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The Incidence of Sex Chromatin in the Amniotic Epithelium of the Domestic Fowl and Japanese Quail

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THE INCIDENCE OF SEX CHROMATIN IN THE AMNIOTIC EPITHELium OF THE DOMESTIC FOWL AND JAPANESE QUAIL

by

Donna Gorecki

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

June, 1969
BIOGRAPHY

Donna Gorecki was born on October 28, 1931 in Chicago, Illinois.

Miss Gorecki is a product of the Chicago Public Schools and was graduated from the Harrison Technical High School, Chicago, Illinois, in June, 1949.

In 1952, she entered the University of Chicago and was awarded the B.A. degree (1956), the B.S. degree (1959) and the M.S. degree (1960). During the years, 1955-1959, she served as Research Technician in the laboratory of Dr. Peter P.H. DeBruyn, Department of Anatomy. In 1959, she joined the staff of Professor William L. Doyle and participated in research on the cytological basis of salt transport mechanisms in different vertebrates. In 1960-1961, she was a Research Assistant in the laboratory of Dr. William Bloom, Professor of Anatomy and Biophysics, Department of Biophysics, The University of Chicago.

In the fall of 1962, Miss Gorecki transferred to the Department of Anatomy, Loyola University Stritch School of Medicine, where she continued her graduate training under the sponsorship of Dr. L.V. Domm, Chairman of the Department. She served as a Graduate Assistant in Anatomy during the 1962-1963 academic year. In 1964 she was awarded a United States Public Health Service, Predoctoral Fellowship, which award she held during the years, 1964-1967. In the fall of 1967 she was appointed a Research Associate in Anatomy and she has served in this capacity in the laboratory.
of Professor L. V. Domm until the present time.

Miss Gorecki has also served as a free-lance science writer with Science Research Associates, Chicago, Illinois and as Assistant Professor of Biology in the Chicago City College.
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ABSTRACT

The incidence of sex chromatin in the amniotic membrane was investigated in the brown and white Leghorn races of the domestic fowl, Gallus domesticus, and in the Japanese quail, Coturnix coturnix japonica. Bits of amnion were excised from 40 Leghorn embryos at daily intervals until the seventeenth day of incubation. Whole-mount and squash preparations and histological sections were made from tissues fixed in Davidson's solution and stained with Harris's hematoxylin. In addition, a modified embedding technique was introduced which involved the preparation of 'membrane sandwiches for the observation of sections containing parallel sheets of amniotic cells.

The nuclear sexing technique of Barr and Bertram ('49) was applied to at least two groups of 100 amniotic cells each for every embryo. A cell was scored 'positive for sex chromatin' if it contained a dense-staining, nuclear chromocenter, at least 0.5μ in breadth, within 0.5μ of the nuclear membrane. Cells scored 'negative' lacked peripheral as well as centrally located nuclear chromocenters. The incidence of sex chromatin was expressed as the percentage of positive cells. From the seventh day of incubation, the gonadal sex of each embryo was determined by visual inspection of the gonads.

When the results from the nuclear sexing technique were compared with the gonadal sex of the Leghorn embryos, a nuclear sexual dimorphism was found, resulting from a bi-modal distribution in the frequency of sex
chromatin. The low peak, with a range of average incidence of sex chromatin from 2.4% to 16.7%, corresponded to male embryos and the high peak, ranging from 27.7% to 52.4%, to females. The average frequency of sex chromatin was consistently, although not significantly, higher in brown Leghorn males and females than in white Leghorn males and females. The incidence of sex chromatin was generally higher in whole-mount and squash preparations than in histological sections although the greatest variation in sex chromatin frequency in any given embryo was found in the sectioned material.

Although sex chromatin studies have not been previously reported for the brown Leghorn fowl, the results of this investigation agree with the observations of Ishizaki and Kosin ('60) on the New Hampshire breed and of Van Limborgh ('64) on the white Leghorn breed of domestic fowl. In addition to the demonstration of a nuclear sexual dimorphism in brown and white Leghorn embryos, this study revealed that the frequency of sex chromatin remained relatively constant in both male and female white Leghorn embryos, in spite of an increasing coarseness in the chromatin pattern, throughout the incubation period.

When identical sexing procedures were applied to squash preparations of the amnion from 42 quail embryos at 2 to 20 days of age, no nuclear sexual dimorphism was apparent. The average incidence of 'sex chromatin' was the same in 16 males (range, 9% to 45%) as in 18 females (range, 7% to 45%). In 8 embryos, whose gonadal sex could not be determined, there was
a progressive increase in 'sex chromatin' frequency during the first week of incubation.

As in the Leghorn fowl, the chromatin pattern in quail embryos increased in coarseness with increasing incubation time in both males and females. Unlike the fowl, however, the averaged male and female frequencies exhibited a curious daily fluctuation during the second half of incubation - with male and female values out-of-phase by a period of 24 hours and differing from each other by 15 percentage units, on the average, at each day.

The cytological disposition of the sex chromatin body appeared identical in the fowl and quail. The only consistent difference noted was that the V-shaped form of the sex chromatin body, associated with the female sex in the domestic fowl, was frequently observed in both sexes of the Japanese quail.

A proposal was made that assumed: (1) an equivalence between the heterochromatic behavior and the late-replicating behavior of the W chromosome in certain avian species reported by previous investigators, and (2) an identity between a W chromosome exhibiting such behavior and the sex chromatin body of the interphase nucleus. It was predicted that avian species, such as the pigeon and domestic fowl, which possess a heterochromatic and late-replicating W chromosome, should also reveal a nuclear sexual dimorphism in tissues suitable for nuclear sexing. Conversely, avian species, in which the W chromosome has been reported to behave euchromatically, such as the budgerigar and Japanese quail, would not be expected to show a
nuclear dimorphism based on the presence of a sex-specific chromocenter in female cells.

The results of this study on both brown and white races of the Leghorn fowl are consistent with the proposal advanced here. The results from the quail study can also be interpreted in support of this proposal if the nuclear chromocenter, identified as sex chromatin in this species, is taken to represent some variable expression of the quail genome or, perhaps, some metabolic activity in the amniotic cells.
I. INTRODUCTION

The discovery of sexual dimorphism in the interphase nuclei of cat neurons by Barr and Bertram in 1949 stimulated numerous studies on the existence and derivation of sex chromatin in a variety of animals. Their nuclear sexing technique, in which a small, densely-staining nuclear chromocenter is identified as sex chromatin, has been used as the basis for diagnosing the genetic sex of an individual. This technique has been extensively applied in the class, Mammalia, where the occurrence of the sex chromatin body in relatively high frequencies in an individual has been found to be associated with the female sex. The most successful application of nuclear sexing has been in the identification of sex chromosome anomalies using large samples of the human population (Ford et al., '59; Ford '60).

The derivation of sex chromatin, or the Barr body as it is frequently called, has been problematic since its discovery. Moore and Barr ('53) had originally suggested that the sex chromatin body was formed from the heterochromatic regions of a pair of X chromosomes. The earlier cytogenetic studies on Drosophila melanogaster had given rise to the notion that "once heterochromatin always heterochromatin" and so it was logical to assume that the mammalian X chromosome also consisted of one part heterochromatin and one part euchromatin. In the light of this assumption, the double-X origin for sex chromatin seemed to be supported by the demonstration of a large
nuclear chromocenter in mouse follicular cells by Ohno et al. ('58). The absence of a corresponding half-sized Barr body in male somatic nuclei was dismissed as a mere technicality. Moore and Barr's interpretation, then was entirely consistent with the findings in several species of Mammalia in which the XX/XY sex determining mechanism is in operation.

In 1959, however, Ohno et al. observed that only one of the X chromosomes in regenerating liver cells from female rats exhibited a positive heteropycnosis along its entire length while the X and Y chromosomes in male cells appeared isopycnotic with the autosomes. These authors suggested that the paternally-derived X chromosome in the female cells forms the sex chromatin of the interphase nuclei since the male cells contain only a maternally-derived sex chromosome. This single-X hypothesis for the origin of sex chromatin received support from studies in the mouse (Ohno and Hauschka, '60) and in man (Ohno and Makino, '61). The idea that the sex chromatin of the interphase nuclei was formed from a paternally-derived X chromosome was later abandoned by Ohno when positive heteropycnosis of the X chromosome was observed in the tetraploid cells of the X\textsuperscript{MO} mouse - while being suppressed in the X\textsuperscript{PO} mouse (Ohno et al., '60d).

The hypothesis that sex chromatin is derived from a single-X chromosome received additional support from a study of Kosin and Ishizaki ('59) in which they reported a high frequency of sex-chromatin-positive cells in a variety of tissues from young female chickens. Since a sex determining
mechanism of the ZW/ZZ type prevails in the class, Aves, this finding came as a surprise. It had been expected that should sex chromatin be present in any of the avian species, it would most likely occur in the male sex with its ZZ complement of sex chromosomes. On the one hand, Kosin and Ishizaki's report was taken as providing additional evidence for the single-X hypothesis for sex chromatin and, further, that the paternally-derived sex chromosome could not form the sex chromatin body since both male and female birds possess a paternally-derived sex chromosome. On the other hand, the report raised doubts concerning the theory of dosage compensation by means of X-inactivation which, in the meantime, had come to be associated with the occurrence of sex chromatin in mammalian species.

Kosin and Ishizaki's report, therefore, served to focus attention on the incidence of sex chromatin in species exhibiting female heterogamety. The results of investigations in avian species were, however, conflicting. In the same year and in the same tissues, Ashley and Theiss ('59) reported the absence of sexual dimorphism in the intermitotic nuclei of the chicken, duck, parrot and parakeet. Similarly, in 1962, Miles and Storey reported negative findings for sex chromatin in a variety of cultured tissues from the adult Leghorn.

Ohno et al. ('60a), however, found a sex-specific chromocenter in cultured cells from skin and liver of embryonic chicks. The most convincing
study on sex chromatin in birds, however, was reported by Ishizaki and Kosin ('60). These investigators were able to separate chick embryos into two groups on the basis of the presence or absence of sex chromatin in cells of the area opaca prior to gonadal differentiation. They found complete agreement between the nuclear sex assigned to each embryo at two days of age and the gonadal sex assigned to the same embryo following an additional seven days incubation.

The only positive finding of a nuclear sexual dimorphism in an adult bird was made by Moore and Hay ('61) who reported a fairly distinct sexual dimorphism in epidermal and smooth muscle cells of the adult white Leghorn.

Confirmation of nuclear sexual dimorphism in avian embryonic tissues was made by Van Limborgh in 1964 who verified Ishizaki and Kosin's observations on the chick amnion and extended the study to the amniotic epithelium of the duck with almost identical results.

Preliminary studies in this laboratory on the incidence of sex chromatin in thionin-stained, squash preparations of embryonic chick liver cells gave negative results. Similarly, no sexual dimorphism was found in liver nuclei from the brown Leghorn rooster, poulard and adult hen. There was, however, a suggestion of sexual dimorphism in thionin-stained, whole-mounts of amnion cells from 8-day-old chicks. This finding coupled with the positive results of Ishizaki and Kosin ('60) cited above, prompted the selection of amnion for the study on the distribution of sex chromatin in the white and brown Leghorn
races of the domestic fowl (Gallus domesticus) reported here.

The diagnosis of genetic sex based on chromosome counts has been extremely hazardous in avian species because of the numerous microchromosomes included in most avian karyotypes. Although there had been general agreement that the female is the heterogametic sex in the class, Aves, uncertainty prevailed as to whether the sex determining mechanism was of the ZO/ZZ or ZW/ZZ type. Only within the past decade has the Z chromosome been unequivocally identified in the domestic fowl (Ohno et al., '60a) and in the pheasant and turkey (Stenius et al., '63). A W sex chromosome has recently been identified in birds belonging to the orders Galliformes, Columbiformes, Psittaciformes, Passeriformes and Strigiformes so that it can be assumed that, at least for the subclass, Carinatae, the female possesses the ZW sex chromosome constitution. Unfortunately, no reports have appeared as yet concerning the mechanism of sex determination among the ratites.

It has been known since the beginning of the century that the mammalian X chromosome becomes heteropycnotic during spermatogenesis while both X chromosomes of the female remain isopycnotic during oogenesis. This contrasting behavior led Mohr in 1915 (cited by Mittwoch, '67) to suggest that the heteropycnosis of the male X chromosome was due to the necessity of its undergoing meiosis without a partner. The discovery of sex chromatin in the
homogametic female and the subsequent interpretation that this chromocenter represents one heteropycnotic member of a pair of chromosomes has led to a paradoxical situation in mammals.

If Mohr's hypothesis had been extended to include avian species it would have received support from Ohno's study on spermatogenesis in both embryonic and adult chicks in which no heteropycnosis was observed in the ZZ bivalent of both rat and mouse oocytes, Ohno ('61) concluded that "regardless of which sex is homogametic, the homozygous sex bivalent does not display any of the allocyclic behavior so commonly associated with sex chromosomes in meiosis . . ." Furthermore, in birds, Mohr's hypothesis would not have been required to account for the occurrence of sex chromatin in the homogametic sex inasmuch as the high frequencies of this chromocenter have only been reported for the heterogametic, female sex. On the other hand, any theory capable of explaining the allocyclic behavior of the univalent sex chromosome in meiosis would also have to account for the apparent absence of such behavior in the oogonia and during meiosis of the embryonic chick cells studied by Ohno ('61).

One of the remarkable achievements of modern cytogenetics is the synthesis of Ohno's single-X hypothesis for the derivation of the sex chromatin body in mammals with the inactive-X hypothesis of Lyon which provides an explanation for the dosage compensation observed for the X-linked genes in mammalian species. The apparent absence of dosage compensation for the
Z-linked genes in avian species coupled with reports of the existence of sex chromatin bodies in female birds raises the possibility that it is the W chromosome rather than the Z chromosome which gives rise to the typical sex chromatin body observed in the resting cells of some avian species.

Soom after the chick study reported here was undertaken, the observations of Vegni-Talluri and Vegni ('65) on the karyogram of the Japanese quail, Coturnix coturnix japonica, became available. Although the idiogram of this quail species was singularly similar to other Phasianidae studied by Ohno et al. ('64 b), these investigators never observed positive heteropycnosis of any sex chromosome in either male or female somatic cells. The metacentric Z chromosome had an average length of 2.8 μ and a ratio of arms equal to 1.15 in cultured cells from the testes and kidneys of young males and females. The W chromosome was telocentric with a length almost six-tenths that of the Z chromosome.

These observations on the quail contrast sharply with those of Ohno ('61) on the domestic fowl in which 50% of the embryonic follicular cells exhibited a univalent element in positive heteropycnosis. This element was identified as the Z chromosome seen in earlier studies (Ohno et al., '60 a).

Vegni-Talluri and Vegni's findings in coturnix were not unprecedented since, in a study of cultured kidney cells, Rothfels et al. ('63) found that both the Z and the W chromosomes were negatively heteropycnotic at metaphase in both sexes of the budgerigar, Melopsittacus undulatus. In this species, the
unpaired Z chromosome was the 5th largest element of the female complement while the W chromosome, with an arm ratio of 1:1.5, was intermediate in size between the 9th and 10th chromosomal pairs. The sex chromatin body in the interphase nuclei of these cells, if present, was not conspicuous although Rothfels and his coworkers did not make a point of searching for it.

As in the case of mammalian species, the application of the autoradiographic technique, labeling chromosomes with tritiated thymidine, has been particularly helpful in avian species. Schmid ('62) showed that in cultured bone marrow cells of the white Leghorn and brown Brahma fowl, both Z chromosomes of the male sex were found to "replicate DNA and to cease replication at a rate and time comparable to other macrochromosomes."

