THE CHROMOSOMES OF THE COTTON RAT
(SIGMIDON HISPIDUS TEXIANUS) WITH
CRITICAL NOTES ON SOME PHASES OF
CYTOLOGICAL TECHNIQUE.

BY
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MASTER OF ARTS

Approved by: ___________________________
Head of the Department.

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11/9 - [Handwritten notation]
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THE CHROMOSOMES OF THE COTTON RAT
(SIGMODON HISPIDUS TEXIANUS) WITH
CRITICAL NOTES ON SOME PHASES OF
CYTOLOGICAL TECHNIQUE.

Introduction

In the study of mammalian chromosomes, the greater part of the work has been concerned with the primates, the rodents, and the marsupials. Of these, the rodents are perhaps better known than any of the others.

The primary purpose of the writer in undertaking this study was the desire to master or at least obtain a knowledge of the technique used by our best cytologists, and to apply this technique to the study of a rodent from a group which had not before been examined.

The Chromosomes of the Kangaroo Rat

In surveying the field it was discovered that most of the families of Rodentia had been examined with the exception of the Heteromyidae. Among the sand dunes and arid stretches of southwestern Kansas there lives a member of this family, Dipodomys ordii richardsoni, commonly called the Richardson Kangaroo Rat. The kangaroo rats as a whole have been the subject of many interesting anatomic and taxonomic papers but they had never been studied from the standpoint of cytology. While studying at the
University of Kansas I obtained a number of kangaroo rats and attempted to breed them for a complete cytological study. Suffice it to say that my investigations into the chromosomes of this creature had to be abandoned, for I found evidence of an exceedingly high number of chromosomes, somewhere in the eighties (see plate 1, a and b); it therefore presented a problem much too complicated for an initial venture into this difficult field, even had adequate microscope equipment and an abundance of material been available.

Survey of work already done, and problems.

Considering the vast number of species and varieties of rodents in the world, the study of their chromosomes is still in its infancy for, of the hundreds of rodents, there are chromosome maps for only some 12 species. Thus our present knowledge is hardly sufficient to enable one to predict the value of such an undertaking as the survey of the chromosome numbers of the members of a family or order. Neither have we yet reached the point of being able to conduct parallel cytological and genetical studies of mammals, a work with which much has been done in the field of plant genetics and cytology, particularly with *Oenothera* and *Datura*. So far as mammals are concerned, the work of Painter ('27) is unique in that a cytological examination of Gates' v-0 mice showed a visible absence of a part of a chromosome. This was the first time that a cytologist has been able to locate a definite gene in a definite chromosome in any species of Vertebrata.
Before much progress had been made a move from Kansas to South Texas made it still more difficult to obtain material, and it seemed advisable to work on a locally common wild rodent. I therefore decided to study the East Texas cotton rat, Sigmodon hispidus taxianus, a member of the family Cricetidae.

In reading the literature the following problems in regard to the cytology of the cotton rat presented themselves:

1. A critical study of Cytological technique to determine the possibilities of improving B15 as a fixative.
2. A determination of the number and morphology of the chromosomes of the cotton rat.
   (a) from the spermatogonial cells.
   (b) during 1st and 2nd maturation division
   (c) from the somatic cells of the female.
3. A determination of the nature of the sex complex.

General Methods

Tissue was examined from animals which had been in captivity for some time and from animals killed soon after trapping, but no observable difference was noted. Some 14 cotton rats were used in this study along with a number of kangaroo rats, white rats and white mice. The white rats and mice used served as a check on methods.

The details of technique which were finally decided upon as giving best results are to be found in an appendix to this paper, to which references are made in the text.
Acknowledgments

The background for this study was obtained at the University of Kansas where a study of mammalian cytology was begun in 1928 under Dr. W. J. Baumgartner, and later, during his absence, continued under Dr. H. H. Lane. The writer wishes to acknowledge his appreciation for the help and encouragement of these two men. I wish also to thank Miss Marie Scott who assisted with many of the details of technique and without whose willing assistance the early work would have been much delayed.

In taking up the study again at the Rice Institute, the writer is indebted to Dr. Asa C. Chandler for the laboratory facilities and encouragement offered during this investigation; to Dr. T. S. Painter of the University of Texas for suggestions during the progress of the work; to Dr. C. E. McClung for technical advice; to Mr. J. I. Davies, who from time to time made many helpful suggestions in technique; and to Mr. Homer T. Matthews for furnishing a plentiful supply of cotton rats.
PART I: A critical discussion of some Phases of Cytological technique.
A critical discussion of some phases of cytological technique.

