STUDIES ON THE DEVELOPMENT OF PRIMORDIAL GERM CELLS IN *XENOPUS LAEVIS*.

A thesis submitted by
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degree of
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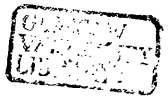

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INTRODUCTION

In 1885 Weismann proposed a theory of continuity of the germ line through particulate elements which were passed from generation to generation via egg cytoplasm. This theory has gained support with the identification in many eggs of cytoplasmic inclusions which determine primordial germ cells.* (E.g. Boveri, 1909 on Ascaris; Geigy, 1931 on Diptera; Bounoure, 1934 on Rana; Blackler, 1958 on Xenopus; Smith, 1966 on Rana.)

Early segregation of primordial germ cells.

In many animals the future cells of the germ line are distinguishable from somatic cells very early in development. The segregation may start as early as at the first cleavage division, as in Ascaris (Boveri, 1899) and appears to be complete in some insects (Geigy, 1931) and anurans

*Footnote The term "primordial germ cell" will be used throughout this study for those cells which are already completely segregated from the somatic line, but are not yet qualified to be called oogonia or spermatogonia.

(Bounoure, 1934) before somatic cells have undergone visible cytodifferentiation. Despite earlier work which claimed to show a secondary formation of germ cells from somatic cells in mammals (Allen, 1904) most of the recent evidence suggests that in vertebrates (Blackler and Fischberg, 1961; Smith, 1964) and insects (Hathaway and Selman, 1961) interconvertion of germ cells and somatic cells after segregation is not possible.

The embryology of the germ line seeks both to establish the cell lineage of the gametes, and to discover the nature of the agents determining their germinal fate. Therefore when it appeared that a cytoplasmic localization in the fertilized egg of some insects and anurans became enclosed in cells which then became primordial germ cells two questions arose. In the first place, was the cytoplasmic inclusion a determinant of germ cells, and if so, what was its mode of action? In the second place, what advantage was conferred upon an animal by such precocious determination of its germ line, since in cytogenetic theory all cell nuclei may be expected to be able to support germ cell development except in cases where chromatin elimination is known to occur?

In many animals the primordial germ cells are first identifiable after cytodifferentiation has started in some somatic tissues, e.g. mammals,

birds and urodeles, but in these cases the germ line is already completely segregated from the somatic line when it is first detected. It may be that the process of segregation begins very much earlier, and since the eggs of these animals do not appear to have a localized cytoplasmic determinant which is detectable by present techniques, the germ cell precursors cannot be traced. This does not preclude the possibility of a cytoplasmic determinant for primordial germ cells. It may be that in these animals the cytoplasmic agent is dispersed or undetectable for other reasons.

Nevertheless, early segregation of the germ line is almost universal. In mammals the primordial germ cells arise in the yolk sac endoderm of pre-somitic stages (Allen, 1904; Everett, 1943) in birds they arise in the vitelline endoblast of primitive streak stages (Swift, 1914) and in many fish (Eigenmann, 1891) and urodeles (Nieuwkoop, 1947) they are first detected at gastrula or early neurula stages. In many anurans, insects, copepods, coelenterates and nematodes primordial germ cell precursors or determinants can be identified at early cleavage stages. (For review see Wolff, 1964).

That the primordial germ cells of so many different groups of animals are segregated

so early in development suggests some important biological significance. An answer to the question "What advantage does precocious segregation of the germ line confer upon an animal?" may be found in considering the nematode Ascaris and some insects, e.g. Miastor. In both these cases chromatin elimination occurs in somatic cell nuclei (Boveri,1909; Kahle,1908) and in both cases primordial germ cells are protected from chromatin loss by a special area of cytoplasm. It may be that in all animals the germ line has to develop early so that it does not come under the same morphogenetic influences (e.g. embryonic induction) that cause somatic cells to differentiate along a particular pathway. Although there is no evidence in most animals to suggest that chromosomal material is lost in somatic cell nuclei, it may be that part of the genome e.g. that which is necessary for meiosis, is inactivated in somatic cells. Germ cells would obviously have to be properly protected from any such action.

Protection against somatic influences may be due in part to the extra-embryonic position of primordial germ cells in some animals.

In some insects (Metshnikoff,1866), birds (Swift,1914) and mammals (Everett,1943) the primordial germ cells are spatially segregated, arising in areas outside or on the edge of the embryo proper, and they migrate into the embryo

and towards the gonadal sites at a later developmental stage.

This argument, for the protection of primordial germ cells being due in part to their position in regions far from the morphogenetic influences of somatic differentiation, cannot be used in many cases. For example, in amphibians at gastrula and neurula stages the primordial germ cells are found deep in the endoderm (Bounoure, 1934) or in the lateral mesoderm (Nieuwkoop, 1947) where morphogenetic influences may be expected to reach them. However, in these cases it may be that the primordial germ cells are physiologically isolated, immune from the processes of differentiation which are affecting their somatic neighbours. Certainly in anurans the primordial germ cells appear more embryonic than the somatic endoderm cells from which they migrate at tail bud stage. They are larger and contain more undigested yolk platelets. (Blackler, 1962)

Germ plasm determines fate.

That cytoplasm can affect nuclear function is now well established. (For review see Gurdon and Woodland, 1968)

In many insects there is an area of cytoplasm at the posterior pole, called pole plasm, which determines primordial germ cells. Cauterisation

(Hegner,1911) and treatment of this area with ultraviolet light (Geigy,1931) causes sterility in otherwise normal insects. Similarly in anurans there is an area of egg cytoplasm located at the vegetal pole, called germ plasm, which determines primordial germ cells. Pricking the egg in this area to cause loss of cytoplasm (Blackler and Buehr, 1970) or treating the vegetal pole with ultraviolet light (Bounoure,1939; Blackler,1958) causes partial or total sterility in an otherwise normal frog. Replacement of germ plasm from a normal egg into one which has been irradiated restores fertility. (Smith,1966) These experiments prove that there exists a substance in the pole plasm of insects and in germ plasm of anurans necessary for the development of germ cells. That the substance is a nucleic acid is suggested by the fact that the wavelength most effective in sterilizing eggs is also that which is absorbed by nucleic acid. (Padoa,1963)

Life history of germ cells in anurans.

In 1934 Bounoure first described primordial germ cells in Rana temporaria. Since then Blackler (1958) working on Rana temporaria, R. esculenta, Bufo bufo and Xenopus laevis, L.D. Smith (1965,1966) on Rana pipiens, Gipouloux (1966,1970) on Discoglossus pictus, Mahowald and Hennen (1971) on

Rana pipiens, Czolowska (1972) on Xenopus laevis and Kalt (1973) also on X. laevis have increased our knowledge.

Areas of cytoplasm at the vegetal pole of the unfertilized egg in the cortical region can be identified histochemically. After fertilization these areas coalesce, and at the first cleavage division they are carried a little way into the egg close to the newly formed cell membrane, and evenly distributed between the two blastomeres. As cell division continues the germ plasm which increases in volume till about mid-blastula stage, comes to lie at the periphery of a few cells deep in the egg. In ranids, Bufo and Discoglossus the primordial germ cell precursors may reach the floor of the blastocoel, but in Xenopus laevis their position in the blastula is about mid way between blastocoel and vegetal pole. Their number varies from animal to animal, but the average for late blastula stage is about eight.

As gastrulation begins the germ plasm moves from the edge of the cell towards the nucleus, over which it forms a cap. From this stage onwards primordial germ cells can be considered segregated from somatic cells. At the same time the morphogenetic movements of the egg cause the primordial germ cells to be displaced so that at neurula stage the group of germ cells is located

deep in the endoderm. They remain in this position during neurulation and early tail bud stages but then migrate through the somatic endoderm, round the archenteron to its dorsal crest. Here they leave the endoderm, migrate along the dorsal mesentery to lie under the dorsal aorta in a position where later the gonads will form. Migration is thought to be induced by a specific chemical substance released by dorsal mesodermal organs, notochord, myotomes and pronephric ducts. (Gipouloux, 1970; Giorgi,1974) When they reach the gonadal sites there are some 20 to 40 germ cells. No mitotic figures have been seen in germ cells between gastrula and late tail bud stages so it is thought that they do not divide during those stages

Morphology, chemical nature and fine structure.

Primordial germ cells in many animals are slightly larger than somatic cells, with a large clear nucleus. In many birds, urodeles and invertebrates this has been a means of their identification. However in some animals the primordial germ cells have unique staining properties which make them identifiable before their morphological difference develops.

The primordial germ cells of some mammals are rich in alkaline phosphatase (Mintz,1960), those of birds are rich in glycogen (Dubois,1968) and

those of anurans contain a localized area of germ plasm rich in RNA and mitochondria. (Bounoure,1934; Blackler,1958)

The RNA stainability of Xenopus laevis primordial germ cells fades during development, indicating that the RNA content falls. (Blackler, 1958) Similarly in insects (Mahowald,1971) an ultrastructural study has shown that the RNA content of polar bodies, a component of pole plasm, remains high during early embryogenesis, at least until the germ cells reach the gonadal ridges. At this stage the cells are rich in large yolk platelets and have a large clear multilobed nucleus.

Electron microscopical studies on the germ plasm of Rana pipiens (Mahowald and Hennen,1971) and Xenopus laevis (Czolowska,1972; Kalt,1973) confirm the earlier light microscope work. Germ plasm has abundant mitochondria and "dense bodies" which are rich in RNA. Although the effective agent in determining germ cells is thought to be nucleic acid (Padoa,1963) it is not known whether it is the mitochondrial DNA or "dense body" RNA that is important. The "dense bodies" are no longer seen when the primordial germ cells reach the gonadal ridges (Camber et al,1970), although an elecron dense material appears again in close association with mitochondria during oogenesis (Al-Mukhtar and Webb,1971).

The object of this present study was to confirm the observations made by Bounoure (1939) and Blackler (1958) that primordial germ cells do not divide while the nucleus is capped by germ plasm, and to discover whether they are metabolically inactivated in some way by germ plasm. This was done by the use of radio-active isotopes to compare the rates of DNA, RNA and protein synthesis of germ cells with those of somatic cells. It was thought that if there was a real difference in the rates of division and differentiation between the two groups of cells it would be detectable by this method.

Also an electron microscopical study was made of normal germ plasm during early development, special interest being shown in the gastrula stages when the germ plasm is in contact with the nucleus. This was compared with germ plasm of eggs which had been treated with ultraviolet light. It was hoped that a comparison might elucidate which component of germ plasm is the effective agent. It was also hoped to discover the fate of cells containing irradiated germ plasm.

MATERIAL AND METHODS

Eggs of Xenopus laevis were obtained by injecting chorionic gonadotrophin into the lymph spaces of hind limbs of both male and female adults. Fertilized eggs were colected and kept at room temperature ($18^{o}C$ - $22^{o}C$) till they reached the developmental stage required for experimentation. Only those eggs and embryos which developed normally were used. The stages referred to in this study are those of Nieuwkoop and Faber (1956).

1. Histological techniques

For the identification of primordial germ cells in early embryos of Xenopus laevis mitochondrial stains proved more successful than RNA stains. Best results were obtained by fixing in Smith's fixative and embedding in soft (m.p. $46^{o}C$) paraffin wax, using Peterfi's celloidin method. Two staining methods were used.

a) Aniline acid fucshin and Methyl green. This stain, an adaptation of Altmann's technique used by Bounoure (1929) stains mitochondria. Unfortunately it also stains yolk platelets, but by reducing the time of staining, the mitochondrial accumulation can be distinguished from the surrounding yolk. The nuclei stain green. This stain is especially successful when used in conjuntion with autoradiography, as the emulsion is not stained at

all, and the silver grains are not affected.

b) Luxol fast blue and Phloxine. (Takaya, 1967) This stain is more specific for mitochondria, and does not stain the yolk platelets so strongly. Using this method the primordial germ cells are relatively easy to identify even in migrating stages. However the strong acidic differentiating steps in the staining schedule make it unsuitable for use with autoradiography, as the silver grains are removed.

See plate 1 on page 17 and plate 2 on page 18 for a comparison of these two staining methods used.

2. Autoradiography

Radioactive isotopes were obtained from the Radiochemical Centre, Amersham. Although it is possible for molecules of thymidine to penetrate early embryonic stages by culturing in a strong salt solution (Loeffler and Johnston, 1964) this method becomes less efficient as development proceeds. Injection of soluble material into early stages of X.laevis has been successfully accomplished, and has the advantage of penetrating into the cells in higher concentrations and more quickly.

Injections were made using a very fine needle attached to an Agla syringe into the blastocoel or archenteron of animals which had been manually de-jellied. The vitelline membrane was

left intact on the young stages, i.e. up to gastrula stages. Although it was impossible to judge how much injected material escaped when the needle was withdrawn, loss was kept to a minimum by leaving the needle in the egg for about 30 seconds after injection, then holding closed the vitelline membrane with fine forceps as the needle was slowly withdrawn. Injections were carried out in 100% Holtfreter solution to accelerate healing, and the animals transferred to 10% Holtfreter after 1 hour, and kept at 20°C. They were fixed in Smith's fixative, embedded in wax, cut at 5μ, dewaxed, coated with Ilford L2 emulsion (Rogers, 1969), dried and left for periods of up to six weeks in the dark. Liquid emulsion eliminated the problem of air trapped between sections and photographic film, which was found to occur when strip film was used. Silica gel was added to the dark storage boxes for the first 24 hours of storage to absorb the last traces of moisture from the slides, as water reduced the number of silver grains which finally remain on developing. The slides were then developed in Kodak D19, fixed in SolFix, and stained with Aniline acid fucshin and Methyl green.