Contrary to the practice of other investigators, Schmid restricted the term, macrochromosome, to the six largest pairs of chromosomes. In female birds the single Z chromosome had a replication rate and relative timing similar to the two Z chromosomes of the male. However, the cells also showed a striking labeling pattern that was sex limited. A larger microchromosome, intermediate in size between the 7th and 9th chromosomal pairs, showed a heavy and comparatively late uptake of tritiated thymidine. This microchromosome, which was consistent in behavior and easily detected in all metaphase figures, was always single and, therefore, identified as the W chromosome.
A later thymidine-labeling study on the pigeon by Galton and Bredbury ('66) revealed that while both Z chromosomes behaved euchromatically in male somatic cells, the W chromosome of the female was heterochromatic and distinctly late-labeling. The Z chromosome in female cells, on the other hand, behaved euchromatically as in male cells and its DNA synthesis pattern was synchronous with that of the autosomes.

Since it is the W chromosome that has been found to be late-replicating in two avian species, the pigeon and the domestic fowl, it seems reasonable to assume a cytological equivalence between the manifestation of positive heteropycnosis and a late DNA-replication pattern for the W chromosome only. On the basis of such an assumption, the appearance of a dense nuclear chromocenter in female avian cells, identified as the sex chromatin body, might well represent the W chromosome in positive heteropycnosis. If such an interpretation is correct, then it may be deduced that: (1) avian species which possess a W chromosome that is both late-replicating and heteropycnotic in its mitotic behavior, should be expected to have a typical sex chromatin body in the interphase nuclei of female cells, and that (2) avian species which possess a normal-replicating, euchromatic W chromosome should not be expected to have a sex-specific chromocenter in the interphase nuclei of female cells.

The genetic consequences of such a thesis would not be in the least
inconsistent with what is known about the absence of an effective dosage compensation mechanism for the Z-linked genes of birds. However, should a strict mother-to-daughter inheritance be proved for any of the avian species, it should, on the basis of this interpretation, be restricted to those species which lack a sex chromatin body.

Since some progress has been made in describing the behavior of the W chromosome in female somatic cells of the Japanese quail and budgerigar, a study of the incidence of sex chromatin in either of these species would be desirable. The Japanese quail has many features which make it an ideal laboratory animal (Padgett and Ivey, '59). This fact coupled with its ready availability prompted the selection of the coturnix species for the investigation reported here. If our thesis is borne out in this species, the quail should not exhibit a nuclear sexual dimorphism based on the presence of sex chromatin in females owing to the euchromatic behavior of the W chromosome during mitosis reported by Vegni-Talluri and Vegni ('65).

Since avian embryonic tissues, particularly the amniotic epithelium, appear more likely to exhibit the presence of the sex chromatin body, if it exists, the study of sex chromatin undertaken here will focus on the presence or absence of this nuclear chromocenter in the developing amnion of the coturnix species as well as on the white and brown Leghorn races of the domestic fowl.
In any event, it should be interesting to compare the sex chromatin pattern in a species possessing a late-replicating and heteropycnotic W chromosome, *Gallus domesticus*, with a species possessing a euchromatic W chromosome in somatic cell divisions, *Coturnix coturnix japonica*. 
As early as 1937, Geitler (cited by Mittwoch, '67) discovered sexual dimorphism in the interphase nuclei of insect species belonging to the orders Hemiptera and Diptera. In these species, in which the male is the heterogametic sex, two heteropycnotic bodies were found in the somatic nuclei of females while male interphase nuclei displayed only one such chromocenter. It was thought that each X chromosome formed a dense chromocenter in the resting nuclei of certain somatic cells.

A. The Discovery of Sex Chromatin in Mammals

The discovery of sexual dimorphism in the interphase nuclei of mammals may be described as a singular instance of serendipity. While investigating the response of hypoglossal cell nuclei to accelerated nucleoprotein synthesis, Barr and Bertram ('49) noted a conspicuous 'paranucleolus' in the neurons of the cat which migrated away from the nucleolus during Nissl depletion and during the early stages of Nissl restoration. Soon they encountered animals whose nuclei lacked these 'nucleolar satellites'. Checking back into their data for the cause of this discrepancy, they discovered that the presence of this satellite was always associated with the sex of the animal, that is, the satellite was found only in the nuclei of female cats. Although the paranucleolus had been seen before, and even drawn by Cajal in several mammalian species, including man, it remained for Barr and Bertram to show that this nuclear chromocenter provided the basis for a sexual dimorphism in nerve cell nuclei (Moore, '66).
In time, it became apparent that the term, 'nucleolar satellite', was not appropriate for this sex-specific chromocenter and in later studies it came to be known as the 'Barr body' or 'sex chromatin body'. Investigations utilizing the sex chromatin body have proceeded along two main lines since the discovery of sexual dimorphism in mammalian interphase nuclei. In one, clinical studies based on the nuclear sexing technique of Barr and Bertram or "sex chromatin test", have been directed toward detecting errors in the sex chromosome constitution in individuals with aberrant sexual development. In the other, cytological studies based on the frequency, structural characteristics, location, staining properties and distribution of the sex chromatin body, have been oriented toward elucidating the derivation of this chromocenter. Fortunately, advances in each line of investigation have enhanced and refined the knowledge being accumulated in the other.

1. Characteristics of the Sex Chromatin Body

   a) Structural Characteristics

   The identification of sex chromatin in living cells by Schwarzacher ('63) and others has ruled out the possibility that this nuclear chromocenter is a fixation artifact. In the in vitro studies of human fibroblast-type cells of female origin, the sex chromatin body usually measured from one to two microns in diameter, and following fixation and staining, was shown to be Feulgen positive.
In cultured human cells, Miles ('60) described a variety of shapes assumed by the sex chromatin body. The peripheral clumps at the nuclear membrane ranged from a plano-convex body with a hollow center to a bipartite form connected to the nucleolus by a faintly-visible, linin thread.

In his cultures, Miles found that the percentage of peripheral clumps ranged from 51% to 85% in female cells. The few peripheral clumps found in male nuclei appeared to have half the volume of the clumps found in female cells. These female clumps were seldom seen in prophase and were entirely absent during the mitotic cycle until after the ensuing telophase.

Miles also reported that the sex chromatin disappeared when hypotonic saline was added to these cultures. This finding is in sharp contrast to the behavior of the heteropycnotic X chromosome which, at metaphase, is not significantly changed by exposure to hypotonic media. If the sex chromatin body of the interphase nucleus is identical to the heteropycnotic X chromosome, as current opinion suggests, then this observation paves the way for speculation concerning the physical properties of the heteropycnotic X chromosome during mitosis and during interphase.

In fixed and stained preparations from a variety of normal cell types, the sex chromatin body measures approximately 0.8 x 1.1 micra. It is typically seen as a planoconvex structure along the inner aspect of the nuclear membrane but may also appear as a U- or V-shaped body with its apex directed toward the center of the nucleus. Frequently, the sex chromatin assumes a
bipartite structure with its 'arms' resting on the nuclear membrane. When the sex chromatin body is not located peripherally along the nuclear membrane, as in the neurons of the cat studied by Barr and Bertram, it tends to assume a spherical shape. Unfortunately, electron microscopic observations have not, as yet, yielded any additional significant information on sex chromatin morphology (James, '60, cited by Barr, '66).

b) Staining Characteristics

The staining characteristics of the sex chromatin body, which depend upon its DNA content, are the same as those for chromatin and other nuclear chromocenters. It is for this reason that efforts to produce a specific stain for sex chromatin have been largely unsuccessful, although some innovations on Guard's method made by Beckert and Garner ('66) may prove to be fruitful.

According to Culling ('66), Basic dyes such as cresyl violet, thionin, gallocyanin, methyl green, basic fuchsin, orcein and pinacyanole may be used to demonstrate sex chromatin, presumably because of their affinity for the phosphate radical in the DNA molecule. However, the only really specific method for chromatin is the Feulgen reaction for nuclear DNA. In sex chromatin studies, this method has the advantage of detecting this chromocenter more readily since the nucleolus which remains unstained is less likely to obscure the sex chromatin body. Also, in various types of smear preparations, the sex chromatin body is not likely to be masked by the
RNA-containing bacteria.

Hematoxylin and eosin staining has also been used successfully for the demonstration of sex chromatin and the non-specific staining of the nucleoli in these preparations may be avoided by pretreatment with ribonuclease.

It might be noted that in addition to its DNA component, the sex chromatin body may, at least in some tissues, also contain RNA. In nuclei from the adrenal cortex of female cats, James ('60) demonstrated the staining of the sex chromatin body with gallocyanin and methylgreen-pyronin following treatment with DNA-ase. A similar chromocenter was not observed in preparations treated with DNA-ase and RNA-ase followed by gallocyanin staining.

c) Cytological Orientation

There is general agreement that the sex chromatin body is usually found in a peripheral region of the nucleus adjacent to the nuclear membrane, although the reason for this preferred position remains to be found. It should be kept in mind, however, that because of limitations in resolving power of the light microscope, sex chromatin in spherical nuclei, if randomly located at the nuclear membrane, would appear to have a peripheral location in about fifty per cent of the cells. Miles ('61) calculated that for buccal smear preparations, the sex chromatin, if randomly located at the nuclear membrane, should appear at the periphery of the nucleus in approximately forty per cent of the sex-chromatin-positive cells. His actual count, however, revealed that over eighty per cent of the sex chromatin clumps in these cells were
located at the nuclear membrane, a result, for which there is still no adequate explanation.

In nerve cells, the position of the sex chromatin body may vary from cell to cell in a particular neuronal group, from one region of the nervous system to another within an individual, and from one species of mammal to another (Barr et al., '50; Moore and Barr, '53).

In non-nervous tissues, Graham ('54) showed that the intranuclear location of the sex chromatin body may vary with the developmental stages of the cell. In the cat, the sex chromatin body tends to assume a more peripheral position in the course of embryonic life, reaching its adult pattern when the kitten is three weeks of age. During the maturation of the nervous system of the cat, however, the sex chromatin displays a reverse tendency and when the adult pattern is reached at two months of age, the greatest proportion of cells exhibit a juxtanucleolar position of the sex chromatin body.

In a similar manner, the position of the sex chromatin body in the nucleus may vary in response to pathological or experimental conditions (Barr and Bertram, '49). This movement, which may also be observed in living cells, indicates that the sex chromatin body is not in any way anchored to a particular region of the nucleus nor to a particular nuclear structure but, whether this movement is the passive result of other cellular rearrangements or whether the sex chromatin body is capable of initiating and maintaining an independent migration throughout the nucleus, remains to be determined.
d) Frequency

Although the nuclear sexing technique is highly subjective, there appear to be some real variations in the frequency of sex chromatin which depend upon the cell type, the nature of the preparation and the mitotic activity of the tissue. Sections of nervous system, for example, which can be cut at a thickness of twelve micra may yield counts of sex-chromatin-positive cells up to 95%. In other tissues, however, depending on the criteria used to identify the sex chromatin body, the counts may range from 20% to 96% in female cells and from zero to 30% in male nuclei except in those species with unusual sex chromosomes to be discussed below (Graham and Barr, '52).

The use of whole mounts of thin membranes avoids the problems inherent in the study of tissue sections and, as could well be expected, substantially higher counts were reported by Graham ('54) for the cat amnion and by James ('60) for the mesentery of the rabbit. Although smear preparations appear to have the same advantage as whole mounts in permitting the observer to view the entire nucleus, they generally suffer from the inclusion of many unhealthy cells and bacterial contamination. It is not surprising, therefore, that nuclear sexing studies based on buccal smears, for example, yield wide ranges for the incidence of sex-chromatin-positive cells.

The variation of sex chromatin with mitotic activity has attracted considerable interest in recent years. In attempting to account for the absence of sex chromatin in a number of female cells grown in tissue culture, James
suggested that many of these cells have an abnormal karyotype. Another explanation was advanced by Miles ('60 cited above) who found that the incidence of sex-chromatin-positive cells varied inversely with the mitotic activity of the culture so that female cells were not sex-chromatin-positive throughout interphase. Miles's explanation was endorse by Therkelsen and Petersen ('62) who found a low incidence (60%) of sex-chromatin-positive cells in the logarithmic- and a high incidence (almost 100%) of sex-chromatin-positive cells in the post-logarithmic growth phases of fibroblasts from human embryos and adults.

These investigators tried to explain this inverse correlation between mitotic growth activity and the frequency of sex-chromatin-positive cells by suggesting that the sex-chromatin-negative cells might be in a period of DNA synthesis and that, since this period is likely to be a greater fraction of the total intermitotic time in the logarithmic than in the post-logarithmic growth phase, a low incidence of sex-chromatin-positive cells could be expected in the logarithmic growth phase. Presumably, the heteropycnotic X would uncoil during the S phase and the nuclei undergoing DNA replication would appear to be sex-chromatin negative.

In 1964, DeMars reported that 90% to 95% of the cells were sex-chromatin positive in maximal density populations of human fibroblasts while in rapidly proliferating populations, only 10% of the cells exhibited sex chromatin. In those cultures where the cell density increases, the mitotic index
decreases so that the rise in sex-chromatin frequency could be the result of decreasing mitotic activity. Klinger ('66) was able to maintain a nearly constant mitotic rate while increasing the cell density of the culture and, by so doing, showed that the rise in sex-chromatin frequency was not due to the mitotic activity alone. In a further study on cultures derived from human females, Klinger and his colleagues found that sex chromatin was as frequent in nuclei during the S phase as in those before (G1) and after (G2) DNA synthesis. Moreover, there was no difference in the average DNA content between the sex-chromatin-positive nuclei and the sex-chromatin-negative nuclei of the same phase. In summarizing these results, Klinger concluded that the negativity of the female nuclei in these cultures could not be due to the fact that the heteropycnotic X chromosome, which is thought to form the sex chromatin body, uncoils during DNA replication.

A recent report by Therkelsen and Lamm ('66) showed that the frequency of sex-chromatin-positive cells was statistically higher among DNA-synthesizing than among non-synthesizing, cultured human cells. While this result invalidates the notion that cells in the S phase are sex-chromatin negative, it does suggest that the frequency of positive cells is different in different mitotic phases. Their preliminary studies of synchronized cultures showed that a steep fall in the frequency of positive cells occurs simultaneously with the first burst of mitosis. These investigators suggest that this result can be explained on the assumption that all post-mitotic (G1) cells are
sex-chromatin negative, as originally proposed by DeMars ('64) or, possibly, that all cells are sex-chromatin negative in late S phase and G2 phase.

From the brief review just given, it can be seen that the variation in sex chromatin frequency with the mitotic cell cycle is still an open question. It is hoped that future investigations in this area will elucidate the physiological role of heterochromatin in the life cycle of the cell so that we may gain some insight into the genetic activities of the sex chromosome complex.

Although there have been numerous reports linking reversible alteration in sex chromatin frequency in human oral and vaginal smears with fluctuations in physical and mental health, seasons of the year, toxic reactions and allergies, ACTH treatment, menstrual cycles etc., none of these has received confirmation. Similarly, a report of a size reduction in the sex chromatin body in the cells of the buccal mucosa following treatment with antibiotics awaits verification.

2. Characteristics of the Drumstick Appendage

Sexual dimorphism represented by a 'drumstick appendage' was demonstrated by Davidson and Smith ('54) in the polymorphonuclear leucocytes from the peripheral blood of human females. Since then, these dense-staining chromocenters have been found in monocytes and metamyelocytes (Davidson, '66) but it has not been possible to use their occurrence in these latter cell types for sexing blood films. Sexual dimorphism has been reported to be absent in myeloblasts and erythroblasts. It is hoped that further studies of
these blood cell progenitors will provide some evidence on the question of how single-X inactivation gives rise to the natural mosaicism seen in female mammals.

