye (Benjamin, 1970). The d gene in the homozygous condition results in a reduction in the numbers of melanophores and xanthophores, and restricts them to the dorsal surface, giving the animal a white appearance .. This mutation is such that it is the skin of the white animal that prevents the migration of the propigment cells into it, or prevents their subsequent differentiation (Dalton, 1949). A second mutation is the melanoid variant. Homozygous m/m larvae develop a velvety black appearance due to an increase in the numbers of melanophores and a decrease in the number of xanthophores. The genes d and m are non-allelic, and animals that are homozygous d/d and m/m are recognizable by their lack of iridophores (Humphrey and Bagnara, 1967). A third gene that affects the pigmentation of the axolotl is the recessive gene determining the albino phenotype, designated a. Since the albino gene was known in Ambystoma tigrinum, but not in Ambystoma mexicanum, Humphrey devised and carried out a brilliant series of experiments utilizing the techniques of artificial insemination, nuclear transplantation from non-viable hybrid blastula, and gonadal transplants, to introduce this gene into the axolotl genome (Humphrey, 1967). Like the m gene, the a gene is

recessive. The only known effect of the homozygous a/a condition is to prevent melanogenesis in axolotls of any genotype, although the affected animals do differentiate melanoblasts. The a gene appears to have no effect on either xanthophores or iridophores (Benjamin, 1970). The fourth gene which affects pigmentation is also a simple, Mendelian recessive, axanthic (ax). Homozygous ax/ax animals appear normal except for their lack of visible xanthophores and iridophores. It appears that the axanthic trait acts at the level of the xanthoblasts, blocking the synthesis of pteridines which are responsible for the yellow colour of the xanthophores (Lyerla and Dalton, 1970). In addition to these four characterized colour variations, a fifth has been observed in our lab. The melanoproteins produced by this variant appear to be identical in number and distribution to those of a homozygous (d/d) white larva, but the xanthophores appear to be either normal or increased in numbers, and there is an increase in the numbers or iridescence of iridophores. The result of these variations in pigments is a gold or yellow colored larva with extremely iridescent areas, particularly on the gills, flank, and in the iris of the eye. Should further genetic and experimental studies establish this variant as a mutant, it could prove valuable in determining the ultimate phenotypic expressions of the genes involved in pigmentation in the axolotl.

These pigment cells provide model systems for the study of cell differentiation and cell-cell interactions. They are useful for studies on melanogenesis, and can be used as indirect markers for embryological analysis, and direct markers for such techniques as gynogenesis, androgenesis, and nuclear transplantation. In addition, the complete absence of melanin granules in the albino facilitates the examination of histological sections with various autoradiographic techniques (Malacinski and Brothers, 1975).

The fifth group of genes is those classified as autonomous lethals. Each mutant in this group consistently exhibits its own syndrome of abnormalities, but all are unable to be rescued by parabiosis with normal embryos, nor will grafts of mutant organ primordia survive on normal hosts. This indicates that mutants of this group are suffering from alterations, presumably deficiencies, in essential cell components which are common to most or all parts of the embryo, and which cannot move from cell to cell (Briggs, 1973). The gene l is such a recessive autonomous lethal. Homozygous l/l embryos develop normally to an advanced embryonic stage (Harrison stage 40), at which time they begin to show abnormally small heads, small and poorly developed eyes, poorly developed gills, undifferentiated limb buds, and reduced growth rate. They may feed briefly, but soon stop and invariably die within a few weeks of hatching. Reciprocal grafting and

parabiosis have demonstrated that this is indeed an autonomous lethal. The molecular nature of the effect of the gene l is unknown, as is that of most of the other autonomous lethals in the axolotl (Chung and Briggs, 1975). The ability of homozygous recessive mutants to develop to advanced embryonic stages, and their ability to differentiate functional cells of many types in the absence of the normal alleles of the lethal genes can be explained by two alternate theories. The first is that the various cell types come to require new gene products at the time when the lethal effects are expressed. This cannot be excluded, but seems somewhat unlikely since there is no evidence that the lethality is expressed only at transition points, when new cell functions are developing which might require new gene products (Briggs, 1973). The second explanation is that substances produced and stored in the egg during oogenesis by a heterozygous female support development, and development halts when additional synthesis of these essential substances is required (Humphrey, 1975). It is possible that after further investigation many of the mutant genes now classified as cell lethals will be found to have more subtle cell or tissue specifities and be of considerable value in embryological analyses (Malacinski and Brothers, 1975).

At present, thirty-three genes are known in the axolotl, many of which may be useful in elucidating the normal path

of embryonic development. The characterization of each genetic mutation is a prerequisite for determining the usefulness of each new gene for studies in developmental genetics. Part of this thesis is devoted to a preliminary morphological and histological description of a previously discovered but as yet undescribed mutation, premature death, (p), in the axolotl.

In recent years, interest in vertebrate genetics has increased as many biologists turn from relatively simple organisms to the study of such complex problems as development. Attempts to adapt the powerful techniques of bacterial and fungal genetics have met with some success for the study of somatic cells in culture (Davidson, 1974), but as yet have been of little use to "whole animal" genetics.

Although the axolotl is the best studied genetically of all amphibians (Humphrey, 1975), mutant isolation has generally resulted from chance observation or inbreeding programs. As shown in Table I, if one starts with a single mutant sperm carrying a recessive mutation a, several generations are required, even with full-sib inbreeding, before a small percentage of mutant individuals would be homozygous for the mutation. One possible alternative to such prolonged inbreeding is parthenogenesis (Tyler, 1941). Parthenogenesis is the production of an embryo from a female gamete without any genetic contribution from a male gamete, and with or without eventual development into an adult. This deliberately wide definition includes the special case of gynogenesis,

Table I

Mutant isolation - a comparison of inbreeding and parthenogenesis

Inbreeding

mutant sperm a egg A

adult Aa

eggs 1/2A 1/2a

sperm A

adults 1/2AA 1/2Aa

inbreeding

25% of matings are Aa x Aa

1/4 of the progeny from these matings are aa

2 generations required, and a maximum of 1/16 of the progeny will be mutants

Diploid Parthenogenesis

mutant sperm a egg A

adult Aa

eggs 1/2A 1/2a

parthenogenesis

progeny 1/2AA 1/2aa

Requires 1 generation, 1/2 progeny are mutants.

in which a spermatozoon does enter the egg and activate it into development, but does not exercise its second role of contributing genetic material (Beatty, 1967). If homozygous diploids can be induced from unfertilized eggs, then mutants should be obtained in much shorter time than that required for conventional inbreeding for uncovering recessive mutations (Table I).

There are two main experimental requirements in the induction of parthenogenesis. First, the egg must be activated to begin development as a potentially haploid parthenogenome. If diploid parthenogenesis is required, it is necessary also to double the chromosome number. In induced gynogenesis, a genetically inactive spermatozoon is used to activate the egg, the principal modes of inactivation of spermatozoon being by X-rays, radium irradiation, ultraviolet rays, photodynamic chemicals in the presence of light, and by use of nitrogen mustard; all except the last involve radiation of some kind (Beatty, 1964). At least two routes are open for the induction of diploidy in a haploid parthenogen (Beatty, 1957). One is the suppression of first cleavage, where the resultant animals would be homozygous for all loci (type IC1). The other is suppression of meiosis II (type 2PB, retention of the second polar body). In this case progeny may be heterozygous if crossing over has occurred, and in fact this method has been used to determine gene kinetochore (G-K) linkage (Volpe, 1970; Nace, Richards and Asher, 1970). The utility of the second

method is based on the fact that for most vertebrates, mature eggs have not extruded the second polar body, an event that occurs at some interval after fertilization. In the axolotl, Fankhauser and Humphrey (1942) showed that cold treatment of eggs after fertilization led to triploid larvae, presumably by suppression of meosis II. Heat shock has also been used to produce the same effect in the axolotl (Sladacek and Lanzova, 1959; Rott and Betina, 1964).

Cold shock has been used with radiation induced gynogenesis to produce diploid trout, plaice and flounder (Purdom, 1969). Mortality of the gynogenetic diploids was greater than for the controls, and the incidence of abnormalities was high. Purdom (1969) supposed that this was due to increased homozygosity and consequent "inbreeding depression", but no genetic markers were used to demonstrate this.

In some strains of turkeys, parthenogenetic development occurs without cold shock or similar treatments (Olsen, 1969). Olsen (1966) demonstrated that females heterozygous for plumage colour produced parthenogens of both types of homozygote, plus one individual out of thirty-three that was heterozygous and could have resulted from crossing-over. Olsen(1969) concluded that diploidy was restored through retention of the second polar body.

In the frog, Tokunaga (1949) produced albinos from a wild type female by parthenogenesis. Though these were presumably diploid, the chromosomes were not counted, and there was no treatment to increase the incidence of diploids.

Volpe and Dasgupta (1962) produced gynogenetic diploids by fertilizing eggs with sperm of another species, followed by heat shock to induce retention of the second polar body. Of 811 treated eggs, only 70 were diploid, and all but 17 died before metamorphosis. The authors postulated that most had died as a result of being homozygous for lethal mutations. Nace et al. (1970) report carrying out a large number of similar experiments for the purpose of developing "inbred" lines. They reported zero to thirty-four percent viable embryos in 65 experiments. As part of these studies, Richards, Tartof, and Nace (1969) isolated a melanoid variant.

The sex of a diploid gynogenome is dependant on whether the female parent from which it is derived is homogametic or heterogametic. Where the female is homogametic (X.X), all normal diploid gynogenomes will be of the same genetic constitution with respect to sex chromosomes. However, when the female is heterogametic (Z.W, or Z.O in birds), several possibilities can arise. Where prereduction occurs, the secondary oocyte is of either Z.Z or W.W constitution, and diploid gynogenone produced by either the type 2PB or type ICI route will have the same sex chromosomes. However, where postreduction occurs, crossing-over can produce a secondary oocyte of Z.W constitution, and diploid gynogenones produced by the type 2PB route will also be Z.W. The type of reduction may be obligatory to the species or may vary within or among a species (Beatty, 1967). In spontaneously occurring turkey diploid parthnogens, all viable offspring are

males of Z.Z constitution. Since these parthenogens are thought to arise by suppression of meiosis II (Olsen, 1966), the implication is that the 0.0 genotype is not viable. In the axolotl, however, Humphrey (1945) has shown that the W.W genotype is a viable and fertile female so that diploid gynogenones from a normal Z:W female could be males (Z.Z) or females (W.W).

In addition to providing a shortcut for detecting mutations, Olsen (1969) cites several other potential uses for parthenogens. Artificial parthenognesis can be a useful tool for examination of questions concerning the relative roles of nucleus and cytoplasm in development and heredity, the mechanism of cell division, and problems of embryonic and sex determination (Tyler, 1941). Since genetic material from only one parent is involved in the production of parthenogens, this type of animal is ideal for demonstrating various principals in immune reactions. Because of its origin, the parthenogen should be carrying no antigens that are not found in its parent. Since the parthenogen receives some, but not all the genes of its parent, it will therefore be lacking in some of the antigens found in that parent. These facts make parthenogens a good system for the study of histocompatability and graft rejection. Another potential use of parthenogens is in the development of inbred lines. One obstruction in the establishment of inbred lines is the high mortality encountered as a result of the expression of recessive lethal genes. Because the method of inducing

diploid parthenogens results in an animal homozygous at most, if not all loci, any normal, fertile diploid parthenogen must be relatively free of recessive lethals. Use of such an animal as one of the parents in a mating should reduce the lethal load of its progeny, and with the gradual reduction of lethals, the establishment of inbred lines should become less difficult. A normal mating of two diploid parthenogens produced by the type 2CL route would result in a completely homogenous population of progeny, making them an ideal system for testing the effect of environmental variables on a genetically identical population.

