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City University of New York, Ph.D., 1976
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1976

AN ANALYSIS OF THE EFFECTS OF HOMOZYGOSITY FOR A LETHAL ALLELE (p^1) IN THE MOUSE

by

LAURA JOAN STRAUSS

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1976

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ACKNOWLEDGEMENTS

I wish to thank my husband and children for their help and patience, and our family for their encouragement.

I greatly appreciate all the help given me by the people at the University, especially Dr. L. J. Smith, Dr. L. Marcus, and Dr. U. Behrens.

Part of my graduate education was supported by a N.D.E.A. Fellowship.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

LIST OF TABLES AND CHARTS 2

LIST OF MICROGRAPHS 3

INTRODUCTION 4

METHODS AND MATERIALS 8

Light Microscopy 8

Electron Microscopy 9

RESULTS 12

Light Microscopy 12

6½ Days 12

5½ Days 13

4½ Days 15

3-3/4 Days 17

3½ Days 20

Electron Microscopy 23

Normal 23

Mutant 28

DISCUSSION 33

TABLES AND CHARTS 50

LIST OF ABBREVIATIONS USED IN MICROGRAPHS 60

MICROGRAPHS 61

ADDENDUM 91

Tables 95

REFERENCES 98

LIST OF TABLES AND CHARTS

TABLES

1. Distribution of Embryos in Control and p-Lethal Litters (6½-4½ Days P.C.) 50

2. Frequency of p^1p^1 Embryos in p-Lethal Litters 51

3. Distribution of Embryos in Control and p-Lethal Litters (3-3/4 and 3½ Days P.C.) 52

4. Distribution of Blastocysts by Morphological Type at 3-3/4 and 3½ Days P.C. 53

5. Mitochondrial Values for Normal and p^1p^1 Embryos 55

6. Abnormal Characteristics of p^1p^1 Mutants as Related to their Morphologies 57

7. Abnormal Characteristics of p^1p^1 Mutants as Determined by Electron Microscopy and their Frequencies 58

8. Chi-Square Tables on Genotypes 95

9. Test on Seasonal Variation 96

10. Mean Litter Sizes 97

11. Analysis of Variance 97

CHART

1. Difference Between Young and Well-Expanded Normal Blastocysts of p-Lethal and Control Series 59

LIST OF MICROGRAPHS

LIGHT MICROGRAPHS 62

6½ Day Normal Embryo 62

5½ Day Normal Embryo 62

5½ Day Mutant Embryo 64

4½ Day Normal Embryo 66

4½ Day Mutant Embryo 68

3-3/4 Day Normal Embryo 70

3½ Day Normal Embryo 70

3¼ Day Normal Embryo 72

3-3/4 Day Mutant Embryo 72

3½ Day Mutant Embryo 76

ELECTRON MICROGRAPHS 78

Normal Embryos 78

Mutant Embryos 84

Introduction

The initiation and control of gene action is one of the more interesting problems in developmental biology today. A lethal trait can be useful in the study of genetic control of development since each new lethal allele represents a highly specific experiment: shedding light on the functional relationships between individual mutational states and the process leading to the formation of character (Hadorn, 1961). The study of developmental lethals acting at early embryonic stages can lead to the clarification of processes necessary to normal development at this most crucial time. The effects of lethal genes will manifest themselves as soon as the specific function of the affected structure or enzyme becomes indispensible, and have been found to cluster around certain sensitive periods: 1) implantation, 2) gastrulation, 3) system formation (Hadorn, 1961). The particular lethal allele under study was found to act between 3½ and 5½ days of development, encompassing the time of the change from morula to blastocyst. On the microscopic level the lethal condition was found to be associated with abnormalities in several cytoplasmic organelles, which suggests an abnormality in energy and protein synthetic mechanisms.

The lethal (p^1) used in this study is an allele of the P locus. The locus affects coat and eye color. Work by E. Russell (1949) indicated that the p allele dilutes coat color by limiting the size of the pigment granules in all non-agouti animals, changing the granules to shred shapes which form floccular clumps, and altering the deposition of eumelanin. Later studies by Moyer (1963, 1966) suggested that the P locus controlled the cross-linking and orientation of the fibers making up the matrix of the melanoblast. In the normal melanin granule the

shape is assumed to be maintained by the compound fibers and their cross-linkages (Moyer, 1966). The recessive p allele may prevent the normal parallel arrangement of the matrix fibers by causing fewer cross-linkages between the fibers (Moyer, 1966). The abnormal arrangement of the fibers could produce a distortion of shape and lead to the shred-like granules seen by Russell (1949). Rittenhouse (1968) noted that the pp pigment granules failed to grow beyond the size of developing, lightly melanized PP granules, and this was accompanied by failure of pigmentation. The rate of tyrosine incorporation into pp melanoblasts is less than one-half normal, causing a decreased amount of melanization (Searle, 1968). Apparently the p allele alters the framework of the granule so that the melanin binding capacity of the matrix is greatly reduced. Compounds of p^1/p do not have as diluted coat or eye color as homozygous pp mice. This indicates that the p^1 allele does not affect the matrix, especially in the retinal melanoblast, to the extent that pp does.

The matrix was found to be a complex of protein subunits, with different enzymatic properties, bound to a lipid component (Moyer, 1966, Searle, 1968). The comparison of melanosomal fine structure and the ontogeny of melanoblasts in mice of different genotypes demonstrated that the protein products of several loci are involved (Moyer, 1963). The three fundamental attributes of the melanosomes--ability to produce melanin, appearance of the melanin produced, and cross-linking of the fibers and their orientation--appear to be under control of the C, B, and P loci respectively (Moyer, 1963). Each of these melanosomal attributes were assumed to be based on some specific structural property of the matrix protein, and the protein was assumed to be an ordered aggregation of polypeptides of the loci involved (Moyer, 1963).

The alteration of a single amino acid may alter the cross-linkages of the matrix fibers interfering with their parallel array (Moyer, 1963).

Many recessive alleles of the P locus were radiation induced and have pleiotropic effects on fertility, viability, behavior, coat color, and eye color (Melvold, 1971). The pp genotype results in smaller body size (Searle, 1968), a greater sensitivity to ovulatory stimuli (Wolfe and Coleman, 1966), and pigmentation dilution. Of the studies done on radiation induced P alleles, only in some p-sterile mutants was a common factor found which appears to link sterility and pigmentation. These p-sterile mutants have germinal hypoplasia and abnormal spermatids (Hunt and Johnson, 1971). The authors suggested that the abnormalities in pigmentation and spermatogenesis could be linked by the normal involvement of the Golgi body in both processes (Hunt and Johnson, 1971). In recent studies on p^{25H} (sterile) homozygotes, Johnson and Hunt (1975) report that these mice had abnormally small pituitary glands and that some of the pleiotropic effects of the p alleles, notably male sterility and female fecundity may have an endocrinological basis. While the association of the endocrinological effect with the pigment defect is still unclear, Johnson and Hunt (1975), explained the variability found in the endocrine and gonad development by saying that the P dilution locus is involved in the synthesis of a common α sub-unit of follicle-stimulating hormone, thyroid-stimulating hormone, and luteinizing hormone. Mutations at various points in this structure, expressed as P alleles, might be expected to have differing effects on the hormones involved, leading to a wide spectrum of phenotypic diversity (Johnson and Hunt, 1975).

The particular P allele used in this study arose as a radiation induced lethal and was used in L. B. Russell's studies on X chromosome inactivation at Oak Ridge. Russell (1964) determined that p^1/p^1 embryos elicited an implantation reaction, but died between 2½ and 6½ days post coitum. In order to elucidate the time of initial action of p^1 a retrograde analysis was done on embryos starting from day 6½. Results from this study were inconclusive as to the mode of action of the lethal. However, it was determined that abnormalities could be found in p^1p^1 embryos as early as the late morula stage. Some cells in the late morulae and early blastocysts found at 3½ days were already dead or dying. The embryos continued to die through the blastocyst stage until they reached a morphology characteristic of a 4½ day embryo at which time development ceased and death occurred rapidly. An electron microscopic analysis was undertaken to determine if any abnormalities on the cellular level would explain the lethal effect. This analysis of the p^1p^1 embryos indicated abnormalities of several organelles concerned with energy metabolism and protein synthesis possibly caused by a defective common structural protein.

Initial statistical analysis done on litter sizes indicated that the expected 25% reduction in p-lethal litter sizes was not present and, therefore, a new statistical analysis was undertaken to determine why these unexpected results were found. The procedure and results of this analysis are presented in the addendum.

Methods and Materials

Mice used in this study were maintained on a light-dark cycle automatically controlled to provide 14 hours of light and 10 hours of darkness. For light and electron microscopic studies two types of crosses were employed. To provide animals that were either homozygous for the p allele or heterozygous for the p and p^1 alleles, females of the p^1p type were crossed with males of the pp genotype. Litters from this cross were called control litters. Females heterozygous for the p and p^1 alleles crossed to males of the same genotype provided the p-lethal litters, in which, at conception, one quarter of the mice were expected to be p^1p^1 homozygotes. At birth litter sizes were determined by counting the number of offspring as soon as litters were observed. Post-natal data were obtained by examining mice at 14 days after birth at which time the two viable genotypes, pp and p^1p, could be identified by their coat color. Females used in the light and electron microscopic studies had had at least one previous litter or were at least three months old before being sacrificed.

Light Microscopy

To obtain embryos, two males were placed in female cages housing three to five females. Males were introduced between 4-5 P.M., and the females were examined for vaginal plugs between 9 and 10 A.M. on the following day. Day 1 was considered as beginning at 1 A.M. on the day on which the plug was found, that being the presumed time of mating.

Mated females were killed by cervical dislocation at 6½, 5½, 4½, 3-3/4, and 3½ days post coitum (p.c.). Their uteri, oviducts, and ovaries were removed and placed in a solution of 0.9% saline. Corpora lutea were counted on all days studied. On 6½ and 5½ days p.c. counts

of implantation sites were made. Uteri were fixed in Carnoy's fixative, without chloroform, for a period of two hours. Following fixation, uteri were held in 70% alcohol until dehydrated and embedded in paraffin. All sections were cut at 8μ on an A.O. Spencer microtome. Sections were stained with either Azure B (0.25%) at pH 4.00 at 40° C (Flax and Himes, 1952), or by the Feulgen and Fast Green method (Humason 1962). The Fast Green was used at pH 3.00 and a concentration of 0.2%. Under these conditions Fast Green will stain proteins with a low isoelectric point (Alfert and Geschwind, 1953). The conditions employed for Azure B staining allowed the identification of RNA by its blue-green color (Flax and Himes, 1952). For analysis, embryos were given both chronological and morphological ages.

Electron Microscopy

The same mating procedure was used to obtain embryos for the electron microscopic study. Mice were killed by cervical dislocation on day 3-3/4. The uterus was removed from the mouse and placed in a modified Brinster's culture media (Brinster, 1965), in which water was substituted for bovine serum albumin (Calarco, 1968). In the early experiments the uterus was cut into pieces of less than 1 mm before fixation and embedding in Epon 812. This method proved to be very unsuccessful since few embryos were found, presumably having been lost when the uterus was cut for fixation.

Embryos were obtained by flushing the uterus. Initially gluteraldehyde was the flushing medium, but it was found that more embryos could be obtained if the modified Brinster's medium was used. Even so, a large discrepancy was found between the number of embryos flushed from 3-3/4 day uteri and the number of corpora lutea. It is

possible that at this time some embryos were already beginning to implant and were not readily flushed out.

All flushed embryos from one litter were simultaneously fixed on ice with 3% gluteraldehyde buffered to pH 7.40 with 0.1M sodium cacodylate and refrigerated overnight in the fixing medium. Embryos were post-fixed under refrigeration with 1% osmium tetroxide (0.2M cacodylate buffer) at pH 7.40 for 45-60 minutes. Passing the embryos through alcohols and propylene oxide caused many to be lost, so at this point the embryos were embedded in warmed liquid 1% purified agar in water, in Grobstein culture dishes (Grobstein, 1956), and arranged so that they could be individually cut out for subsequent embedding in Epon blocks. Embryos so embedded were preserved as well as those done in the usual fashion. In a study concerned with size changes of mouse ova during electron microscopic preparation, Konwiński et al. (1974) determined that the use of agar before dehydration resulted in a smaller final diameter of the ova, but that it also facilitated cutting of the Epon block. Entire agar blocks with several embryos were stained with a 1% solution of uranyl acetate for 20 minutes (Hayat, 1970). This procedure gave improved contrast to the embryos and also allowed the agar blocks to be seen more clearly in the alcohols and Epon. The agar blocks were passed through the alcohol series at room temperature, and then cut into smaller pieces containing one or two embryos. These smaller blocks were placed in flat embedding dishes which were then filled with Epon 812. After 48 hours at 60° C (Luft, 1961), the flat blocks were reembedded in Beem capsules for easier cutting. Initially Araldite was tried as the embedding medium, but was found to give poor contrast.

Serial sections were cut on a Porter Blum MT-1 ultramicrotome at 1 or 2μ with a glass knife. These sections were placed on slides, stained with Azure II (0.2% in 1% sodium borate) and dried on a hot plate. The slides were made permanent by placing a cover slip with mounting media over the section. Ultra-thin sections were cut on a Porter Blum MT-2 ultramicrotome with a diamond knife. Several sections were placed on formvar coated grids without carbon shadowing. Since the grids were not carbon coated, it was necessary to use a thick layer of formvar (1.5%). Grids were either 100 mesh or hexagonal. Contrast was enhanced by post-staining with uranyl acetate (3% uranyl acetate in 50% alcohol) for 10 minutes and lead citrate for 5 minutes (Venable and Coggeshall, 1965). Grids were examined in a Jeolco T-7 and Phillips 300, at 60 kV, electron microscopes. Photographs were made at a low magnification of the whole embryo, and a medium and high magnification of various embryonic areas. Areas chosen represented both embryonic cell types and contained all the organelles under study.

Organelles were studied in chance selected cells of all embryos. A grid was used to give equal areas of measurement at all magnifications studied. The basic area was arbitrarily decided to be 4 square inches at 7,700 magnification, or a 2 by 2 inch square. Equivalent areas were calculated for the various magnifications studied and these areas were cut out on the grid sheet. The appropriate grid area was placed over the cells under study and notation made of organelles present. In some cases, the concentration of organelles was determined. Mitochondria were counted in all cells to determine their distribution in relation to cell type. This method provided a rough estimate of the concentration and distribution of organelles in the cells.

Results

Light Microscopy

This study was carried out using retrograde analysis and the results are presented in the manner in which the embryos were analyzed. The range in stage of development (morphological type) for any one time post coitum (age) was wide.

6½ Day Embryos

In both control and p-lethal litters all living embryos were well implanted in their crypts. For a description of a typical embryo see Figure 1. The least developmentally advanced normal embryo resembled the most developmentally advanced 5½ day old embryo.

In the control series the average litter size was 8.00 (Table 1). All the embryos appeared normal and 16 out of 24 (66.7%) were at the most advanced developmental stage for 6½ day embryos. In the p-lethal series the average litter size was 7.00 (Table 1) and 25 of 35 (71.4%) embryos were identical to those in the control series, and so classified as normal. The average number of normal embryos per litter (5.00) was in reasonable agreement with the average at birth litter size (Table 1). Fifteen of the 25 (60.0%) normal blastocysts were at the most advanced developmental stage. Of the ten (28.6%) abnormal embryos two were morphologically abnormal, while eight were represented only by capsules, i.e., normal looking uterine stromal swellings (decidua). Some capsules contained embryonic cells some of which were giant trophoblastic cells; others were not identifiable. Normal giant trophoblastic cells are readily identified after Azure B staining by their large size, and large, lobular intensely basophilic nucleoli, while those cells in

the mutant embryos were similar except that their nucleoli were smaller and more spherical.

The frequency of empty capsules (22.9%) in p-lethal uteri and their absence from the control series uteri indicated that the "empty" capsules were induced to form by p^1p^1 conceptuses, most cells of which had died and disintegrated by 6½ days post coitum (Table 2). Conceptuses so degenerate as to be represented only by a capsule or a few cells within a capsule will be called Group I type abnormals. These findings limit the viability of the mutant to less then 6½ days, confirming the results obtained by L. B. Russell (1964).

5½ Day Embryos

All conceptuses in both groups, with the exception of one abnormal embryo, had either implanted or had initiated epithelial breakdown and were at the attachment phase. For a description of the most developmentally advanced embryo of this age see Figure 2. The least developmentally advanced embryos classed as normal resembled the most developmentally advanced 4½ day old embryo.