Although an absolute frequency of the drumstick in the human female has not been determined, a rough estimate indicates a frequency of about one drumstick in 36 neutrophils.

That the drumstick appendage is not a fixation artifact has been shown by its presence in wet films. Moreover, the extrusion and re-entry of the drumstick into the nucleus has been described in phase contrast observations. Unlike the sex chromatin body, an increased frequency of the drumsticks has been reported at birth but a subsequent decrease with advancing age remains to be confirmed.

When allowance was made for the flattening of the head of the drumstick in blood film preparations, Davidson ('66) calculated the volume of the drumstick appendage to be comparable to that of the sex chromatin body.

It is the relationship of the drumstick appendages to the sex chromosomes that has provided the strongest argument for the common assumption of an identity between the sex chromatin body and the drumstick appendage. In a typical case of Turner's syndrome, where the sex chromosome constitution is XO, the leucocyte drumsticks are absent. They may, however, be present in relatively low frequencies if mosaicism of the XX/XO type is present. Similarly, in Klinefelter's syndrome with its XXY constitution, single drum-
sticks may be found although their frequency will be lower than in the normal female.

In contrast to the sex chromatin body which increases in frequency and also forms multiple masses corresponding to the number of X chromosomes exceeding 1/2 the ploidy, a simple increase in the number of X chromosomes does not give rise to a significant increase in the frequency of drumsticks. And, although cells with double and triple drumsticks have been found, they are extremely rare. Similarly, in the presence of a Y chromosome, increasing numbers of X chromosomes will be accompanied by an increase in the number of sex chromatin bodies per nucleus while both the frequency of the drumsticks and their number per nucleus will remain unaltered.

Typical drumstick appendages have been found in the leucocytes of different mammals where their presence in females forms the basis of a nuclear sexual dimorphism in species belonging to the Primates, Chiroptera, Lagomorpha, Carnivora, Perissodactyla and Artiodactyla. These drumsticks appear similar in form to those of man and their size ranges from 1.3 micra in sheep, rabbits and bats to 1.5 micra in goats, horses and dogs. Their frequency varies from 7.0% in the rabbit to 0.4% in the giant panda. In the mouse, hamster, guinea pig and chinchilla, multiple, pedunculated nodules in both sexes preclude sexing the leucocytes.

In metatherian species, no sexual dimorphism could be found in the leucocytes from the kangaroo or the wallaby. In birds, too, considerable doubt
exists as to whether chromatin nodules can be recognized in the leucocytes (Ashley and Theiss, 1959).

While considerable evidence from studies on human cells has accumulated in support of the idea that the drumstick appendage and the sex chromatin body are homologous structures, some discrepancies have been noted (Ashley and Jones, '58). Disagreement between the incidence of sex chromatin and the occurrence of drumsticks in an individual has sometimes been explained in terms of faulty techniques and, more recently, by Ford ('63) and other investigators in terms of a mosaicism with respect to the sex chromosome constitution found in human females with sex chromosome abnormalities. A more divergent point of view has been advanced by Beckert ('62) who does not regard the drumstick appendage and sex chromatin body as homologous nuclear structures. Instead, he claims that the drumstick is associated with the homogametic sex while the typical sex chromatin body is associated with the female sex.

Whatever the case may be, the term, 'sex chromatin', as it is used today includes both the Barr bodies found in a variety of tissues as well as the drumstick appendages of the polymorphonuclear leucocytes.

B. The Distribution of Sex Chromatin in Mammals

Although nuclear sexual dimorphism is widespread in the Mammalia, there is considerable variation in sex chromatin patterns from one order to another, from one species to another, and, in some cases, within the cells
of the same individual. The reason for this variation lies in the fact that while some nuclei are open-faced or vesicular (as in nerve or buccal mucosal cells) where autosomal chromatin is virtually absent, other nuclei contain coarse, autosomal chromatin (as in some kidney or hepatic cells) making it extremely difficult to distinguish the chromatin derived from the sex chromosomes from that derived from the autosomes. Occasionally, nuclei contain so much coarse chromatin or numerous chromocenters, that the presence of sex chromatin, if it exists, is obscured and it becomes impossible to determine any nuclear sexual dimorphism. Sometimes, too, the chromatin is so condensed (as in some splenic and hepatic cells) that sex chromatin cannot be identified. Such cells are referred to as pycnotic and the nuclei of poorly-fixed tissues will also have this appearance.

In man, the presence of sex chromatin is most clearly illustrated in oral epithelial cells (Marberger et al., '55) and in cells of the zona fasciculata of the adrenal cortex (Moore, '66). In some tissues whose cells have a rather coarse chromatin pattern, as in the thyroid epithelium, the incidence of positive cells in males is as high as 21%.

Of particular importance for the development of the sex chromatin theory are the observations of Ohno and his co-workers ('61b; '62b) who have noted an absence of sex chromatin in human oocytes where both X chromosomes are isopycnotic with the autosomes.
In apes, a nuclear sexual dimorphism, resembling that in humans, was found in oral epithelial cells and in cultured dermal cells. Similarly, the Rhesus monkey and several species of Marmoset also possess a sex-specific chromocenter although it is more difficult to distinguish than in other primates because of the coarse chromatin pattern in these species.

Different tissues in species belonging to the order, Carnivora, display a nuclear sexual dimorphism which is most striking in neurons where the position of the sex chromatin body varies considerably from species to species. In non-nerve cells the disposition of sex chromatin is similar to that of primates.

Among species belonging to the order Artiodactyla, a nuclear sexual dimorphism has been identified in the nerve cells of domestic cattle, deer, goats, sheep and swine. The sex chromatin body in other cell types, if present, is masked by a coarse chromatin pattern. The oral epithelial cells of the American bison, for example, have been examined but sexual dimorphism was not recognizable in these cells owing to the presence of multiple chromocenters in both sexes.

Of special interest, is a study conducted by Moore, Graham, and Barr ('57) in which the nuclear sexing technique was applied to tissues of the bovine freemartin - a sterile heifer born as a co-twin with a normal male. Although there was no doubt concerning the genetic "femaleness" of the freemartin when the study was undertaken, the stability of the sex chromatin
body in a 'foreign' hormonal environment posed a provocative question. These investigators were able to demonstrate the presence of a typical sex chromatin body in various nerve cell nuclei in six freemartin calves and in two normal female calves between one week and four months of age. The nuclear chromocenter was absent in the male co-twins and in four normal male calves at a corresponding age. These observations, therefore, were consistent with the theory advanced by Lillie ('16; '17) that the freemartin is a genetic female that has become masculinized under the influence of a hormone from a male twin. Moreover, the sex chromatin body in these freemartins was not altered in any morphological manner following early exposure to hormones capable of altering other sexual characteristics. This demonstration of sex chromatin stability provides comfortable reassurance in the application of the nuclear sexing technique in general medical practice.

An alternate view, namely, that the freemartin condition may be caused by sex chromosome mosaicism found some support in the work of Ohno et al. ('62c). These investigators found a number of female (2A-XX) diploid metaphase figures in the testes of bulls born as co-twins to freemartins. Apparently the 2A-XX germ cells remain in the seminiferous tubules as primordial germ cells without differentiating into oocytes or spermatocytes. The erythrocyte chimerism, which is well known in dizygotic cattle twins, was thought to be accompanied by germ cell chimerism in these cases. The possibility of a reverse migration of male germ cells across the anastomosed chorioallan-
toic vessels from the normal male twin into the freemartin has given rise to speculation that a germ cell chimerism may be responsible for the freemartin condition. However, the possibility that sex chromosome mosaicism may come about by mechanisms other than germ cell chimerism has been suggested recently and a renewed controversy is currently underway concerning the origin of freemartinism in cattle.

Among species belonging to the order, Perissodactyla, sexual dimorphism was found in nerve cells and in intestinal epithelial cells of the horse and in skin biopsies from the horse, donkey, mule and zebroneky.

In Insectivora, species, such as the mole and shrew, have a recognizable sexual dimorphism in nerve cell nuclei which appears to be lacking in the hedgehog.

In the order, Chiroptera, the bat has a detectable sexual dimorphism in spinal cord neurons. The nine-banded armadillo (Edentata), on the other hand appears to be lacking nuclear sexual dimorphism, although sex differences have been reported in buccal cells, cardiac cells and embryonic connective tissue cells in this species.

A different type of sexual dimorphism has been found in the rabbit (Lagomorpha) where Purkinje cells of the female contain two sex chromatin bodies while the same cells in males have only one such chromocenter. In addition, the nuclear sexual dimorphism characteristic of most mammals was found in liver, mesothelial and pancreatic islet cells and in cultured
fibroblasts.

It seems ironic that rodent species, such as the mouse, which have provided much information concerning the genetic characteristics of the sex chromosomes, should possess nuclei with such coarse chromatin patterns as to completely obscure any sexual dimorphism. This situation seems to prevail in cells that have been examined from the muskrat, prairie dog, gopher, ground hog, guinea pig, squirrel, bank vole and beaver. It might be noted in passing, that while Moore ('66) reported the absence of sex chromatin in the beaver, Davidson ('66) found a nuclear sexual dimorphism in this species based on the presence of the drumstick appendage.

The presence of multiple chromocenters in neurons and other cell types has complicated the detection of a nuclear sexual dimorphism in the rat. However, a sex-specific chromocenter has been identified in female rats in a variety of cells from the nervous system, kidney and liver. Interestingly, it is the embryonic cells of the species which most clearly reveal a sexual dimorphism. In squash preparations of liver cells, for example, Ohno et al. ('61a) were able to determine the sex of rat embryos prior to gonadal differentiation.

In 1959, Ohno and his colleagues made a most important discovery while examining regenerating liver cells of the Norwegian rat. In studying prophase figures in the female, they noted that the sex chromatin body of the preceding interphase was not resolved as the heterochromatic regions of two chromo-
somes but, rather, as a single, large chromosome condensed along its entire length. Moreover, the two sex chromatin bodies in the tetraploid cells of these preparations became resolved into two such condensed chromosomes at prophase. In contrast, neither the diploid nor the tetraploid nuclei in male preparations contained a condensed chromosome of comparable size. These investigators concluded that the sex chromatin body seen in interphase nuclei actually represents a single-X chromosome manifesting positive heteropycnosis along its entire length. The genetic implications of this single-X hypothesis for the derivation of sex chromatin will be discussed in the next section.

Sexual dimorphism was found in liver nuclei of the chinchilla and golden hamster but not in other cell types. It might be noted that both of these species possess the duplicate-type X chromosome. According to Ohno ('67), a clear-cut sexual dimorphism in these species should not be expected since "inactivation by heterochromatization extends to one-half of the functional X chromosome." He suggested that both male and female nuclei of the golden hamster should contain two sex chromatin bodies. In the male, one of these chromocenters should represent one-half of the duplicate-type, X chromosome and the other the very large Y chromosome (Ohno and Weiler, '62). Similarly, in the female, one of the chromocenters should represent one-half of one of the X chromosomes while another, larger chromocenter, should consist of an entire X chromosome.
The creeping vole, *Microtus oregoni*, possesses the triplicate-type X chromosome (Ohno et al., '63) along with a unique sex-determining mechanism. The female vole, originating from an XO zygote, has an XO sex chromosome constitution in its somatic cells. Yet the size of the single sex chromatin body in the interphase nuclei is approximately twice that found in species possessing the original-type (normal) X chromosome. Ohno explains this fact on the basis that heterochromatization has extended to two-thirds of the single, triplicate-type X chromosome in these cells. The male of this species, which has an XY sex chromosome constitution, displays two large chromocenters in its intermitotic nuclei. This condition is thought to result from the heterochromatization of two-thirds of the triplicate-type, X chromosome, as in the female, and the entire Y chromosome.

In the field or bank vole, *Microtis agrestis*, which has the quadruplicate-type, X chromosome, sexual dimorphism could not be detected in various cell types. Instead, the nuclei of both sexes contain two enormous chromocenters of approximately the same size. This species probably contains the largest X chromosome of all the placental mammals. It comprises 20% of the genome, while the Y chromosome of the male, which is also very large, makes up 12% of the genome. In considering the behavior of these elements during replication, Ohno ('67) inferred that one entire X chromosome along with three-fourths of the other X chromosome have become inactivated by heterochromatization in female cells and give rise to the two enormous
chromocenters found in the intermitotic nuclei. A similar inactivation is thought to occur in male cells in which three-fourths of the X chromosome as well as the entire Y chromosome become heterochromatic and are seen as two huge chromocenters in resting cells.

Among the metatherian mammals, the Virginia opossum, *Didelphys virginiana*, appears to be the only marsupial investigated for the presence of the sex chromatin body. Although Davidson in his review (cited above) noted the absence of sexual dimorphism based on leucocyte morphology in the kangaroo and wallaby, Graham ('56) reported the presence of a nuclear chromocenter in both sexes of the Virginia opossum. The sexual dimorphism noted in this species stems from the fact that the sex chromatin body was larger in female neurons (0.8 x 1.1 micra) than in male neurons (0.6 x 0.8 micra). A similar discrepancy in size was found between male and female sex chromatin in other cell types. These results were confirmed by Ohno et al. ('60b) in squash preparations of bone marrow cells. From their examination of prophase figures, these investigators interpreted the large chromocenter of females as a single-X chromosome and suggested that the smaller chromocenter found in male cells might represent the Y chromosome which is heteropycnotic during interphase.

Thus, in mammals, the female-specific sex chromatin body seems to be a characteristic of all nuclei in which the disposition of chromatin is not too coarse. The identity of the sex chromatin body, with a single-X chromosome
in female cells, is apparently associated with the XX/XY sex-determining mechanism in mammals.

C. The Derivation of Sex Chromatin and its Functional Significance

1. The Origin of Sex Chromatin

When Barr and Bertram discovered a distinct sexual dimorphism in the somatic interphase nuclei of the cat in 1949, mammalian cytogenetics was still in its infancy. It was quite natural, therefore, that these workers should seek an explanation for this phenomenon in the already well-developed cytogenetic model of Drosophila melanogaster. In this insect, the X chromosome is structurally divided into a proximal, heterochromatic region and a distal, euchromatic region. During somatic pairing, the heterochromatic regions of the two X chromosomes in female cells become closely paired chromocenters attached to the nucleolus. The genetic studies on this insect, which paralleled these cytological observations, implied that the heterochromatic region of the X chromosome was a genetically empty 'dummy' and that the X-linked genes were located almost exclusively in the euchromatic region. Thus, the Drosophila studies gave rise to the concept "once heterochromatin, always heterochromatin." Since this notion appeared unchallengeable, it was assumed that the mammalian X chromosome must also consist of hetero- and euchromatic regions.

It was in this context, then, that Barr and his coworkers suggested that the sex chromatin body of female mammalian cells represented the fused
heterochromatic regions of the two X chromosomes (Barr et al., '50; Moore and Barr, '53). This double-X hypothesis appeared to receive support from the occasional bipartite form of the sex chromatin noted by Klinger ('58) and from the demonstration of a large nuclear chromocenter in follicular cells of the mouse by Ohno et al. ('58). That the nuclei of male mammals lacked a chromocenter half as large as that of the female was dismissed as a mere technicality. Thus, the double-X hypothesis appeared to be entirely consistent with the XX/XY sex determining mechanism in mammals and also in accord with what was known in Drosophila.

In 1959, however, Ohno and his collaborators observed that only one of the X chromosomes in regenerating liver cells from female rats exhibited a positive heteropycnosis along its entire length while the X and the Y chromosomes in male cells appeared isopycnotic with the autosomes. In the tetraploid cells of these preparations, two heteropycnotic chromosomes were seen and these were interpreted as X chromosomes. The location and staining properties of these heteropycnotic bodies led Ohno to suggest that it was a single-X chromosome that formed the sex chromatin body of the preceding interphase. This single-X hypothesis for the origin of the sex chromatin body received support from later studies by Ohno and Hauschka ('60) in normal and tumor cells in female mice and by Ohno and Makino ('61) in prophase figures from human females.