A part of this thesis is devoted to describing attempts to develop a reliable method for obtaining gynogenetic axolotls. The method, basically similar to those previously described, involves fertilization with radiation inactivated sperm, followed by heat shock. The remainder is a preliminary morphological and histological description of the premature death mutant (p), in the axolotl.

MATERIALS AND METHODS

Animals

Animals were gifts from R.R. Humphrey (Indiana University), H.C. Dalton (Pennsylvania State University), L. DeLanney (Ithaca College), or had been raised in our laboratory. The following genetic markers were involved in some of the experiments: d, white; a, albino; ax, axanthic. The melanoid phenotype was assessed in newly hatched larvae primarily on the basis of the presence or absence of iridophores in the iris. One of the gynogenesis experiments involved an animal heterozygous for the gene p, premature death.

Histology and Cytology

For routine histological studies, whole embryos were fixed in Smith's fixative for 24 hr, washed in tap water for 24-48 hours, and kept in 5% formaldehyde. Embryos to be sectioned were dehydrated in a graded ethanol series and were transferred to a 1:1 ethanol-ether mixture for 24 hrs. Specimens were then infiltrated for 24 hrs or longer in a 3-5% collodion solution, and were vacuum imbedded in Paraplast (Gomori, 1952; Burstone, 1962). Sections were cut at 10μ and mounted on albumized glass slides. Sections were stained with Harris' haematoxylin and eosin, dehydrated, and mounted in Permount.

Ploidy was determined at hatching, when tailtips were removed from the larvae and squashed in 5% formaldehyde, or alternatively, whole embryos were fixed in 5%

formaldehyde so that epithelial tissue from both sides of the embryo could be taken and squashed to aid in the identification of mosaics. These squash preparations were then examined under a phase contrast microscope to determine ploidy based on the number of nucleoli (Fankhauser and Humphrey, 1943). Chromosome counts were also made from the tailtips of newly hatched larvae. The distal 2-3 mm of the tails were removed from the larvae and placed for 3 hrs in 3-5 ml amphibian culture media containing 45mg/100ml colchicine. Tailtips were then washed in tap water and squashed on a subbed slide in a drop of 50% acetic acid with a siliconed cover slip. The cover slips were removed by means of a dry ice-methanol bath and the slides were allowed to air dry. Chromosomes were stained for 10 min. in 2% aceto-orcein, dehydrated, and mounted in Permount.

Parabiotic Twins

Parabiotic combinations from a spawning in which both parents were heterozygous for gene p were made, using embryos at stages 24-25 of the Screckenberg and Jacobson (1975) series. Embryos were manually dejellied with watchmakers forceps and allowed to remain in calcium free 10% modified Holtfreter's containing 400mg/l each of penicillin and streptomycin for several minutes before the operation. Ectoderm was removed from the appropriate sides of the prospective twins, which were then placed side to side in a narrow groove cut in an agar lined petri plate, and

allowed to remain in the calcium free modified Holtfreter's solution for several hours while wound healing took place. This calcium free solution was then gradually removed and replaced by 10% modified Holtfreter's with 400mg/l each of penicillin and streptomycin, and the agar was removed from the plates.

Artificial Insemination

The artificial insemination procedure was based on that previously described (Trottier and Armstrong, 1975), but with a number of modifications. Instead of one large dose of follicle stimulating hormone (FSH), the female was given a combined dose of 250 IU of FSH intramuscularly and 50 µg of luteinizing hormone intraperitoneally the morning prior to the experiment, and a second dose of 250 IU of FSH intramuscularly in the afternoon. The double injection seemed to make the interval between injection and laying more reproducible. The female usually began to lay eggs 24 hr after the first injection. At the time of the second injection the males were injected with 500 IU of chorionic gonadotropin, and the dummy females were given 80 IU of FSH intramuscularly and 50 µg LH intraperitoneally. Although it was previously reported (Trottier and Armstrong, 1975) that sperm remained viable up to 8 hr after collection, in some recent experiments sperm viability appeared to decrease with time. For this reason the male was not placed with the dummy female until the

experimental female began to lay eggs. If more than one male was used, the second was not placed with the dummy female until all the sperm from the first had been used. Spermatophores were collected as previously described, except that only 2-3 spermatophores were used with 1.0 ml of culture medium, and they were not homogenized until immediately before use.

As many eggs as were produced in a twenty minute period were placed directly in the tube with the sperm suspension. The stoppered tube was gently agitated at room temperature (18°-20°C), generally for one hour, although 15-20 min appeared to be sufficient for successful fertilization.

For ultraviolet inactivation, sperm were collected as described above, placed in a hemicylindrical plastic trough on ice, and irradiated at a distance of 4 cm from the light source of a Raytech model G-1 ultraviolet light, to give ultraviolet doses ranging to 8000 ergs/mm^2 X 10^{-3}. Ultraviolet intensity was measured with an Ultra-Violet Products, Inc. (San Gabriel, California) model J-225 short wave meter. Immediately after the irradiation, artificial insemination was carried out as described above. After a one hour insemination period, the eggs were transferred to a 10% modified Holtfreter's solution and either heat shocked or allowed to continue developing at room temperature.

Heat shock was used to induce triploidy in normally fertilized eggs or to restore diploidy in eggs artificially inseminated with ultraviolet inactivated sperm; and was done in 10% modified Holtfreter's solution in a water bath at 36.0 $\pm$ 0.2°C for the desired length of time and the desired time after oviposition.

Materials

Chorionic gonadotropin was obtained from the Sigma Chemical Co. (St. Louis, Mo.). Follicle stimulating hormone was from Ormond Veterinary Supply (Hamilton, Ont.). Luteinizing hormone was purchased from Schwartz/Mann (Orangeburg, N.Y.). Amphibian culture medium was from Grand Island Biological Co. (Grand Island, N.Y.). Penicillin G and streptomycin sulfate were from the Sigma Chemical Co. Our modified Holtfreter's solution contained 3.46 g NaCl, 200 mg $MgSO_4$, 200 mg $NaHCO_3$, 100 mg $Ca\ Cl_2$, and 50 mg KCl per liter distilled water.

Results

I p/p mutant

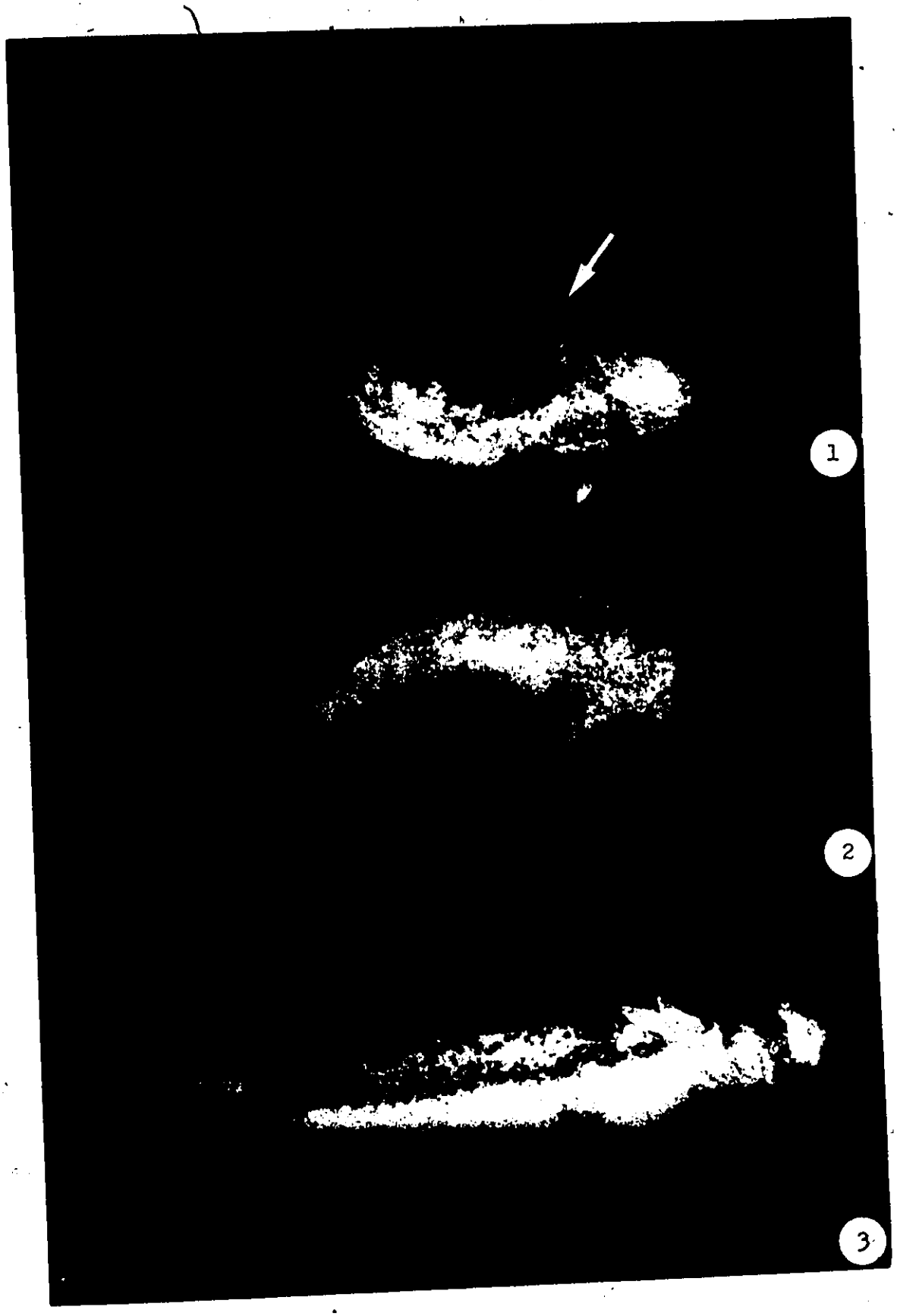
A previously undescribed mutant appeared in two separate spawnings of the same animals. In the first, 71 of 261 progeny or 27.2% showed the mutant phenotype, and in the second, 162 of 602 were mutants, for a total of 233 of 863, or 27.0%. These results indicate that the gene in question is inherited as a typical Mendelian recessive trait (p=.170). The parents of these spawnings, kindly donated by R.R. Humphrey, were sibs designated 3719-2 and 3719-5. In addition to being heterozygous for the gene p, they were also heterozygous for the gene axanthic (ax). The male was heterozygous for the gene melanoid (m), while the female was homozygous for that gene. The gene p had previously been discovered by R. Thompkins, whose description of the mutant was simply that homozygotes reached stage 37 of Harrison's series (simple unbranched gill rudiments), and that heart action continued for a time after disintegration of superficial tissue had begun (Humphrey, 1975). Lack of further published data concerning this mutant prompted us to consult Dr. Thompkins, wherupon we found that he had lost all of his animals carrying this gene. Part of this thesis will be concerned with a preliminary description of the mutant.