In the control series the average litter size was 6.60 (Table 1), and 51 out of 62 (82.3%) normal blastocysts showed maximum development for this age. The four (6.1%) abnormal embryos were present as two "empty" capsules, and two embryos which were morphologically abnormally retarded. The average litter size in the p-lethal group was 6.82, with 107 out of 149 (71.8%) embryos classified as being normal. Eighty-eight out of 107 (82.2%) normal blastocysts reached the maximum stage of development described for this age. Among the 42 (28.2%) abnormal embryos there were a morphologically normal appearing 5½ day old egg cylinder which was lying free in the lumen, a vesicle made up solely of

trophoblast cells of 4½ day type, a 4½ day old blastocyst with a possible twin inner cell mass, and a 3½ day old blastocyst.

The remaining 38 embryos classified as abnormal were divided into three groups. Group I, the most abnormal, was made up of 17 capsules, some of which contained a few isolated embryonic cells. The cells were either dead and unidentifiable, or were primary trophoblastic giant cells (Fig. 3). Group II consisted of 15 embryos which appeared as shapeless or morula-like solid masses of cells (8), or masses of cells with cavities too small to be considered a blastocoel (7) (Fig. 4). In some masses inner cell mass cells were recognizable and trophoblast cells which had invaded the uterine epithelium were seen; other masses were made up of trophoblast tissue alone. While many of these cells showed signs of degeneration in the form of net-like degenerative nucleoli, numerous small round nucleoli, pycnotic nuclei, and vacuolated cytoplasm, most cells stained as intensely as normal. These masses appeared to be in an intermediate stage of breakdown between the stage seen in Group I capsules and the following Group III blastocysts. Group III, the least abnormal group, was made up of six 4½ day blastocysts whose blastocoels had either collapsed or were in the process of collapsing (Fig. 5). All of these embryos were strongly basophilic as evidenced by the intensity of their staining by Azure B. Nucleoli were bright blue (Azure B) and mostly oval. Four embryos contained cells with pycnotic nuclei. One embryo had begun to show signs of degeneration as indicated by abnormal vacuolation, especially in the trophoblast cells. In three of the six embryos one could clearly discern hypoblastic endoderm.

Analysis of their morphology, indications of degeneration, and

frequency (25.5%) led to the conclusion that the empty capsules (Group I, 11.4%), masses (Group II, 10.1%), and collapsed and collapsing blastocysts (Group III, 4.0%) represented the p^1p^1 embryos or their implantation sites (Table 2). The morphological state of the least abnormal of the 5½ day old mutant embryos (Group III) suggested that the limit of development of the p^1p^1 embryo is the 4½ day blastocyst. Additionally, these observations suggested that the time of death of the inner cell mass cells and the trophoblast varied in different embryos.

4½ Day Embryos

Aside from three embryos in the p-lethal series, the surrounding stromal reaction of which was less than expected for the age of the embryo, all other embryos had induced implantation reactions typical of their ages. "Primary invasive cells" (Wilson, 1963) characteristic of 4 day embryos were found both in trophoblastic and uterine epithelium. For a description of a normal 4½ day old embryo see Figure 6.

The average litter size for the control group was 7.83 (Table 1), with 87 of 94 (92.6%) embryos classed as normal. Eighty-three (95.4%) of these were blastocysts; four were morulae from a very young litter. The abnormals were represented by three morphologically abnormal blastocysts, a vacuolated morula, a mass with pycnotic nuclei, and two groups of dying cells. Of the 83 normal blastocysts, 47 (56.6%) were at the most advanced 4½ day stage. Embryos were classified as 4½ days old if they contained recognizable endoderm and the mural trophoblast was greatly expanded. Hypoblastic endoderm was distinguished from the remaining inner cell mass cells by its greater affinity for Fast Green and its lesser affinity for Azure B. In one quarter of the embryos one could differentiate polar trophoblast cells from the inner cell mass cells.

The average litter size for the p-lethal group was 6.58 (Table 1), with 58 out of 79 (73.4%) embryos classed as normal. Thirty-six of the 58 (62.1%) normal blastocysts had reached the most advanced developmental stage. Twenty of the 21 abnormal embryos showed one or more of the following abnormal characteristics: 1) pycnotic nuclei, 2) net-like nucleoli, 3) numerous small round nucleoli per nucleus, 4) nucleoli which stained bright blue with Azure B against a pale cytoplasm, 5) spotty cytoplasmic staining, including clumps of blue stain (Azure B), 6) granular staining, 7) more round than oval nucleoli, 8) dark blue outlines on large cells, 9) vacuoles. These abnormal embryos were further classified into three groups, each of which appeared to represent a progressively more advanced stage of blastocyst degeneration. The most normal group (Group III) was made up of three collapsing or collapsed blastocysts. The blastocysts were oval, with a small cell bound cavity (Fig. 7). "Primary invasive cells" were found in two blastocysts. The next most normal was a group of 14 masses (Group II) which appeared to be fully collapsed blastocysts (Fig. 8). Their cell number and the presence of "primary invasive cells" in two embryos indicated that at some time they had been blastocysts. Six of these masses were small disorganized collections of cells, loosely held together. Seven were long ovular cell groups with a small intercellular space, and one was round with a small intercellular space. The cells of the ovular masses were larger, had fewer vacuoles, and appeared healthier than those in the disorganized masses. The smaller size of the disorganized masses could be accounted for by fluid loss by the cells as they started to break down. Finally there was a group (Group I) consisting of three capsules containing only separated dying embryonic cells. The remaining

abnormal blastocyst was oddly shaped and had extensive trophoblast.

The abnormal embryos comprising Groups I (3.8%), II (17.7%), and III (3.8%) were considered to be 4½ day old p^1p^1 mutants on the basis of their common abnormalities, their frequency (25.3%), and the fact they they constituted a continuous series, that is, they ranged from abnormal blastocysts (Group III) made up of fairly normal cells surrounding a cavity, to masses made up of fairly normal cells with intercellular gaps (which may represent the last trace of the collapsed blastocoel), to masses made up of many abnormal cells separated from each other (Group II), to empty capsules (Group I). This pattern was consistent with degeneration occurring over a period of time. This conclusion was supported by the increasing frequencies of the more abnormal types seen at 5½ days (compare the 4½ and 5½ day frequencies for Groups I, II, and III, Table 2).

3-3/4 Day Embryos

In both control and p-lethal litters, well-expanded blastocysts were often found in groups of two to five rather than spaced out in individual crypts. A typical normal embryo from a p-lethal litter is shown in Figure 9. In some of these embryos a distinction could be made between inner cell mass and polar trophoblast cells. In those embryos where polar trophoblast could be differentiated from inner cell mass region, the trophoblast was seen to stain more intensely with Fast Green. In 3½ and 3¼ day embryos the inner cell mass region stained more intensely with Azure B than the trophoblast. It was also noted that as the embryo started to implant the trophoblast cells had a higher concentration of vacuoles than the inner cell mass cells.

The average litter size for the control group was 8.75 (Table 3), with 31 out of 35 (88.6%) conceptuses being classed as normal. These 31 blastocysts were classified as 3-3/4, 3½ or 3¼ day morphological types and the number and percent of total blastocysts of each type is given in Table 4. The 3½ day type embryos were distinguished from the 3-3/4 day blastocysts by their rounder blastocoel, thicker inner cell mass, and more rounded shape (Fig. 10). The 3¼ day type embryo was identified by its round, morula-like shape and small eccentric blastocoel (Fig. 11). These embryos also had larger cells than were seen at the 3-3/4 day stage. Of the remaining control embryos, all from one litter, one was an abnormal morula the cells of which were falling apart, and three were dead morulae.

The average litter size of the p-lethal group was 8.25 (Table 3). Of 65 embryos, 54 (83.1%) were blastocysts with blastocoels ranging in size from that in the normal 3-3/4 day embryos to that of the size seen in normal 3¼ day embryos. In Table 4 the number and percent of total blastocysts for each morphological type into which these 54 blastocysts fall is recorded. Of these blastocysts, 36 (66.7%) were considered to be normal since they were the same with respect to morphology and staining pattern as those found in control litters. A comparison of the frequency distribution of the normal blastocysts in both control and p-lethal litters (Table 4) with respect to stage of development (age) indicates that the average stage of development was the same in each.

Of the abnormal blastocysts, 14 were classified as p^1p^1 mutants of Group III type (Table 2 and Figs. 12, 13, 14). These blastocysts possessed: 1) some cells which were larger than normal for this developmental stage, 2) abnormally round nucleoli for this stage of development,

3) nucleoli which appeared extremely basophilic against a poorly basophilic cytoplasm, 4) striking variation in staining of adjacent cells, 5) gaps between cells, 6) disintegrating cells, 7) some of the other abnormalities seen in 4½ and 5½ day mutants. These mutant blastocysts appeared developmentally retarded when compared with the normal blastocysts in both the p-lethal and control series, that is, the percentages of them which were classed as having reached the 3-3/4 and 3½ day stage were considerably lower than among the normal blastocysts (Table 4). Of the remaining p-lethal series embryos, six were morulae. Four of these possessed some of the same type of cellular abnormalities of 5½ and 4½ day mutant embryos. Additionally, the frequency of morulae in the p-lethal series (9.2%) was considerably greater than in the control series (2.8%) (Table 3). For these reasons four of the total six morulae were also classified as p^1p^1 mutants. This new class of mutant is identified as Group IV type (Table 2).

The remaining four blastocysts and one morula were considered as non-p^1p^1 abnormals since their only abnormalities were either a pale stain, or the presence of large cells. The remaining conceptuses included a normal morula, an embryo of less than 16 cells, and four dead embryos. The latter five embryos were arbitrarily classified as non-p^1p^1 because the percentage of them was greater in the control litters than the p-lethal litters (Table 3). The frequency of embryos classed as non-p^1p^1 conceptuses (15.4%), therefore, was high but comparable to the frequency of embryos classified as abnormal in the control litters (11.4%). It was assumed that the large number of embryos classed as non-p^1p^1 embryos in both series was either due to difficulty in differentiating some slightly different looking normal embryos from abnormals,

or to chance variability within the limited number of litters examined.

In summary, the Group I and Group II type mutants seen in older litters were not found at 3-3/4 days. However, 27.7% of the conceptuses having distinctive cellular abnormalities could be described as Group III type mutants (abnormal blastocysts) or assigned to a new mutant group, Group IV (abnormal morulae) (Table 2). A comparison of the frequency distribution of the 3-3/4, 3½ and 3¼ day type normal and p^1p^1 blastocysts and of the normal and abnormal morulae in both types of litters suggested that at least some of the mutants were developmentally retarded even at this age. Additionally, the presence of isolated pycnotic nuclei and of strikingly different cytoplasmic staining of adjacent cells suggested that cell death had begun in the mutant blastocysts and morulae. While some of the embryos did go on to become 4½ day types, others were found in the groups of p^1p^1 masses and dead embryos at 4½ days.

3½ Day Embryos

Three observations suggested that the average developmental age of the p-lethal series litters timed to be 3½ days p.c. was greater than that of the control series litters. This difference was not seen in older stages. The three observations were: 1) a larger percentage of p-lethal series blastocysts had reached the 3½ day morphological type (Table 4), 2) more blastocysts of the p-lethal series than the control series showed differentiation of the polar trophoblast from the inner cell mass, 3) in the p-lethal series only nine out of 21 (42.9%) litters showed clumping of embryos, while in the control group the numbers were eight out of 13 (61.5%). Clumping suggests that the embryos have recently entered the uterus. This apparent difference in developmental

age between control and p-lethal litters complicated comparisons between them.

The average litter size of the control series was 7.46 (Table 3). Sixty-four of 96 (66.7%) embryos were blastocysts which could be classified into 3½ and 3¼ day types (Figs. 10, 11 and Table 4), and 18 (18.7%) were morulae (Table 3). The remainder (14.6%) included ten embryos of less than 16 cells and four dead embryos (Table 3). Sixty-two blastocysts and 15 morulae (80.2%) were normal. Two abnormal blastocysts contained pycnotic nuclei, and three abnormal morulae showed apparent cell wall breakdown, pycnosis and green-blue dots in the cytoplasm. All embryos of less than 16 cells were considered abnormal.

The average litter size of the p-lethal series was 6.95 (Table 3). Of a total of 145 classifiable embryos, 114 (78.6%) were blastocysts whose blastocoels ranged in size from the 3½ to the 3¼ day types seen in the control group (Tables 3 and 4) and 21 (14.5%) were morulae (Table 3). Three morulae and 81 blastocysts were classed as normal (57.9%). The remainder (6.9%) were eight embryos of less than 16 cells and two dead embryos (Table 3). Neither the dead embryos, nor the embryos of less than 16 cells were considered to be p^1p^1 mutants, since these were present in lower frequency in these litters than in the control series litters. Eleven blastocysts and four morulae were also classified as non-mutant abnormals. These were abnormal based on one of the following pale stain, abnormal morphology, pycnotic nuclei, or multiple nucleoli.

The p^1p^1 mutants were found among the blastocysts and morulae with a frequency of 24.8% (Table 2). In addition to the p^1p^1 abnormalities previously noted the following were found at this age: 1) accumulations

of stain, e.g. irregular distribution of RNA, accumulations of blue-green dots, 2) odd cytoplasmic inclusions seen as dots and lines. Of the 114 blastocysts, 22 were considered to be p^1p^1 mutants because they had various combinations of the cellular abnormalities seen in older mutants (Figs. 15, 16). Two blastocysts appeared to be collapsing. All mutant blastocysts were placed in the Group III type category. Of the 21 morulae, 14 were considered to be p^1p^1 mutants and placed in Group IV. These mutant morulae were unique because some of their inner cells were abnormally large and/or showed pale staining, or they contained cells whose cytoplasm was unevenly stained. Eleven of these morulae were shaped like normal morulae, two looked like the disorganized masses seen at 4½ days (although with more cells), and one with distinct trophoblast cells appeared to be a collapsed blastocyst.

From these data it was clear that at least some of the 3½ days blastocysts and morulae could be identified as p^1p^1 conceptuses on the basis of several cytoplasmic characteristics. These were: 1) the abnormally low cytoplasmic staining with either Azure B or Fast Green of some of their cells, 2) different staining of adjacent cells of the same cell type, 3) odd clumps and streaks of material in the cytoplasm of some cells, 4) nucleoli inappropriate for their developmental stage, i.e., rounder, smaller, more numerous and net-like. The gross morphology of 3½ day old blastocysts also differed from normals in that some had large cells and, in two cases, collapsed blastocoels.

Electron Microscopic Analysis

Three and three-quarter day embryos were used for electron microscopic analysis. This was one of the earliest stages at which one could identify mutant embryos by light microscopy. Of the 32 embryos studied, 19 were normal blastocysts, eight were determined to be p^1p^1 mutants, two were otherwise abnormal due to the presence of several large lysosomes in the inner cell mass region, and three were normal morulae. The latter five embryos will not be discussed in this paper. The normal embryos came from both control and p-lethal litters. Normal appearing embryos from p-lethal litters were first compared to normal embryos from control litters before placement in the normal category. Those embryos determined to be p^1p^1 mutants had a pattern of abnormalities which will be detailed later. Of a total of 23 embryos from p-lethal litters ten were found to be abnormal (43.5%) and eight of the abnormal embryos (34.8%) were thought to be p^1p^1 mutants. The high frequency of abnormal embryos was probably due to the small sample size.

3-3/4 Day Normal Embryos

As in the light microscopic study, the 19 3-3/4 day normal blastocysts showed different degrees of blastocoel expansion. In all but one case the blastocysts were enclosed in a zona pellucida.

The presence of the following structures and inclusions, previously described for this stage by Enders and Schlafke (1965), Potts and Wilson (1967), Calarco and Brown (1969), Hillman and Tasca (1969), Enders (1971), and Nadijcka and Hillman (1974), was corroborated in the normal embryos and also found to be characteristic of the mutants: 1) golgi material which occurred infrequently (Fig. 17); 2) clear membrane bound vesicles (Figs. 18, 19); 3) pinocytotic vesicles found along the

periphery of cells and associated with the cell membrane from which they were formed (Fig. 27); 4) round and oval nucleoli; 5) "doughnut" structures associated with mitochondria and found in agranular endoplasmic reticulum, described by Calarco and Szollosi (1973) and Chase and Pikó (1973) and thought to be viruses by Biczysko et al. (1973), Biczysko et al. (1974), and Solter et al. (1974) (Fig. 42); 6) microvilli (Calarco and Epstein, 1973) (Figs. 20, 27); 7) multivesicular regions (Fig. 18); 8) "jig-saw" bodies (Figs. 19, 30); 9) density differences between cells (Fig. 20); 10) size of crystalloid and filament structures (Figs. 23, 24). It should be noted that microfilaments (Ducibella and Anderson, 1975) were not present in the photomicrographs. Lipid, crystalloid concentration, granular and agranular endoplasmic reticulum, ribosome concentration, mitochondria, and lysosomes were found to differ in the normal and mutant embryos. For the purpose of comparison they will be described in detail.