Introduction

The problem offered by technique alone is the bête noire of most of those who would do creditable work in cytology. As noted by McGregor, (18) progress in microscopic anatomy is largely dependent upon the refinement of present methods and the invention of new ones. Rigid attention to all of the details of procedure is absolutely required in cytological investigation, and this may rightfully be demanded of each worker as a prerequisite to the acceptance of his results.

Recent developments

With the development in the Zoological laboratory of the University of Pennsylvania (1916-'19) of the modification of Bouin's fixing fluid which resulted in the formula known as B15, many of the difficulties involved in the technique were banished. Developing this method further, Painter was enabled to survey the general

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1. After experimenting with various combinations of substances in use for fixation, Allen found that his mixture labelled B25 in the plan of his experiment gave the best results. This mixture consisted essentially of Bouin's fixing fluid to which 1.5% chromic acid and 2% urea had been added. For convenience, and for want of a better name, this method is referred to either as B15, or as Allen's modification of Bouin.
field of mammalian cytology with reference to the chromosomes. Painter (125) came to the conclusion that 48 chromosomes is the typical number for the Eutheria, and that when a number in some species differed from this the condition was due either to end-to-end fusion or fragmentation. The interest aroused by Painter, and others inspired by him, opened up a new field of study for the cytologist who, in the words of Painter, must be in the future a "super-technician".

Previous work

The following tabulation (table I), serves as a general review of the literature on the subject of rodent chromosomes since 1889, giving the important facts brought out, the names of the observers, and references to their papers.

As will be seen from table I, the study of rodent chromosomes dates back almost half a century, the first published work being that of Tafani, (189) who studied the house mouse. In view of the developments since this work of Tafani the study of rodent chromosomes might be divided into two eras; the pre-B15 era, a period of some 27 years during which time workers used Flemming, Bouin, Carnoy, etc. as fixing agents; and the B15 era (since 1918) which was brought about by the use of modifications which improved the actions of the fixing agents. This improvement, together with increased technical skill, marks the work of this period as superior to that of previous years.
<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Sex</th>
<th>Age</th>
<th>Tissue</th>
<th>Bands</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mus musculus</td>
<td>Wild mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>White rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>watusi</td>
<td>White mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cavia porcellus</td>
<td>Guinea-pig</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Leucopus americanus</td>
<td>White rabbit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dasyproctta variegata</td>
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**Chromosome Numbers of Rodents**

<table>
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<th>Bands</th>
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<td>Mus musculus</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>watusi</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cavia porcellus</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucopus americanus</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dasyproctta variegata</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This table was compiled from recent literature and published by [source](#).
The work done on the mouse and the rat may be taken as an illustration of the work done in these eras. Table 2 gives a comparison of the reliability of results obtained with and without the use of $B_{15}$ as a fixative, as gathered from the literature of the past forty-one years. Successful confirmation with improved technique is accepted as a justifiable criterion of reliable work.

Table 2. Tabulation of work done on mouse and rat with regard to fixation.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Number of times reported</th>
<th>Reports</th>
<th>Confirmation</th>
<th>No. Gs5 eva.</th>
<th>Observations confirmed</th>
<th>Gs5 eva.</th>
<th>Observations confirmed</th>
</tr>
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<tbody>
<tr>
<td>Mouse</td>
<td>23</td>
<td>9</td>
<td>20</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>12</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>17</td>
<td>25</td>
<td>8</td>
<td>10</td>
<td>9</td>
<td></td>
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The improvement in results brought about by the modification of technique is rather striking, and indicates the primary importance of proper fixation.

Despite the picture as revealed in Table 2, Osmic acid fixation still has virtues; although its action is not so consistent as one would hope. Nance, ('18), Masui, ('23) and Von Winiwarter and Oguma, ('26) are among those who have achieved remarkable success with various modifications of the Osmic acid technique. In text figure A, the results of the use of Osmic Acid by Nance ('18) and Von Winiwarter and Oguma ('26) are compared with the $B_{15}$ technique in the hands of Painter, ('23).
I believe that in view of the success of various persons with different techniques it is well for a beginner to experiment with a fixative in order to gain a first hand knowledge of its range of usefulness.