The most obvious feature of the mutant was stasis of development with simple unbranched gill rudiments, at about stage 37 of Schreckenberg and Jacobson's (1975) normal table for Ambystoma mexicanum. Figures 1 and 2 show the dorsal and ventral aspects of the mutant at about the time it first becomes recognizable. The mutants were recognizable by their gills and their lateral curvature at about eight days after fertilization (at 18-20°C), at which time development was arrested. In addition to remaining unbranched, the gills frequently developed a small bulb at the distal end. The mutants survived for about a week after recognition, when disintegration of superficial tissue began. During this period, most showed edema or epidermal blistering of some sort. The swelling was most often localized in the area of the presumptive forelimb or pronephros, but was not restricted to that area, as older mutants often showed blistering along the fin fold and sides caudal to the pronephric region. The head appeared somewhat microcephalic, and eye structure was apparent in some, but not all, of the mutants. The external nares were well developed and the cloacal margins were generally slightly swollen. Chromatophores in the mutant were sufficiently well developed so that ax/ax embryos could be distinguished from wild type or m/m embryos, but it was difficult to distinguish

Fig. 1 Dorsal view of a p/p mutant. Arrow indicates the small bulb that frequently develops at the distal end of the gills. X20.

Fig. 2 Ventral view of the p/p mutant showing the external nares and the enlarged cloacal margins. X20.

Fig. 3 Side view of a normal stage 37 embryo. The gills are as yet unbranched, and the external nares and eyes are visible at this stage. X20.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between the latter two groups. Melanophores tended to be contracted in the caudal areas and expanded in the cranial areas.

The heart could be seen to be beating feebly from the time the mutant could be detected, but microinjection of a small amount of dye into the heart at this stage showed no circulation. The blood islands were distinct, but at no time before death did blood appear elsewhere than the islands. The mutants showed no righting reflex or swimming movements, but could be stimulated to move their tails feebly by irritation of the gill area with a probe.

Of several pairs of animals joined in parabiosis, four pair were of the desired mutant-normal combination. Figure 4 is a photograph taken three days after joining the embryos, at which point mutants could be distinguished from normals. The mutant animal in each of the pairs was easily detected by the above criteria. Circulation was well established in the normal twin, but no red blood cells were observed in the circulating fluid. The blood islands were ~~still~~ evident and had become concentrated at the join between the two twins. At this time the mutants had established a heartbeat which was rhythmic and slower than the normal, although there was no evidence of a circulating fluid in the mutants. Figure 5 is a photograph of the same parabionts taken one week later

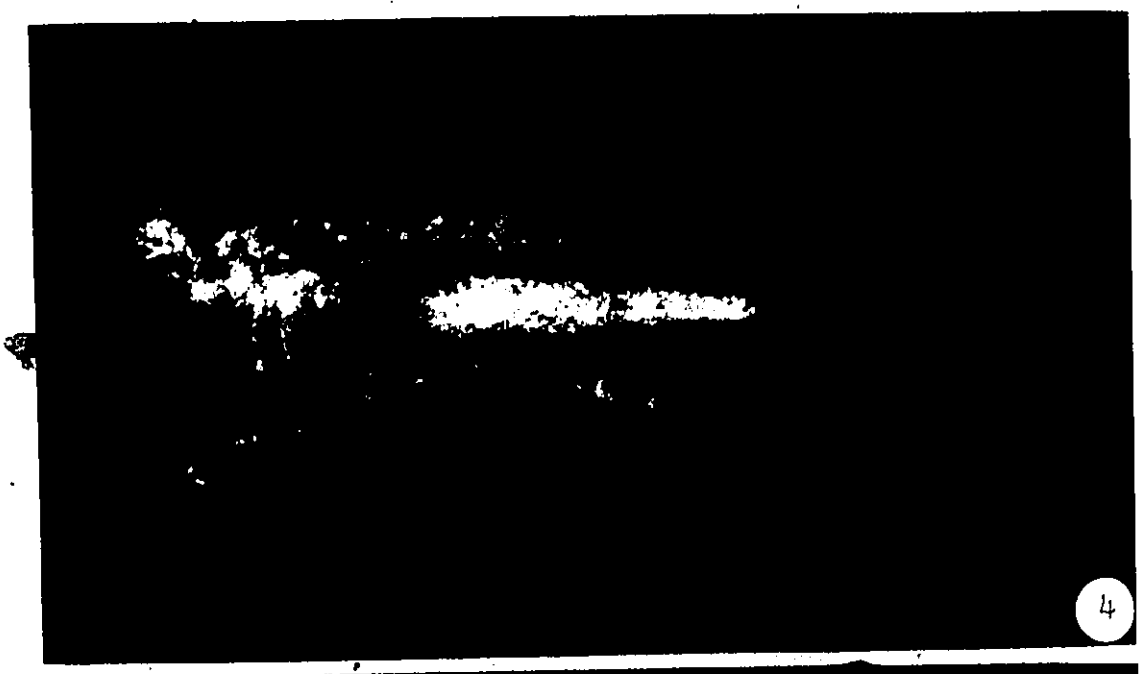

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Fig. 4 Parabiotic combination of mutant and normal embryos. This photograph was taken three days after the embryos were joined. The normal twin is at stage 38, and the mutant is at the stage where development normally arrests. X20.

Fig. 5 Parabiotic combination of mutant and normal embryos at ten days after joining. The normal embryo has reached stage 40, but the mutant partner has not changed appreciably. An epidermal blister has formed on the side of the mutant. X10.

than figure 4. At this time the normal member of each pair had progressed in development to about stage 40 of the Schreckenberg and Jacobson (1975) series, while mutants had progressed no further than they would have otherwise. There was no definite evidence of red blood cells in either the mutant or normal partner, and the mutants had begun to blister as previously described. Superficial tissues of the mutants had begun to disintegrate and all four pairs of the parabionts died shortly after the photograph was taken.

A microscopic examination of serial sections of mutants and stage 37 normal embryos showed that the tissues of the mutant embryos had little affinity for the haematoxylin stain. In addition, mutants were more difficult to section, and artifacts that didn't occur in the normal embryos occurred frequently in mutants processed in the same manner. This explains the somewhat poorer quality of the photographs of the mutants compared to those of the normals. Examination of serial sections of the mutant in frontal, saggital and transverse planes revealed that it suffered from several abnormalities, when compared to a normal stage 37 embryo. The most obvious defects in the mutant are: abnormal gill pouches; abnormal heart structure; abnormal liver structure; swollen and distended vendus vessels and pronephroi; poorly differentiated and

disintegrating myotomes; and gut abnormalities. Mutants were examined on the first day they became recognizable as mutants. In addition some were allowed to continue "developing" for two or four days. This gave four groups of embryos for comparative purposes: mutants isolated on the day they were discerned to be mutants, designated M-0; normal stage 37 embryos, which was the stage attained by most normals when mutants were recognizable, designated N-0; mutants isolated two days after the mutant phenotype had been expressed, designated M+2; and finally, mutants isolated four days after recognition, designated M+4. As all mutants were morphologically at stage 37 when development ceased, they were compared to normal stage 37 embryos for levels of development and organization of tissues and organs.

Perhaps the most striking feature of the mutant is the abnormal structure of the heart. The gross morphology of the heart of the M-0 mutant appeared as complex as that of the normal, with good distinction between conus arteriosus, ventricle, atrium, and sinus venosus (Figures 6-9). However, a recurring feature in the mutant was an additional loop at the conus end of the heart (Figure 8). This additional loop was observed most often and most strikingly in the M+4 mutants, with an intermediate number of the M+2, and few of the M-0 series showing this trait. In

Fig. 6. Frontal section of an N-0 embryo showing the endocardium, myocardium and the pericardial lining of the heart. X75.

Fig. 7. Frontal section of a M-0 mutant showing the endocardial "plug" present in the conus and ventricular portions of the heart. X75.

Fig. 8. Frontal section of a M+2 mutant. Arrows demonstrate the additional loop in the anterior end of the heart of later mutants. X75.

Fig. 9. Frontal section of a M+4 mutant. Arrows show the separation normally apparent between the endocardium and the myocardium, and the more normal appearance of the endocardia lining in the posterior parts of the heart. X75.


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addition, the mutant heart displayed obvious abnormalities as regards the establishment of a normal endocardial tubular lining. In all of the mutants examined the cephalic parts of the heart (conus arteriosus, ventricle) contained a plug of undifferentiated cells rather than the thin walled endocardial tube displayed by the normal embryo. Although there was some variation in the degree to which this plug extended, it rarely continued to the level of the atrium. There, endocardial cells began to show more normal differentiation and the single celled endocardial lining was practically indistinguishable from that of the normal (Figures 6,8,9). Caudal to the atrium, the mutant heart possessed a well established sinus venosus which received the vitelline veins much as did the normal, with the exception that these vessels were often swollen and distended. This fact often aided in identification of the major venous vessels of the mutant (Figures 12 and 13). The vitelline veins and the anterior and posterior cardinal veins were at least present, even though they were abnormal looking because of their distension.

The arterial system was considerably more difficult to examine in the mutant, partially due to the lack of red blood cells which aided in the identification of these structures in the normal embryo, but also because even the major arteries were either absent or so poorly developed that they escaped detection. The aortic arches

themselves were generally present, but the dorsal aorta was usually evident only as paired, shapeless, thin walled vessels ventral to the notocord. (Figures 12 and 13). In none of the mutants examined was there any communication between the aortic arches or ventral aorta, which were tubular structures when present, and the conus arteriosus of the heart, which invariably ended as a mass of undifferentiated cells at the cephalic limits of the pericardial cavity, where the conus arteriosus meets the pericardium (Figures 10 and 11). The endocardial plug in the anterior of the heart was usually well separated from the myocardium, but this distinction was less obvious where the conus arteriosus terminated in the body wall. Neither the myocardium nor the pericardium appear to be substantially less differentiated in the mutant than in the normal, and those differences that can be noted are most likely attributable to the poorer staining characteristics of the mutant tissue rather than to actual differences from the normal embryo (Figures 6 and 9).

Examination of sections revealed abnormalities in the branchial region of the mutant as early as in the M-0 series. Figures 14 and 15 illustrate the structure of gill pouches in both normal and mutant embryos. Instead of outpocketings of pharyngeal endoderm in the mutant, this tissue appeared to have formed the finger-like projections seen in figures 8 and 15. There was little evidence of

Fig. 10. Cross section of an N-0 embryo showing the ventricle, conus arteriosus, and part of an aortic arch. X55.

Fig. 11. Cross section of an M+4 embryo through approximately the same region as that of figure 10. Arrow indicates the typical undifferentiated appearance of endocardial and myocardial cells at the point where the conus arteriosus meets the pericardium. X55.

Fig. 12. Cross section through an N-0 embryo showing the liver diverticulum, dorsal aorta, and the vitelline and posterior cardinal veins. X55.

Fig. 13. Cross section through an M+4 mutant at the same level as figure 12. The liver diverticulum is poorly developed and the vitelline and posterior cardinal veins are extremely distended. Arrows indicate the left half of the unfused dorsal aorta rudiment. X 55.

