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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CYTOGENETIC, HISTOLOGIC AND ANATOMIC STUDIES OF HETEROCHROMIA IRIDIS IN THE RESTRICTED RAT

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

ROSEMARY K. HARKINS

Oklahoma City, Oklahoma

1972

CYTOGENETIC, HISTOLOGIC AND ANATOMIC STUDIES OF HETEROCHROMIA IRIDIS IN THE RESTRICTED RAT

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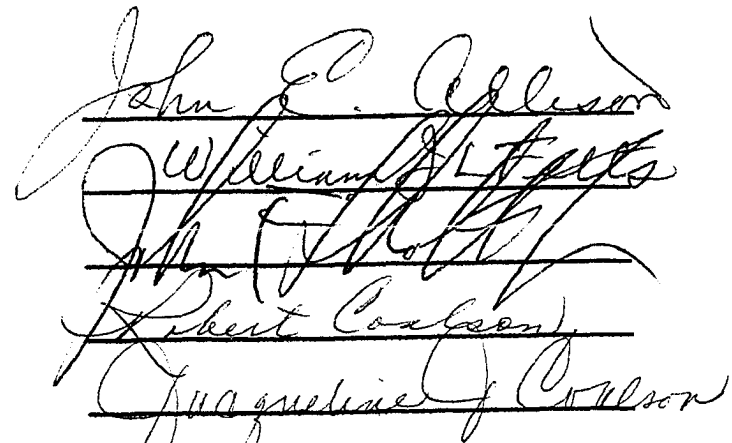

DISSERTATION COMMITTEE

TO MY MOTHER AND FATHER

ACKNOWLEDGMENTS

I wish to thank Dr. John E. Allison for his advice, critical reading of the manuscript, and continual support and encouragement in directing the course of this research.

For his interest and conscientious efforts in behalf of this project, sincere gratitude is expressed to Dr. R. M. Macy who gave many valuable suggestions bearing on the genetics of this problem.

Dr. Allen J. Stanley and Dr. Laurence G. Gumbreck kindly supplied the animals used in this investigation.

Dr. Robert E. Coalson has rendered invaluable assistance with the cytology and photography of the chromosomes.

I am indebted to Dr. William J. L. Felts, Chairman of the Department of Anatomical Sciences, for his cooperation in making available the equipment and facilities of the Department.

I am especially grateful to Dr. John F. Lhotka, Jr., Dr. Ernest Lachman, and Dr. Jacqueline J. Coalson for their encouragement during my graduate studies.

I acknowledge the technical advice of Mr. Thomas M. Sprowl during the histological preparation of the eye material. I wish also to thank Miss Dorothea Lofquist and the Department of Medical Illustration and Photography for photography of the eye material.

I am especially grateful to Mrs. Patricia Friedel for her characteristic accuracy and competent secretarial assistance.

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CHAPTER I

INTRODUCTION

Description of the Sterile Male Rat

A variety of genetic anomalies have been described in the Stanley-Gumbreck colony of King-Holtzman hybrid rats maintained and propagated at the University of Oklahoma Health Sciences Center (Stanley and Gumbreck, 1964; Allison et al., 1965; Allison, 1966; Gumbreck et al., 1967; Stanley et al., 1968; Gumbreck et al., in press).

Within this population of rats a mutant type has arisen presenting a restricted coat color pattern which is present shortly after birth. Gumbreck and associates (1971) propose a new allele of the pattern series in this strain of rats which causes spotting and restriction of the colored areas of the body in hooded, irish and self-colored animals. The new dominant gene has been designated restricted (H^{re}).

In experimental breeding studies, female rats heterozygous for the H^{re}

pattern were mated to normal males homozygous for alleles of the pattern series designated as follows: H/H, self, full color (see Plate I, Fig. 1, in Appendix); h^i/h^i, irish, white belly (see Plate II, Fig. 3, in Appendix); and h/h, hooded (see Plate III, Fig. 5, in Appendix). Depending on the animals involved in the cross, siblings show variable expanses of pigmentation, but all those carrying the H^{re} gene show a restricted area of pigmentation including an area of white or unpigmented hair on the forehead (see Plate I, Fig. 2; Plate II, Fig. 4 and Plate III, Fig. 6, in Appendix). Male rats inheriting this restricted pattern exhibit tubular dysgenesis (Allison et al., 1968) with consequent sterility at or shortly after puberty.

Many of these animals, both male and female, which are also homozygous for the color genes red-eyed yellow and nonagouti, show bicolored eyes or heterochromia. Stanley and his associates (1971) suggest that the heterochromia is an expression of the restricted color pattern.

Matings of restricted females ($H^{re}/+$) to restricted males ($H^{re}/+$) produce 34% homozygous normal (+/+) and 66% heterozygous restricted ($H^{re}/+$) offspring for an approximate ratio of 1:2. When compared to normal litter size, that of the restricted matings is reduced by approximately 26%, suggesting embryonic lethality as far as homozygous restricted (H^{re}/H^{re}) embryos are concerned (Gumbreck et al., 1971).

These recent investigations have tended to emphasize that heterochromia and sterility are pleiotrophic effects of the restricted coat color gene. Since both sterility and heterochromia have been pointed out as being congenital

anomalies, it is interesting to note their coexistence in these animals.

Comparative Incidence of Color Restriction and Heterochromia in Animals

It has been established in lower animals that iris color is closely related to skin color and hair color and that both can be influenced by genetic factors. Davenport and Davenport (1907) first determined that iris color in man is inherited in a Mendelian manner.

According to Mitchell (1935) determination of coat color in the dog is the result of the interaction of allelomorphic pairs of genes. The blue-merle factor of the collie (M) behaves as an autosomal dominant gene of variable expression. In the heterozygous state (Mm) the gene acts to dilute the ground color of the coat. The iris is often affected; heterochromia may be present, or one iris may be multicolored while the other is brown. In the homozygous state (MM) the coat color is almost white and the dog manifests severe eye and ear defects.

Dunn and Mohr (1952) described heterochromia iridis in a strain of mice which showed piebald spotting. They ascribed this variation in eye color and spotting to a combination of two mutant genes. Ophthalmic examination of these piebald mice revealed asymmetry in pupil size ranging from slight enlargement (anisocoria) to complete absence (aniridia).

Sorsby (1954) described heterochromia iridis in dogs with hereditary factors that dilute the color of the hair coat. In a variety of breeds of dogs, heterochromia has been shown to be associated with merle or dappled coat color

which is controlled by an autosomal dominant gene. In addition to merling of the coat color, heterozygous dogs consistently have tapetal abnormalities as well as heterochromia iridis (Lucas, 1954). Mann (1957) stated that in striped animals, the color stripe on the skin may run across the iris.

Kamer (1960) reported similar findings of bicolored eyes in roan and piebald horses, cattle, dogs and cats. Whether heterochromia is actually controlled by the gene for merling or by a separate, closely linked gene has not been conclusively determined and is purely speculative at this time (Burns and Fraser, 1966).

Color Dilution and Hereditary Disease in Man

There are hereditary syndromes in man in which color dilution of the hair and skin is associated with abnormalities of the sense organs. The Chediak-Higashi syndrome (Spencer et al., 1960) and Waardenburg's syndrome (Waardenburg, 1950; 1951) are well-recognized examples of this association.

In 1950, Waardenburg began to associate certain developmental anomalies of the eyelids, eyebrows and nose root with pigmentary defects of the irides and head hair. In 1951, he documented the syndrome and concluded that it is genetically determined and transmitted in an autosomal dominant manner with varying degrees of penetrance of the individual characteristics. The features of Waardenburg's syndrome suggest an anomaly in the neural crests occurring in the third month of gestation (Ray, 1961).

Historical Descriptions of Heterochromia

Heterochromia is a condition characterized by marked differences in the color of the two irides or in the segments of one iris.

Aristotle referred to the condition and called it heteroglaucos. Anastasius, Byzantine emperor in the fifth century, A.D., was called Dicorus, the Greek word for different colored eyes, because of the inequality in the color of his eyes. Plutarch states that Alexander the Great had the anomaly also. Amadeo Modigliani, an Italian artist, depicts heterochromia in his painting of the Polish poet Leopold Zborowski (Duke-Elder, 1964).

The etiology of heterochromia is disputed and remains obscure. It was originally presumed that this pecularity was un jeu de nature often with an hereditary tendency (Duke-Elder, 1964). Jonathan Hutchinson (1867) gave the first scientifically reported cases. Sym (1889), Gunn (1889) and others reported cases in which the condition was accompanied by cataract and cyclitis. Weill (1904) suggested that a circulatory disturbance in fetal life interfered with pigment development and later led to cyclitis. Fuchs (1906) concluded that cyclitis was the essential feature and was due to an unknown toxin which was somehow functional in intra-uterine or infantile life, thereby causing the inflammation. He claimed this to be the only etiologic agent of the phenomenon.

After Fuchs' investigations, heterochromia cyclitis was generally accepted as a clinical entity. Several observers later complicated the issue by associating heterochromia with a derangement of the sympathetic nervous system.

Galezowski (1911), Bistis (1912) and Scalinci (1915) reported clinical cases in which heterochromia was associated with the ptosis, enophthalmos and miosis of a typical Horner's syndrome. In 1918 von Herrenschwand suggested that this type of heterochromia should be classed as a separate entity. Bistis (1928) corroborated this theory stating that heterochromia cyclitis did not exist because the keratic precipitates, the cloudy vitreous and the lenticular disturbance were not cyclitic in nature but were instead the result of a protein-rich aqueous exudate from dilated and abnormal vessels deprived of sympathetic control.

In the opinion of several observers, the etiologic factor was tuberculosis (King, 1927; Lloyd, 1931). Even the sympathetic type has been associated with tuberculosis (Giannantoni and Possenti, 1933). Maternal rubella has also been reported to cause heterochromia.

Types of Heterochromia

Heterochromia may take two forms: a hypopigmentation of the iris with iris hypoplasia or a hyperpigmentation with iris hyperplasia. The degree of involvement of the irides is variable. The color change may involve one eye alone or both eyes and may be either partial or complete. When different parts of the same iris are of different colors, this uniocular type is called partial heterochromia iridis, piebald iris, variegated iris or iris bicolor. If the complete iris ring in one eye is of a different color than that of the other eye, the term complete heterochromia iridis is applied. Binocular heterochromia or heterochromia iridum are the terms applied when both eyes have areas of different colors.

Three main types of heterochromia are admitted: 1) simple heterochromia, 2) sympathetic heterochromia and 3) complicated heterochromia.

Simple Heterochromia

Simple heterochromia, which is usually benign, is the most common type. It is sometimes transmitted as an autosomal dominant trait. It may be a congenital, sporadic or familial alteration in iris color, the etiology of which is not completely resolved. Fuchs (1906) and Streiff (1919) assumed that its occurrence was due to the fact that the two parents had differently colored eyes and that this condition may have been a manifestation of the crossed inheritance of eye pigments. Giblett et al. (1963) cited it as an example of mosaicism, while Mann (1957) cited it as an arrest of local iris development, a view supported by Amalric et al. (1959). It is more common in domestic animals than in man and is strongly hereditary. The phenomenon is found frequently in rabbits, cats, dogs, sheep, pigs and dairy cattle (Pearson et al., 1913; Koby, 1923; Huston et al., 1968).

The abnormal eye shows hypopigmentation associated with hypoplasia of the stromal elements of the iris or hyperpigmentation with its hyperplasia. It may also be associated with a piebald iris in the other eye (Haessler, 1960).

Sympathetic Heterochromia

Sympathetic heterochromia is associated with a neurogenic lesion of the sympathetic nerve supply to the iris. Both sympathetic paralysis and

associated heterochromia may be transmitted as irregular autosomal dominant traits, according to François (1961). Calhoun (1919) reported the occurrence of four cases in one family and Durham (1958) found five cases in two generations.

It has been repeatedly demonstrated that changes in the color of the iris may follow a sympathetic lesion. Angelucci (1893) noted a depigmentation in the uveal tract of dogs and rabbits after cutting the superior cervical ganglion. Calhoun (1919) produced heterochromia by the same method in rabbits. Mayou (1910) reported a similar case after an injury at birth. Lazarescu (1933) reported a case of heterochromia following a bayonet wound in the neck.

In man, if the sympathetic nervous system is intact, the normal iris becomes fully pigmented after birth, but pigmentation is not complete until about two years, although in dark-skinned races it may be completed earlier. Since this occurs at childhood, hypochromic heterochromia occurring in adult life can only arise through depigmentation. For this reason hypochromic heterochromia may be seen with congenital Horner's syndrome but rarely with Horner's syndrome acquired in adult life. In sympathetic heterochromia, the affected iris is always hypochromic and atrophic, so sympathetic heterochromia allows the differentiation of congenital Horner's syndrome from a more recent acute lesion (Walsh, 1957). Congenital Horner's syndrome with associated heterochromia iridum is usually caused by birth injury to the brachial plexus but other causes have been described (Passow, 1933).

According to von Herrenschwand (1924) the architecture and trabeculae of the deeper layers of the iris remain definite and clearly demarcated. Corneal precipitates and lens changes occur in some cases, while in others the eye appears otherwise normal.

It is supposed by some that the characteristic color is directly due to the influence of the sympathetic nervous system on the chromatophores themselves, but the more general opinion is that the primary cause of the anomaly is vascular. It has been experimentally demonstrated that section of the cervical portion of the sympathetic trunk causes vasodilatation with increased permeability of the capillaries of the eye (Bistis, 1928).

Complicated Heterochromia

Complicated heterochromia or the heterochromic cyclitis of Fuchs was first recognized by Lawrence in 1853 but was described by Fuchs in 1906. It is usually associated with ocular disease and is a relatively common condition. It is usually of congenital or early origin (François, 1946; 1954); however, cases may develop later in life. It is almost always unilateral, although bilateral cases have been reported. It occurs ten times more frequently in men than women (Duke-Elder, 1966).

The condition is characterized by an insidious origin and is likely to become obvious only when the typical clinical signs of heterochromia, secondary cataract and glaucoma develop, usually in the third and fourth decades.

There is a loss of chromatophores and the affected iris loses color and

the stromal architecture atrophies as a consequence of connective tissue overgrowth. The pigment epithelium shows through and appears defective and moth-eaten in places. The capillary network of the iris is extremely fragile and susceptible to filiform hemorrhage, particularly after paracentesis of the anterior chamber. Nonpigmented and confluent precipitates are formed on the posterior corneal surface, the endothelium of which becomes edematous. Frequently the cyclitis is associated with cataract formation (Sugar, 1965).

An hereditary element also has been observed in the transmission of complicated heterochromia. Also it appears to be transmitted as an irregular dominant trait. Several pedigrees demonstrating this type of transmission have appeared (Fuchs, 1906; Bistis, 1912; Koby, 1921 and others). Makley (1956) observed the condition in monozygotic twins.

Heterochromia Associated with Other Disorders

Heterochromia iridum has been associated with several other systemic disorders. It is not uncommonly found in patients with Romberg's syndrome often with localized scleroderma in the form of a saber (en coup de sabre). This is usually due to a defect of the first branchial arch (Klingman, 1907; Franceschetti and Maeder, 1958). Simple heterochromia also has been associated with arachnodactaly (Pino et al., 1937) and cutaneous xanthomatosis (Lees, 1957). In association with other ocular defects, heterochromia is usually of a secondary nature.

Simple heterochromia may occur as part of Waardenburg's syndrome

(Ray, 1961; Partington, 1964; Goldberg, 1966). This condition is characterized by hypertelorism, congenital deafness, dystrophia canthorum and punctorum, hypertrichosis and white forelock. Fisch (1959) postulated that the abnormalities found in Waardenburg's syndrome were due to a genetic defect of the neural crests occurring in the third month of gestation.

Heterochromia also may occur in association with status dysraphicus or Bremer's syndrome (Bremer, 1926; 1927). The axial skeletal anomalies are secondary to a failure of proper closure of the neural tube. Georgiades (1956) found frequent anomalies of the spine in heterochromic patients.

The occurrence of different types of heterochromia in the same pedigree has suggested a common etiological factor. Gossage (1908) reported nine cases in one family; van den Breggen (1915) found five cases in three generations and Nowak (1932) found one case in each of three generations. The simple and complicated forms have been seen in different members of the same pedigree (Larmande, 1949). Amalric et al. (1959) reported the simple and sympathetic forms in a father and son.

Mechanisms Determining Eye Color

Melanin Metabolism

Melanin seems to be formed as a biological adaptation for the absorption of radiant energy. It is formed in the cytoplasm of melanocytes as an end product of the enzymatic oxidation of the amino acid tyrosine in the presence of tyrosinase. Tyrosinase is a copper-containing enzyme located in the mitochrondria.

It is generally accepted that the colorless substances of the nature of 3,4-dihydroxyphenylalanine (dopa) are brought by the bloodstream to specific cells in which they are oxidized and turned into colored pigment (Duke-Elder, 1964). A cell which synthesizes pigment in this way is called a melanoblast or chromatoblast. A cell containing the formed pigment is called a melanocyte, melanophore or chromatophore.

Metabolic defects are known to influence definitive color. Certain evidence suggests that pigment dilution is a sign indicating absence of or diminution of certain essential growth factors. These unknown growth factors may be required for normal pigmentation and therefore may be the substrates or enzymes concerned with melanogenesis.

In phenyl-pyruvic oligophrenia, an hereditary disorder of intermediate metabolism in man, there is a defect in the conversion of phenylalanine in the blood. Hypopigmentation of the hair, skin and eyes is a common sign in afflicted infants and has been attributed to inhibition by phenylalanine of melanin formation by tyrosinase (Mason, 1948).

Distribution and Nature of Ocular Pigment

Melanin is found primarily in the pigmented layer of the neural epithelium of the eye, particularly in the retina, ciliary body, iris and in the melanocytes of the uveal tract. Neural crest-derived pigment cells are also typically found in the skin and hair and to a lesser extent in the substantia nigra, pia mater and in association with sympathetic nerves supplying the heart and great vessels,

the gut and mesenteric and retroperitoneal tissue.

Origin and migration of ocular pigment. The grafting experiments of Rawles (1940) showed that the area of the blastula potentially capable of forming pigment cells corresponds exactly with the area capable of forming neural tissue, a potentiality which later becomes restricted to the neural crest. In this regard, the stimulus or lack of stimulus to these tissues that induces abnormal pigmentation in one part of the eye apparently affects corresponding parts of the eye in a similar fashion.

In most vertebrates, the embryonic cells cannot be differentiated morphologically or histologically from the other embryonic cells of the neural crest, but they eventually migrate as nonpigmented amoeboid cells into the regions destined to become pigmented and the pigment ultimately develops when the cells have reached their destination.

The exact pathways of migration are unknown in most cases. But once these cells have reached their destination, the pattern of pigment laid down would appear to depend upon an interaction between the melanoblasts themselves and between their tissue substrates. Like any other embryonic cell the melanoblast carries a complement of genes which would appear to determine its particular fate, while the physiological condition of the substrate may be influenced by numerous environmental factors such as vitamins, temperature or light. One of the most important influences is that of hormonal control, particularly through the melanocyte-stimulating hormone (MSH) of the intermediate

lobe of the pituitary gland (Williams, 1968). Although changes in the physiological condition of the substrate can modify melanoblastic response, including the amount of melanin produced, the specific response is always in accordance with the genetic constitution (Rawles, 1948).

Embryogenesis of melanoblasts. Because the choroid itself is primarily of mesodermal and mesenchymal origin, there has been controversy concerning the origin of melanoblasts of the uveal tract. Experimental data from amphibians, birds, and mammals show that the retinal and choroidal pigments have independent origins, the choroidal melanoblasts originating from the neural crests and the retinal pigment developing in situ in the ectodermal cells of the outer layer of the optic cup (Barden, 1942; Ris, 1941; Brini, 1950; 1953).

Disorders of pigmentation of the iris including albinism, melanosis, melanoma and heterochromia have been attributed to abnormalities of the mesodermal pigment (Mann, 1957). According to Zimmerman (1965) the temporal development and the tendency to undergo reactive neoplastic proliferation are distinctly different in the uveal and the retinal melanocytes.