The time of appearance and differentiation of junctional complexes have only recently been well described. Such complexes were not specifically studied, but their presence was noted in these embryos. Typical tight junctions were seen between trophoblast and inner cell mass cells. Predesmosomal junctions and interdigitations of a lock and key fashion were also found (Fig. 28). These two primitive junctions were initiated in the morula stage (Calarco and Brown, 1969). Ducibella et al. (1975) noted that tight junctions could be seen on the outer cells of 16-32 cell morulae. They also noted that when the blastocoel appeared desmosomes could be observed for the first time. However, desmosomes were only seen between trophoblast cells and not between inner cell mass cells. In this study the presence of different cellular

junctions was used as a guide in aging the embryos.

Lipid was seen as medium density, round or oval granules of varying size, usually but not always bounded by an electron dense line (Figs. 21, 22). The granules were most often found in aggregates, but single ones were also seen. Enders and Schlafke (1965) noted that lipid droplets may be numerous in some cells of blastocysts in which total lipid concentration was sparse. Lipid granules were found to be associated with mitochondria (Fig. 28) as had been noted by Calarco and Brown (1969) and Hillman, Hillman and Wileman (1970). In embryos with small blastocoels the trophoblast had more lipid per unit area than the inner cell mass cells.

A prominent feature of the blastocysts was the presence of two types of fibrous structures, one of which was in the shape of a crystalloid and the other of which assumed a filamentous form. The crystalloids were found in all cells, and in approximately equal frequency in the inner cell mass and trophoblast regions. Their size varied and they were usually found in aggregations, though single crystalloids were seen. Granular endoplasmic reticulum was closely associated with crystalloid structures (Figs. 23, 25). While no difference in fiber size and spacing was found between mutant and normal embryos, the values obtained in this study differed from those obtained by Enders and Schlafke (1965). The fibers making up the crystalloid ranged in size from 6 mμ to 9 mμ while the space between fibers ranged from 5 mμ to 7 mμ (Fig. 23). The other fibrous inclusion, filaments, were arranged in curved or straight packs of two to eight parallel filaments per pack. The filaments were found in cytoplasmic areas lacking other organelles. Cross striations were visible along the filaments formed by a fiber that ran perpendicular

to the parallel filaments (Fig. 24). As with the crystalloids, the size of the filaments and the spatial relationship in the normal and mutant embryos did not differ; however, again the values obtained in this study differed from those of Enders and Schlafke (1965). The filaments ranged in size from 14 mμ to 24 mμ and the spaces between them from 9 mμ to 28 mμ. Calarco and Brown (1969) have given the symbols α to the parallel filaments, and β to the cross strand (Fig. 24). The β fibers ranged in size from 9 mμ to 18 mμ and the space between these cross strands gave the unit its periodicity ranging from 10 mμ to 27 mμ. These filamentous packs were found in approximately equal concentration in the trophoblast and inner cell mass cells.

Granular and agranular endoplasmic reticula were found, with the granular form being more common. The granular form was found in the trophoblast and inner cell mass cells with equal frequency and usually associated with mitochondria and crystalloids (Figs. 25, 26). The part of the membrane juxtaposed to the mitochondrion was often found to be free of ribosomes along the length of its association with the organelle (Figs. 25, 27). In one case a region of granular endoplasmic reticulum had completely surrounded a crystalloid (Fig. 25). The arrangement of ribosomes on the reticulum varied: in some cases they were equally spaced, or in others gaps of various length occurred between them. Some granular endoplasmic reticulum had wide cisternae, a feature more common in the infrequent agranular form.

Few free ribosomes were found in these embryos. Ribosomes were either aligned along the endoplasmic reticulum, or aggregated as clusters of polysomes (Figs. 22, 23, 25). The trophoblast had a higher concentration of polysomes than the inner cell mass region, but the

number of ribosomes per polysome was approximately the same: 4.93 (mean value) for the inner cell mass and 4.86 for the trophoblast cells.

The mitochondria were unlike typical mitochondria seen in adult animal cells: most were round, had cristae that were not lined up in lamellar fashion, and contained varying numbers of vacuoles, e.g., one or two very large vacuoles, several small ones, or none at all (Fig. 26). This type of mitochondria has been described for the mouse ovarian oocyte by Wishnizter (1967) and Szollosi (1972) and for the mouse from cleavage to blastocyst stage by Stern, Biggers and Anderson (1971). In the blastocyst stage the mitochondria had started to take on their adult morphology, though many were still vacuolated (Figs. 25, 27). Some of the mitochondrial vacuoles contained filamentous or thread-like material, suggested by Stern, Biggers, and Anderson (1971) to be mitochondrial desoxyribonucleoprotein, while others had a clear matrix (Fig. 26). The mitochondria were very numerous at this stage and were often seen in aggregates, rather than being evenly distributed throughout the cytoplasm. In normal blastocysts from both control and p-lethal litters, the concentration of mitochondria in the trophoblast cells was less per unit area than the concentration in the inner cell mass cells (Table 5). Lipid granules, granular endoplasmic reticulum, and occassionally crystalloid material were usually associated with mitochondria (Figs. 25, 27, 28).

While lysosomes were found in normal embryos, they were not present to any great extent in either trophoblast or inner cell mass cells. Their frequency in both cell types was approximately equal. Lysosomes were small, with either medium or heavy density material inside (Fig. 29). Degradation bodies were vesicles which contained parts of

cytoplasmic material or myelin figures (Fig. 29). Autophagic vesicles (Wilson, 1963), cytoplasmic inclusions with a double membrane structure, were also found containing parts of cytoplasmic material (Fig. 19). It appeared that these vesicles might have been formed from pieces of granular endoplasmic reticulum whose ribosomes were lost leaving a double membrane structure surrounding cytoplasmic material, as shown in Figure 30.

When normal blastocysts were subdivided into young and well-expanded blastocysts, differences in certain organelles were apparent (Chart 1). The well-expanded blastocysts of both control and p-lethal series reflected their advanced developmental age in their organelles (Chart 1). These results were in keeping with those found by Calarco and Brown (1969).

3-3/4 Day Abnormal Blastocysts

In the mutant embryos the same three morphological types that were seen in the light microscopic studies were found: Group IV abnormal morulae, and Group III conceptuses composed of collapsed blastocysts, and collapsing or abnormal blastocysts. The eight mutants studied were characterized as two abnormal morulae (morula 1 and 2), two collapsed blastocysts (blastocyst 1 and 2), and four collapsing or abnormal blastocysts. While collapsed blastocysts were not found at 3-3/4 days in the light microscopic study, they were seen at 3½ and 4½ days, and collapsing probably continues throughout that time period. Only abnormal and relevant normal features will be discussed (see also Tables 6 and 7).

Light microscopic studies established that the elongated morulae represent the most abnormal form of the mutant embryos. The presence of tight junctions in both morulae indicated that they were either late

morulae or had entered the blastocyst stage and collapsed. Both morulae had large accumulations of lipid in the inner cell mass and trophoblast regions (Fig. 31). In both morulae the morphology of the granules was abnormal: they were either larger than normal, irregular rather than round, or the lipid material was separated from the granule border (Fig. 31 compare to Fig. 21). For this stage of development both the morulae had an abnormally low concentration of crystalloid material. Both also showed a lower concentration of granular endoplasmic reticulum than expected; in one case this was crystalloid associated granular endoplasmic reticulum; and in the other case mitochondrial associated granular endoplasmic reticulum. Morula 1 had an abnormally high polysome concentration as seen in Figure 32, and an increased number of ribosomes per polysome as compared to control embryos. Both embryos showed higher concentrations of mitochondria than normal, either in the inner cell mass cells, or in the trophoblast cells (Table 5). The mitochondria of morula 2 were immature, being round and vacuolated. Both embryos had above normal concentrations of lysosomes. In morula 1 these were filled with an electron dense material and found in cells with atypical lipid. Cytoplasmic inclusions, e.g., myelin figures, were found in morula 2. Morula 2 consisted of three large inner cell mass cells surrounded by a trophoblast ring. Broken cell membranes and disorganized cytoplasmic material indicated that the trophoblast cells of this embryo had undergone some disintegration. In summary, these embryos exhibited abnormalities in their lipid material, decreased concentrations of crystalloid material and granular endoplasmic reticulum, and increased concentrations of mitochondria and lysosomes. Abnormalities of polysome concentration, mitochondrial morphology, and trophoblast

disintegration were present in one or the other embryo, but not in both.

One of the two collapsed blastocysts, blastocyst 1, had larger than normal cells for its apparent morphological stage. Both embryos were undergoing degeneration as seen by disorganized cytoplasmic material in some cells and disintegrating trophoblast cells in blastocyst 2. Excess lipid accumulation and abnormal lipid granules were found in blastocyst 1, while the only lipid abnormality in blastocyst 2 was the large size of some of the granules (Fig. 33 compare with Fig. 21). In both collapsed blastocysts there were abnormally low concentrations of crystalloid material and of both types of reticulum. The granular endoplasmic reticulum was especially sparse in the inner cell mass cells. In both embryos the mitochondrial associated granular endoplasmic reticulum was abnormally low, and in blastocyst 1 the crystalloid associated granular endoplasmic reticulum was also low (Figs. 34, 35 compare to Figs. 23, 26). Blastocyst 1 had some wide cisternae in the granular endoplasmic reticulum. A greater than normal ratio between the number of polysomes in the trophoblast and the inner cell mass was seen in the second embryo, the trophoblast having a much higher value than expected. Both embryos had decreased numbers of mitochondria when compared to normal, however, in blastocyst 1 this applied only to the trophoblast cells (Table 5). Blastocyst 2 had greater vacuolation of mitochondria than blastocyst 1. The concentration of electron dense lysosomes was increased in both embryos over the normal value. Blastocyst 2 also had degradation bodies and autophagic vesicles. This embryo had a decreased concentration of filaments.

Both embryos had the following abnormalities in common: degeneration, lipid abnormalities, decreased concentrations of crystalloid

material, granular and agranular endoplasmic reticulum and mitochondria, decreased association between granular endoplasmic reticulum and mitochondria, increased lysosome concentration. The individual embryos demonstrated the following abnormalities: abnormal lipid accumulation, wide cisternae in the granular endoplasmic reticulum, vacuolated mitochondria, abnormal ratio of polysomes in the trophoblast cells, a decreased concentration of filaments.

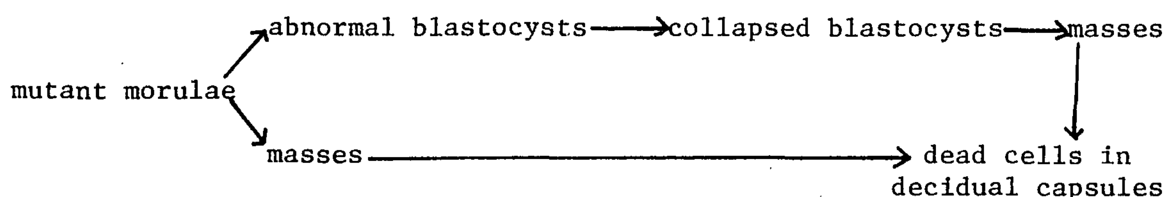
The four blastocysts that were in the process of collapsing were one of the least degenerate mutant forms, and therefore, likely to show the initial effects of the p^1p^1 genotype. Normal concentration of lipid was found in all embryos. All four blastocysts had morphologically abnormal lipid. Three embryos had abnormally shaped lipid granules (Figs. 36, 37 compare to Fig. 21) and two embryos had abnormally large lipid granules (Fig. 38). All of the blastocysts had low amounts of granular endoplasmic reticulum. Two of the embryos had abnormally low amounts of mitochondrial and crystalloid associated granular enoplasmic reticulum (Figs. 39, 40, 41 compare to Figs. 23, 26). In a third embryo only the mitochondrial associated endoplasmic reticulum was abnormally low, and in the fourth, only the crystalloid associated granular endoplasmic reticulum was reduced. There were several pieces of granular endoplasmic reticulum with wide cisternae in two embryos (Fig. 42). This form of reticulum was found in dead cells by El-Shershaby and Hinchliffe (1974), and thus may be indicative of the degenerative state of these cells. Three of the four embryos had a lower concentration of mitochondria than normal (Table 5). In two cases this was seen as a slight reduction of mitochondria in the inner cell mass cells and a larger reduction in the trophoblast (Table 5).

The other embryo had a greater reduction in the inner cell mass cells than in the trophoblast (Table 5). Unexpectedly high numbers of mitochondria in both cell types were found in the fourth embryo (Table 5). The mitochondria of two embryos were highly vacuolated (Fig. 43). Only one had an exceptionally high number of lysosomes present, and one lacked crystalloid material in the inner cell mass region. Two of the embryos had trophoblast cells that were disintegrating as seen by cell membrane breakdown. One embryo had an oval shape, reflecting the state of the blastocoel. This embryo had very large cells, with two cells making up the inner cell mass region. This was the only abnormal blastocyst in which too few inner cell mass cells was noted. The cells of the inner cell mass and trophoblast had spaces between them, while the trophoblast cells were joined by junctional complexes.

Tables 6 and 7 summarize the common abnormalities found in these embryos as related to mutant morphology and frequency of abnormality.

Discussion

The analysis of the light and electron microscopic studies revealed both the morphological and cytological abnormalities associated with the p-lethal embryos. The pattern of lethality was found to be:



It was clear from the light microscopic analysis that at least some blastocysts and morulae could be identified as mutant conceptuses at 3½ days. Several cytoplasmic characteristics form the basis for such identification: 1) the pale cytoplasmic staining of some of their cells, 2) different staining of adjacent cells of the same cell type, 3) odd clumps and streaks of basophilic material in the cytoplasm of some cells, 4) nucleoli inappropriate for the developmental age of the embryo, i.e., rounder, smaller and more numerous. The gross morphology of 3½ day blastocysts also differed from normal ones: some had large cells, and in two cases collapsed blastocoels. By 3-3/4 days the retardation in development seen in some p^1p^1 embryos at 3½ days was characteristic of most or all p^1p^1 embryos. Only one of the 14 mutant blastocysts had a morphology typical for 3-3/4 days; all others were either 3½ or 3¼ day types (Table 4). The electron microscopic analysis of 3-3/4 day mutant embryos revealed abnormalities in mitochondria, granular endoplasmic reticulum concentration, crystalloid and lipid material. The increased amounts of lysosomes, disintegration of trophoblast cells, and disorganized cellular material reflect the degenerative state of

these embryos.

The retardation of the mutant embryos became increasingly evident in older litters. The most developmentally advanced morphological type found among the mutants was a 4½ day blastocyst found at 5½ days. Collapsed and collapsing blastocysts were found at all days studied. Abnormal masses, some with intercellular spaces, were found at 4½ and 5½ days. The masses were considered to be collapsed and degenerative forms of the 3½, 3-3/4, and 4½ day blastocysts based on their cell numbers, cell types, and because they had elicited decidual reactions. The masses were smaller than normal 4½ day blastocysts. At 5½ days all inner cell mass cells were missing from some masses. Some of the mutant morulae found at 3½ and 3-3/4 days resembled the masses found at 4½ days and may be blastocysts which have already collapsed. There was evidence for this from the electron microscopic study, in which both mutant morulae had tight junctions which were indicative of the blastocyst stage. However, all masses found at 3½ and 3-3/4 days will be referred to as morulae. Other mutant morulae were more morula-like in morphology, i.e., round, and having more round than oval nucleoli. The decrease in frequency in mutant morulae between 3½ and 3-3/4 days indicated that some mutant morulae formed blastocoels. The frequency of embryos classed as most abnormal and assigned to Group I increased between 4½ and 5½ days, with a concomitant decrease in the frequency of masses. By 6½ days only a few cells remained in the decidual capsules, some of which could be identified as trophoblast giant cells. From these observations it became apparent that the mutant embryos were undergoing gradual death, passing through two morphological stages, over a period of 3½ to 6½ days.