Three experiments were carried out using three different radioactive isotopes.

a) Injection of 0·04μCi/animal of ^{3}H-thymidine (methyl-T), specific activity 5c/mM,

to compare the rates of DNA synthesis in primordial germ cells and somatic endoderm cells.

b) Injection of 0·04μCi/animal of ^{3}H- uridine of specific activity 5c/mM, to compare the rates of RNA synthesis in primordial germ cells and somatic endoderm cells. Both t-RNA and m-RNA are soluble so only those RNAs which are actually bound to protein while the embryo is being fixed are likely to remain on the section during histological procedure.

c) Injection of 0·08μCi/animal of ^{3}H-Leucine of specific activity 23c/mM to compare the rates of protein synthesis in primordial germ cells and somatic endoderm cells. The amino acid leucine was considered best (Deuchar,1966) for Xenopus laevis embryos, but an identical experiment was done using ^{3}H-methionine. Results were similar.

The migratory pathway of primordial germ cells was confirmed by autoradiography. Animals were injected at blastula stages and again at gastrula stages with ^{3}H-thymidine in order to label heavily every cell, even the slowly dividing endoderm cells. At Stage 22 an area of the endoderm, anterior to the anus, was excised and grafted onto an uninjected normal animal of the same stage which had a similar part cut out. The area of grafted endoderm is that which contains the primordial

germ cells. The animals were then left to develop to Stage 40, at which stage they were fixed and prepared for autoradiography. This method allows the primordial germ cells, which are labelled, to be easily identified as they migrate through the unlabelled host tissue.

3. Ultraviolet irradiation

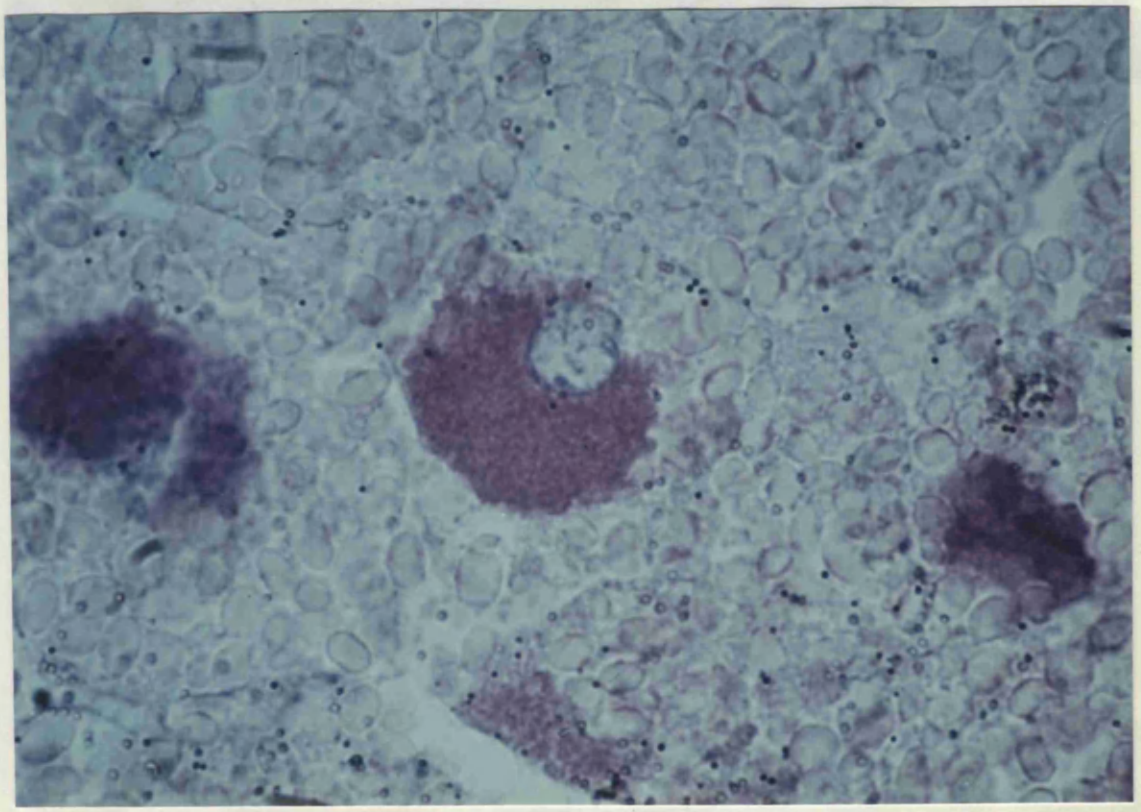
Eggs were picked out at Stage 1 and de-jellied manually. Just as the first cleavage furrow was forming they were placed on a nylon film, vegetal pole downward, and placed directly over a source of ultraviolet light at a distance of $2\frac{1}{2}$ to 4 cms. Each egg was exposed for 5 to 6 minutes, which was found experimentally to be the best time to produce partial or total sterility in tadpoles. Smith (1966) working on Rana pipiens has measured this optimum dose to be between 8,000 and 30,000 ergs/mm^2. Eggs were then allowed to develop normally till they reached the required stage.

4. Electron microscopy

Normal and ultraviolet irradiated eggs of Xenopus laevis at the following stages were treated for electron microscopy. Stages 2,4,7,9,$10\frac{1}{2}$,22,42 and 45. All eggs were de-jellied manually, and in later stages, i.e. gastrula onwards, the vitelline

membrane was removed as well. All eggs were fixed in 1% osmium tetroxide in 0·2M Sorensen's phosphate buffer, pH 7·6 at 4°C for 2 to 4½ hours. The longer fixation time was required for late gastrula and neurula stages, when the primordial germ cells are found deep in the endoderm, but penetration of fixative was encouraged by cutting a slit through the archenteron. Thick 1μ single sections were cut on an LKB ultratome, placed on a glass slide and stained with 1% Methylene Blue, and 1% Azure Blue in a 1% borax solution to locate the germ plasm. when it was found the block was trimmed and thin (400 Å to 600 Å) sections were cut, mounted on formvar coated copper grids, stained for 15 minutes each with uranyl acetate followed by lead citrate (Reynolds, 1963) and examined on an AEI 6b or 801 electron microscope.

PLATE 1. Primordial germ cell of a gastrula (Stage 10½) X.laevis embryo stained with Aniline acid fucshin and Methyl green. The nucleus stains blue/green and the germ plasm red. The yolk platelets are only very lightly stained.



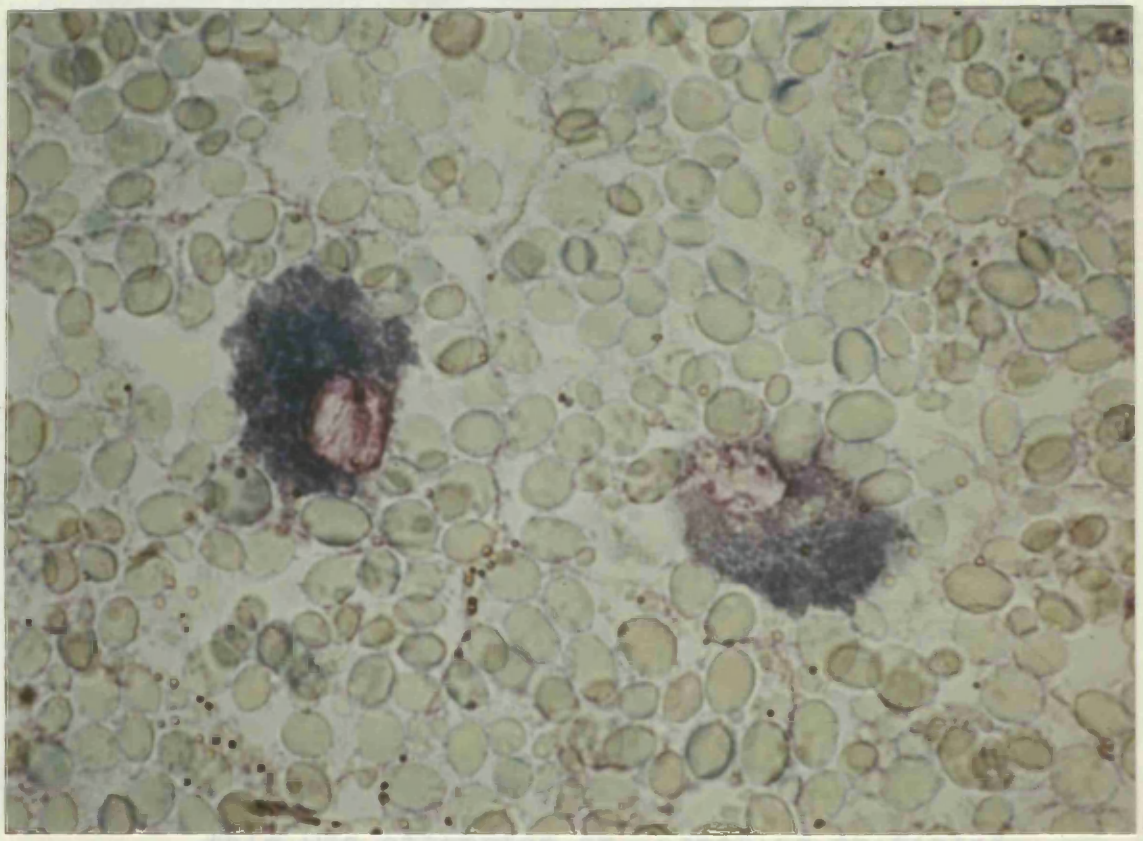


PLATE 2. Primordial germ cells of a gastrula (Stage 10½) X.laevis embryo stained with Luxol fast blue and Phloxine. The nucleus stains red and the germ plasm blue. The yolk platelets hardly stain at all.

RESULTS 1

Comparison of synthetic activity of primordial germ cells and somatic cells as measured by their DNA, RNA and protein synthetic rates.

If as has been suggested, primordial germ cells are protected from the morphogenetic influences that are affecting the somatic cells around them, and protection takes the form of physiological isolation, it might be possible to measure at least a temporary difference in metabolic activity between somatic cells and primordial germ cells.

This was attempted by injecting tritiated thymidine, uridine or amino acid into embryos of various ages and assessing the incorporation into DNA, RNA, and protein by autoradiography.

Comparison of DNA synthesis in the two cell types.

Tritiated thymidine (methyl-T) which incorporates into DNA during DNA synthesis was injected into embryos at stages shown in Table 1, page 21. The embryos were then left to develop normally at room temperature for various lengths of time before being killed by fixation and treated for autoradiography. The importance of using

thymidine labelled in the methyl position is discussed by Evans (1966). In conditions where thymidine is demethylated to uridine the label is lost, so ^{3}H-thymidine (methyl-T) can be used as a specific label for DNA. In preliminary trials the times chosen for exposure of the embryo to radioactive thymidine were 2,5,10 and 20 hours. The results shown on Table 1 and Figure 1 are those of a five hour exposure. Two hours was found to label too few cells, especially at tail bud stages, to make accurate counts and the longer exposures of 10 and 20 hours had the opposite effect, of labelling too many cells. A cell was considered labelled if the nucleus had eight or more grains over it.

Assuming that incorporation of thymidine occurs during the S-phase of the cell cycle and that the S-phase precedes cell division, then it follows that cells which are dividing quickly and have short G-phases will incorporate more radioactive thymidine in a given length of time than those which are dividing slowly, i.e. they will have passed through more S-phases, so will have incorporated more ^{3}H-thymidine and will therefore be more heavily labelled than more slowly dividing cells. In cases where some cells are labelled and some are not, the complete cell cycle is obviously longer than the exposure time. However, any

difference in the cell cycle times of the two cell types under observation should show up as a difference in the proportion of cells labelled.

A five hour exposure to tritiated thymidine caused all mesoderm and ectoderm cells to be very heavily labelled. The more slowly dividing endoderm cells showed a considerable variation in the proportion of cells labelled at various stages of development. See plate 3 on page 31.

The results on Table 1 are those obtained after a 5 hour exposure to ^{3}H-thymidine. At each stage shown 5 animals were examined and all primordial germ cells seen were counted, as well as 50 somatic endoderm cells from each of the same 5 animals.