In view of the demonstration of the nervous origin of the cutaneous melanoblasts in mammals, the hypothesis of a common origin of the melanoblasts of both the skin and the uvea is accepted. Because the uveal melanocytes are richly supplied with sympathetic nerves and because the sympathetic system is derived from the neural crests, this theory is supported by the fact that a sympathetic paralysis alters the characteristics of the uveal melanocytes,

leading to their depigmentation, loss of shape and characteristic processes (Komoto, 1915; Collins and Adolph, 1926). Similarly, in the integument of some lower animals, particularly the frog, the rapid pigmentary transformations are determined by neural influences and also can be readily brought about in the iris of this animal if the nerves to the eye are sectioned (Hogben and Winton, 1922; 1923; Kropp, 1927).

The current consensus is that all the cells in the eye which elaborate melanin are of neural (ectodermal) origin and trace their origin to the embryonic neural crests. The cells of the pigmented epithelium of the retina were originally the cells of the optic vesicle which migrated from the neural tube at an early stage. The "clump cells" of the iris represent a restricted and later migration of these cells into the neighboring mesoblast. The double layer of pigmented cells on the posterior aspect of the iris and the muscles derived from it are of neuroectodermal origin, while the stroma originates from the mesoderm. The epithelium on the anterior aspect of the iris is also considered to be of mesenchymal origin (Bloom and Fawcett, 1962; Mann, 1964).

Types of pigment cells. In the eye, two main types of pigment cells are found. The most characteristic are the chromatophores of the uveal tract. This includes the choroid, the stroma of the ciliary body and the iris. They are characterized as dendritic melanocytes on the basis of their morphology. These are large, flat cells possessing a variety of branched processes frequently forming a syncytium and with granular pigment scattered throughout.

These cells are richly innervated by the sympathetic nervous system and are responsible for the changes in the iris color seen in infants after birth in white races. If the sympathetic innervation is intact, pigmentation of the iris is completed in about two years.

The cells of the second type are designated epithelial melanocytes. They constitute the pigmented epithelium of the retina and of the retinal derivatives, the pars ciliaris and pars iridica retinae. They arise in situ. These are small round cells devoid of processes and in which the pigment is rod-shaped and concentrated near the nucleus.

Post-natal differentiation. At the time of birth melanocytes are present in all of the potentially pigmented sites. Melanin granules are numerous in cells of the pigmented epithelium of the retina and of the outer epithelium of the pars ciliaris and pars iridica retinae. A few small granules are present within cells of the inner epithelium of the pars iridica and only in cells close to the pupillary margin. Dendritic melanocytes are found throughout the uveal tract but are not very numerous and are especially few in number in the choroid, except for the area surrounding the optic disc. Eye pigmentation during the post-natal period includes an increase in the number of pigment cells as well as an increase in pigment content of some cells already present.

In the mouse, the development of eye pigmentation occurs mainly after birth. In the newborn mouse, the pigmented epithelium of the retina is probably completed, but the pars ciliaris and pars iridica retinae are fairly rudimentary

structures. During the first week after birth, the pars iridica increases in size and the pars ciliaris is transformed into a complex structure possessing ciliary processes. Since all of the cells present in the outer layer of the pars iridica and pars ciliaris contain pigment granules, a concomitant increase in epithelial melanocyte number occurs. The spread of pigment granule synthesis into more and more cells of the inner epithelium of the pars iridica also contributes to this increase. Epithelial melanocyte number continues to increase for some time, as indicated by an increasing complexity of the ciliary processes, the growth of the iris and the number of pigment granule-containing cells found in the inner epithelium.

Dendritic melanocytes also show obvious increase during the first week after birth, particularly in the choroid. This increase becomes difficult to detect as the uveal melanocyte number increases and it is likely that the level of dendritic melanocytes does not increase very much beyond that number which is established during the second week of life (Lucas, 1961).

Primary Indications for Chromosome Analysis

Punnett (1911) has stated that unit characters in the zygote are represented by factors in the gamete which behave as indivisible entities in the process of heredity. There are certain facts about the irregular forms of eye color and piebald coat color in animals that suggest that the presence or absence of certain gametic factors are essential in the determination of the behavior of unit characters. These facts suggest that much depends on the

manner in which any factor is present in the gamete which carries it, and on the way in which it incorporates itself with, or is incorporated by the gamete which bears the alternative factor during the process of gametic union.

Complications have arisen in irregular types of chromosomal transmission and have in some cases proven to be the modifying factor introduced by one or the other parent into the zygote which causes an alteration in composition or a rearrangement of the chromosomal material. It could also be responsible for the deficiency of or the intermediary action of restraining factors, but it would be best to leave the final conclusion until all the evidence has been considered.

Chemical Nature of Genetic Material

The analytical methods of Kossel (1928), Levene and Bass (1931), Behrens (1938) and Gulick (1941) have established the polynucleotide structure of the deoxyribonucleic acid (DNA) molecule in association with the chromosome. Gradually, the study of chromosome structure has shifted from a purely cytogenetical level to chemical and histological ones.

According to the model suggested by Watson and Crick (1953a, 1953b) the DNA molecule is of the nature of a double stranded helix composed of two complementary polynucleotide strands connected with each other by hydrogen bonding between purine and pyrimidine bases. The helix may be compared with a ladder in which base pairs form the rungs and phosphoric acid forms the uprights. The arrangement of the four bases is a distinct orderly

pairing between adenine and thymine on the one hand and guanine and cytosine on the other. These base pairs form the genetic code. As such, all hereditary information is stored in this alphabet of four letters. It is not precisely known how many base pairs constitute a functional gene, but there is no doubt that they provide many diversities in form for the control of diverse characters.

The DNA determining and transmitting the genetic code for protein configuration is situated in the nucleus while the ribosomal ribonucleic acid (r-RNA) required for protein synthesis is located in the cytoplasm. Between the two there must be an intermediary to carry the required instructions. The intermediary is messenger RNA (m-RNA) which is synthesized in the nucleus. When m-RNA is synthesized in the nucleus, its base sequence is determined by the base sequence of the DNA. The m-RNA then moves toward the metabolically active cytoplasm. Here the soluble or transfer RNA (t-RNA) becomes attached to the appropriate amino acid when activated by a specific enzyme. The ribosomes, using the m-RNA as a template, ensure that the appropriate protein is synthesized with the characteristic sequence of amino acids.

Gene action. In each cell, the process of coding and transcription is regulated by genes which block the activity of a specific part of the code, but allow the remainder to function, so that cells are produced with the properties characteristic of specialized organs. The genes presumably affect the rate at which genetic information is transferred to the sites in the cell where appropriate proteins are manufactured (Jacob and Monad, 1961).

The nature of the code is probably of great complexity. The suggestion put forward by Wright (1934) was that the essential feature in the process was the determination of the pattern of the enzymes within the cell which control the ordered sequence of chemical reactions. However, Beadle (1945) and Horowitz (1951) claim that a single gene (or cistron) determines the formation of a single specific enzyme which can be formed only if the appropriate gene is present.

If a gene mutation results in the absence of an indispensable enzyme the mutation is lethal; when the enzyme is of less importance, so that development may proceed without it or if its activity is merely impaired, an "inborn error of metabolism" results. Such examples are albinism, which is due to the absence of one of the enzymes required to convert phenylalanine into melanin or Tay-Sach's disease, which is due to the lack of the enzyme necessary to oxidize spingomyelin.

In dominant mutations all offspring carrying the mutant gene possess a new phenotype. In recessive mutation there is no phenotypic effect unless the offspring receives the recessive mutant gene from both parents, or unless in the male offspring the recessive mutant gene is on that portion of the X-chromosome that is unmatched by genes on the Y-chromosome. The classic examples of X-chromosome sex-linkage are color blindness and hemophilia (Stern, 1957; 1959).

If an abnormal genetic balance is present because of any rearrange-

ment or loss of chromosomal material, then gonadal sex may be abnormal. Autosomal genes may, in addition, influence the formation of abnormal gonadal development even in the presence of normal sex genes. The possibility of an abnormal hormonal environment or other environmental factors affecting the primordial gonads to produce abnormal gonadal differentiation must be considered, but these possibilities are probably less likely in the light of mounting experimental evidence.

In the female with two normal X-chromosomes, one of the two is inactivated at random early in embryonic development (Lyon, 1961; Russell, 1961). Once it has occurred in any cell it becomes a fixed property of the chromosomes concerned in each derivative somatic cell line. Recent evidence has accumulated suggesting that genetic inactivity not only affects the presence or absence of sex chromatin, but also other heterochromatic areas not associated with the nucleolus as well. Hsu (1962) noted differential RNA metabolism to occur between the euchromatin and heterochromatin of a mouse cell line in culture. In this cell line DNA-dependent RNA synthesis occurred in the euchromatic regions rather than in the heterochromatic regions. This observation suggests, in agreement with Moore and Barr (1953) that no obvious sex chromatin is seen in interphase nuclei of the mouse because it is obscured by the presence of other heterochromatic regions in the complement. They suggested that clumps of heteropyknotic material, which are variable in size and number, aggregate in interphase nuclei.

On this basis, the interdependence among genes is necessary. Its chemical activity may depend on the product of the action of another gene. Moreover, this relatively simple picture may be complicated by interaction between genes and their environment through "feedback" mechanisms (Potter, 1957).

Determination of genotype by gene assortment. The factors which determine dominance are unknown but presumably concern the effectiveness of the enzyme controlling a particular action in the chemistry of the cell. The fact that enzymes are usually effective in minute quantities probably accounts for the frequence of dominance (Haldane, 1941).

Dominance is not necessarily uniquely characteristic or exclusive. No gene acts in isolation but only as a member of an integrated gene-complex.

An enzyme may catalyze more than one chemical chain of reactions so that a single gene may determine more than one character. Since the gene complex may interact in different ways, it follows that all the characters determined by a gene may not have the same mode of inheritance. The most obvious character for which the gene is responsible may be recessive while a minor character is dominant. Moreover, the dominant trait may not always express itself completely. This is occasionally seen in pedigrees wherein it would appear that the homozygotes show a more marked defect than the heterozygotes so that in such cases the two may be distinguishable.

It was originally supposed by Mendel (1866) that his "factors" acted as

the determinants of the adult characteristics but it seems apparent that these genetic changes are induced only in the early stages of the development of the organism before differentiation has taken place. Waddington (1947) has shown that cellular differentiation is controlled by non-vital chemical substances, probably of the nature of sterols, called "organizers" which are found in the cytoplasm. These vary in the cells of different tissues and since all cells contain the normal complement of chromosomes and genes, it was postulated that these organizers activitate the particular genes which are responsible for determining the particular cellular characteristics of the tissue in question.

The highly complex existence of genes, hypothetical though they are as structural entities, must be admitted and it would seem that they are the determiners which direct development along different paths and so fashion cells and individuals, as well as determine differences between individuals within a species and between species - how an eye becomes an eye and whether it is blue or brown. Occasionally, but periodically, unavoidable chance changes the key molecule giving rise to more dramatic changes, sometimes subtle and sometimes gross.

Chromosome Aberrations

The movements and reactions of chromosomes during cell division are very exact, and in the majority of cases there is orderly segregation. There are, however, occasional deviations from the normal procedure and

aberrant forms and arrangements of chromosomes result.

There has been much speculation regarding the mechanisms of chromosome abnormalities and the exact stage at which these errors arise. Primarily, the errors must be caused by improper separation of the chromosomes at meiosis or mitosis, resulting in daughter cells containing abnormal numbers or forms of chromosomes.

In the animals under investigation several types of chromosome defects are suspected, each of which could conceivably give rise to aberrant phenotypes. These cause either change in the arrangement of the gene loci in the chromosome or a numerical change in the chromosome complement.

Types of Chromosome Defects

Deletion. When a chromosome suffers breakage, either reunion occurs or one of the fragments may be lost through subsequent cell division. It is probable that the effect of deletion of chromosomal material is far more deleterious to subsequent development than is added material (Bridges, 1917).

In man, mutation or deletion of the X or Y chromosome is postulated as the cause of certain congenital gonadal defects in individuals with Klinefelter's syndrome, germinal aplasia, Turner's syndrome, low fertility, congenital anorchia or "pure gonadal dysgenesis" (deGrouchy,1961). Autosomal mutations may result in hypogonadism in certain individuals with non-sex-linked congenital defects (Griboff and Lawrence, 1960).

Inversion. Sturtevant (1921) discovered an inversion of linked

genes. This process is due to a break across the chromosome length with rearrangement of the pieces. This does not result in a loss or gain of genes. If the centromere is not involved in this rearrangement, it is referred to as paracentric inversion. If the centromere is involved, it is called pericentric inversion. Inversions are known to be quite common in Drosophila. Subjects who are heterozygous for a pericentric inversion usually show reduced fertility. Complete synapsis of the homologous chromosomes at meiosis leads to a reversed-loop formation in the region of the inversion. A single chiasma within the loop will produce two abnormal chromatids lacking one end region and carrying the other in duplicate. The presence of a pericentric inversion leads effectively to suppression of crossing over in the inverted region, partly because of incomplete pairing within the loop and partly because of the non-viability of zygotes from crossover gametes with gene duplications and deficiences. The arrangement thus has the effect of holding genes together but the inversion seems to have no detectable effect on the individual carrier. Inversion is detected only at mitosis if the morphology of a chromosome is substantially altered. Several possible instances of inversion in man are now recorded, most of them in normal people (Carr, 1962; Grey et al., 1962; Ellis et al., 1962; Chandra and Hungerford, 1963). Lele et al. (1965) studied the chromosomes in five cases of coloboma and found pericentric inversions of the No. 1 chromosome in two of them.

Translocation. Translocation was first described by Bridges in 1919.

It involves the shift of a segment of a chromosome to a new position in the chromosome complement. Only those translocations which produce morphologically unusual chromosomes can be recognized.

Simple translocation may be defined as fusion of the long arms of two acrocentric chromosomes at or near their centromeres in such a manner as to prevent them from separating during subsequent divisions. Thus the simple translocation carrier has 45 chromosomes. Such individuals are usually phenotypically normal. The affected offspring has 46 chromosomes but is trisomic for one of the two chromosomes involved in the translocation (Lejeune, 1964).

Balanced translocation occurs when two chromosomes are broken, and the resulting pieces are reciprocally exchanged. As a result, no loss or gain of genetic material has occurred and two new hybrid chromosomes are formed. Each contains parts of the two original chromosomes. The unbalanced translocation carrier has 46 chromosomes and is phenotypically normal. The offspring of such a carrier may be 1) chromosomally normal, 2) a balanced translocation carrier, 3) an unbalanced translocation carrier with duplication of chromosomal segment A and deficiency of segment B, or 4) an unbalanced translocation carrier with duplication of B and deficiency of A.

Snell (1933), Hertwig (1940), Koller and Auerbach (1941) and Russell (1952) pioneered work on radiation-induced translocations in the mouse. Snell detected his translocations by the semi-sterility they caused.

He stated that if a mouse of either sex were heterozygous for a translocation, abnormally small litters would be produced when mated to a normal mouse. He reasoned that a translocation zygote forms two types of gametes with euploid and aneuploid chromosome complements, respectively. Snell et al. (1934) and Hertwig both found that about half of the zygotes, presumably from the aneuploid gametes, die during embryonic development. Koller and Auerbach obtained cytological confirmation of this by examining meiotic figures in semi-sterile males. Russell found two stocks in which some of the translocation heterozygotes were semi-sterile and others were completely sterile.

Cattanach (1961) produced an X-autosome translocation in the mouse. The translocation was non-reciprocal; a piece of autosome was inserted into the X but the X was not divided into separate parts.

Searle (1962) also produced an X-autosome translocation during an experiment with XO mice by using a post-fertilization irradiation method. This translocation resulted in a division of the X into separate entities, one part illustrating positive heteropyknosis and one part remaining euchromatic during prophase. A wild-type female heterozygous for the sex-linked coat color marking Tabby (Ta) was produced. Breeding studies showed that a portion of the X carrying the wild-type allele of Tabby (+Ta) had been translocated to an autosome. In its new position (after removal from the X destined to be rendered inactive in genic function) the translocated piece expressed itself fully in the heterozygous female.

Females heterozygous for Tabby normally have variegated coats with a black transverse banding against a background of wild-type fur. After translocation, the animal completely lacked the black transverse banding of the coat. The males carrying this translocation were sterile, although at onset of puberty, some meiotic activity did take place.

A cytological study by Ohno and Cattanach (1962) on Cattanach's translocation in the mouse showed that a piece of an autosome inserted onto the X behaved as an integral part of the X. The normal X-chromosome of the mouse (X^n) is one of the longer members of the complement but is not morphologically distinguishable. However, it has been found that females heterozygous for Cattanach's translocation have an insertion of an autosomal segment into an X, making the translocated chromosome (X^t) clearly the longest chromosome of the set.

A translocation in the mouse is inherited in a quasi-Mendelian manner (Snell, 1935; 1946).

Nondisjunction. Abnormalities in chromosome number arise through errors in the normal chromosome migratory patterns during cell division. Failure of the duplicated chromosomes to separate and migrate to the opposite poles of the cell during either mitosis or meiosis is known as nondisjunction. This process is the most widely accepted explanation of the majority of sex chromosome abnormalities. Bridges (1913; 1916), while conducting genetic studies with Drosophila, first described nondisjunction. He reported an individual whose

sex-chromosome constitution was XXY. Hecht et al. (1963), Penrose (1961) and Smith et al. (1961) have established that in man there is an increasing frequency of occurrence of nondisjunction leading to conditions called trisomy 13, trisomy 17 and trisomy 21 with advancing maternal age.

Developmental anomalies of the eye, ranging from anophthalmia to iris coloboma are found in cases of 13 and 15 trisomy (Smith et al., 1963). Angelman (1961) described four cases of a syndrome of congenital colobomata associated with developmental errors, particularly of the brain and heart. Preliminary investigations pointed to an unspecified chromosome anomaly in one case.

Monosomy is generally considered to be the result of nondisjunction (Polani, 1962) occurring during gametogenesis, or in early cleavage. Normally, chromosome pairs segregate during meiotic division so that daughter cells receive equal chromosome complements. If at the first meiotic division a pair of homologous chromosomes fails to separate, the result is an imbalance in the daughter cells: one has both chromosomes of the pair or pairs involved, whereas the other has neither. This imbalance is carried over to the second division. If the cell containing the excess of chromosomes is fertilized by a normal gamete, the result is a trisomic zygote. If the cell lacking a chromosome is fertilized by a normal gamete, the result is monosomy. Whereas trisomy is compatible with life, monosomy, when it occurs in the autosomes is not (Hirshhorn, 1970). In nondisjunction involving sex chromosomes, ova may

form without an X-chromosome. If fertilized by a normal sperm bearing an X-chromosome, an XO zygote is formed (Ford, 1960). This is the only monosomy in mammals compatible with life. It involves the loss of an entire sex chromosome in all cells. This is the Turner syndrome (Turner, 1938) with 45 chromosomes. Apparently, at least one X-chromosome is necessary to support life since the YO deficiency has not been found and is probably lethal.

In contrast to the autosomes, the addition of an X-chromosome or an X-monosomy produces mild phenotypic effects. This is attributed to the heteropyknosis and the late DNA replication of all X-chromosomes in excess of one at an early period of embryogenesis. It is presumed that late-replicating X-chromosomes are in a highly condensed state in interphase nuclei of somatic cells and have little gene expression. Y-chromosomes show heteropyknosis in early prophase and also have late DNA replication. The addition of extra Y-chromosomes produces no measurable phenotypic effects. The chromosomes or chromosomal segments involved in all viable chromosome abnormalities have been found to have late replicating DNA. It is postulated that these chromosomes or segments are not as deleterious as early replicating ones when present in excess, because late replicating DNA may be an expression of complete or partial gene inactivation (Yunis, 1965).

The XO zygote would be likely to develop into a phenotypic female having gonadal dysgenesis. However, about 5 percent of such embryos abort

spontaneously (Carr, 1965). The XO individual develops a female reproductive system because of the inherent tendency of the fetus to feminize in the absence of masculinizing inductor substances of testicular origin (Jost, 1950).