The evidence found in the electron microscopic study done on 3-3/4 day embryos suggests abnormalities in energy, protein and lipid metabolism, all of which may be interrelated. Excessively vacuolated mitochondria were considered abnormal in the collapsed and collapsing blastocysts. Stern, Biggers and Anderson, (1971) reported that normally a change in mitochondrial morphology takes place between the eight cell stage and morula at which time two mitochondrial forms are found. The forms found are the younger, round, vacuolated mitochondria, with concentric cristae and dense matrices, and the older, more elongated mitochondria with transverse cristae (Stern, Biggers, and Anderson, 1971). By the late blastocyst stage the mitochondria were all in their mature form. Nadijcka and Hillman (1974), however, found mixed mitochondrial populations in the blastocysts they studied. The change in morphology of the mitochondria at the eight cell stage has been correlated with the change in substrates which can support embryonic development and the increase in metabolic function (Biggers, 1972, Ginsberg and Hillman, 1973). The changes which occur in the internal structure of the mitochondria might allow for changes in the transport mechanism of the substrates (tricarboxylic acid cycle [TCA] intermediates) into the mitochondria; special carrier molecules for these compounds are located on the inner mitochondrial membrane and the mitochondrial cristae (Biggers, 1972). The excessive vacuolation found in p^1p^1 mitochondria suggested failure to change to the mature form, possibly restricting the availability of substrates to the TCA cycle and thus causing a reduction in energy production. Abnormal, round mitochondria with electron dense matrices, rather than mixed populations, were found in t^{w32} mutants about the time of their death: the eight

cell stage and early and late morula stages (Hillman and Hillman, 1975).

Brinster (1967a) found that there was a large increase in the metabolic rate from eight cell stage to morula, which he suggested was due to an increase in the activity of the TCA cycle. This increase in metabolic rate could reflect an increase in respiratory capability of the individual mitochondria, or an increase in the total number of mitochondria, the individual capability of which had not changed (Stern, Biggers, and Anderson, 1971). That individual mitochondria have increased their capability was related to their structural changes. The time at which mitochondrial synthesis begins in mouse embryos is not known. Pikó (1970) noted a lack of circular mitochondrial DNA replication in the blastocyst stage, indicating a lack of mitochondrial synthesis in early embryos. The reappearance of the mitochondrial form of aspartate-amino transferase after implantation suggests the development of new mitochondrial activity (Engel, 1973). It has been suggested that the earlier mitochondrial form present until the early blastocyst stage, arose from maternal mitochondria or cytoplasmic storage (Engel, 1973, Moore and Brinster, 1973). The decrease in the number of mitochondria found in the collapsed and collapsing blastocysts (Table 5) could cause a decrease in metabolic activity which would decrease the amount of available energy needed for synthetic processes in the embryo, e.g., protein synthesis. The reduced numbers of mitochondria could have been caused by a decrease in synthesis of these organelles, or by mitochondrial breakdown secondary to the primary action of p-lethal. There was no explanation for the increased number of mitochondria in three of the mutants. Based on

the finding of significant correlation between ATP levels in two cell embryos and those embryos which go on to form blastocysts, Quinn and Wales (1973b) concluded that the developmental potential of an early embryo is linked to its levels of ATP. Thus, as the mutants do form blastocysts, the mitochondrial abnormalities seen in mutant embryos probably developed in post-cleavage stages.

Studies of RNA synthesis using various techniques have determined that synthesis of RNA is low until the eight cell stage then increases rapidly, reaching its highest level in the blastocyst stage (Flax, 1953, Mintz 1964a, Monesi and Salfi, 1967, Ellem and Gwatkin, 1968, Pikó, 1970, Tasca and Hillman, 1970, Olds, Stern and Biggers, 1973). The synthesis of r-RNA dominates this period, but other RNAs were also synthesized (Mintz, 1964a, Ellem and Gwatkin, 1968, Woodland and Graham, 1969, Pikó, 1970, Wolf and Engel, 1972). The findings in this study of clumping of stain and dark cellular outlines seen with Azure B and Fast Green indicated abnormal distribution of RNA and protein in the mutants. The pale staining with Azure B and Fast Green found in 3½ and 3-3/4 day mutants probably signifies a reduction or slowing down in RNA and/or protein synthesis. At 4½ and 5½ days the mutant embryos were normally stained. While reduction of Azure B stain in some of the mutants indicated abnormalities in RNA synthesis, the number of ribosomes, seen as polysomes, in the electron micrographic study of mutant embryos, was not reduced. In two embryos there was even a rise in the number of ribosomes over control figures. In a similar case, Mintz (1964c) noted that the t^{12} morulae, when stained with Azure B were less basophilic than normal morulae of the same age. No reduction could be found in the number of ribosomes in the electron microscopic studies (Calarco

and Brown, 1968, Hillman, Hillman and Wileman, 1970). The only decrease noted in relation to p^1p^1 ribosomes was the decrease of granular endoplasmic reticulum, but it is unlikely that the reduction in this material with its associated ribosomes would be responsible for the large decrease of stain seen in the young mutants. The absence of Azure B stain could be due to a decrease in synthesis of other classes of RNA, but their concentration is such that their absence would probably not be reflected in a different staining pattern.

Nucleoli are present in mouse eggs from the pronuclear stage, and their size, shape and structure change as development progresses. Several spherical pronucleoli are present in the fertilized egg (Calarco and Brown, 1969). At the early two cell stage there are three to 11 dense, round primary nucleoli which are composed of tightly packed fibrils (Hillman and Tasca, 1969). The replacement of these multiple nucleoli by definitive, reticulated nucleoli starts to occur just prior to second cleavage, and by late two cell stage both fibrillar and reticulated nucleoli are present (Hillman and Tasca, 1969). These nucleoli are still spherical in shape (Calarco and Brown, 1969). At the four cell stage the reticulated nucleoli are not as round as before (Calarco and Brown, 1969). By eight cells two reticulated, definitive nucleoli per cell have formed, and as development progresses, they become more reticulated (Hillman and Tasca, 1969). At the morula stage they are almost totally reticulate (Hillman and Tasca, 1969). The two to three nucleoli per cell present in the morula vary in shape from round to oval (Calarco and Brown, 1969, Hillman and Tasca, 1969). The two blastocyst nucleoli are seen as elongated and totally reticulated (Nadijcka and Hillman, 1974).

In the mutants the nucleolar fine structure pattern was normal, and the presence of ribosomes indicated normal nucleolar maturation, but various other nucleolar abnormalities were noted. The ratio of round to oval nucleoli in p-lethal embryos when compared to controls was large. The presence of both round and oval nucleoli in mutants suggested that the round nucleoli may have become oval, and then, during embryonic degeneration, reverted back to their round form. Hillman, Hillman and Wileman (1970) suggested this process to explain the presence of round nucleoli in t^{12} homozygotes. Under the light microscope degenerative nucleoli were seen as numerous, small round nucleoli and netlike nucleoli. At 3-3/4 days, the only indication of nucleolar abnormality in the electron microscopic study was the round shape of some. The appearance of the nucleoli seems to result from cellular degeneration rather than a specific abnormality associated with their structure or function.

Abnormalities in protein metabolism were also found in mutant embryos. In normal embryos, protein synthesis was found to occur before cleavage (Mintz, 1964b). Synthesis increases gradually until the third cleavage when a significant rise was noted (Quinn and Wales, 1973a) which becomes more pronounced at the blastocyst stage (Mintz, 1964b, Brinster, 1967b, Monesi and Salfi, 1967, Epstein and Smith 1973, Weitlauf, 1973, Van Blerkom and Brockway, 1975). Therefore, the intensity of the stain for protein should increase as the embryos develop. The pale staining pattern with Fast Green found in young mutants indicated some type of inhibition of protein synthesis or increased degradation. Interpretation of protein synthesis studies using labelling and actinomycin-D indicates that half the protein synthesized in the early embryo is dependent on continuous formation

of m-RNA and the other half depends on long-lived m-RNA (Monesi and Molinaro, 1971, Graham, 1973). Epstein and Smith (1974) using gel electrophoresis and labelled amino acids found five major protein components to be synthesized during the preimplantation period. Each of the components may consist of a mixture of several different protein species, and changes in the composition of the mixture may reflect the relative proportions of the proteins in it which could change during development (Epstein and Smith, 1974). Any abnormality in one of these protein species could lead to a number of cellular abnormalities, especially if the protein was essential to more than one major component. Decreased activity of protein synthesis in young mutants could be a general phenomenon based on delayed initiation of m-RNA synthesis, or could be caused by a lack or decrease of a common structural protein, an explanation which would link the abnormalities found in the mitochondria, crystalloid material and granular endoplasmic reticulum.

At 3-3/4 days there was a decrease in overall concentration of granular endoplasmic reticulum, as well as a decreased association of granular endoplasmic reticulum with mitochondria and crystalloid material. Abnormal granular endoplasmic reticula with swollen or wide cisternae were also seen. El-Shershaby and Hinchliffe (1974) found this form of endoplasmic reticulum in dead embryonic cells and considered it a sign of degeneration. The association of granular endoplasmic reticulum and mitochondria was noted by Odor and Blandau (1969) and Szollosi (1972) to start in the oocyte. Szollosi (1972) suggested that the association could be interpreted to mean that the mitochondria provided the rough endoplasmic reticulum with the high energy required for synthetic activity. The association disappears prior to ovulation,

but returns at the eight cell stage (Szollosi, 1972), when protein synthesis becomes more active. Decreased amount of granular endoplasmic reticulum were found in t^{12} homozygotes during degeneration (Hillman, Hillman, and Wileman, 1970), and granular endoplasmic reticulum was found lacking in parthenogenones (Solter, et al. 1974a). Reduction of granular endoplasmic reticulum at the blastocyst stage when protein synthesis is at a high level could cause a decrease in a number of essential proteins and lead to eventual death of the embryos. The decrease in agranular endoplasmic reticulum in mutant embryos may not be an abnormality as very little was found in normal early mouse embryos by Calarco and Brown (1969).

The decreased association between rough endoplasmic reticulum and crystalloid material, and reduced crystalloid concentration also could reflect an abnormality in protein synthesis. In normal embryos crystalloid material was seen to increase from cleavage to the blastocyst stage (Enders, 1971). The fixation of this material with permanganate and its association with the granular endoplasmic reticulum suggested a proteinaceous nature (Calarco and Brown, 1969, Szollosi, 1972). The association of crystalloid with the granular endoplasmic reticulum and its continued presence during the preimplantation period indicated that it could represent a product to be used in later development (Calarco and Brown, 1969, Szollosi, 1972). The absence of crystalloid in parthenogenones may be related to the abnormalities associated with protein synthesis in those embryos (Solter, et al. 1974a).

Another very prominent abnormality in the mutant enbryos involved lipid material. Normally very little lipid is present in mouse embryos (Mintz, 1964c, Potts and Wilson, 1967, Calarco and Brown, 1969, Enders,

1971). Lipid concentration was found to decrease as the embryo matured, with only a few granules found in the blastocyst stage (Calarco and Brown, 1969). Solter and others (1974a) noted numerous lipid droplets in parthenogenetic embryos up to the eight cell stage. In the present study abnormal appearance of lipid granules was found more frequently than the abnormalities of large lipid granules and increased lipid concentration (Table 7). Both the collapsed blastocysts and morulae had increased lipid concentration when compared to normal embryos. Similar lipid abnormalities have been seen in t^{12}, t^{w32}, and t^{6} mutant embryos (Hillman, Hillman and Wileman, 1970, Hillman, and Hillman, 1975, Nadijcka and Hillman, 1975a). The presence of excessive lipid in the t^{12} and t^{w32} embryos was believed to result from excessive carbohydrate oxidation leading to high levels of ATP synthesis and excessive amounts of acetyl-CoA, which are the prerequisites for excessive lipid synthesis (Ginsberg and Hillman, 1975). Nadijcka and Hillman (1975b) have demonstrated that the mutant t^{12} and t^{w32} embryos were synthesizing excessive lipid; however, they could not determine if a lack of utilization of lipid material was also involved. Hensleigh (1971) indicated that increased lipid may be related to degenerative changes in the embryo. In the case of $p^{1}p^{1}$ mutants it is probable that the excessive lipid and lipid abnormalities were reflective of the total degeneration of the embryo, rather than related to excessive ATP synthesis, as the mitochondria did not contain crystalloid deposits which are associated with excessive ATP synthesis. In normal and mutant embryos one finds mitochondria associated with lipid material, and this may be related to the metabolism of lipid products in the TCA cycle, or to lipid synthesis.

The abnormalities associated with energy and protein metabolism probably lead to the death of the embryo. The various other abnormalities found on electron microscopy, including: lysosomes, autophagic vesicles, degradation bodies, and disintegrating cells are part of the degenerative process. Disintegrating cells were also noted in the light microscopic study. The large cells found in the collapsing and collapsed blastocysts, noted in both light and electron microscopic studies, may be the result of lack of cell division caused by retardation of cell growth or lack of energy to carry out cell division.

The abnormalities of metabolism result in gradual cell death extended over a period of several days. Other early embryonic lethal genes produce the same pattern. Genetically determined incompatibility between spermatozoa and egg resulted in dead embryos seen as retarded blastocysts and morulae (Wakasugi, 1974). In two homozygous lethals at the t locus, the period of death ranged from the eight cell stage through morula, with most embryos dying as early morulae for t^{w32} (Hillman and Hillman, 1975), and from the eight to twelve cell stage until blastocyst formation for t^{12} (Hillman, Hillman, and Wileman, 1970).

Evidence from both light and electron microscopy in the present study indicated that both healthy and unhealthy cells, i.e., cells in mitosis as well as degenerating cells, were present in p^1p^1 embryos as early as 3½ days. In the case of the two t lethals the proportion of healthy to unhealthy cells present in the embryos determined the time of death (Hillman, Hillman, and Wileman, 1970). The ratio of healthy to unhealthy cells in the p^1p^1 morulae and young blastocysts could determine whether degeneration would be expected to occur or development continue in either a normal or retarded fashion. The lack of necessary

energy and cell products resulting from metabolic abnormalities could cause a slowing down of cell division which would result in the large cells found in some embryos. The various shapes seen in mutant blastocysts and masses in the present study could be explained by the number and location of abnormal cells, as proposed by Hillman, Hillman and Wileman (1970) to explain the abnormal shapes associated with the t^{12} mutant embryos. By the 4½ day blastocyst, the proportion of unhealthy cells in these embryos was so great as to cause embryonic degeneration. The presence of healthy and unhealthy cells in the t^{12}, t^{w32}, and t^{6} embryos indicated to the authors (Hillman, Hillman and Wileman, 1970, Hillman, and Hillman, 1975, Nadijcka and Hillman, 1975a) that t^{12}, t^{w32}, and t^{6} were cell lethals. L. B. Russell (1964) concluded that p^{1} was not a cell lethal on the basis of the presence of variegated $R(+)/p^{1}$ females after x-autosome translocations. If p^{1} were a cell lethal, those cells which were $0/p^{1}$ after X-inactivation would die and have no effect on coat color, leaving animals with a wild phenotype.

The presence of healthy and unhealthy cells in the mutants could explain why some morulae did not cavitate and why the blastocysts collapsed. Normal cell association and the formation of a compact group of cells, a prerequisite for the normal secretion of blastocoel fluid (Stern, 1972), may have been prevented by the presence of unhealthy cells. Stern (1972) noted that reaggregating disaggregated embryos formed either blastocysts or irregular vesiculated masses. If the cells were not in a compact mass they would accumulate fluid within themselves (Stern, 1972). Thus, this might explain why $p^{1}p^{1}$ morulae with a large proportion of abnormal cells would not cavitate. The number and location of abnormal cells may affect the collapse of the blastocoel. As

the number of abnormal cells increased more strain would be put on the blastocyst wall, and eventually the integrity of the blastocoel could no longer be maintained. In the A^y/A^y lethal embryo, asynchronous cell death caused the arrest of several blastomeres during late cleavage, which resulted in the collapse of the blastocysts formed from these embryos (Pedersen, 1974). Mintz (1964d), found that in t^{12} mosaics the mutant cells could not hold the blastocyst together under the increasing pressure of expansion, and the blastocyst collapsed due to breaks in cell connections.

Another explanation for the collapse of the blastocyst was the improper sealing of the junctional complexes. It was noted by Ducibella et al. (1975) that the appearance of a continuous junctional complex between the trophoblast cells was a prerequisite for the establishment and expansion of the blastocyst. The desmosome, first found at the blastocyst stage, was thought to counteract the hydrostatic pressure which builds up inside the blastocyst as the cavity increases in size (Ducibella et al., 1975). However, while some mutant embryos appeared to have gaps between cells when viewed under the light microscope, cellular junctions were found intact on electron microscopic study. Calarco and Brown (1968) found the same difference in the t^{12} mutant. It may be while complexes were present, they were not properly sealed. In a study on rabbit junctional complexes Hastings and Enders (1975) suggest that the more complete the complex seal, the more efficient the mechanism of accumulation and retention of fluid in the cavity. A greater amount of energy expenditure would be needed for the blastocyst to achieve and maintain the same expansion with incomplete seals (Hastings and Enders, 1975). The paucity of mitochondria and thus decreased

available energy may lead to the failure of the mutants with incomplete junctional complexes to maintain blastocoel expansion.

