SPERMATOGENESIS IN THE HUMAN INFERTILE MALE

by

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A thesis presented in partial fulfillment of the ordinances for the degree of Doctor of Philosophy of the University of Sheffield

January 1979

SUMMARY

The different cellular stages of the spermatogenic cycle in man were identified and categorised from the array of cells observed in preparations obtained from whole tubules of the testis. This included description of meiotic figures. An attempt to establish the dynamics of the spermatogenic process was then made by two complementary approaches.

Experimental work, initiated to establish and maintain differentiation in tissue culture, was successful. In vitro labelling with tritiated thymidine identifed S phase spermatogonia and S phase primary spermatocytes and followed the fate of these cells. At ten days, labelled primary spermatocytes were distinguishable from labelled spermatogonia and at 10-12 days a surge of unlabelled primary spermatocytes, identified at diakenesis, was observed. Changes in cell category frequencies were recorded with time in tissue culture and between individuals.

Cell category frequencies were also studied in a series of infertile males. This revealed very considerable variation, with some categories not represented in some individuals. Significant relationships were found between some cell categories in the series. These trends allowed the cell categories to be assigned to the sequence of events within the spermatogenic cycle with some confidence. Reasons were given for assigning differences in cell category frequencies to a rate effect due to a combination of cell division events and the proportional duration of each cell stage. Previous models of spermatogenesis have concentrated on multiplication due to division to explain ratios between cell categories. One cell category was a particularly important factor for describing the activity of the germinal epithelium. A practical method of identifying points of lesion in the infertile spermatogenic cycle was described and compared with established methods for expressing the status of the infertile testis.

ACKNOWLEDGEMENTS

I would like to record my appreciations of the many persons who have contributed in various ways to the presentation of this thesis.

Professor I D Cooke, Head of the Department of Obstetrics and Gynaecology, University of Sheffield, initiated a multifaceted investigation into male infertility. He obtained funding from the Former Trustees to the United Sheffield Hospitals to cover my employment as the Cyto-geneticist. He has maintained a vigorous interest in the project as it has progressed.

Mr J L Williams, Consultant Urologist, Hallamshire Hospital, Sheffield, was responsible for the clinical investigations and surgery of the infertile patients described in this study. This investigation would have been impossible without the freely given co-operation and interest of Mr Williams.

Dr Adrian Thomas, formerly Research Assistant, Jessop Hospital for Women, obtained and kept the clinical records on the infertile population. We exchanged many hours of discussion on infertility. He was responsible for reminding me that testicular biopsies can also be patients.

Several members of various Hospital and University departments have generously co-operated in this work. Mr M Fox, Consultant Urologist, Hallamshire Hospital, Sheffield, referred three patients to me for chromosome analysis and has kindly permitted their inclusion in the series. Dr Elizabeth Lenton, Biochemist, University Department of Obstetrics and Gynaecology, was responsible for the plasma gonadotrophin estimations and generously allowed me access to her results.

Mr Graham Anderson, Chief Technician, Department of Pathology, Hallamshire Hospital, provided extra histology slides of each biopsy for my use. In addition, he prepared sections from some of my experimental samples which were specially cut and stained for me. My colleagues, at the Centre for Human Genetics, Sheffield, have been generous with their co-operation and information whenever I have asked for help. In particular, Mr Bill Crowe and Mrs Audrey Briscoe have provided technical and clerical assistance whenever requested.

Dr Maj Hultén, East Birmingham Hospital shared her enthusiasm and expertise with me when I first started working with meiotic chromosomes. Dr Pat Cooke, City Hospital, Nottingham helpfully offered expert advice on my use of statistics and we spent exciting hours discussing this work in general. Mr Tony Potter, Centre for Human Genetics, Dr Charles Curtis, University of Sheffield and Ms Rose Evison have critically read, commented on and argued about the presentation and content of this thesis. Some of my personal friends have listened at length whilst I have been involved in this work. Charles, Sarah and Kate Curtis have found other things to do whilst I have been engrossed in preparing this thesis for presentation. This thesis has been skillfully typed by Miss Barbara McNeill.

Finally, I thank my supervisor, Dr C E Blank, Reader in Medical Genetics, Subdepartment of Medical Genetics, University of Sheffield, for his support and interest whilst I was exploring the experimental work. I particularly appreciated his help and patience whilst I was preparing this thesis for presentation.

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Forethought :-

"We can never be prevented from thinking
nor from choosing
a good viewpoint from which to think."

Harvey Jackins

CHAPTER 1

INTRODUCTION

1. Aims

2. Reasons why programme was initiated

3. A logical view of the investigation

   A. How has the process of spermatogenesis been studied previously?

   B. New methods for studying spermatogenesis can be devised

   C. What does this work mean?

4. Restrictions placed on the investigation

5. Description of the investigation

1. AIMS

In 1953, Roosen-Runge and Barlow said that, "the process of spermatogenesis in any individual is recorded in the cells of the testicular epithelium, the problem is to translate the record". The aim of this thesis is to study the sequence of spermatogenesis recorded in the cells of the testicular epithelium in a series of infertile males. If the cycle can be understood, points of impairment can be recognised.

2. REASONS WHY THE PROGRAMME WAS INITIATED

In 1974 I was employed on a research contract to study meiotic chromosome division in a series of infertile males. This was part of a wider investigation into male infertility. My part in the programme was to screen the population for chromosome abnormality. There were two immediate research implications resulting from the preliminary studies undertaken :-

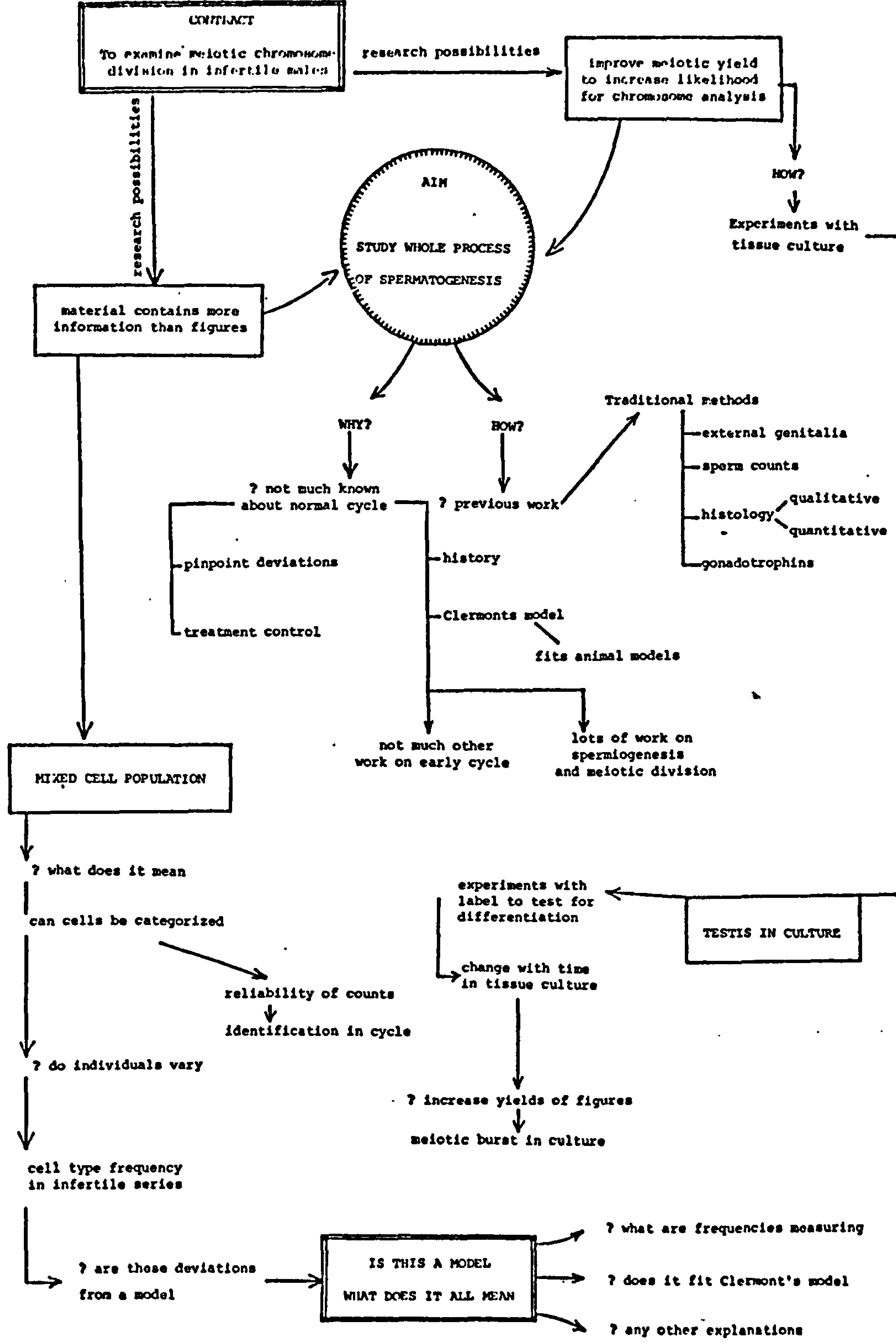
1. Was it possible to improve yields of meiotic figures for analysis by culturing testis tubules in tissue culture?
2. The material studied contained more information than was necessary for chromosome analysis. Could this material be used to study the spermatogenic cycle as a whole?

These two research propositions can be linked in the aim of this thesis, outlined in Section 1.

3. A LOGICAL VIEW OF THE INVESTIGATION

The investigation is outlined in Figure 1.1 which shows how the two research propositions were linked together so that experiments designed to test one area of work provided information relevant to the other area of work. The following paragraphs briefly describe Figure 1.1.

Figure 1.1



A. How has the process of spermatogenesis been studied previously?

(i) Traditional methods used to study male infertility

1. Case history-taking and examination of the external genitalia.

2. Sperm count analysis

3. Histological examination of testis biopsies which include qualitative assessments via traditional pathology laboratory reports and quantitative scores of tubule function (eg Johnson's Mean Testicular Biopsy Score)

4. Estimation of gonadotrophin function

(ii) What previous work has been reported on the spermatogenic cycle in man?

1. Histological review of previous work

2. Clermont's model of spermatogenesis in man and other animal models

3. Other authors' studies of parts of the cycle, notably meiotic division and post-meiotic maturation to spermatozoa

B. New methods for studying spermatogeneis can be devised

(i) Work designed to improve the yields of meiotic figures by growing testicular cells in culture

1. Experiments to grow testis in culture. There is no concensus of opinion on best methods for culturing testis in vitro.

2. Experiments to establish continued differentiation in vitro using labelled testicular cells. Previous

work on maintaining meiotic division during culture experiments report widely varying success rates.

(ii) Can the information present in the preparations be used to study the spermatogenic cycle as a whole?

1. What cell types are present in the preparation and can they be recorded and categorised?
2. If cell counts are made are they reproducable and thus reliable?
3. Do cell counts change with time in tissue culture?
4. Do cell counts vary from individual to individual?
5. Can cell types be assigned to the sequence of events occurring during spermatogenesis?
6. What type of cell count variation is observed in infertile individuals?

C. What does this work mean?

1. Does the use of testis culture improve the yield of meiotic figures which are suitable for analysis?

2. Does the data obtained from recording cell types provide information relevant to understanding a model of spermatogenesis?

3. Does the data obtained record deviations from the model of spermatogenesis in infertile individuals?

4. RESTRICTIONS PLACED ON THE INVESTIGATION

1. Priority was given to the contract to study chromosome

abnormality in the series of infertile males. The individuals sampled were selected because of their infertility problem. Thus experimental work could only be set up when preparations had been made for meiotic chromosome analysis.

2. There have been many other studies of meiotic division in man, therefore this part of the spermatogenic cycle would receive less attention than the cycle prior to division.
3. Maturation of post-meiotic division stages to mature spermatozoa, sometimes referred to as spermiogenesis, would be excluded from this work. This part of the cycle is well documented.

5. DESCRIPTION OF THE INVESTIGATION

Because of the restrictions outlined in 4.1 the work was developed and is presented in this thesis in the following manner which broadly corresponds to Chapter sequence.

1. Techniques for preparing meiotic chromosomes for analysis were studied and developed to give good meiotic figures under local laboratory conditions. The analysis of meiotic figures was learnt and developed using various staining techniques.
2. The results of other investigations together with the results of the cytogenetic screening of the infertile population are presented in detail.
3. Experiments to set up testis biopsies in culture are described. The results of labelling studies to follow differentiation in vitro are presented.
4. Analysis of the cell types observed in the slides prepared

for chromosome analysis is recorded. Questions relating to reproducibility, differences between individuals and changes in cell type frequency with time in tissue culture are discussed.

5. An analysis of the labelled cell types present in samples grown in tissue culture is recorded. This data is used to set up a model of sequential events within the spermatogenic cycle and the problems of correlating these results with those of other workers are discussed.

6. An analysis of cell types present in the infertile population is recorded. This data is used to clarify the sequential location of cell types within the spermatogenic cycle. The frequency of cell types recorded is used to test for numerical relationships between the sequential stages of the cycle. Possible models of spermatogenesis are discussed and a method of recording deviations from the model is presented.

   The implications of being able to study the whole of the spermatogenic cycle are discussed and methods for further investigations are suggested.