Mosaicisms involve a mitotic error in the early cleavage division of the zygote, thereby giving rise to individuals possessing two or more stem lines, each with a different chromosome constitution. If in the adult there are more than two cell types, the error would have occurred subsequent to the first cleavage division during embryogenesis. In some cases, one tissue may contain one type only, while other tissues contain an entirely different type or types. In still other instances, the tissues are composed of all of the cell types but the ratios will vary markedly from one tissue to another. Mosaicisms sometimes produce atypical sex chromatin results.

Many theoretical postulations have been proposed for the association of hypopigmentation of the eye and coat color dilution but the pathogenetic mechanisms remain unknown.

The present study is an attempt to investigate hereditary factors that might influence the relationship of sterility, the restricted color pattern and heterochromia iridis in Rattus norvegicus and to compare pigment-forming capabilities in irides of various genotypes within a common stock of animals.

CHAPTER II

MATERIALS AND METHODS

Mitotic Chromosome Preparations from Corneal Epithelium

Experimental Animals

Seventeen King-Holtzman rats from the Stanley-Gumbreck strain were used for this study. Of this group, seven of the animals were sterile males with bicolored eyes (see Plate IV, Fig. 7, in Appendix); five were normal males and five were normal females. All of these animals possessed the genotype H^{re}/h^{i}; r/r; a/a. The normal animals had neither reproductive impairment nor bicolored eyes and were therefore used as controls. In each of the two control groups three of the animals had dark red eyes (see Plate IV, Fig. 8, in Appendix) and two of them had pink eyes (see Plate V, Fig. 9, in Appendix). Littermates were used whenever possible. All animals used were weanlings aged 30-35 days old and weighed 70-85 grams.

Methodology

The procedure for examination of chromosomes from the epithelial cells of the cornea is a slight modification of that used by Fredga (1964). The

method combines hypotonic pre-treatment before fixation in order to spread the chromosomes (Hsu, 1952) and the orcein staining method based on the technique by Tjio and Levan (1954). Preparations were always made in the morning because patterns and peaks of diurnal mitotic cycles fluctuate with the time of day and are influenced by light, environmental temperature and bodily activity. Bullough (1962) and Bertalanffy and Lau (1962) found a high rate of mitotic activity in the corneal epithelium of the rat, particularly in the morning.

Reagents and Stain Used

Reagents. Isotonic saline was prepared by adding 0.9 gm of sodium chloride to 100 ml of distilled water.

A 0.04% colchicine solution was prepared by dissolving 0.2 gm of colchicine[1] in 500 ml of distilled water. The solution was stored at room temperature and warmed to 37° C before use. This mixture allows the eye to be treated with a colchicine and strong hypotonic solution simultaneously (Ford and Hamerton, 1956). Colchicine, by interfering with spindle formation, arrests mitosis at metaphase. The hypotonic solution produces swelling of the cells and spreading of the chromosomes.

The fixative was a mixture of 90 ml of 50% acetic acid and 10 ml of 1N hydrochloric acid.

Stain. The stain was prepared by dissolving 2 gm of synthetic orcein

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[1] K & K Laboratories, Inc., Plainview, New York.

in 100 ml of 50% acetic acid.

Technical Procedure and Slide Preparation

The animals were first anesthetized with ether and then killed by cervical dislocation. All animals were sacrificed during the morning. Immediately after the animals were killed, both eyes were removed using bent forceps and a pair of fine scissors. Care was taken to avoid touching the cornea and to include a piece of the optic nerve. The eyes were placed in a beaker containing isotonic saline (37^0 C) and rinsed briefly to remove any hair or debris that may have accumulated during enucleation. The eyes were then transferred immediately into a beaker of 0.04% colchicine-hypotonic solution warmed to 37^0 C. The beaker was placed in a 37^0 C incubator for 40 minutes after which the eyes were placed in a beaker containing fixative and left for 5 minutes. After removal from the fixative, an entire eye was grasped by a piece of the optic nerve and held cornea-downward into 2% orcein stain for 2 minutes. It was then removed and, using a dissecting needle, pieces of the epithelial layer were scraped from the surface of the cornea into a drop of stain on a precleaned slide.

A No. 1 1/2 coverslip (22 x 40 mm) was placed over this material. The covered slide was placed on four layers of Whatman No. 1 filter paper. These were then folded over so that the slide had four layers under it and four layers on top. The squashing process was begun by using the thumbs to gently press against the top four layers. When stain could no longer be pressed from

underneath the coverslip, a final strong pressure was applied for 1-2 minutes.

Each slide was sealed around the outer edges with fingernail polish to prevent dessication. The slides were examined immediately under the microscope since these were semipermanent preparations. It was possible to obtain three to four slides from each eye. From each slide, the best mitotic figures were chosen. Those exhibiting the least overlapping chromosomes were chosen for photography.

Photography

Photomicrographs were taken under oil immersion with a Baush and Lomb camera on a Leitz Wetzlar Labolux Standard Microscope. Kodak Contrast Process Panchromatic 2 1/4 x 3 1/4 film was exposed using maximum light through a white filter at 2/10th second. The film was developed in Kodak D-11 for four minutes at 68°F, then placed in a 2% acetic acid stop bath for 30 seconds. Next it was immersed in Kodak rapid fix for 5 minutes, followed by 10 minutes in hypo clearing agent and 15 minutes in a water bath. Prints are on single weight F-5 Kodabromide paper.

Meiotic Chromosome Preparations from the Testes

Experimental Animals

Two groups of animals were used for this study. Six sterile males with bicolored eyes of genetic designation H^{re}/h^{i}; r/r; a/a (see Plate IV, Fig. 7, in Appendix) comprised the test group. Six normal males (h^{i}/h^{i}; r/r; a/a, see Plate V, Fig. 10, in Appendix) functioned as the control group. These animals

were 60-90 days old and weighed 170-220 grams.

Methodology

A technique for obtaining satisfactory cytological preparations from mammalian gonads was first developed by Makino and Nishimura in 1952. The method used in this study was originally developed by Ford et al. (1964) for the study of mice testes and was modified by Evans et al. (1964) for examination of the germ cells of other mammalian species. Following hypotonic treatment chromosome spreading is further enhanced by the air-drying technique of Rothfels and Siminovitch (1958).

Reagents and Stain Used

Reagents. The anesthetizing agent was prepared by dissolving 1.6 gm of sodium pentobarbital in 100 ml of distilled water.

The hypotonic solution was prepared by dissolving 1 gm of sodium citrate in 100 ml of distilled water. This solution should be prepared fresh just prior to use.

Carnoy's fixative was made by preparing a solution of three volumes of absolute methanol to one volume of glacial acetic acid.

A 60% solution of acetic acid was prepared by diluting 60 ml of glacial acetic acid to 100 ml with distilled water.

Stain. Lactic-acetic orcein stain was made by adding 2 gm of synthetic orcein to a mixture of 50.0 ml of glacial acetic acid, 42.5 ml of 85% lactic

acid and 7.5 ml of distilled water.

A 45% acetic acid solution prepared by diluting 45 ml of glacial acetic acid to 100 ml with distilled water was used as a rinse following staining.

Technical Procedure and Slide Preparation

The animals whose cells were to be examined were injected intraperitoneally with sodium pentobarbital at a final concentration of 4 mg per 100 gm of body weight. Immobilization took place within 5-10 minutes. A longitudinal incision was made in the abdominal wall. The testes were forced into the abdominal cavity and out the incision. Each was then removed by severing the spermatic cord, and attached vessels and tissue.

After extirpation approximately 100 mg of testicular material was placed in a small petri dish containing fresh 1% sodium citrate solution at room temperature. The ratio of fluid to tissue was kept at about 20:1 throughout the procedure. The testicular tunic was incised and the tubular mass was rinsed in this solution to remove loose cellular debris and any adherent fat. The tubules then were placed into another dish of the citrate and gently teased apart using fine straight forceps in one hand and fine curved forceps in the other, thus allowing adequate penetration of the citrate solution. Vigorous handling at this stage destroys the cells most suitable for study. As the interstitial cellular debris fell into solution the citrate became cloudy and subsequently the tubules were removed to a third 1% sodium citrate solution. Here they were thoroughly but gently dissected apart. The duration of the hypotonic treatment

was found to be critical. The hypotonic treatment through the three changes of citrate solution was eight minutes. Immediately following the hypotonic treatment the tubules, a few at a time, were placed in a screw cap test tube containing Carnoy's fixative. The tubules were refrigerated overnight in the fixative. Ohno et al. (1958) found that extended cold fixation improves the differential staining of chromosomal regions. The following day a small amount of the fixative and a few of the tubules were transferred into a small petri dish. About two inches of the tubular material was separated out and placed in a 12 x 75 mm tube containing 0.5 ml of 60% acetic acid.

As the spermatogenic cells fall into suspension the tubules quickly become transparent. Each tube was tapped lightly with the forefinger to ensure suspension of the cells within the acid solution. Using a disposable Pasteur pipette one drop of the cell suspension was transferred onto a precleaned slide warmed on a hot plate to 60° C. Each drop was quickly withdrawn into the pipette and placed on an adjacent part of the slide. This was repeated with several successive drops on each slide in order to obtain adequate distribution and concentration of cells per slide. The procedure described in the preceding paragraph must be carried out over a period of not more than 5 minutes. Each drop dries rapidly to produce a series of concentric rings in and around which the chromosomal configurations may be found. The cells in suspension begin to disintegrate after about five minutes; however it was possible to prepare 5-10 slides within this period.

After becoming thoroughly dry, the slides were stained with lactic-acetic orcein for 30 minutes. Lactic-acetic orcein gives better staining of this type of material than does acetic orcein. Lactic acid prevents the occurrence of too quick drying and consequent precipitates of stain (Welshons et al., 1962). Excess stain was washed off with three successive changes of 45% acetic acid and the slides were allowed to dry overnight. The slides were then placed in xylol for five minutes and subsequently made into permanent preparations with Permount[2] and a coverslip.

The slides were examined microscopically under high power for the presence of mitotic metaphase of spermatogonia and meiotic metaphase of spermatocytes. When a favorable spread was selected for photography, the coordinates in relation to the microscope stage were recorded to allow future relocation of the same area.

Photography

Photomicrographs were taken by phase-contrast optics using a 50 power oil objective. A Wild MKa4 automatic camera on a Wild M20 microscope was used. Kodak Panatomic-X 135 mm film was exposed using maximum light through a green filter. The film was developed in Kodak Microdol X for 9 minutes at 68° F, then placed in a 2% acetic acid stop bath for 30 seconds. Subsequently, it was immersed in Kodak rapid fix for 5 minutes, followed by

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[2]Fisher Scientific Company, Fair Lawn, New Jersey.

10 minutes in hypo clearing agent and 15 minutes in a water bath. Prints are on single weight F-4 Kodabromide paper.

Enumeration and Construction of the Karyotype

In order to establish a representative karyotype for each type of animal used in the cytogenetic studies, the chromosomes in twenty well-spread and intact metaphase plates from each animal studied were counted under oil immersion by direct microscopic inspection. The number of chromosomes present in most cells became the modal number.

The diploid chromosome number of Rattus norvegicus has been established as 42 (Makino and Hsu, 1954). In the male, the complement is composed of 20 pairs of autosomes and the heteromorphic sex pair. The small Y chromosome, in both meiotic and mitotic metaphase preparations, stands out because of its positive heteropyknosis. The autosomes of the largest pair have prominent constrictions at their subterminal regions whereas several pairs of the medium sized autosomes are divided into two arms of equal or almost equal length by median or submedian constrictions. The remaining autosomes appear to have centromeres at a position extremely close to the proximal end. Some do and some do not exhibit the very short second arms. The X-chromosome resembles other telocentric members of chromosome alignment and therefore can only be recognized as such after the karyogram has been constructed. It appears to be equal in size to the third or fourth largest autosome. The centromere of the X-chromosome

is located extremely close to the proximal end. During mitosis, whether of a somatic cell or of a gonium, all of the 42 chromosomes except the heteropyknotic Y are clearly two chromatid structures and the connection between any two sister chromatids is maintained at the centrometric locus.

Initially each chromosome of the complement in the enlarged photograph of a metaphase plate from each subject involved in the present study was cut out. The homologues of each group were matched according to size and shape in descending order of length. The chromosomes from each subject were then arranged in a karyogram.

In karyotypes in which a chromosome appeared defective, additional cells of the subject were examined under oil immersion. Thus, if the defect of the chromosome in the karyotype in question had been the result of actual alterations in the chromatid, it became apparent.

In each karyogram the total length was derived from measurements of each individual arm of each chromosome made directly from phase photomicrographs. The relative length and arm ration were calculated for each chromosome on each subject. The arm ratio is an expression of the length of the longer arm relative to the shorter one. The reported values for each chromosome number represent a mean adjusted from the lengths of the chromosome pair. This was done in order to allow for variations in contraction state between the different metaphase plates. No attempt was made to allow for bent or twisted chromatids; measurements were straight line point-to-point distances. These indices were

calculated on the basis of the total X-containing haploid set in order to allow direct comparison between sexes.

Gross Anatomic Examination of the Eye

Four types of male animals were used for these studies: 1) H^{re}/h^{i}; r/r; a/a with bicolored eyes (see Plate IV, Fig. 7, in Appendix; 2) H^{re}/h^{i}; r/r; a/a with both eyes dark red (see Plate IV, Fig. 8, in Appendix); 3) H^{re}/h^{i}; r/r; a/a with both eyes pink (see Plate V, Fig. 9, in Appendix) and 4) h^{i}/h^{i}; r/r; a/a with both eyes dark red (see Plate V, Fig. 10, in Appendix). Because of the absorption of light and the manner in which light rays are reflected from the eye, the very dark irides of the H^{re}/h^{i}; R/R; A/A (see Plate II, Fig. 4, in Appendix) and the H/H; R/R; A/A (agouti) (see Plate VI, Fig. 11, in Appendix) were examined with the opthalmoscope but not recorded photographically. Very little detail is apparent on the anterior surfaces. The animals were 60-90 days old and weighed 190-225 grams.

The eye color or pigmentation and ray patterns in the iris of the study subjects were examined first with an ophthalmoscope and later recorded by photographs. These photographs were taken by the Medical Photography Department of the University of Oklahoma Health Sciences Center. The eyes were photographed at 2X and 3X magnification with a Medical Nikkor lens on a Nikon camera.

Histologic Studies of the Iris

Six types of male animals were used for histologic studies:

1) H^{re}/h^{i}; r/r; a/a with bicolored eyes (see Plate IV, Fig. 7, in Appendix); 2) H^{re}/h^{i}; r/r; a/a with both eyes dark red (see Plate IV, Fig. 8, in Appendix); 3) H^{re}/h^{i}; r/r; a/a with both eyes pink (see Plate V, Fig. 9, in Appendix); 4) h^{i}/h^{i}; r/r; a/a with both eyes dark red (see Plate V, Fig. 10, in Appendix); 5) H^{re}/h^{i}; R/R; A/A with both eyes black (see Plate II, Fig. 4, in Appendix) and 6) H/H; R/R; A/A (agouti) with both eyes black (see Plate VI, Fig. 11, in Appendix).

Fixative and Stains Used

Fixative. The solution used for preliminary treatment of the tissues was 10% neutral buffered formalin which was made by placing 2 gm of monobasic sodium phosphate, 3.25 gm of anhydrous dibasic sodium phosphate and 50 ml of 40% formalin in 450 ml of distilled water.

Stains. Harris' hematoxylin was prepared by dissolving 5 gm of hematoxylin crystals in 50 ml of absolute ethanol. One hundred gm of ammonium alum was dissolved in 1000 ml of distilled water with the aid of heat. After removal from the heat, the two solutions were mixed and 2.5 gm of red mercuric oxide was added slowly. The mixture was then simmered until a dark purple color was reached after which it was removed from the heat and placed in an ice bath to cool. Forty ml of glacial acetic acid was added to increase the precision of the nuclear stain. The stain was filtered before use.

Eosin was used as a counterstain. A 1% stock alcoholic solution was prepared by dissolving 1 gm of water soluble eosin Y in 20 ml of distilled

water after which 80 ml of 95% ethanol was added. A working eosin solution was prepared by mixing one part of the stock eosin solution and three parts of 80% ethanol. Just before use 0.5 ml of glacial acetic acid was added to each 100 ml of stain used.

Preparation of Tissue

The respective animals were injected intraperitoneally with sodium pentobarbital and their eyes removed. The eyes were quickly examined grossly for variations in size and for abnormal appearance, then placed immediately into 10% neutral buffered formalin for 48-72 hours. Fixation was carried out at room temperature in at least a 50:1 ratio of fluid to estimated volume of the material.

After adequate fixation, each eye was opened dorsoventrally with a sharp razor blade by removing the two sides of the globe with the blade passing on either side of the optic nerve and through the cornea. The tissue was then held in 60% alcohol until ready to process. The processing schedule began with dehydration through a series of graded alcohols from 80% to 100%. The tissue was then cleared in several changes of chloroform, impregnated with paraffin and finally embedded in paraffin. Each eye was oriented in the paraffin block so that the scleral sides would be parallel to the knife. The tissue was serially mounted on albuminized slides.

Photography

Photomicrographs were taken with a Leitz Ortholux microscope and Aristophot stand using a Leica 35 mm camera by the Medical Photography Department of the University of Oklahoma Health Sciences Center.

CHAPTER III

RESULTS

Chromosome Analysis

Mitosis

In all animals studied, the serial alignment of the chromosomes show 20 pairs of autosomes and the sex pair which consists of a large X and a small Y in the male (see Plates VII, IX, X, XI and XII, in Appendix) and two large X's of equal size in the female (see Plate VIII, in Appendix). Abnormalities of the autosomes such as enlarged satellites, unusual secondary constrictions or deletions were not seen. By counting chromosomes from 340 cells from corneal epithelium and 240 cells from seminiferous tubules containing mitotic metaphase figures, it was determined that the diploid (2n) number is 42 (see Tables 1 and 2). There was no discrepancy of chromosome number found in cell populations from either the corneal epithelium or the gonadal tissue.

Relative length measurements obtained from metaphase plates of corneal epithelium of normal and heterochromic animals and from spermatogonial metaphase figures of normal and sterile testes were compared to data previously published on the rat karyotype. The measurements of Tjio and Levan (1956) are based on material from ten karyotypes of spermatogonial cells of the normal

TABLE 1

DETERMINATION OF MODAL NUMBER

Distribution of Mitoses from Corneal Epithelium

Type of Animal	Total # of Metaphase Cells Counted	Number of Chromosomes Counted									% Hypo-modal	% Hyper-modal	Modal number
		<39	39	40	41	42	43	44	45	>45			
Normal males H^{re}/h^{i}, r/r, a/a Dark red eyes	60		1	1	3	51	2	2			8.3%	6.7%	42
Normal males H^{re}/h^{i}, r/r, a/a Pink eyes	40			1	1	36	2				5.0%	5.0%	42
Normal females H^{re}/h^{i}, r/r, a/a Dark red eyes	60		1	2	2	53	1	1			8.3%	3.3%	42
Normal females H^{re}/h^{i}, r/r, a/a Pink eyes	40	2		1	1	35		0		1	10.0%	2.5%	42
Sterile males H^{re}/h^{i}, r/r, a/a Bicolored eyes	140	1		2	6	125	4	1	1		6.4%	4.3%	42
Total # of mitoses counted	340	3	2	7	13	300	9	4	1	1			42

TABLE 2

DETERMINATION OF MODAL NUMBER

Distribution of Mitoses in Spermatogonia

Type of Animal	Total # of Metaphase Cells Counted	Number of Chromosomes Counted								% Hypo-modal	% Hyper-modal	Modal number
		<39	39	40	41	42	43	44	45 >45			
Normal males h^i/h^i, r/r, a/a	120	3	4	4	5	96	2	2	4	13.3%	6.7%	42
Sterile males (Bicolored eyes) H^{re}/h^i, r/r, a/a	120		4	8		103	2	2	1	10.0%	4.2%	42
Total # of mitoses counted	240	3	8	12	5	199	4	4	5			42

Norway rat. Nowell et al. (1963) published data obtained from chromosome analysis of six karyotypes in Norway rats with primary granulocytic leukemia. Vrba (1963) studied chromosomes of twenty-two metaphase plates of bone marrow cells in Rattus norvegicus. Our results are based on twenty-nine karyotypes and indicate that there is similarity of relative lengths between the morphological groups and homologous chromosomes from cell to cell. No significant differences were detected between the various animals studied. Table 3 permits a quantitative comparison of our results with those of other investigators.