Since all masses at 4½ days in this study induced a decidual reaction it was concluded that they differentiated into cells normal enough to behave as trophoblast cells in eliciting decidual reactions (Gardner, 1971, 1972). Gardner (1975) suggested that inner cell mass and trophoblast are determined to be different between the eight and 16 to 64 cell stage. Thus, it is probable that the embryos did not become markedly abnormal until after trophoblast determination. The partial implantation of collapsed blastocysts at 4½ and 5½ days indicated that even at such a late stage in mutant development the trophoblast was functioning normally. Eaton and Green (1963) noted that collapsed A^y/A^y blastocysts would attach themselves to the uterine wall. Gardner and others have determined that inner cell mass tissue was essential for trophoblast proliferation, that is, in the absence of inner cell mass cells the trophoblast cells stop dividing and assume the giant form (Gardner, Papaioannou, and Barton, 1973). This finding may explain why mostly giant cells were found in decidual capsules at 6½ days, as some masses seen at 5½ days did not contain inner cell mass tissue.

The pattern of p-lethal action that emerges from this study portrays a deficiency in energy metabolism perhaps causing limitations on RNA and protein metabolism. Whether the deficiency in energy metabolism was due to the direct action of the p^1 allele or to degenerative changes in the dying embryos was not clear. At 3½ days the beginning of embryonic degeneration was seen in the mutant embryos. While there were no signs of embryonic disintegration, the presence of vacuoles, granular staining, round and net-like nucleoli, and pycnosis indicated

cellular degeneration. By 3-3/4 days the mutant embryos have started to disintegrate as evidenced by gaps between cells and degenerating cell walls. The retardation of RNA and protein synthesis indicated by pale staining patterns found in embryos at 3½ and 3-3/4 days may reflect impoverished energy supply due to abnormalities in and decreased numbers of mitochondria in some mutants. The reduced granular endoplasmic reticulum concentration observed in most mutant embryos could result in decreased protein synthesis. The abnormal staining patterns, e.g., clumping of stain, which appeared in mutant embryos probably were directly related to cellular degeneration.

By 4½ days the embryos showed the most advanced signs of degeneration and masses began to appear. While some of the morulae seen at 3-3/4 days may really be masses, the frequency of the masses reached its highest level at 4½ days. The dark staining pattern found in some 4½ and 5½ day mutants indicated possible retardation of RNA and protein synthesis rather than absence of these synthetic processes. The presence of asynchronous cell death in the mutant embryos could result in the variety of mutant shapes seen, the lack of cavitation of some morulae and collapse of the blastocoels. The similarities between some of the mutant characteristics seen in p^1p^1 embryos with those of other early lethals, as well as with the abnormal control embryos, indicated that many of these characteristics may be degenerative changes common to dying embryos.

The nature of pleiotropic effects of radiation-induced mutations suggest that the primary mutational effect is an abnormality of cell membranes (Gluecksohn-Waelsch and Cori, 1970). A mutation in a single membrane protein could cause abnormalities of various membranes.

Woodward and Munkres (1967) found that the replacement of a single amino acid in the structural protein of the mitochondrial membrane of Neurospora modified the activity of the enzyme MDH. From their data they concluded that a component of the mitochondrial structural protein was common to other cellular structures (Woodward and Munkres, 1967), thus the mutation in a single membrane protein would result in abnormalities of various membranes, and the behavior of the enzymes bound by the respective protein. A protein which is basic to organelle membrane structure, and which is synthesized in early developmental stages as well as during melanosome formation could link the abnormalities seen with p^1 and the action of the P locus. Changes in the structure of a basic protein could be responsible for the abnormalities seen in the mitochondria, granular endoplasmic reticulum, and melanosomes. Kiehn and Holland (1968) suggested that there are similarities between proteins of nuclear and plasma membranes, mitochondria, and microsomal membranes. They argue that all these membranes possess a number of related or identical proteins as part of their basic structure (Kiehn and Holland, 1968). The concept of multiple protein subunits is in harmony with the multiple functions of membranes (Kiehn and Holland, 1968).

The relationship of energy metabolism and protein synthesis is linked to the eight cell stage of the embryo, when there is an increase in the embryo's ability to oxidize glucose followed by in increase in the ability to synthesize protein (Mintz, 1964a, Brinster, 1967a). At this time the amount of granular endoplasmic reticulum increases in normal embryos (Hillman and Tasca, 1969). Considering these relationships and the findings of the microscopic studies it is possible that

the mutant starts to act sometime soon after the eight cell stage, probably just before morula formation.

TABLE 1

DISTRIBUTION OF EMBRYOS IN CONTROL AND p-LETHAL LITTERS
(6½-4½ DAYS P.C.)

Cross	Age (Days)	Total Litters	Total Embryos	Average Embryo/Litter	Average Normal Embryo/Litter	Total Abnormal (%)
Control	at Birth	130	955	7.35		
p-Lethal		130	729	5.61		
Control	6½	3	24	8.00	8.00	0(0)
p-Lethal		5	35	7.00	5.00	10(28.6)
Control	5½	10	66	6.60	6.20	4(6.1)
p-Lethal		22	150*	6.82	4.86	42/149(28.2)
Control	4½	12	94	7.83	7.25	7(7.4)
p-Lethal		12	79	6.58	4.83	21(26.6)

*One embryo could not be identified.

TABLE 2

FREQUENCY OF p^1p^1 EMBRYOS IN p-LETHAL LITTERS
CLASSIFIED ACCORDING TO DEGREE OF ABNORMALITY

Day	Total Embryos*	p^1p^1 (%)	Group I		Group II	Group III		Group IV
			Empty Capsules (%)	Dying (%)	Masses (%)	Collapsing and Collapsed Blastocysts (%)	Abnormal Blastocysts (%)	Abnormal Morulae (%)
6½	35	8 (22.9)	8 (22.9)	0	0	0	0	0
5½	149	38 (25.5)	17 (11.4)	0	15 (10.1)	6 (4.0)	0	0
4½	79	20 (25.3)	0	3 (3.8)	14 (17.7)	3 (3.8)	0	0
3-3/4	65	18 (27.7)	0	0	0	0	14 (21.5)	4 (6.2)
3½	145	36 (24.8)	0	0	0	2 (1.4)	20 (13.8)	14 (9.7)

*Total number of embryos which could be classified.

TABLE 3

DISTRIBUTION OF EMBRYOS IN CONTROL AND p-LETHAL LITTERS
(3-3/4 AND 3½ DAYS P.C.)

Cross	Age (Days)	Total Litters	Total Embryos	Average Embryo/ Litter	Average Normal Embryo/ Litter	Total Blastocysts (%)	Total Morulae (%)	Total 2-16 cells (%)	Total Dead (%)	Total Abnormal (%)
Control	3-3/4	4	35	8.75	7.75	31 (88.6)	1 (2.8)	0 (0)	3 (8.6)	4 (11.4)
p-Lethal	3-3/4	8	66*	8.25	4.63	54/65 (83.1)	6/65 (9.2)	1/65 (1.5)	4/65 (6.2)	28 (43.1)
Control	3½	13	97*	7.46	5.92	64/96 (66.7)	18/96 (18.7)	10/96 (10.4)	4/96 (4.2)	19 (19.8)
p-Lethal	3½	21	146*	6.95	4.00	114/145 (78.6)	21/145 (14.5)	8/145 (5.5)	2/145 (1.4)	61 (42.1)

*One embryo could not be identified and percentages were based on number of total embryos minus one.

TABLE 4

DISTRIBUTION OF BLASTOCYSTS BY MORPHOLOGIAL TYPE AT 3-3/4 AND 3½ DAYS P.C.

Cross	Age (Days)	Total Blastocysts	Normal (%)	3-3/4 Day Type			
				Normal (%)**	p^1p^1 (%)**	Abn.* (%)**	Total (%)**
Control	3-3/4	31	31 (100)	12 (38.7) [38.7][1]	0	0	12 (38.7)
p-Lethal	3-3/4	54	36 (66.7)	14 (25.9) [38.9][1]	1 (1.9)	0	15 (27.8)
Control	3½	64	62 (96.9)	. . .	. . .	. . .	. . .
p-Lethal	3½	114	81 (71.1)	. . .	. . .	. . .	. . .

*Abnormal group (abn.) is made up of non-p^1p^1 mutants.

**% as % of total blastocysts found.

[1][]% as % of total normal blastocysts found.

TABLE 4--Continued

3½ Day Type				3¼ Day Type			
Normal (%)**	p^1p^1 (%)**	Abn.* (%)**	Total (%)**	Normal (%)**	p^1p^1 (%)**	Abn.* (%)**	Total (%)**
14 (45.2) [45.2][1]	0	0	14 (45.2)	5 (16.1) [16.1][1]	0	0	5 (16.1)
17 (31.5) [47.2][1]	9 (16.7)	3 (5.5)	29 (53.7)	5 (9.2) [13.9][1]	4 (7.4)	1 (1.9)	10 (18.5)
47 (73.4) [75.8][1]	0	1 (1.6)	48 (75.0)	15 (23.4) [24.2][1]	0	1 (1.6)	16 (25.0)
76 (66.7) [93.8][1]	14 (12.3)	4 (3.5)	94 (82.5)	5 (4.4) [6.2][1]	8 (7.0)	7 (6.1)	20 (17.5)

TABLE 5

MITOCHONDRIAL VALUES FOR NORMAL AND p^1p^1 EMBRYOS

Morphology	Number of Embryos	Trophoblast: Unit Areas[1]	Trophoblast: Average[3] Mitochondria[2] per Unit Area	Inner Cell Mass: Unit Areas[1]	Inner Cell Mass: Average[3] Mitochondria[2] per Unit Area
Normals					
Control series					
Well-expanded	2	19 [10, 9]	3.90 [2.78, 4.90]	7 [3, 4]	6.00 [5.00, 6.75]
Small blastocoels	5	39	5.03 [9.17 ↓ 3.67] *	37	6.68 [8.71 ↓ 4.17] *
p-Lethal Series					
Well-expanded	9	90	4.02 [2.40 ↓ 8.00] *	41	5.73 [3.28 ↓ 7.33] *
Small blastocoels	3	34 [11, 15, 8]	4.94 [4.64, 4.40, 6.75]	34 [14, 12, 8]	6.79 [7.36, 7.25, 5.12]

TABLE 5--Continued

Morphology	Number of Embryos		Trophoblast: Unit Areas[1]	Trophoblast: Average[3] Mitochondria[2] per Unit Area	Inner Cell Mass: Unit Areas[1]	Inner Cell Mass: Average[3] Mitochondria[2] per Unit Area
p^1p^1 Embryos						
Morulae	2		12	7.17	7	8.00
		#1	8	4.48	4	9.00
		#2	4	11.75	3	6.67
Collapsed blastocysts	2		18	3.06	10	3.40
		#1	6	2.33	1	6.00
		#2	12	3.42	9	3.11
Collapsing blastocysts	4		24	4.46	29	6.03
		#1	5	2.60	7	5.43
		#2	7	2.57	10	5.80
		#3	6	4.50	10	4.80
		#4	6	8.17	2	16.50

[1]One unit area per cell was examined, so that the number of unit areas is equal to the number of cells studied.

[2]In the above calculation the unit area was adjusted to represent a constant size despite differences in magnification.

[3]The average number of mitochondria is represented by weighted averages.

*These numbers represent the range found in the weighted averages for those groups.

TABLE 6

ABNORMAL CHARACTERISTICS OF p^1p^1 MUTANTS AS RELATED TO THEIR MORPHOLOGIES*†

Masses	Frequency	Collapsed Blastocysts	Frequency	Collapsing Blastocysts	Frequency
Abn. shaped lipid	2/2	Fewer agran er	2/2	Fewer g er	4/4
More lysosomes	2/2	Fewer g er assoc. mitochondria	2/2		
Fewer crystalloids	2/2			Abn. shaped lipid	3/4
More lipid	2/2	Fewer crystalloids	2/2	Fewer g er assoc. mitochondria	3/4
Fewer g er	2/2	More lysosomes	2/2		
		Fewer mitochondria	2/2	Fewer g er assoc. crystalloids	3/4
		Fewer g er	2/2		
				Fewer mitochondria	3/4
Large lipid	1/2	Large lipid	1/2		
Large cells	1/2	Abn. lipid	1/2	Large lipid	2/4
Fewer g er assoc. crystalloids	1/2	Fewer g er assoc. crystalloids	1/2	Wide cisternae in g er	2/4
				Abn. vacuolated mito- chondria	2/4
		More lipid	1/2		
		Large cells	1/2		
		Wide cisternae in g er	1/2	More lysosomes	1/4
		Abn. vacuolated mito chondria	1/2	Fewer crystalloids	1/4
				Fewer agran er	1/4
				Large cells	1/4

*The frequency of abnormal characteristics was determined through chance sampling of cells in the eight mutant embryos. Abnormalities with less than 3/8 frequency in all p^1p^1 mutants are not included.

†The terms fewer and more refer to the relative frequency of the organelles when compared to control embryos.

Abbreviations used: agran er - agranular endoplasmic reticula; g er - granular endoplasmic reticula; abn. - abnormally; assoc. - associated with

TABLE 7

ABNORMAL CHARACTERISTICS OF p^1p^1 MUTANTS AS DETERMINED BY ELECTRON MICROSCOPY AND THEIR FREQUENCIES*

Abnormal Characteristic	Frequency
Fewer total granular endoplasmic reticula[1]	8/8
Abnormally shaped lipid granules	6/8
Fewer granular endoplasmic reticula associated with mitochondria[1]	6/8
Fewer mitochondria[1]	5/8
Fewer crystalloids[1]	5/8
More lysosomes[1]	5/8
Fewer granular endoplasmic reticula associated with crystalloids[1]	5/8
Large lipid granules	4/8
Disintegrating trophoblast cells	4/8
More lipid material[1]	3/8
Fewer agranular endoplasmic reticula[1]	3/8
Large cells in the embryo	3/8
More mitochondria[1]	3/8
Wide cisternae in the granular endoplasmic reticula	3/8
Abnormally vacuolated mitochondria	3/8

*The frequency of abnormal characteristics was determined through chance sampling of cells in the eight mutant embryos. Abnormalities with less than 3/8 frequency are not included.

[1]The terms fewer and more refer to the relative frequency of the organelles when compared to control embryos.