Studies of DNA replication, using tritiated thymidine and autoradiography
of individual chromosomes in a number of placental mammals, indicated that late replication of DNA is characteristic of an individual X chromosome that behaves in a heterochromatic manner. In cultures from mouse embryos, for example, Galton and Holt ('65) were able to identify the Y chromosome in male cells and one of the X chromosomes in female cells on the basis of their late replication. In 1963, Schmid had also reported the asynchronous replication pattern of the heteropycnotic X or 'hot' chromosome in cultures of human female cells.

At about the same time, persons with XXX chromosome constitutions came under observation and the sex chromatin findings in these were inconsistent with a double-X hypothesis for the origin of sex chromatin. The single-X theory, on the other hand, was not only compatible with the sex chromatin disposition in these cases but was also found to be capable of diagnosing the genetic sex in a large variety of conditions caused by sex chromosome abnormalities (Barr, '60).

When the single-X origin of the sex chromatin body was put on a firm basis, the question naturally arose as to which of the X chromosomes in female cells was likely to acquire the property of heteropycnosis. Since both of the X chromosomes of oocytes are isopycnotic with the autosomes in the rat (Ohno et al., '60c; '61a) and in man (Ohno et al., '61b), it was logical to suppose that the heteropycnotic X chromosome of female somatic cells was of paternal origin. It is not surprising, therefore, that in the rat, Ohno et al.
('59) suggested that the paternally-derived X chromosome in female cells forms the sex chromatin body of the interphase nuclei since the male cells contain only a maternally-derived X chromosome. Ohno later abandoned this view when he and his coworkers ('60d) observed positive heteropycnosis of the X chromosome in the tetraploid cells of the $X^M$O mouse and suppression of X chromosome heteropycnosis in the $X^P$O mouse.

An alternative view, namely, that either the $X^M$ or $X^P$ chromosome may acquire positive heteropycnosis has gained increasing acceptance. Cytological evidence for a random inactivation of either X chromosome has been provided by a very interesting study of DNA replication in the female mule by Mukherjee and Sinha ('64). The normal diploid complement of the mule is 63 chromosomes, 32 of which are derived from the mother, *Equus caballus*, and 31 from the father *Equus asinus*. The maternally-derived X chromosome has a submedian centromere while the paternally-derived one has a subterminal centromere. In a blood culture with 33 metaphase figures showing heavy labeling of one chromosome, the 'hot' chromosome was identified as the X chromosome of *E. caballus* in 16 cells and as the X chromosome of *E. asinus* in 17 cells.

Presumably, once either the $X^M$ or the $X^P$ acquires heteropycnosis or isopycnosis during embryogenesis, this property is perpetuated in all of its descendants - a principle now referred to as 'fixed differentiation.' Cytological support for this concept has been provided by DeMars ('63a, b) who
showed that cultured polyploid human cells frequently display paired sex chromatin masses. Such a spatial orientation led him to deduce their derivation from a single heteropycnotic X chromosome of a normal, diploid cell thus reflecting a fixed differentiation of the X chromosome.

2. The Genetic Significance of Sex Chromatin

The idea that the phenomenon of heteropycnosis is equivalent to genetic "inertness" grew out of classic studies on the heterochromatic chromosomes and heterochromatic regions of chromosomes in various plants and insects such as Drosophila. It is obvious that the behavior of the mammalian X chromosome is quite different from the heterochromatic chromosomes in these lower forms. First of all, the mammalian X chromosome may be heteropycnotic or isopycnotic on the basis of chance alone and depending on the cell type in which it is located. Secondly, the mammalian X chromosome is entirely heterochromatic during male meiosis and entirely euchromatic during female meiosis. And finally, either the $X^M$ or the $X^P$ may assume the property of heteropycnosis during the interphase of somatic nuclei. Thus a temporary, heterochromatic condition need not necessarily reflect genetic emptiness. Ohno has expressed the view that heterochromatization is not the consequence of genetic deterioration but, rather, the "functional" state assumed by individual X chromosomes. He calls attention to the fact that there are still a few functioning genes on the Drosophila Y chromosome which is entirely heterochromatic in the true sense of the word. According to Ohno
"the clue to the single-X nature of the sex chromatin body lies in the ambivalent behavior of the mammalian X chromosome."

A similar view was expressed by Hsu ('62) who felt that neither of the X chromosomes in the mammalian female is genetically inert. The "inertness", he suggested, was a metabolic expression and "heteropycnosis is not a unique property of heterochromatin." In his autoradiographic studies, Hsu demonstrated a differential rate in RNA synthesis between euchromatin and heterochromatin and suggested that the so-called genetic inertness of heteropycnotic chromosomes may be largely the result of interference with the production of a special messenger.

There has been some speculation concerning the biological importance of X chromosome differentiation in the somatic cells of females. Barr ('66) suggested that the heterochromatization of the mammalian X in female cells permits a "dosage compensation" capable of modifying undesirable genetic differences between XX and XY individuals "while at the same time allowing mammals to take advantage of the biologically efficient XX/XY sex-determining mechanism." Ohno and his collaborators ('64a) have taken the view that X chromosome heterochromatization provides a mechanism in which a constant optimal ratio is maintained in somatic cells between the functional X chromosome and the autosomes. Thus, in placental mammals, the functional X chromosome material in both male and female somatic cells constitutes 5% of the genetic material in the haploid set. This 5% level is still
maintained in those species with duplicate-, triplicate-, and quadruplicate-
type, X chromosomes.

According to Ohno's scheme, specialization of the Y chromosome during
the course of evolution left the X-linked genes of the male in a hemizygous
condition and created a gross disparity between male cells with a single-X
and female cells with two X chromosomes. A need for effective dosage
compensation of X-linked genes in the female was met by the heterochromat-
ization of one of the X chromosomes early in embryonic life which may be
seen then as the sex chromatin body of the interphase nuclei.

At the beginning of this decade, English investigators (Russell, '61; Lyon,
'60; '62; '63) had formulated an inactive-X hypothesis in order to account for
certain mosaic effects in female mice heterozygous with respect to X-linked
genes affecting coat color. These females have patches of normal (wild-
type) color and patches of lighter (mutant) color which correspond to the
lighter color of the mutant males. These observations, coupled with the
finding of normal, fertile, female mice with an XO sex chromosome con-
stitution led Lyon ('62) to formulate the following theory:

"1. One of the two X chromosomes in the cells of normal
female mammals is genetically inactivated.

2. The inactive-X can be either the maternal or the
paternal one in different cells of the same animal.

3. The inactivation occurs early in embryonic development
and, once it has occurred, remains fixed throughout the
further development of each cell line."
Effects similar to the mosaicism described above and sometimes referred to as "variegated position effects" have been found in female mice heterozygous for some X-autosome translocations in which the wild-type alleles of certain autosomal coat color genes are translocated onto the X chromosome. In each case, the color of the patches corresponds to that of the mutant gene involved. According to the inactive-X hypothesis, then, the cells in the mutant color patches are descended from cells in which the X-chromosome carrying the translocated, wild-type allele was inactivated while the cells in normal-colored patches are descended from cells with the mutant gene inactivated.

When individuals with abnormal numbers of X chromosomes came under observation, the inactive-X hypothesis was extended so that the number of inactivated X chromosomes, or sex chromatin bodies, would be equivalent to the number of X chromosomes minus one-half the degree of ploidy. Thus, according to the theory, phenotypic abnormalities should not be expected in females with supernumerary X chromosomes. This expectation seems to be borne out in human females with multiple X chromosomes. However, slight abnormalities have been noted in human males with an XXY constitution (Klinefelter's syndrome) and became more pronounced in individuals as the number of X chromosomes increased (as in XXXXY individuals). Similarly, human females with an XO complement of sex chromosomes have shown serious phenotypic defects (Turner's syndrome) although such a finding was not expected on the basis of the Lyon hypothesis. These facts have been
interpreted by Lyon ('66) as an indication that the second X chromosome in man cannot be completely inactive at all stages of development. This later interpretation is in accord with the findings of Ohno et al. ('61b; '62b) who noted that both X chromosomes were isopycnotic in the germ cells of human females. It has been suggested that an incorrect dosage of X-linked genes in these cells may be responsible for the sterility found in individuals with anomalous X chromosome numbers.

Deductions made from the inactive-X hypothesis have been tested successfully in the mouse. Thus, a female mouse heterozygous for two non-allelic genes acting through the same cells and borne one on each X chromosome would be expected to show the manifestation of one or the other gene in all cells. Lyon ('63) was successful in breeding female mice that had the mutant, color gene, dappled (Modp), on one X chromosome and Cattanach's translocation (the wild-type allele of the gene for pink-eye, p, and albino, c,) on the other X chromosome. Although three kinds of color patches were possible (the wild-type color due to Cattanach's translocation, the white color due to the Modp gene, and the orange color due to the autosomal color genes, pp) only the wild-type patches and the white patches were actually observed indicating that either the mutant color gene, dappled, on one X chromosome or the wild-type allele of Cattanach's translocation on the other X chromosome were active in all cells.
This finding received support from a cytological study (Ohno and Cattanach, '62) of skin cells in female mice with a normal X chromosome and an X chromosome carrying Cattanach's translocation. In these cases, the autosomal insertion behaved as an integral part of the X chromosome—manifesting heteropycnosis whenever the X chromosome itself became heteropycnotic. Thus, the white patches in these animals were populated by cells containing a condensed X chromosome carrying the translocation, while in the wild-type patches, the translocation-bearing chromosome (which is 20% longer than the normal X in this species) was isopycnotic and behaved in the same manner as the euchromatic autosomes.

Conversely, it was expected on the basis of the Lyon hypothesis, that if the two genes were carried on the same X chromosome, then both genes should act in some cells and neither gene should be expressed in the remainder of the cells. This test was carried out with the two genes, tabby (Ta), and striated (Str) which affect the texture of the coat in mice. It was found that in female mice with the StrTa/++ sex chromosome constitution, both the Ta and Str genes were active in some patches and neither gene in other patches, whereas, in females with the Str+/+Ta constitution, the gene, tabby, acted only in the non-striated patches.

A further demonstration of the alternate action of the two X chromosomes was provided by Searle's translocation in the mouse in which a piece of X chromosome is inserted into one of the autosomes and, reciprocally, a
piece of the autosome is translocated onto the X chromosome. Females with Searle's translocation that were heterozygous for the mutant gene, tabby, displayed a wild-type phenotype instead of the mosaic effect predicted by the inactive-X hypothesis. A cytological study reported by Ohno and Lyon ('65) revealed that in 88% of the early prophase figures from diploid liver cells, the normal X chromosome manifested positive heteropycnosis. The normal X chromosome was identified in this species on the basis of its nucleolus-organizing ability (Ohno et al., '57). In 10% of the cells, one chromosome, half as large as the normal X chromosome, was positively heteropycnotic along its entire length while the normal X chromosome remained euchromatic. This smaller, condensed body was identified as the X chromosome which had received a small autosomal piece. In only 2% of the cells examined, were the autosome-bearing X chromosome and the X-portion of the X-bearing autosome both condensed. (It might be noted that the autosome which carried the non-centromeric portion of the X chromosome remained euchromatic even when the translocated portion of the X chromosome became heteropycnotic.) The authors argue, therefore, that females with Searle's translocation such that the mutant gene, tabby, is on the normal X chromosome and the wild-type allele is on the translocated X chromosome (Tx+/+Ta) can be expected to show the wild-type phenotype owing to the extensive heteropycnosis of the normal X chromosome. By the same token, females that have the gene, tabby, on the translocated X chromosome (TxTa/
++) can be expected to exhibit the tabby phenotype. Therefore, since either tabby itself or its wild-type allele on the normal X chromosome will be inactivated in most cells, females heterozygous for Ta should not be expected to show the variegated phenotype. Furthermore, in double heterozygotes where the gene, tabby, was located on one of the X chromosomes, and the coat-color, mutant gene, blotchy, on the other, either of these genes is fully expressed in female mice depending on which gene happens to be on the X chromosome involved in the translocation.

Ohno ('67) has interpreted this preferential inactivation of the intact X chromosome as incompatible with successful speciation in placental mammals. Inasmuch as this kind of X-autosome translocation deprives the female of its natural mosaic status, it represents a complete breakdown of the dosage compensation mechanism. Should a female become homozygous for this kind of translocation, presumably none of the four separate pieces would become inactivated, leaving the individual exposed to a double dose of all X-linked genes.

The inactive-X hypothesis appears to be substantiated in other mammalian species where X-linked genes which affect coat color are known. In both cats and golden hamsters, females heterozygous for these genes display a mosaic, tortoise-shell phenotype. Moreover, the male XXY cat also exhibits the mosaic, tortoise-shell color.
Recent studies on DNA synthesis utilizing the uptake of tritiated thymidine and autoradiography have provided a cytological correlation for some of the genetic observations made in mammalian species which display a phenotypic mosaicism in females heterozygous for X-linked genes. In the Syrian hamster, for example, Galton and Holt ('64) have described a late-labeling pattern of DNA synthesis in parts of both X chromosomes of the female. This finding corresponds well with that of Ohno et al. ('64a) who observed that one-half of one of the large, duplicate-type X chromosomes was heteropycnotic while the other X chromosome was entirely heteropycnotic in prophase figures from the female golden hamster.

Cytological evidence in support of the inactive-X theory has also been inferred from the asynchronous replication of the sex chromosomes of both sexes in the chinchilla by Galton, Benirschke and Ohno ('65). As in the Eurasian hamster, the X chromosomes of the chinchilla are of the duplicate type although the Y chromosome in this species is exceedingly small. Similarly, one of the X chromosomes in female cells, and the Y chromosome in male cells, were identified by Galton and Holt ('65) from their late-replication pattern of DNA synthesis in cultured embryonic mouse cells.

Another consequence of the inactive-X hypothesis is that since both males and females have only one functional X chromosome, they should have equal amounts of direct gene products that are capable of quantitative measurement. The most elegant evidence in support of the Lyon hypothesis was presented
by Grumbach et al. ('62) who found that eight out of nine human females with three or four X chromosomes had normal enzyme levels of erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) activity. The exception, an XXXX-female, had a slightly higher level of activity for this enzyme. Moreover, Beutler et al. ('62) were able to demonstrate two populations of erythrocytes in women that are heterozygous for a mutant allele responsible for G-6-PD deficiency. In one population, the enzyme was measured at the normal 100% level, while in the other, the level was nearly zero.

And finally, Davidson et al. ('63) demonstrated two distinct populations of cultured fibroblasts in skin biopsies from six heterozygous women carrying the normal G-6-Pd variant (B) and the G-6-Pd deficiency allele (A). Clones derived from single cell platings were found to contain either the normal B variant or the A variant. Since the skin specimens were very small (3 mm in diameter), the mosaicism apparently involves extremely small patches of cells and, presumably, X-inactivation occurred relatively late in development.

Although the evidence supporting the inactive-X theory has been accumulating, there remain some troublesome discrepancies. In particular, mosaicism has not been demonstrated for the Xg\(^a\) blood group resulting from an X-borne gene in humans. The red blood cells of males carrying the Xg\(^a\) (+ve) allele are agglutinated by suitable antisera whereas those of males with the Xg(-ve) allele are not. The fact that the Xg\(^a\)Y male reacts more strongly
to the antisera than the $Xg^aXg$ female and that the $Xg^aXg^a$ homozygotes react as strongly as the hemizygotes and more strongly than the heterozygotes has been taken by Ohno ('67) as an indication that dosage is compensated for the $Xg$ locus.