Experiments with $B_{15}$

The general problem of fixation has been fairly well covered in the papers of Hance ('19), Allen ('20), and Painter (24). However, in the light of the failure of several workers, e.g., Root and Harmon ('24), and Weedsdalek ('24), to obtain good results with the use of $B_{15}$, and in view of some of my own failures in developing a satisfactory technique, it was thought advisable to experiment with the various proportions of formalin, picric acid, and glacial acetic acid used in $B_{15}$ to see if another combination would give better results. The technique as outlined in the appendix was followed in all parts with the exception of the mixture used for fixation. Twelve different combinations of the reagents used in $B_{15}$ were tried as fixatives.
The impression gained from the study of the tissue fixed in these various combinations suggested the general conclusion that the exact proportions of reagents used in the fixative, while important, are not so vital as the physical preparation of the material so that each cell may be killed and at the same time preserved without undergoing changes. Failure with a fixative properly balanced so as not to cause shrinkage or swelling of the tissue would seem to be due to poor penetration. It would seem that some combinations of reagents used as fixatives, when acting upon some tissues effect only a partial penetration, causing the cell to die without immediate fixation, therefore not preserving the nuclear structure in detail until after the nucleases, which act upon the nucleo-proteins immediately after death, have begun their hydrolysis. This gives the appearance of "fluidity" to the chromatin so often seen in material which has been poorly fixed.

Causes of faulty fixation

In general faulty fixation is due to one or more of the following factors: (1) delayed preservation of the tissue; (2) intervening stroma which separates the cells from the action of the fixative; (4) improper temperature: when a slowly penetrating fixative is used, such as strong Flemming, a low temperature (40°C) seems to give best fixation, while with B15 a temperature of 37°C is best and a temperature higher than this causes shrinkage; (5) the power of selective adsorption which enables elements of the tissue to combine with constituents in the fixative so that an impermeable surface is presented beyond which fixation is poor if it occurs at all.
PART II. The Chromosomes of the Cotton rat.
The Chromosomes of the Cotton Rat

Introduction

This study has been more or less superficial, being directed primarily at the number and morphology of the chromosomes and the nature of the sex complex. So far as has been observed the cytology of the Cotton Rat differs in no way from that known of mammals in general. Thus to avoid repetition of observations already made, this paper gives only a brief account of the observations of interest made in the course of this investigation. Material from the testes, ovaries and uteri of some fourteen cotton rats, prepared by the technique outlined in the appendix, form the basis of this study.

Description

Diploid chromosome number

Excellent spermatogonial divisions were found, and a large number of counts were made. The chromosomes have a tendency to condense into rods which are short and rather thick, but occasionally a cell is encountered in which these are elongated as shown in text figure D (1 and 4). These latter cells are used for drawings and for the study of the morphology of the chromosomes. As can be seen from Plate II (figures 6a, 8a, 9a), the number of the chromosomes appears to be forty-two.
An effort was made to obtain somatic tissue which would give the diploid chromosomes of the female to serve as a check on the observations recorded from the male. Since no embryonic tissue was available at the time this study was made, various organs from the female were examined in hopes of finding suitable somatic cells in process of division. The tissue found most suitable was that of the uterus. During the estrus cycle, after the casting off of the epithelium of the uterine mucosa there follows a period of rapid growth by which a large number of cells are produced in a short time. Since these epithelial cells are on the inner surface of the uterus their fixation is excellent. With considerable search plates of equational divisions are to be found (fig. 7a). The somatic cells from the female show the diploid number of chromosomes to be forty-two.

Types of Cells observed other than normal germ cells.

As the study of the testicular tissue progressed several types of cells were noticed aside from those which appeared to be normal germ and sertoli cells.

First, in about half of the rats examined, there were noticed cells (text figure 6) which after staining appeared opaque. The distinguishing feature of these cells seems to be the manner in which their cytoplasm retains the stain which in normal cells disappears in the course of destaining. These cells seldom show visible nuclear differentiation either because there is none or because the cytoplasm has taken the stain so deeply that the nuclear structures cannot be
observed. When present they are found in all parts of the tubule. No explanation could be found concerning the nature of these cells. If they were consistently found in a rather definite location with respect to the tubule they might be interpretable.

Text figure C. Photomicrograph of Cotton Rat testis x 1000

A. Cells of unknown nature. They are found in the tissue of some animals and seem to be absent in others. Are these cells in the process of dissolution or are they transforming spermatids?

B. Resting cells showing Heterochromosome

C. Beginning of diffuse stage

D. Developing spermatocytes.
In many ways these cells seem to fit into the description of the spermatogonial formation as given by Wilson ('25, p. 369); however, the process of spermioteleosis for mammals merits re-investigation for in the case of the guinea-pig Duesberg ('08) gives a description of the earlier stages of sperm formation which Harman and Root ('24) were unable to confirm. For the present these cells are of uncertain nature and future study may show them to be developing sperm or cells which have died and are in the process of dissolution.

Another type of cell observed while making spermatogonial counts were dividing cells which had an unusually high number of chromosomes (Text figure D, figs. 2 and 3), and showed a great many short, thick rods. Counts of the chromosomes of these cells range all the way from forty-eight to seventy-two. This deviation from the normal number of chromosomes is thought to be due to fragmentation, or better called, "precocious splitting".