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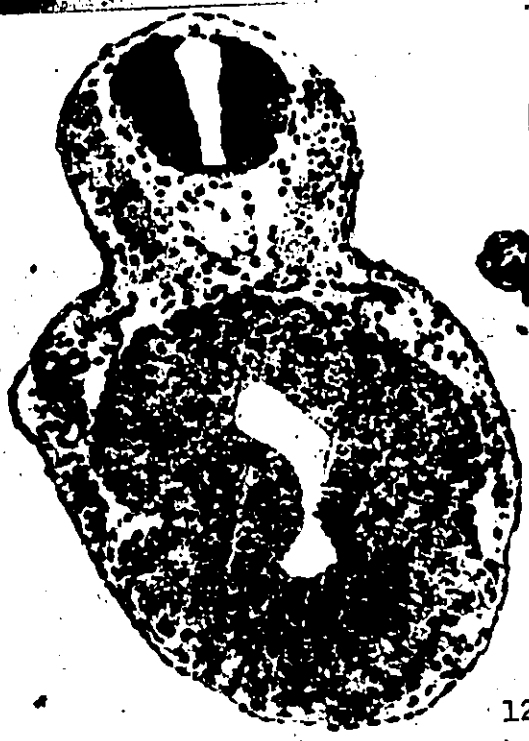

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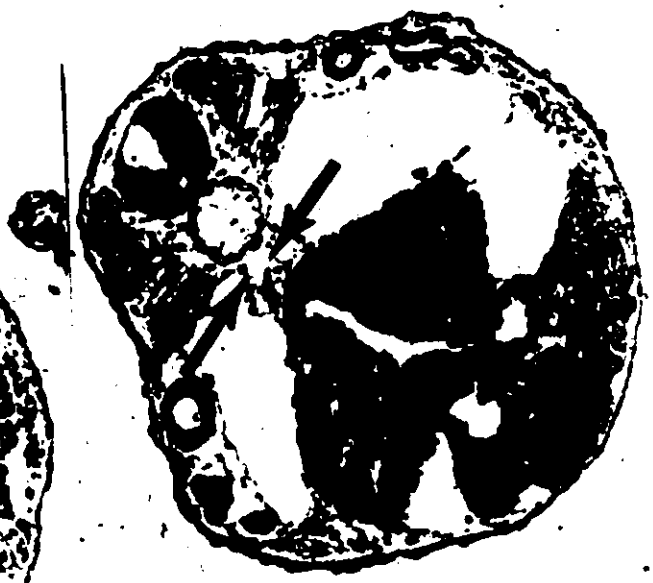

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Fig. 14. Sagittal section through an N-0 embryo. Gill pouches and aortic arches are evident. X55.

Fig. 15. Sagittal section through an M-0 mutant. Arrow indicates the endodermal projections which normally form the lining of the gill pouches. X55.

Fig. 16. Cross section through an N-0 embryo. The normal aspects of the pronephros, dorsal aorta, posterior cardinals, and myotomes is shown. X65.

Fig. 17. Cross section through an M+4 mutant at the same level as seen in figure 16. The pronephros and the posterior cardinal vein are swollen, and the myotome is degenerating. The arrows indicate the pockets that occur more frequently and more extensively in the mutant than in normal. X65.

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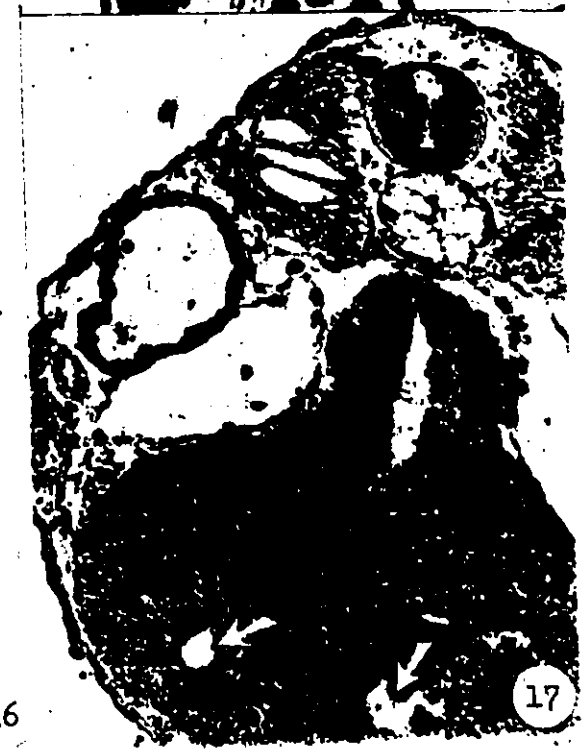

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gill pouches having been extensively formed in the mutant. As a result, the pharyngeal cavity in the mutants was a more continuous, oval shaped cavity than that found in normal embryos which had well established gill pouches and gill clefts.

It has already been pointed out that the mutant at all stages exhibited swollen and distended vessels, particularly venous vessels. In addition to this, there was an accumulation of fluid and subsequent swelling and distension in the coelom and pronephroi of the mutant (Figures 16, and 17). The extent to which this accumulation of fluid occurred, and the subsequent edema and epidermal blistering caused by it are further illustrated in figures 18 and 19. Little more will be said of this trait, as it is patently obvious in almost any of the figures of the mutant.

There was considerable variation in the integrity of the gut throughout the mutant embryos. In general, the gut was either abnormal, or portions of it have regressed in all the mutants. Further reference will not be made concerning the pharyngeal cavity in light of the previous discussion of gill clefts. Of the foregut, the liver diverticulum appeared to have the least normal appearance when compared to a normal embryo. Figure 13 illustrates what is perhaps the best developed liver

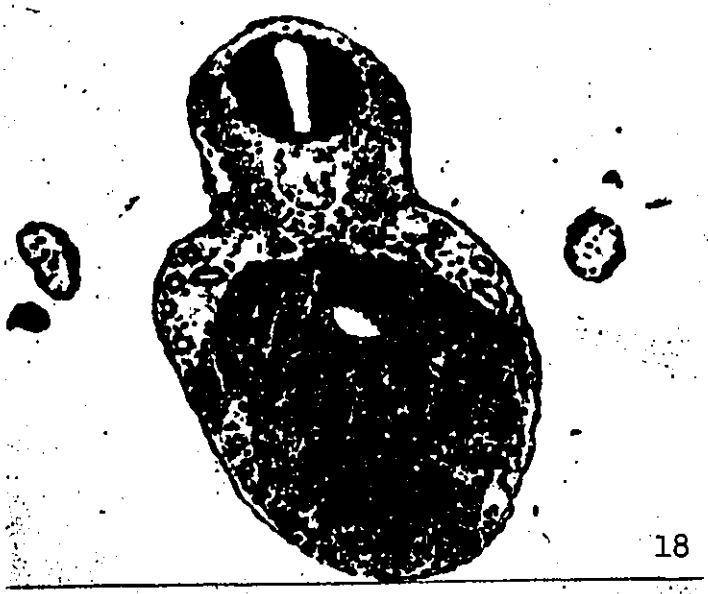
diverticulum in all of the mutants surveyed for this study, and even it was decidedly lacking in organization when compared to the normal (Figure 12). More often, the liver diverticulum was only a short, slender, blind pocket proceeding caudoventrally from the anterior part of the foregut, rarely extending as far as the presumptive liver cells. The midgut was rarely evident in any of the mutants, though on occasion it appeared as a small slit in the dorsal part of the gut endoderm. There were often blind pockets of varying sizes lying lengthwise in the gut endoderm, usually more ventrally than where the gut normally occurred (Figures 19 and 20). In the embryo shown in figure 19 there was an accumulation of red blood cells in one of these pockets, and isolated groups of these cells were often found in indentations, perforations, or pockets in the endoderm. The cloaca and the hindgut of the mutants were usually comparatively normal, with the exception of the swelling of the epidermal portions of the cloaca, and a continuity between these two was fairly easy to establish. The nephric ducts appeared to open into the cloaca normally.

A comparison of myotome structure between the mutant and normal embryos showed that mutant cells remained less differentiated than those of a normal embryo, and that mutant myotomes also underwent considerable progressive degeneration during the observation period (Figures 21, 24).

Figure 18. Cross section through mid gut area of an N-0 embryo. X50.

Figure 19. Cross section through mid gut area of an M+4 mutant showing a blister and a cavity in the endoderm containing red blood cells. As in most cases for the mutant, the mid gut is not evident. X50.

Figure 20. Cross section through an M+4 mutant showing the extent to which the epidermal blistering can occur. X50.



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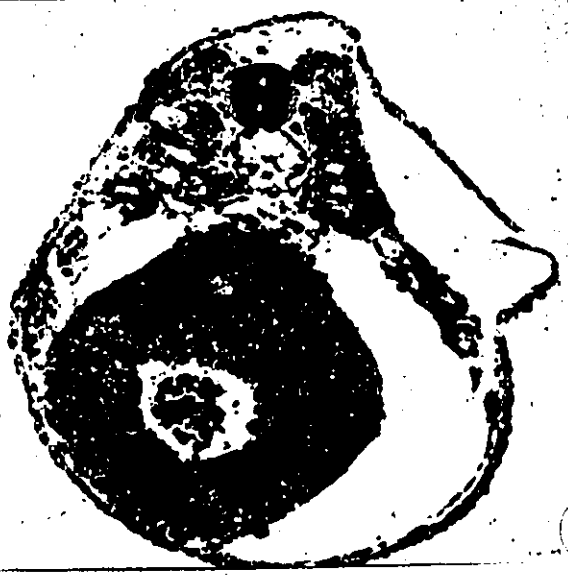

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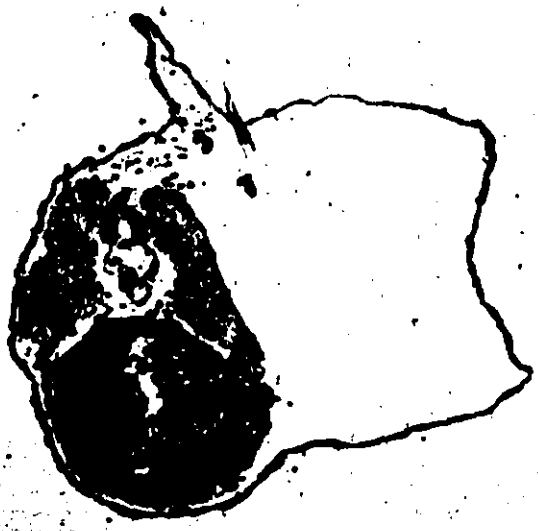

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Figure 21. Sagittal section through the myotomes of a N-0 embryo. The cells are well differentiated and the myotomes are losing their segmental characteristics. X50.

Figure 22. Sagittal section through the myotomes of an M-0 mutant. The cells are less differentiated and the segmental nature of the myotomes is preserved by the vertical myocomma (arrow). X50.

Figure 23. Sagittal section through the myotomes of an M+2 mutant. Numerous tears (arrow) are beginning to appear between the cells. X50.

Figure 24. Sagittal section through the myotomes of an M+4 mutant. Degeneration of the myotomes is quite extensive now. X50.


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Figure 21 shows the degree of differentiation the myotomal cells have undergone in the normal stage 37 embryo. The nuclei of these cells had become elongated longitudinally, and the vertical myocomma, the connective tissue which earlier in development maintains the segmental arrangement of myotomes, was not particularly evident, as the primitive segmentation of somatic muscles has somewhat regressed at this time. Figure 22 shows the status of the myotomes in the M-0 mutant. The nuclei of the myotomal cells were less elongated and the cells in general were less differentiated in the mutant than in the normal. The vertical myocomma was quite obvious and maintained the segmental arrangement of the myotomes that had already degenerated in the normal. Figures 23 and 24 illustrate the degeneration of the myotomes during the four day period between the M-0 and M+4 mutants. In the M+2 mutant, several "tears" can be seen between the myotomal cells, and by the M+4 stage this progressive degeneration has led to the scarcely recognizable structure seen in figure 24. It is somewhat surprising that the mutant can move its tail at this stage. In neither the M+2 nor the M+4 mutants did there appear to be any further differentiation of myotomal cells beyond what was observed in the M-0 mutant. The vertical myocomma was still apparent in the M+2 mutants, but because of the degeneration this structure

was not obvious by the M+4 stage. A high power light microscopic examination of myotomal cells in the normal embryo revealed that the myofibrils produced by these cells were highly organized and arranged in a longitudinal fashion conforming to the arrangement of the cells themselves. There appeared to be myofibrils in the mutant cells as well, but they were neither as numerous nor as well defined as those in the normal embryo, and by the M+4 stage they were as disrupted as the myotomes themselves.