The arm ratio indices show a range of variation and indicate that interchange of homologous chromosomes within groups not showing great differences in length is highly probable (see Table 4).

Meiosis

All figures studied in first meiotic metaphase demonstrated 20 autosomal bivalents and the X and Y chromosomes in end-to-end association. Autosomes are paired in the usual manner and the formation of chiasmata is seen in the cells which are at diakinesis. The results of the meiotic pairing studies are consistent with those from cytological observations of mitosis. With the methods used, there was no indication of translocation of other gross chromosomal anomalies.

The heavily condensed sex chromosome pair seen in the meiotic metaphase contrasts sharply with the extended isopyknotic autosomes. Their consistent terminal association is an outstanding feature. The X and Y chromosome

TABLE 3

RELATIVE LENGTH DISTRIBUTION OF CHROMOSOME PAIRS

Chromosome Pair Number	(29 Cells) Normal Cornea	Full Color Cornea	Non-pigmented Cells	Normal Testes	Sterile Testes	(6 Cells) Tjio & Levan 1956	(10 Cells) Nowell et al. 1963 Measurement 1	2	(22 Cells) Vrba 1963 Measurement 1	2
1	9.0	9.5	10.0	9.2	9.3	9.3	9.9	10.3	9.9	9.9
2	8.2	8.3	8.8	8.3	8.2	8.3	8.4	8.7	8.3	8.1
3	6.3	6.7	6.9	6.5	6.3	6.5	6.0	6.1	6.1	5.9
4	6.2	6.6	5.8	5.6	5.8	6.5	6.9	7.0	6.8	6.7
*X	5.0/4.8	5.2	5.3	5.4	5.3	6.0	6.2	6.2	5.5	5.4
5	5.5	5.8	5.8	5.4	5.5	5.6	5.5	5.3	5.3	5.1
6	5.2	5.2	5.1	4.6	4.9	5.3	4.8	4.8	4.7	4.6
7	4.8	4.8	4.7	5.0	4.8	5.0	4.6	4.5	4.2	4.2
8	4.6	4.4	4.5	4.6	4.6	4.8	4.3	4.3	4.1	3.9
9	4.5	4.1	4.3	4.4	4.0	4.6	4.0	3.9	3.8	3.7
10	4.2	4.2	4.0	4.0	4.0	4.4	3.8	3.7	3.4	3.5
11	4.1	4.0	3.7	3.7	3.8	4.3	4.4	4.4	4.6	4.6
12	4.0	3.5	3.7	3.8	3.8	4.1	4.3	4.2	4.4	4.5
13	4.2	4.1	4.0	4.3	4.1	3.9	4.0	4.0	4.2	4.3
14	4.0	3.9	4.1	4.4	4.1	3.9	3.7	3.8	3.9	4.1
15	4.0	3.7	3.9	3.8	4.0	3.6	3.7	3.7	3.8	4.0

TABLE 3 continued

RELATIVE LENGTH DISTRIBUTION OF CHROMOSOME PAIRS

Chromosome Pair Number	(29 Cells) Normal Cornea	Full Color Cornea	Non-pigmented Cells	Normal Testes	Sterile Testes	(6 Cells) Tjio & Levan 1956	(10 Cells) Nowell et al. 1963 Measurement 1	2	(22 Cells) Vrba 1963 Measurement 1	2
16	3.9	3.6	3.7	3.9	3.9	3.3	3.5	3.5	3.6	3.8
17	3.7	3.5	3.4	3.7	3.8	2.8	3.3	3.3	3.5	3.7
18	3.3	3.2	3.0	3.6	3.3	2.8	3.0	3.1	3.3	3.5
19	3.0	2.9	2.8	3.0	3.4	2.6	2.7	2.8	3.1	3.2
20	2.7	2.6	2.3	2.9	3.1	2.4	2.6	2.5	2.9	2.9
Y	2.6	2.7	2.5	3.9	2.9	2.1	2.4	2.4	2.9	2.9

Chromosome length expressed in percent of total length of X-containing haploid set.

*Represents two X-chromosomes from female rat.

TABLE 4

COMPARATIVE ARM RATIO DISTRIBUTION OF CHROMOSOME PAIRS

	(29 Cells)				
Chromosome Pair Number	Normal Cornea	Full Color Cornea	Non-pigmented Cornea	Normal Testes	Sterile Testes
1	5.6	5.5	5.6	4.1	5.3
2	10.1	12.6	11.6	10.7	16.5
3	8.1	9.3	9.6	4.0	6.8
4	9.1	8.0	11.8	7.2	11.1
*X	6.5/7.8	9.5	10.9	8.7	9.3
5	10.5	12.1	12.4	7.9	9.6
6	10.9	11.6	13.3	5.9	9.9
7	8.0	12.5	11.5	5.8	8.9
8	7.5	10.7	11.4	5.7	7.6
9	8.2	11.0	9.1	6.3	7.3
10	7.1	9.1	7.4	4.9	7.1
11	6.5	5.4	5.0	4.5	6.1
12	4.7	6.6	5.4	2.1	3.4
13	1.5	1.7	1.3	1.5	1.4
14	1.3	1.3	1.5	1.2	1.4
15	1.3	1.6	1.4	1.4	1.3
16	1.2	1.2	1.2	1.2	1.7
17	1.4	1.4	1.3	1.3	1.5
18	1.6	1.6	1.3	1.2	1.5
19	1.4	1.5	1.5	1.3	1.3
20	1.5	1.5	1.3	1.3	1.2
Y	4.3	3.3	4.4	3.1	3.6

pair was always in close proximity to one of the smaller autosomal bivalents (see Plate XIII, in Appendix).

Gross Anatomic Examination of the Eye

Ophthalmoscopic examination revealed that the pupils of all animals studied were regularly rounded and that they responded normally to changes in light intensity. All of the animals readily found the opening to their watering device, never bumped into the side of their cage, and always jumped back to the cage instead of to one side or the other when they were held slightly above and to one side. They also blinked when an object was rapidly passed close to their eyes. Thus, their visual acuity seemed unimpaired. None of the animals exhibited anisocoria or aniridia, such as that found in the house mouse (Dunn and Mohr, 1952).

Animals carrying the restricted gene alone (H^{re}) without association of r/r; a/a (see Plate II, Fig. 4, in Appendix) as well as agouti animals (see Plate VI, Fig. 11, in Appendix) presented extremely deeply pigmented irises. The pupils appeared black. The pigment in both eyes of both animals was evenly distributed. The color was so intense as to make the vasculature completely obscure.

Animals carrying the r/r and a/a genes only without association of the restricted gene (see Plate V, Fig. 10, in Appendix) also showed no sectoring in the iris pattern (see Plate XIV, Fig. 20, in Appendix). The pigment was not as concentrated however as that found in the two types of animals

just described and appeared to be very dark red rather than black.

In the pink or nonpigmented eyes of the heterochromic and non-heterochromic animals carrying a combination of r, a, and H^{re} genes (see Plate IV, Fig. 7, and Plate V, Fig. 9, in Appendix), the iridal vasculature is obvious (see Plate XIV, Fig. 21, in Appendix). The dark red eye of the heterochromic animal as well as the full-colored eyes of the non-affected animals carrying the combined genes (see Plate IV, Fig. 7, and Plate IV, Fig. 8, in Appendix) exhibit irregular pigmentation of the iris in many but not all cases examined. The asymmetry or sectoring of pigmentation in these animals, which can be seen only under magnification, was not always in the same quadrant of the iris. Several different patterns were noted. In some, the involved area is more or less triangular in shape widening laterally as it extends from the margin of the pupil to the periphery (see Plate XV, Figs. 22 and 23, in Appendix). In other animals the pigment was distributed throughout the iris in islands of irregular size and shape, so that the entire iris surface presented a mottled appearance (see Plate XVI, Fig. 24, in Appendix). In others, a fringe of deeper pigmentation was found to be confined in a ring-like pattern around the pupil (see Plate XVI, Fig. 25, in Appendix). These pigment distribution patterns in H^{re}/h^{i}; r/r; a/a animals appear to be a random phenomenon so that there is no proclivity for a specific area of the iris to be involved. No other gross eye defects were noted. Thirty day-old weanlings exhibited the same pigment patterns as have been described for adults.

In the rats with bicolored eyes, the condition does not manifest itself on one side in preference to the other or in one sex more than the other (Gumbreck et al., in press). However, it occurs only in animals which have a very light pelage pattern. Since the coat hairs are so lightly colored it is difficult or impossible to determine the pattern of coat pigmentation in the animals showing heterochromia.

Histologic Studies of the Iris

All histological sections may be referred to Plate XVII, Figs. 26 and 27, in Appendix for more specific localization of the areas illustrated.

Morphology of the Full-Color Iris

In the full-color iris of the restricted animal with both eyes dark red (H^{re}/h^{i}; r/r; a/a) and in the irish animal (h^{i}/h^{i}; r/r; a/a), many of the basic histological characteristics are the same.

In man and certain other animals, the anterior surface of the iris is classically described as being covered by a layer of inconspicuous squamous endothelial cells (Wolff, 1968); however, no specialized surface endothelium could be identified in any of the rats. The existence of this endothelium is controversial. Many other investigators accept only the presence of an anterior limiting membrane and define it as the anterior border layer (Kestenbaum, 1963). Beneath this limiting border is the dense anterior part of the stroma, containing many pigment cells. Deep to this, the remainder of the stroma forms the greater

part of the thickness of the iris (see Plate XVIII, Fig. 28, in Appendix). Chromatophores are scattered throughout and are the major structural constituents. Their cyton and processes contain aggregates of pigment granules. The numerous processes of any one pigment cell touch the cell bodies and interdigitate with the processes of adjacent pigment cells of the anterior epithelial layer. Nuclear morphology of the cells in this area is obscured by the heavy deposition of pigment and the cell boundaries cannot be clearly delineated (see Plate XVIII, Fig. 29, in Appendix).

The dilator muscle is not noticeable in many areas because of the overlying melanocytes. The sphincter muscle is particularly prominent in the stroma near the pupillary margin. It consists of longitudinal bundles of smooth muscle cells.

The posterior surface of the iris is covered by a double layer of cuboidal epithelial cells containing granules of a finer calibre than those found in the anterior layer of the stroma (see Plate XIX, Fig. 30, in Appendix). This layer is directly continuous with the pigmented epithelium of the ciliary processes and the pigment layer of the retina.

A striking feature of the iris stroma in the animal carrying r/r; a/a without association of the restricted gene is the presence of so-called "clump cells" in the stroma (see Plate XIX, Fig. 31, in Appendix). These cells, also pigmented, are rounded and lack protoplasmic processes. The pigment appears to be concentrated near the nucleus. They are found in the

area of the sphincter muscle and throughout the stromal element toward the ciliary border. They are not seen in the posterior epithelial area (see Plate XX, Fig. 32, in Appendix)..

The iris of the agouti animal (H/H; R/R; A/A) is thick and densely pigmented. Chromatophores and round pigment cells or "clump cells" form a profuse syncytium and are particularly prominent throughout the stroma from the pupillary to the ciliary margin. The posterior epithelium can be differentiated, but is not sharply demarcated from the stroma. It is dark-brown to black in appearance and otherwise shows no striking deviations in structure (see Plate XX, Fig. 33, in Appendix).

The iris of the animal carrying the restricted gene (H^{re}/h^{i}; R/R; a/a) contains the greatest amount of pigment of all the animals studied. The stroma is quite dense and is remarkable for the large number of pigment granules which appear in section as large, irregular structures. There is very little intercellular space and blood vessels are nearly invisible. Behind the iris stroma the posterior pigmented epithelium is extremely heavily pigmented and nontransparent (see Plate XXI, Fig. 34, in Appendix). The degree of pigmentation in the iris is consistent with that of the pigmented portions of the ciliary epithelium, choroid and retina.

Morphology of the Nonpigmented Iris

The anterior epithelial layer and stroma in the nonpigmented iris are noticeably devoid of pigment (see Plate XXI, Fig. 35, in Appendix). The

stroma is made up of cells resembling fibroblasts and have been termed amelanotic melanocytes in the albinoid iris (Silvers, 1958; Takeuchi, 1964). Dendritic melanocytes and "clump cells" are not present. Blood vessels and intercellular spaces are especially prominent (see Plate XXII, Fig. 36, in Appendix). The bulk of the iris is made up of blood vessels held by a loose stroma of collagenous fibers and cells. The posterior epithelial layer is poorly pigmented but in the region of the angle of the anterior chamber the amount of pigment increases slightly so that there is some pigment present in the epithelium of the ciliary processes (see Plate XXII, Fig. 37, in Appendix). Beyond the ora serrata the retinal epithelium is pigmented. The pink color of the nonpigmented iris is obviously due not only to the decreased deposition of pigment but also to exposed blood vessels on its anterior surface.

The dilator muscle consists of a few layers of elongated cells, loosely interdigitated. It is closely applied to the adjacent posterior pigmented epithelium and frequently contains pigment granules. The sphincter muscle shows no deviations from that described in the full-color iris (see Plate XXIII, Fig. 38, in Appendix).

Morphology of the Irides in the Heterochromic Animal

The morphologic characteristics of each eye of the heterochromic animal were compared to those of the nonaffected eyes in the control animals of the same stock. In the light eye of the heterochromic animals the histologic characteristics

of the iris, ciliary processes and choroid are comparable to that in the restricted animal with unpigmented irides.

The anterior epithelium and stroma of the contralateral eye are similar to that described for the full-color eyes of the nonheterochromic H^{re}/h^{i}; r/r; a/a animals. Histologic examination failed to reveal differences or alterations in structure of the posterior pigmented epithelium in either eye when compared to those from nonaffected animals.

CHAPTER IV

DISCUSSION

In a strain of Norway rats exhibiting heterochromia iridis, the irregular pigmentation of the irides is congenital and in the male rat, so affected, is associated with sterility (Stanley et al., 1971). Sterility in other mammals is often associated with discrepancies in eye and coat color (Bergsma and Brown, 1971). A similar situation involving coat color appears in mice exhibiting X-autosome translocation (Russell and Montgomery, 1969). Occasionally heritable anomalies arise because a piece of the chromosome carrying the gene or genes involved become translocated to another chromosome in the metaphase plate. Koller (1944) showed that translocation could be detected in the male mouse at the late prophase or during metaphase of the first meiotic division. Since the characteristics of the male rats being studied resemble some of those caused by the X-autosomal translocation in mice and because of Koller's results, it seemed logical to look for aberrations in their germ and somatic cells.

If in the formation of the metaphase plate of the first meiotic prophase, two tetrads come into close association (contact), the chance for translocation of parts between the two chromosomal units involved is enhanced. A configuration wherein this is possible may be identified as a ring or chain of four dyads. If

the larger chromosomes of the complement are involved the quadrivalent is easily identified. If very small chromosomes are involved, the chain could easily be confused with a normal bivalent and the presence of a translocation must be determined by a reduction in count of bivalents. An exchange of segments of equal length will remain unnoticed. Transposition of chromosomal material to another site within the nucleus does not alter the total gene dosage of the affected cell but still may result in an alteration of the characteristics of individuals carrying cells so affected.

A cell-to-cell variation in chromosome number is said to occur normally in some mammalian tissue (Beatty, 1954). The variation can be either hyperdiploid or hypodiploid or some of both. Tanaka (1953) recorded a moderate degree of hypodiploid and hyperdiploid inconsistancy in various tissues of the embryo rat. Sachs (1953) and Boothroyd and Walker (1954) showed that extensive hypodiploid variation does not occur in the corneal epithelium of the mouse. They found that the mean value of hypodiploid cells counted in 340 cells from the corneal epithelium of the rat was 6.7%. In the cornea, cell division occurs mainly in the basal layer (Arey and Covode, 1943). Beatty (1954) also indicated that the basal layer in corneal epithelium and the few outer layers of more flattened cells are its specialized cells. He suggested that this so-called germinal epithelium, i.e. the restricted zones of proliferation from which the specialized cells of an organ develop may be regularly diploid, and the inconstancy of chromosome number is to be sought among the differen-

tiated cells arising from this epithelium. In the squash technique used in this study the outer layers of the corneal epithelium were utilized and the modal number of chromosomes was found to be 42.

According to Vrba (1963), similarities or variations in relative length of chromosomes depend not only upon the nature of the biological specimen examined but also upon the method used for making chromosome preparations. It is now recognized that differences in arm length and arm ratio between two homologous chromosomes occur rather frequently, but are not linked to any form of pathology (Court-Brown et al., 1966). The measurements which have been made were directed at determining whether any aberrations exist in the spermatogonial metaphase and/or the somatic metaphase figures of the sterile male rats. Hirschhorn (1964) illustrated that the relative sizes of the chromosomes alter according to the stage of metaphase in the cell which is selected for study. The largest chromosomes may contract more in later stages than the smaller ones. Thus any mean value must not be considered merely as an average for the particular population of cell division selected for convenience. In the present study, the relative lengths and arm ratios emphasize that there are no essential differences in structure between the metaphase cells of the corneal epithelium or gonadal tissue. Differences large enough to be significant are also large enough to be recognized without measurements; however, length measurements were done in order that chromosome identification would be on a more objective basis.

Variegated spotting in some cases is due to color mosaicism. This type of mosaicism can be explained on the basis of autosome to X translocation. Lyon (1961; 1962) advanced the theory that one X-chromosome in each cell is genetically active and the other X is genetically inactive or partially inactive. The postulate states that the decision as to which of a pair of X-chromosomes is to be inactivated is made early in embryonic life and that the descendents of each X-chromosome will be like the parent chromosome. The original inactivation in each cell occurs at random, so that in some cells a paternal and in others a maternal X-chromosome will be inactiviated. Females heterozygous for pigment genes linked to the X-chromosome or for pigment genes located on autosomal segments translocated to the X-chromosome exhibit phenotypic expression of different degrees of mosaicism. Robinson (1957) theorized that animals presenting a variegated coat color are the result of mosaicism which expresses itself by gene mutation, gross chromosome change or cellular dynamics. The mosaics may be somatic, somatic-gonadal or gonadal in type. Presumptive evidence for mosaicism would lie in a significant discrepancy within the chromosome complement in the various types of animals karyotyped. Using this criterion, mosaicism could not be demonstrated in any of the cells of the restricted or bicolored rats and no abnormalities were seen in the X or Y chromosomes. Also, abnormalities of the autosomes, such as enlarged satellites, unusual secondary constrictions, translocations, deletions or inversions were not seen. The chromosomes were shown to pair normally at meiosis. Evidence accumulated

with the methods used in these cytogenetic studies indicate that apparently a gross chromosomal abnormality plays no role in the causation of heterochromia iridis in the Norway rat.

The fundamental cause of morbidity and the lethal state that has been observed in animals homozygous for color restriction is poorly understood. It has been found that the homozygous embryos have severe macrocytic anemia (Peeples and Gumbreck, 1971).

Hurst (1908) described spotted iris patterns in human species and concluded from his studies that genetic factors which control eye color operated in both eyes equally, i.e., they are bilateral or bi-iridal in action. The results of this present study indicate that these genetic factors appear to control independently different areas of the body. Spotting does not occur regularly in both eyes in the same animal but may affect one eye only. It may affect the entire or only a portion of the same iris with the remainder of the iris showing even distribution of pigmentation. Unilateral manifestation of heterochromia iridis is not readily explained.

Russell (1948) established that dilute hairs in adult mice contain as many granules as do nondilute. He found that dilution in pigment was related to the clumping of the individual granules. These clumps originate in the follicular melanocytes which are morphologically abnormal in that they lack dendritic processes.