Morphology of the Chromosomes

In lining up the chromosomes in the approximate order of their size and shape (figs. 6b, 7b, 8b, 9b), it is found that there is a seriation, graduated in size of paired, i.e., homologous, chromosomes. After a serial alignment of the chromosomes of a cell is made, there is noticed one large element and one rather small element which are unpaired. These are labelled "X" and "Y" in all figures. This constancy of the unpaired condition, together with the even number of chromosomes, is taken as evidence for the presence of a sex chromosome of the X-Y type in the cotton rat.
Text figure D. The chromosomes have been separated in drawing so that underlying elements would not be obscured. Drawn from testis of the Cotton rat, x 3600.

Fig. 1 and 4. Equatorial views of normal chromosomes showing 42 chromosomes.

Figs. 2 and 3. Cells showing an abnormal number of chromosomes, Fig. 2 showing 59 elements and Fig. 3 showing 60 elements.

Figs. 5 and 6. Showing precocious division of the elements of the Primary spermatocytic cells. A couplet indicates the possible pairs of chromosomes which have split.
Growth Period

Early in the growth period a large heterochromosome is observed. This element appears first as a simple irregular mass of chromatin and later, as the cell emerges from the protobrach, i.e., resting stage, this element may be found divided into two parts (fig. 16, 17, 18), which are connected by a fine thread of chromatin. Guthertz ('22), has described in detail the behavior of these two elements during the growth period for vertebrates in general. A complete account of this so-called, "chromatin-nucleolus", is also given for the opossum by Painter ('24).

First spermatocyte division

With the microscopic equipment at hand the study of the morphology of the haploid chromosomes was very difficult. A number of spindle dissections were attempted, two of which are figured (figs. 10a and 11a) on plate II. A serial alignment of the elements (fig. 10b and 11b) was attempted, however a comparison of this nature is of little value unless the elements of five or six cells can be given. Each tetrad has a more or less characteristic shape, and specific individual complexes may be recognized in cells from tissues of various cotton rats.

One element noticed always divided early (fig. 20), after division which is always unequal, i.e., resulting in a large and a smaller mass of chromatin, the parts separate with the exception of a fine connecting thread. It is probable that this is the sex complex.
The number of haploid chromosomes found was between 18 and 21, but this part of the work must be carefully checked before much can be said of it.

The number of elements found in a normal polar group is 21. The changes taking place between the completion of the primary spermatocyte division and the beginning of the second spermatocyte division have not been studied. Occasionally there were noted cases of splitting of the chromosomes of the polar groups in the telophases of the primary spermatocytes (text figure D, 5 and 6). This precocious splitting was not observed in the secondary spermatocytic division.

Secondary Spermatocyte Division

In general cells showing the secondary spermatocyte division are rare and are found only after long search. From the number of dividing cells found per field and the scarcity of such groups of cells, it would seem that the period is of very short duration. The chromosome count from the secondary spermatocyte division is twenty-one. The cells in this stage are easily recognized because of their small size, reduced amount of chromatin, and their position usually near the inner portion of the tubule.

Discussion and Summary

The germ cells and somatic cells of the Cotton rat have been studied to discover the number and morphology of the chromosomes. From the equatorial plates of the dividing cells of the testis and the uterus a count of forty-two chromosomes has been determined.
Upon studying the serial alignment of these elements there was found to be a gradation in the size of the chromosomes much like that found in the rat, Pincus ('27) and the mouse, Cox ('26).

A study of the first and second maturation divisions indicated that the probable haploid chromosome number is 21.

The even number of chromosomes, and the presence of a pair of elements, which in the male, fail to match, indicates that the cotton rat possesses an X-Y type of sex complex. The "X" chromosome is one of the large elements in the cell and the "Y" is one of the smaller. In the heterotypic division the "X" and "Y" are found together (figs. 10a, 11a), but separate to opposite poles (fig. 20), so that half of the sperm should carry the "X" (fig. 19), and half the "Y" chromosome (fig. 21).
PART III: Appendix. Giving details of Cytological technique used for investigation of mammalian chromosomes.
Appendix

Methods of technique Adopted in this Work

Introduction

The contents of this appendix are not original. They have been gathered together from the literature, by word of advice, and learned by trial and error. They form a portion of the common knowledge which grows upon one working with cytological technique. However, in view of the fact that it has taken several years to learn what now seems absurdly simple, it was thought not amiss to give the procedure in reasonable detail in hope that it might be of aid to anyone just beginning work in cytology.

Mastery of technique needed

It is best first to master the technique on some common Rodent, say the white rat. In this way one may become accustomed to the motions etc. which must be made during the technique. Thus unforeseen needs for equipment and materials will arise and may be met by the time one has reached that degree of skill which makes the study of rare or valuable material justifiable.