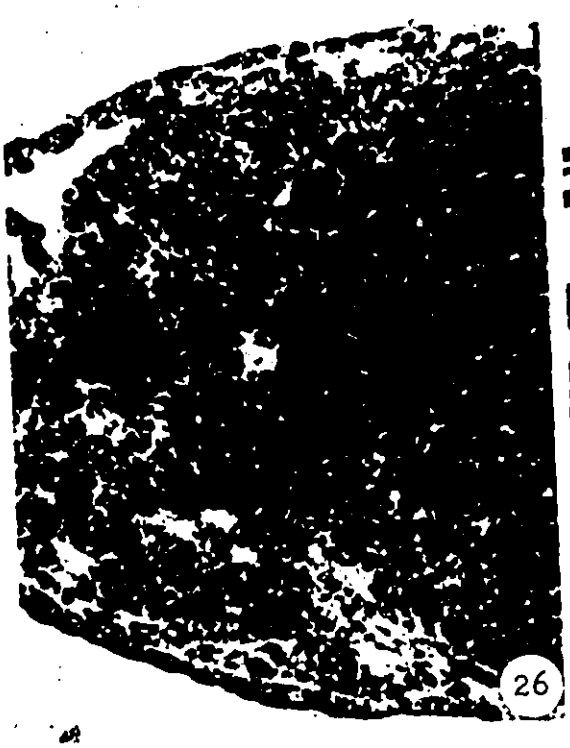
Figures 25 to 28 demonstrate the primitive state at which the liver remains in all of the mutants. All of these sections transected the ventricular portion of the heart, at which level the liver normally showed a high degree of differentiation and organization (Figure 25). The hepatic cavity was evident and the cells appear well differentiated. In the mutants there was little evidence of the hepatic cavity, and the greatest extent to which that structure appeared can be seen in figure 27. The cells in the presumptive liver area showed almost no differentiation and it was almost impossible to distinguish between these cells and the endodermal cells caudal to the presumptive liver area. Figures 29 and 30 further illustrate these phenomena, observed in cross section. As well as failure to establish or maintain

Fig. 25. Frontal section through an N-0 embryo. The hepatic cavity is evident and the liver cells are more differentiated than the more posterior endodermal cells. X75.

Fig. 26. Frontal section through an M-0 mutant. There is no obvious hepatic cavity and the presumptive liver cells are no more differentiated than the yolky endodermal cells. X75.

Fig. 27. Frontal section through an M+2 mutant. This section is slightly more dorsal than those in figures 25, 26, and 28, and there is some evidence of a hepatic cavity. X75.

Fig. 28. Frontal section through an M+4 mutant. There has been no further differentiation of liver cells. X75.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a hepatic cavity, the mutants failed to establish or maintain a liver diverticulum, as has been previously pointed out (Figures 12 and 13).

In addition to those mutants isolated at stage 37, nine embryos from the heterozygous P/p parents were fixed and sectioned at stage 25. All nine of these embryos were essentially identical when examined (Figures 31 and 32). All looked normal for an embryo at this stage of development, with well defined pharyngeal cavities, liver diverticuli, cardiac primordia, foreguts (Figure 32), midguts, hindguts, and segmental myotomes (Figures 31).

In none of the serial sections examined was there any indication of abnormalities in the developing nervous system, and the brain and spinal cord, as well as associated structures, were essentially the same in both the normal and mutant embryos. It must be pointed out, however, that this study was done at a fairly gross level and cannot rule out the possibility that the nervous system, or any other system or tissue, may suffer abnormalities not observed in this preliminary investigation. A high power light microscopic comparison of mutant and normal cells in various of the investigated tissues revealed no differences that could not be attributed to the differences in staining characteristics.

Figure 29. Cross section through an N-0 embryo showing the gut and the liver. X50.

Figure 30. Cross section through an M+4 mutant. Neither the liver nor the gut resemble the normal in terms of organization or differentiation of cells. X50.

Figure 31. Sagittal section of a stage 25 embryo from a mating of heterozygous P/p parents. Liver diverticulum, cardiac primordia, pharynx, and gut are all normal for an embryo of this stage. X65.

Figure 32. Sagittal section of a stage 25 embryo from heterozygous P/p parents showing the normal appearance of the myotomes. X65.

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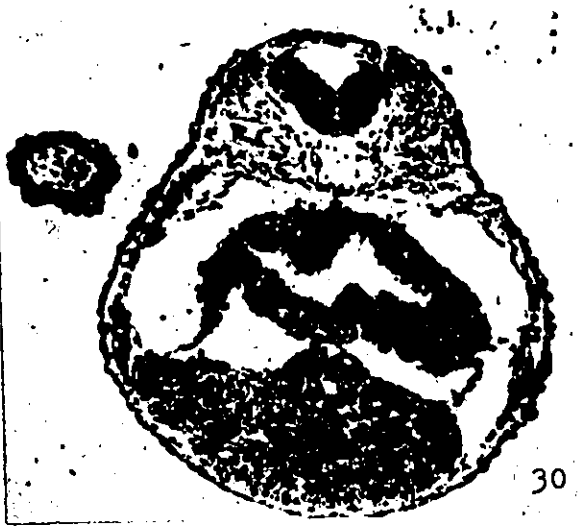

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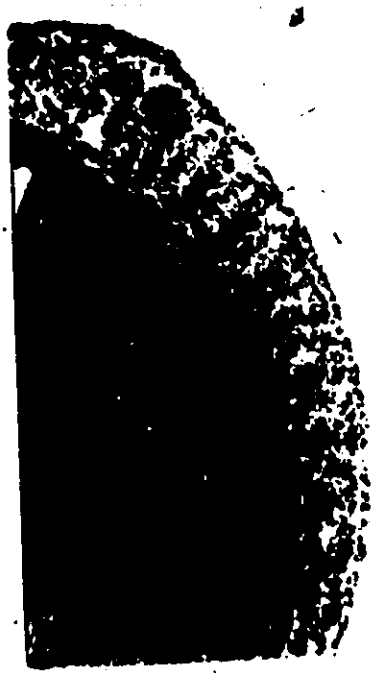

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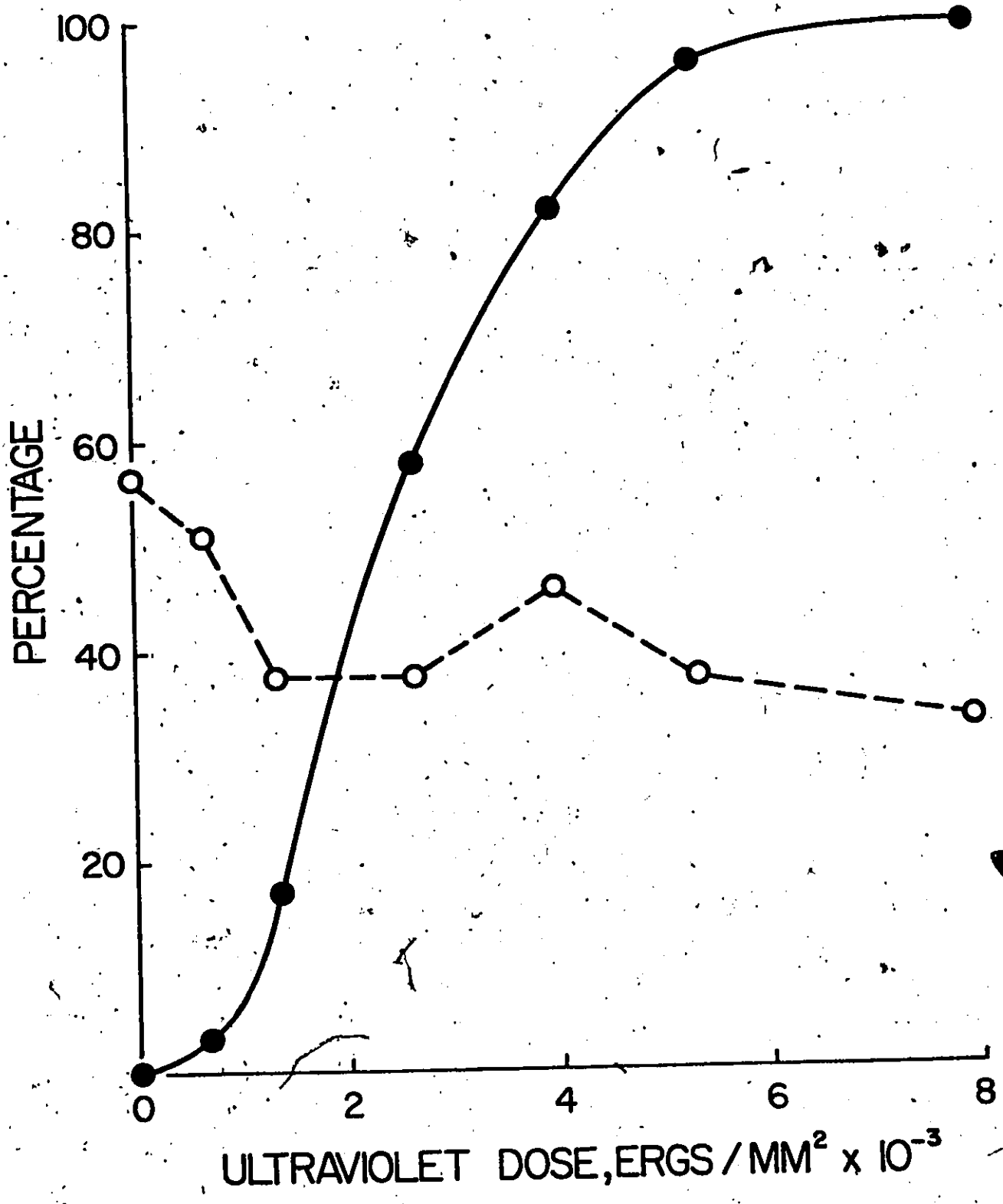
II diploid gynogenesis

Our method of inducing diploid gynogenesis in the axolotl is similar to that used by Purdom (1969), Volpe and Dasgupta (1962), and Nace et al. (1970) for other species. It involved inactivation of the sperm by ultraviolet irradiation, followed by heat shock to suppress meiosis II and restore diploidy. In order to establish optimal conditions for gynogenesis, optimal conditions for ultraviolet inactivation of the sperm and for suppression of meiosis II were separately examined.

For the ultraviolet inactivation experiments, sperm from several males was irradiated with doses ranging from 660 to 7920 ergs/mm^2, and then used to artificially inseminate a total of 656 eggs from 4 females. As controls, an additional 205 eggs were inseminated with unirradiated sperm. For the control, 60% were judged fertile when examined after 24 hr. This is comparable to the 51% fertility obtained in earlier artificial inseminations (Trottier and Armstrong, 1975). For the experimental groups, fertility averaged only 44%, but was substantially higher (70%) for the shortest dose. Survival, calculated as the percentage of fertile eggs which hatched, is shown in Figure 33. In these experiments, three of the four females were melanoid, and the males were wild-type (non-melanoid). All of the haploid larvae from such inseminations were melanoid, confirming sperm inactivation.