CHART 1

DIFFERENCES BETWEEN NORMAL YOUNG AND WELL-EXPANDED

BLASTOCYSTS OF p-LETHAL AND CONTROL SERIES

Organelle or Feature	Young Embryos	Well-Expanded Blastocysts
Jig-saw regions	present	absent
Multivesicular bodies	present	infrequent
Granular endoplasmic reticulum	short pieces	long pieces
Polysomes	low number	higher number
Crystalloids found in the inner cell mass	fewer	more

ABBREVIATIONS USED IN MICROGRAPHS

B - Blastocoel

C - Crystalloid material

C V - Cytoplasmic Vesicle

D - Degradation body

D En - Distal Endoderm

E C - Ectoplacental Cone

Em C - Embryonic Cells

Em Ec - Embryonic Ectoderm

En - Endoderm

Ex Ec - Extraembryonic Ectoderm

F - Filaments

G - Golgi

G ER - Granular Endoplasmic Reticulum

G T - Trophoblastic Giant Cell

I C M - Inner Cell Mass region

J - Jig-Saw region

L - Lipid

Ly - Lysosome

M - Mitochondrion

Mat - Dense material

M R - Multivesicular Region

Mv - Microvillus

N - Nucleus

P C - Proamniotic Cavity

Pd - Predesmosome

P En - Proximal Endoderm

P V - Pinocytotic Vesicle

S - Stroma

T - Trophoblast

T J - Tight Junction

Ut - Uterine epithelium

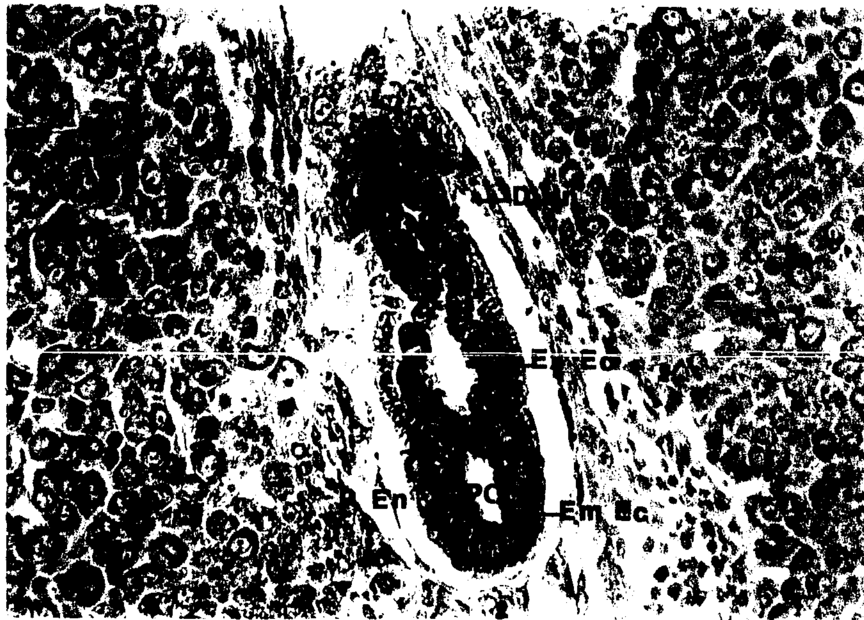
V - Vacuole

Z P - Zona Pellucida

Light Micrographs

1. Most developmentally advanced normal 6½ day embryo from a control litter. The embryo has a developing ectoplacental cone. The endoderm is divided into proximal and distal regions. The proximal endoderm is thinner along the lower margin of the egg cylinder, more cuboidal laterally. Distal endoderm covers the full length of the trophoblast. The proaminotic cavity is present. The post-amniotic fold is beginning to divide the internal cavity into the proamniotic and ectoplacental cavities. Azure B. 350X

2. Most developmentally advanced normal 5½ day embryo from a control litter. The ectoderm is divided into embryonic and extraembryonic regions and the ectoplacental cone is just forming. The long axis of the embryonic ectodermal cells is perpendicular to the proamniotic cavity. Extraembryonic ectoderm is less well organized. Nucleoli range from round to oval. The endoderm has differentiated into highly columnar vacuolated proximal and squamous distal regions. The proximal endoderm surrounds both ectoderms, except in the region of the ectoplacental cone, and is thinner at the embryonic end of the egg cylinder. Distal endoderm lines the trophoblast. Azure B. 450X



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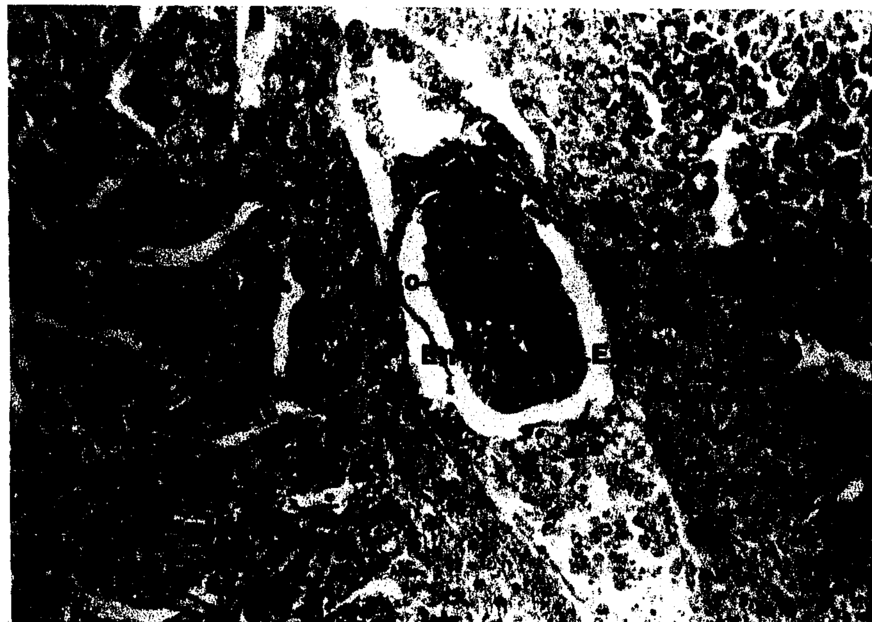
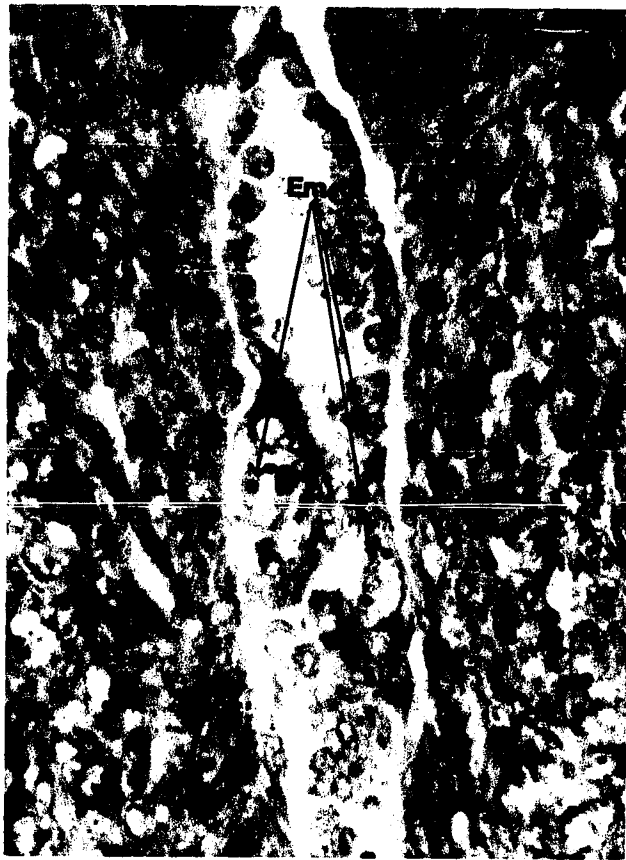


2

3. A capsule with embryonic cells (arrows) from a 5½ day p-lethal litter. Note the round darkly staining nucleoli. The uterine epithelium is missing at the level of giant trophoblast cells as is normal at this age. Azure B. 520X

4. A 5½ day mass from a p-lethal litter. Note the round nucleoli and large vacuoles present in cytoplasm. Uterine epithelium, although abnormal, is still present. The presence of the epithelium is characteristic of the polar trophoblast level. Azure B. 1575X



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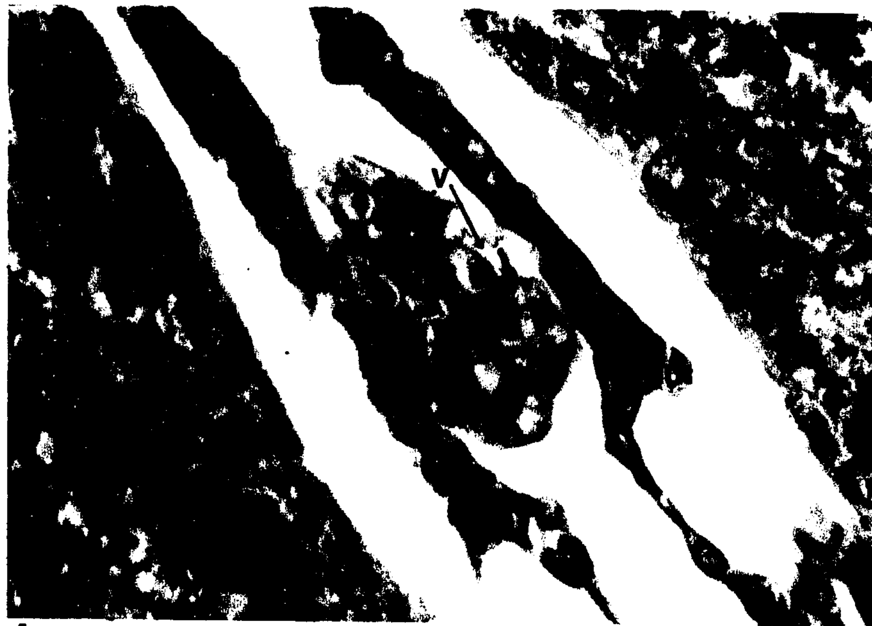


4

5. A 5½ day collapsed blastocyst from a p-lethal litter. The blastocoel is seen as a small space (arrows). Trophoblast cells have invaded the uterine epithelium. Some areas of the embryo have darker stain than others. Azure B. 1575X

6. Most developmentally advanced normal 4½ day embryo from a control litter. This embryo shows differentiation of the proximal endoderm from the inner cell mass region. The proximal endoderm is somewhat vacuolated at this time. It is difficult to distinguish between the inner cell mass and trophoblast regions. Note the round to oval nucleoli. Trophoblast was pulled away from uterine epithelium during fixation. Azure B. 520X

5

6

7. A 4½ day collapsed blastocyst from a p-lethal litter. Note the long, thin, shape similar to that of the ovular masses. Note the numerous round, darkly staining nucleoli in some cells. There is also uneven staining seen as pale and dark areas. Azure B. 1040X

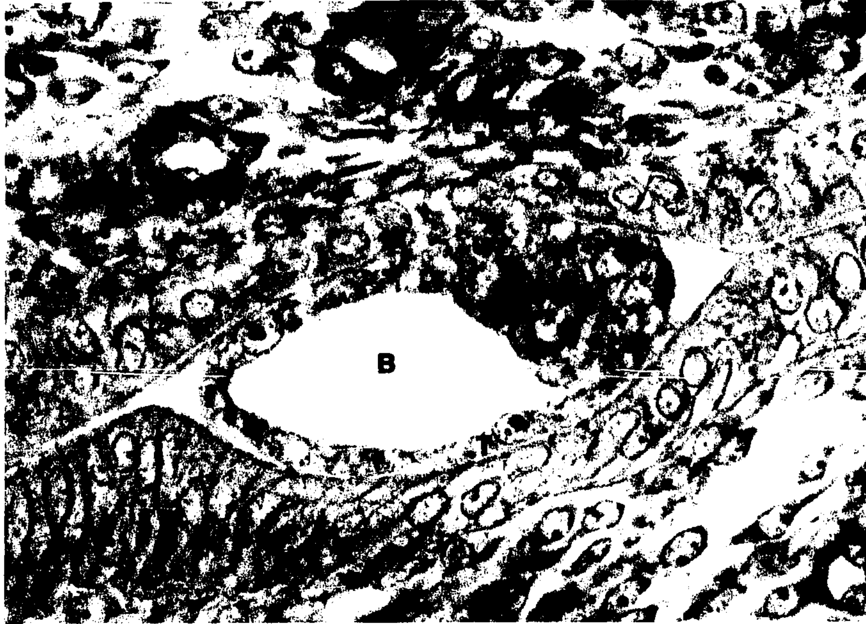
8. 4½ day ovular mass from a p-lethal litter showing small dark round nucleoli, uneven cytoplasmic staining pattern of light and dark areas. One cell has a dark blue clump of stain. Note decidualization reaction, i.e., typical detachment of uterine epithelium from stroma. Former is disorganized, cells in latter are enlarged. Azure B. 780X

7

8

9. A 3-3/4 day normal embryo from a p-lethal litter. It is difficult to distinguish the trophoblast from the inner cell mass region. Note the enlarging capillary wall cells. Decidualization has not yet begun. Azure B. 780X

10. Most developmentally advanced normal $3\frac{1}{2}$ day embryo from a $3\frac{1}{2}$ day p-lethal litter. This embryo has round to oval nucleoli, and a suggestion of distinction between the inner cell mass and trophoblast regions. Note the typical elongated nucleoli. Azure B. 1575X



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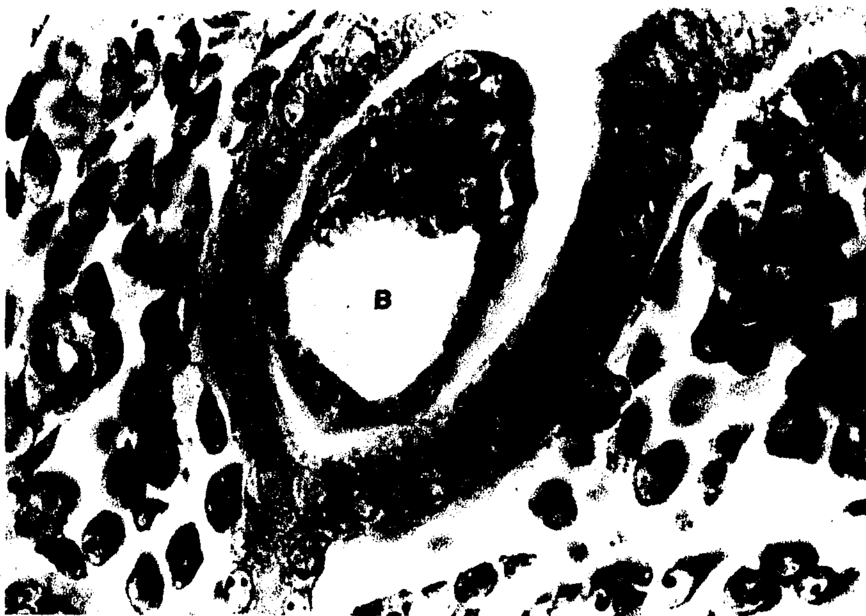
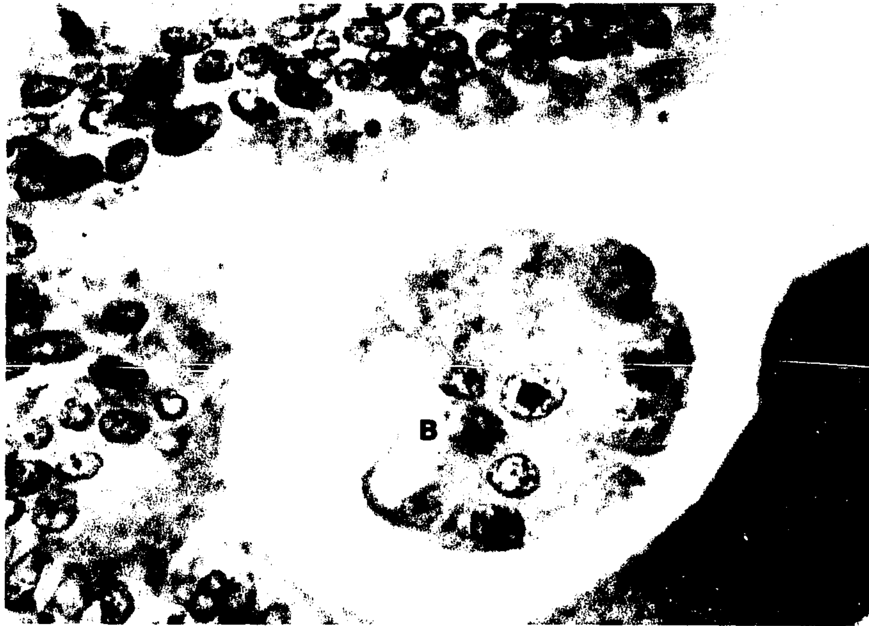


10

11. A 3¼ day normal embryo from a 3½ day control litter. Note the large cells, round nucleoli, and small, eccentric blastocoel. Feulgen and Fast Green. 780X

12. A mutant blastocyst from a 3-3/4 day p-lethal litter. This embryo displays the following abnormalities: a large pale cell, bright, darkly staining round nucleoli, uneven staining pattern, granularity of stain. Azure B. 780X



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13. A mutant blastocyst from a 3-3/4 day p-lethal litter. This embryo displays pale cytoplasmic staining with dark, bright round nucleoli, a large central cell, uneven staining pattern with some areas being paler than others. Azure B. 780X

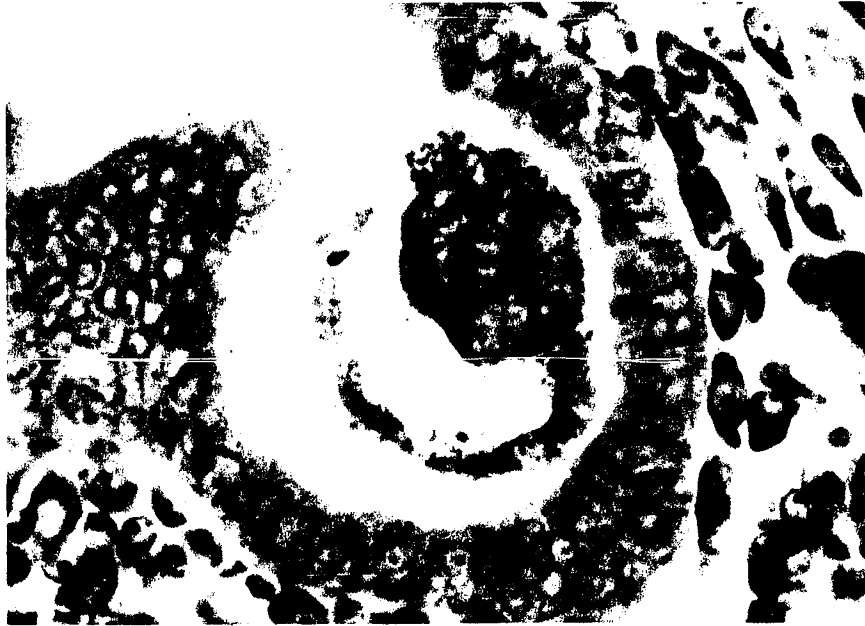
14. A mutant blastocyst from a 3-3/4 day p-lethal litter. Note the pale stain, dark, bright round nucleoli, vacuoles, uneven stain, and pycnotic nucleus. Azure B. 780X