1 It is taken for granted that the reader has some knowledge of histological technique, for the writer has only given phases of the technique in detail where cytological methods differ from those in common use for the preparation of animal tissues for Histology.
Careful preparation for the work of carrying out the steps involved aids greatly in saving time and avoiding mistakes. It is well to have on hand instruments, warm Petri dishes (37° C), numbered vials with corks to fit, and lastly a record sheet for keeping notes on all tissue fixed with the detail of procedure. If the animal is given a serial number and each bit of tissue used placed in a numbered vial, and later transferred to a bag bearing the proper number or symbol, much time is saved in referring to steps taken and in searching for weak points in technique. It is desirable to have one or more assistants to aid with the operating so that one's entire attention may be devoted to the details of the fixation.

When one’s technique becomes consistently good it is then well to prepare a series of slides from opossum material. Since this marsupial has only twenty-two chromosomes it presents a source of material which is excellent for beginning study. Having at hand a good set of opossum slides to which one can refer, the interpretation of some of the figures found in the cells of mammals having a difficult chromosome complex is simplified.

Modus operandi

After experimenting with various methods of technique the writer came to the conclusion that a tried and relatively simple technique, yielded better results than any other method known today. Other techniques, while giving favorable results,
can not be said to excel those obtainable with B15 as used by Painter.

To make the procedure easy to grasp and simple to follow, the major phases have been included in the following outline which may conveniently pinned to the wall in the laboratory for reference until the whole process becomes second nature.

Value of Complete Records

In the use of the record sheet, it is always important to record, among other things, the source of the animal, its condition of health, etc. While there is scant evidence to believe that the abnormal conditions of captivity have any effect on the germ cells, there is no evidence to the contrary. What this effect is and its extensiveness remain problems for further investigation.

Methods of Killing

In killing small rodents the method used is debatable, either from a humane standpoint or from the standpoint of obtaining good material. The methods in popular use are: (1) decapitation, by means of a pair of bone forceps; (2) killing by means of a blow on the head; and (3) severing the spinal column by means of bone forceps. The main object in killing is to render the animal insensible to the pain caused by removal of the testes, while at the same time not introducing into the blood stream any substance such as chloroform, ether, etc. which might alter the germ cells. McClung
Outline of Cytological Technique using Bi5 for Demonstration of Mammalian Chromosomes.

1. Have work planned and instruments, etc., in readiness.
2. Fixative is mixed just before use and is at 37°C.
3. Kill small animals by decapitation and place or shake a portion of the tissue in a vessel with the fixator.
4. Fix at 37°C for 90-120 minutes.
5. At 6:30 PM, place tissue in bags of cheese cloth and wash in warm 5% Alc. 2 hrs.
6. At 8:30 place bags in dehydrator over night with Siphon dripping 12 drops 93.2% Alc. per minute for a mixing vessel of 25 cc. Capacity.

Table Showing paraffin mixtures used for Embedding:

<table>
<thead>
<tr>
<th>Vial No</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parts A</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Parts B</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Parts C</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table shows time in minutes between bags. After each vial is used, it is stopped and stored in the dark. Keep fresh paraffin in vials 11-13.

Aniline oil may be purified by distillation with zinc dust (5 grams dust per 500 cc. Aniline Oil) collecting portion coming off at 163°C. Store in amber bottles.

Material is now ready for use.

To dehydrate

Alcohol-Xylo Series used in Cytology

- Alcohol
- Xylo

To Shrink

Solutions

- 95% Alcohol
- 80% Alcohol
- 70% Alcohol
- 50% Alcohol
- 38% Alcohol
- 30% Alcohol
- 15% Alcohol
- 7% Alcohol
- 3% Alcohol
- 2% Alcohol

Neutral Balsam

Cylinder for washing bags of balsam.

Agitator or dehydrator

Various solutions in the drying oven at 50°C.

To dry slides overnight in incubator at 37°C.

Remove slides from stain, wash in 5 changes of 70% Alcohol, x 1 min each.

33° C

Place slides in methyl alcohol for 4 hrs.

Remove paraffin from slides. Soak slides into Xylo. Using series given above.

Cryostat in 80% Alcohol, x 1 min each.

To destain one slide at a time, under observation with microscope.

When all stain is removed with exception of Xylo in Tubules, dividing cells, and sperm heads, remove slides from destaining.

To wash in 5 changes of 70% Alcohol, x 1 min each.

Remove from oven, clean, label, and store in box bearing proper serial number.

Chemical and instruction given by

P. Parmer
1939.
(16) states that in working on the effects of killing grasshoppers, chloroform, decapitation and cyanide tended to cause clumping of chromosomes, while a drop of xylol place on a grasshopper and used as a means of killing the insect gave cells with well separated chromosome figures. This effect of killing is an interesting study and merits further investigation.