In rabbits, Heydenrich (1958) showed that injuries to the iris produce

a grossly and microscopically visible depigmentation of the stroma. This is characterized first by a transformation of chromatophores in the anterior portion of the iris into the so-called "clump cells". An increase in pigmentation two to three weeks after the injury is the result of migration of chromatophores from the center of the lesion to tissue at its periphery. This often resulted in local hyperchromia. The remaining "clump cells" are surrounded and overgrown by the new chromatophores. Heydenrich pointed out that in human eyes the "clump cells" stem not so much from the chromatophores of the iris as from its pigment epithelium. The migration of chromatophores in the anterior iris may be especially stimulated in some cases by traumas associated with inflammation such as a perforating injury to the crystalline lenses.

Our histological studies have shown that "clump cells" are particularly prominent in animals which carry r/r; a/a and lack H^{re} but not in animals carrying H^{re} and r/r; a/a. The r/r; a/a seems to be a less intense gene than the H^{re}. In contrast to the action of H^{re}, the r/r; a/a genotype reduces the level of pigmentation in the rat as a whole. There is increased clarity of dilution when the H^{re} gene is combined with r/r; a/a. The anomalous hypopigmentation of body hair appears to be a component part of the condition. The combined effect of H^{re} and r/r; a/a not only dilutes the quantity of pigmentation but also restricts the presence of the "clump cells". It dilutes the color of the entire iris or part of it. The degree of penetrance or expressivity is not predictable. The structure of the developing iris is affected by alteration of pigment distribution and inter-

ference with the appearance of melanocytes in the epithelial components of the eye. The reduction of pigment in these tissues may be explained on the basis of pattern modifier genes which act to limit the distribution of pigment-bearing epithelial components in the full-colored animal (Macy, 1971, Personal communcation).

The morphological basis of pigment accumulation is found in the differentiation of melanoblasts into melanocytes and the continued production of pigment granules in the melanocytes. The relationship which exists between the extent to which the epithelial melanocyte number is reduced and the degree of interference with pigment accumulation is indicated in photomicrographs in the Appendix.

Chase (1958) and Chase et al. (1963) suggest that radiation-induced greying or depigmentation of the coat in the mouse is due to the inability of follicular melanocytes to deposit pigment in the hair cells. He also found that irradiation during the first eight days after birth inhibits pigment accumulation in the eye. This inhibition is associated with a reduction in epithelial melanocyte number and under certain conditions with interference of pigment granule synthesis in certain epithelial melanocytes of the ciliary-iris region. In extreme color dilution, the neuroepithelium of these animals is also affected. In this case, it appears that aberrant pigment changes in the light-colored eye of bicolored animals is influenced by the amount or quality of pigment received by the prospective melanocyte in the tissues concerned.

It has been postulated that a mutant gene causing restriction of pigment may express its influence at any stage from the time that melanoblasts differentiate from the neural crest to the time of final differentiation of melanoblast to melanocyte. This is true whether it be found within the hair follicle, in the skin or in the iris. Theoretically then, the melanocytes in white spotted areas are either absent, undifferentiated or abnormally differentiated. Billingham and Silvers (1960) and Silvers (1961) list three possible reasons for the absence of melanoblasts from a white spot. These are 1) a neural crest defect, such as might result from a disturbance of the neural crest and which would therefore interfere with the differentiation of its cells, 2) a migratory defect which would result in the melanoblast not reaching all or some areas of the embryo or 3) failure of potentially pigmented cells to survive in the "spotted environment". The absence of pigmentation would not necessarily mean that melanoblasts were unable to reach a given area, but rather that conditions for melanogenesis were unfavorable in that region. Markert and Silvers (1956) and Silvers (1957) studied several hybrid genotypes and suggested that failure of melanoblast differentiation is due to latent factors within the tissue environment and therefore to varying cellular environments among different tissues at some early stage in the development of mutant spotting genes.

It is known that white spotting in the skin is influenced by various modifier genes (Charles, 1938). In analyzing the action of white spotting

genes in the hooded rat, Rawles (1955) exchanged skin grafts between potentially pigmented areas and potentially spotted regions just after birth. Melanoblasts from the adjacent pigmented skin migrated into the spotted skin graft where pigment was formed in the developing hair. This gave clear evidence that the region could suport melanogenesis. Mayer and Reams (1962) state that although the initial migration of melanoblasts into all major regions of the mouse embryo is completed by the end of the 12th day of development, a critical and transitory period affecting melanoblast maintenance in the spotted areas may occur shortly after this time. Further studies by Mayer (1967) confirm this earlier speculation and he found that the factor preventing melanoblast differentiation may play a role in the development of the piebald spotting pattern. Spotting and iris defects are closely associated and the hypothesis that they are due to the action of a spotting gene or spotting genes cannot be excluded.

Many investigators claim that all melanin pigment is formed by ectodermal cells, while others feel that melanocytes have two embryonic origins - the neural crest and the wall of the optic cup. Our studies show heterochromia iridis to be due to a defect in the mesodermal portion of the iris. Animals with this iridal defect demonstrate an absence of pigment granules in the stroma and the anterior pigmented layer of the iris. Cells containing some immature pigment granules are present in the posterior epithelial layer. There is a gradual increase in the number of melanocytes in the region of the ciliary processes so

that the remainder of the uveal tract shows definite pigmentation. This observation favors the theory that nondendritic melanocytes are found only in retinal pigment epithelium and arise from a source different from that of the dendritic type which are usually found in hair, skin, irides and connective tissue of other organs. Failure to demonstrate melanocytes in color-dilute irides also suggests or infers prenatal interruption of maturation of the melanocytes. In animals showing segmental distribution of pigment, the ray of pigment appears to be the result of the ingrowth of mesoblastic tissue which eventually becomes pigmented with subsequent spreading out around the globe of this tissue in a somewhat diffuse radial manner to form the iris stroma.

The etiology and pathogenesis of heterochromia iridis remain unsettled. However, it appears that irregular pigmentation of the iris is congenital. Whether the variable phenotypic expressions are controlled or influenced by a gene for merling, multiple alleles, multiple foci for genes or a pattern modifier gene has not been determined. If, indeed, all pigment cells are of ectodermal origin and normally migrate to their final location, failure of this migration does not seem probable as the sole mechanism in extreme color dilution. If pattern modifier genes are involved they may cause an enzymatic defect involving clones of cells in the developing embryo or in postnatal development. This is assumed because of the mild to moderate defects found in the animals which carry the combined genes. The site of action of the hereditary factors for pigment restriction may be in the substrates or enzymes concerned with melano-

genesis. Thus, such a deficiency in the region under study might produce a localized effect by reducing pigmentation in a specific area. It is difficult to correlate and trace a pathway between primary gene product and effect in animals whose phenotype is a function of the melanocyte. It is also hard to determine which of the mechanisms is responsible for variations in iridal components. The information and evidence available on the pattern formation remains largely speculative and indirect. Our data does not permit a decision between these alternatives to explain any developmental phenomenon.

CHAPTER V

SUMMARY

A dominant allele of the pattern series (H^{re}) of rats causes restriction of the colored areas of the body in hooded, irish and self-colored animals (Gumbreck et al., 1971). Male animals inheriting this restricted pattern exhibit tubular dysgenesis (Stanley et al., 1968) with consequent sterility at or shortly after puberty. The H^{re} animals, both male and female, which are also homozygous for the red-eyed yellow and nonagouti genes (r/r; a/a) show a high incidence of heterochromia.

The present study deals with several factors which bear on the hereditary aspect of heterochromia iridis. In order to determine if the mosaicism is due to a gross chromosomal change, chromosome analysis was performed on corneal epithelium and testicular tissue. Counts obtained from corneal and spermatogonial mitoses indicate that the diploid number is 42 in all animals studied. With methods employed, no physical alterations between either meiotic or mitotic chromosomes from normal and sterile animals were detected. Also, there are no essential differences in chromosomal structure of one of a pair of bicolored eyes as opposed to the other.

Ophthalmoscopic examination of the eyes of rats carrying both the

H^{re} and the r/r; a/a genes reveal dehiscences of pigmentation in the full-color eye. This iridal defect varies in individuals and the irregular pigment distribution pattern is not predictable. The spotted or ray patterns are not seen in either the control animals with bilateral albinoid irides or in the unpigmented eyes of the bicolored animals. No other gross eye defects were noted in either of the animals.

Histologic studies show similarities of the irides of the restricted (H^{re}/h^{i}; r/r; a/a) and normally colored (h^{i}/h^{i}; r/r; a/a) animals each exhibiting dark red eyes; however because the effect of the r/r; a/a genes is less intense than that of the H^{re}, "clump cells" are strikingly evident in the connective tissue stroma of the irides of the unrestricted animal.

In the nonpigmented eye there is a marked deficiency of pigment granules over the anterior surface and within the stroma of the iris. Fine pigment granules are present in the posterior epithelial layer of the irides of these animals. This pigment gradually increases in the region of the ciliary processes so that the retinal epithelium shows substantial pigment saturation.

The irides of both the H/H; R/R; A/A and the H^{re}/h^{i}; R/R; a/a animals show extremely full pigmentation.

The histologic appearance of the irides of the heterochromic animals show no structural deviations from that described in the full color and nonpigmented irides of control animals carrying the H^{re}/h^{i}; r/r; a/a genotype.

These investigations present evidence to show that hypopigmentation

is determined by genes that dilute pigmentation within the Stanley-Gumbreck colony of King-Holtzman rats. Our studies indicate that the r/r; a/a genes when combined with the H^{re} gene reduces the level of pigmentation in the hair coat and also affects the color of the iris.

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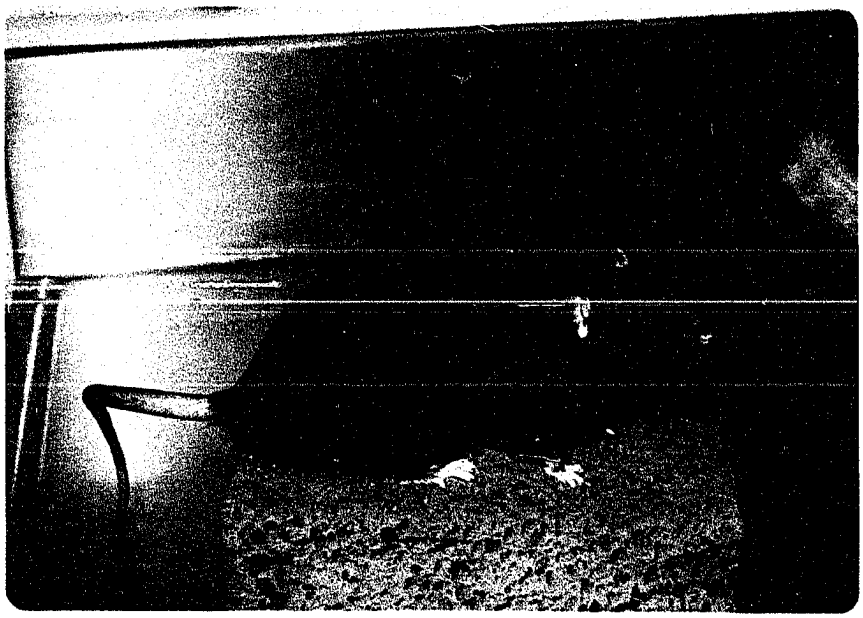
APPENDIX

PLATE I

Figure 1. Coat pigmentation pattern of the self, full-colored animal (H/H; R/R; a/a). The hair coat is made up of black pigmented hairs. The eyes are black.

Figure 2. Coat pigmentation pattern produced by the restricted gene (H^{re}) in combination with the self (H) allele. The restricted gene causes restriction of the coat color. The restricted self animal (H^{re}/H; R/R; a/a) has a variable expanse of white on the ventral surface and limbs. White hairs are also interspersed among pigmented hairs on dorsal and lateral surfaces of the body. The animal shows a tuft of white hair on the forehead which is a characteristic effect of the restricted gene. The eyes are black.

PLATE I



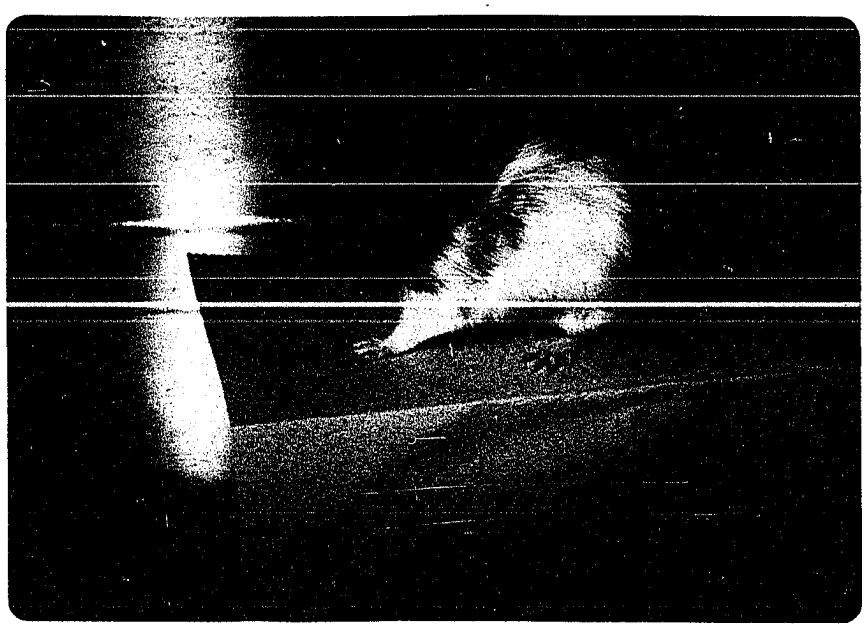


PLATE II

Figure 3. Coat pigmentation pattern of the irish, white-belly animal (h^i/h^i; R/R; a/a). The irish gene (h^i) produces variable white spotting ranging from a small spot to that in which the entire ventral surface of the animal is unpigmented. This animal shows an extreme irish pattern. The eyes are black.

Figure 4. Coat pigmentation pattern produced by the restricted gene (H^{re}) in combination with the irish (h^i) allele. This animal is a restricted irish agouti (H^{re}/h^i; R/R; A/A) and shows variable reduction in the area of lateral pigmentation as well as the white spot on the forehead. The extent of reduction in pigmentation is much greater in this animal than in the restricted self animal in Fig. 2. The eyes in the restricted irish agouti are black.

PLATE II

PLATE III

Figure 5. Coat pigmentation pattern of the hooded animal (h/h; R/R; a/a). The amount of white is increased over that seen in Fig. 3. Pigmentation is limited to the head, shoulders and a small mid-dorsal stripe. The eyes are black.

Figure 6. Coat pigmentation pattern produced by the restricted gene (H^{re}) in combination with the hooded (h) allele. The restricted hood animal (H^{re}/h; R/R; a/a) shows reduced pigmented areas so that the white spot on the forehead is continuous with nonpigmented areas over the rest of the body. The eyes are black.

PLATE III

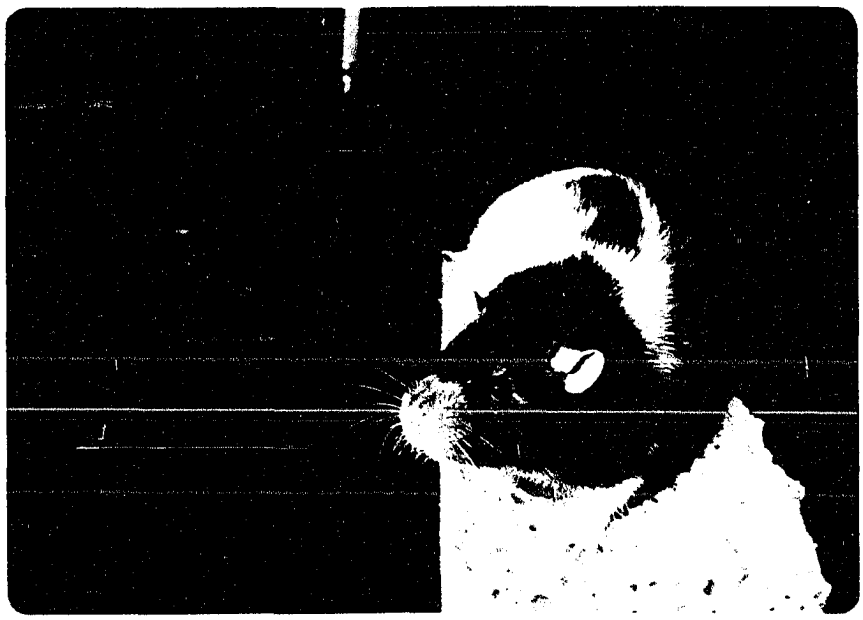


PLATE IV

Figure 7. Restricted animal (H^{re}/h^{i}; r/r; a/a) with bicolored eyes or heterochromia iridis. This animal is homozygous for the color genes red-eyed yellow and nonagouti (r/r; a/a). The r/r; a/a genes are not linked to restricted (H^{re}) and have the ability to further reduce the amount of black pigment in the restricted irish animal so that the unpigmented frontal head spot is not evident. The affected eye is unpigmented or pink, whereas the contralateral eye is dark red.

Figure 8. Restricted animal (H^{re}/h^{i}; r/r; a/a) with dark red eyes. The degree of coat pigmentation is slightly increased in this animal over that seen in Fig. 7.

PLATE IV

PLATE V

Figure 9. Restricted animal (H^{re}/h^{i}; r/r; a/a) in which both eyes are unpigmented or pink.

Figure 10. Irish animal (h^{i}/h^{i}; r/r; a/a) carrying the color genes red-eyed yellow and agouti without association of the restricted (H^{re}) gene. This animal does not exhibit the unpigmented frontal head spot. The eyes are dark red.

PLATE V

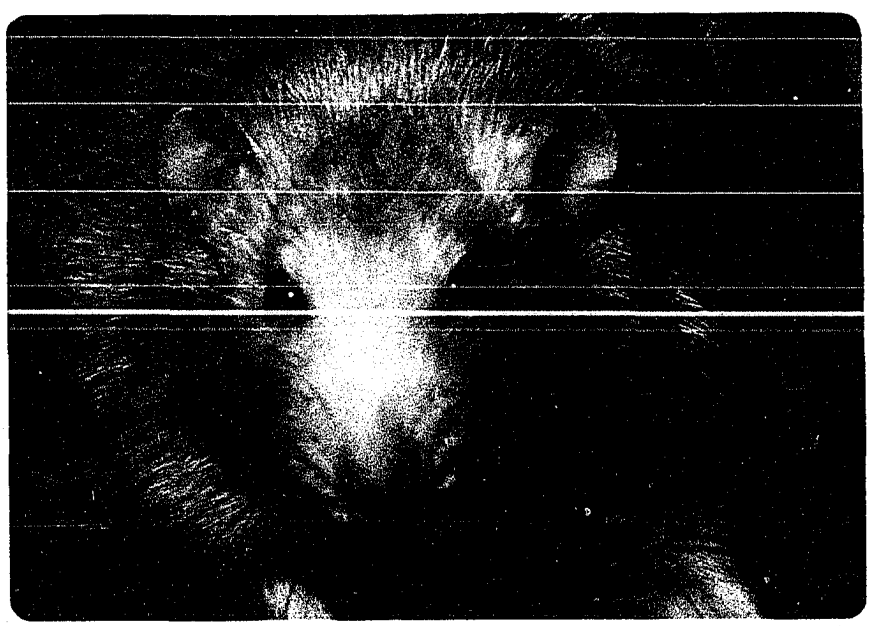


PLATE VI

Figure 11. Agouti animal (H/H; R/R; A/A) in which each hair has alternating bands of black (eumelanin) and reddish yellow (phaeomelanin). The eyes are black.