7. The results are summarised.

CHAPTER 2

PREVIOUS CYTOLOGICAL STUDIES OF THE SEMINIFEROUS TUBULES WITH PARTICULAR REFERENCE TO PREMEIOTIC DIFFERENTIATION

1. Introduction

2. Early studies on premeiotic cells of the seminiferous epithelium

3. Early attempts to describe the process of spermatogenesis in humans

4. Early theories of spermatogonial renewal

5. Stem cell renewal theory

6. The stem cell renewal theory in a number of animals

7. The stem cell renewal theory in man

8. Alternative points of view

9. Work utilising knowledge of spermatogenesis

10. Conclusions

1. INTRODUCTION

The testicular system in general attracted the attention of the early microscopists and cytologists. The earliest relevant cytological study was probably the description of sperm by Leeuwenhoek in the eighteenth century. Then, since spermatozoa were readily accessible in the seminal fluid, spermatozoa were described in detail both qualitatively and quantitatively. During the nineteenth century, the differentiation of spermatids to spermatozoa was described. Towards the end of the nineteenth century the development of primary spermatocytes received considerable attention which was focused on the mechanism of chromosome division. During this stage in the history of cytology, which culminated with the "chromosome theory of inheritance" workers were beginning to comprehend that meiotic chromosome division was a process whereby the chromosome number of the species was maintained and whereby genetic information was passed from generation to generation. In general, the study of the premeiotic cell components of the seminiferous tubule received less attention than the study of cells in meiotic division or the development of postmeiotic cells to sperm. This latter process became known as spermiogenesis.

2. EARLY STUDIES ON PREMEIOTIC CELLS OF THE SEMINIFEROUS TUBULE

Sertoli in 1875 described two types of cell lining the seminiferous epithelium. He recognised a new cell type (which now bears his name) which he distinguished from the germinal cell type. La Valette Saint-George (1876) described the two cell types respectively as "follicular" and "nourishing". He coined the term "spermatogonia", but he mistakenly applied this term to the new cell type of Sertoli which he believed was the ancestral cell of the sperm. In 1866 Sertoli described the structure and development of the "germinative" cell

type. In 1887, Waldeyer sorted out the confusion in terminology by retaining the term "spermatogonia" and defined its role as ancestor of sperm. He clearly pointed out that the term spermatogonia referred to Sertoli's "germinative" cell and th Saint-George's "nourishing" cell. The term "Sertoli cell" was retained to describe Sertoli's new cell type which had been mistakenly described as "follicular" by Saint-George.

Since this naming of spermatogonia in 1876 and the rapid subsequent acceptance that spermatogonia are the ancenstral cells of sperm, there have been numerous attempts to decipher the mechanism of spermatogenesis. The actual process of spermatogenesis is recorded in the cell types which we can observe, in the sequential relationship between the different cell types and in the quantitative relationship between the different cell types. The problem is to decipher the record in order to understand the process. Our present state of knowledge of the process of spermatogenesis in humans is very far from complete. For instance, when confronted with the multitude of testicular lesions of the infertile male, our knowledge of the process of spermatogenesis does not often enable us to define precisely the location of the lesion within the process. Nevertheless, the process of spermatogenesis is recorded within the seminiferous tubule if we can interpret the record.

3. EARLY ATTEMPTS TO DESCRIBE THE PROCESS OF SPERMATOGENESIS IN HUMANS

Waldeyer (1906) described large "stem cells" which divided to give somewhat smaller spermatogonia. The spermatogonia, which had relatively lighter staining cytoplasm with clumps of chromatin in the nucleus compared to the "stem cells", gave rise by division and differentiation to spermatocytes. The spermatocytes were considered the direct

ancestors of dividing meiotic nuclei which in turn differentiated to mature spermatozoa. Branca (1924) wrote an extensive and detailed description of the whole of human spermatogenesis. His drawings are delightful and excellent (Figure 2.1), and include descriptions of the wide variation between and within the cell types of spermatogenesis. It is interesting to notice that his excellent drawings pick out the essential cell type differences in a visually descriptive manner which is not always accomplished by photomicroscopy. In particular Branca noted that some of the distinctive features which differentiate spermatogonial types in certain mammals (such as rodents) are not seen in man. He also noted that human material is highly variable between individuals.

Stieve (1930) described the prepubertal testis as containing undifferentiated small germ cells. At puberty these germ cells gave rise to all other cells of the seminiferous tubule, i.e. Sertoli cells and spermatogonia. After puberty only a small number of undifferentiated cells remained. Stieve described the spermatogonia and primary spermatocytes as very similar but slightly larger than the undifferentiated germ cells. He believed that these three cell types could be identified in human material and that the size progression was from small undifferentiated germ cells to large primary spermatocytes.

Roosen-Runge and Barlow (1953) attempted a quantitative description of spermatogonia in human seminiferous tubules following earlier work on the rat.

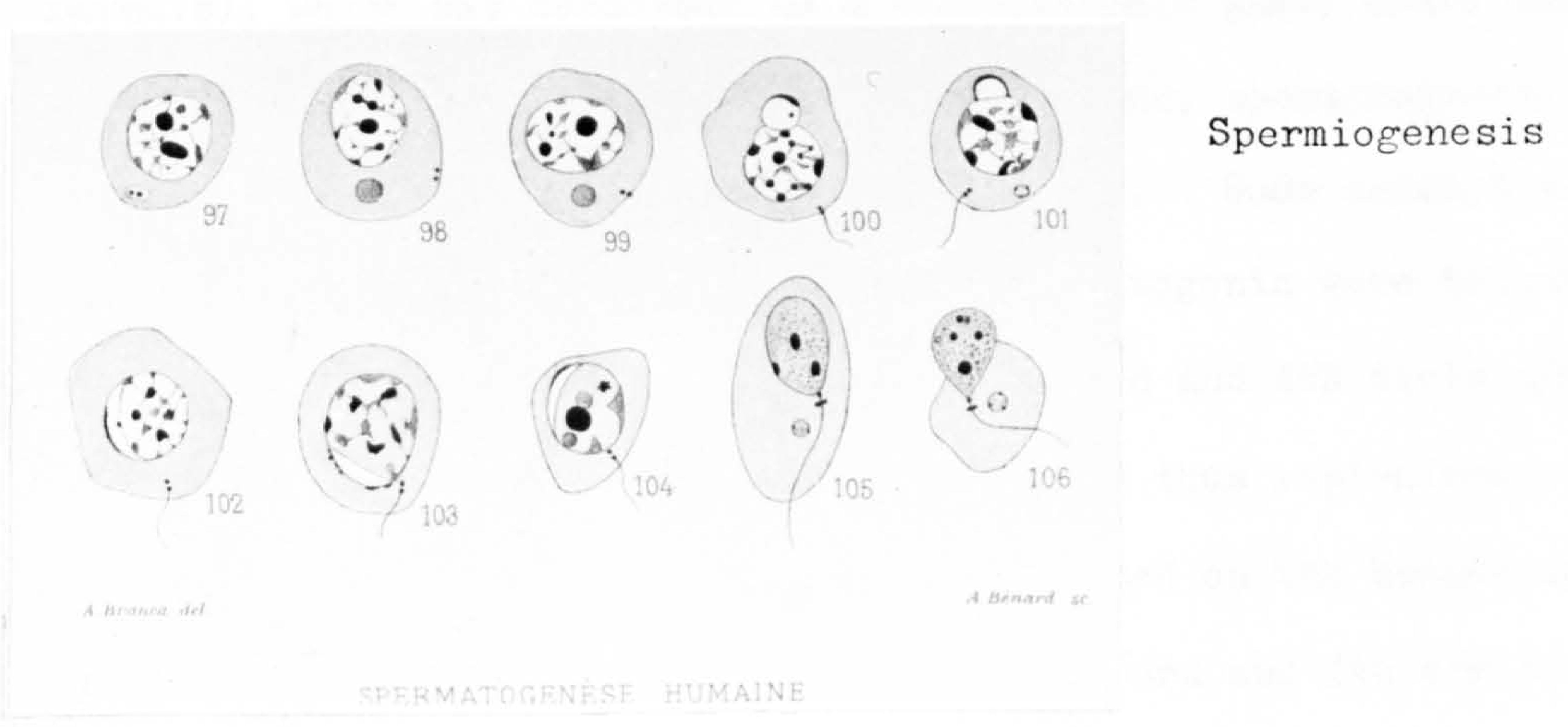
These workers described human spermatogonia as occurring in a large variety of sizes. The largest cells were defined as stem cells with a range of cell diameter of 19.6 - 18.1μ. Studies of aggregates of nuclei in mitotic division suggested that spermatogonia went through some seven divisions before transformation to primary spermatocytes.

Spermatogonia
"Large and small"

Primary spermatocytes

Secondary spermatocytes

Spermatocytes inter M I, M II



Spermiogenesis

BRANCA'S DRAWINGS, CHOSEN TO ILLUSTRATE SELECTED STAGES THROUGH SPERMATOGENESIS

They thought that stem cells usually divided singly or occasionally in pairs or quadruplets. The first three spermatogonial divisions were usually synchronous but after 3 - 4 divisions the synchrony broke down. At the last spermatogonial division, the spermatogonia was some four times smaller than the stem cell. During spermatogonial divisions they noted that the appearance of the nucleus changed from the large pale nucleus of the stem cell, through a somewhat smaller pale nucleus with chromatin clumps, to an even smaller nucleus with chromatin clumps. All spermatogonial types had a clear well defined nuclear membrane. The distinction between the smallest spermatogonial type and the similar sized primary spermatocyte (particularly when spermatocytes were still found adjacent to the periphery of the tubule rather than more centrally placed) was based on differences between the nuclear membranes. The primary spermatocyte had a poorly defined nuclear membrane and thus the periphery of the nucleus appeared indented in contrast to the clearly defined periphery of the smallest spermatogonial nucleus.

These workers noted that human spermatogonia did not resemble the spermatogonial types recorded in the Rat. In addition, the synchronous development of spermatogenesis in the Rat (and other mammals), which was described as a spermatogenic wave, could not be identified in Man. The concluded that, in man, spermatogenesis was initiated in large palely staining stem cells. Some seven divisions of the spermatogonia took place before spermatogonia were transformed into primary spermatocytes. At about the 3rd and 4th divisions some spermatogonia reverted to stem cell types and thus replenished the stem cell line. This interpretation was based on the breakdown of mitotic synchrony which they recorded at the 3rd and 4th divisions of spermatogonia. These results and conclusions largely agreed with

the observations of Waldeyer (1906) and Branca (1924) but were not in agreement with Stieve (1930).

Thus by the early 1950's workers were extending descriptions of spermatogonial cell types towards a search for a model of spermatogonial renewal. Whilst interest in human spermatogenesis continued, many workers turned to animal models to study the process of spermatogenesis. At this time there were several theories of spermatogonial renewal in existence and under investigation.

4. EARLY THEORIES OF SPERMATOGONIAL RENEWAL

Some early workers believed that spermatogonial renewal was accomplished by transformation of Sertoli cells or cells from the wall of the seminiferous tubule to spermatogonia. As late at 1950, Roosen-Runge and Giesel still considered this possibility as a tenable model. However, by 1953, Roosen-Runge and Barlow had revised this hypothesis to take account of their work described in the preceding section.

A second model held up to the early 1950's, which again was the revival of a much earlier view, was that each spermatogonial division produced two morphologically different cells called respectively a spermatogonium and a spermatocyte. This model was generally known as the theory of "bivalent mitosis".

In contrast to these theories, Regaud (1901) had observed two types of spermatogonia in the Rat. The first "A type" spermatogonia (or "dust-like") were the more primitive cells which divided to give more differentiated "B type" (or "crust-like") cells. Then in turn, "B type" cells divided to give rise to spermatocytes.

These observations of Regaud (1901) were developed by later workers into the hypothesis that A type cells were the stem cells from which B type cells arose. A type cells divided to give further A type cells as well as B type cells which proceeded to

differentiate. Thus Roosen-Runge and Barlow (1953) in their paper described in the preceding section, had experimental evidence that fitted this model in man.

This theory of the maintenance of the stem cell line was tentative, since workers had difficulty in determining the state of differentiation of the spermatogonia. This stumbling block remained despite recognition that, in animal models such as Rat, large numbers of cells differentiated synchronously in what was known as the "spermatogenic wave".

The work of Leblond and Clermont (1952) provided the key to the understanding of spermatogonial renewal in the Rat. These workers used the periodic acid - Schiff reaction to stain the acrosomal system of the maturing spermatid. The precisely defined maturation of the acrosomal system was used to indentify 14 successive stages in the cycle of the seminiferous epithelium. This key, together with the previously observed synchronisation of large numbers of cells in the Rat system, allowed spermatogonial development to be recorded accurately. As a result of this work Clermont and Leblond (1953) were able to make quantitative determinations of the spermatogonial types present at each stage in the seminiferous cycle. They described three types of spermatogonia as follows :-

1. Type A Spermatogonia (Regaud's "dust-like" spermatogonia) These cells had an ovoid, pale nucleus with a thin nuclear membrane. Chromatin material was present as fine dust-like granules; sometimes one or more larger chromatin granules could be seen. This type of spermatogonium was recorded at all stages of the seminiferous cycle.

2. Intermediate type Spermatogonia

These cells were like Type A but the nuclear membrane appeared thicker, larger chromatin granules became more numerous, the nuclear stain was deeper and the nuclear shape was more obviously round. This type of spermatogonium appeared at the end of Stage I of the cycle when they were difficult to distinguish from Type A. They were present throughout Stages II, III and IV and graudally changed to Type B spermatogonia.