Decapitation of small animals seems to be the best and most painless method of killing small rodents for the purposes of cytology.

Teasing of tissue

After decapitation, a gonad is removed from the scrotal sac or body cavity and the tunica surrounding the testis slit and a portion of the tubules which are forced out are cut off with a pair of scissors and either placed in a Petri dish of warm fixative and quickly teased apart, or in a vial of the fixative which is corked quickly and shaken vigorously in order to cause the tubules to separate. Either method results in the rapid penetration of the tissue by the fixative, a step of primary importance, for if this separation of tubules is not through the tissue is likely to be poorly fixed and any subsequent treatment is a waste of time. Some testes, like those of the horse, are so constructed that the tubules can only be separated by teasing.

When the connective tissue of the testis is quite resistant to teasing in a dish of the fixative, the tissue may be frayed by teasing a bit of it on a piece of cardboard and then placing the
resulting strands of testis in the fixative. The cells themselves live for some time after removal from the body of the animal. Care in seeing that the testis is thoroughly teased apart seems of more importance than the speed with which tissue is transferred from the body cavity to the fixative.

Mixing of the fixative

The fixative is that as given by Painter '24.

A. Saturated aqueous solution of Picric acid, C. P. • • • • • • • • • • • • • • 75 c.c.
Formalin Merks Blue Label, 40% • • • • • • • • 25 c.c.
Glacial acetic acid • • • • • • • • • • • • 10 c.c.
B. Chromic acid crystals C.P., & Ury • • • • 1.5 gms.
Urea crystals, C. P. • • • • • • • • • • 2.0 gms.

The flask containing solution A. is placed in a water bath and warmed to 35° C. just before using. The chromic acid is added first and dissolved before the urea is added. Since the addition of the chromic acid raises the temperature about 2 degrees, care must be taken not to have the final mixture over 37° C. Excessive heat causes shrinkage, which is to be avoided.

Since the chromic acid used in the mixture is a powerful oxidizing agent the fixative usually becomes a dark green color in about 3 hours. In order to complete fixation before this change comes about it is desirable to mix the fixative within a few minutes before using.
Bagging tissue

After fixation for about 90-120 minutes at 37° C, the tissue is removed from the vial of fixative and tied in a small bag of cheese cloth by means of a fine copper wire, in such a manner that a slight pull will remove the wire and release the corners of the bag so that the tissue is easily accessible. A tag bearing the series number and any other information necessary is attached to the free end of the wire used to fasten the bag. The tissue need not be removed from the bag until ready for embedding. This method of handling tissue is of great aid when a number of various tissues, fixed with different agents, are to be washed, dehydrated and embedded, all at the same time.

If squares of cheese cloth, tags, and lengths of wire (3") are at hand the "bagging" may be completed in less than a minute. The tissue is now ready to be washed.

Washing

Washing is accomplished by hanging the bags, by means of their wires, over the mouth of a 500 c.c. or liter cylinder filled with 5% alcohol at about 30° C. (see outline for technique). The picric acid has a tendency to diffuse out of the tissue and settle to the bottom of the cylinder. This washing is continued for about 2 hours, the alcohol being changed as often as it becomes yellow, depending on the amount of tissue being washed.
Dehydration and Clearing

Subsequent dehydration and clearing are carried on henceforth by a very careful process which insures the most gradual change from one fluid to another; thus reducing any shrinkage which might be caused by the too violent action of the dehydrating or clearing agent. At first glance the whole scheme might seem to be a great deal of needless attention to detail; however it is this extreme care in detail which makes all the difference between excellent and indifferent results.

The Mixing Device

The diagram in the outline of technique illustrates the construction of an agitator useful for the gradual changing of tissue from one fluid to another.

A tank of compressed air, A, or any other source of compressed air furnishes the energy needed to mix the liquids thoroughly. The flow of air is regulated by means of a needle valve on the tank so that no more air than is needed to mix the fluids thoroughly, is allowed to escape. A drying tower B, filled with calcium chloride, is necessary to remove all traces of moisture from the gas used.

Mixing of the various fluids is carried out in the vessel C. The bags of tissue are suspended by means of their wires from the sides of the mixing chamber. The fluid into which the tissue is being changed is allowed to enter the mixing chamber by means of a capillary siphon from the reservoir, D. All excess liquid is carried off into the waste jar E, by means of the waste outlet on the mixing chamber.
Various sizes of mixing vessels and reservoirs are needed so that a combination suitable for the work at hand may be used. The siphons should be made in quantity and roughly calibrated in drops per minute. Usually the most convenient capillary siphon is one giving from 9 - 12 drops per minute for a mixing vessel holding from 35-40 c.c.