TABLE 1

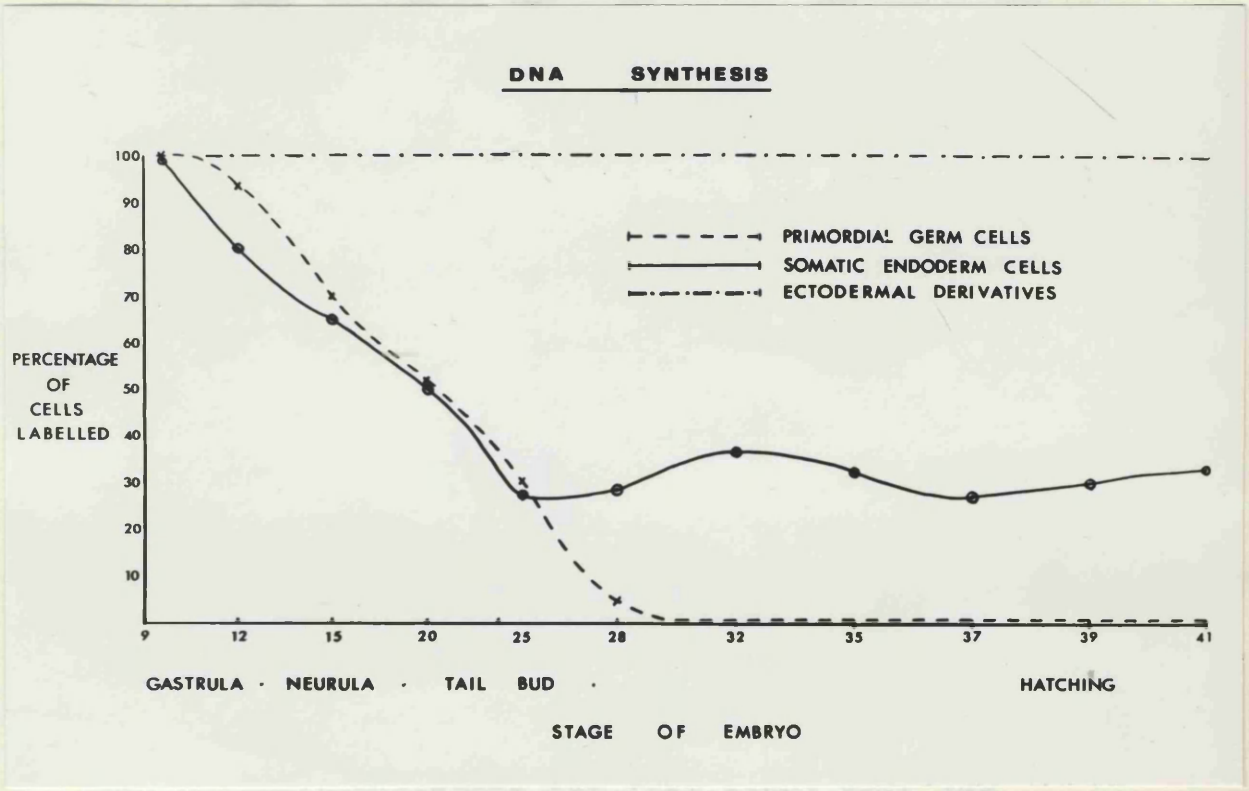
Stage of embryo	No.of pgcs* labelled	No.of pgcs seen	No.of secs* labelled of 250	%age pgcs labelled	%age secs labelled
9	15	15	250	100	100
12	38	40	203	95	80
15	32	46	162	70	65
20	25	47	131	53	52
25	15	50	77	30	31
28	1	17	80	6	32
32	0	34	95	0	38
35	0	25	88	0	35
37	0	81	81	0	32
39	0	98	85	0	34
41	0	78	90	0	36

*pgcs = primordial germ cells

*secs = somatic endoderm cells

Figure 1 shows the results of Table 1 plotted in graph form, with the additional line for cells of ectodermal origin, which were all labelled.

FIGURE 1



The results show that there is a difference in thymidine incorporation in the two cell types. In the graph both lines fall sharply during gastrulation and neurulation but about 30% of endoderm cells always become labelled. The primordial germ cell line, on the other hand, continues to fall, till at Stage 28 the cells are no longer incorporating thymidine at all. This shut down co-incides with the start of migration. Ectoderm cells and their derivatives are always 100% labelled.

Comparison of RNA and protein synthesis in the two cell types.

The same experimental procedure was used as for thymidine incorporation. In this case the precursor used for measuring RNA synthesis was tritiated uridine-5-T. Under biological conditions when uridine is methylated to thymidine it occurs at the 5 position so the tritium atom at that position is lost. Uridine-5-T can therefore be used as a specific label for RNA (Hayhoe et al.,1965). The histological procedures used which precede autoradiography cause all the RNA which is not complexed with protein to be washed out. In this way all cytoplasmic t-RNA is lost, and it seems likely that some r-RNA is also lost (Rogers,1969).

The results for a five hour exposure were different from the thymidine results in two respects.

1. The label was very light over the endoderm. This either means that the cells are not synthesizing RNA very fast, or else much of the RNA which is being synthesized is washed out in the histological process.

2. All cells were labelled. See Plate 4 on page 32. Since 100% of cells were labelled the amount of tritiated uridine incorporation per cell, in the form of number of grains per nucleus, was compared.

There were two reasons for counting nuclear grains rather than cytoplasmic grains. In the first place the amount of label in the cytoplasm of the endoderm cells was very small, whereas in the nucleus the grains were numerous enough to be counted easily and accurately. In the second place it was considered likely that background contamination would be greater in the cytoplasm than in the nucleus. Although background was not a serious problem in this series of experiments Rogers (1969) has suggested that one of the causes of background contamination is shrinkage of the photographic emulsion as it dries. Since the effects of this are seen to a greater extent in areas where the thickness of the emulsion changes rapidly, for example between relatively large objects such as yolk platelets it was felt that results based on cytoplasmic grain counts would have a greater degree of error. Shrinkage artefacts do not prove a problem over a relatively large, "flat" area, such as the interphase nucleus.

In order to minimise the error due to different nuclear size grain counts were taken from somatic cell nuclei of approximately the same cross-sectional area as those of primordial germ cells. Although the sizes were not measured accurately it was felt any slight difference would even out in the sample taken. There was certainly no noticable difference in the size of the nuclei of the two cell types during the stages of this experiment.

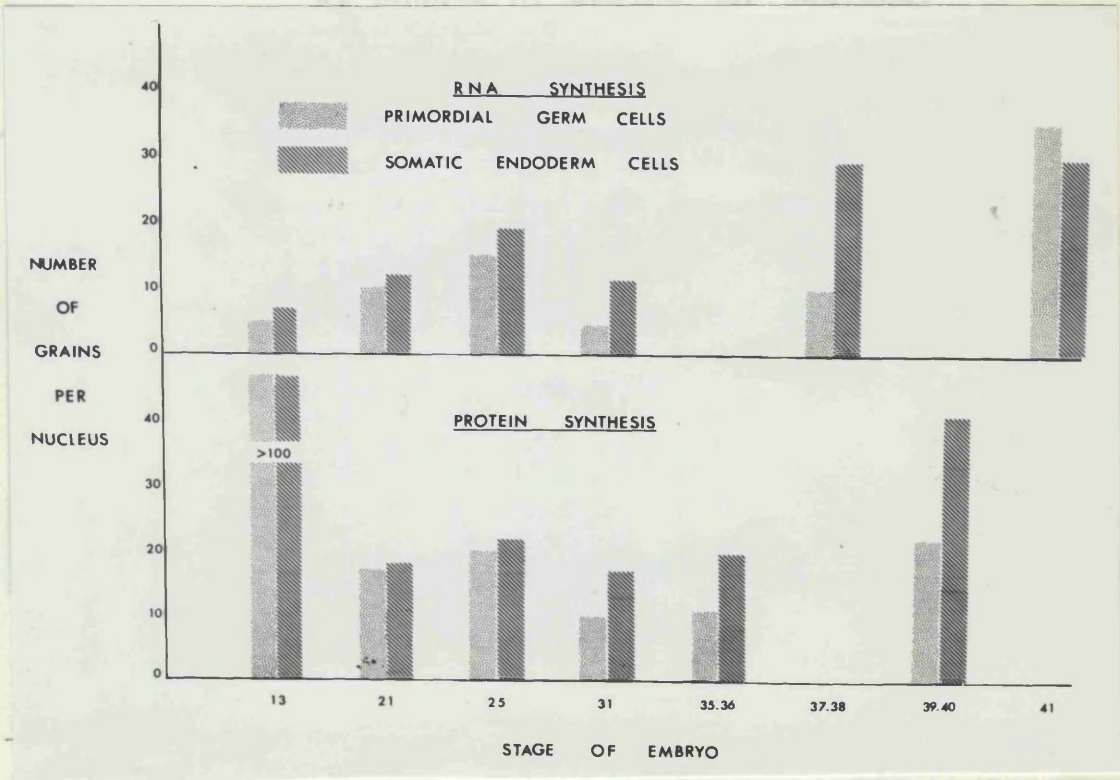
β-particles of low energy, as emitted by tritium have a track length of about 2μ when passing through emulsion. The emulsion thickness is estimated to be about 4μ, so no particles would be expected to pass right through and not be recorded. (Rogers, 1969) However the sections used in this experiment were all cut at 5μ so those β tracks originating in the lower half of the section, nearest to the slide, would never reach the emulsion to be recorded. As embryonic cell nuclei are estimated to be approximately 5μ in diameter only that part of the nucleus lying 2μ below the emulsion will be contributing to the results. The only consistent source of error in these conditions would occur if one cell type had a larger nucleus than the other, so that over equal areas the grain counts over the larger nucleus might be less, being more thinly spread. However the situation where nuclei of different volume appeared to have the same area in section would only occur if the larger, or both, of the nuclei were sectioned. To reduce this possibility grain counts were only taken from nuclei wholly visible in one section. If part of the nucleus was visible in the sections on either side of it, it was not used.

Thus although the absolute values of grain counts cannot be sensibly interpreted, the comparisons between cells of different type and

between earlier and later stages may well indicate real differences.

The results shown on Figure 2 were obtained from five primordial germ cells and five endoderm cell nuclei from each of three animals at each stage. The results were drawn in histogram form as a direct correlation could not be made between the different stages of embryonic development in this case where individual grains were being counted, rather than the proportion of cells showing label.

FIGURE 2



The pairs of results at each stage shown in Figure 1 were treated statistically using the t-test, to see whether the difference between the two cell lines was significant.

The formulae $t = \frac{\bar{x}_1 - \bar{x}_2}{S_{\bar{x}}}$, $S_{\bar{x}} = \sqrt{2.\frac{p.v.}{n}}$, $p.v. = \frac{Sx^2}{d.f.}$ were used to calculate 't' and t-tables were consulted to evaluate 'p.'

d.f.= degrees of freedom = 8.

Sx^2 = Sum of the squares of the results

p.v.= Pooled variance.

n = Number of results from each group = 5.

$\bar{x}_1$ and $\bar{x}_2$ = The average of the results for somatic endoderm cells and primordial germ cells respectively, as counts of grains per nucleus.

TABLE 2

Stage of embryo	$\bar{x}_1$	$\bar{x}_2$	Sx^2	$S_{\bar{x}}$	t	p
13	6.3	4.9	170	2.9	0.48	.6
21	11.9	9.9	737.1	6.06	0.33	.7
25	18.9	14.5	1814.6	9.5	0.44	.6
31	11.2	3.9	450.1	4.74	1.54	.05
37/8	28.6	9.6	2875.5	11.9	1.6	.05
41	29.1	34.6	Not calculated			

From these calculations it is seen that the difference in the pairs of results of uridine incorporation are only significant, $p < 0.05$, at Stages 31 and 37/8 which is while the primordial germ cells are migrating.

For protein synthesis the same method was used as for RNA, in this case the precursor being tritiated leucine. Again grain counts were taken from the nucleus only. In this experiment the cytoplasmic grains, between the yolk platelets, were so dense as to form solid black lines in many cells, and were therefore uncountable.

The results shown on Figure 2 were obtained after a five hour exposure to tritiated leucine, from five primordial germ cells and five somatic endoderm cells from each of three animals at each stage shown.

As for RNA, these results were treated statistically at each stage shown, using the t-test, as shown in Table 3, to see at which stages the difference between the two cell types was significant.

TABLE 3

Stage of embryo	$\bar{x}_1$	$\bar{x}_2$	Sx^2	$S_{\bar{x}}$	t	p
13	100	100	not calculated			
21	18	17	1839	8.5	0.12	.9
25	22.7	20.0	2741	11.7	0.23	.8
31	17.1	9.4	1157	7	1.1	.05
36/37	20.7	10.7	1632	9	1.1	.05
39/40	41.0	22.0	6671	18.3	1.04	.05

The abreviations and notations used are explained on page 27.

As counts were taken from the nucleus only either protein which is synthesized in the nucleus, or else protein which has moved into the nucleus after synthesis in the cytoplasm, is being measured. That there is an active passage of protein from cytoplasm into nucleus is well established (Arms, 1968). In either case the protein has been recently synthesized, so it was felt that a comparison of nuclear counts would give a meaningful picture of the relative protein synthetic activities of the two cell types, or the relative active passage of protein into the nucleus of the two cell types.

The results for protein synthesis show that the significant difference, $p < 0.05$, between primordial germ cells and their somatic neighbours is during stages 31 to 40, while the primordial germ cells are migrating. The very high incorporation of amino acid at gastrula stage in the absence of excessive uridine incorporation could be explained if maternal RNA, already present in the egg, is being used for protein synthesis.