A further indication that the $Xg$ locus undergoes inactivation (Lyon, '66) is suggested by the fact that three instances of phenotypically $Xg$-negative mothers gave birth to $Xg^a$-positive sons. Since the genotype of these mothers had to be $Xg^aXg$, apparently the $X$ chromosome carrying the $Xg^a$ allele became inactivated at a very early stage in a progenitor cell that gave rise to the entire blood-forming system.

However, the fact that two distinct populations of erythrocytes have not yet been demonstrated in females heterozygous for the $Xg^a$ allele, may be due either, to technical difficulties, or to differential rates in the formation of the two cell types, to unequal life-spans of the cell types, or to a transfer of a gene product from cell to cell.

Thus, although there is no clear-cut example in mammals indicating that an $X$-linked gene has completely escaped inactivation, the possibility always exists, as in the case of the functional gene, **bobbed**, on the *Drosophila* Y chromosome that at least in man, some of the $X$ linked genes are not entirely inactivated by the heterochromatization of the $X$ chromosome.

D. The Distribution of Sex Chromatin in Species Showing Female Heterogamety
1. Insects

In 1945 S. G. Smith advanced the idea that the genetic sex of individuals could be determined by means of heteropycnotic chromocenters in the resting nuclei of somatic cells. His conclusion was based on a study of the larval cells of the spruce budworm in which one dense chromocenter was found in female interphase nuclei but lacking in the nuclei of males. Since female heterogamety prevails in the order, Lepidoptera, Smith identified the dense chromocenter as the single Z chromosome in positive heteropycnosis.

2. Birds

A similar situation prevails in the class, Aves, where female heterogamety of the ZW/ZZ type is in operation. Measurements on the DNA content of avian erythrocytes given by Allfrey et al. (’55) indicate that the DNA content of several of the avian species is approximately half that found in diploid nuclei from various placental mammals (7.0 mg x 10^-9/cell). A comparative study of the Feulgen stain content of various cell types in placental mammals, reptiles and birds by Atkin et al. (’65) is in substantial agreement with the biochemical data presented by Allfrey and his colleagues.

Since the avian Z chromosome comprises almost 10% of the genome (Ohno et al., ’64b). While the X chromosome of placental mammals makes up only 5% of the genome (Ohno et al., ’64a), it would seem that birds require an effective dosage compensation mechanism even more than mammals.
a) **Sex-linked Genes**

All of the known Z-linked traits in the well-studied avian species are morphological in nature, usually affecting plumage color, so that any homology between Z-linked genes is based on finding extreme similarity in phenotypic expression rather than on the identification of the direct product of the gene in question.

Sex-linked albinism has been reported in the Japanese quail by Lauber ('64) and in the domestic fowl, turkey, canary and budgerigar by Hutt and Mueller ('42). These authors found that the effects of the sex-linked mutation, al, causing albinism in the domestic fowl, differed from those in the albinotic turkey in the following respects: (1) the fowl had some melanin in the pigment epithelium in the posterior eye which is lacking in the turkey, (2) the viability of albinotic embryos and pullets is unimpaired in the fowl but sub-normal in the turkey, and (3) vision is apparently normal in the fowl while complete blindness results in the turkey.

Because of the difference in the distribution of the melanin in the eye, Hutt and Mueller ('42) did not think that the sex-linked genes causing imperfect albinism in the two species are homologous. Attempts to test whether these genes occupy homologous loci in the Z-chromosomes by interspecific matings of albino fowl and albino turkeys have not been successful. Although the linkage relationships of the gene, al, have been found for the domestic fowl, the only other known sex-linked gene for the turkey, Narragansett, has
not been proven to be homologous with any Z-linked gene of the fowl. Thus it has not been possible, as yet, to compare the linkage relationships of al in the fowl with those of al in the turkey.

Interestingly, the sex-linked albino gene, al, does not appear to show the dosage effect typical of other sex-linked genes in avian species. In the fowl, quail and turkey, the hemizygous female is just as white as the homozygous male, carrying two doses of al. While sexual differences in the expression of a sex-linked gene constitute prima facie evidence that a dosage compensation mechanism is absent or incomplete, the apparent absence of such sexual differences does not constitute proof that a dosage compensation mechanism exists because an amorphic mutant allele (amorph) would not be expected to show a dosage effect under any circumstance. According to Cock ('64), "there is no positive evidence to suppose that dosage compensation occurs outside Drosophila and Mammals."

b) Sex Chromatin

Thus, while there are many known sex-linked color genes, none of these shows the kind of mosaic effect in heterozygotes which is typical of similar X-linked genes in mammals. Since it was generally agreed that dosage compensation does not exist in birds, it was surprising when, Kosin and Ishizaki ('59) using hematoxylin, RNA-ase and Feulgen preparations, reported the presence of sex chromatin in the intermitotic nuclei of three-week-old New Hampshire chickens. They found that the frequency of this chromocenter in
females was at least ten times its occurrence in males, ranging from 22% in duodenal muscle cells to 52% in the epidermal cells of growing feathers.

Conflicting results were reported in the same year by Ashley and Theiss ('59) using the Feulgen method and routine hematoxylin and eosin preparations. These investigators were unable to detect any sexual dimorphism in cells from the epidermis, liver, heart or epithelium from the alimentary tract of the domestic fowl, duck, parrot and parakeet. Miles and Storey ('62) also reported the absence of sexual dimorphism based on a nuclear chromocenter in white Leghorn chicks ranging in age from two weeks to two months. These authors studied thionin-stained cultures of heart, spleen and kidney cells as well as sections of heart, spleen, liver, skin, feathers and smooth muscle of the duodenum.

On the other hand, Ohno et al. ('60a) reported a nuclear sexual dimorphism in Feulgen squash preparations of liver and skin cells from white Leghorn embryos, ten to seventeen days of age. These workers recognized a large, bipartite, nuclear chromocenter (almost 2μ in length) only in female cells. (The sex of these chicks was determined from the number of Z chromosomes in metaphase figures.) Early and late female prophase figures revealed the presence of one chromosome, positively heteropycnotic along its entire length. Since this phenomenon was never observed in male nuclei, the bipartite sex chromatin body of female interphase nuclei was interpreted as representing a single, mediocentric Z chromosome in positive hetero-
pycnosis, whose arms are bent back upon each other. Although these investigators had previously suggested that the sex chromatin seen in mammalian nuclei was derived from the paternal X chromosome (Ohno et al., '59), they abandoned this position as a result of this study since both male and female birds possess a paternally-derived Z chromosome.

In 1961, Moore and Hay confirmed Kosin and Ishizaki's ('59) observations in the white Leghorn. They reported the first positive finding of a nuclear sexual dimorphism in an adult bird. The sex chromatin in female epidermal cells and smooth muscle cells of the duodenum was observed to be fairly distinct while no clear-cut dimorphism could be detected in epithelia of liver, kidney or duodenum nor in nervous tissue, cartilage and skeletal or cardiac muscle.

Ishizaki and Kosin ('60) performed a convincing experiment in which they removed a piece of area opaca from New Hampshire eggs at 48 hours of incubation. The eggs were then sealed and incubated for an additional seven days. Each "biopsy" specimen was fixed in Davidson's solution followed by Harris's hematoxylin staining of 5μ sections. Cytological examination of the sex chromatin patterns from the biopsies enabled these investigators to predict the sex of each embryo. Of the sixteen embryos that survived the additional incubation, nine were diagnosed as males and seven as females by inspection of the gonads. When this sex classification was compared with that of the same embryo, predicted at two days of incubation, a perfect
agreement was found.

Ishizaki and Kosin ('60) also found a clear-cut nuclear sexual dimorphism in amniotic cells from chick embryos 9 to 14 days of age. In females the sex chromatin frequency ranged between 45% and 68% while the corresponding range in males was from 3% to 12%. A similar "bi-modal" pattern of sex chromatin distribution was observed in amniotic cells from 5-day-old embryos in which sex could not be established even following histological examination of the gonads. In four of the embryos, the frequency of sex chromatin ranged from 63% to 76% while in seven other embryos, the corresponding range was 8% to 23%.

An examination of fifteen blastoderms from unincubated eggs, revealed the sex chromatin frequencies to be sufficiently discrete so as to permit identification of seven blastoderms as "females" and eight as "males." Similarly, out of eleven embryos incubated for two days, five showed large complements of sex chromatin in 65% of the cells from the area opaca (range = 59% to 71%) while in the other six, a comparably large chromocenter was found in only 11% of these cells (range = 7% to 16%). The existence of such a bi-modality in sex chromosome frequency in embryos as young as the gastrula stage led Ishizaki and Kosin to exclude the involvement of sex hormones in the formation of sex chromatin in the chick.

Van Limborgh ('64) verified Ishizaki and Kosin's observations on the amniotic epithelium of the white Leghorn chick embryo. Using Carnoy's
fixative with Delafield's hematoxylin staining of 10μ sections. Van Limborgh showed that the incidence of sex chromatin in 4 and 15 day-old embryos ranged from 40.0% to 70.5% in female nuclei while the corresponding range in male nuclei was from 17.0% to 26.5%.

Van Limborgh also extended his observations to duck embryos (Khaki Campbell) at 10 - 12 days of incubation and at 5 days of incubation. In addition he performed "biopsies" (similar to those of Ishizaki and Kosin) in which a small piece of amnion was removed from twenty duck embryos at 80 hours of incubation. The eggs were then sealed and incubated for an additional 6 to 18 days. Cytological study of the biopsies showed that eight embryos had a sex chromatin frequency ranging from 55.0% to 69.5% while the corresponding values for the other twelve embryos ranged from 16.5% to 28.0%. Microscopic examination of the gonads of these embryos following continued incubation showed that the gonadal sex of the former embryos was female and that of the latter male. Van Limborgh felt that the nuclear sexual dimorphism in both chick and duck embryos was so clear-cut, that he used it as the sole criterion for determining the genetic sex in a later study (Van Limborgh, '68) on the distribution of primordial germ cells in white Leghorn embryos at 100 hours of incubation.

c) Sex Chromosomes

That the female is the heterogametic sex in Aves, has been known for some time but the type of sex-determining mechanism in this class has only
been elucidated within the past ten years. The numerous microchromosomes
in most avian karyotypes (2n = 58-82) prevented the unequivocal identifica-
tion of the sex elements in many species of birds. Although these micro-
chromosomes have been regarded as supernumeraries by some researchers
and thought to be unnecessary for the normal, functioning avian genome,
Ohno et al. ('62a) have shown that, at least, twelve of these microchromo-
somes are important genetic components inasmuch as they participate in
nucleolus-formation.

(1) The Z Chromosome

Improved cytological techniques have enabled Ohno and his colleagues
('60a) cited above, to identify the ZZ sex chromosome complement of male
cells in the domestic fowl although they were unable to demonstrate a W sex
chromosome with certainty in female cells. In somatic prophase figures
from embryonic skin and liver cells, neither of the Z chromosomes exhibited
positive heteropycnosis in male cells while one positively-heteropycnotic
chromosome, identified as the Z chromosome, was found in female prophase
figures.

In 1961, Ohno found that in both the somatic elements of the embryonic
testis in the white Leghorn and in the spermatogonia of the adult testis, the
ZZ bivalent remained euchromatic. Similarly, in the early prophase of
oogonia in this species, Ohno found that the single Z chromosome was not
heterochromatic. Only in the embryonic follicular cells could a positively-
heteropycnotic chromosome, identified as the Z chromosome, be distinguished at the periphery of the nucleus. In contrast to the extensive occurrence of this phenomenon in skin and liver cells, only 50% of the follicular cells displayed this heterochromatic element. This investigator was unable to determine whether the Z chromosome existed singly in these preparations or as a ZW bivalent.

In artificially-induced testicular teratomas in the white Leghorn strain, Guthrie ('62) was able to corroborate the findings of Ohno and his group. He showed that the ZZ chromosomal pairs in the diploid cells of these teratomas closely resembled those of the male host. Moreover, he did not see anything akin to sex chromatin in his preparations.

The karyotype of Gallus domesticus was further elucidated by the electron microscopic study of testicular chromosomes by Ford and Woolam ('64) who found that the number of chromosome pairs at meiosis, probably 40, was constant and that the macro- and microchromosomes differed only in size, not behavior. Their behavior was identical at mitosis and meiosis. They also found that the short bivalents contained less material per unit length than the long ones and suggested that this may be the reason why these chromosomes could not always be resolved by light microscopy when fully contracted.

A comparative cytological study by Stenius et al. ('63) of the karyotypes of the domestic fowl, pheasant (Phasianus colchicus) and turkey (Meleagris
revealed the presence of a Z chromosome, apparently identical in all three species, while the autosomal complement of the pheasant and turkey differed radically from that of the chicken. Intergeneric crosses between pheasant and chicken usually produce sterile hybrids with a high mortality rate. Similarly, crosses between turkey and chicken produce interfamilial male hybrids only and very few of these survive. Stenius and his coworkers, however, predicted that interfamilial crosses between pheasant and turkey should prove to be more successful because of the very similar autosomal complements of these species. Apparently, this proved to be the case because Ohno et al. ('64b) reported that an interfamilial hybrid resulting from a cross between *M. gallopavo* (male) and *P. colchicus* (female) was found to have a chromosome complement indistinguishable from that of either parental species.

(2) The **W** Chromosome

A W sex chromosome was, at last, identified in the domestic fowl, *Gallus domesticus*, by Schmid ('62) and later confirmed by Owen ('65). Unfortunately, only metaphase figures from spleen, thymus and bone marrow were used in Owen's study so that he was unable to describe the behavior of this element prior to and during early prophase. A W chromosome was also demonstrated by Rothfels et al. ('63) in the budgerigar, *Melopsittacus undulatus*. In both the canary, *Serinus canarius*, and pigeon, *Columba livia domestica*, Ohno et al. ('64b) found a W chromosome with two-thirds the
length of the Z chromosome. Krishan et al. ('65) were able to demonstrate a W sex chromosome in the great horned owl, Bubo v. virginianus, while in the red-breasted parakeet, Psittacula s. striatus, the W chromosome was identified by Ray-Chaudhuri et al. ('66). Similarly, Vegni-Talluri and Vegni (65) found a W chromosome of about half the size of the Z chromosome in the Japanese quail (Coturnix coturnix japonica).

In summarizing these findings, therefore, it may be said that the W chromosome has now been identified in eight species belonging to six different orders of the avian subclass, Carinatae. Although the type of sex-determining mechanism that operates in the Ratitae remains to be explored, it seems reasonable to assume that for Carinatae, a sex-determining mechanism of the ZW/ZZ type is in operation and that a physical basis for strictly maternal inheritance has been demonstrated.
A. Preliminary Investigations

A preliminary investigation in this laboratory on the incidence of sex chromatin in the domestic fowl, *Gallus domesticus*, revealed that no nuclear sexual dimorphism was apparent in the hepatic epithelium from brown Leghorn embryos at 8, 10, 14, 16, 18, 19, and 20 days of incubation and at the time of hatching. Similar results were obtained in squash preparations and sections of liver from a two-year-old rooster and a one-year-old poulard.

A variety of techniques were used for the fixation and staining of these tissues. It was found that fixation in 95% ethanol resulted in better staining with the thionin technique of Klinger and Ludwig ('57) than fixation in Davidson's solution. However, Davidson's solution proved to be the better fixative when blocks of liver tissue were used. Most of the tissues were stained with thionin, pinacyanole and the Feulgen method. For each technique employed, male and female tissues from each age group were arranged back-to-back and processed simultaneously. In order to determine the effectiveness of these stains, smears of buccal mucosa from males and females were subjected to the same treatment as the embryonic chick tissues.