3. Type B Spermatogonia (Regaud's "crust-like" spermatogonia)

The nuclei of these spermatogonia were spherical, darkly stained and contained coarse chromatin masses. They were present at Stages IV, V and VI of the seminiferous cycle. At the end of Stage IV, division of Type B spermatogonia produced small cells with dark nuclei identified as the new generation of spermatocytes. These cells were designated "R" indicating that the cells were resting before entering meiotic prophase. Clermont and Leblond (1953) correlated information on the presence of mitotic peaks with the appearance of the different spermatogonial cell types and concluded that mitosis produced two identical daughter cells which thereafter transformed into the succeeding generation. In addition 4 of the 5 mitotic peaks produced only spermatogonia whilst the 5th peak produced only spermatocytes. This was clear evidence against the theory of "bivalent mitosis" mentioned earlier. Thus, Clermont and Leblond extended the work of men such as Regaud (1901), Branca (1924), Roosen-Runge and Giesel (1950) and Roosen-Runge and Barlow (1953) and, basing

their hypothesis on their own observations, formulated the "stem cell renewal theory".

5. STEM CELL RENEWAL THEORY Clermont and Leblond (1953)

Spermatogonia are replenished whilst producing generations of spermatocytes.

The process in Rat can be explained as follows :-

Some of the Type A spermatogonia, present at Stage I, are referred to as dormant (Ad) since they do not divide until Stage IX of the cycle. Other Type A (A) spermatogonia transform to Intermediate Type and in turn to Type B spermatogonia. Mitotic peaks at Stage I and IV precede this transformation. The mitotic peak at Stage VI precedes transformation of Type B spermatogonia to resting spermatocytes. At Stage IX of the cycle the dormant type Ad divides once again and again at Stage XII to produce 4 type A spermatogonia. One of these 4 cells becomes the Ad of Stage I thus replenishing the spermatogonial "stem cell" line. The other 3 spermatogonia (A) divide at Stage I and transform to Intermediate type spermatogonia. Figure 2.2 illustrates this model.

6. THE STEM CELL RENEWAL THEORY IN A NUMBER OF ANIMALS

In 1956, Oakberg published a study of spermatogenesis in the mouse. His experimental work was comparable with that of Clermont and Leblond and he tried to explain his data using the model of stem cell renewal proposed for the Rat by Clermont and Leblond.

One of the interesting points of his work was his clarification of the nature of division of Type B cells. Roosen-Runge and co-

Figure 2.2

"MODEL OF SPERMATOGENIC RENEWAL IN THE RAT"

(from Clermond and Leblond, 1953)

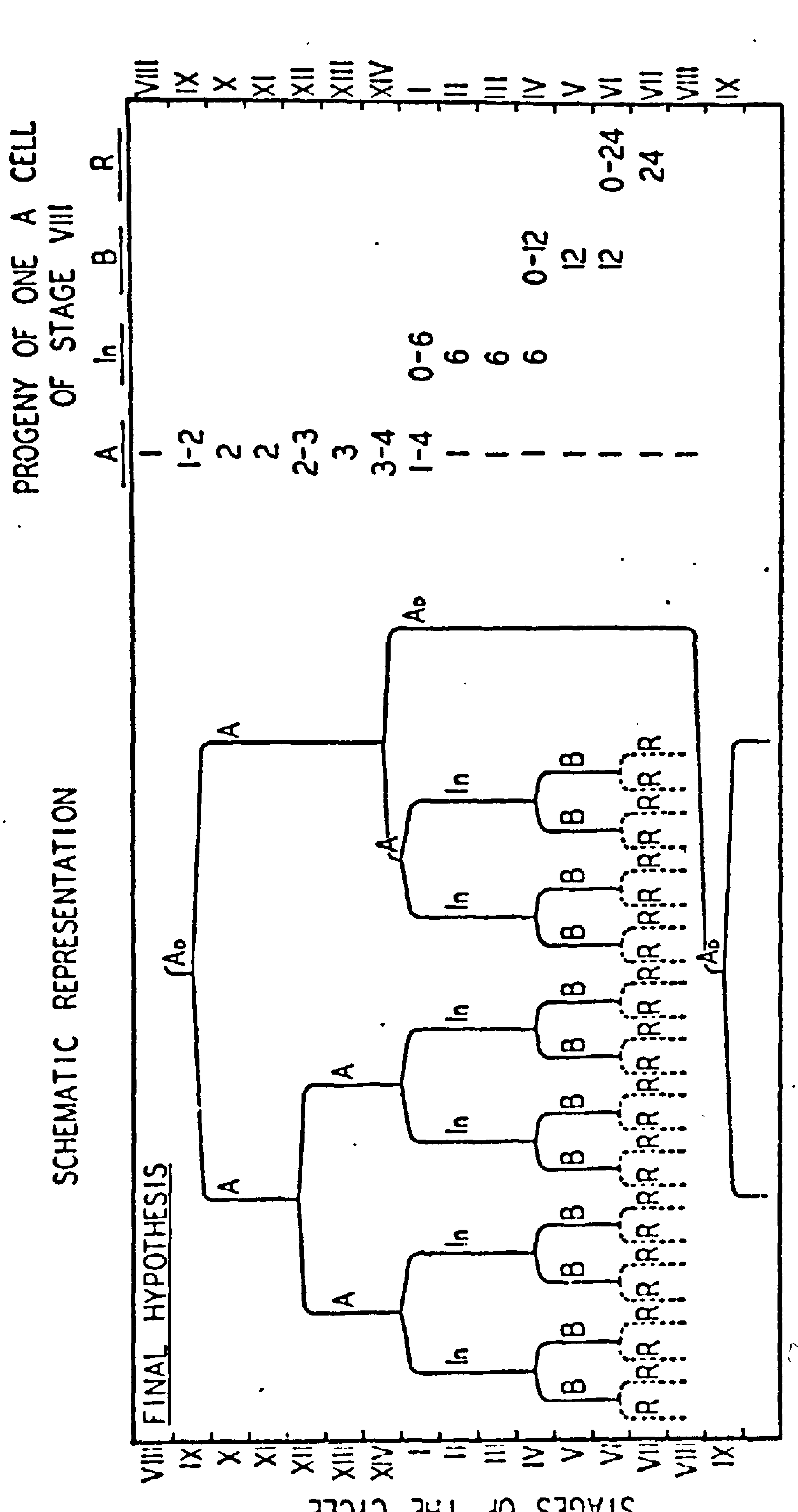


Fig. 4 Diagrammatic representation of the most probable pattern for the development of spermatogonia (or "Stem Cell Renewal Theory"). The Roman numerals on either side of the diagram indicate the stages of the cycle. Lettering: A, type A spermatogonia; A_D, dormant type A spermatogonia; In, intermediate type of spermatogonia; B, type B spermatogonia; R, resting spermatocyte.

workers (1950, 1953), referred to the products of Type B division as spermatogonia. Oakberg said that in mouse it was clear that the small round cells which were the product of Type B spermatogonial division, enlarged and immediately entered the Leptotene stage of meiotic prophase. Thus Oakberg agreed with Clermont and Leblond (1953) who believed that Type B spermatogonia divided to produce resting primary spermatocytes. Oakberg investigated with thoroughness the observation of spermatogonial and spermatocyte degeneration in the mouse. He claimed that necrotic nuclei were transitory in nature being rapidly removed from the system. Calculations of expected numbers of Type A cells with observed numbers of cells, suggested that there was an approximate 25% deficiency of Type A cells during Stages XII and XIV of the cycle. Degeneration also occurred at the first and second meiotic division with a resulting 13% loss of spermatids relative to primary spermatocytes. Degeneration of spermatocytes during premeiotic division was not observed. Oakberg noted that these degenerative changes made absolute comparisons with Clermont and Leblond's model difficult. Nevertheless he saw mouse and rat as having the same model of stem cell renewal.

In 1962, Monesi extended his own earlier work, Oakberg's (1956) work and that of other workers, to publication of a model for mouse stem cell renewal. In order to achieve this, Monesi used autoradiographic studies of DNA synthesis following the incorporation of tritiated thymidine into replicating DNA during the synthetic phase of the cell cycle. Other workers had previously used these autoradiographic techniques employing a variety of labelled substances in a number of different animal systems. For instance as early as 1950, Howard and Pelc published some preliminary observations on the timing of spermatogenic stages in mouse, following labelling with P^{32}.

Monesi was able to identify 4 different sub-classes of Type A spermatogonia by identifying their presence at different stages of the cycle of seminiferous epithelium. The stages of the cycle were identified by correlation with the development of the acrosomal system. Morphologically the 4 classes of Type A nuclei are indistinguishable. He was also able to calculate the relative lengths of the phases of the synthetic cycle in each spermatogonial class.

LENGTH OF PHASES OF THE SYNTHETIC CYCLE IN EACH SPERMATOGONIAL CLASS

(Monesi 1962)

Cell type Spermatogonial Class	Average G_1 in hours	Range S in hours	Variability	Range G_2 in hours	Variability	Average life span in hours
A_1		7.0– 7.5	Low	8–19	High	
A_2	7.5	6.0– 7.5	Low	8–19	High	28–30
A_3	8.0	7.5– 8.0	Low	7–16	High	26–28
A_4	9.5	12.0–13.0	High	5–10	Low	30–31
Intermediate	8.5	12.5–14.0	High	3–10	Low	26–28
B	10.5	14.5–18.0	V.High	3– 7	V.Low	29–30
R		14.0				

From these results it can be seen that the average life span of all spermatogonial types was more or less constant. Variation in the length of the cycle occurred in the ratio between S and G_2. From these results Monesi worked out a model of spermatogonial renewal in

the mouse, which differed from that in the Rat. The difference lies in the fact that at Stage VIII, division of Type A spermatogonia produced functionally different daughter cells, one of which was established as the new dormant (Ad) or stem cell renewing line. The other cell differentiated to an A_2 type and subsequently gave rise to spermatocytes. Oakberg used degenerating spermatogonia to explain the discrepancies between his observations and Clermont and Leblond's (1953) model for the Rat. Monesi did not deny the presence of degenerating spermatogonia. He found that his model, see Figure 2.3, could take into account degenerating nuclei whilst fitting the observed numbers of nuclei in each class to the expected numbers in each class, if the stem cell maintenance line arises earlier in the cycle in mouse than it does in Rat. In fact, Monesi's model for the mouse is one of the models postulated by Clermont and Leblond (1953) and discarded by them for Rat spermatogonial renewal.

Thus these workers postulated two different models of stem cell renewal for Rat and Mouse respectively. The models were similar but differed in the time at which stem cell renewal was accomplished. In other respects, such as the descriptions of the morphology of spermatogonial types A, Intermediate and B, there was little difference.

During the late 1950's Clermont and other workers studied spermatogenesis in a number of other animals such as hamster, ram, duck and monkey. The first three have models of spermatogonial renewal similar to those described for Rat and Mouse. In the monkey, however, the possibility remained that differential or bivalent mitosis occurred when Type A cells gave rise to Type A and Type B spermatogonia. Clermont and Leblond (1959) studied spermatogenesis in Macacus rhesus in detail and were able to elucidate a model of spermatogonial renewal which fitted their stem cell renewal theory and involved no differential mitosis. In this monkey Clermont and Leblond noted three basic

Figure 2.3

"MODEL OF SPERMATOGENIC RENEWAL IN THE MOUSE"

(from Monesi, 1962)

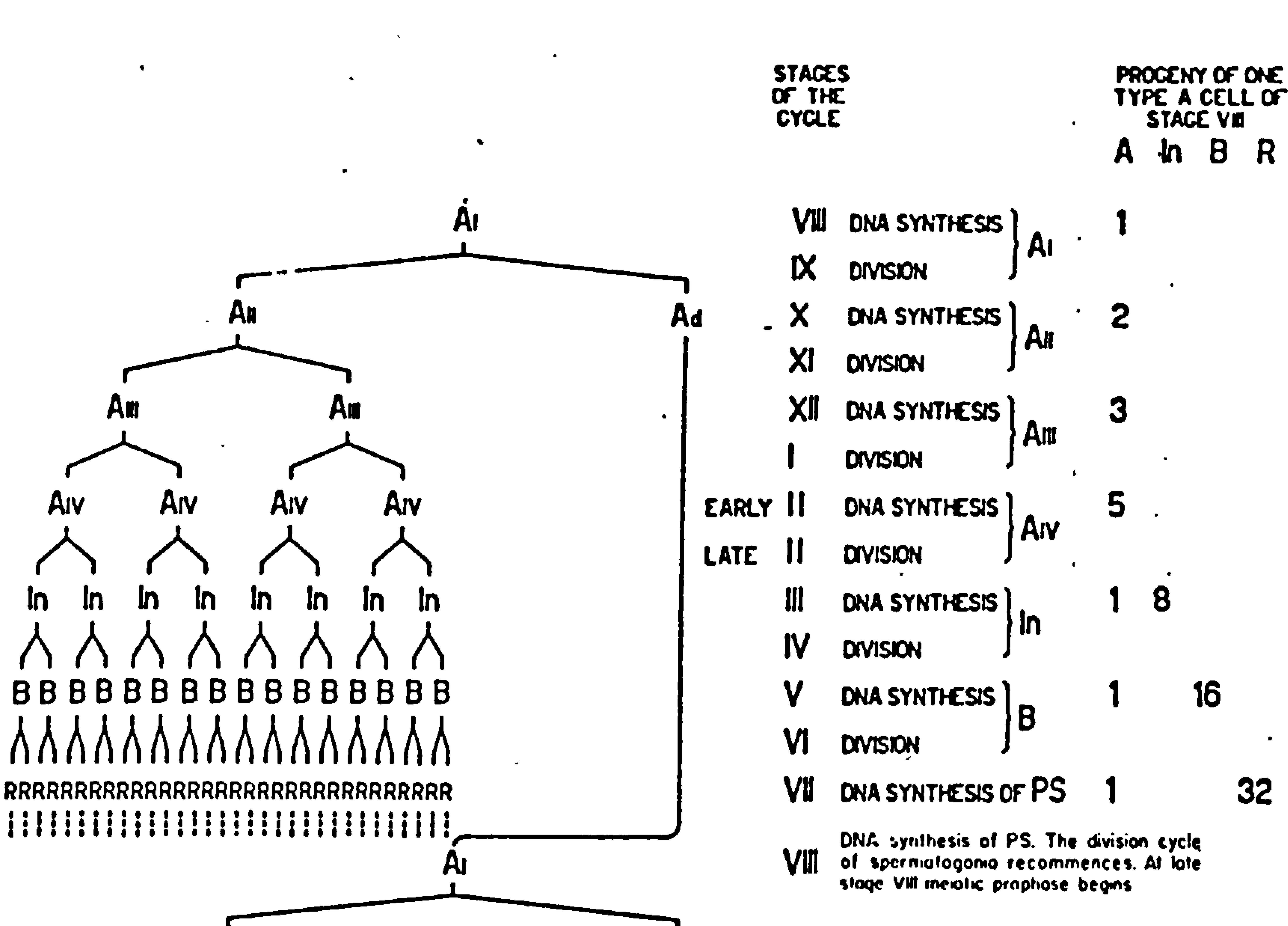


FIGURE 3

Diagrammatic representation of the proposed pattern for the renewal of spermatogonia in the mouse. The method of representation is taken from that used by Clermont and Leblond (2). Abbreviations: AI, AII, AIII, AIV, four successive generations of type A spermatogonia; Ad, "dormant" type *A* spermatogonia; *In*, intermediate type spermatogonia; *B*, type B spermatogonia; *R*, resting primary spermatocytes; *PS*, primary spermatocytes. DNA synthesis for each generation of spermatogonia occurs also at stages IX, XI, I, IV, and VI, but the peaks of the synthesis are localized at stages VIII, X, XII, II, III, and V, as indicated (see Fig. 1).