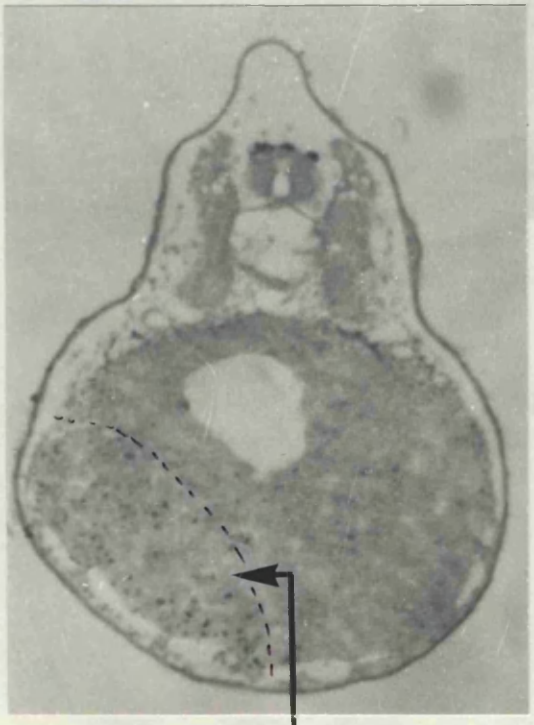
See Plate 5 for autoradiograph of ^{3}H-leucine incorporation into Stage 42 embryo.

Migratory pathway of primordial germ cells.

Plate 6 shows the results of grafting tritiated thymidine labelled endoderm cells into a normal host at early tail bud stage, and allowing the animal to develop normally to Stage 40, then treating the animal autoradiographically to trace the original grafted cells.

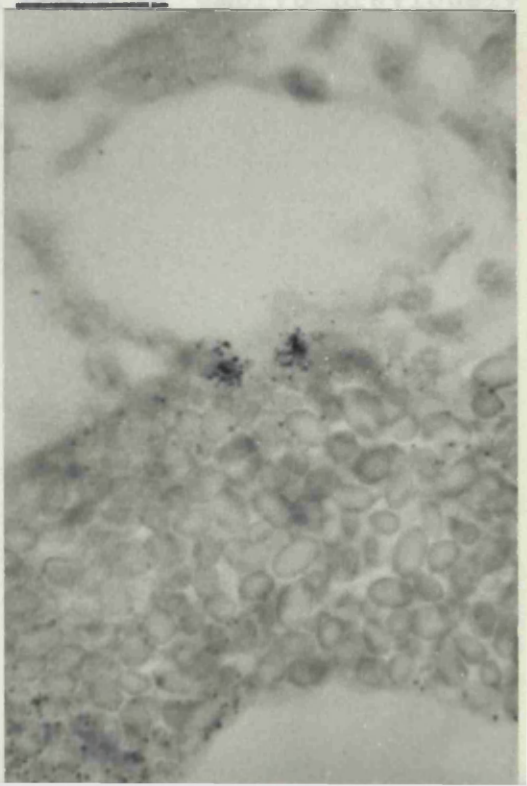
Plate 6a shows the area of the graft in the endoderm, distinguished by the labelled nuclei. Plate 6b shows two labelled primordial germ cells which have migrated from the grafted area to the dorsal crest of the endoderm.

PLATE 6a



Area of grafted, labelled cells.

PLATE 6b



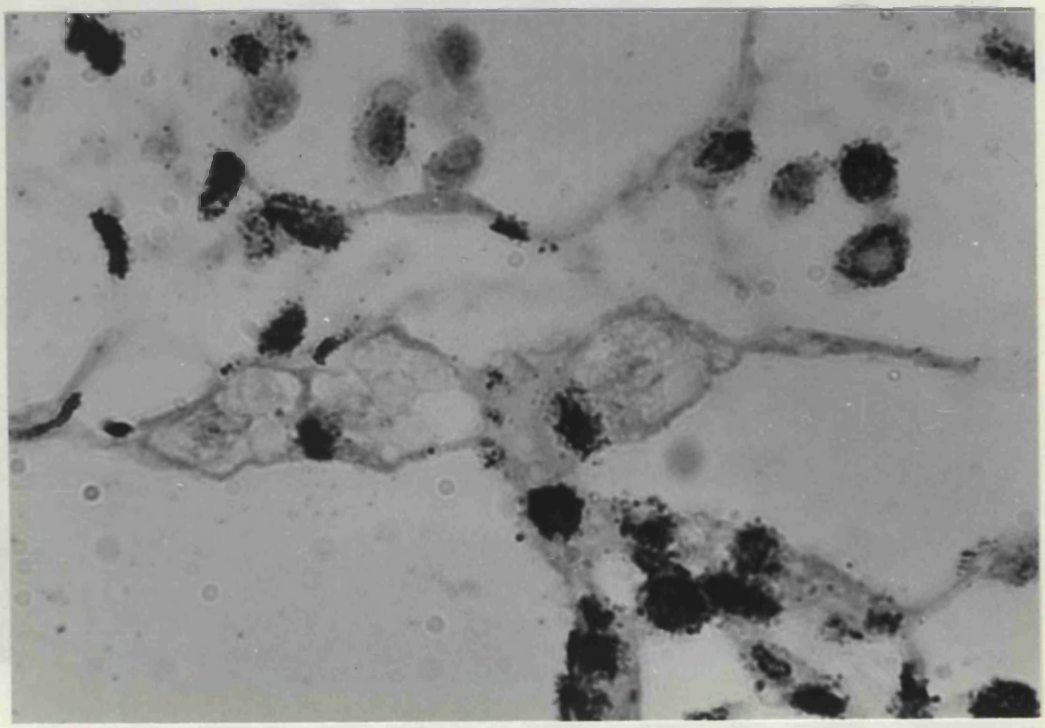


PLATE 3. Autoradiograph of a Stage 42 embryo previously injected with ^{3}H-thymidine. 3 or 4 unlabelled primordial germ cells are visible in the gonadal ridge. All other cells are heavily labelled.

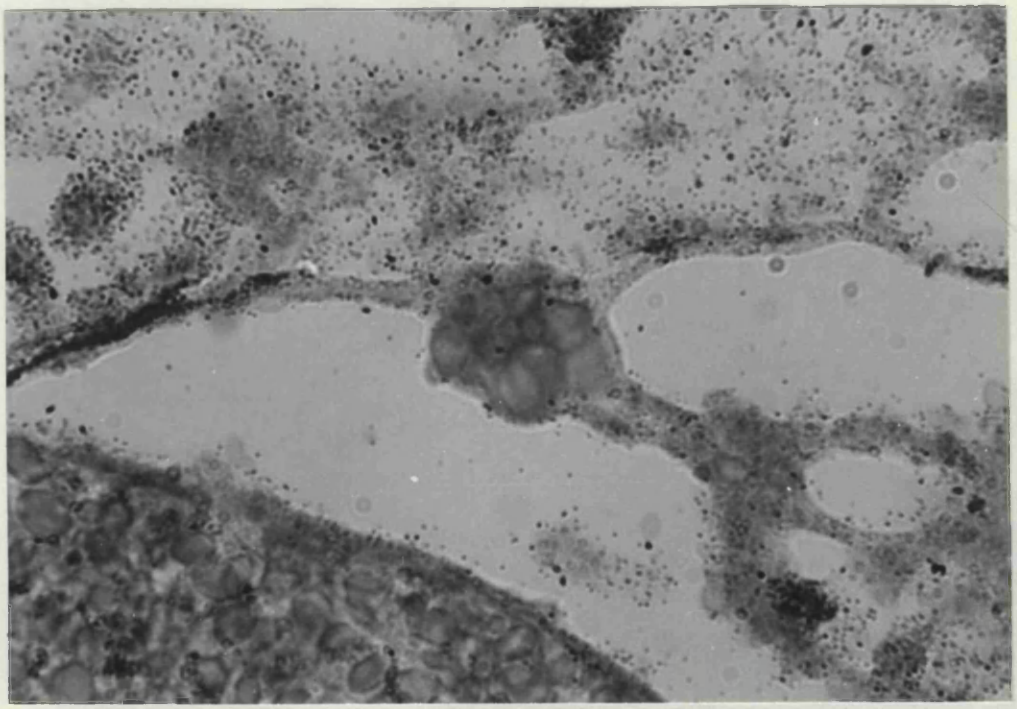


PLATE 4. Autoradiograph of a Stage 42 embryo previously injected with ^{3}H-uridine. One primordial germ cell is visible in the gonadal ridge.

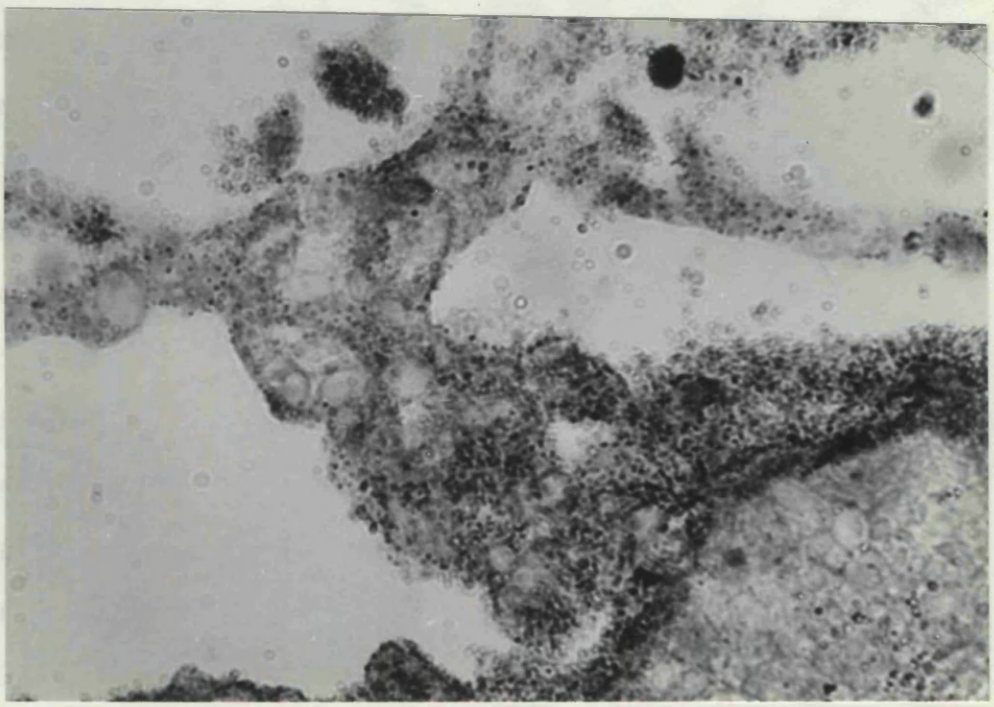


PLATE 5. Autoradiograph of a Stage 42 embryo previously injected with ^{3}H-leucine. Two or three primordial germ cells are visible in the gonadal ridge.

RESULTS II

Comparison of germ plasm from Stage 2 to Stage 42 in normal eggs, and in eggs which have been treated with ultraviolet light.

Controlled doses of ultraviolet light, directed at the vegetal pole of a just fertilized anuran egg causes sterility in an otherwise normal embryo. (Bounoure, 1934; Blackler, 1958) Replacement of germ plasm from a normal egg into one which has been so treated with ultraviolet light restores fertility (Smith, 1966). The wavelength of ultraviolet light most effective in causing sterility is 260 nm, the same as that which is absorbed by nucleic acids. This suggests that there is a nucleic acid component of germ plasm which is essential for determining germ cells. However since proteins are also destroyed by ultraviolet light (Deering, 1962) the role that protein plays in determining germ cells may be equally important.

It is known that germ plasm is rich in RNA and in mitochondria, so there are two main candidates for the sensitive agent.

1. The RNA or protein, or both, of "dense bodies."
2. The DNA or protein, or both, of mitochondria.

It was the object of the present study to try to elucidate what the role of mitochondria

and "dense bodies" was in germ cell determination, and if possible to see what effects ultraviolet light had on those components of germ plasm, at the light microscope and electron microscope levels.

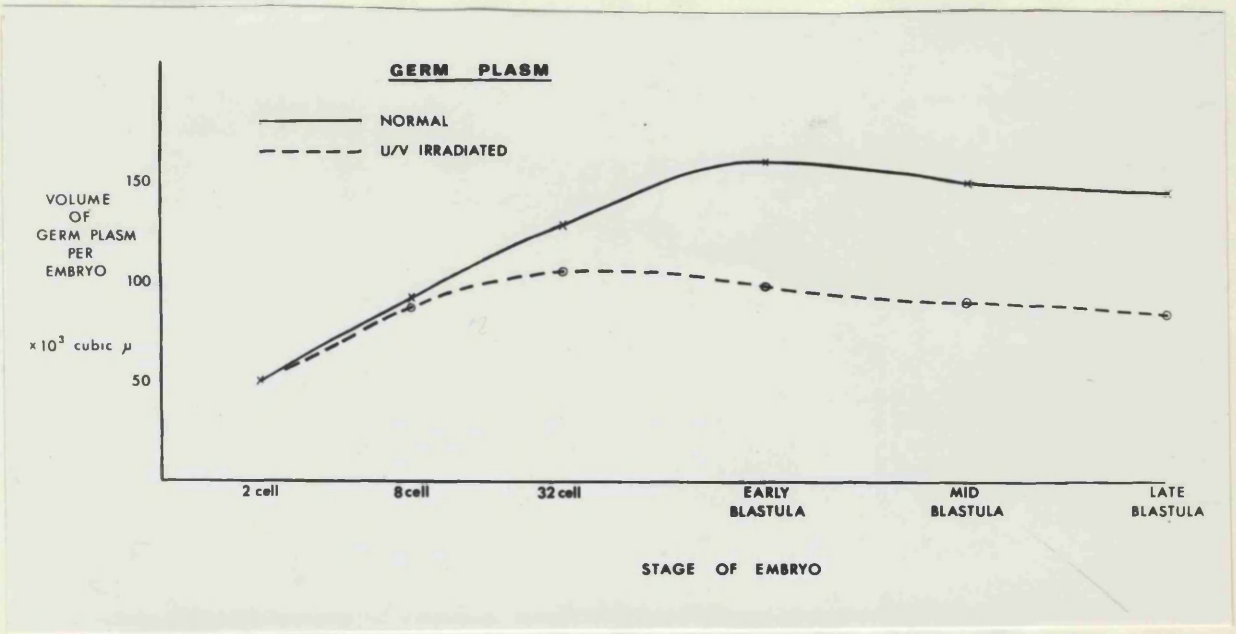
Light microscope observations.