PLATE VI

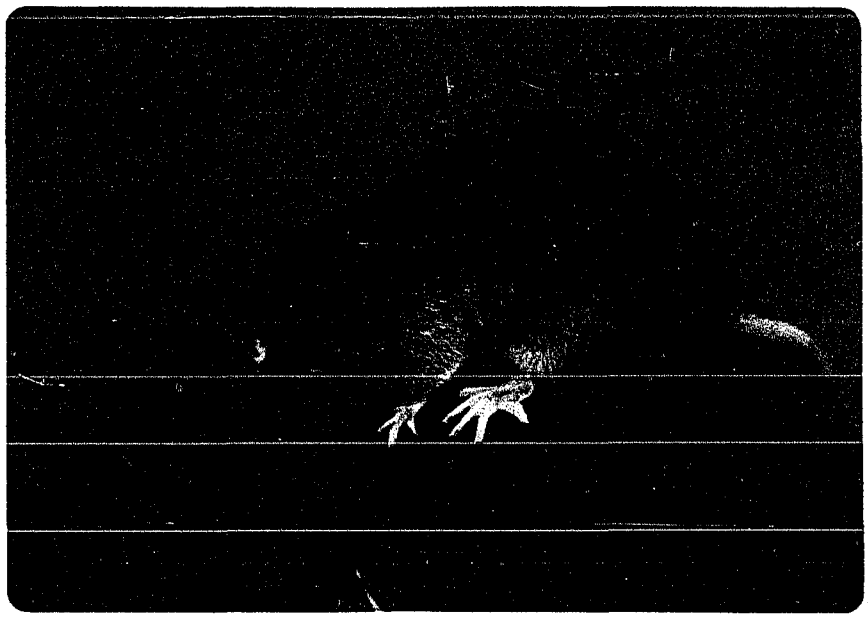


PLATE VII

Figure 12. Photomicrograph of karyotype and metaphase plate of chromosomes from squashed corneal epithelium of normal male rat. The serial alignment shows 20 pairs of autosomes and the heteromorphic sex pair which consists of unequal X and Y chromosomes. (Aceto-orcein, X 1250).

PLATE VII

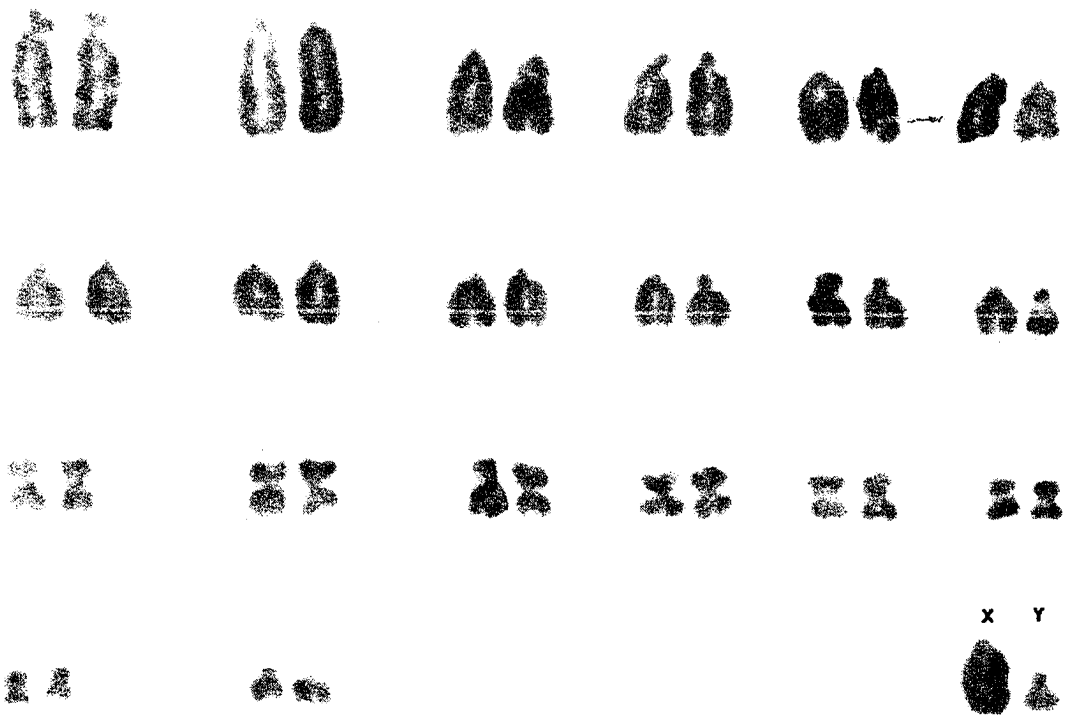


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PLATE VIII

Figure 13. Photomicrograph of karyotype and metaphase plate of chromosomes from squashed corneal epithelium of normal female rat. Female karyotype shows 20 pairs of autosomes and the sex pair consisting of two X-chromosomes of equal size. (Aceto-orcein, X 1250).

PLATE VIII

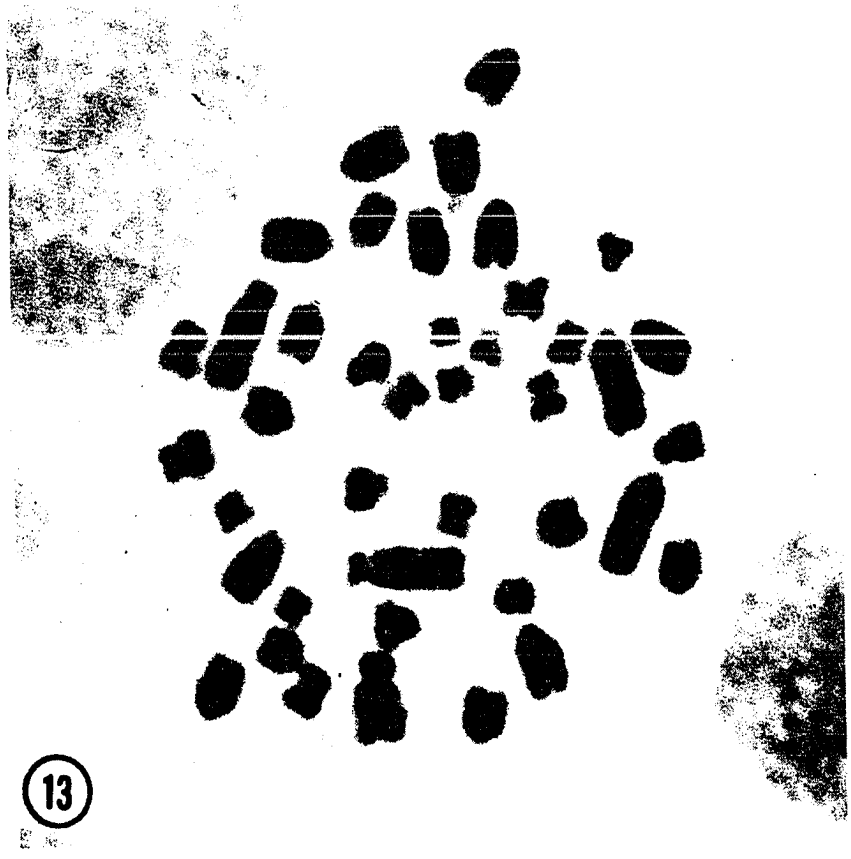

PLATE IX

Figure 14. Photomicrograph of karyotype and metaphase plate of chromosomes from squashed corneal epithelium from dark red eye of male rat with heterochromia iridis. (Aceto-orcein, X 1250).

PLATE IX

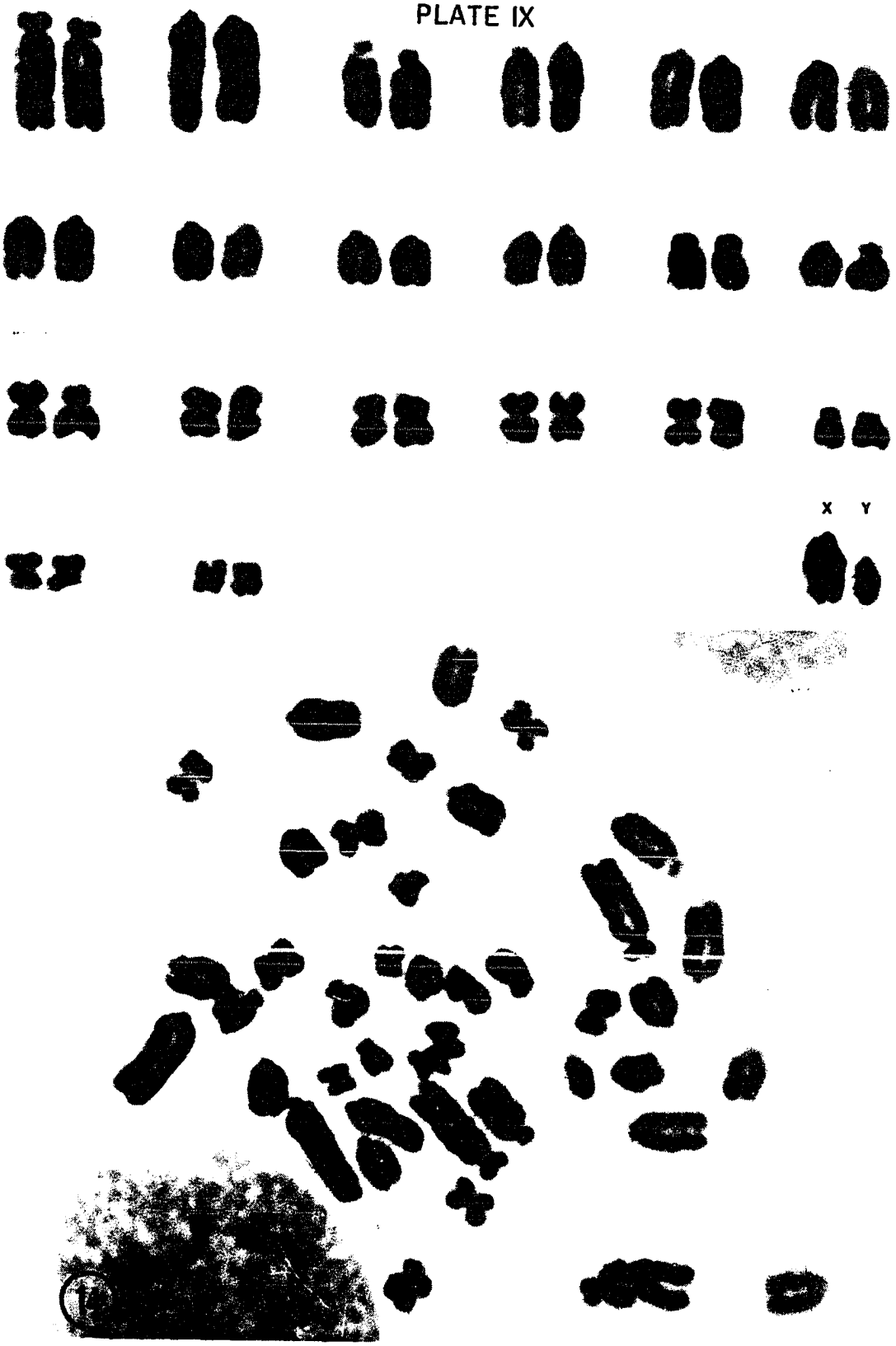

PLATE X

Figure 15. Photomicrograph of karyotype and metaphase plate of chromosomes from squashed corneal epithelium from pink or unpigmented eye of male rat with heterochromia iridis. (Aceto-orcein, X 1250).

PLATE X

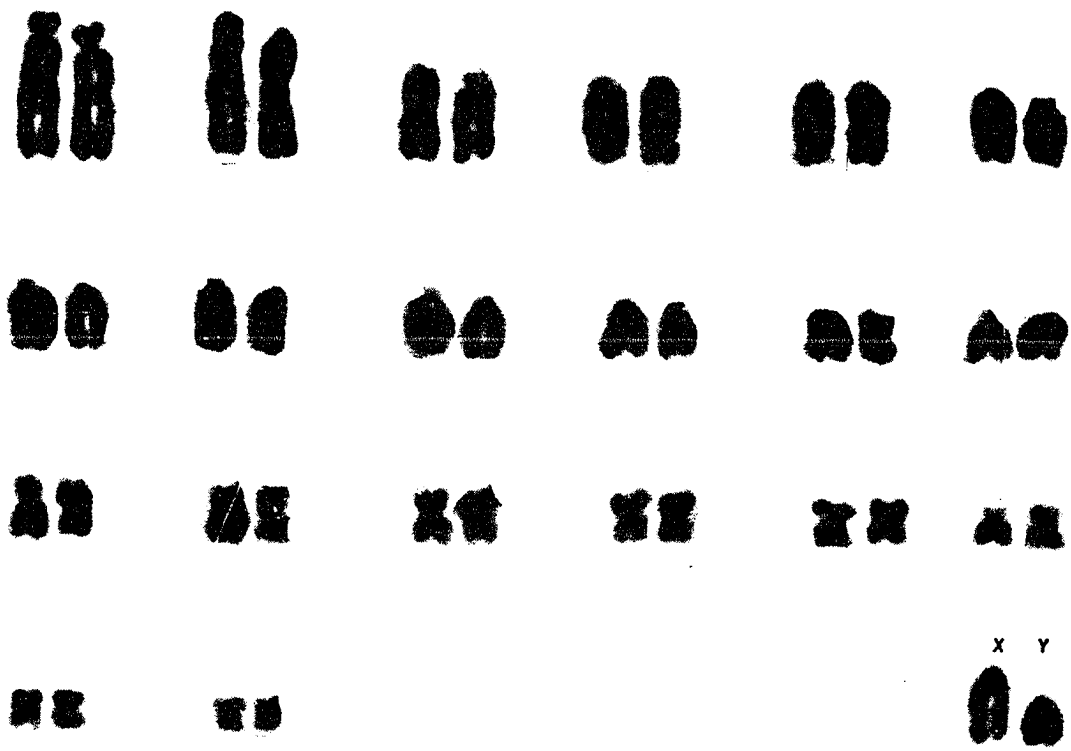

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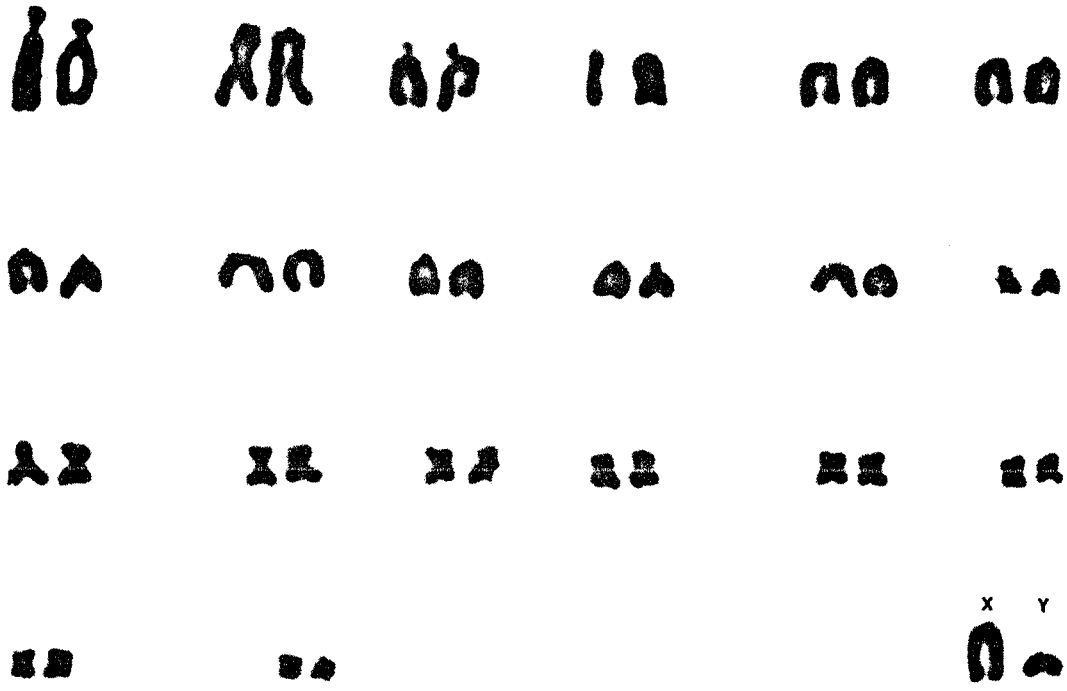
PLATE XI

Figure 16. Photomicrograph of karyotype and metaphase plate of air-dried spermatogonial chromosomes from testis of normal male rat. (Lactic-acetic-orcein, X 500).

PLATE XI



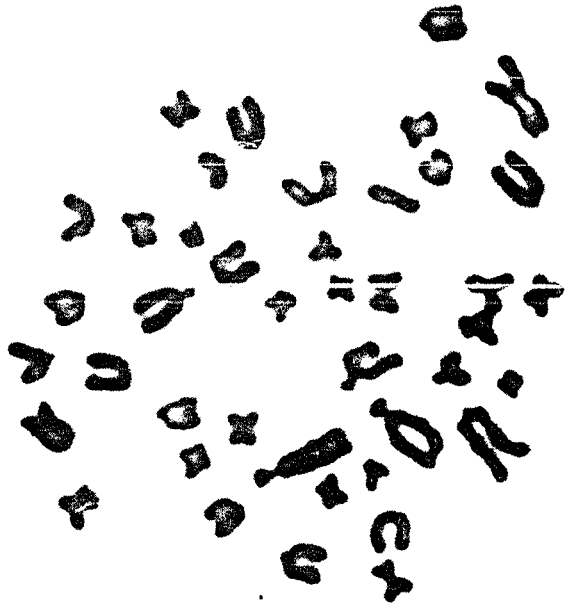


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PLATE XII

Figure 17. Photomicrograph of karyotype and metaphase plate of air-dried spermatogonial chromosomes from testis of sterile male rat. (Lactic-acetic-orcein, X 500).