The Feulgen technique produced too much variation in nuclear size and 'apparent' amount of chromatin in cells from the liver, testis and allantois of the chick embryo. And, since the technique produced a very granular disposition of the chromatin, it was discontinued. Both pinacyanole and
thionin-stained sections and squash preparations of poulard liver nuclei presented an extremely varied picture of the chromatin. Similarly, in thionin-stained squashes of liver cells from a rooster, many of the nuclei contained single, large, chromatin masses, some contained two, densely-stained bodies, and some showed only pale, homogeneous concentrations of chromatin.

Sections from the spinal cord of a male chick at hatching gave different results with thionin and pinacyanole. The ventral horn cell nuclei and Nissl substance remained unstained with thionin while other nuclei showed very discrete dense chromocenters. When hydrolysis was omitted from the thionin preparations, good nucleolar staining was observed. Pinacyanole, on the other hand, stained the nucleoli and Nissl substance of the ventral horn cells while the nuclei of surrounding cells remained extremely pale. There was also an interesting metachromasia in that the nucleoli stained a deep blue while the Nissl substance stained purple. No definite chromocenter was distinguishable in these preparations, although some of the ventral horn cells contained two or three, tiny, nucleolar satellites.

A somewhat varied picture was also seen in the thionin-stained amniotic nuclei of a female chick embryo at eight days of incubation. In this case, some of the nuclei had very discrete vacuoles of obscure origin. Many nuclei, however, contained V-shaped chromatin bodies; a configuration not found in the 8-day-old male counterpart.
Of the different tissues examined, therefore, the chick amnion appeared most promising for the detection of a sex-specific, nuclear chromocenter. Since both Ishizaki and Kosin ('60) and Van Limborgh ('64) successfully demonstrated a nuclear sexual dimorphism in amniotic cells from the domestic fowl (New Hampshire and white Leghorn races) and the duck (Khaki Campbell) with hematoxylin staining, it seemed advisable to apply this stain to our brown Leghorn embryos. Also, in view of the varied results obtained from our preliminary investigation of brown Leghorn tissues, it was decided to include white Leghorn embryos in the present study in order to determine if there was a significant difference in the incidence of sex chromatin between these two races of domestic fowl.

B. Preparation of Material for Cytological Study

Small pieces of amnion were excised from 13 brown Leghorn embryos at 56 and 77 hours, and at 9, 10 and 14 days of incubation. Similarly, amnion was excised from 27 white Leghorn embryos at 70 hours and at 4, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 17 days of incubation. The tissue was usually removed from the head amnion and immediately immersed in Davidson's solution which consists of:

- glacial acetic acid - . one part
- formaldehyde . . . - . two parts
- 95% ethanol . . . - . three parts
- distilled water . . . - . three parts

Three types of preparations were used throughout the chick study. In the
squash preparations, the excised amnion was placed in a few drops of fixative on a glass slide and covered with another glass slide. Firm pressure was applied to the slides so that the tissue was forced to spread out between the slides. Both slides were immediately placed into the fixative and, after a few minutes, were separated. Generally, both slides were carried through the remaining procedures since it was found that some cells tended to adhere to the applied (upper) slide.

In the whole-mount preparations, a small piece of amnion was placed into the fixative and carried through the remainder of the solution by means of a fine, camel's hair brush. The stained and dehydrated tissue was finally mounted on a glass slide with Harleco Synthetic Resin (HSR) and covered with a #1 cover slip.

In the sectioned preparations, the amnion, which had been stored in 75% ethanol for varying periods of time following fixation, was dehydrated and embedded in paraffin and sectioned at a thickness ranging from 3μ to 8μ. The sections were subsequently dehydrated in ethanol and mounted in HSR. Inasmuch as the nuclei were usually seen in profile in these sections (owing to the haphazard arrangement of the membrane in the paraffin block), it was decided to orient the tissue prior to sectioning so that sheets of amniotic cells could be observed parallel with the surface of the membrane. This modification was utilized for three white Leghorn embryos (WL299, WL300 and WL301).
Prior to fixation, the amnion was inserted in a triangular, metal clip with a central open area in each arm. The amnion surrounding the clip was trimmed away and the clip with its "membrane sandwich" was placed in the fixative. The clip was then transferred to the various solutions by means of forceps and finally embedded in a paraffin boat which had previously been fitted out with a paraffin platform. When the wax had hardened, the entire clip was cut away from the boat and the central triangular area dissected free of the clip - giving a central layer of amnion covered on each surface with a layer of paraffin. This "sandwich" was then attached by means of a warm spatula to the surface of a paraffin block and inserted into the chuck of a microtome. The sandwich was oriented in such a way that the knife sliced through layers of amnion parallel with the flat surface of the membrane.

All three types of preparations were stained in Harris's hematoxylin for periods of from 5 to 15 minutes, dehydrated in ethanol, cleared in xylol and mounted in HSR. Various combinations of the three types of preparations were used for many of the chick embryos in this study.

Since the squash preparations gave the best staining results and the most uniform quality in the chick study, only this type of preparation was used throughout the study of the quail amnion. Small pieces of amnion were excised from 40 quail embryos of the species, Coturnix coturnix japonica, following incubation periods of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17 days. The quail amnion was processed for each embryo in the same
manner as in the chick squash preparations, except that the staining in
Harris's hematoxylin was increased to twenty minutes.

C. Sexing Techniques

1. Nuclear Sexing

   a) Chick Embryos

   Since the nuclear sexing technique is a subjective procedure, all pre-
parations were studied with their identifying labels obscured. Two counting
areas were selected in preparations from each chick according to uniformity
in thickness, differentiation of stain and mitotic activity. At least 100 cells
in each area were scored as 'positive for sex chromatin' or as 'negative for
sex chromatin'. Arbitrary criteria were stipulated for the identification of
the sex chromatin body. Thus, for a cell to have been scored as 'positive
for sex chromatin', it must have had a dense-staining, nuclear chromocenter
either attached to the nuclear membrane or not more than 0.5μ away from
this membrane. The breadth of the chromocenter had to be at least 0.5μ
and its density must have been equal to or greater than that of the nucleolus(i).
Although the disposition of the centrally located chromocenters was noted in
all preparations, a cell containing such a chromocenter(s) was not scored as
'positive' unless it had, in addition, one, dense-staining, chromatin body of
appropriate dimensions at the nuclear membrane. Cells scored as 'negative
for sex chromatin' included only those nuclei lacking the peripheral as well
as the centrally-located chromocenters. The incidence of cells scored pos-
itive for sex chromatin was expressed as the percentage of positive cells in a count of 100 cells scored as either positive or negative for sex chromatin. Cells in which both the nuclear membrane and one nucleolus were not clearly visible were omitted from the counts. All counts were made with an oil immersion objective at a total magnification of 980X.

b) Quail Embryos

In addition to the cells scored as 'positive' or 'negative' for sex chromatin, as in the chick study, counts were also made of those nuclei with one central, chromatin body only, two central chromatin bodies only, two peripheral chromatin bodies only, and of nuclei with one peripheral and one central body, herein referred to as a 'split'. The ratio of cells scored positive to a total number of 100 cells scored as positive and negative, was expressed as the percentage of cells positive for sex chromatin in each preparation.

In order to check on the effectiveness of the stain, smears of oral epithelium from human males and females were examined every time a new staining solution was used. A typical 'unhealthy', desquamated oral epithelial cell from a human adult female is included here (fig. 23) for convenience in the comparison of the relative staining characteristics of the sex chromatin body in humans with that of the avian species studied in this investigation.

2. Gonadal Sexing
Following removal of amnion, the gonadal sex of the white and brown Leghorn embryos was determined by inspecting the area of the gonads under a dissecting microscope. A brief sketch was made of the gonadal area showing the relative size of the gonads, their form, and their relations to the mesonephroi, post-caval vein, and Müllerian and Wolffian ducts. In four cases, at six and seven days of incubation (WL305, WL306, WL307 and WL308), where the sex of the gonads could not be determined with certainty, the gonads were removed, fixed, and prepared for histological examination. No attempt was made to determine the sex of the gonads of Leghorn embryos prior to six days of incubation.

Gonadal sexing of quail embryos was performed in the same way except that the entire embryo was fixed in Davidson's solution at the time the amnion was removed. Examination of the gonads was, therefore, postponed until a later time. Because of the precocious development of quail embryos when compared with the Leghorns studied, the gonadal sex could be determined with certainty in some of the embryos as early as five days of incubation.
IV RESULTS

Nuclear chromocenters, identified as sex chromatin bodies according to the criteria adopted for this study, were found in both the brown and white Leghorn races of the domestic fowl, *Gallus domesticus*, and in the Japanese quail, *Coturnix coturnix japonica*. Moreover, these dense, peripheral chromatin bodies were present in the amniotic cells of embryos of both sexes of these species.

A. Incidence of Sex Chromatin in the Domestic Fowl

The incidence of sex chromatin found in brown Leghorn embryos and in white Leghorn embryos is tabulated in table 1. The sex chromatin frequency for each embryo is expressed as the percentage of cells scored as 'positive for sex chromatin' out of every group of 100 cells counted. Since at least two such groups of cells were counted for each embryo, the sex chromatin frequency is also expressed as the range of positive cell percentages from the lowest value in one group to the highest value in the other. The average frequency of sex chromatin and the ranges for both brown and white Leghorn races of the domestic fowl are related to the time of incubation in figures 1 and 2, respectively.

Since some variation was found in the appearance of the cells from different types of cytological preparations from the same embryo, the percentage of cells was averaged for each preparation type. Comparisons can, therefore, be made between the incidence of sex chromatin in squash prep-
arations, in whole-mount preparations, and in sectioned material. These relations are illustrated graphically in figure 3, where the white and brown Leghorn embryos are treated as a single group. It can readily be seen that the whole-mount and squash preparations generally gave higher values than sections of amnion from the same embryos. This result was to be expected since the whole mount and squash preparations were one or more cell-layers thick, permitting observation of an entire cell, while the histological sections varied from 3µ to 8µ and, therefore, restricted observation to, at most, approximately three-fourths of any given cell.

Plate VI illustrated the differences found in an histological section of amnion from a brown Leghorn female and in a squash preparation from the same embryo. When nuclei showing a maximum area in the sectioned tissue (fig. 6) are compared with those in the squash preparation (fig. 7), the sectioned nuclei exhibit a consistently smaller area. While this may be due, in part, to the random sectioning of the nuclei inherent in the sectioning technique, other factors, such as shrinkage incurred during embedding, probably contribute to the apparent reduction in the nuclear area of the sectioned cells.

In order to display the nuclei with a maximum area in sections of amnion, the 'membrane sandwich' modification of the embedding technique, outlined in the previous section, was utilized. Some of the results from this modification are shown in figures 9 and 10. The low magnification view in figure 10
shows the orientation of the nuclei in sheets similar to that observed in whole-mount and squash preparations. When figure 9 is compared with a conventional section of the same embryo (fig. 8), it can be seen that the parallel orientation of the nuclei facilitates the scoring of cells with respect to the presence or absence of the sex chromatin body. It was not surprising, therefore, when counts based on material similar to that seen in figure 9, indicated a sex chromatin frequency of about 9% for this 13-day-old white Leghorn male while the counts based on conventional sections (similar to that in fig. 8) showed a frequency of about 6%.

A similar comparison between embedding procedures in another white Leghorn embryo (WL 301, a 14-day-old male) revealed a frequency of sex chromatin that was about 1% greater in the oriented sections than in the random sections. The discrepancy between these embedding techniques was even greater in the case of WL300, a 13-day-old white Leghorn female where the incidence of sex chromatin was found to be 38% in the conventionally-sectioned material and 43% in sections from the modified embedding procedure.

Finally, the average frequency of sex chromatin was calculated for each embryo irrespective of the type of preparation and tabulated (table 1). These average values were plotted against the time of incubation as shown in figure 1 where it can be seen that the average frequency of sex chromatin in embryos whose gonadal sex was identified as "female" ranged from about
28% to 52%. In embryos identified as "male" the corresponding range was from 2.4% to 16.7%. This distribution of the average frequency of sex chromatin, therefore, displays a bi-modal pattern in the domestic fowl with a low peak corresponding to male embryos (arithmetic mean, 9.6%) and a high peak corresponding to female embryos (arithmetic mean, 40.2%).

When the range of the average frequency of sex chromatin in all male embryos (2.4% - 16.7%) is compared with that of all female embryos (27.7% - 52.4%), an area of "no-overlap" can be seen extending from 17% to 27%.

When the "t" test of significance between two sample means was applied to the average frequency of sex chromatin in males and females of the domestic fowl, $P < 0.01$ when the null hypothesis assumes that the male and female embryos belong to the same population with respect to the incidence of sex chromatin in their amniotic cells. Thus, there is only a very small probability that the deviation observed in the average frequency of sex chromatin between male and female embryos is due to chance alone and, therefore, the null hypothesis may be rejected.

In order to facilitate a comparison of the sex chromatin frequency between the two races of Leghorn fowl, a brief summation of the data follows:
## AVERAGE FREQUENCY OF SEX CHROMATIN

<table>
<thead>
<tr>
<th>Brown Leghorn Males</th>
<th>Brown Leghorn Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range: 8.6% - 16.7%</td>
<td>Range: 39.1% - 52.4%</td>
</tr>
<tr>
<td>$P &lt; 0.01$</td>
<td></td>
</tr>
<tr>
<td>$N^a = 4; \ X^b = 13.3%$</td>
<td>$N = 4; \overline{X} = 43.4%$</td>
</tr>
<tr>
<td>$0.02 &lt; P &lt; 0.05$</td>
<td>$0.2 &lt; P &lt; 0.3$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>White Leghorn Males</th>
<th>White Leghorn Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range: 2.4% - 15.6%</td>
<td>Range: 27.7% - 44.8%</td>
</tr>
<tr>
<td>$P &lt; 0.01$</td>
<td></td>
</tr>
<tr>
<td>$N = 12; \overline{X} = 8.5%$</td>
<td>$N = 9; \overline{X} = 39.1%$</td>
</tr>
</tbody>
</table>

### TOTAL MALES

<table>
<thead>
<tr>
<th>Range: 2.4% - 16.7%</th>
<th>Range: 27.7% - 52.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P &lt; 0.01$</td>
<td></td>
</tr>
<tr>
<td>$N = 16; \overline{X} = 9.6%$</td>
<td>$N = 13; \overline{X} = 40.2%$</td>
</tr>
</tbody>
</table>

### Maximum Variation Per Embryo

- Brown Leghorn: 6.4% - 22.9%
- White Leghorn: 10% - 39.6%

---

*a The letter, $N$, represents the number of embryos  
*b The symbol, $\overline{X}$, is the arithmetic mean of the average percentage of positive cells from $N$ embryos.
The tabulation above shows that the average frequency of sex chromatin is consistently higher in brown Leghorn males and females (13.3% and 43.3%, respectively) than in white Leghorn males and females (8.5% and 39.1%, respectively). While this difference is not statistically significant for the females (P > 0.2) the significance for the males is doubtful (0.05 > P > 0.02). This difference in the males may merely reflect the small number of embryos used (N = 16) or it may, on the other hand, reflect a real difference in the incidence of sex chromatin associated with these races of Leghorn fowl. Additional observations should resolve this question.

There was no striking difference in the cytological appearance of the sex chromatin body between brown and white Leghorn males or between brown and white Leghorn females. Figure 7, may be compared with figure 11, both of which illustrate the typical appearance of nuclei in amniotic cells of the domestic fowl. These figures were taken from 14-day-old females of the brown Leghorn race (figure 7) and the white Leghorn race (figure 11). By varying the focus on the nucleus centered in figure 11, the V-shaped form of the sex chromatin body can be distinguished. This particular appearance of the sex chromatin is associated with the female sex in Leghorn embryos. Among male embryos, the more spherical form of this nuclear chromocenter predominates.