Eggs treated with ultraviolet light and normal eggs of the same batch were fixed at various stages of development for light microscpe observation. The stain used for primordial germ cell identification was Luxol fast blue, which stains mitochondria specifically.

Experimentally it was found that 6 minutes of ultraviolet light directed at the vegetal pole of a Stage 1 egg, at a distance of 2½ cms. results in almost total absence of germ cells from the gonadal ridge of Stage 45 animals, which are otherwise normal.

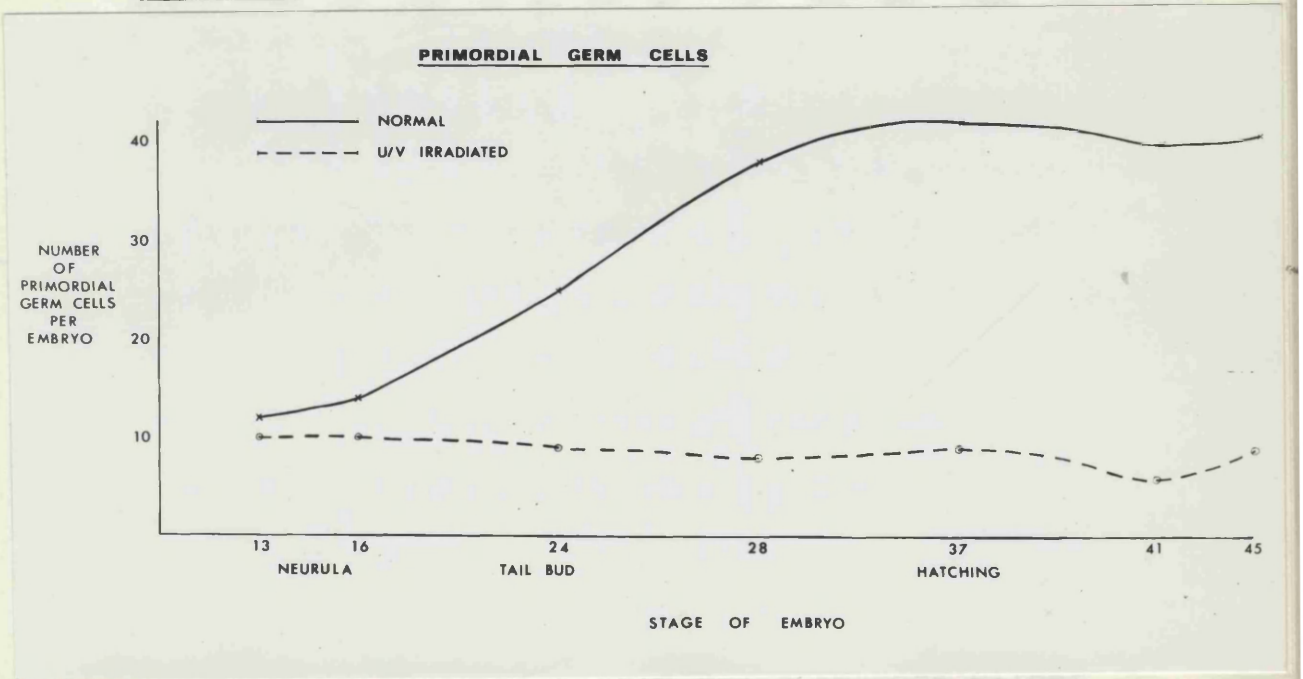
A measure was made of the amount of germ plasm visible in normal and ultraviolet irradiated eggs at various stages of development by measuring the total area of germ plasm in each of three animals cut at 5μ. The total volume of germ plasm was calculated, the average for each stage was found and the results plotted in graph form in Figure 3. From gastrula stage onwards numbers of primordial germ cells were counted, rather than the amount of germ plasm. These results are shown in Figure 4.

FIGURE 3.



Volume of germ plasm of normal and U/V irradiated animals at different stages of development, up to late blastula stage.

FIGURE 4.



Number of primordial germ cells of normal and U/V irradiated animals at different stages of development From late gastrula stage to Stage 45.

Ultraviolet irradiation has the effect of inhibiting the increase in volume of germ plasm that occurs in normal eggs during blastula stages, although the number of primordial germ cells visible at Stage 13 is not significantly different in the two groups. However by Stage 28 the number of primordial germ cells in the irradiated group is very small and their appearance is similar to those of normal gastrula stage. Also they are found together in a group, deep in the endoderm, and have not started to migrate. As well as the few apparently healthy primordial germ cells there are patches of germ plasm and perhaps whole cells which look necrotic, stain more deeply than the healthy cells and are sometimes found in or near the archenteron. These are thought to be dying primordial germ cells.

It appears therefore that ultraviolet radiation affects the mitochondriogenesis which normally occurs at blastula stages, does not affect the migration towards the nucleus of the germ plasm, does not significantly decrease the number of primordial germ cells at gastrula stage, but may affect the number of mitochondria each primordial germ cells has. However, from Stage 13 onwards ultraviolet irradiated germ plasm appears either to die or to cause the arrest of development of primordial germ cells, and finally causes their

death, or their continuance as somatic endoderm cells.

It was impossible to determine clearly whether irradiation caused the death of germ plasm alone, allowing the cells which incorporated germ plasm to continue their existence as somatic cells, or whether irradiated germ plasm caused the death of the whole cell. These studies indicated that both conditions probably occured together, as both germ plasm alone and germ plasm, yolk platelets and nucleus toghther were seen in the archenteron of irradiated animals.

Electron microscope observations.

Normal

The only previous studies of the ultrastructure of germ plasm in Xenopus laevis have been made by Czolowska (1972) and Kalt (1973) who confined their attention to early cleavage and blastula stages.

In general the ultrastructural study has confirmed observations made by light microscopy. Germ plasm consists of regions of yolk-free cytoplasm varying in size from about 20μ across at the 2 cell stage, to 50μ across at the 32 cell stage. This cytoplasm contains numerous mitochondria, "dense bodies," particles of two distinct kinds, classified as β-glycogen and ribosomes according to

their size, small vesicles and a very few lipid droplets. "Dense bodies" are thought to be unique to germ plasm (Mahowald and Hennen,1971) so their ultrastructural changes were carefully noted through the developmental stages studied. Mitochondria on the other hand although not unique to germ cells are generally thought to play some special role in germ cell development, by virtue of their very large numbers. The mitochondria of germ plasm of early cleavage stages vary in size from 0.5μ to 1μ, have clear external and internal membranes, have electron dense matrices, and are in the "condensed" state (Hakenbrock,1972). This is a physical state identified by clear, distinct cristae often showing a zig-zag pattern, and a well defined matrix. See Plate 7. The association between mitochondrial structure and energy state will be discussed later. Electron-dense spots on the matrix, thought to be complexes of stain and divalent cations, probably calcium, and also characteristic of the energy state of the mitochondrion are rarely seen in the two cell stages, but increase in number during cleavage stages, till at late blastula stage they average four per mitochondrial profile. This average remains the same through the following developmental stages studied. The internal structure of the mitochondria changes very little during cleavage and early blastula stages. They retain their clear

internal structure and distinct membranes. At late blastula stage there is a slight breaking down of the internal morphology, the cristae have become less clear, and the matrix has developed "empty" spaces. One striking feature of blastula stages is the vast increase in mitochondrial numbers. Not only does germ plasm increase in volume up to mid-blastula stage, but the number of mitochondria per unit area in section increases by 25% from the 2 cell stage to late blastula stage. From gastrula stage onwards the mitochondria are so tightly packed as to leave little room between them for other cell constituents. Whether the increase in mitochondrial number is due to mitochondrial division or "de novo" synthesis has not been clearly established.

At gastrula stage there is a change in the morphology of the mitochondria. Their internal and external membranes become less distinct and as the mitochondria are tightly packed together they are difficult to distinguish individually. They press closely against the nuclear membrane, which has numerous pores.

In late neurulae (Stage 22) the mitochondria are still found in large numbers, tightly packed together, and against the nucleus. At this stage their external membranes are clear and distinct. Their internal structure, although clear, differs from that described for early cleavage stages. In

this case the cristae are narrow, sometimes in the form of straight tubes or plates, sometimes arranged in a zig-zag pattern. The matrix is electron dense, and has a finely granular appearance. There are numerous calcium spots. See Plate 8.

The nuclear membrane does have pores but no electron dense material is seen in them.

At Stage 42, the next stage studied, when the germ cells have migrated out of the dorsal crest of the endoderm, mitochondria are still present in large numbers, but their appearance is not clear. External membranes, and to a lesser extent, internal membranes have broken down and in many cases the mitochondrial matrix merges with the general cytoplasm. See Plate 9. Some cells contain whorls of membranes, characteristic of degenerating mitochondria.

At Stage 45 the germ cells have large bi-lobed nuclei, surrounded by mitochondria, but not in the vast numbers of previous stages. Mitochondria are abundant but not excessively so. They have clear internal and external membranes, and look normal and healthy.

That large numbers of mitochondria disappear so suddenly between Stages 42 and 45, just as the primordial germ cells are coming to the end of their migratory phase suggests that the main function of the mitochondria is to support migration, perhaps by providing the necessary energy. However there

are other possible roles for large numbers of mitochondria, and these will be discussed later.

"Dense bodies" which are characteristic of germ plasm and found as early as at the first cleavage stage are small areas, 0·2μ to 0·3μ across, of elecron dense granular and fibrillar material, lying in the cytoplasm between the mitochondria. They are not membrane bound, and their outer edge consists of fibrils spreading out and in contact with mitochondrial outer membranes, or particles, probably ribosomes. See Plate 10.

At the 32 cell stage the "dense bodies" continue to have a granular core, but the fibrillar halo is now more distinct. As blastula development proceeds the "dense bodies" become less dense, but do not diminish in size. Their close association with mitochondrial outer membranes persists. At late blastula stage "dense bodies" have a large, in some cases 0·5μ, finely granular core, with a large halo of electron dense material around them, clear of other cell constituents, such as glycogen and ribosomes. A large area of cytoplasmic material very similar to the core of the "dense body" and associated with small vesicles, was seen in one cell very close to the nucleus. See Plate 11.

At gastrula stage some few "dense bodies" are visible between the now very tightly packed

mitochondria. At neurula stage there are no "dense bodies" visible at all, nor are they seen in the later stages studied. A similar electron dense material appears again near the nucleus in germ cells during oogenesis (Al-Mukhtar and Webb,1971)

Camber et al. (1970) previously reported that primordial germ cells of Rana lacked "dense bodies" when they migrated out of the dorsal crest of the endoderm, whereas they were present in germ plasm of early cleavage stages. In Xenopus laevis it appears as if the "dense bodies" disappear from the cytoplasm at gastrula stages. It has not been clearly established whether they enter the nucleus or simply change their form so that they are no longer visible in the electron microscope. However since their disappearance co-incides with the movement of germ plasm towards the nucleus, and since similar material has been seen near the nucleus at a time when nuclear pores are abundant and filled with similar electron dense material the possibility exists that "dense body" material enters the nucleus. See Plates 11 and 12.

Particles of β-glycogen and ribosomes are present in germ plasm through all stages studied. β-glycogen particles are 30nm in diameter and stain heavily. Ribosomes are smaller, 18nm, and stain more lightly. At mid-blastula stages there is a sudden increase in particles of 30nm size, and

although they are light staining when they first appear they may be β-glycogen, as they become darker at later stages. They are not unique to germ plasm but appear in the cytoplasm throughout the cell, and also in somatic cells. Both glycogen and ribosomes remain as single units free in the cytoplasm and do not form rosettes. Also there is no endoplasmic reticulum in the primordial germ cells of the embryos studied. The small vesicles found in germ plasm, and in the general cytoplasm of eggs are thought to be the embryonic equivalent of endoplasmic reticulum (Balinsky and Devis, 1963). At early cleavage stages they are bound by a singe membrane and are empty, or contain only a few fibrils. At mid-blastula stage many of them in the germ plasm have deep infoldings of their membrane so they appear double membraned, see Plate 13, and at late blastula they appear to have electron dense material between their membranes. This may be the way in which new mitochondria are formed by "de novo" synthesis (Leninger, 1964). As has been stated before, at these late blastula stages the density of packing of mitochondria increases, so the theory that mitochondria are formed from the vesicles is a possibility. Also the vesicles in germ plasm from gastrula stage onwards are greatly reduced in number.