When using the agitator on a humid day it is well to have covers of cardboard or glass to fit over the mixing vessel and reservoir so that the alcohol used in dehydration will not absorb moisture from the air by means of its exposed surface.

Cleaning, infiltrating and Embedding

After the tissue is brought into 80% alcohol as indicated in the outline it is cleared by the drop method, using aniline oil. Aniline oil as found commercially is usually of dark color and contains considerable water. By distilling with zinc dust, 3 to 5 gms. per 500 c.c. of aniline oil, and collecting that fraction coming off at 18° C., a pure clear fluid results. This is stored in an amber bottle until used. As a medium for infiltration with paraffin the tissue is brought into wintergreen oil. The "methyl salicylate of Merck" is very satisfactory for this purpose. The process of embedding is accomplished by the gradual infiltration of paraffin as the tissue is moved from one vial to another in the infiltration series as given in the outline for technique.
When the tissue is ready for embedding, the wire holding the corners of the cheesecloth bag is removed and the tissue is embedded in small paper boxes upon which are written the serial number, etc., useful in later identification. The block containing tissue is then cooled quickly. The tissue may now either be stored away or sectioned and mounted on slides, by means of Meyer's egg albumen, preparatory to staining.

Staining Slides

Of equal importance with the chemical process of fixation and the physical process of dehydration and embedding, is that of staining the preparation. The best results in staining are obtained by the use of iron haematoxylin. This stain is superior because, when properly used, it leaves the chromosomes and tetrads a very deep purple or black and all of the surrounding cells colorless, or nearly so.

For mordanting a fresh solution of 3% Ferric alum is made from selected crystals of the salt. After the paraffin is removed from the tissue and the slides are brought into water they are transferred to the mordant for about 12 hrs. After this period of treatment with iron alum, the slides are washed in about 5 changes of distilled water to remove all of the excess iron alum, which if not removed will have a deterring effect upon the subsequent staining action.

After washing, the slides are placed in a 0.5% aqueous solution of certified Haematoxylin. This is best made up and allowed to
ripen 3 or 4 days before use. So long as the stain is a deep cherry to a mahogany color it is suitable for staining, but when it turns a gray black or a precipitate forms, then it is best to discard it.

The slides are allowed to remain in the stain for about 18 hrs. after which they are washed in distilled water and then destained one at a time.

Destaining

Destaining is accomplished in a 2% solution of Fe-alum and controlled under the microscope. This is a very important step and cannot be rushed or done "en masse". Slides are suitable for study only if the structures being investigated are clearly shown, and with this staining method, the destaining must be carefully controlled if good results are to be hoped for.

The only criterion useful in destaining is, as Painter suggests, to destain until the "tetrads are transparent", that is, all of the stain is removed from the tissue with the exception of that in the tetrads, dividing figures and sperm heads. Inspection under a low power will show the cells of the tissue to be almost colorless with the exception of those mentioned above.

Final washing

After destaining the iron alum must be removed from the tissue before mounting or else it will cause the stain to fade. Washing
first in distilled water and then for about 20 minutes in running tap water is sufficient. The slides are then, carefully dehydrated, cleared in Xylol, and mounted in neutral balsam. After drying for about 48 hrs. at not over 53° C, the slides are ready to be cleared, labelled, and stored away until opportunity for their study presents itself.
Reference to most of the papers reviewed will be found in Table I, which gives a condensed picture of the present status of rodent cytology.

Allen, Ezra.


'19. A technique which preserves the normal cytological conditions in the testes of the albino rat. Anat. Rec., Vol. 16.

Cox, Elizabeth K.


Duesberg, J.

'03. La spermagenese chez le rat. Archiv. fur. Zellforsch., Bd. 2.

Evans, H. M. and Olive Swezy.


Gotherz, S.

Hance, R. T.


Harman, H. T. and Root, F. P.


Harvey, Ethel Browne.


McClung, C. A.


McClung, C. A. (Cont'd)


Miller, G. S. Jr.


Moore, J. C. S., and Arnold, G.


Painter, T. S.


Pincus, G.

Ragaud, G.*


Swezy, Olive.

'28. Two chromosome numbers in a mixed strain of rats.


Wilson, E. B.

'25. The Cell in development and heredity.


Winiwarter, H.v., and Oguma, K.


Wedssedalek, J. E.

'20. Spermatogenesis of Cattle.

EXPLANATION OF PLATES

All figures, with the exception of those on Plate I, which are of Kangaroo Rat, are taken from Cotton Rat material which was fixed in Borax and stained with iron haematoxylin. Drawings were made with the aid of a camera lucida. For the finished drawings, the paper was shifted slightly so that all of the chromosomes might be shown in greatest detail with regard to size and shape.
Figures from the testis of the kangaroo rat. x 3000 diameters.