PLATE XII

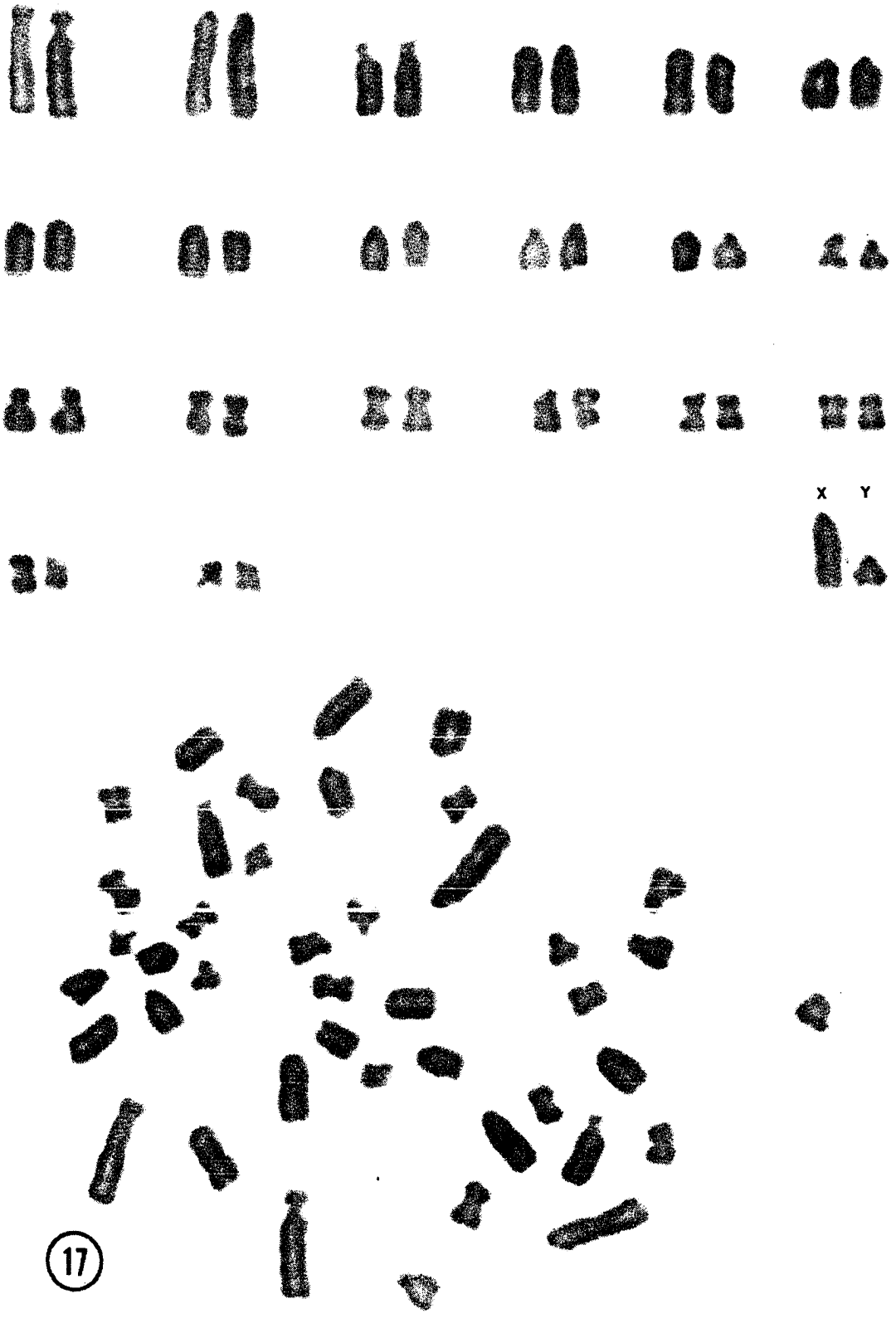
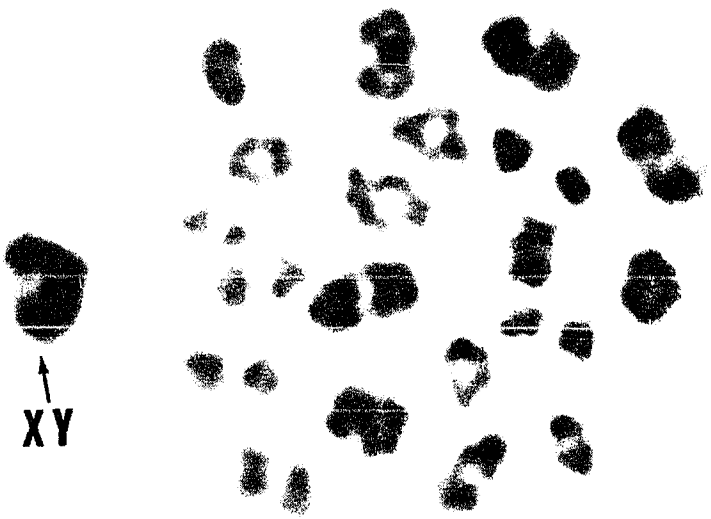


PLATE XIII

Figure 18. First meiotic metaphase figure of a primary spermatocyte from a normal male rat. In addition to the 20 autosomal bivalents, the X and Y are aligned end to end. This association appears to be due to terminal chiasmata. In this figure the XY pair is heavily condensed but easily recognized and clearly distinguishable from the autosomal bivalents. The intimately paired chromosomes are beginning to separate except at points of interchange. Note the close proximity of the XY chromosome pair to one of the smaller autosomal bivalents. (Lactic-acetic-orcein, X 500).

Figure 19. First meiotic metaphase figure of a primary spermatocyte from a sterile male rat. Several terminal chiasmata can be seen in the larger autosomal bivalents. The short bivalents are less distinct. The X appears as a long rod and the Y as a shorter elliptical rod. (Lactic-acetic-orcein, X 500).

PLATE XIII



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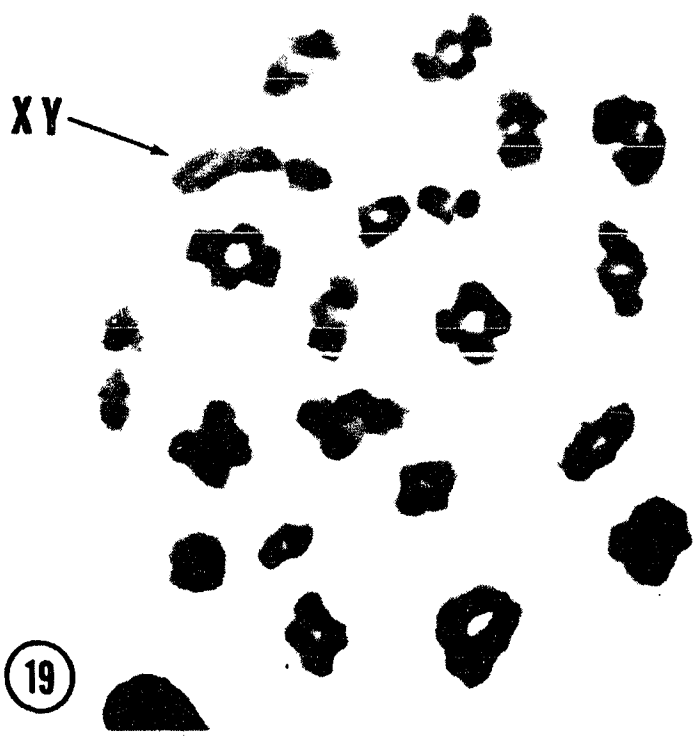

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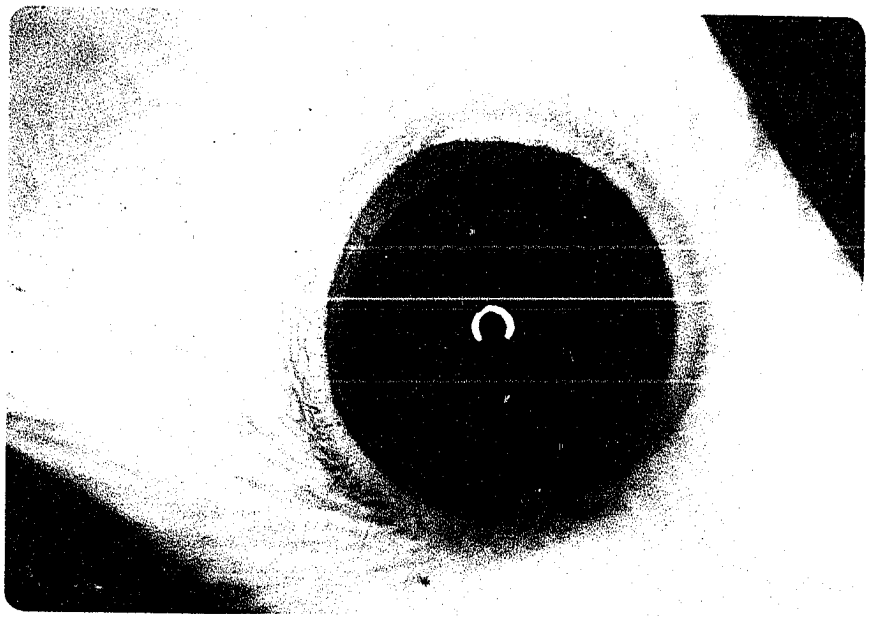
PLATE XIV

Note: The photographic artifacts present in Figs. 20-25 are due to slight drying of the cornea while the eye was being held open during photography. The white circular spot near the pupil is due to light reflection from the ring light of the camera.

Figure 20. The pigment in the iris of the h^i/h^i; r/r; a/a animal is evenly distributed and does not show the sectoring pattern. X 2.

Figure 21. Iris of H^{re}/h^i; r/r; a/a animal with both eyes pink or unpigmented. The iridal vasculature is prominent. X 2.

PLATE XIV



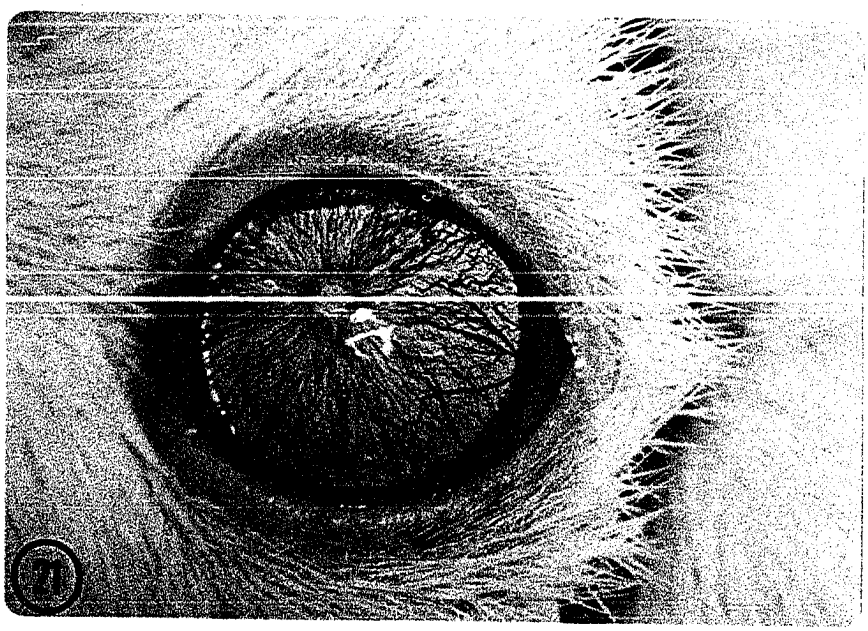
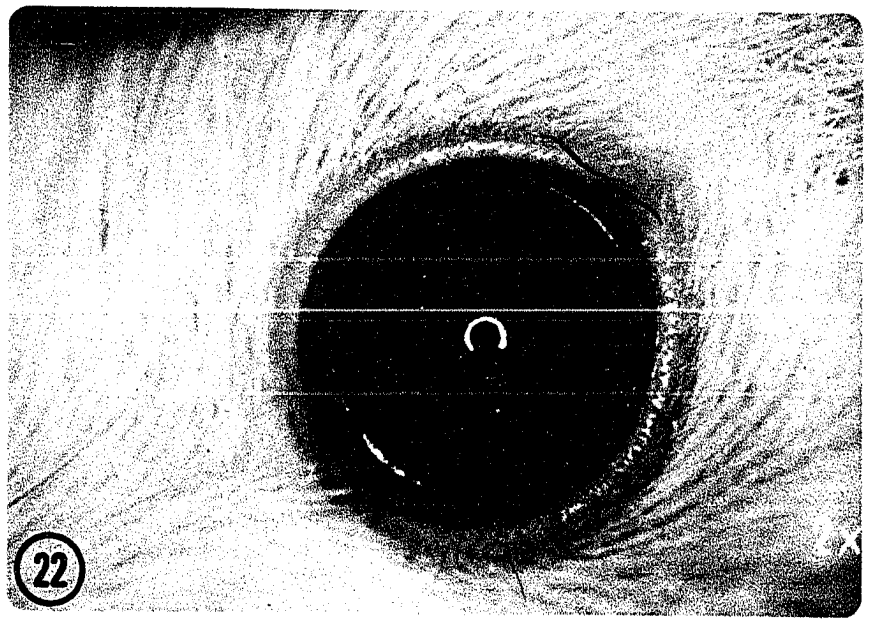


PLATE XV

Figure 22. Full-color or dark red eye from H^{re}/h^{i}; r/r; a/a animal with heterochromia iridis. The iris shows an asymmetrical sectoring pattern in which a triangular area of hypopigmentation extends laterally from the pupillary margin to the periphery. X 2.

Figure 23. Full-color or dark red eye from H^{re}/h^{i}; r/r; a/a animal with heterochromia iridis in which nearly half of the iris is affected by hypopigmentation. X 3.

PLATE XV



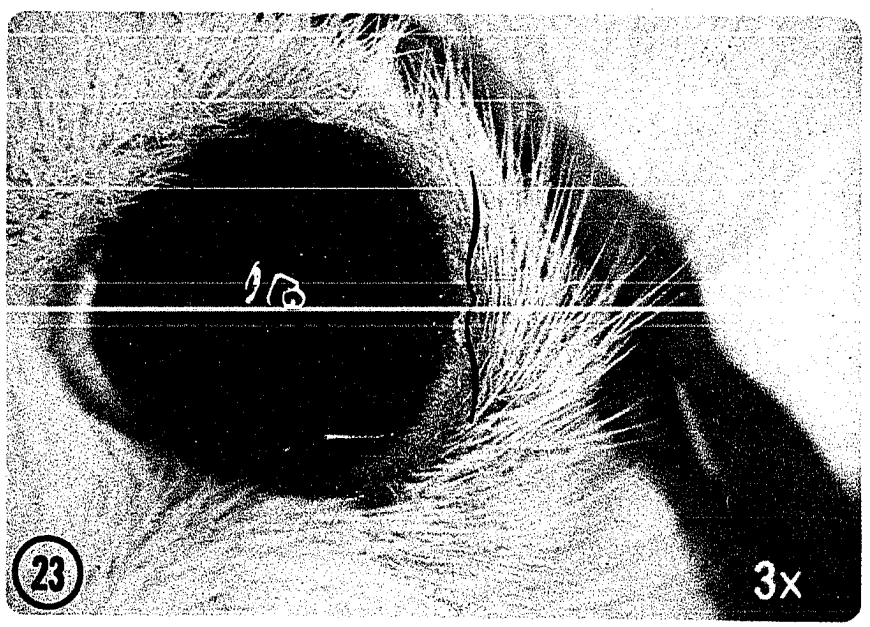
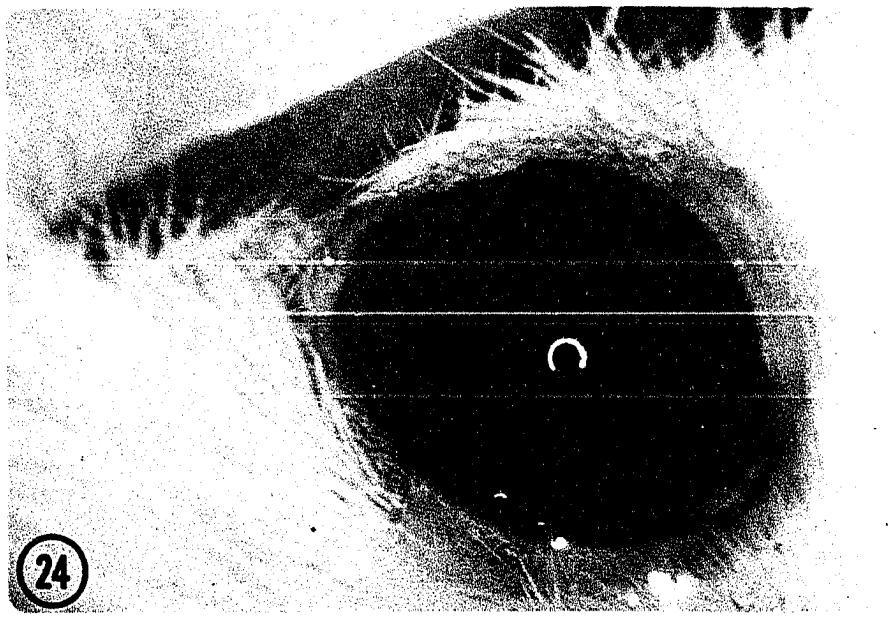


PLATE XVI

Figure 24. Full-color or dark red eye from H^{re}/h^{i}; r/r; a/a animal with both eyes dark. Patchy islands of pigment are distributed throughout the iris giving it a mottled and splotchy appearance. X 2.

Figure 25. Full-color or dark red eye from H^{re}/h^{i}; r/r; a/a animal with both eyes dark. The area of deeper pigmentation appears as a ring-like collarette around the pupil. X 2.

PLATE XVI



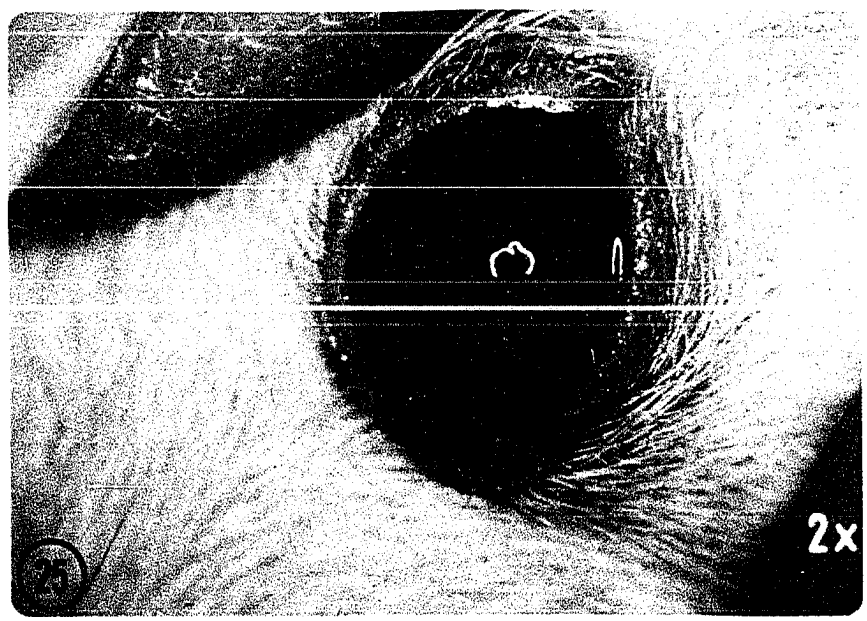


PLATE XVII

Figure 26. Ciliary processes. (H & E, X 575).

Explanation of Figures

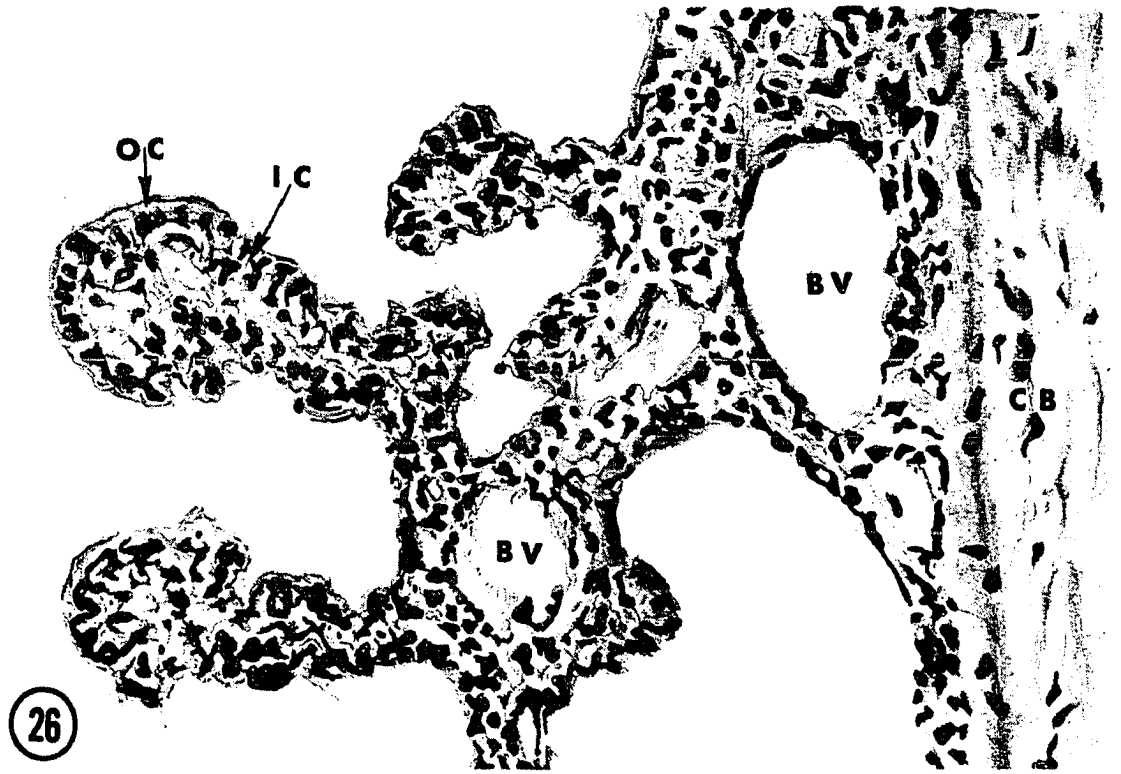
BV	Blood vessels
CB	Collagenous tissue of the ciliary body
IC	Inner ciliary epithelium
OC	Outer ciliary epithelium
S	Vascular stroma

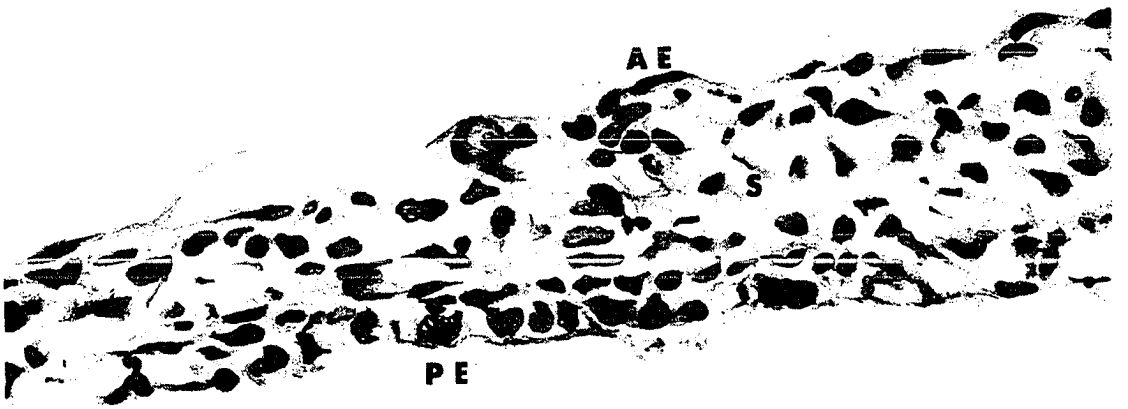
Figure 27. Iris and lens. Saggital section (H & E, X 613).