Rod-like inclusions found in some mitochondria.

In one clutch of eggs rod-like structures were seen in the mitochondria of the germ plasm. Since they were only seen in the eggs of one pair of animals, and then in only about 1 to 2% of the mitochondria, their existance is probably abnormal, although similar structures were noted in the adrenal cortical cells of the rat (Lin, 1965).

They appear as long, sometimes 1μ, rods composed of 7 fibrils, six of which are arranged hexagonally round a central one. The whole fibre is 32nm in diameter, each individual fibril being 8nm in diameter. The whole fibre sometimes appears slightly twisted in longitudinal section. They always appear in the cristae of the mitochondrion, never in the matrix, and were more commonly seen in large, long mitochondria which were apparently dividing along the long axis. See Plates 14 and 15.

What the function of these inclusions is, or what causes them to appear in only some mitochondria is not known.

Ultraviolet irradiated

There has been no previous reports of an ultrastructural study of germ plasm of an embryo previously irradiated with ultraviolet light.

This study of irradiated germ plasm began at the 2 to 4 cell stage, just 15 minutes after irradiation. At this stage there was no visible difference between normal and irradiated germ plasm. Mitochondria, "dense bodies," ribosomes, β-glycogen and small vesicles all appeared identical with normal 2 cell stages.

However by the 8 cell stage, 1½ hours after irradiation, the mitochondria are already showing signs of change. Many of them look swollen with little internal structure. The matrix is thin in areas, and appears clumped round broken and incomplete cristae. "Dense bodies" appear unaffected. See Plate 16.

By mid-blastula stage the effects of early irradiation are more noticable. The size of patches of germ plasm is not as great as in normals and the number of mitochondria per unit area is not so great. The mitochondria themselves vary in size from 0·4μ to 2μ, a wider range than normal, and in many the internal structure is indistinct. The matrix is thin and the cristae only clear at the edge.

The most striking difference lies in the

cytoplasm, where at this stage the normal animal shows an increase in the glycogen content. In the irradiated animals, however, there is no great increase in the number of particles and the background remains relatively empty. This dirth of glycogen particles remains a feature of irradiated germ plasm through all future stages studied. See Plate 17. "Dense bodies" remain unaltered as electron dense areas of finely granular consistency.

By gastrula stages the mitochondria appear round and swollen, average diameter 1μ, and the matrix is thin round degenerating cristae, with "empty" areas fairly abundant. "Dense bodies" are only rarely seen.

At neurula stage the irradiated egg contains primordial germ cells with mitochondria similar in structure but fewer in number than normal. The mitochondria are in the "orthodox" configuration, with narrow cristae and a large volume of matrix. Whorls of membranes are commonly seen, often attached to the mitochondria themselves. See Plate 18. There are no "dense bodies."

Later stages of ultraviolet irradiated eggs were not studied, as light microscope observations indicated that primordial germ cells do not develop further.

Conclusions

From these observations, made at the cellular and sub-cellular level, certain conclusions can be drawn. Normal germ cell development is characterised by a great increase in the number of mitochondria in the germ plasm during blastula stages, a large increase in the number of primordial germ cells during gastrula and neurula stages, maintenance of these large numbers of mitochondria throughout embryogenesis till at Stage45, when the primordial germ cells have reached the site of the future gonads, many of the mitochondria break down and disappear. "Dense bodies" present during blastula stages disappear at gastrulation.

Ultraviolet irradiated germ plasm does **not** show the large increase in mitochondrial numbers seen in normals, nor is there the increase in primordial germ cell numbers seen in normals. The mitochondria themselves change in form to become swollen with an indistinct internal structure. This condition lasts until late gastrula stage. At neurulation the mitochondria again look normal, but are present in very much fewer numbers. There are also numerous membranous whorls, evidence of degenerated mitochondria. "Dense bodies" are apparently unaffected.

These observations might imply that mitochondria play a more important role than

"dense bodies" in the formation of germ cells in Xenopus laevis and if damaged by ultraviolet irradiation, not only are fewer primordial germ cells formed, but those that are, are retarded in development and unable to migrate.

The different possible roles for mitochondria and "dense bodies" will be discussed in the next chapter.

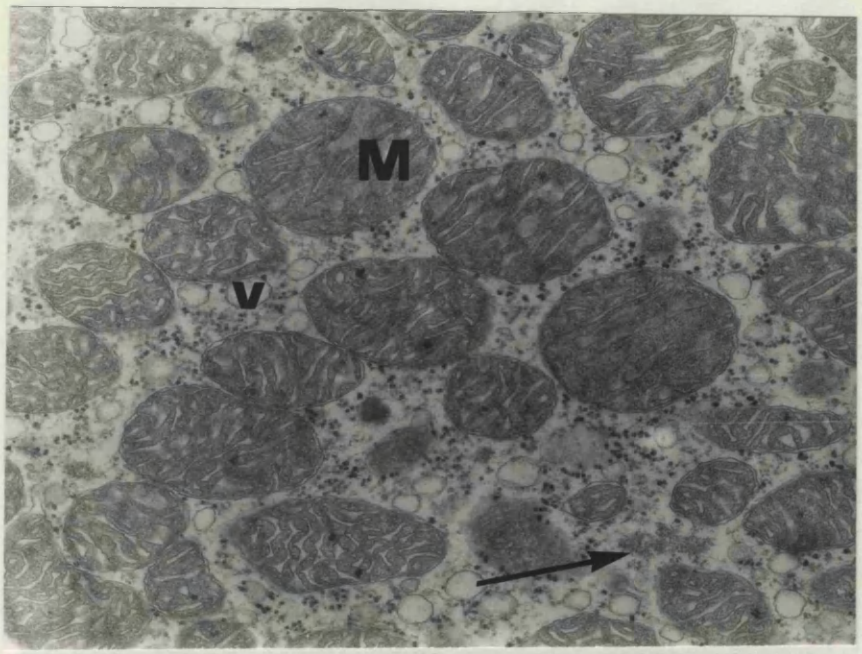


PLATE 7. Germ plasm of an 8 cell stage X. laevis embryo showing (M) mitochondria, (V) vesicles, dark and light particles and "dense bodies" (arrow).
x21,300.

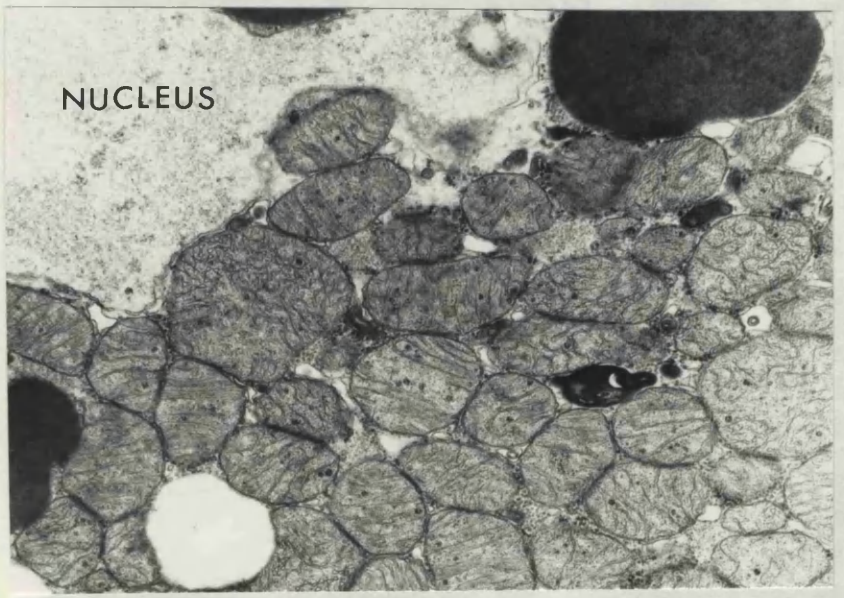


PLATE 8. Germ plasm and nucleus of a neurula (Stage 22) embryo. Mitochondria are in the orthodox configuration and have numerous calcium spots. There are no "dense bodies."
x20,240.

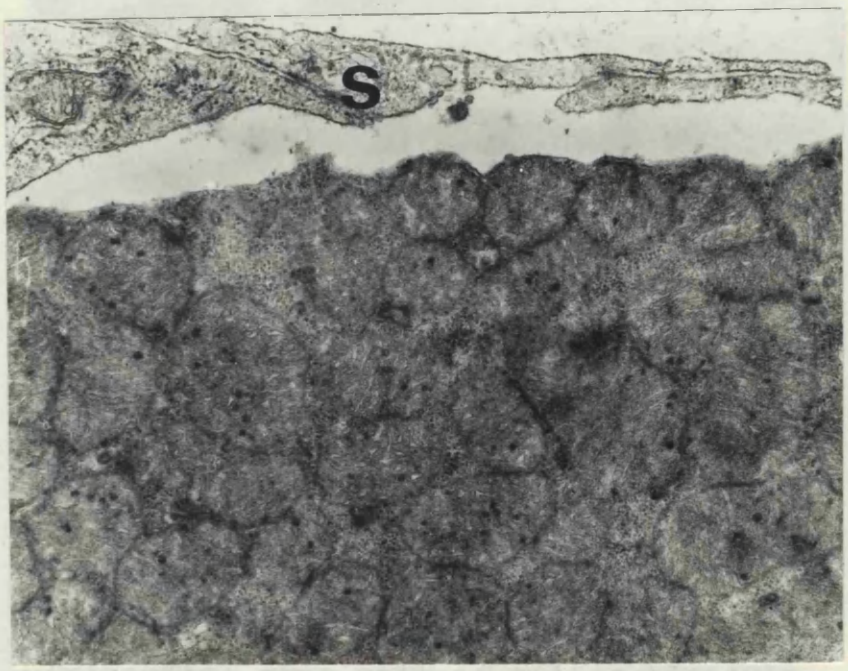


PLATE 9. Edge of a primordial germ cell of Stage 42 embryo. The mitochondrial stucture is breaking down. Membranes are indistinct. (S) Somatic mesentery cells.

x25,240.

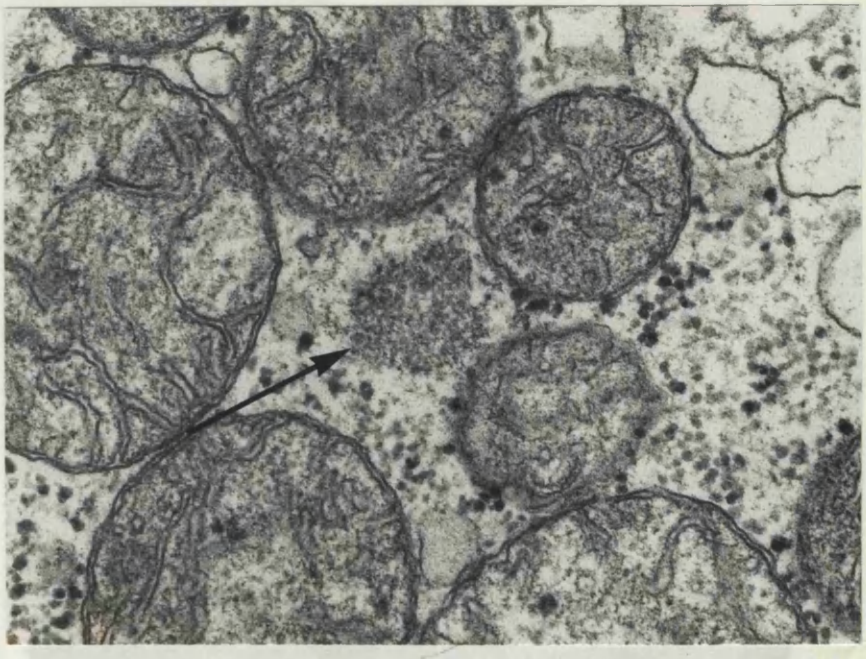


PLATE 10. "Dense body" (arrow) of a mid-blastula (Stage 7) embryo. It is closely associated with the mitochondrial outer membrane and cytoplasmic particles.

x 65,170.