Fig. 33. Effect of ultraviolet irradiation of sperm on their ability to fertilize eggs in an artificial insemination, and on subsequent development. Eggs were considered fertile i there was evidence of cleavage. Percent hatching (o) was calc ulated as no. hatching/no. fertile X 100. Ploidy (% haploid embryos, •) was determined only for embryos which hatched.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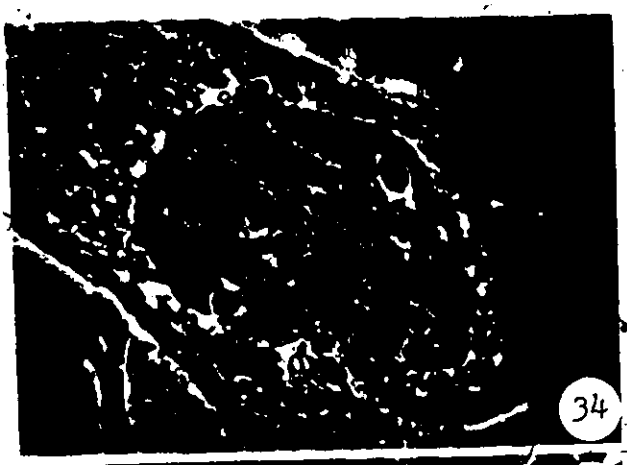
ULTRAVIOLET DOSE, ERGS / MM² x 10⁻³

The optimal conditions for suppression of meiosis II were established on the basis of the efficiency of triploid induction after heat shock to normally inseminated eggs. Eggs were collected at 20 min intervals, kept at room temperature for various periods of time, then exposed to 36°C for 5 min, and returned to room temperature. The maximum yield of triploids was obtained when the shock was applied 60 min after the eggs were collected (Table II). The actual time after oviposition would be 60 to 80 min. This does not seem unreasonable, as Fankhauser and Griffith (1939) found that the second polar body was released about 1 hr after fertilization in the axolotl.

When the duration of heat shock was increased to 7.5 min, 40% triploids were obtained (Table III). A further increase to 10 min yielded 75% triploids, but reduced survival substantially. Figures 34 to 39 show the numbers of nucleoli and the corresponding numbers of chromosomes found in haploid, diploid, and triploid cells. Chromosome counts were conducted periodically, and in all cases confirmed the ploidy of the larva based on nucleolar counts.

The diploid gynogenesis experiments combined the optimal conditions established above. Sperm was collected, irradiated with an ultraviolet dose of 5280 ergs/mm^2, and then used to fertilize eggs laid by a hormonally stimulated female. After 1 hr the fertilized eggs were heat shocked

Figures 34-39. Chromosomes and nucleoli of haploid, diploid and triploid larvae. Chromosome counts were conducted periodically, and in all cases confirmed ploidy determined by number of nucleoli. There is also a correlation between nuclear size and numbers of chromosome sets (Fankhauser and Humphrey, 1943). Whole cells by phase contrast. X1000.



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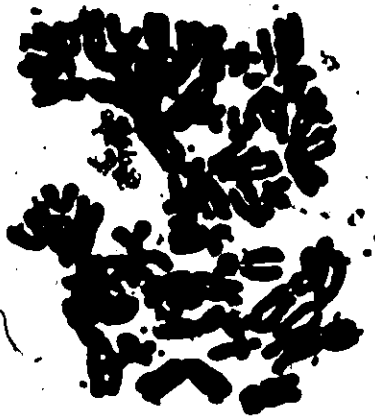

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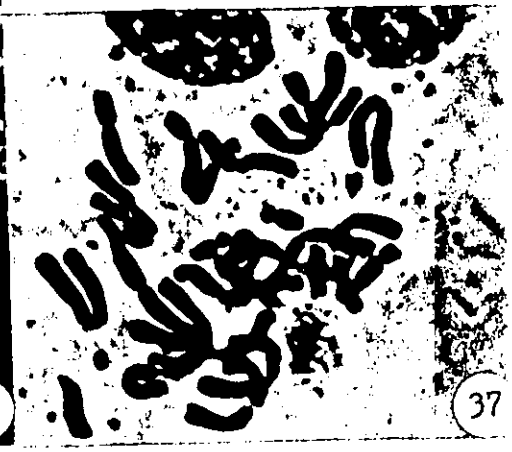

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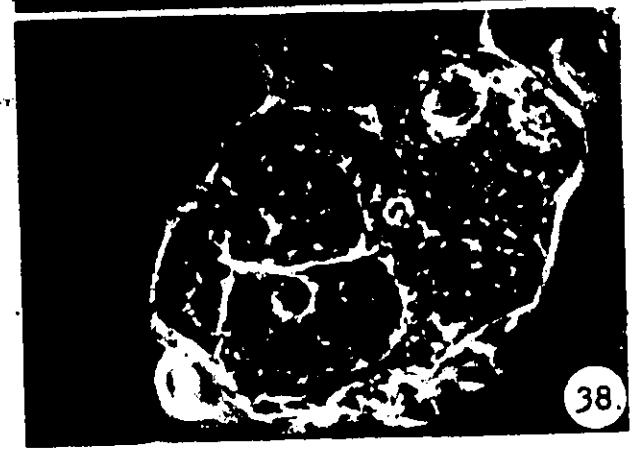

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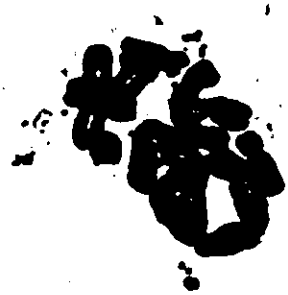

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Table II

Effect of heat shock at different times after fertilization[1]

Time of shock[3]	No. of eggs shocked	No. of larvae[4]	Survival (% unshocked control)	Percent triploid
0 min	92 (2)	64	106	4.9
15	58 (2)	41	100	12.2
30	83 (3)	62	122	6.5
45	189 (5)	100	91	16.5
60	117 (3)	49	63	26.5
75	53 (1)	12	41	8.3

[1]Heat shock was 5 min at 36°C.

[2]The numbers in parentheses are the number of different females from which the eggs were obtained.

[3]After oviposition.

[4]Heat shocked eggs which hatched.

Table III

Effect of duration of heat shock on triploid yield[1]

Duration of shock	No. of eggs shocked[2]	No. of larvae[3]	Survival (% unshocked control)	Percent triploid
5 min	117 (3)	49	63	26.5
7.5	66 (2)	30	79	40
10	50 (2)	4	17	75

[1]Eggs were shocked 60 min after collection.

[2]The numbers in parentheses are the number of different females from which eggs were obtained.

[3]Heat shocked eggs which hatched.

for 7.5 min at 36°C. Control eggs were inseminated with non-irradiated sperm and allowed to develop without heat shock. Daily records were kept of developing embryos, and both ploidy and phenotype were determined at hatching. Eight separate experiments were run, with varying degrees of success. Quantitative aspects of the experiments are summarized in Table IV. The success of the artificial inseminations (54.9% fertile) compared favourably with results obtained previously (Trottier and Armstrong, 1975), but mortality was extremely high. Excluding experiment 3, only 24% of the fertile embryos survived to hatching, and only 7.4% were normal diploids. Mortality, compared to the control embryos, is shown on a daily basis in Figure 40.

Experiments 1 to 4 were of the same design. In all four the female was homozygous melanoid, and the males were wild-type. Of 48 larvae which hatched in these experiments, 2 were wild-type, but grossly abnormal. These likely arose from incomplete inactivation of the sperm. All other progeny were melanoid, indicating a gynogenetic origin.

Experiment 5 was designed as the same type of experiment, but yielded two surprises. Of 29 gynogenetic diploid progeny for which colour could be determined, 8 were axanthic, indicating that the female was Ax/ax. Of the total of 34 diploid larvae, 3 were grossly abnormal and 14 showed a particular mutant phenotype characterized by a pear-shaped form resulting from an accumulation of

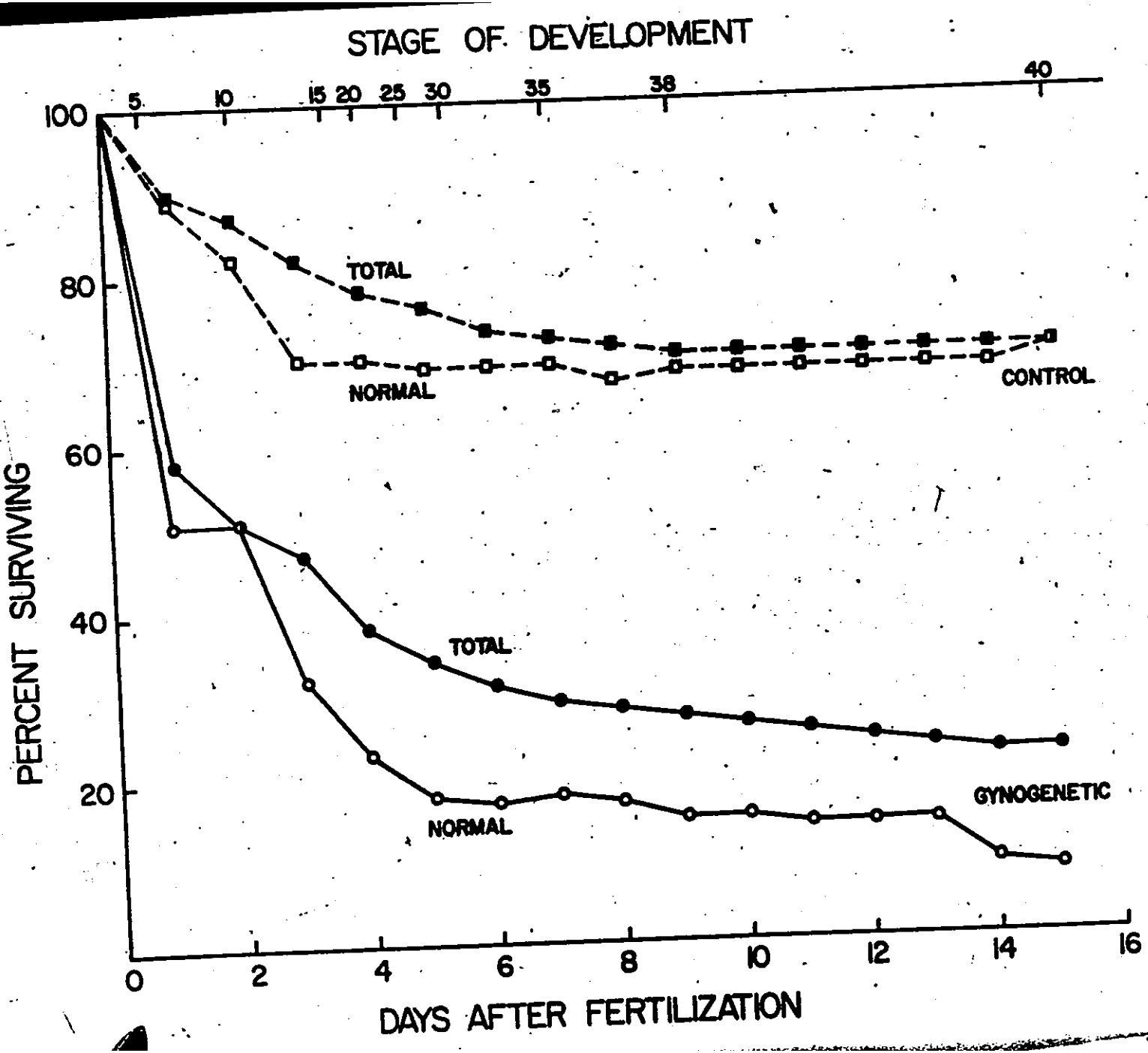
Table IV

Results of diploid gynogenesis experiments

Experiment	No. of eggs: Inseminated	Fertile	Normal (day 1)	No. of larvae at hatching: Total	Normal	Diploid (%Total)
1	88	61	31	24	4	24%
2	127	44	17	8	2	25
3	112	65	34	(all but one diploid died of a fungal infection)		
4	141	69	34	13	9	85
5	159	126	79	44	12	83
6	73	54	21	4	3	75
7	48	24	8	1	0	0
8	100	26	12	3	0	100
Totals:	848	469	236	97*	30	66%

*Excluding experiment 3.