spermatogonial types :-

(a) Type A_1 cells which had relatively small nuclei with darkly stained uniformly distributed chromatin;

(b) Type A_2 cells which had relatively large nuclei with pale staining chromatin;

(c) Type B cells were characterised by nuclei with darkly stained chromatin; granules. Thus the chromatin appeared much coarser than Type A_1 nuclei.

Clermont and Leblond described spermatogonial renewal as being initiated by division of an A_1 type cell. The division of the A_1 cell gives two daughter A_1 type cells. At the next division one of the daughter A_1 cells gave rise to a pair of A_1 cells which proceeded to differentiate; the other daughter A_1 cell gave rise to a pair of A_1 cells which maintained the stem cell line, see Figure 2.4.

In conclusion, Clermont noted that there was a general pattern of spermatogonial renewal which emerged after studies on rat, hamster, ram, bull, monkey, mouse and rabbit. This pattern was that spermatogonial stem cells gave rise to a new generation of stem cells and to spermatogonia which differentiated to yield spermatozoa. This is the basic tenet of the stem cell renewal theory. Beyond this general theory each species has its own particular model of stem cell renewal.

7. THE STEM CELL RENEWAL THEORY IN MAN

In 1963, Clermont turned his attention to spermatogenesis in man. He noted that, amongst others who had worked on man, Branca (1924) and Roosen-Runge and Barlow (1953) had commented that unlike other mammals there was little synchronisation of developmental stages within the

Figure 2.4

"MODEL OF SPERMATOGONIAL RENEWAL IN THE MONKEY (MACACUS RHESUS)"

(from Clermont and Leblond, 1959)

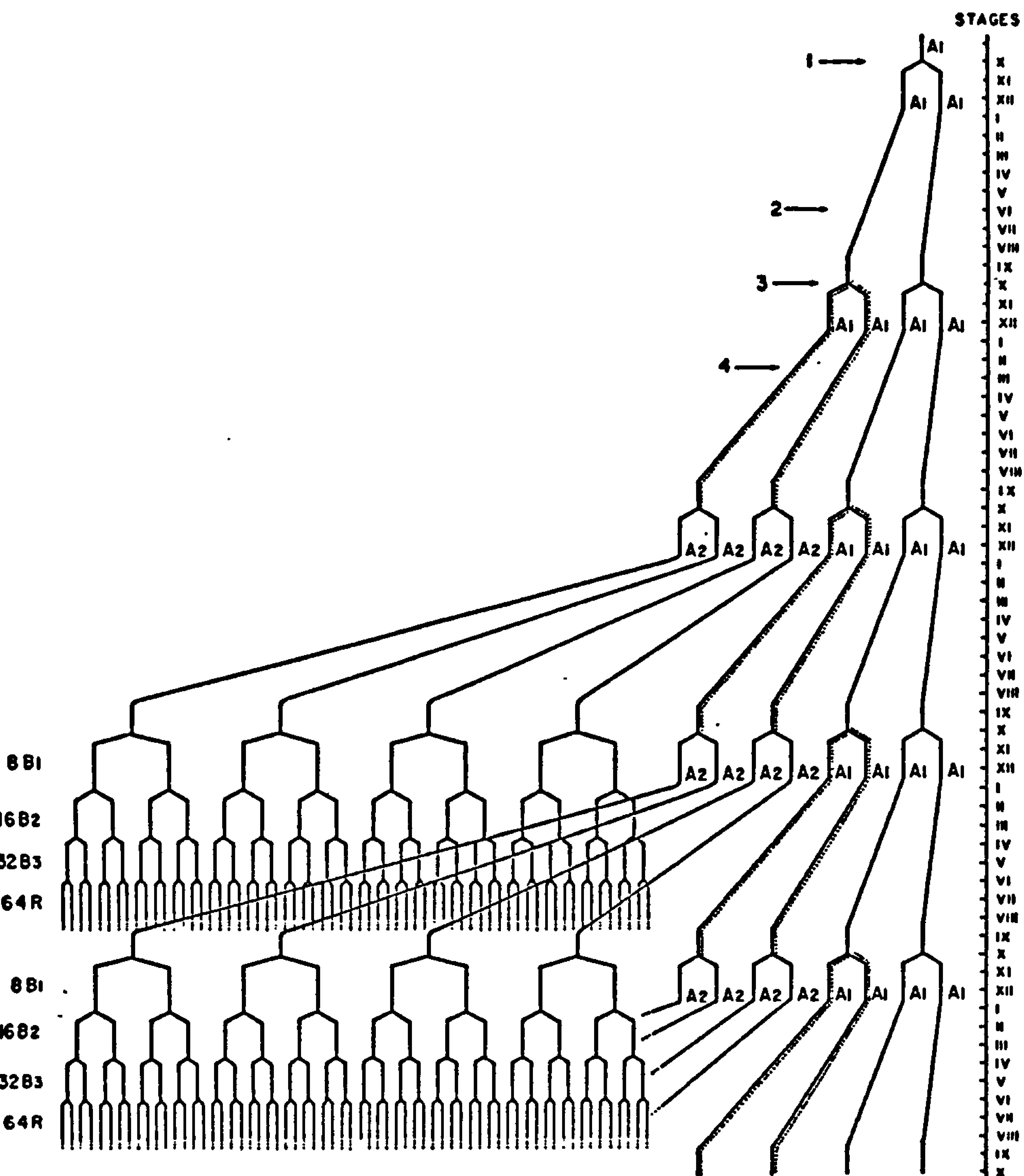


Fig. 14 Detailed diagram representing the evolution of the progeny of one type A_1 cell under scheme 4. The diagram encompasses 5 consecutive cycles of the seminiferous epithelium. The two cycles at the base of the diagram show the behavior of all spermatogonial types. Arrows 1–4 show 4 possible sites for the initiation of differentiation.

tubule and in addition there was considerable variability between tubules and between different individuals. Nevertheless, Clermont, with his usual thoroughness, began his dissection of spermatogenesis in man with a description of the three main spermatogonial classes.

1. <u>Dark Type A Cells (Ad)</u>

   The nucleus was sperical/ovoid. The finely granular chromatin stained deeply and looked homogeneous. The main characteristic of the nucleus was described as a large vacuole like cavity.

2. <u>Pale Type A Cells (Ap)</u>

   The nucleus was ovoid and uniformly lightly stained. As differentiation proceeded a few dark staining chromatin clumps became visible. These cells differentiated into Type B cells.

3. <u>Type B Cells</u>

   The nuclei could be distinguished from Ap types by the fine nuclear granulation and the presence of several darkly staining chromatin clumps.

Spermatocytes contained spherical nuclei with well stained chromatin granulation. These cells (R) were usually detached from the basement membrane and thus distinctive from Type B cells. When they were adjacent to the basement membrane this distinction was less easy to make. At leptotene the R nuclei became filamentous and by prophase, the primary spermatocytes were readily distinguishable from other cell types.

Clermont recognised a similarity between spermatogonial types in man and monkey. He chose to place his human spermatogonial types within the sequential relationship worked out for monkey. Clermont obtained his samples from patients undergoing surgery on the testis, from autopsies and, with the co-operation of Dr C G Heller, from healthy prisoner volunteers. Both workers were scrupulously careful in taking testicular biopsies (Rowley and Heller, 1966) since they believed that human seminiferous tubules were peculiarly delicate compared with other animals. Both workers also believed that fixation in Zenker's formol solution or Cleland's (a modified Bouin's) fixative preserved nuclei with better retention of morphological detail than other more commonly used fixatives.

As a result of this attention to detail Clermont felt able to describe six characteristic cellular associations in man. These associations are shown in Figure 2.5. He described the six cellular associations as succeeding each other in a fixed cycle of the seminiferous epithelium which constantly repeated itself. In this respect, therefore, man was no different from the other animals studied. Clermont suggested three ways in which the evidence for this cycle was obscured.

1. In man, any given cell association covered a very small area of the seminiferous epithelium. For instance, in any one TS tubule, several associations may be observed. Thus synchronisation in man was between rather few cells rather than between the many cells of the mouse, for example. The effect was that the borders between such cell associations or areas of cell synchrony were far more numerous than in other species.

Figure 2.5

"STAGES OF THE SPERMATOGONIAL CYCLE IN MAN"

(from Clermont, 1966b)

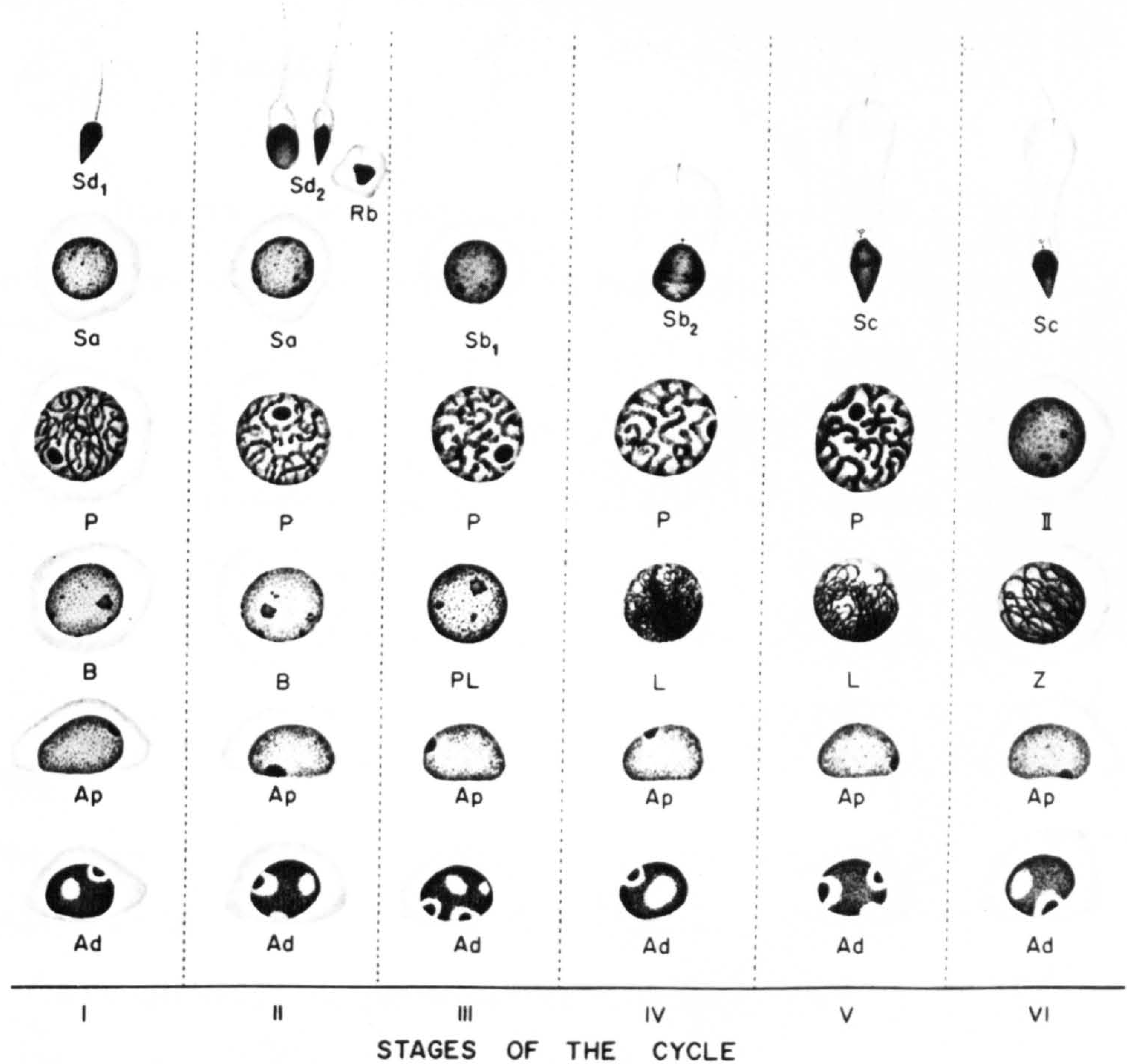


Fig. 5. Composition of six typical cellular associations observed in seminiferous epithelium of man. Each column consists of various cell types constituting such a cell association, which succeed one another in time in any given area of seminiferous tubule according to sequence indicated from left to right. Transformation of Type B spermatogonia into spermatozoa can be followed by reading along row from *B* and going successively to row above. Cellular associations, identified by Roman numeral, give "stage of cycle." *Ad,* dark Type A spermatogonia; *Ap,* pale Type A spermatogonia; *B,* Type B spermatogonia; *Pl,* preleptotene primary spermatocyte; *L,* leptotene primary spermatocyte; *Z,* zygotene primary spermatocyte; *P,* pachytene primary spermatocyte; *II,* secondary spermatocytes; *Sa* to *Sd,* consecutive steps of spermiogenesis; *Rb,* residual cytoplasmic body.