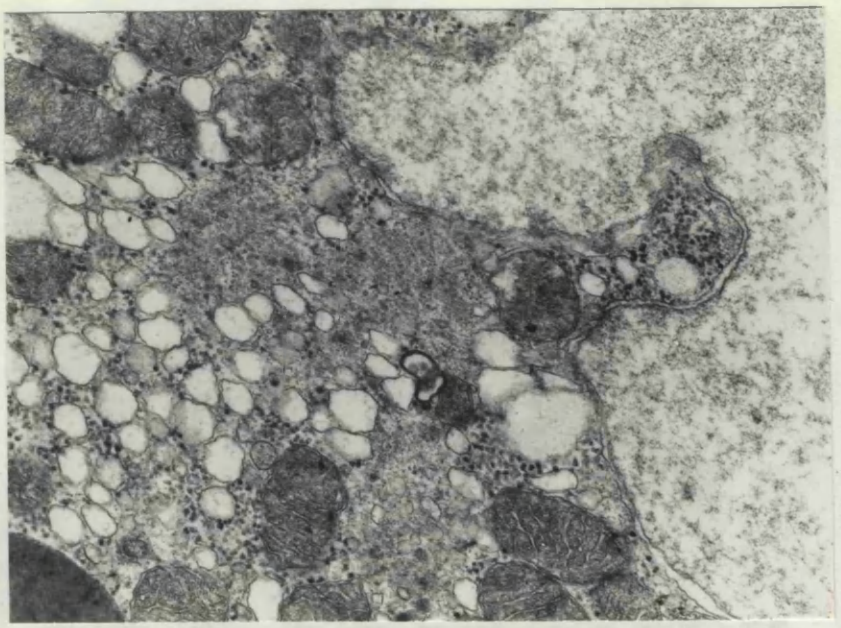


PLATE 11. Primordial germ cell of a late blastula (Stage9) embryo, showing finely granular material similar to "dense body" close to the nucleus.

x26,720.

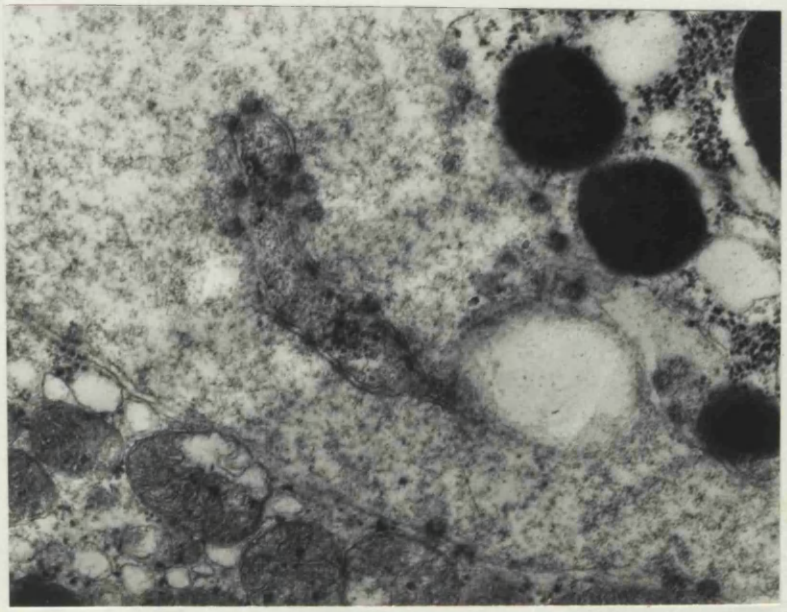


PLATE 12. The same cell as that shown on Plate 11. This micrograph shows the numerous pores in the nuclear membrane through which the granular material may be passing.

x26,000.

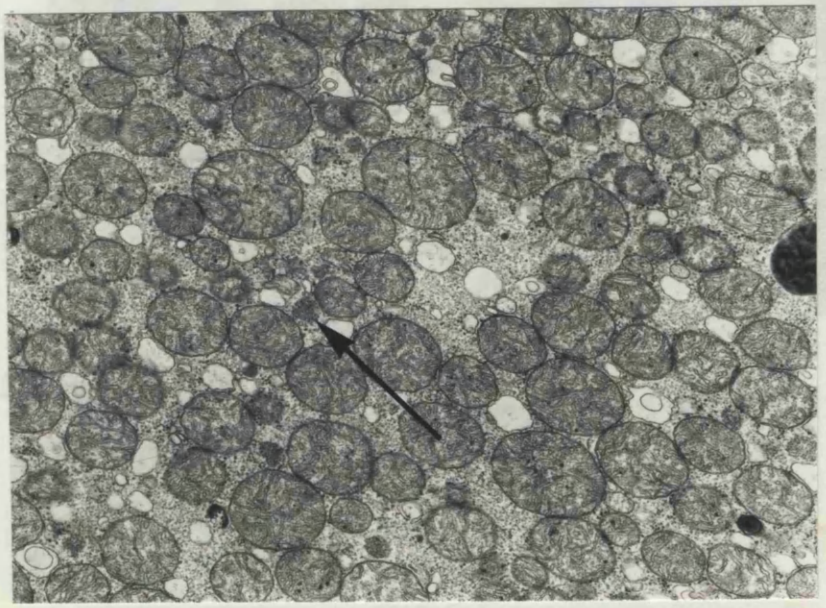


PLATE 13. Germ plasm of a mid-blastula (Stage 7) embryo. The cytoplasm is filled with numerous particles, the "dense bodies" are still present (arrow) and the vesicles often have deep infoldings of their membranes.

x13,800.

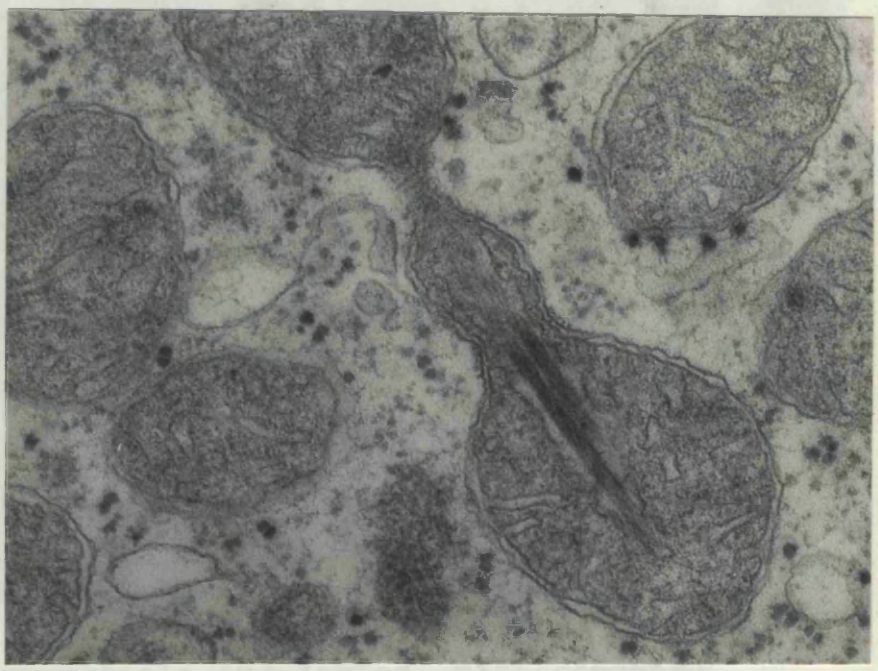


PLATE 14. Longitudinal section through a long, perhaps dividing, mitochondrion containing two "rods."
x63,600.

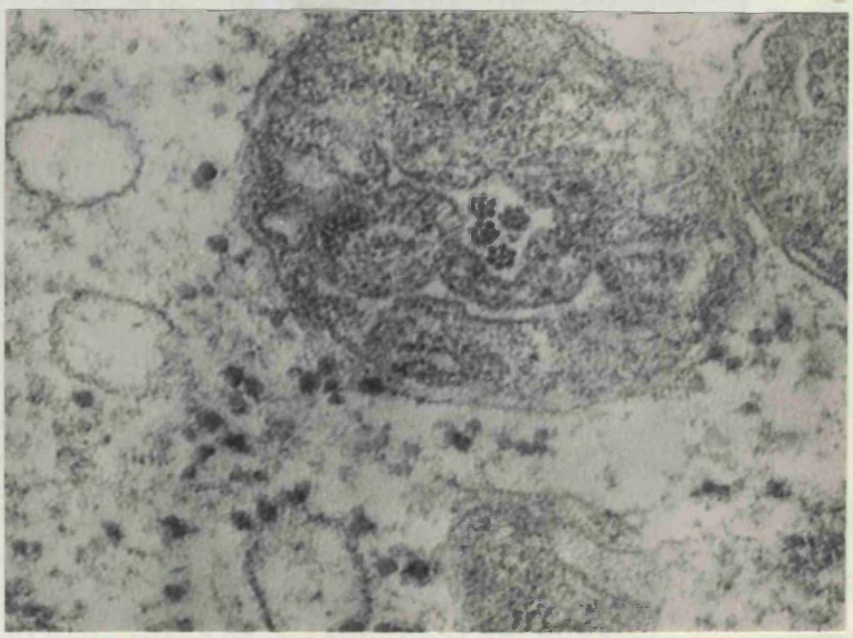


PLATE 15. Transverse section through 4 "rods" in the crista of a mitochondrion. The hexagonal arrangement round a central fibril can be seen.
x100,000.

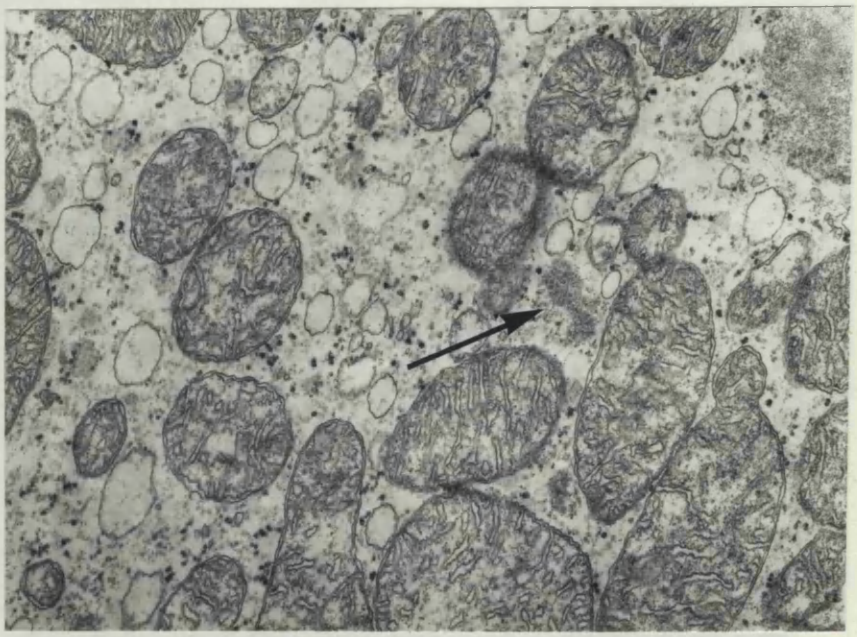


PLATE 16. Germ plasm of an 8 cell stage embryo previously irradiated. Mitochondrial internal structure shows signs of breaking down, c.f. Plate 7. but "dense bodies" are unaffected.(Arrow).

x17, 430.

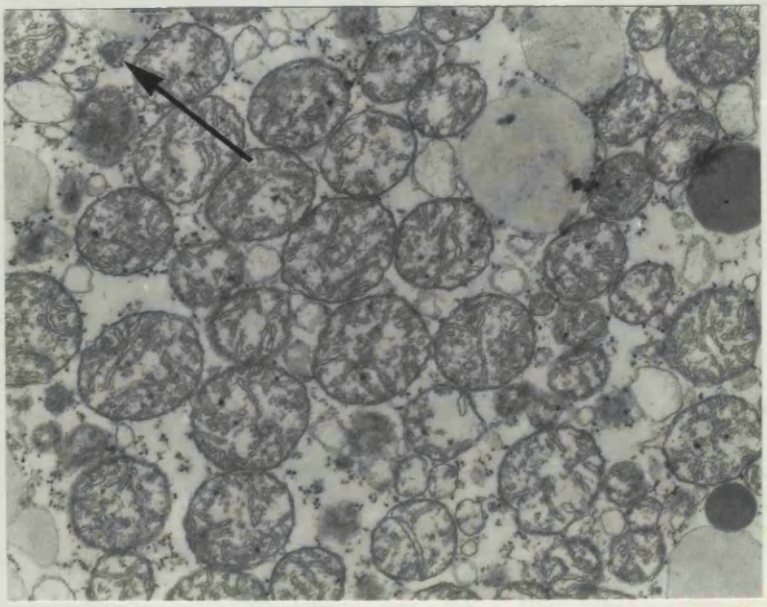


PLATE 17. Germ plasm of mid-blastula (Stage 7) embryo previously irradiated. c.f. Plate 13. The mitochondria show further signs of internal structural damage. There are very few particles in the cytoplasm. "Dense bodies" (arrow) are unaffected.

x16,350.