13

14

15. A mutant blastocyst from a 3½ day p-lethal litter. This embryo demonstrates the following abnormalities: uneven staining, dark areas of clumped stain, blue dots, some large cells, round nucleoli, and a pycnotic nucleus. Azure B. 780X

16. A mutant blastocyst from a 3½ day p-lethal litter. This embryo demonstrates the following abnormalities: bright, round nucleoli, numerous nucleoli in one cell, vacuoles, granular staining, and a pycnotic nucleus. Azure B. 780X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16

NORMAL EMBRYOS

17. A juxtanuclear golgi body consisting of a group of vacuoles. Note the mitochondria and associated granular endoplasmic reticulum. 24,000X

18. A multivesicular region on a dense matrix. These organelles were found in both young and well-expanded blastocysts. 33,250X

19. Jig-saw regions, one of which is incorporated into an autophagic vesicle. These regions were found in young embryos. Note the denser material at the junctions of various segments (arrows). Also note the clear cytoplasmic vesicles associated with the jigsaw region. 31,800X

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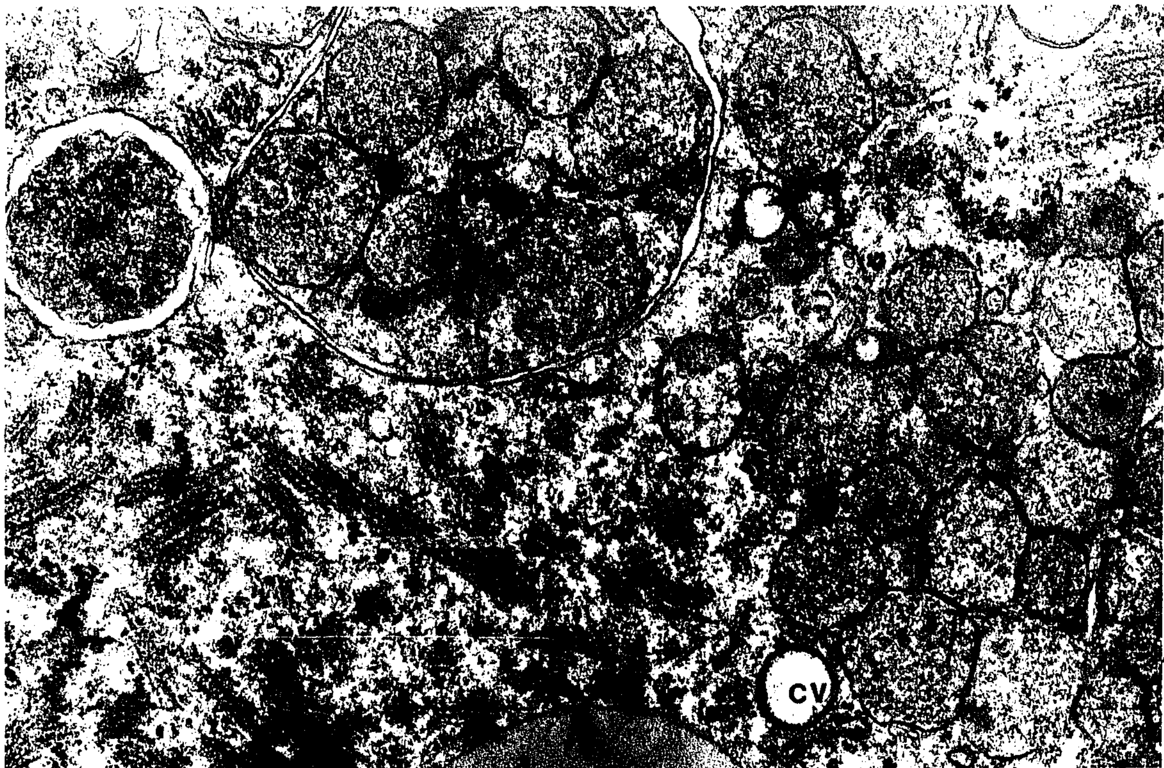

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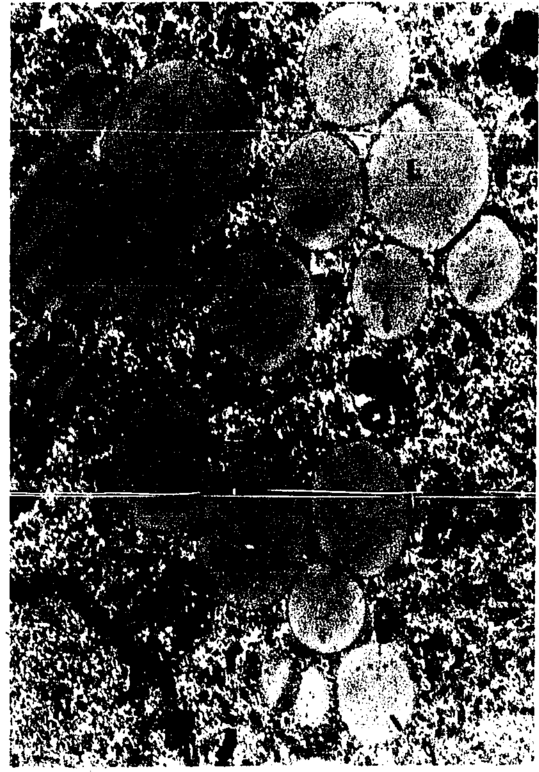
20. Light inner cell mass cells seen in comparison with dark trophoblast. Density differences between cells were found in both normal control and mutant embryos. Microvilli are present on cell surface. 5,075X

21. An aggregation of lipid granules each bound by an electron dense line. Degradation bodies are also present. 10,650X

22. A lipid granule without an electron dense line. Note the polysomes. 28,650X

23. Crystalloid material in blastocyst. The fine structure and its association with granular endoplasmic reticulum are shown. Polysomes are also seen. 40,800X

20



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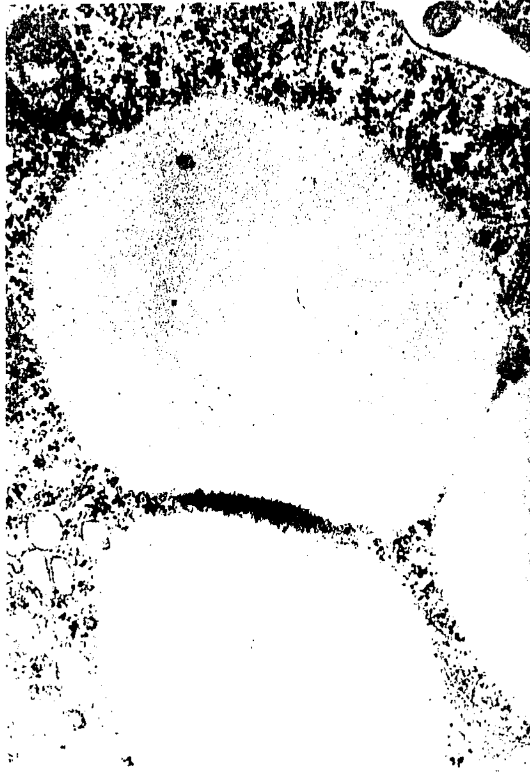

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24. Fine structure of the filaments showing the α and β fibers. 81,180X

25. Mitochondria and associated granular endoplasmic reticulum. Note a crystalloid surrounded by granular endoplasmic reticulum. The part of the granular endoplasmic reticulum adjacent to the mitochondrion is relatively free of ribosomes. 37,240X

26. Highly vacuolated mitochondria of oval and round shape. Cristae are not always parallel. Some of the vacuoles contain a medium dense substance and fibrous elements. 8,700X

27. Older type of mitochondria with normal alignment of cristae. The granular endoplasmic reticulum associated with it does not have ribosomes on the side apposing the organelle. Also indicated is a pinocytotic vesicle and a microvillus. Note the cellular junction (in square). 14,500X

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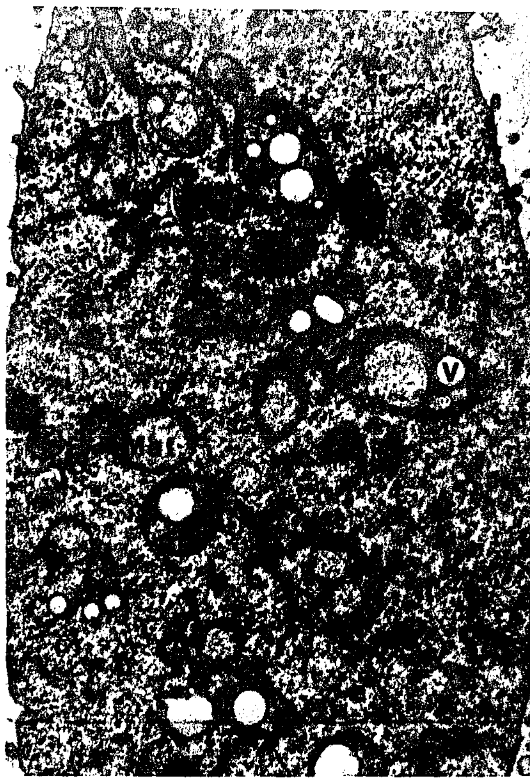

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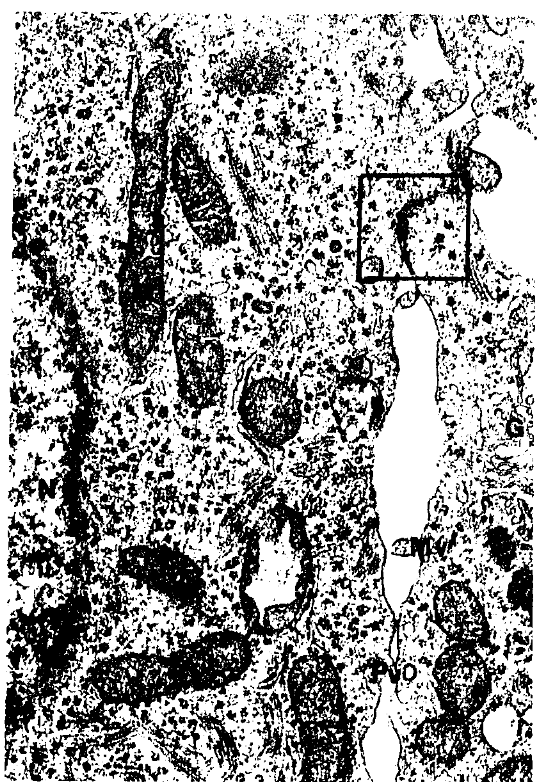

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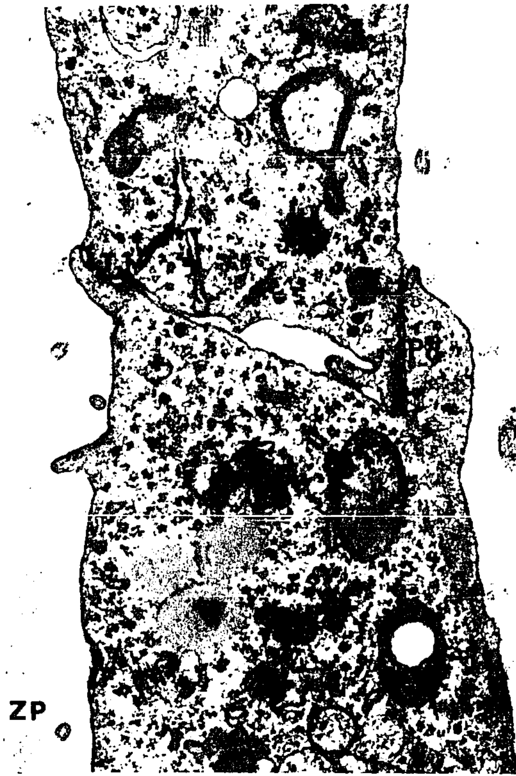
28. The association of mitochondria, lipid and crystalloid material is seen in the lower trophoblast cell. Note also predesmosome junction and a tight junction. 16,250X

29. Lysosome with heavy density inclusion and a degradation body. 29,400X

30. Formation of an autophagic vesicle by association of granular endoplasmic reticulum. A jig-saw body is contained in the vesicle. 39,900X

MUTANT EMBRYOS

31. Large lipid aggregation seen in a morula. The lipid material is pulled away from the granule border. 14,625X



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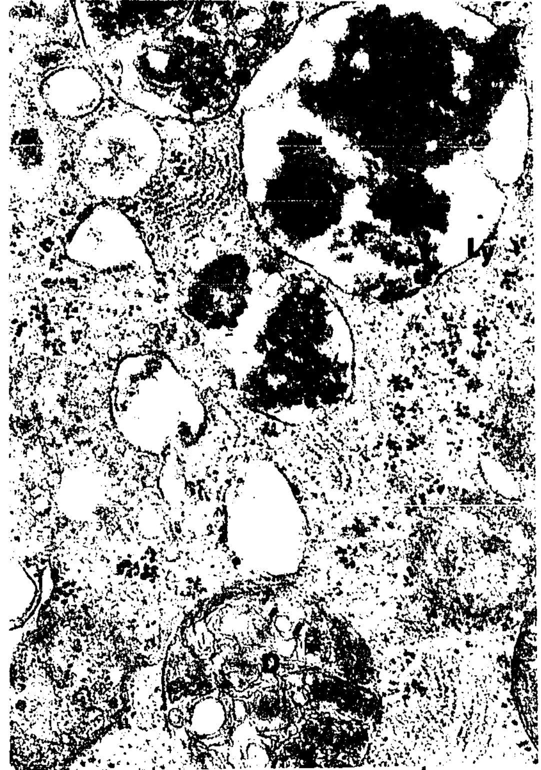

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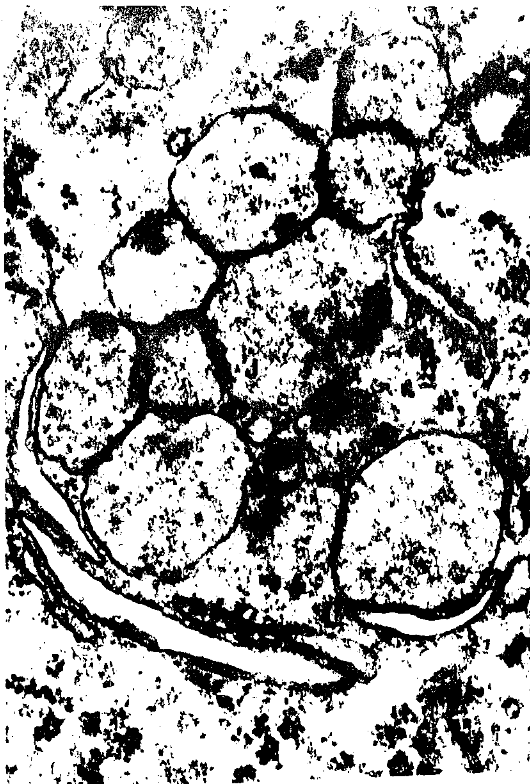

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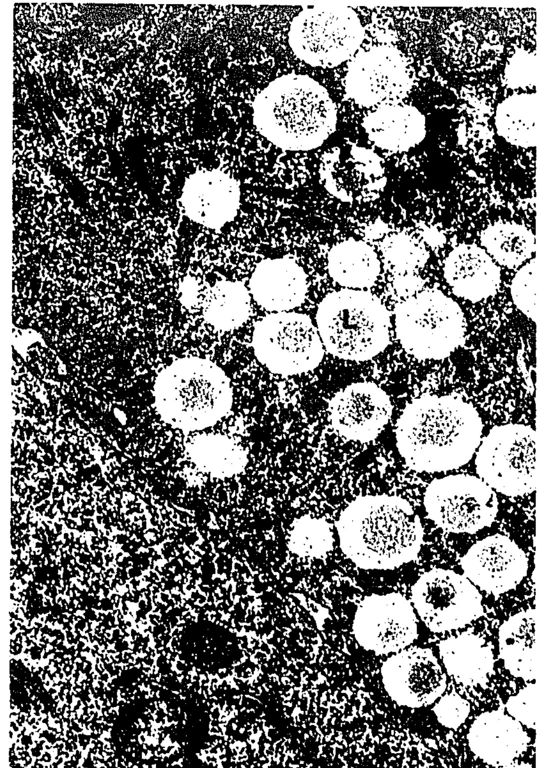