The results from the embryos, whose sex could not be determined from the visual inspection of the reductive tract or from histological examin-
ation of the gonads, were inconclusive because of the lopsided sex ratio which was found among the embryos used for this study. During the first half of incubation, the ratio of males to females was 10:5 but during the last half of incubation, however, the ratio shifted to 5:9. It was not known when this study began what sex ratio to expect in the domestic fowl either at the time of laying or at the hatching period. Naturally, it was hoped that an even distribution of males and females would be found throughout the incubation period.

Among those embryos of unknown sex necropsied during the period of incubation between 2 and 7 days, 9 embryos showed an average incidence of sex chromatin in the 5% - 20% range, 1 embryo showed a frequency of 22% and another of 37% and there was no overlap in the individual ranges for these three groups. Since chromosome preparations were not made for any of these embryos, it is not known whether these frequencies correspond to 'genetic males' or 'genetic females'. There was a general tendency for the average frequency of sex chromatin to increase slightly for the white Leghorn female embryos during the second week of incubation. The significance of this finding will be discussed with the results of our study on the Japanese quail embryo.

B. Cytology of Sex Chromatin in the Domestic Fowl

The cytological disposition of the nuclear chromocenter identified as sex chromatin did not appear to change in either male or female embryos during the course of incubation. There was, however, an increase in the
coarseness of the nuclear chromatin pattern in both male and female embryos with increasing time of incubation. A comparison of cells from a one-week-old white Leghorn female (fig. 12) with those of a white Leghorn female at two weeks of age (fig. 11) although at different magnifications, gives some suggestion of this change in chromatin patterns.

While these results indicate a clear-cut, nuclear sexual dimorphism in embryos of the domestic fowl based on the average incidence of sex chromatin the maximum variation exhibited by the sex chromatin frequency of individual counts indicates an area of overlap between the male and female ranges, particularly for one embryo, WL236 (plate II). Although the range of values is an awkward statistic, in studies of this nature, the range provides the most useful measure of dispersion. Thus, if the nuclear sexing technique were to be applied to embryos of the domestic fowl as the sole estimate of genetic sex, then only those values below 20%, based on individual counts, could safely be assumed to represent males during the last two weeks of incubation. Similarly, only values greater than 35% could be assumed, with certainty, to correspond to the genetic female sex.

That the ranges of sex chromatin percentages are greater for female embryos of both races of Leghorn fowl than for male embryos is not surprising since the individual counts of female cells yielded much higher percentages of cells scored as 'positive for sex chromatin'. The greatest variation that occurred in any one male, for example, was 16.5 percentage
units (WL 306) whereas a variation of 29.6 percentage units occurred in one of the female embryos (WL236).

C. Incidence of Sex Chromatin in the Japanese Quail

The squash preparations of the amniotic membrane in 42 embryos of the Japanese quail were of better quality in comparison with similar preparations for the domestic fowl. Moreover, these preparations were relatively uniform throughout the incubation period which facilitated application of the nuclear sexing technique. The criteria used to determine whether or not a cell was 'positive for sex chromatin' were the same as those employed for the chick study. The results, expressed as the percentage of cells scored as 'positive' for every 100 cells counted, are tabulated in table 2. Since inspection of the reproductive tract of quail embryos was performed quite apart from the nuclear sexing, all sex chromatin frequencies were determined without prior knowledge of the embryo's gonadal sex.

The percentage of positive cells for each sex is shown at corresponding days of incubation (plate IV). The range of positive cell percentages for 17 female embryos from the 6th to the 17th day of incubation varies from 7% to 45%. The corresponding range for 16 male embryos varies from 9% to 45%. The differences in positive cell percentages between female embryos on any particular day of incubation vary from as little as 2 percentage units on day-16 to as much as 23.5 units on day-9. In a similar manner, the differences between male embryos varied from 2 units on day-11 to 26 units on
When the positive cell percentages were averaged for 18 female quail embryos, the arithmetic mean, $\bar{X}$, was found to be $26.0\% \pm 2.5\%$. The mean for the 16 male embryos was $26.8\% \pm 2.6\%$. Application of the "t" test to these two means revealed no significant difference between them ($P > 0.8$). It was concluded, therefore, that the difference between the percentage of positive cells in female and male embryos was due entirely to chance and that a nuclear sexual dimorphism based on the presence of a sex-specific chromocenter does not exist in the amnion of the Japanese quail.

The only general tendency noted in the distribution of positive cell percentages among the quail embryos was that during the first week of incubation, the values for 12 embryos were all below 31%. After this period, the values for all 30 embryos were found to be between 9% and 45%. However, when the values for male and female embryos were averaged according to sex at each day of incubation, a curious daily fluctuation became apparent for both male and female embryos (plate V). During the second half of incubation, the averaged percentages of positive cells in female embryos were found to be between 35% and 45% on the odd-numbered days while the averaged percentages ranged from 19% to 28% on the even-numbered days. Similarly, in the male embryos, the corresponding values were found to be in the 36% - 45% range on the odd-numbered days - as though the males and females were out-of-phase by a period of 24 hours. Whether this cyclic pattern in both
sexes is the result of averaging disparate values at each day of incubation or whether it represents a real fluctuation in mitotic activity and/or nucleo-protein synthesis remains to be explored.

D. Cytology of Sex Chromatin in the Japanese Quail

The cytological findings in the amniotic nuclei of quail embryos were quite similar to those in the domestic fowl. Figures 13 and 17 illustrate the typical forms of the nuclear chromocenter identified as the sex chromatin body found in quail embryos. Unlike the situation in the domestic fowl, however, the V-shaped form of the sex chromatin body was frequently observed in male embryos of the Japanese quail.

In young quail embryos, the amniotic nuclei had a more vesicular appearance than those in embryos during the last week of incubation - an observation paralleling that made in brown and white Leghorn embryos (figures 14, 15 and 22). During the course of incubation, however, a coarser chromatin pattern became apparent and, as in the chick study, it became increasingly difficult to distinguish a sex chromatin body from the multiple nucleoli and other dense chromocenters (figures 16 and 21)

When male and female nuclei at the same stage of incubation were compared, no real difference in the disposition of the nuclear chromocenters was observed. In figure 18, the sex chromatin frequency for the 9-day-old male was 23% while a 45% incidence of sex chromatin was found in the cells of the 9-day-old female shown in figure 19. The sex chromatin frequency in the
13-day-old male (figure 21) on the other hand, was almost 39% while that of
the 12-day-old female was only 20% (fig. 20).

The mitotic activity of the amniotic cells was observed in the counting
areas and recorded for most of the quail embryos studied. However, it
was found that the number of mitoses per counting area did not accurately
reflect the overall mitotic activity of this tissue. This was due, in part, to
the avoidance of areas with intense mitotic activity in the selection of the
'counting sites'. Very early prophase figures, in particular, display numer-
ous small, dense chromocenters easily confused with the sex chromatin body
of the interphase nuclei identified in this study. It would, of course, be
interesting to relate the incidence of sex chromatin to the mitotic activity of
the avian amnion, but such a study would require some means of accurately
estimating the mitotic index for this tissue.
A. Correlation of Sex Chromatin Frequency and Gonadal Sex

The results of this study indicate that a sexual dimorphism exists in the amniotic nuclei of both the brown and white Leghorn races of the domestic fowl. This conclusion was based on the bi-modal distribution of a small, dense-staining, nuclear chromocenter located on or within 0.5μ of the nuclear membrane. The frequency of this 'sex chromatin body' ranged from 27.7% to 52.4% in embryos that were identified as females by visual inspection of the reproductive tract. The corresponding range for male embryos varied from 2.4% to 16.7%. The average frequency of sex chromatin from 13 female embryos from 7 to 17 days of incubation was 40.2% while the average frequency for 16 males during the same period of incubation was 9.6%. This difference in the incidence of sex chromatin between the sexes was significant (P< 0.01).

These results are in substantial agreement with those of Ishizaki and Kosin ('60) who found a nuclear sexual dimorphism in the amniotic cells of New Hampshire embryos from 9 to 14 days of incubation. These investigators reported a range of sex chromatin positive cells from 45% to 68% in 10 female embryos and from 3% to 12% in 10 male embryos. Van Limborgh ('64) reported a similar result for the amniotic cells of white Leghorn embryos in which the range of positive cells varied from 60.5% to 70.5% in 10 females at 15 days of incubation and from 17.0% to 24.0% in 10 males during the same
period. The results from the present study of the amniotic cells of the white Leghorn fowl at 13-14 days of incubation ranged from 14.8% to 44.8% for 3 females, while the corresponding range for 3 males during this period varied from 7.5% to 10.0% for sectioned material only. The only brown Leghorn embryo examined at this period for which sections were made had a frequency of 44.6%. Thus while a nuclear sexual dimorphism was found in all three studies, the interval between the male and female ranges was 33 percentage units in the New Hampshire chicks, 46 units in the white Leghorn chicks (Van Limborgh) and 31 units in both the white and brown Leghorn chicks found in this study. That the frequency of sex chromatin positive cells reported here for the female embryos is consistently below that found in the other studies was to be expected since one of the criteria used to identify the sex chromatin body in this study was that it be located within 0.5μ of the nuclear membrane. None of the other investigators had placed this restriction on the identification of sex chromatin in their studies and have, presumably, included in their positive scores, various small, dense chromocenters observed throughout the nucleus -- as is commonly done in sex chromatin studies of mammalian species.

B. Relation of Sex Chromatin Frequency and Incubation Time

A comparison can also be made with the findings of these investigators for the early period of incubation prior to the time when the gonads can be sexed from the visual inspection of the gonads. At four days of incubation,
Van Limborgh (’64) found two discrete ranges of sex chromatin frequencies: one at 49.0% to 69.5% and another at 18.0% to 26.5% for 20 white Leghorn embryos. A similar bi-modal distribution in the incidence of sex chromatin was observed by Ishizaki and Kosin (’60) in 11 New Hampshire chicks at 5 days of incubation. The "peaks" of this distribution corresponded to ranges of 63%-76% and 8%-23%. In this study, the results from histological sections of the amniotic cells from two white Leghorn chicks at 4 days of incubation gave 36.8% for one embryo and 5.2% for the other. For this incubation period, therefore, the interval between the "peak" ranges was 40 percentage units for the New Hampshire, 22 for the white Leghorn reported by Van Limborgh, and 31 for the white Leghorn embryos reported in this study.

It might be noted that there was an increased (7%) in the frequency of sex chromatin at our 4-day "peaks" to that of the male and female peaks at 14 days. Similarly, in Van Limborgh's study (’64) the range at the high "peak" at 4 days increased about 11% to the female range at 15 days although there was not a significant change to the male range at 15 days from the low "peak" at 4 days. Ishizaki and Kosin's (’60) findings were just the reverse. These investigators observed an 18% decrease for the range of the presumptive females and a 5% decrease for that of the presumptive males between the two periods of incubation. Thus, for the white Leghorn embryos in our study and that of Van Limborgh (’64), there was an increase in the incidence of sex chromatin with increasing incubation time. In the amniotic cells from
the New Hampshire chick embryos, on the other hand, the frequency of sex chromatin decreased with increasing time of incubation. That this inverse relation found in Ishizaki and Kosin's study reflects a species difference is doubtful, since an overall increase in sex chromatin frequency with increasing incubation time was found for the female Japanese quail embryos observed in this study. Similarly, in the Khaki Campbell ducks studied by Van Limborgh ('64), the sex chromatin frequencies remained relatively constant for both males and females from 80 hours to 5 days to 10-12 days of incubation. On the other hand, recollection of the study by Stenius et al. ('63) in which autosomal complements were found to differ more between two genera (pheasant and chicken) than between two families (pheasant and turkey), suggests caution in the interpretation of what might appear to be 'species differences'.

C. Comparison of Sex Chromatin in Domestic Fowl and Japanese Quail

Observations on the amniotic cells of Japanese quail embryos from 2 to 20 days of incubation gave no indication at any age of a bi-modal distribution in the incidence of the nuclear chromocenter, identified as sex chromatin, that could be associated with the gonadal sex of the embryos. Indeed, when the frequency of this chromocenter was averaged in known males and females, the average values for each sex were almost identical.

In view of the great similarity in size, staining characteristics, and location in the nucleus between the sex chromatin body in chick embryos and
and that in quail embryos, this result may appear somewhat surprising. The long axis of the sex chromatin body at the nuclear membrane was found to range from 1.12μ to 1.66μ in white Leghorn squash preparations (1.73μ in the comma-shaped variety) and from 1.37μ to 1.82μ in similar preparations from the Japanese quail. However, these measurements were made for only a few cells so that it cannot be concluded that these is a consistent size difference between these chromocenters in the two species.

The only consistent cytological observation that may provide a morphological correlate for some of the results obtained for the incidence of sex chromatin is the apparent absence of the V-shaped form of the chromocenter in Leghorn males. While this absence may help to explain the disparity in sex chromatin frequencies between males and females in the domestic fowl, it does little to account for the wide range of frequencies encountered in both male and female quail embryos. Another possibility that can be ruled out in the female quail embryos is that the low incidence of this chromocenter at the nuclear membrane was accompanied by its high incidence elsewhere in the nucleus. In most quail embryos, the incidence of the chromocenter scored as sex chromatin was very similar to that of another chromocenter scored as 'one central body'. In only one case, that of a 49-hour embryo, did the single central body have a greater frequency than the peripheral body identified as sex chromatin.
D Interpretation of 'Sex Chromatin' in the Japanese Quail

The conclusion that a nuclear sexual dimorphism does not exist in the amniotic cells of the Japanese quail does not preclude the possibility that a sex-specific chromocenter does exist in the interphase nuclei of this species and that, for any number of reasons, it was not detected. However, because of the very similar appearance of chick and quail amniotic nuclei, and also because the increase in coarseness of the overall chromatin pattern, with age, in the quail, parallels that observed in the chick, it seems reasonable that if a nuclear sexual dimorphism could be detected in the chick the same phenomenon in the quail should be capable of demonstration by the same technique if, indeed, it does exist.

The results from the quail study can also, however, be interpreted that a sex-specific nuclear chromocenter is absent in the intermitotic nuclei of the amniotic cells of the quail - leaving open to question the real identity of the so-called 'sex chromatin body' in this species. Such an interpretation would lend support to the thesis which predicts that species with a W chromosome that behaves euchromatically at mitotic metaphase will be lacking a sex-specific nuclear chromocenter in the intermitotic nuclei. Such species would include the Japanese quail (Vegni-Talluri and Vegni, '65) and the budgerigar (Rothfels et al., '63) for which both the W and Z chromosomes were reported to be negatively heteropycnotic at metaphase. Inasmuch as Galton and Bredbury ('66) reported a late-labeling, W chromosome for
Columba livia domestica that was also heterochromatic, this species of pigeon should also, according to the thesis proposed here, possess a nuclear sexual dimorphism based on the presence of a dense-staining, sex-specific nuclear chromocenter in female birds. The brown Brahma is another species of domestic fowl reported to have a late-labeling W chromosome (Schmid, '62) but for which the sex chromatin pattern has not yet been determined.

A further interpretation for the difference in results obtained in the two species investigated in this study may be provided by the frequency of sex chromatin during the early stages of incubation. The fact that the incidence of this chromocenter did not increase with time for the "unknown" Leghorn embryos during the first week of incubation and, since such an increase was noted for this period in the "unknown" quail embryos suggest that, perhaps, two different entities were scored as "sex chromatin" in these two species. In the domestic fowl, this entity was a sex-specific chromocenter whose frequency remained fairly constant throughout the incubation period (although variable within the cells of a given embryo). Such a chromocenter possibly represents the W chromosome of the interphase nucleus in a heteropycnotic condition. In the Japanese quail, on the other hand, this entity varied with the age of the embryo. During the first week, the frequency increased progressively but thereafter, it fluctuated on a daily basis in both males and females. In any event, the "variable" entity in both sexes of the Japanese quail appeared to be morphologically identical with the "constant" entity.
found at a relatively high incidence in female embryos of the domestic fowl.