2. Secondly, cell associations were often incomplete with specific stages either very much reduced in number or missing altogether.

3. Finally, synchronisation appeared less complete than in other animal models. Thus any cellular association was likely to be more variable in man than in the mouse, for example.

Despite these drawbacks, Clermont believed that the cycle of the seminiferous epithelium could be recorded.

In 1963 Heller and Clermont published results on the duration of the spermatogonial cycle in man. These results were achieved by injecting tritiated thymidine locally into the testis of vasectomised prisoner volunteers and taking biopsies at subsequent time intervals. When they examined Stage III of the cycle, which typically had preleptotene, mid-pachytene and mid-spermatid cell type associations, they found that preleptone nuclei were labelled one hour after injection of labelled thymidine. They concluded that preleptotene spermatocytes were in S phase of meiotic division at the time of injection. They observed that Stage III, mid-pachytene nuclei, were labelled 16 days after injection (but not at days 12 or 14) and Stage III spermatids were labelled 32 days after injection. Thus they concluded that one complete cycle of the seminiferous epithelium took 16 days to complete differentiation. Further, they deduced that spermatogenesis as a whole takes some 64 days to complete differentiation. However, they point out that termination of the cycle is hard to estimate. Moreover, initiation depends on whether, like Heller and Clermont (1964) and Oakberg (1956) it is calculated from the first spermatogonial mitosis leading to

spermatocyte formation or whether it is seen as the point at which the stem cell divides. They gave a minimum time estimate of 48 days for spermatogenesis.

By 1966a Clermont was ready to consider a model for spermatogonial renewal in man. As in the Rat, Clermont's model was based on quantitative estimates of different spermatogonial types. These were deduced as a ratio of :-

$$Ad : Ap : B : Pl = 1 : 1 : 2 : 4$$

Mitotic counts indicated that nuclei divided preferentially at Stages II and V of the cycle. The preliminary model deduced by Clermont is shown in Figure 2.6. The model assumed that once spermatogonia were embarked on differentiation the process continued without reversion to a more primitive stem cell type. There was evidence for this from Clermont's autoradiography. The second assumption was that spermatogonia of one type divided once only during the cycle. This was based on finding a ratio of 1 stem cell to 4 spermatocytes and was difficult to ascertain; other animal models suggested that this was likely. Finally it was based on the assumption of little spermatogonial degeneration during the cycle, only very small numbers of degenerating nuclei were recorded. Oakberg (1956) had difficulty in calculating spermatogonial degeneration due to their apparent rapid removal from the system. Therefore, the assumption of very little degeneration must be questioned.

This initial model required a "differential mitosis" at Stage V of the cycle whereas the evidence of other animal models made this unlikely. Therefore, Clermont subjected this aspect of his model to further scrutiny. If differential mitosis occurred then, assuming that little migration of cells took place away from the area of division, Ad and Ap nuclei should be found distributed at random or in pairs of

Figure 2.6

"<u>INITIAL MODEL OF SPERMATOGENESIS IN MAN</u>" (from Clermont, 1966b)

Fig. 7. Preliminary model to illustrate possible development of spermatogonia. On left, stages of cycle are indicated in Roman numera's. Spermatogonial divisions illustrated by junction of three lines, occurring in Stages II and V. According to this scheme, divisions of dark Type A spermatogonia (*Ad*) would be "differential." Divisions of Ap and B spermatogonia would be "equivalent." (From Clermont.[6])

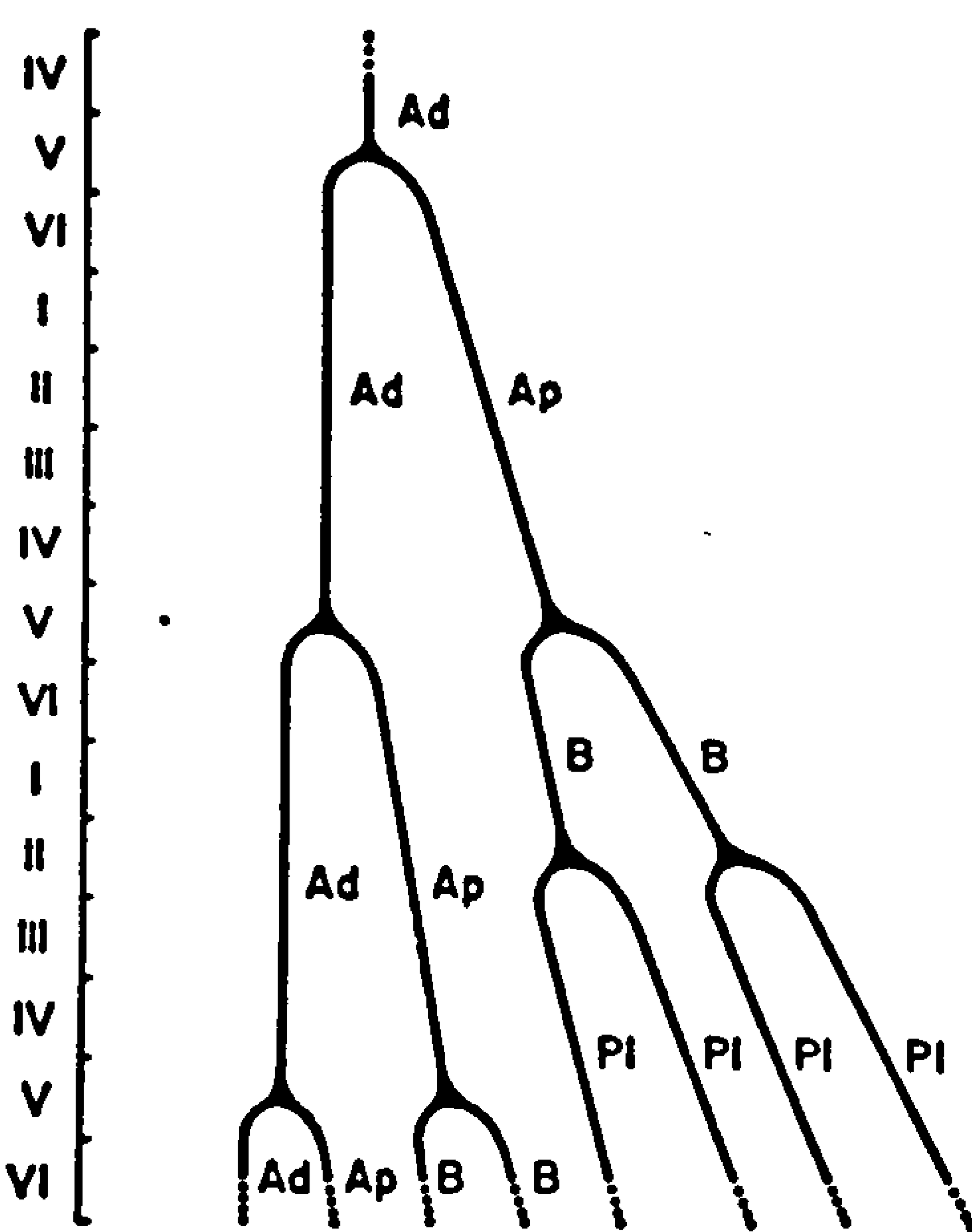


Figure 2.7

"<u>FINAL MODEL OF SPERMATOGENESIS IN MAN</u>" (from Clermont, 1966b)

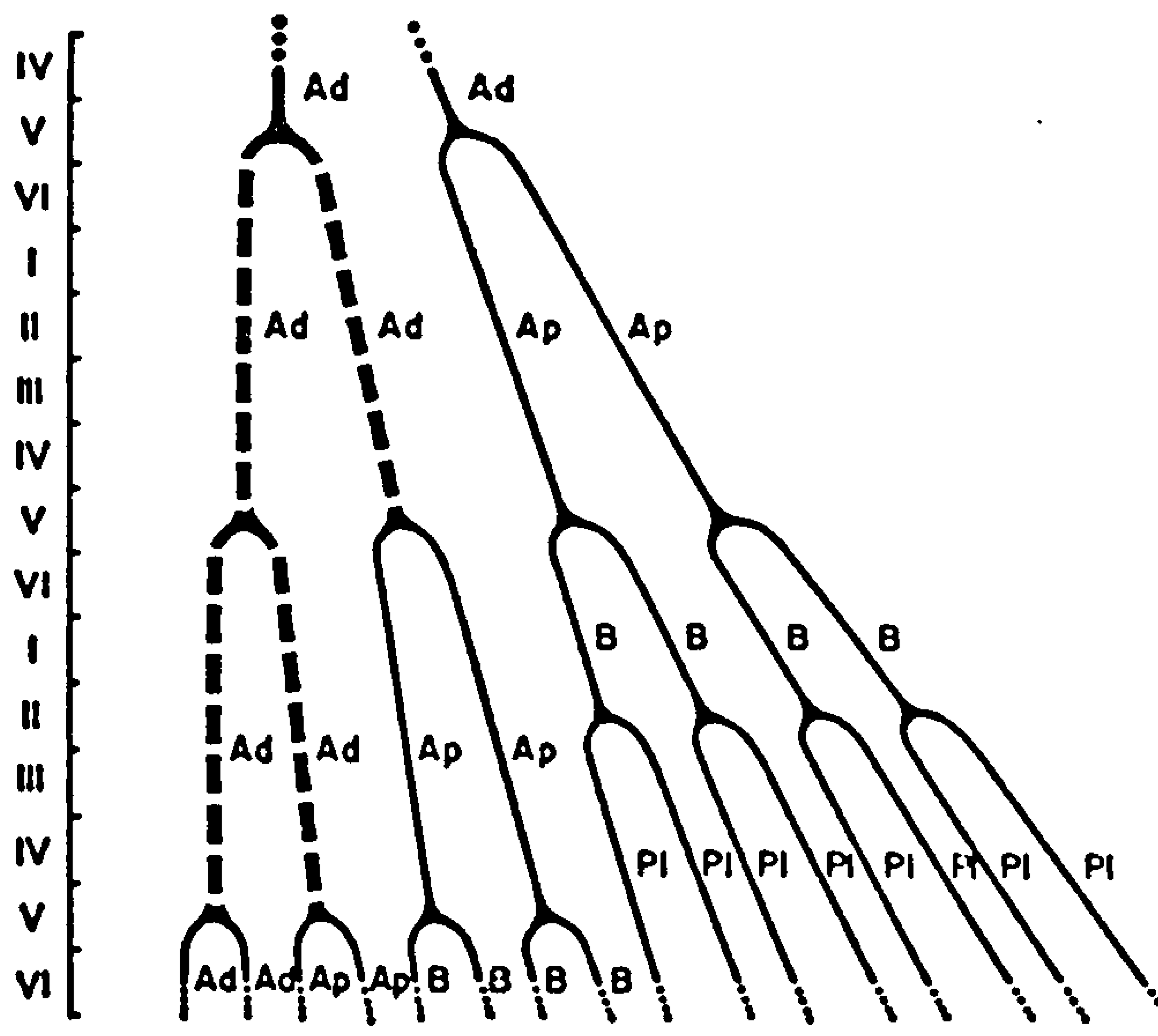


Fig. 10. Tentative model for mode of development and renewal of spermatogonia in man. Roman numerals on left indicate stages of cycle. (From Clermont.[6])

Ad/Ap association. Clermont therefore prepared maps of Ad/Ap distribution and noted that, rather than random association, aggregates of Ad or Ap nuclei could be distinguished. He thus concluded that differential mitosis did not occur in man and he amended his model accordingly. Figure 2.7 describes this second model. In this model the ratios of Ad : Ap : B : Pl were maintained as 1 : 1 : 2 : 4. It was also interesting to note that the Stage II mitotic peak was composed of divisions producing spermatocytes whereas the Stage V mitotic peak accounted for all spermatogonial divisions.

Clermont returned to the questions posed by Branca in 1924 and answered them thus :-

1. The spermatogonial stem cell passes through three successive divisions before giving rise to spermatocytes.

2. This number of mitosis is fixed.

3. The first two spermatogonial divisions give rise to spermatogonia; the third spermatogonial division gives rise to spermatocytes.

Clermont's work differed from the earlier work of Roosen-Runge and Barlow (1953). Clermont commented that Roosen-Runge and Barlow based their deduction of seven spermatogonial mitosis on the presence of occasional clusters of approximately 64 mitotically dividing nuclei. In addition they thought that stem cells entered spermatogenesis singly rather than (as Clermont deduced) in pairs. Further, Roosen-Runge and Barlow believed that there were seven classes of size within the mitotic

cells, and size diminished with differentiation. Conversely Clermont observed that Ad cells were the smallest spermatogonial type and B cells were the largest spermatogonial type.

Clermont's model was confirmed by the work of Steinberger and Tjioe in 1968.