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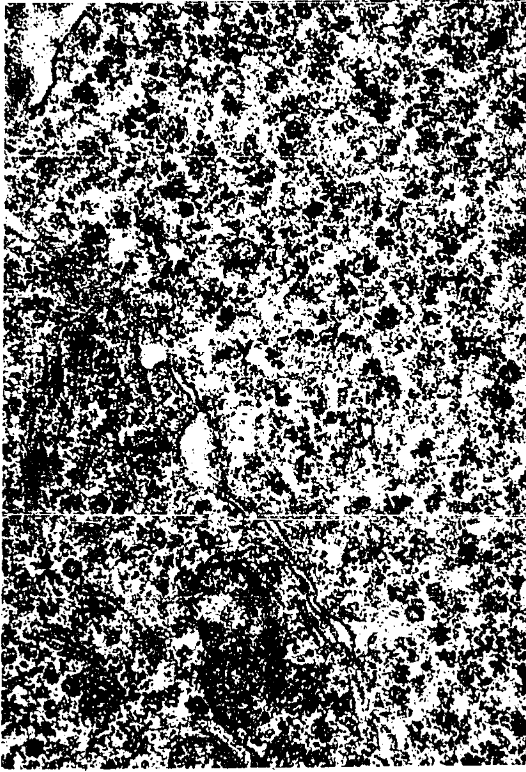
32. High concentration of polysomes seen in morula 1. 29,250X

33. Collapsed blastocyst showing lipid accumulation in three cells. 3,800X

34. Crystalloid material showing a lack of association with granular endoplasmic reticulum. 42,700X

35. Mitochondria showing a lack of association with granular endoplasmic reticulum. Also note round, vacuolated mitochondria. 8,125X



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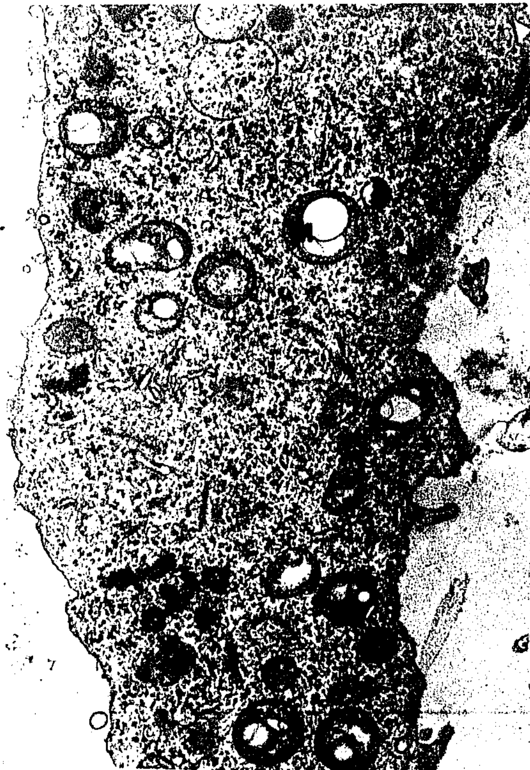

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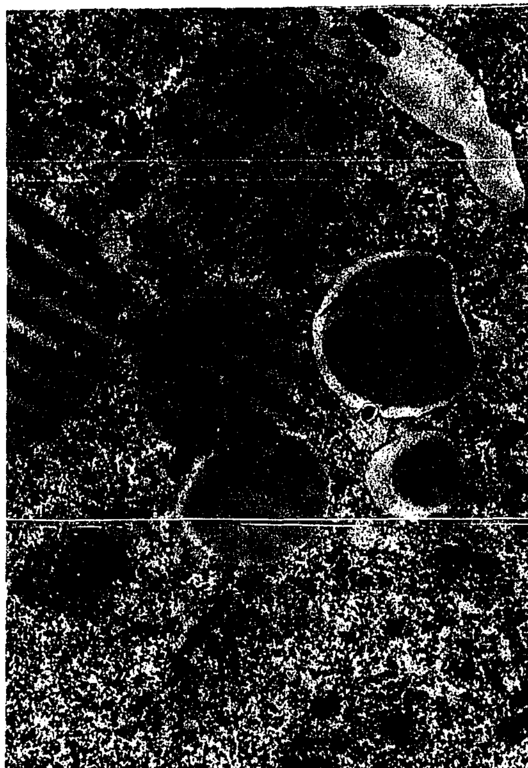
36. Lipid material shrinking from granule border in a collapsing blastocyst. 11,360X

37. Lipid material in a collapsing blastocyst which seems to be losing its round shape. 28,560X

38. Large lipid granules found in a collapsing blastocyst. 6,815X

39. Crystalloid material lacking normal association of granular endoplasmic reticulum. 13,135X



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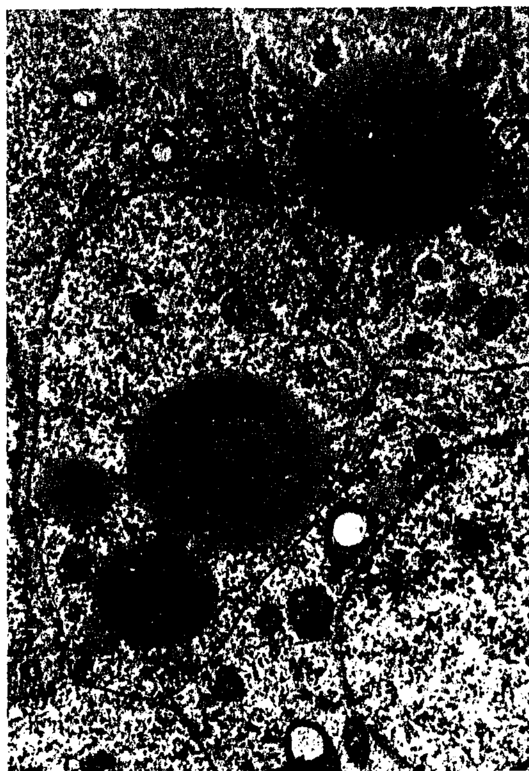

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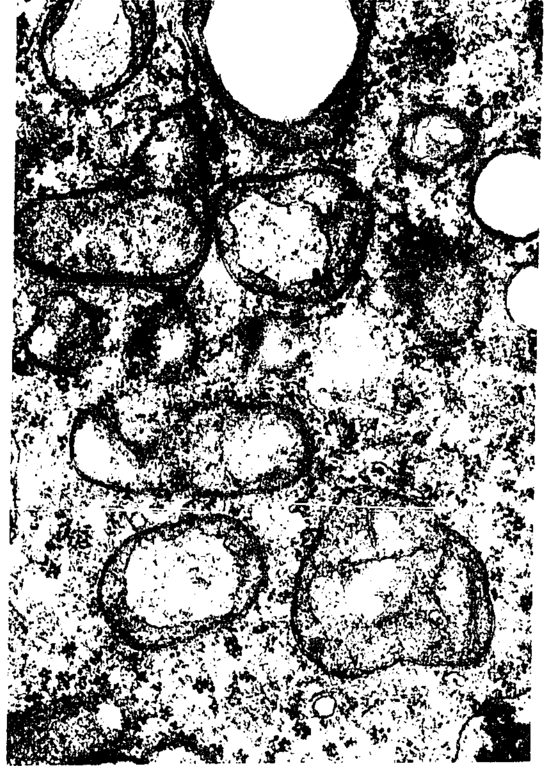
40. A different collapsing blastocyst demonstrating crystalloid material without the normal association with granular endoplasmic reticulum. 30,600X

41. Lack of normal association of granular endoplasmic reticulum and mitochondria 28,560X

42. Wide cisternae found in some granular endoplasmic reticulum. Doughnuts can also be seen. 46,550X

43. More mitochondria are seen in the inner cell mass cells than in the trophoblast cells. Note the highly vacuolated state of some of the mitochondria. 3,625X

40



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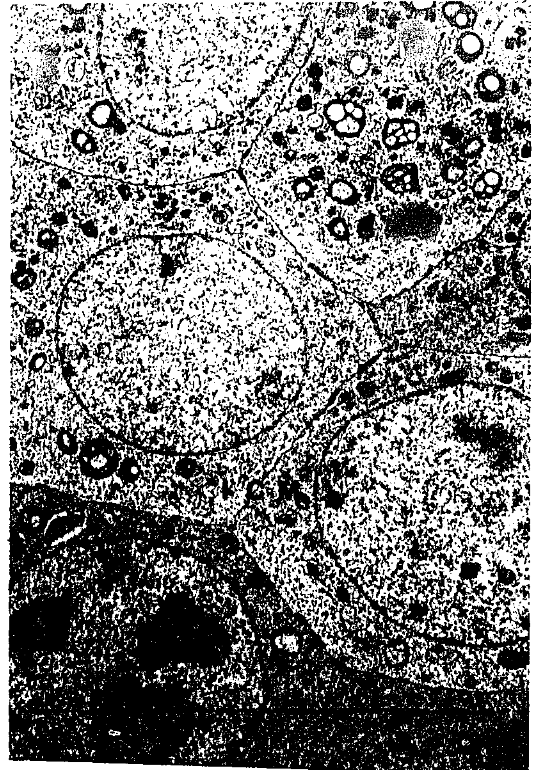

43

Addendum

The standard method of identifying embryos homozygous for recessive lethals acting before birth was used in the light microscopic study. Litter sizes and condition of embryos of female mice heterozygous for p^1 (p^1p) were compared at 6½ through 3½ days after mating to males heterozygous for p^1 and homozygous for p. In this method if the average litter size in utero from the p^1p x p^1p matings is 75% of that in the uteri of p^1p x pp matings, and all embryos appeared normal then it could be concluded that the p^1p^1 embryos died and were reabsorbed at an earlier stage of development. This comparison is continued through progressively earlier stages of development until a stage is reached at which the average number of embryos is the same, but the number of normal embryos in the p^1p x p^1p cross is 75% that in the p^1p x pp matings, and 25% are uniquely abnormal. The latter embryos are assumed to be representative of the p^1p^1 group genotype. Next, younger uteri are examined to determine the first visible manifestation of the p^1p^1 genotype. This is assumed to lie between the time the percent of abnormal embryos from both kinds of matings is the same and the time an additional 25% of them are of the uniquely abnormal type.

The 25% reduction in total embryos due to the loss of p^1p^1 embryos is expected to be reflected in a 25% reduction in the average litter size at birth from the p^1p x p^1p matings, as compared to the average litter size at birth from the p^1p x pp matings. It was, therefore, unexpected when the initial t-test done on the at-birth data from the two matings did not show a significant difference between the litter sizes of the two crosses. The mean litter size for the control cross

(p^1p x pp) was 6.53 (s.e. 0.92), and for the p-lethal cross (p^1p x p^1p) 6.37 (s.e. 0.29), and the t value was 0.208. The possibility that p^1 was not segregating in Mendelian fashion was examined by a chi-square test done on the ratios of p^1p and pp mice at weaning. The pp mice could be easily identified by their pink eyes and diluted coat color. The expected ratios of 2 p^1p: 1 pp offspring in the p^1p x p^1p matings and 1 p^1p: 1 pp in the p^1p x pp matings was found to fit the data well (Table 8). The replacement of the p^1p^1 embryos by p^1p and pp embryos leading to approximately equal litter sizes in the two kinds of matings was considered as a possible explanation of the unexpected results. If this were the case there would be difficulties in the use of retrograde analysis aimed at identifying the p^1p^1 mutants. To clarify the discrepancy between the expected and observed values of the litter sizes additional mating experiments were designed to determine if there was any difference in total number of embryos a heterozygote female would deliver when mated to the following males: 1) a wild type male (PP), 2) a pink-eyed male (pp), and 3) a pink-eye lethal heterozygote (p^1p). Additional information concerning order of matings, and variation within males of the strain would also be obtained. In order to use as many mice as possible two experiments were established; one running from October through February and the other from February through July.

For both experiments three types of males were used: 1) BBPP (strain $0112C_3$He b/FeJ), 2) BBpp, and 3) BBp^1p, the two latter groups of males were segregants from an inbred strain. All mice used were approximately the same age. In both experiments it was attempted to randomly distribute the females among the males. In all cases two males were placed in a cage with five to six females. If mice became

sick or died during the experiment, they were not replaced. Each female (p^1p) was mated to each type of male, and her litter size at birth was recorded. Young were removed from female pens as soon as the births were recorded, usually within less than twelve hours after birth. Females were then held separately for at least one week before being introduced to males of the next genotype. After three matings, one to each male, the female was destroyed. All females were at least three months old, and in all except six cases virgins.

In the fall experiment (October-February) a total of 64 females were used. There were 12 cages of males, four for each of the three types of crosses. The pattern of mating was $PP \rightarrow p^1p \rightarrow pp$. Using this system one could determine if there was any effect on the litter size due to mating pattern, since one-third of the females were mated to PP males first, another third to p^1p males first, and the last third to pp males first. One could also determine if a p^1p mating had any effect on subsequent matings.

The spring experiment (February-July) was conducted in the same manner as the fall. New females were used all of which were virgins and at least three months old. Males were used from the first experiment with the addition of at least one new male pair in each genotype group. Five cages of each type of male were set up with five to six females in each cage, for a total of 83 females and 30 males. In this experiment the order of mating was reversed to $PP \rightarrow pp \rightarrow p^1p$. The reversal provided six mating combinations over both experiments.

Initially the data for the two experiments was analyzed separately, but there were not enough mice in the fall or spring experiments to detect differences. The results of the two experiments were pooled.

This was justified because there was no seasonal variation in the phenotypic distribution for the two experiments (Chi-square =0.69, 3df Table 9). The analysis of variance based on the pooled data indicated no significant variation among male cages within a genotype (Table 10). There was a significant difference ($p < 0.001$) in average litter size for the three genotypes. Order of mating was not analyzed in detail but did not appear to have any effect on litter size or variation.

Mean litter size was not significantly different for PP and pp male cages, which indicated that females were carrying an equal number of embryos whether mated to the PP or pp males, and that the males of the strain used in the original experiment probably had no inhibitory effect on the number of embryos that a female of that strain could carry. There was no evidence for replacement of p^1p^1 embryos by normal ones. There was a significant difference ($p < 0.10$) between mean litter sizes for p^1p and pp matings. The hypothesis that p^1p matings had an average litter size 75% of the pp average litter size was accepted ($p > 0.5$).

The results in this analysis indicated that the litters from the heterozygote cross did have a 75% reduction in litter size when compared to control matings. As there was no variation found in the males, the only explanation that can be suggested for the lack of a 25% reduction in the original data was that the recording of the litter sizes and care of the animals was not as carefully done.

TABLE 8

CHI-SQUARE TABLES ON GENOTYPES*

CONTROL EXPERIMENT

Hypothesis: The number of pp mice is equal to the number of p^1p mice

	pp	p^1p	Total
Obs.	38	39	77
Exp.	38.5	38.5	77

$X^2 = 0.012$

$p > .9$

p-LETHAL LITTERS

Hypothesis: The number of p^1p mice is twice the number of pp mice

	pp	p^1p	Total
Obs.	128	257	385
Exp.	128.3	256.7	385

$X^2 = .0023$

$p > .5$

*Original data

TABLE 9

TEST ON SEASONAL VARIATION

Hypothesis: There is no seasonal variation

df=3

Observed Values	PP	p^1p	pp	Total
Fall	436	323	405	1164
Spring	560	406	550	1516
Total	996	729	955	2680

Expected Values				
Fall	432.59	316.63	414.78	1164
Spring	563.41	412.37	540.22	1516
Total	996	729	955	2680

$X^2 = 0.69$

$p > .75$

TABLE 10

MEAN LITTER SIZES

Litter Sizes: mean ± se* (data pooled from spring and fall experiments)

	Cages	Litters
PP		
7.66±0.62	6	130
P^1P		
5.61±0.68	5	130
pp		
7.35±0.68	6	130

*Note se is conservative as it is based on Mean Square for male cages; a value of 0.12 is obtained if the Mean Square 'among cages' is pooled with the 'among female mean square' (see Sokal and Rohlf, 1969).

TABLE 11

ANALYSIS OF VARIANCE (POOLED FOR FALL AND SPRING)

Source	DF	Mean Square	F
Among genotypes	2	167.05	72.42 ($p < .001$)
Among male cages within genotypes	13	2.31	.60 (ns)
Among females within male cages	374	3.86	

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