Figure 40. Survival of control and gynogenetic embryos from fertilization to hatching. Both total survivors and those which looked morphologically normal are indicated. Calculations were made from the combined data of experiments 5 to 8 only, since no control groups were run for the first three experiments. The staging is that of Schreckenberg and Jacobson (1975).


STAGE OF DEVELOPMENT
5
10
15
20
25
30
35
38
40
100
80
60
40
20
PERCENT SURVIVING
TOTAL
NORMAL
CONTROL
TOTAL
NORMAL
GYNOGENETIC
0
2
4
6
8
10
12
14
16
DAYS AFTER FERTILIZATION

fluid. No heart beat or circulation of red blood cells was apparent. The larvae did not feed, and died several days after hatching. The mutation seems to be similar, if not identical to the cardiac lethal, c, described by Humphrey (1972). Of the other 17, only 12 appeared truly normal. Eleven survived to feeding, and any mortality after this stage was more likely due to infection than to expression of lethal genes.

Experiment 6 was similar, except that the female was known to be heterozygous Ax/ax and P/p as well as m/m. Because the developing embryos showed a range of abnormalities, p/p homozygotes could not be identified with complete certainty. However, of 13 embryos which developed past stage 30, 4 to 6 appeared to show the characteristic phenotype of p/p animals, and died before hatching. Five larvae did hatch, one of which appeared to be a pentaploid, and one a wild-type diploid, presumably not of gynogenetic origin. Of the remaining three, two were axanthic.

The last two experiments lacked a suitable genetic marker to indicate that the progeny were purely gynogenetic. In experiment 7 the female was D/d. Unfortunately a very high percentage of the embryos were abnormal. Many stopped development at the 64 to 128 cell stage, and the only one to hatch was a haploid. In experiment 8 the female was D/d, Ax/ax, and A/a. Again, mortality was high, and only 3 hatched. All three displayed a mutant phenotype similar

to that observed in experiment 5 (cardiac lethal), and none survived to feed. Two of the three were albino, with apparently normal xanthophores, while the third was wild-type. The females from both experiments 5 and 8 were subsequently sent to R.R. Humphrey at Indiana University, where matings between these females and males known to be heterozygous for the cardiac lethal, c, demonstrated that both of these females were heterozygous for gene c.

DISCUSSION

I p/p mutant:

Malacinski and Brothers (1975) have classified the p gene as an autonomous lethal. Although the sample size is relatively small, results of the parabiosis experiments in this report support the view that this gene is an autonomous lethal. However, since reciprocal transplants between mutant and normal embryos have not been carried out, it is possible that this classification as an autonomous lethal is incorrect. The gene p is the earliest acting of the thirteen genes classified as recessive autonomous lethals in the axolotl; all others express themselves at some time after hatching (Humphrey, 1975).

Several of the abnormalities that occur in the p/p embryo can be related to either lack of circulation, or edema, or both; others cannot be directly attributed to these conditions. Fortunately, two well characterized mutants are available in the axolotl that demonstrate the abnormalities that arise from lack of circulation and/or edema. The cardiac lethal mutant (c) suffers from lack of circulation and concomitant edema, and the fluid imbalance mutant (f) suffers from edema and blistering, often accompanied by failure to establish a circulatory system. Those abnormalities in the p/p mutant that cannot be attributed to lack of circulation and/or edema will be

discussed with reference to the normal development of the affected organs or tissues.

Several of the abnormalities observed in the p/p mutant are almost identical to those encountered in the c/c mutant. Melanophore expansion, under control of the hypophyseal secretion, intermedin, is retarded in the caudal parts of both the c/c and p/p mutants, as this humoral agent is distributed by the circulatory system. The gills in the p/p and the c/c mutants are similar, and the gut in both is poorly developed, conditions which Humphrey (1972) ascribes to lack of circulation. Microcephaly is another condition which Humphrey (1960) relates to the absence of circulation in f/f embryos. The fact that the blood islands remain in the abdomen and in pockets of the endoderm can be attributed to failure to establish a complete circulatory system and/or the accumulation of excess fluid in the region of the blood islands (Humphrey, 1960, 1971). The additional loop often observed in the cephalic part of the heart is similar to the elongated, tortuous structure observed by Lemanski (1971) in the c/c mutant. In addition, the heart primordium seems to have a labile determination for curvature at an early age, and this curvature can be influenced by the shape of the pericardial cavity and by other mechanical factors (Copenhaver, 1955). The edema and blistering that occur in the p/p mutant are commonly encountered in any of the mutants that lack circulation

(Humphrey, 1975). Swollen pronephroi can also be attributed to lack of circulation, and this situation is probably compounded by the fact that the nephric ducts, though opening into the cloaca normally, are often convoluted and blocked as a result of the edema and blistering in the abdomen and flanks of the embryo. The swollen venous vessels likely arose as a result of the fact that the heart does beat, albeit feebly, and based on the assumption that these vessels are fluid filled, this heartbeat in a closed system (anterior end of the heart is plugged) could create a sufficient pressure to cause the observed swelling.

The myotomes and muscle cells appear to remain in a more primitive state in the mutant than in the normal embryos. Lemanski (1973) states that skeletal muscle in the c/c embryo was unaffected by the c/c mutation, based on the fact that c/c embryos were capable of normal swimming movements, as well as on the fact that skeletal muscle could be induced to contract vigourously by electrical stimulation. Since the musculature of the c/c mutant was unaffected by lack of circulation, it would seem that these abnormalities encountered in the p/p mutant are also unrelated to lack of circulation. The tears and degeneration observed in the musculature of the p/p mutant may well be related to the general lack of integrity of all the tissues of the p/p mutant, particularly those of later (M+4) mutants.

The plug in the anterior end of the heart of p/p mutants is the best example of a condition that cannot be easily explained as arising from lack of circulation or edema. The first visible structures representing heart primordia in urodeles are not present until early tailbud, and even at this stage only the presumptive endocardium, in the form of loosely organized mesenchymal-like cells, can be discerned. In early tailbud stages the free edges of the mesodermal mantle gradually converge towards the midline, thicken, and give rise to a small number of loose pre-endocardial cells. These cells detach and accumulate in the myocardial trough as a longitudinal strand which later hollows out and forms a thin-walled tube of endocardium. The myocardium continues growth and eventually surrounds the endocardium completely, forming the tubular heart. The cardiac primordia develops and remains as paired structures until late neurula stages, at which time the fusion of the bilateral heart primordia takes place in a cephalocaudal direction, with the bulboventricular region forming first, and the sinus venosus forming last. Differentiation also occurs in a cephalocaudal direction. In urodeles, the "tubular heart", at the stage at which the beat is initiated, contains the conus, the ventricle, and the anterior portion of the atrium. At this stage, only the most cephalic portion of the heart is completely tubular with respect to the myocardium. It must be stressed that during amphibian

development, all four regions of the heart are not formed simultaneously. The cephalic parts differentiate first, and then the more caudal regions (Lemanski, 1971, 1973). Heart determination in a urodele is dependant on the suppressive influence of the anterior neural plate, the inductive influence of the anterior endoderm, and the competence of the heart forming mesoderm (Jacobson, 1961). Transplantation experiments by Jacobson have shown that an inductive interaction between the anterior endoderm and the presumptive heart mesoderm, which overlies this endoderm, brings the heart to a state of determination such that early neurala stage heart explants will form beating heart tissue. The presumptive heart mesoderm requires further interaction with the anterior endoderm beyond early neurala stages, because there is a suppressive effect on heart formation from the anterior neural plate. Not until mid tailbud stages is the presumptive heart mesoderm sufficiently determined to form a heart in the absence of endoderm and in the presence of anterior neural tissue. Continued association of the heart mesoderm with anterior endoderm is essential if the heart is to acquire its proper form (Jacobson, 1961).

Heart development in the p/p embryo is perplexing in that the plug is in the endocardium of the cephalic parts of the heart, the part of the heart that first develops in urodeles. This means that either the early development

of the heart was normal and endocardial plug developed later, or that this endocardial plug does not interfere with the subsequent (more or less) normal development of the caudal portions of the heart. There is no ready explanation for the presence of the plug in the mutant but it would seem reasonable to assume that this condition did not arise from lack of circulation or edema in these affected embryos. Whether or not this abnormality has a genetic basis, or is a result of abnormal development of other parts of the embryo can be determined only by further study.

The poor state of development of the liver in the p/p mutant cannot be related to circulatory malfunction or edema, but may be explained by considering its normal development in urodeles. The liver in all vertebrates develops from the endodermal epithelium on the ventral side of the duodenum. In the anterior part of the mass of yolk cells, little cavities arise. These cavities, primitive blood islands, join and form at once the vitelline veins. The duct system common to liver and pancreas arises as a dumbbell shaped body, differentiating from primitive liver cells at the anterior pole of the liver anlage. The liver of amphibians is a conversion of the anterior part of the yolk mass into a liver (Elias, 1955). Nieuwkoop (1973), as a result of various transplantation experiments, concluded that the regional differentiation

of the endoderm depends on two sets of factors, one residing in the mesoderm, particularly in the mesenchyme, and the other in the endoderm itself. The regional determination of the endoderm gradually becomes firmer during gastrulation and neurulation. The actual stage of determination of the endoderm at the beginning of gastrulation, however, is still largely unknown. Balinski (1965) states that liver tissue rarely develops outside its normal position in experiments involving explantation and transplantation of endoderm. In isolation experiments, liver differentiated only as a rare exception, and then only atypically. In transplantation experiments liver often differentiated if endodermal grafts came to lie in the liver region. As a result of these experiments, Balinski concludes that quite special conditions are necessary for the development of the liver rudiment and these special conditions are found only in the region where the liver develops normally. These conclusions suggest that the poor development of the liver in the p/p mutant is due to either an inability of the hepato-cardiac area mesoderm to induce differentiation of liver tissue from the endoderm, or the defect is in the presumptive liver endoderm itself. As has been previously noted there are abnormalities in the mesoderm of the cardiac area and this may account for the lack of differentiation of the presumptive hepatic endoderm, although it does not rule out the possibility that the defect is in the hepatic

endoderm itself. In any event it is unlikely that the poor liver development in the p/p mutant contributes much to its overall poor development as Copenhaver (1943) found that removal of the liver anlage from Ambystoma embryos did not produce any detrimental effects during the embryonic stages.

Another abnormality in the p/p mutant that cannot be explained as arising from lack of circulation or edema, is the poor development in the branchial region. Normally, the endoderm plays the leading part in the development of the branchial region in urodeles. The endodermal cavity in this region is distended in a transverse direction. In the stage immediately following the closure of the neural tube, the lateral walls of the pharyngeal cavity bulge out and produce a series of outwardly directed pockets on each side. These pockets are the gill pouches or branchial pouches. The gill pouches are developed one after another beginning with the pair lying posterior to the mandibular arch. As the endodermal gill pouches reach the epidermis, having pushed aside the intervening mesoderm, the epidermis becomes folded inward, to meet the gill pouches. A series of branchial grooves is thus developed on the surface of the embryo, each groove corresponding to an endodermal pouch. The outer wall of the endodermal pouch and the inner wall of the epidermal groove fuse into a closing plate. A branchial cleft is formed where the closing plate becomes

perforated, so that open communication is established between the pharyngeal cavity and the outer medium. For the development of external gills in the urodeles, it is also necessary that the endodermal gill pouch reach the epidermis; otherwise the gills fail to appear. The external gills in the urodeles are later supplemented by internal gills that develop on the branchial arches (Balinski, 1965). In the stage 37 p/p embryo there is no evidence disproving that these pouches may have formed normally, and filled in afterwards, either from a proliferation or a migration of endodermal cells. Again, whether this defect has a genetic basis or is simply an indirect effect of the overall poor development of other parts of the embryo will require further study.