8. ALTERNATIVE POINTS OF VIEW

One further piece of work requires brief mention. In 1960, Mancini et al published descriptions of spermatogonia which differed from those of Clermont. Clermont (1953) commented that it was not possible to correlate Mancini's spermatogonial types with those which Clermont observed. One major difference between these workers was that Mancini studied only three adult samples out of a total of 60 samples. Mancini's work was in fact largely concerned with the origin and development of the seminiferous epithelium during prepubertal life and during pubertal change.

In 1970 Clermont presented his model at a workshop devoted to the study of the testis. E. Steinberger, in the discussion, challenged Clermont's supposition that the Ad type was the stem cell type on two points :-

1. In tissue culture the ratio between Ad/Ap types shifted towards greater numbers of Ap types. With time Ap types kept on dividing whilst Ad types disappeared.

2. In two oligospermic individuals, where spermatocytes and spermatids were absent, the tubules were lined with Ap cell types.

Steinberger added that there was definite evidence for the supposition that Ap rather than Ad was the stem cell type. In reply, Clermont neither confirmed nor denied Steinberger's observations.

9. WORK UTILISING KNOWLEDGE OF SPERMATOGENESIS

It might appear from the preceding sections that the spermatogenic cycle in man was well understood. One obvious application of this knowledge would clearly be in the field of infertility investigations where the spermatogenic cycle is faulty. Steinberger and Tjioe (1968) compared cell types (using Clermont's descriptions) from normal individuals with cell types from infertile individuals. They found that a numerical analysis of cell types in 25 tubule cross sections was statistically adequate for estimating the cell types present in the tubule. In normal individuals the cell types were stable between individuals but the Sertoli cell showed significant variation between individuals. In the two infertile individuals studied, the numbers of cell types present were similar in both cases, but these infertile individuals differed from the normal controls in the ratio of cells present as Ap and Ad. Steinberger and Tjioe said that there was a failure of Ad cells to transform to Ap cells in both these infertile males. These authors believed that this method enabled lesions of spermatogenesis to be localised accurately.

When the majority of literature on infertility studies was examined there was a very large gap in relating infertility to specific lesions of the cycle. For example, since publication of Clermont's model, a number of studies on populations of infertile males have been published (Kjessler, 1966; McIlree et al, 1966; Meinhard et al, 1973; Koulischer and Schoysman, 1974; McDermott, 1974; Chandley et al, 1975; Hendry et al, 1976). The primary concern of these authors was to

ascertain abnormal meiotic karyotypes. Most of these authors included a histological analysis of their cases and to do so, they used a qualitative description of the state of the testicular epithelium. Hendry et al (1976) used a quantitative analysis of the histology of his cases. This quantitative method was developed by Johnsen (1970) and is referred to as Johnsen's Testicular Biopsy Score Count. The method assigned a score to each tubule based on the most advanced cell type observed and thus recorded the most advanced stage of spermatogenesis reached in a sample of tubules. Johnsen considered the work of both Clermont (1966) and Steinberger and Tjioe (1968) and came to the conclusion that neither authors methods were suitable for clinical biopsies. Whilst Johnsen's method was able to put a numerical value on histological samples, it was not able to take account of the relationship between the stages of the cycle. Therefore the method failed to analyse the dynamic nature of spermatogenesis and concentrated on recording the results of events subsequent to the lesion. In addition, this method could not identify the proliferative stages of the spermatogonia. It seems unlikely that lesions of spermatogenesis occur only during meiotic division and spermatogenesis and are absent from earlier parts of the cycle. If the results of infertility investigations are examined there are many records of early failure described in qualitative terms as Grade III or IV on McIlree's (1966) scale.

Skakkebaek et al (1973a) analysed chiasmata counts, X and Y association rates and the ratio of MI/MII divisions in a series of infertile and normal individuals. In this work Skakkebaek related his results to qualitative histological assessments of the testis. However, Skakkebaek and Heller (1973) had already published a quantitative study of the testicular epithelium using this same normal control series, and had extended this quantitative work to a series of XYY men

(Skakkebaek et al, 1973b). In this work the quantitative counting of cell types was based on the method developed earlier by Rowley and Heller (1971), which will be discussed later in this section.

Another major approach to understanding male infertility has been through investigations which are primarily concerned with studying the hormonal control of spermatogenesis. In a series of papers de Kretser, in collaboration with a number of other authors (de Kretser et al, 1972a; 1972b; 1974) had attempted to correlate hormonal, histological and chromosomal studies in infertile males. In his earlier work (1972a,b) de Kretser used qualitative histological categories but in his 1974 paper de Kretser made use of Clermont's (1966) description of testicular cells. However, de Kretser did not discriminate between the different types of spermatogonia which Clermont described.

In 1970, Heller and Heller examined the effect of the drug Clomiphene Citrate on spermatogenesis in normal men. They based their analysis of histological changes in the testicular epithelium on quantitative counts of all cell types in the germinal epithelium (Rowley and Heller, 1971). This method was essentially a development of the earlier work of Clermont and Heller but used the Sertoli cell as a constant. These workers believed the Sertoli cell was relatively resistant to change in the normal adult testis which was in contrast to the work of Steinberger and Tjioe (1968).

Steinberger et al (1970) studied steroid metabolism in human testis maintained in culture over long periods of time. They monitored differentiation by examining histological sections for the presence of specific changes of spermatogenesis. In order to do so, they utilised Clermont's description of spermatogonial cell types and noted an increase in the ratio of Ap : Ad types with time in culture. They were unable to explain these ratio changes.

10. CONCLUSIONS

Early workers provided a descriptive framework for the process of spermatogenesis. A number of theories were proposed which took into account the need to replenish the testicular epithelium whilst maintaining sperm production. Work on animal models provided evidence for the stem cell renewal theory of spermatogenesis. From 1963 to 1966 Clermont studied spermatogenesis in detail and proposed a model of stem cell renewal in man. His work was repeated and confirmed by Steinberger and Tjioe in 1968 and has not to date been seriously challenged.

Clermont's description of the spermatogenic cycle in man has been utilised by some workers whose primary interest is in the hormonal control of spermatogenesis. In contrast, Clermont's work appears to have been largely neglected by workers primarily interested in the cytogenetics of male infertility.

CHAPTER 3

THE INFERTILE POPULATION

1. Introduction

2. Patient selection for testicular biopsy

3. Patient data collection

   A. Case histories

   B. Examination of external genitalia and sperm counts

4. Conclusions

1. INTRODUCTION

The process of patient referral is reviewed briefly in order to understand how patients were selected for Testicular Biopsy (TB).

In the Sheffield area, couples who were experiencing difficulty with conception were generally referred by their General Practitioner to the Jessop Hospital for Women (JHW). It is right to keep in mind that couples with an infertility problem frequently delegate the women to initiate such investigations. Professor I D Cooke, at JHW, recognised that male infertility played an important part in the fertility of any couple and he was responsible for obtaining a grant from the Special Trustees to the Former United Sheffield Hospitals in order to investigate this problem. He envisaged the investigation as a multi-faceted approach to male infertility which would include investigation of the female partner together with clinical studies, hormonal investigation and chromosome studies on the male partner. The grant to Professor Cooke was largely made available for my employment from October 1974 - October 1976 to carry out the chromosome investigations on these male patients and from which the results and investigations reported in this thesis are drawn. In order to carry out the investigation of the male partner, the support and co-operation of Mr J L Williams, Consultant Urologist at the Hallamshire Hospital, Sheffield was sought. Mr Williams freely gave his support, enthusiasm and time to the subsequent investigations.

2. PATIENT SELECTION FOR TESTICULAR BIOPSY

Couples with an infertility problem were referred by their GP to JHW. If the male partner had a sperm count of less than 20×10^6 sperm/ml or had a sperm profile with less than 40% motility at two hours (Rehan and Sobrero, 1975) he was referred to the

Infertility Clinic at the Hallamshire Hospital where investigations were taken over by Mr J L Williams. Dr A Thomas, Research Assistant, JHW, liaised between JHW and the Hallamshire Hospital and he was largely responsible for obtaining and keeping the clinical records for the male patients. Occasional patients were referred directly to Mr Williams, mainly because previous investigations by their GP or other local hospitals had revealed abnormal sperm counts. Other occasional patients were referred for chromosome investigations by Mr M Fox, Consultant Urologist, Hallamshire Hospital, Sheffield.

Patients No. 1-43 were selected for TB by Mr Williams during the period October 1974 - October 1976 out of a total referred infertile population of 82 cases. Therefore 39 cases were investigated without a biopsy being taken. During this period patient selection was on the basis of a TB providing information which would contribute substantially to the picture of the patients infertility. From October 1976 - October 1977 the selection of patients continued due to Mr Williams's enthusiasm for the work, although at this time the work was unfunded. It is important to notice that during this time selection of patients for TB changed slightly. This can be seen in the higher frequency of patients with varicocoeles. Some of this bias was due to the pressure for bed space necessitated by surgery for varicocoele repair. Varicocoele repair required a two to three day hospital stay whereas TB was performed on a day admission basis. Thus the series of cases No. 44-68 (25 cases) which was selected from a total referred population of 135 patients has a higher frequency of patients with varicocoele and higher sperm counts than the earlier series. This backlog of patients requiring varicocoele repair, from whom TB's were taken, meant the subsequent and inevitable exclusion of cases where TB was the sole surgical event.

A further alteration in selection procedure occurred starting October 1976 which was concommitant with funding ceasing. From October 1976 all patients attending the Male Infertility Clinic at the Hallamshire Hospital were offered mitotic chromosome analysis. Thus the population was effectively screened for somatic chromosome abnormality before the gametic genotype was examined.

To sum up, patients were selected for TB when they had a sperm count of less than 20×10^6 sperm/ml and/or a motility of less than 40% at two hours after sampling. Most patients had at least two semen samples taken at intervals of two to three months and after two - three days abstinence from ejaculation. Patients were selected for TB when it was thought that TB would contribute an important direct inspection of the patients testicular state thereby adding to the picture of the patients infertile status. Prior to October 1976 the patients somatic genotype was unknown at TB. Post October 1976 the somatic genotype was known. The series consists of 68 patients who had TB performed.

3. PATIENT DATA COLLECTION

A data sheet was specifically designed for this project in order to facilitate the collection of information from the 68 patients who had TB's taken. An example of the data sheet is appended. These data sheets were used to record physical measurements, clinical histories, sexual histories and details of life style. Patients were questioned about the clinical histories and fertility record of the immediate family. Information about the partners history of infertility investigation was noted. This data collection was additional to the normal hospital case taking procedure.

A. Case Histories

The main features of the general clinical information and of the partners history of investigation are recorded in Table 3.1. The average age of the population was 28.6 years with an approximate average period of infertility of 3.5 years. Three patients were over 6 feet in height. Nine patients had some previous or continuous clinical feature including one spastic quadriplegic, one treatment for venereal disease and one asthmatic. Seven patients had diabetic relatives. Five patients had a family history of infertility. Four patients did not require to shave daily and one patient had a female distribution of pubic hair. One patient had a previous marriage of some length which was childless. One female partner had a child by a previous marriage and one had a pregnancy terminated (her husband was not the father of this conception). Five patients period of infertility included one or more conceptions which had terminated in abortion. Two patients reported previously fathering a child, one following a previous period of infertility.

Amongst the female partners of these patients, three had abnormal uteruses, nine had some irregularity of the menstrual cycle, three had anovulatory cycles and one was amenorrhoeic. During these investigations three women were on drug therapy for infertility. Examination of life styles and sexual behaviour demonstrated one patient who worked unsociable shift hours and one who was reported as ? abnormal sexuality - no details were available.

B. Examination of genitalia and sperm counts

Table 3.2 records specific abnormalities of the genitalia and sperm counts from the 68 patients together with any previous history of intervention with the genital system. 23 patients (34%) had varicocoeles which were repaired surgically. Nine patients had small

TABLE 3.1

68 INFERTILE MALES : PHYSICAL DESCRIPTION, FAMILY HISTORY AND PARTNERS INFERTILE STATUS

Case No	Age	Years of Infert	Height	Body Hair Dist	Shaving	Previous Clinical History	Family Clinical History	Family History Infertility	Previous Fertility of Patient + Partner	Investigation of ♀ Infertility	Other
6	25	2							♀ child previous marriage		
7	25	2									
8	26	3				Gonorrhoea treated 8 years ago					
9	27	2								Ooc anovulation	
10	32	4				Cholecystectomy					
11	24	4								NK	
12	20	3					1 sister diabetic				
13	26	3									
14	30	3	6'1"		Does not have to				3 abortions	Bicornuate uterus	? abnormal sexuality
15	26	2								NK	
16	23	2									
17	32	3									
18	38	2									
19	26	2			Alt days	Asthma				Bicornuate uterus defective luteinisation	
20	23	3	6'5"	♀ pubic	1/week					NK	
21	37	4							2 abortions		
22	23	2									
23	35	6									
24	27	2.5							? abortion		
25	32	1								Irregular cycle	
26	40	4.5							Child 6½ years		
27	23	3.5									
28	23	1									
29	28	3.5								Double uterus	
30	30	8				Bowel resec. 19yr Laparotomy 23yr					
31	28	1								NK	
32	29	2						2 cousins infertile			Works shifts
33	31	5					Father diabetic				
34	26	4	6'1"								
35	31	2				Spastic quadriplegia					
36	34	6.5						Sister infertile		anovulatory cycle	
37	27	3								NK	
38	29	3									
39	29	2.5				? Bells palsy					
40	27	2.5									
41	27	1									