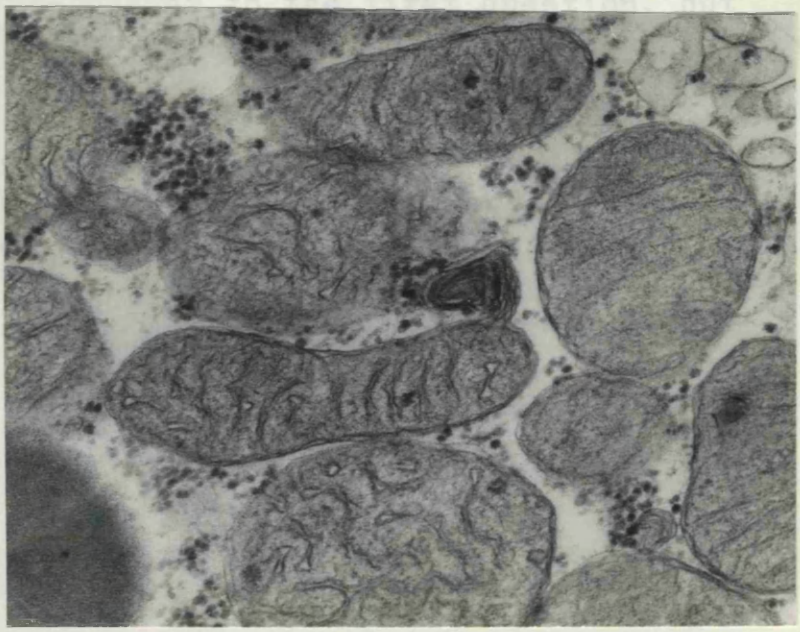


PLATE 18. Mitochondria of a primordial germ cell of a neurula (Stage 22) embryo, previously irradiated. c.f. Plate 8. The mitochondria are not so tightly packed but are otherwise similar in configuration. The membranous whorl is characteristic of irradiated mitochondria. x 56,360.

DISCUSSION

That germ cells of many animal species are segregated very early in development is undisputed. The questions that remain unanswered are "Why do animals set aside the cells of their germ line so early?" and "How do they do it?" we can at present only guess the answer to the first question, but the answer to the second may be found by studying germ cell development.

The first part of this study was done to confirm that in Xenopus laevis the segregation of primordial germ cells from somatic cells involves some measurable change in cell behaviour. Blackler (1958) noted that he never saw mitotic figures in primordial germ cells between gastrula stages and Stage 45, when the germ cells have reached the gonadal sites. Smith (1966) working on Rana pipiens showed that primordial germ cell nuclei of post migratory stages were significantly more likely to be totipotent than somatic endoderm cell nuclei of the same stage. These observations and results prompted this present study on synthetic activity of primordial germ cells in X.laevis to confirm that segregation of the germ line from the somatic takes some physiological form. The results indicate that for DNA synthesis there is a clear-cut difference between the two cell types during migration.

Primordial germ cells do not, at that time, synthesize DNA to any measurable extent, whereas somatic cells do. Similarly for RNA and protein there appears to be a reduced rate of synthesis in primordial germ cells during migration. These results are particularly striking when compared with those of Flickinger et el (1970) who showed that in both Rana pipiens and Xenopus laevis embryos the cells that are dividing quickly synthesize less RNA per cell than the more slowly dividing ones. If Flickinger's result is general then the changes in RNA synthesis in primordial germ cells noted in this study cannot be a consequence of reduction in DNA synthesis. That both DNA and RNA synthetic rates are reduced together implies that primordial germ cells are affected in a more specific way. It therefore appears that segregation of the germ line in X.laevis, which is probably complete by mid-gastrula, results in a real physiological difference from Stage 31 onwards. The fact that the difference is a reduced DNA and RNA synthetic rate makes possible the suggestion made earlier (See Introduction) that primordial germ cells are not responding to the morphogenetic influences around them, while somatic endoderm cells are responding by continuing to differentiate. In other words, it appears that primordial germ cells are unable to respond to signals to differentiate along somatic pathways, and this may

be associated with their inability to synthesize DNA, RNA or protein actively during this stage of development.

The results of the light microscope study show that ultraviolet light can affect the increase of mitochondria that occurs in the germ plasm of normal eggs, that some germ plasm apparently "dies," that germ cells which are formed do not increase in number and appear retarded in development, and that migration does not occur at all. Electron microscope studies suggest that mitochondria are apparently more affected than "dense bodies." Mitochondrial numbers are very much smaller in the ultraviolet irradiated eggs. These observations immediately raise the question "Are large numbers of mitochondria needed for migration, and does ultraviolet light sterilize by destroying mitochondria so that primordial germ cells cannot migrate?" There are several reasons for supposing that large numbers of mitochondria are necessary for migrating primordial germ cells. In the first place it may be assumed that migrating cells use more energy than those which are immobile, and since mitochondria are associated with energy conversion, their presence in large numbers would be in keeping with the behaviour of germ cells. Also the mitochondria themselves change in configuration during development. Throughout blastula stages the mitochondria are in

the "condensed" configuration, corresponding with a de-energized state (Hackenbrock,1972). At tail bud stages just before the primordial germ cells begin to migrate the mitochondria are found in the "orthodox" configuration with numerous calcium spots on the matrix, both conditions of a highly energized state. As this is the stage one would expect the mitochondria to be storing energy, prior to migration, the observations are consistent with the possibility that large numbers of mitochondria are needed for energy production for migration. Also the fact that the large numbers disappear at about the same time that the primordial germ cells reach their destination points in the same direction.

However these arguments are by no means conclusive. Only the primordial germ cells of some anuran amphibians have been reported to be rich in mitochondria, but many different species have primordial germ cells which migrate. Also Xenopus laevis embryos have other cells which migrate, such as neural crest cells, and these are not known to be exceptionally rich in mitochondria.

There are many other possible roles for the mitochondria in this situation. For example, they may be rich in an enzyme which inactivates an inductive substance before it can induce somatic differentiation in the primordial germ cells. Or the mitochondria may produce a substance, perhaps

with the help of "dense bodies" which acts specifically on the germ cells to determine their fate. The fact that the mitochondria are found grouped round the nucleus rather than scattered throughout the cytoplasm lends support to the idea that they are "protecting" the nucleus or acting directly on it, rather than that energy production is their sole function.

In 1934 Bounoure showed that ultraviolet light directed at the vegetal pole of early eggs caused sterility in otherwise normal frog tadpoles. Since then ultraviolet light of appropriate frequency has been shown to destroy nucleic acids and proteins (Deering, 1962). The object of the present study was to try to elucidate what role germ plasm played in determining germ cells and which of its principle components, namely mitochondria or "dense body" was the more important. "Dense bodies" were thought to be the principle determining constituent as they are unique to germ plasm (Mahowald and Hennen, 1971) and are found in the germ cells of some other animals, e.g. insects (Mahowald, 1968). Also they have been found to be rich in RNA in anurans (Mahowald and Hennen, 1971) and insects (Mahowald, 1971) and during early development in both these groups of animals they and their RNA content disappear. So it appears as if "dense bodies" contain accumulations of RNA which is biologically

effective early in germ cell development, but are later destroyed, dispersed or changed to some unrecognisable form. It would seem likely therefore that ultraviolet irradiation if it destroys the RNA of "dense bodies" would result in the non-production of a protein or proteins which might finally result in the malfunction of the germ plasm or primordial germ cell.

However, the ultrastructural observations of this study show no obvious differences in the "dense bodies" of normal and ultraviolet irradiated germ plasm. This either means that the ultraviolet light has no effect on them, perhaps because they are well protected with protein, or else the effect is not demonstrable by normal electron microscopy.

On the other hand, comparison of the ultrastructure of normal and ultraviolet irradiated mitochondria does show very clear differences. Not only are the mitochondrial numbers very greatly reduced but also their structure alters with irradiation. They swell in volume, their matrix becomes thin and in many cases the cristae break down into vesicles and tubes and eventually disappear. Associated with this mitochondrial degeneration is a lack of glycogen production, as revealed by accumulations of glycogen bodies, which occurs in the normal egg.

This appears to confirm the suggestion that

ultraviolet irradiation interferes with the production by mitochondria of an enzyme or other substance which is necessary for germ cell determination.

The role of "dense bodies" in the normal development of primordial germ cells is still a matter of speculation. Since they are rich in RNA it is almost certain that their function is to provide information which directly or indirectly influences primordial germ cell development. It may be that the "dense bodies" themselves as they move towards the nucleus with the germ plasm at late blastula stage, continue to migrate into the nucleus itself, and so directly effect a change in nuclear function. It may be that some protein, perhaps made in the cytoplasm from the RNA of "dense bodies" enters the nucleus and acts as a specific repressor, or de-repressor, of gene function. One piece of evidence in favour of material passing into the nucleus was found in electron micrographs of very late blastula stages where electron dense material lying in the cytoplasm close to the nucleus is also seen in the nuclear pores. See Plates 11 and 12. This material may be passing out of the nucleus into the cytoplasm but since the material is ultrastructurally very similar to "dense body" and since "dense body" disappears from germ plasm very shortly after, it

seems more likely that it is "dense body" or a product of it passing into the nucleus.

At later stages of germ cell development in Xenopus laevis, during oogenesis (Al-Mukhtar and Webb, 1971) similar electron dense material has been observed in the cytoplasm, close to the nucleus, possibly passing out of it through nuclear pores.

Therefore it appears that "dense body" material passes into the nucleus from the cytoplasm at very early gastrula stages, causes some change in nuclear function which determines germ cells, and then passes out to the cytoplasm again at the start of meiosis. In spermatogenesis the material would be lost during cytoplasmic degeneration, whereas in oogenesis the material and closely associated mitochondria are thought to move to the periphery of the cell, and lie there until ovulation and fertilization, at which time it forms the next generation's germ plasm (Balinsky and Devis, 1963).

What the action of the material is while it is in the nucleus, or around it, can only at the present time be speculation. It may protect the primordial germ cell from differentiating along somatic pathways, it may make them receptive to the attractive influences from the mesoderm and therefore able to migrate, or it may be a pre-requisite for meiosis.

In 1971 Wallace, Morray and Langridge

proposed a model for gene amplification in amphibian oocytes, which stated that the rDNA of the nucleolar cores are self-replicating particles restricted to the germ line and are not re-iterated copies of the chromosomal nucleolar organiser. These rDNA satellites move to the cytoplasm at the beginning of meiosis, are lost in sperm, but in oocytes they collect as constituents of germ plasm, and in the next generation they again enter the nucleus of the primordial germ cell, where they can replicate, transcribe rRNA and organise accessory nucleoli.

This theory fits very well with observations made on the germ plasm of anurans. The independent self-replicating rDNA could be incorporated into the DNA of the mitochondria of germ plasm and could enter the nucleus of germ cells at any time from early gastrula stages to Stage 45, during which time the numerous mitochondria are close to the nucleus. At about Stage 45 most of the mitochondria disappear from the cytoplasm and soon after there appear extra nucleoli in oogonia and spermatogonia.

According to Al-Mukhtar and Webb (1971) "dense body" leaves the nucleus of oocytes at the beginning of meiosis and is incorporated into mitochondria. If this "dense body" is considered to be the rDNA of Wallace et el's theory, then how can rRNA synthesis continue in the nucleus of

the oocyte throughout meiosis to maturation stage in the absense of rDNA? rDNA is known to self-replicate within the nucleus early in oogenesis, before the lampbrush chromosome stage (Brown and Dawid,1968), so it is not impossible that once duplicated half of this rDNA re-enters the cytoplasm whereas the rest remains to act as template for further rDNA and rRNA synthesis.

Although this theory fits well with the role of mitochondria in germ cells, there is one point which remains unexplained. Why, if ultraviolet light affects mitochondria in primordial germ cells as is implied by electron microscopy, are the effects noticed before rDNA is needed in oogonia? There are three possible explanations;

1. Mitochondria have another role in germ cell formation which is brought into play earlier in development.
2. "Dense bodies," the other possible determinant of germ cells, are also affected by ultraviolet light, though not visibly.
3. "Dense body" is not directly affected by ultra-violet light but depends on mitochondria for its proper production and function.

It would be tempting to suggest separate roles for mitochondria and "dense body" in germ cell formation. Maybe "dense body" rich in RNA and protected by protein, enters the primordial

germ cell nucleus at the gastrula stage and "determines" germ cells by, for example, preventing them from differentiating. Then later, once germ cells are in the gonadal ridges the mitochondrial DNA, incorporating rDNA, enters the nucleus and organises the formation of extra nucleoli and rRNA, while the mitochondria themselves "die." Then later, at the beginning of meiosis, both germ plasm constituents leave the nucleus together.

However, due to the observed close association of these two constituents of germ plasm, it seems unlikely that their function is not in some way related, but it is not impossible that the relationship is close only when they are in the cytoplasm, since both have to be restricted of the germ line of the new embryo.

In conclusion, I would propose, on the evidence of the work described in this thesis, that mitochondria are as important as "dense bodies" in the proper formation of germ cells, that they support "dense body" formation and function, and when treated with ultraviolet light they are so affected as to prevent germ cells from developing normally. Whether they or the "dense bodies" are the determinants of germ cells remains to be resolved. It is probable that their roles are so

closely associated they cannot be separated.

Whether the mitochondrial DNA is also involved in the production of rDNA in gonial cell nuclei remains to be determined, but evidence points in this direction. This doubt could be resolved by determining whether mitochondria of primordial germ cells contain more DNA than the mitochondria of somatic cells.

SUGGESTIONS FOR FURTHER WORK

If Wallace et el. are correct in their theory then the mitochondria of primordial germ cells have a different DNA content from those of somatic cells, and it may be possible to test this suggestion by injecting normal somatic mitochondria into the vegetal pole of an anuran egg that has been sterilized by ultraviolet light, and seeing if normal germ cells are reconstituted.

Ultraviolet light can cause sterility in otherwise normal frogs, yet it is not known whether the cells which incorporate the irradiated germ plasm and fail to become germ cells die or become somatic cells. A very careful light microscope study could probably clarify this, and so tell us something about the mode of action of germ plasm.

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