Explanation of Figures

AE	Anterior epithelial layer
PE	Posterior epithelial layer composed of two layers of cells
S	Stroma of iris containing connective tissue and blood vessels

PLATE XVII





POSTERIOR CHAMBER

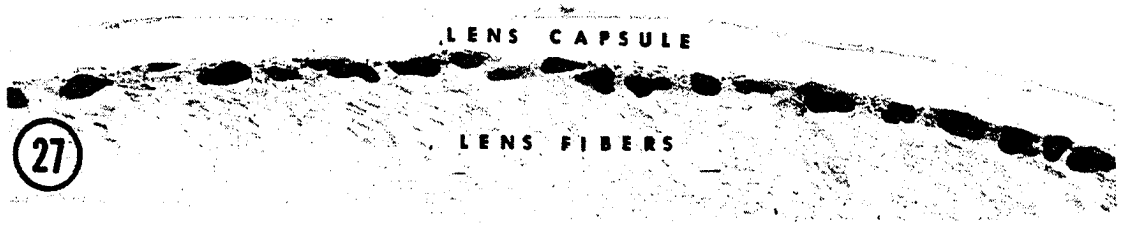
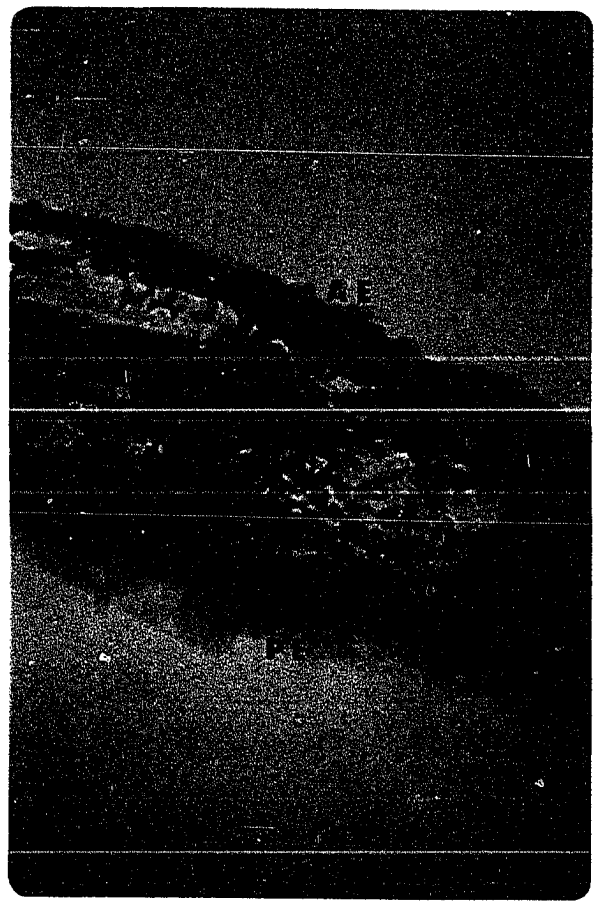


PLATE XVIII

Figure 28. Photomicrograph of iris of an H^{re}/h^{i}; r/r; a/a animal with both eyes dark red. The stroma (S) forms the greater part of the thickness of the iris. Chromatophores are the major structural constituent of this loose, pigmented, highly vascular connective tissue. (H & E, X 300).

Figure 29. Photomicrograph of a small area of the stroma of the same section as in Fig. 28 illustrating dendritic melanocytes (chromatophores) with many branched processes containing granular pigment. The heavy deposition of pigment obscures the outline of individual cells. (H & E, X 500).

PLATE XVIII



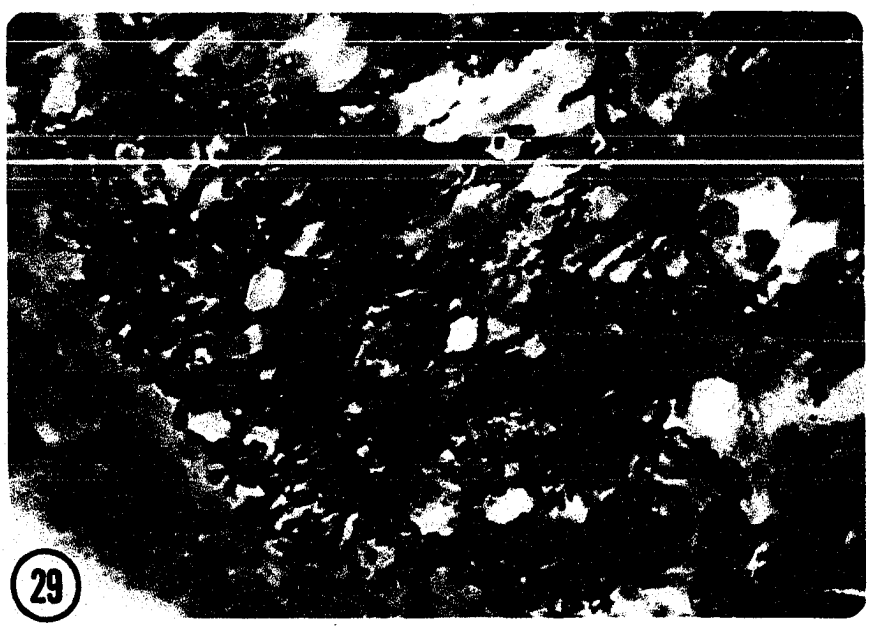


PLATE XIX

Figure 30. Photomicrograph of iris of H^{re}/h^{i}; r/r; a/a animal. The double layer of epithelium on the posterior surface (PE) contains pigment granules of a finer calibre than that found in the stroma (S) and the anterior epithelial layer (AE). (H & E, X 400).

Figure 31. Photomicrograph of iris stroma (S) from h^{i}/h^{i}; r/r; a/a animal in which small rounded clump cells are prominent. These pigment cells are devoid of branching processes and contain large granules which appear to be concentrated near the nucleus. (H & E, X 400).

PLATE XIX

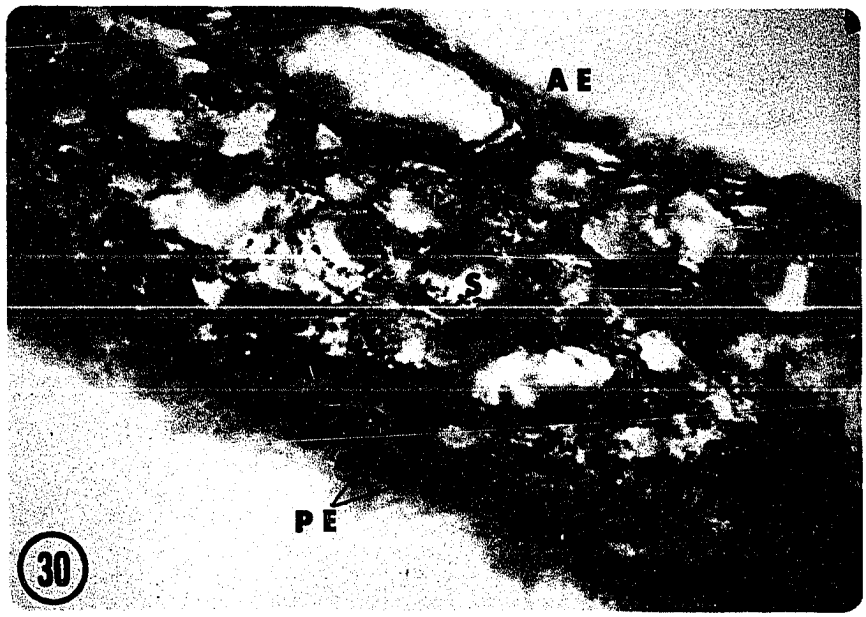
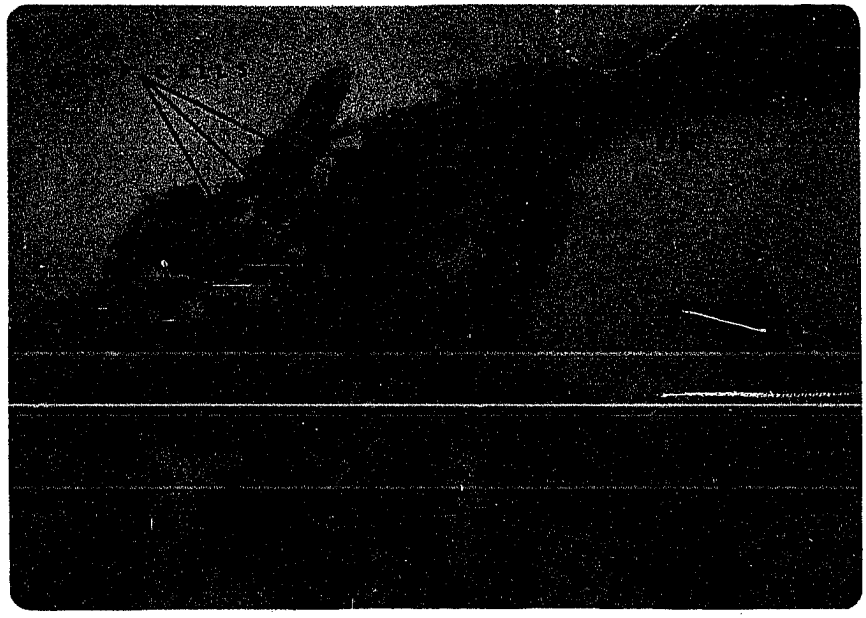


PLATE XX

Figure 32. Photomicrograph of section through the ciliary processes (CP) and iris root (IR) of an h^i/h^i; r/r; a/a animal. Clump cells are present throughout the stromal element (S) and into the region of the ciliary body and root of the ciliary processes. (H & E, X 250).

Figure 33. Photomicrograph of iris from H/H; R/R; A/A animal. The anterior surface (AE) shows irregular crypts extending into the stromal area through which fluid from the anterior chamber of the eye circulates freely in and out of the iris. This section shows several engorged blood vessels. The increased amount of pigment is responsible for the deep color of the irides in these animals. The posterior epithelial layer (PE) is also heavily pigmented with dark brown melanin granules. (H & E, X 400).

PLATE XX



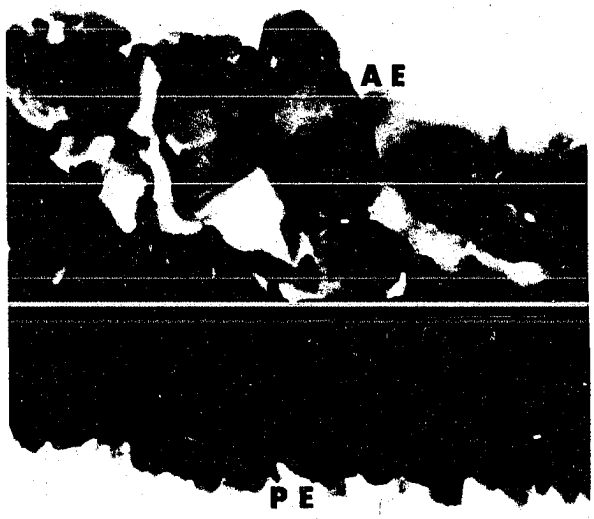


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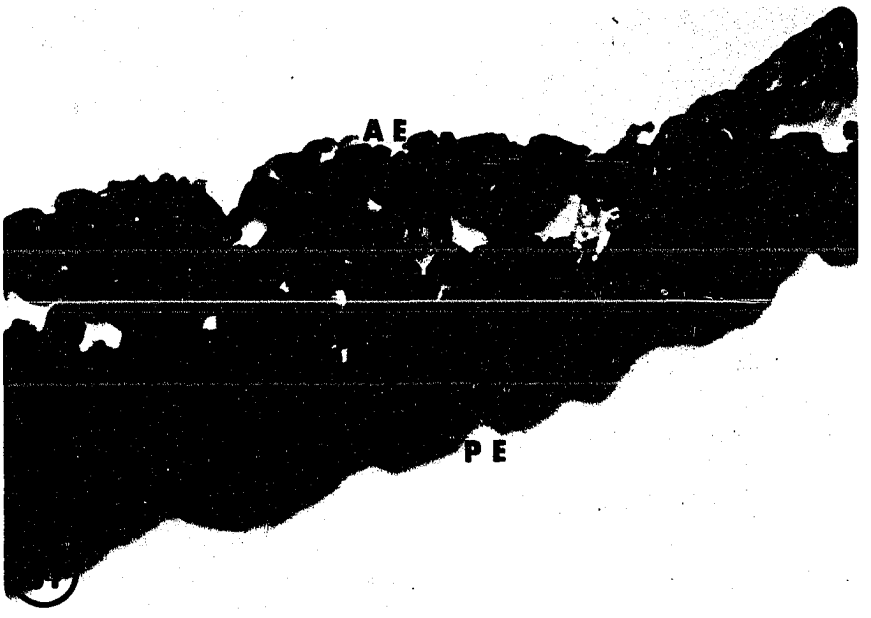
PLATE XXI

Figure 34. Photomicrograph of iris from H^{re}/h^{i}; R/R; a/a animal. The stroma is quite dense and the structural pattern is masked by large irregular melanin granules. There is little intercellular space and blood vessels are nearly invisible. The anterior surface (AE) of the iris is as heavily pigmented as is the posterior surface (PE). (H & E, X 400).

Figure 35. Photomicrograph of unpigmented iris from H^{re}/h^{i}; r/r; a/a animal with heterochromia iridis. Note the marked reduction in pigment and the absence of chromatophores and clump cells. The stroma (S) is loose, highly vascular and contains many amelanotic melanocytes. (H&E, X 400).

PLATE XXI



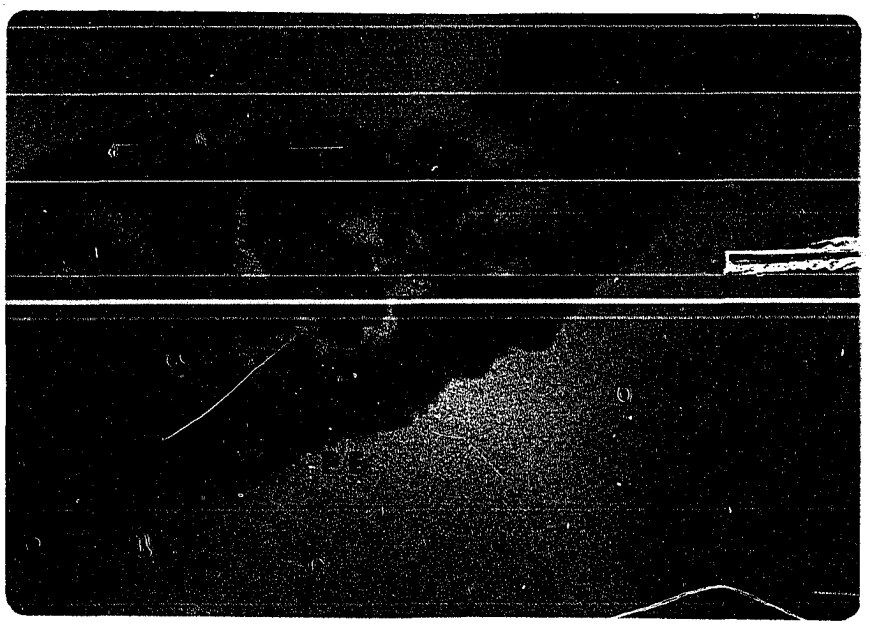


PLATE XXII

Figure 36. Photomicrograph of unpigmented iris from H^{re}/h^{i}; r/r; a/a animal with heterochromia iridis. The stroma shows a thick-walled blood vessel (BV) with a relatively large lumen. Intercellular spaces are especially prominent. (H & E, X 400).

Figure 37. Photomicrograph of section through the ciliary processes (CP) and a portion of the iris root (IR) (upper left) of an H^{re}/h^{i}; r/r; a/a animal with both eyes pink. Note the gradual increase of pigment in the ciliary epithelium. The cells of the posterior epithelial layer of the iris contain granules of a very fine calibre. In the region of the angle of the anterior chamber of the cells gradually begin to accumulate more granules so that the ciliary processes contain substantial pigmentation. (H & E, X 400).

PLATE XXII

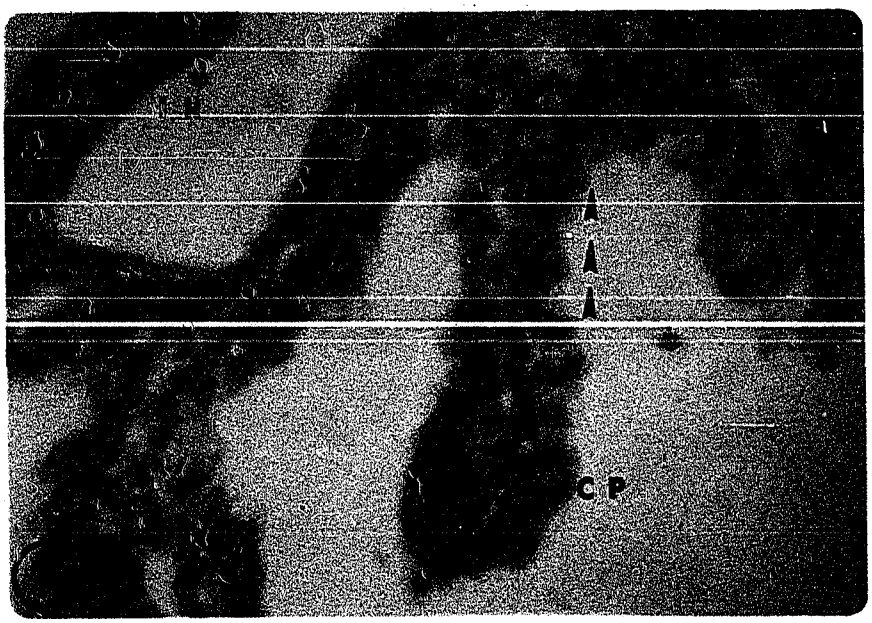


PLATE XXIII

Figure 38. Photomicrograph of section of a small area of the iris near the pupillary margin from an H^{re}/h^{i}; r/r; a/a animal with both eyes pink. The sphincter muscle (Sp) adheres closely to surrounding stromal elements. It consists of longitudinal bundles of smooth muscle cells. (H & E, X 500).

PLATE XXIII