All of the abnormalities that occur in the p/p mutant that cannot be related to a lack of circulation or to edema can be related, at least indirectly, to the endoderm of the developing embryo.

The endocardial plug that occurs in the bulbus of the heart could have arisen through abnormal initial formation of the endocardial tube, or through a normal initial formation followed by development of the plug. In any event it has been shown previously (Lemanski, 1973) that myocardiogenesis is induced by an agent present in anterior endoderm of the embryo. Differentiation of endodermal structures such as the gill pouches and liver

are dependant on two sets of factors; one from the mesoderm in adjacent areas, and one which is found in the endoderm itself. Both of these structures are derived from the anterior endoderm. In addition, the gills of the p/p mutant appear to be abnormal beyond what should result from lack of circulation. In the c/c mutant the gills remain short and stunted, but some branching does occur, whereas the gills of the p/p mutant are short, never branched, and they develop a small bulb at their distal ends. The importance of the branchial endoderm in the formation of gills has been demonstrated by Severinghaus (1930) when it was found that a gill fails to develop after removal or the branchial endoderm from tail-bud stage Ambystoma embryos. Furthermore, Muchmore (1951) and Niewkoop (1973) have utilized transplantation and explantation experiments to determine that muscle or myotome differentiation was very poor in experiments in which endoderm was not included. Both concluded that the dorsal and lateral endoderm is implicated in the induction of the somites.

These observations would seem to justify a tentative hypothesis that the genetic basis for the unexplainable defects in the p/p mutant somehow involves the endoderm. Furthermore, all of the affected structures develop to a certain extent, then cease development. The myotomes form, but myotomes and muscle cells remain in primitive,

or undifferentiated state; gills form but remain unbranched; the endoderm of the presumptive gill pouches reaches the epidermis, as is evidenced by the presence of gills, but the "pouches" are abnormal at the stage examined; the endocardial plug in the heart represents a primitive embryological state of this tissue, or abnormal development of this structure; and the liver remains in a primitive undifferentiated state. These observations at least warrant a further experimental analysis of endodermal and endodermally induced structures in this mutant.

Classification of the p/p mutant as an autonomous lethal is based on rather indirect evidence. Although Malacinski and Brothers (1975) have classified this gene as an autonomous lethal, there are no published reports with any experimental evidence supporting this classification. The four parabiotic pairs discussed in this report can hardly be taken as conclusive evidence, especially in the absence of reciprocal transplants. Furthermore, recent studies on the stasis mutant (gene st) show that it does not produce a lethal metabolic effect involving all cells as it was earlier believed (ie. that it was an autonomous lethal). Transplants of limb and gill primordia from st/st donors have been found to survive and develop on normal recipients (Humphrey, 1975). It is feasible that the p mutation affects some function of the endoderm that cannot be corrected by parabiosis, as

induction by the endoderm requires close association of the endoderm with the tissue that is to be induced, rather than on an agent that can be circulated through a common circulation. Malacinski and Brothers (1975) suggest that it is possible that many genes now classified as autonomous lethals (such as the st mutation) will be found to have more subtle cell or tissue specificities. Further experimental analysis of the p/p mutant, particularly reciprocal transplants, should resolve this question.

In summary, the p gene is a simple mendelian recessive lethal which in the homozygous condition results in a complex of abnormalities. The p/p embryo develops to stage 37 at which time disintegration of superficial tissue begins. Many of the abnormalities observed in sections of the stage 37 p/p embryo are related to the failure of this embryo to establish a functioning circulatory system, or to the resulting edema that distends the abdomen and flanks of the embryo. There are, however, several abnormalities which cannot be attributed to these conditions. All of these abnormalities can be indirectly related to the endoderm, particularly the anterior and dorsal endoderm of the developing p/p embryo.

II diploid gynogenesis

Fankhauser and Humphrey (1942) and other workers have used cold shock with varying degrees of success to produce triploid axolotl larvae from normally fertilized eggs. Humphrey (personal communication) suggested that heat shock might give more reproducible results. Sladacek and Lanzova (1959) using a 10 to 15 min heat shock at 36°C immediately after oviposition, obtained 19% triploids, but only 29% survival. Rott and Betina (1964) obtained better results by delaying the shock to 45 min after oviposition. A 10 min shock at 36°C led to 81% triploids, but survival was not reported. Our value of 75% triploids for a 10 min shock is similar, but survival was reduced to 17%. Thus 7.5 min was chosen, even though it yielded only 40% triploids. In the gynogenesis experiments diploidy was restored in 66% of the embryos which survived to hatching, suggesting that meiosis II may be suppressed more readily in a haploid than in an egg that is already diploid.

Qualitatively, the results of the ultraviolet inactivation experiments were similar to those reported by Pogany (1971) for R. pipiens, though Pogany obtained mainly haploids with a dose of only 465 ergs/mm^2 (assuming that his reported "energy level" of 31 ergs/mm^2 is an intensity of 31 ergs/mm^2 sec), while in this study 96%

haploids were obtained only after a dose of 5280 ergs/mm^2. However, the depression of survival at intermediate doses of irradiation, the "Hertwig effect", was not as marked as in Pogany's experiments. The fact that this "Hertwig effect" is not more pronounced is probably due to inadequate sampling periods during the interval where the depression in survival does occur, as Pogany's experiments indicate that this effect occurs at a rather discrete dose. It was found that intermediate doses also reduced the ability of the sperm to successfully fertilize the eggs. Since Pogany did not indicate how fertility was assessed, this might be accounted for by a difference in procedure.

The relative success of the gynogenesis experiments was similar to that obtained for R. pipiens. At hatching, 16% of the fertile eggs were viable and diploid, though not all were normal. The range was zero to 29%. An additional 8% survived as haploids. Nace et al. (1970) reported that their percentage of viable embryos ranged from zero to 34%. Presumably all were diploids. Volpe and Dasgupta (1962) reported that only 4.4% of the eggs in 8 gynogenesis experiments developed to hatching. About half of the survivors died during larval life (Volpe and Dasgupta, 1962; Volpe, 1970). Because many extraneous factors, such as disease, affect survival after hatching, we have not attempted to compare mortality of gynogenetic larvae to that of normal biparental larvae.

However, of 58 diploids examined at hatching, only 31 appeared even superficially normal, and a number of these died before feeding. Several of these animals have nevertheless been raised to near maturity. To date, the oldest gynogenetic diploid is a one year old female, which, however, has not yet been bred.

Purdom (1969), who was unsuccessful in raising gynogenetic fish, attributed the high mortality to "inbreeding depression". Nace et al. (1970) estimated that the average homozygosity for all genes is .60 to .70 after one generation of gynogenesis. This would mean an increase in homozygosity for recessive alleles. Using this estimate, and our own survival data, we can estimate the genetic load for the axolotl. About 70% of control artificially inseminated eggs developed normally to hatching in the gynogenesis experiments (Fig. 40), while haploids survived about 2/3 as well (Fig. 33). Thus, if only 24 out of 100 embryos hatch, and of these 16 (66%) are diploids and 8 haploids, then there should have been 23 "normal" diploids and 17 haploids at fertilization. If the remaining 60% were diploids which died because of a 32% increase in homozygosity for recessive lethals, then the "average" axolotl must carry about 2 recessive lethals. This compares with estimates of 3 to 8 lethals carried by man (Strickberger, 1968). Although this calculation does not take into consideration causes of death other than homozygosity for

recessive lethals, it must represent the highest estimate for lethal load if gynogenetic death is due to additional factors as well.

The reason for poor survival of parthenogenetic individuals is not clear, though two favourite explanations have been the unmasking of recessive lethal genes (Hamilton, 1966), and, at least in haploids, an altered nucleocytoplasmic ratio (Hertwig, 1913; Briggs, 1949). In an attempt to overcome the problem of a variable genetic background, several groups have created haploid or homozygous diploid clones by nuclear transplantation (Subtelny, 1958; Hamilton, 1966; Dasgupta and Matsumoto, 1972). Prior to hatching, homozygous diploids developed about as well as controls with transplanted nuclei from normal biparental diploid cells, while haploids developed about half as well. However, the majority of homozygous diploids exhibited the "haploid syndrome" and this has been attributed to a lack of heterosis or hybrid vigour, irrespective of whether the embryo is haploid or diploid. All three groups feel that this is not the same as saying that unmasked recessives are expressed, but this is perhaps a subtle and dubious distinction. The distinction might be better made between death due to a single lethal gene, and that due to the combined effect of several sub-lethals.

Unfortunately, these experiments do not explain the very poor survival of gynogenetic diploids, which are still

partially heterozygous. The unmasking of recessive lethal genes is certainly a factor in survival, as is shown in experiments 5 and 8 in the results section for diploid gynogenesis.

A highly inbred line should carry fewer masked recessive lethals than random-bred animals. Thus, parthenogenetic embryos from an inbred line should survive better if the expression of deleterious recessive lethals is an explanation for parthenogenetic death. This has not been tested in an amphibian, possibly due to the lack of inbred lines. However, in the golden hamster, Kaufman, Huberman, and Sachs (1975) were able to perform this experiment, and found better development of haploid animals from inbred animals. In the mouse, Graham (1971) obtained contradictory evidence in a different type of experiment. He created diploid heterozygous embryos from fused blastomeres, but, contrary to expectation, they did not develop better. Graham (1974) proposed that normal development depends on an extra-genetic contribution from the sperm.

In summary, at least three explanations can be considered for parthenogenetic death: expression of recessive lethal mutations; reduced hybrid vigour; an extra-genetic contribution from the sperm. Of these, the first possibility is the only one clearly implicated, but it is probably not sufficient in itself.

The results of the experiments employing heterozygous females strongly supports the hypothesis that the heat shock restores diploidy by suppression of meiosis II. In addition, these results can be used to estimate gene-kinetochore distances. In three experiments where the female was Ax/ax, there were 35 gynogenetic diploid progeny for which colour could be determined, 10 of which were axanthic. The estimated heterozygosity is 43%, based on the assumption that only 10 of the 25 wild-type progeny would be homozygous. The G-K map distance, calculated from the graph of Nace et al. (1970), would therefore be between 23 and 35 map units, depending on the coincidence coefficient. In experiments 5 and 8, where the females were heterozygous for what appeared to be the same lethal gene, c, 17 or 34 progeny were homozygous for the lethal gene, which must therefore be closely linked to the kinetochore.

In the other experiments involving heterozygous females the numbers of progeny were too small or the identification of mutants too uncertain for G-K linkage estimates to be made.

An earlier attempt to determine G-K linkage in the axolotl was based on the frequency of homozygous recessive progeny among cold-shock induced triploid progeny from a heterozygous female and a homozygous recessive male (Lindsley, Fankhauser, and Humphrey, 1956). The genes d,

f, and the gene(s) for sex determination were found to be unlinked to their respective centromeres.

Olsen (1969), Purdom (1969), and Nace et al. (1970) have all stressed the usefulness of diploid gynogenesis to establish "inbred" lines. The same might well be done for the axolotl. However, the main interest in developing the technique reported here has been to use diploid gynogenesis to uncover recessive mutations in a manner similar to that used by Richards et al. (1969) to uncover the melanoid gene in R. pipiens. Though the mutations have not been characterized in detail, experiments 5 and 8 uncovered a mutant affected in late development and the results of experiment 3 suggest that that female may carry a mutation affecting early development. Of course, in the case of recessive lethals, little time will be saved as the female must be bred conventionally, and her progeny inbred, to maintain the mutant stock. However, there is no reason why conditional lethal mutations, such as temperature sensitives, cannot also be sought.

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