Fig. 1. Side view of first spermatocyte division.

Fig. 2. A sex complex taken from the first maturation division.

Figs. 3a-b and 4a-b. Polar views of first spermatocyte division giving 38-44 elements making the diploid number of chromosomes for the kangaroo rat near 82 ± 2.
PLATE Ia
KANGAROO RAT CHROMOSOMES.
Mammalian Spermatogenesis, Kangaroo Rat.

PHILIP A. POWERS.
PLATE I b

KANGAROO RAT CHROMOSOMES.
PLATE Ib.

Photograph of tubule of kangaroo rat testis x460. This shows a few dividing cells. Figure A is a drawing of one of these cell x3600 showing the large number of chromosomes as they appear on the equatorial plate. Only the larger chromosomes show, the small ones being obscured by the larger.
PLATE II

CHROMOSOMES OF THE COTTON RAT.
PLATE II
CHROMOSOMES OF THE COTTON RAT.

Fig. 5. Prophase nucleus of spermatogonia showing 42 chromosomes, one of which is the Y-chromosome.

Figs. 6a., 8a., 9a.; Spermatogonial plates showing 42 chromosomes.

Fig. 7a. A plate of somatic chromosomes from an epithelial cell of the uterus of a female cotton rat.

Figs. 6b., 7b., 8b., and 9b. A serial alignment of the chromosomes of figures 6a., 7a., 8a., and 9a.

Figs. 10a and 11a. Tetrad dissection from cells of the primary spermatocytic division.

Fig. 10b and 11b. An alignment of the tetrads according to their general shape. Note the x-y element.

Figs. 12 and 13. Polar views of elements of the primary spermatocyte showing 21 chromosomes.

Figs. 14 and 15. Equatorial plates of secondary spermatocyte division showing 21 chromosomes.
Chromosomes of the Cotton Rat
Phillip A. Powers

PLATE II

YX

YX

YY

A B C D E F G H I J K L M N O P Q R S T X Y

10b

11b

12 13 14 15
PLATE III

THE SEX-CHROMOSOMES OF THE COTTON RAT.
PLATE III
THE SEX CHROMOSOMES OF THE
COTTON RAT.

Figs. 16, 17, and 18. Cells in various stages of the growth period up to diakinesis, showing the nature of the heterochromosome.

Fig. 19. A side view of a second maturation division showing the probable X-chromosome going to the poles.

Fig. 20. A partial figure from a primary spermatocyte division showing the probable X-Y chromosomes as they separate to go to their respective poles.

Fig. 21. A side view of a second maturation division showing the probable separation of the Y-chromosome.
Chromosomes of the Cotton Rat. Philip QA Powers

Plate III

16

17

18

19

20

21
PLATE IV.
PHOTOMICROGRAPHS OF COTTON RAT TESTIS.

A COMPARISON OF OSMIC ACID AND Bis TECHNIQUES.
PLATE IV.

Photomicrographs from cotton rat testis stained with Fe-haematoxylin.

Fig. 22. From material fixed in B15 note the good general fixation. A bit of shrinkage, due to the use of a fixative the temperature of which was slightly above 37°C., is noticeable.

Fig. 23. Tissue after fixation with Hanco's cold Flemming,
PLATE V.

PHOTOMICROGRAPHS SHOWING THE NATURE OF THE AREAS IN WHICH DIVIDING CELLS ARE FOUND.
Photomicrographs of testicular tissue from three animals showing the appearance of areas in which dividing cells are found.

Figs. 24 and 26. Groups of dividing cells are most frequently found.

Fig. 25. Condition, sometimes common where only one
PLATE VI.

PHOTOMICROGRAPHS OF PRIMARY SPERMATOGONIAL DIVISIONS.
PLATE VI.

Primary spermatogonial divisions.

Figs. 27 and 28. Photographs from the same field at different focus. Showing how good fixation causes the
PLATE VII.

PHOTOMICROGRAPHS OF THE FIRST SPERMATOCYTE DIVISION.
PLATE VII.

Photomicrographs of the first spermatocyte division.

Fig. 29. A low power view of a field showing cells in the stages of the first Spermatocyte division. Observe the arrangement of the tetrads in properly stained material.

Fig. 30. First Maturation division x1000.
PLATE VIII.

PHOTOMICROGRAPHS OF EARLY GROWTH STAGE.

AND

SECOND MATURATION DIVISION.
Fig. 31. Early diakinesis showing (A) the heterochromosome.

Fig. 32. Area showing cells in process of second maturation division.