E. Comparison of Sex Chromatin in Birds and Mammals

When the results reported here for the domestic fowl and the Japanese quail are compared with the overall findings in mammalian species, two major differences are apparent. First of all, it is not usually concluded from mammalian studies that a particular species is definitely lacking in a sex-specific nuclear chromocenter. Rather, when an apparent absence of a nuclear sexual dimorphism is encountered, the results are usually ascribed to a "coarse chromatin pattern" which is thought to 'mask' the sex chromatin body. Such was the interpretation, for example, given to the absence of sexual dimorphism in liver cells of the cat by Graham and Barr ('52) - a species known to exhibit clear-cut sex differences in the cells of the nervous system. Although a few mammalian species, such as the bison, hedgehog and prairie dog, have been reported to lack sexchromatin in female cells(Moore, '66), these species have not received thorough study. In all probability, as different tissues come under observation, a nuclear sexual dimorphism will be established for these mammalian species as well.

The apparent absence of sexual dimorphism in the amniotic cells of the Japanese quail, on the other hand, does not seem likely to be due to a coarseness in the chromatin pattern, particularly during the early stages of incubation. In very young quail embryos, the amniotic nuclei are especially clear or vescicular, and, as in the chick embryos, display an increasing coarse-
ness in the chromatin pattern with increasing incubation time. If a sex specific nuclear chromocenter is present in the quail, it should be detectable, at least, during the first week of incubation.

That the location of the sex chromatin body in the amniotic nuclei of the Japanese quail may vary with the developmental stages of this tissue - as in the cat (Graham, '54), is also doubtful because the low incidence of peripheral sex chromatin in some of the female quail embryos was not accompanied by an increased frequency of small, dense chromocenters elsewhere in the nucleus.

The second major difference between the results reported here and those derived from mammalian studies, concerns the presence of a small chromatin body in the heterogametic sex. Occasionally, a small nuclear chromocenter has been described in male cells of mammalian species and was thought to represent the Y sex chromosome in positive heteropycnosis. For example, the small dense chromocenter, consistently observed by Graham ('56) in different cells of the male Virginia opposum, has been interpreted by Ohno et al. ('60b) to represent the Y chromosome which is heteropycnotic during interphase. In other mammalian species, in which the Y chromosome is unusually large, as in the golden hamster, two large chromocenters are found in male intermitotic nuclei, one of which is thought to represent a portion of the duplicate-type X chromosome and the other, the entire Y chromosome (Ohno and Weiler, '62).

This heteropycnotic behavior of the Y chromosome during cell division
and, presumably, during the intermitotic phases in mammalian cells is consistent with the specialization of this element brought about by heterochromatization during the course of mammalian evolution. Inasmuch as the corresponding W chromosome in cells of the heterogametic female sex of birds has only recently been demonstrated, attempts to draw close parallels between the behavior of this element in avian species and the behavior of the Y chromosome in mammals, appear premature at this time. Suffice it to say, that while the mammalian X chromosome appears to be consistently heteropycnotic during metaphase, the avian W chromosome has been observed to behave euchromatically in some species (Vegni-Talluri, and Vegni, '65; Rothfels et al., '63) and heterochromatically in others (Schmid, '62; Galton and Bredbury, '66).
VI SUMMARY AND CONCLUSIONS

1. The incidence of sex chromatin in the amniotic membrane was investigated in the brown and white Leghorn races of the domestic fowl, Gallus domesticus, and in the Japanese quail, Coturnix coturnix japonica. Small pieces of amnion were excised from 13 brown Leghorn embryos at 56 and 77 hours and at 9, 10 and 14 days of incubation, fixed in Davidson's solution and stained in Harris's hematoxylin.

2. A similar procedure was followed for 27 white Leghorn embryos at 4, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 17 days of incubation. At least two groups of 100 amniotic cells each were counted and scored for the presence of sex chromatin.

3. A cell was scored as 'positive for sex chromatin' only if it contained a dense-staining nuclear chromocenter, at least 0.5μ in breadth and not more than 0.5μ from the nuclear membrane. A negative cell lacked peripheral as well as centrally-located chromocenters. In all cases, the frequency of sex chromatin was expressed as the percentage of positive cells.

4. When the results from the nuclear sexing technique were compared with the gonadal sex of the embryo, determined wherever possibly by visual inspection of the gonads, it was found that a nuclear sexual dimorphism was present in both brown and white Leghorn embryos. This dimorphism resulted from a bi-modal distribution in the frequency of sex chromatin with the high peak corresponding to female and the low peak to male
embryos. The range of the average frequency of sex chromatin throughout the incubation period was 2.4% - 16.7% for males and 27.7% - 52.4% for females. The difference between the averages of all males and all females was statistically significant. The average frequency of sex chromatin, although consistently higher in brown Leghorn than in white Leghorn males and females, was not significantly higher.

5. Three types of preparations were used in the chick study: squashes, whole-mounts and histological sections. In addition, a modified embedding technique was utilized in order to prepare parallel sheets of cells and thereby facilitate the nuclear sexing procedures. Although the frequency of sex chromatin was higher in the squash and whole-mount preparations than in sections, it did not appreciably affect the average incidence of sex chromatin in any one embryo. The greatest variation in the incidence of sex chromatin, for example, was observed histologically in sections from one of the white Leghorn embryos.

6. Identical sex chromatin criteria and sexing procedures were used for the squash preparations of amnion from 42 quail embryos examined daily from 2 to 17 and 20 days of incubation. No nuclear sexual dimorphism was found in this species. The dense-staining body identified as sex chromatin had the same frequency in male cells as in female cells. The range of sex chromatin varied from 7% to 45% in 18 female and from 9% to 45% in 16 male embryos. The incidence of sex chromatin in the
"unknown" embryos showed a progressive increase during the first week of incubation.

7. The results of the chick study were compared with those of other investigations and found to be in agreement with previous observations in the white Leghorn and New Hampshire breeds of domestic fowl. No brown Leghorn studies have been previously reported.

8. The results from the chick study were taken as evidence in support of the thesis that avian species exhibiting a heterochromatic and late-labeling W chromosome may be expected to exhibit a sex-specific nuclear chromocenter derived from the W chromosome in female cells.

9. The results from the quail study, however, may or may not substantiate such a thesis depending on the interpretation placed on the appearance of a relatively high incidence of the so-called sex chromatin body in some of the male and female embryos. This investigator preferred to interpret this chromocenter as a 'variable' entity which, in the quail, may reflect some daily metabolic activity in the amniotic cells during the course of embryogenesis.


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. 1964 Sex chromatin formation during the interphase of human fibroblasts. Ibid., 146: 424.


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. 1956 Sex chromatin in Didelphys virginiana. Ibid., 124:403-404.


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Krishan, A., G. J. Halden and R. N. Schoffner 1965 Mitotic chromosomes and the W-sex chromosome of the great horned owl (Bubo v. virginianus). Chromosoma (Berl.), 17: 258-263.


1958 Somatic association of the positively heteropycnotic X-chromosomes in female mice, Mus musculus. Ibid. 15: 616-618.


1960b The basis of nuclear sex difference in somatic cells of the oppossum, Didelphys virginiana. Ibid., 19: 417-420.


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VIII GLOSSARY

Allocyclic behavior (of the X chromosome): In mammals, the heteropycnotic appearance of the X chromosome in male meiosis contrasted with its isopycnotic appearance in female meiosis.

Amorph (Amorphic mutant allele): A mutant gene whose presence can be detected in the hemizygous or homozygous recessive condition by the absence of a specific gene product that is normally produced by its wild-type allele.

Dosage compensation: The genetic inactivation of X-linked genes on one of the X chromosomes of the homogametic sex.

Euchromatin: Chromosomal regions which comprise the fine network of chromatin in the interphase nucleus.

Genome: The totality of genes in a haploid set of chromosomes.

Heterochromatin: Chromosomal regions which stain darkly and remain condensed to form chromocenters in the interphase nucleus as heterochromatic regions.

Heterogametic sex: That sex which does not carry homologous sex chromosomes. The heterogametic sex may bear sex chromosomes lacking homologous segments or an entire sex chromosome may be absent.

Heteropycnotic: Synonymous with heterochromatic.

Homogametic sex: That sex which bears homologous sex chromosomes.

Inactive-X hypothesis: The theory which states that the single, heteropycnotic X chromosome that forms the sex chromatin of female mammals is genetically inactivated.

Isopycnotic: Synonymous with euchromatic.

Karyotype: The general appearance of the chromosomes with regard to number, size and shape.

S phase: The period of DNA synthesis, between the Gl and G2 phases, in the intermitotic nucleus.
Variegated position effects (V-type position effects): The regional suppression of dominant genes that are brought in close proximity to a block of heterochromatin by translocation, inversion or insertion. The recessive alleles are then expressed in the heterozygote as a mosaic or variegated phenotype with patches of fully expressed recessive phenotype intermingled with patches of the dominant phenotype.
### TABLE 1

INCIDENCE OF SEX CHROMATIN IN THE CHICK AMNION

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<tr>
<th>Chick # Incubation Period</th>
<th>Sex Type of Preparation</th>
<th>Positive Cells Average (Per Type)</th>
<th>Range %</th>
<th>Average (All Types) %</th>
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<sup>a</sup>The letter, S following the preparation number indicates a squash-type preparation.

<sup>b</sup>The letter, C, following the preparation number indicates a preparation consisting of histological sections.
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**White Leghorn Embryos**

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PLATE I

EXPLANATION OF FIGURE

1. Incidence of sex chromatin in the amnion of white and brown Leghorn embryos.

Each symbol represents an average value for the positive cell percentages from each embryo. The averages from brown Leghorn embryos are indicated by the underscored symbols.
PLATE II

EXPLANATION OF FIGURE

2 Incidence of sex chromatin in the chick amnion.

The range of positive cell percentages is plotted against the time of incubation.
Incidence of sex chromatin in the amnion of the domestic fowl according to type of preparation.

The average percentage of sex-chromatin-positive cells in embryos for which two or more types of preparations were used is plotted against the time of incubation.

The identity of each embryo is indicated by the number placed between the values plotted for each preparation type. On days 9 and 14, an average value based on the scores from two embryos was used and the identity of these embryos is given in parentheses.
Incidence of sex chromatin in the Japanese quail.

The percentage of sex-chromatin-positive cells in the amnion of each quail embryo is plotted against the time of incubation.
Relation between sex chromatin frequency and incubation time in the quail amnion.

The percentage of sex-chromatin-positive cells in quail embryos is averaged according to gonadal sex and plotted against time during the last two weeks of incubation.
6 Portion of an histological section of amnion from a brown Leghorn female (286) at 14 days of incubation. Some shrinkage is apparent in this area. X900.

7 This squash preparation was made from the same tissue shown in figure 6. The disposition of the peripheral chromatin bodies is typical of the chromatin pattern in female chick embryos. The black arrow indicates another chromocenter identified as a nucleolus after a thru-focus examination. X900.
PLATE VII

8 This photomicrograph illustrates the random sectioning of the amniotic nuclei that results from conventional embedding procedures. This tissue was taken from a 13-day-old white Leghorn male embryo (399). X1600.

9 This micrograph illustrates the results obtained with the 'membrane sandwich' modification of the routine paraffin embedding technique. Note the parallel orientation of the nuclei, in layers, similar to that seen in squash preparations (fig. 7). The tissue shown here is identical with that shown in figure 8. The frequency of sex chromatin in this preparation was 9% whereas that in the preparation shown in figure 8 was 6%. X1600.
PLATE VIII

EXPLANATION OF FIGURES

10 This is a low magnification view of the preparation shown in figure 9 from a 13-day-old white Leghorn, male embryo (299). Some of these sheets of cells extend the entire length of the section (5mm). The average incidence of sex chromatin in all preparations from this embryo was 7.5%. X720.

11 This squash preparation of amnion from a 14-day-old white Leghorn embryo (311) shows the 'classical' inverted V-shaped sex chromatin body at the nuclear membrane of the cell in the center of the photograph. A tiny vacuole may occasionally be seen between the arms of the "V". About 41% of the cells counted from this animal were positive for sex chromatin. X900.
12 The arrows point to nuclear chromocenters identified as sex chromatin bodies in this squash preparation of amnion from a 7-day-old white Leghorn female embryo (308). The out-of-focus nucleus in the center of the photo displays a chromocenter usually described as a 'nucleolar satellite'. These centrally-located chromocenters were excluded from the definition of sex chromatin used in this study. X1600.

13 This squash preparation of amnion from a 7-day-old, male, Japanese quail embryo (Q72) shows the typical forms of sex chromatin seen in both the fowl and quail. The arrows indicate the spherical and V-shaped forms of this chromocenter common in both male and female quail preparations. One-fourth of the cells counted in this preparation contained similar, dense, chromatin bodies located at the nuclear membrane. X1600.
EXPLANATION OF FIGURES

14 This low power view depicts the vesicular amniotic nuclei typical of young quail embryos. Occasionally, some yolk-granule contamination occurs as in the case of this 3-day-old embryo (Q20). X720.

15 These nuclei are from the same preparation shown in figure 14. The difficulty in scoring cells as 'positive for sex chromatin' can be appreciated from a glance at the overlapping nuclei at the upper right-hand corner. The density of the yolk granules invariably differs slightly from that of the nuclear materials. X1600.
EXPLANATION OF FIGURES

16 The sex chromatin body (arrow) in this cell from a 6-day-old male, Japanese quail embryo (Q23) is quite similar to that shown for the female embryo in figure 17. As incubation progresses, it becomes increasingly difficult to distinguish a "special" nuclear chromatin body from incipient nucleoli and other chromocenters. X1600.

17 The arrow indicates the 'classical' size and form of the sex-specific nuclear chromocenter commonly seen in preparations from mammalian species (fig. 23). This photograph was made from a squash preparation of amniotic cells from a female quail embryo (Q51) at 11 days of incubation. X1600.
This squash preparation is from a 9-day-old, male quail embryo. The cell at the lower right of the photo was scored as 'positive for sex chromatin' because of the size and location of its peripheral chromocenter. A small vacuole may be seen between the sex chromatin body and the large nucleolus - a common finding. Fine, barely discernable, linin threads may be seen extending from the nucleolus to the nuclear membrane. (In some mammals, the sex chromatin body is occasionally found attached to one of these threads but the significance of this arrangement remains obscure.) X1600.

A typical sex chromatin body is shown (arrow) at the nuclear membrane of an amniotic cell from a 9-day-old female, quail embryo (Q56). X1600.
Figure 20

The nucleus in the center of this photograph was scored for two peripheral chromocenters. However, only the one indicated by the arrow is a typical sex chromatin body. The chromocenter to the left, although within 0.5μ of the nuclear membrane, lacked sufficient density at other focal planes to qualify as sex chromatin. This preparation was made from the amnion of a 12-day-old female, quail embryo (Q40). X 1600.

Figure 21

These amniotic cells were typical of a squash preparation from a male, quail embryo at 13 days (Q46). The arrow indicates a cell scored as 'positive for sex chromatin'. X 1600.
The amniotic cells shown in this figure were taken from a female quail embryo (Q68), in good condition, following 20 days of incubation. The coarse chromatin pattern seen in these cells is more pronounced in older embryos. X 1600.

This photo was made of an oral epithelial cell from an adult human female. The smear preparation was fixed in Davidson’s solution followed by staining in Harris's hematoxylin. X 1600.
APPROVAL SHEET

The dissertation submitted by Donna Gorecki has been read and approved by four members of the Department of Anatomy.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 24, 1969

Date

[Signature of Adviser]