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Case No	Age	Years of Infert	Height	Body Hair Dist	Shaving	Previous Clinical History	Family Clinical History	Family History Infertility	Previous Fertility of Patient + Partner	Investigation of ♀ Infertility	Other
43	32	6				Polio 10 years	Mother diabetic				
44	NK	4									
45	NK	2								On endometrium	
46	NK	6								Kidney trouble	
47	30	2					Father diabetic				
48	30	4					Grandmother diabetic	Aunt, 2 mis-carriages		Anovulatory	
49	26	2									
51	29	4				Steroid therapy 1973-75				Endometriosis	
52	28	4			Alt days					Defective luteinisation on Clomiphene	
57	27	1						Brother ? infertile	Termination husb. not father	Irregular cycle	
66	28	2							1 abortion		
67	32	1.5									
68	28	3.5						Sister ? infertile			
69	26	2.5							? abortion	? irregular cycle	
76	24	2.5									
79	32	3.5									
80	29	2.5					Brother diabetic			Anovulatory foll. ectopic pregnancy	
82	30	1.5									
88	31	2									
105	27	4									
106	31	3								? irregular cycle	
107	31	5									
116	31	3.5									
117	24	4							No children of previous 10 yr marriage	NK	
118	30	>10									
121	27	2								Defect.ovulation	
123	29	4								Amenorrhoea	
130	30	6									
133	29	4					Brother diabetic			Treatment fertility drug (JHW)	
135	30	NK				Minor events 1970, 72					
136	24	4							1 child post-natal death This miscarriage, pat. uncertain 1 child previous 15 yr marriage		
137	37	2									

TABLE 3.2

68 INFERTILE MALES : DESCRIPTION OF GENITALIA AND SPERM COUNTS

Case No	Varicocoele present	Abnormal testes		Abnormal epididymus	Previous testicular history	Sperm profile				
		R	L			No. counts	Volume	Count x 10^6ml	% mature forms	% motility at 2 hrs
6				Distended		2	1.65	0	0	0
7				Distended		1	-	0	0	0
8						2	4.5	0.4	-	35
9						3	4.1	1.2	23	34
10				Distended head		3	3.5	1.9	46.5	55
11		small	small			1	-	0	-	-
12		small	small			2	0.45	16.8	41.5	poor
13						2	6.8	4.0	39	15
14	+	± small	± small			2	2.5	8.7	-	45
15						1	-	0	-	-
16				Distended heads		1	-	0	-	-
17		small	small			2	2.0	2.6	39	5
18	+					2	3.8	5.8	34	40
19						2	3.05	6.5	65	55
20						3	3.6	14.0	61.5	20
21				Distended head		2	2.7	101	55.5	30
22		small	small	Blocked		1	-	0	-	-
23						2	5.3	3.75	60	40
24						3	5.0	13.3	63	47
25		Absent			Orchidectomy + bilateral inguinal hernia, 13 yrs	2	2.1	1.5	-	22.5
26				Distended head		2	5.3	0.75	-	30
27						1	3.7	16	69	30
28						2	1.9	16	53.5	30
29						2	2.4	6.4	63.5	55
30						2	1.4	1.85	-	10
31						2	1.6	3.6	25	10
32		small	small			2	2.5	0.75	-	45
33						2	3.45	14.0	71.5	65
34						2	4.35	6	N	35
35						1	-	0	-	-
36		? absent	small		R undescended + hernia 19 yrs	1	-	0	-	-
37					? delayed descent	1	3.8	0	-	-
38	+					2	2.0	22	40	10
39	+					2	5.0	1.6	-	60

Case No	Varicocoele present	Abnormal testes R	Abnormal testes L	Abnormal epididymus	Previous testicular history	Sperm profile: No. counts	Volume	Count x 10^6 ml	% mature forms	% motility at 2 hrs
40					x2 massive testicular swellings in last 6/12	1	2.5	0	–	–
41						1	1.0	10	–	5
43						2	2.1	1.05	–	67.5
44						2	–	4.5	60	47.5
45	+					2	3.5	1.0	67	40
46						2	3.0	9.0	70	35
47	+					2	2.6	2.25	54	52.5
48	+					1	3.0	33	–	10
49	+					NK	–	–	–	–
51	+					2	0.45	11.5	50	5
52						2	1.25	<1.0	–	–
57	+	small	small			3	4.5	6.5	80	30
66	+					1	–	120	–	Low
67	+					2	4.1	7	58.0	70
68	+					2	3.0	37	–	30
69	+					2	3.3	1.3	–	–
76						2	4.0	0	–	–
79	+					2	9.1	11.5	86.5	12.5
80						2	3.3	17.0	88.0	50
82	+					3	3.0	21.8	Low	38
88			Atrophic		Orchidectomy, left undescended in childhood	2	2.4	4	79.0	10
105				Distended, blocked		1	2.0	0	–	–
106				R distended, + 2" vas restricted	Right inguinal herniorrhaphy, 29 yrs	2	4.0	13.5	63	55
107	+					3	2.6	13.0	73	30
116	+					2	3.5	153	79	10
117	+					2	3.3	17.0	85	40
118						3	4.0	0	–	–
121						2	2.2	3.2	83	10
123	+		small			2	4.2	0	–	–
130						2	–	0	–	–
133	+					2	2.2	13.5	64.5	45
135	+					NK oligospermic		–	–	–
136		Atrophic		Left vas occluded	Bilateral herniorrhaphy 13 yrs	2	0.5	0	–	–
137	+					3	3.0	5.6	53	<30

testes, two had testes described as atrophic and two patients had unilateral absence of a testis. Nine patients had evidence of distension or blockage of the epididymis on manual examination. Two patients were thought to have occlusion of the vas unilaterally. Two patients described operative intervention for undescended testes during adolescence and one patient believed testicular descent had been delayed. Three patients had had previous hernia repairs. One patient had massive testicular swellings on two occasions in the last six months before examination, the cause was unknown.

The sperm counts from the patients which are listed in Table 3.2 are summarised in Table 3.3.

Table 3.3

SPERM COUNTS OF 68 PATIENTS WHO HAD TESTICULAR BIOPSIES TAKEN

Sperm count x 10^6/ml	No. of cases	% of population
Azoospermic	16	23.53
< 5	20	29.41
5 - 10	10	14.71
11 - 20	13	19.12
>20	7	10.29 (6 low motility 1 high abnormal forms)
NK	2	2.94
TOTAL	68	100

4. CONCLUSIONS

This chapter sums up the information gathered together as a result of case taking and physical examination. These details provide a description of the population before investigations commenced.

CHAPTER 4

MATERIALS AND METHODS

1. Introduction

2. Preparation of mitotic chromosomes
   - A. Short-term peripheral leucocyte culture
   - B. Slide preparation

3. Preparation of meiotic chromosomes
   - A. Preparation of material
   - B. Slide preparation
   - C. Comments

4. Staining schedules
   - A. Lacto-aceto-orcein staining
   - B. Leishman staining
   - C. Trypsin-Leishman banding
   - D. C-banding
   - E. Quinacrine dihydrochloride fluorescence

5. Photography

6. Autoradiography
   - A. Application of film
   - B. Development of film

7. Chromosome analysis

   A. Mitotic chromosome analysis

   B. Analysis of cell division in testicular biopsies

8. Preparation of histology sections

9. Gonadotrophin estimations

1. INTRODUCTION

The methods used were based on standard laboratory practice. Modifications to these methods have been developed in Sheffield to suit my own preferences for handling material and my own judgement about what constitutes a good preparation for analysis.

These sundry modifications to routine methods for preparing material, techniques for staining material, material analysis, general photography and autoradiographic processing are described in this Chapter.

I have acknowledged all instances where I have not been responsible for material processing and analysis.

2. PREPARATION OF MITOTIC CHROMOSOMES

A. Short-term perhiperal leucocyte culture

Mitotic chromosomes were prepared from short-term peripheral leucocyte cultures using modifications of the method of Moorehead et al (1960). Blood samples were usually received as 20 mls of heparinised venous blood. The preparation was handled sterilely up to fixation. The method follows :-

1. Phytohemagglutinin (Wellcome Reagents Limited) was added to heparinised blood to give a final concentration of 0.02 mls phytohemagglutinin per 1 ml of blood.
2. The sample was allowed to stand for at least 30 mins at 4°C before centrifuging at 400 rpm for 4 mins.
3. The serum and leucocyte cell layer were removed and mixed in a syringe.
4. The resultant cell suspension was distributed between 3

universal containers.

5. 8 mls of TC 199 (Wellcome Reagents Limited) was added to each sample and the samples were incubated for 72 hours at 37°C (culture termination point). During incubation the cultures were shaken once a day to dislodge settled cells and resuspend them in the medium.

6. 4–6 hours prior to culture termination, Colcemid (Grand Island Biological Company, Maryland, US) was added to the culture to give a final concentration of 0.02 μg Colcemid per 1 ml of culture.

7. At culture termination, the universals were shaken to resuspend the cells and the cell suspension tipped into a centrifuge tube. The cell suspension was centrifuged at 1000 rpm for 10 mins to precipitate the cells.

8. The supernatant was withdrawn and the cell pellet resuspended in 2–3 mls of swelling solution at 37°C. The swelling solution consisted of 2 mls Hanks Balanced Salt Solution (Wellcome Reagents Limited), 25 mls 0.075 M Kcl (Analar Grade) and 198 mls deionised water.

9. The cell suspension was incubated for 6–7 mins at 37°C.

10. Following incubation the cells were centrifuged down at 400 rpm for 5 mins and the supernatant was withdrawn.

11. The cell pellet was resuspended in the tiny amount of residual swelling solution. The cells were then fixed by the slow addition of freshly prepared fixative. The fixative was 3 parts absolute alcohol : 1 part glacial acetic acid. 2–3 mls of fixative had been added by the time the cells were fully suspended in fixative.

12. After 15 mins in fixative, the cells were centrifuged down,

the old fixative withdrawn and the cells resuspended in 2-3 mls of freshly prepared fixative.

13. This preparation was then stored at -20°C until required for slide preparation.

B. Preparation of slides

Slides of mitotic chromosome preparations were made as follows :-

1. The cell suspension was precipitated by centrifuging at 1000 rpm for 10 mins and the supernatant was withdrawn.
2. Fresh fixative was added to the cell pellet until the new cell suspension appeared slightly cloudy. The concentration of the cell suspension affects the distribution and spreading of cells on the slide.
3. Drops of cell suspension were pipetted onto grease-free, ice-cold wet slides. Preferably one drop of cell suspension per slide was used. When cell suspensions appeared very thin, more than one drop was pipetted onto each slide.
4. The slides were allowed to dry at room temperature. I believe that the spreading of metaphase cells on the glass slide is facilitated if this drying process is relatively slow rather than rapid.

3. PREPARATION OF MEIOTIC CHROMOSOMES

A. Preparation of material

The testicular biopsy usually measured approximately 0.5 cm^3. After removal, the biopsy was placed immediately in TC 199 and transported to the laboratory as rapidly as possible. This was often within 15 mins

of excision. When the biopsy arrived in the laboratory it was transferred immediately to swelling solution consisting of 0.075 M KCl and the tubules were gently teased apart. Two methods of preparing the material for meiotic chromosome analysis were used.

(i) Modification of the method described by Meredith (1969)

1. The separated tubules, in swelling solution, were cut into lengths of 2-3 cm.
2. After periods of 5 mins, 10 mins and 15 mins in swelling solution, some of the tubules were transferred to freshly made fixative of 3 parts absolute alcohol : 1 part glacial acetic acid.
3. The fixative was changed 3 times and then the tubules were stored at -20°C to await slide preparation.

(ii) Modification of the method described by Evans et al (1964)

1. While the tubules were in swelling solution, the tubule contents were teased out by a combination of stroking out the tubule contents with the aid of two dissecting needles and finely chopping the tubules. This operation was time consuming and took up the time the tubules were left in the swelling solution. Large pieces of empty tubule were usually removed from the swelling solution. The whole operation was carried out under the low power magnification of an inverted microscope.
2. Material remained in swelling solution for 15 mins or 30 mins, giving two samples.
3. After swelling the cell suspension was pipetted off,

avoiding as much as possible of the large debris. The cell suspension was centrifuged down at 1000 rpm for 10 mins.

4. The supernatant was withdrawn and the cells fixed with freshly made fixative consisting of 3 parts of absolute alcohol : 1 part glacial acetic acid.

5. The fixative was changed 3 times and the material stored at $-20^{\circ}C$ to await slide preparation.

B. Preparation of slides

Three methods of preparing slides for meiotic chromosome analysis were used.

(i) Method of slide preparation using whole fixed tubules

Using material preparation method 3A(i) (After Meredith, 1969)

1. A 2-3 cm length of fixed tubule was drained of fixative and placed in a watch glass containing a few drops of 60% acetic acid for 3 mins.

2. The action of the 60% acetic acid caused the tubule contents to be dispelled. This action was assisted by mincing the tubules with dissecting needles and gently sucking the cell suspension in and out of a siliconised pipette.

3. Tiny drops of cell suspension were placed on a clean dry hot slide which was maintained at approximately $60^{\circ}C$ on a hot plate.

4. Surplus 60% acetic acid was sucked slowly off the hot slide leaving a ring of cells on the slide.

(ii) Method of slide preparation for fixed cells in suspension

Using material preparation 3A(ii) (After Evans et al, 1964)

1. Fixed cell suspensions were centrifuged down and the supernatant withdrawn. Fresh fixative was added in sufficient quantity to give a slightly cloudy cell suspension.
2. Drops of cell suspension were pipetted onto clean ice-cold wet slides and allowed to dry at room temperative.

(iii) Third method of slide preparation

Using a combination of 3A(i), 3B(i) and 3B(ii)

1. A cell suspension from whole tubes was obtained following routine 3A(i) and 3B(i).
2. The cell suspension in 60°C acetic acid was flooded with freshly made fixative 3 parts absolute alcohol : 1 part glacial acetic acid, using a quantity of fixative at least 3 times the volume of the 60% acetic acid.
3. The cell suspension was centrifuged at 1000 rpm for 10 mins. The supernatant was withdrawn and fresh 3 : 1 standard fixative added.
4. Slides were then made following routine 3B(ii) for slide making.

C. Comments on preparation of meiotic chromosomes

The early samples in the series were prepared using Evans' method. This method gave extremely good results in terms of well spread figures with clear morphological detail of configurations at diakenesis. The results were also acceptable when diakenesis figures were relatively abundant. However, in this series many of the individuals sampled

had a reduced activity of the germinal epithelium and the biopsy samples themselves were often very small. Preparation of samples based on Meredith's method appeared to solve some of the problems encountered with Evans' method. The advantages noted were :-

1. Swelling and fixation of cells were carried out within the constraint of the tubule. Thus there was likely to be relatively less cell loss with this type of preparation.
2. Once the tubule contents were dispelled they were transferred directly to the slide. Similarly there was less likelihood of cell loss with this method.
3. In order to achieve any spreading of pachytene figures with Evans' method, it was necessary to extend the swelling time to the point where diakenesis figures were apparently destroyed. Meredith's method gave better spreading of pachytene figures without the destruction of diakenesis figures.
4. Meredith's method gave an overall picture of tubule function whether diakenesis figures were present or absent.

The disadvantages of Meredith's method were :-

1. Diakenesis figures were morphologically less clear when this method was used.
2. A greater number of amodal counts of bivalents was obtained when Meredith's method was used in comparison with Evans' method.

Thus it was apparent that compromises had to be made. The third method of slide preparation was a practical attempt at compromise. It

utilised the retention of tubule contents during swelling and fixation thus guarding against loss of cell contents whilst retaining the slide making technique which gave well spread figures with good morphological detail. The results were not wholely satisfactory in that they tended to be worthwhile when there was ample material and the activity of the germinal epithelium was relatively unimpaired.

Overall, the compromise chosen was to use Meredith's method, which conserved the tiny amount of material available and gave an overall picture of tubule function in all individuals studied, in preference to Evans' method, which gave better morphological detail and greater accuracy for analysis of diakenesis figures in relatively few of the individuals studied.

4. STAINING SCHEDULES

A. Lacto-aceto-orcein staining (LAO)

(i) Stain preparation

Synthetic orcein (Searle Diagnostics)	- 2.0 gms
Glacial acetic acid	- 50.0 mls
70% Lactic acid	- 50.0 mls

The ingredients were boiled together for 10 mins. The stain was then filtered and stored at room temperature

(ii) Staining schedule

1. Slides were immersed in LAO for 20 mins.
2. Surplus LAO was washed off in 2 rinses of 45% acetic acid.
3. Slides were washed under running warm tap water and allowed to dry at room temperature.

(iii) Usage

1. The first meiotic preparations were stained with LAO.
2. Some later meiotic preparations were stained with LAO when required for photography.
3. Occasional mitotic chromosome preparations were stained with LAO.
4. All slides processed for autoradiography were restained with LAO.

(iv) Comments

LAO staining gave very clear morphological detail of chromosome structure. In meiotic preparations of exceptional quality cross over strands in chiasmata could occasionally be observed (Figure 4.1). It was useful to stain mitotic chromosomes with LAO when structures such as satellite and secondary constrictions were studied. It seemed essential to restain with LAO for autoradiography since other stains were leached out by the development process. A short LAO staining time was preferred since overstaining obscured morphological detail. The short staining time sometimes meant that phase-contrast microscopy had to be used.

B. Leishman staining

(i) Stain preparation

Leisman stain (G. T. Gurr) = 0.3 gms

Analar grade Methyl alcohol = 200 mls

The ingredients were boiled for 10 mins. The stain was then filtered and stored in a light tight bottle (Curtis and Horobin, 1975).

(ii) Staining schedule

1. The slides, on a staining rack, were flooded with 4 parts of Gurr's pH 6.8 buffer : 1 part Leishman stain.
2. After 1 min, the stain was washed off with Gurr's pH 6.8 buffer.
3. Slides were blotted dry.

(iii) Usage

1. All but the first group of meiotic preparations were stained with Leishman.
2. All mitotic chromosomes were stained with Leishman following banding, see below for banding schedule..

(iv) Comments

Leishman staining of meiotic configurations gave, on average, less distinct morphological detail than LAO staining. The advantages were that Leishman stain can be removed by immersing the slide in 3 parts absolute alcohol : 1 part glacial acetic acid which leaves the material free for further investigations.

Much of the work of this thesis is based on categorising nuclei additional to meiotic configurations in the meiotic preparations. In this respect Leishman gave apparently better morphological detail of the other cell types, although it should be noted that cell cetegorisation was possible with both LAO and Leishman staining.

C. Trypsin-Leishman banding schedule for mitotic chromosomes

(G-banding) Based on method described by Seabright (1971)

(i) Schedule

1. Mitotic chromosome preparations were flooded with ice-cold 0.1% trypsin solution for a time varying between 5-30 seconds.
Freeze dried trypsin (Wellcome Reagents Limited) was dissolved in 10 mls of sterile deionised water to give a 5% stock solution. The stock solution was diluted to 0.1% in Sørensen's phosphate buffered saline solution at pH 6.8 (1 part M/15 Na_2HPO_4 : 1 part M/14 KH_2PO_4) and stored in 5 ml aliquots at $-20^{\circ}C$.
2. The trypsin was washed off with Sørensen's PBS.
3. The slides were Leishman stained.

(ii) Comments

The trypsinisation time was variable between individuals, between cultures and to a lesser extent between slides. I believe that this is due to slight differences between fixation and to subsequent drying of the slides after preparation. Attempts to band meiotic chromosomes were not successful. Any banding which was initiated appeared most developed on pachytene chromosomes.

D. C-banding schedule

Based on methods described by Sumner (1972) and Chandley and Fletcher (1973)

(i) Schedule

1. A saturated $Ba(OH)_2$ stock solution ($\simeq$6%) was prepared

by stirring excess hydroxide with hot water for one hour. The hot saturated solution was then filtered into a dry bottle.

2. The cooled saturated solution was carefully decanted into a staining jar and the solution heated to 60°C.
3. Slides to be banded were dipped into $Ba(OH)_2$ at 60°C for approximately 15 secs (meiotic preparations) or 2-3 mins (mitotic preparations).
4. Treated slides were washed under very hot running tap water.
5. The thoroughly washed slides were then incubated in 2 x SSC (0.3M NaCl containing 0.03M trisodium citrate) for at least one hour.
6. Slides were then washed in Sørensens pH 6.8 PBS and subsequently Leishman stained.

(ii) Comments

C-banding was successful on both mitotic and meiotic chromosome preparations.

Good C-bands were produced when both the $Ba(OH)_2$ solution and 2 x SSC solution were at 60°C when the slides were treated. It was also essential to use very hot running tap water to remove the scum which treatment with $Ba(OH)_2$ left on the slide. Despite these precautions C-banding was often patchy in development over the slide. Sometimes patchiness appeared to be related to the initial deposition of the cell suspension on the slide. C-bands developed in meiotic configurations showed greatest unevenness.

E. Quinacrine dihydrochloride staining

Based on the method described by Bobrow and Pearson (1971)

(i) Stain preparation

An 0.5% solution of quinacrine dihydrochloride (Sigma Chemical Company) was prepared in distilled water.

(ii) Staining schedule

1. Slides were immersed in stain for 10 mins.
2. The slides were washed in running tap water for 3 mins and then mounted in distilled water.

(iii) Comments

This method gave adequate Y chromosome fluorescence but banding could not be reliably obtained. The fluorescent banding methods described in the literature have been fully investigated but have never given good results in the Sheffield Laboratory. Reasons for this are not known.

5. PHOTOGRAPHY

Photographs were taken using a Leitz Orthomat-W fully automatic microscope camera. Exposures were made with the film rated at ASA 50 using a moderately low light intensity (3.5 on the Leitz scale) and with the sub-stage condenser at 4.0. This combination of light intensity and exposure gave a good tonal range on the negative.

Ilfodata HS23 film was used for all photomicroscopy. The film was developed in Ilfodata CP developer diluted 1:3 with water and fixed with Kodafix diluted 1:4.

Prints were exposed at standard enlargement using a Durst 606 enlarger and Kodak Bromide WSG paper. Prints were developed with

Kodak D163 diluted 1:3 with water and fixed with Kodafix diluted 1:7.

A lot of the analysis reported in this thesis depended on photographic prints therefore standard photographic procedures, which take into account temperature control of chemical solutions, print development to completion, correct use of safety lights and care in handling photographic emulsions, were in operation whenever photographs were being prepared.

6. AUTORADIOGRAPHY

The presence of β particles emitted by tritiated thymidine (H^3Tdr), incorporated into DNA as a precursor, was traced using autoradiographic stripping film. The technique follows :-

A. Application of film

1. Kodak fine grain stripping film, supplied as AR10 autoradiographic plates, was used. The film was stored at $4^{o}C$ and was brought to room temperature prior to use.
2. The film was cut into suitable sized portions with a sharp razor before removal from the glass backing plate and dropped emulsion side down onto a tray of water at room temperature. The film expanded slightly after a few seconds contact with the water.
3. The slide to be coated was immersed in the water and then raised under a piece of film. The film was allowed to drape over the slide as the slide was slowly removed from the water. The slide plus film were then left to dry in the dark.

This operation ensured a tight fit of film to slide and it was

important that air bubbles were excluded from the film/slide interface. The slides were stored in a light tight box kept at 4°C for exposure of film to β particles. A common exposure time was approximately 3 weeks.

B. Development of film

1. All solutions for development and fixation were cooled to less than 10°C. This reduced the likelihood of film bubbling off the slide during processing.
2. The film was developed in Kodak D19 for 4 mins, with occasional gentle agitation.
3. The slide was briefly washed in distilled water.
4. The film was fixed in Kodafix diluted 1:10 with water and with the addition of Johnsens Ultrafix Hardener. Time taken for fixation was approximately 10 mins or twice the length of time taken for the film to clear. Gentle agitation during fixation helped to achieve clearing.
5. The slides were washed in two changes of distilled water and then allowed to dry at room temperature.
6. The special precautions taken with photographic processing also applied for autoradiographic film development.

7. CHROMOSOME ANALYSIS

A. Mitotic chromosome analysis

Thirty well spread metaphase cells were recorded for each individual studied. Four metaphase cells from each individual were analysed in detail from photographic prints. All counting and analysis were performed using G-banded material.

In individuals where abnormal chromosome complements were detected,

a repeat blood sample was obtained in order to verify the results. In some cases the C-band karyotype of the individual was also investigated, usually to complement description of a probably abnormal karyotype. In these cases C-banding was carried out on material previously G-banded. In this way, photographic records of G-banded metaphases could be compared with the same metaphase after C-banding.

B. Analysis of cell division in testicular biopsies

The objective, when preparing material for analysis, was material with well spread primary spermatocytes at the diakenesis stage of meiotic division. When this was achieved 22 autosomal bivalents and the XY structure (associated XY or unassociated X, Y) could be clearly identified and counted. In this material well spread diakenesis figures were rare (Figure 4.1). A subjective choice of 10 well spread figures was taken on which to base judgements of normality or abnormality. This figure of 10 cells was sometimes exceeded. When the total number of cells analysed fell below 7, there were too few cells present to be certain about the analysis. The assessment of normal diakenesis was based on the following criteria :-

1. 22 autosomal bivalents + an XY or X,Y structure. Records were kept of the association pattern of the X and Y chromosomes.
2. Bivalent configuration appeared normal with bivalents showing approximately bilateral symmetry, i.e. contain one mirror plane.
3. Structures such as chains, larger than normal ring formations or several univalents were absent from figures.

Other stages of meiotic division were noted in these preparations.

Figures in pachytene were often plentiful and occasionally were partially analysable, (Figure 4.2). Complete analysis was not possible due to many crossovers and complex associations often involving the persistent sex vesicle. The tiny amount of biopsy available for study prevented any persistant attempt to prepare samples for pachytene analysis (Hungerford, 1971; Burdick et al, 1974). Occasional figures in Metaphase II were identified and unlike Figure 4.3 were rarely scoreable.

Spermatogonial mitoses (Figure 4.4) were observed but were not usually plentiful in these preparations. Analysis of spermatogonial mitosis characteristically gave counts which deviated from the expected count of 46 chromosomes. It was not possible to use these counts to clarify possibly abnormal gametic karyotypes, although occasional counts corresponded to the possible abnormality under examination.

8. PREPARATION OF HISTOLOGY SECTIONS

At testicular biopsy a second sample of the testis was sent directly to the Pathology Laboratories of the Royal Hospital, Sheffield. The biopsy was fixed in Bouin's solution and processed routinely. Sections were cut at 6 microns and stained in Haematoxylin and Eosin. The histological preparation of the biopsy was prepared and handed over to me through the courtesy of Mr Graham Anderson, Chief Technician at the Royal Hospital.

9. GONADOTROPHIN ESTIMATIONS

In the first two years of this study serum FSH and LH values were obtained as part of the larger study of male infertility.
Dr Elizabeth Lenton, Biochemist, University Department of Obstetrics and Gynaecology, Jessop Hospital for Women, Sheffield made the determinations using a modification of the method of Saxena et al (1968).

Figure 4.1

Primary spermatocyte from infertile male. Diakenesis stage of meiotic division. 22 autosomal bivalents and an XY bivalent. Orcein stain.

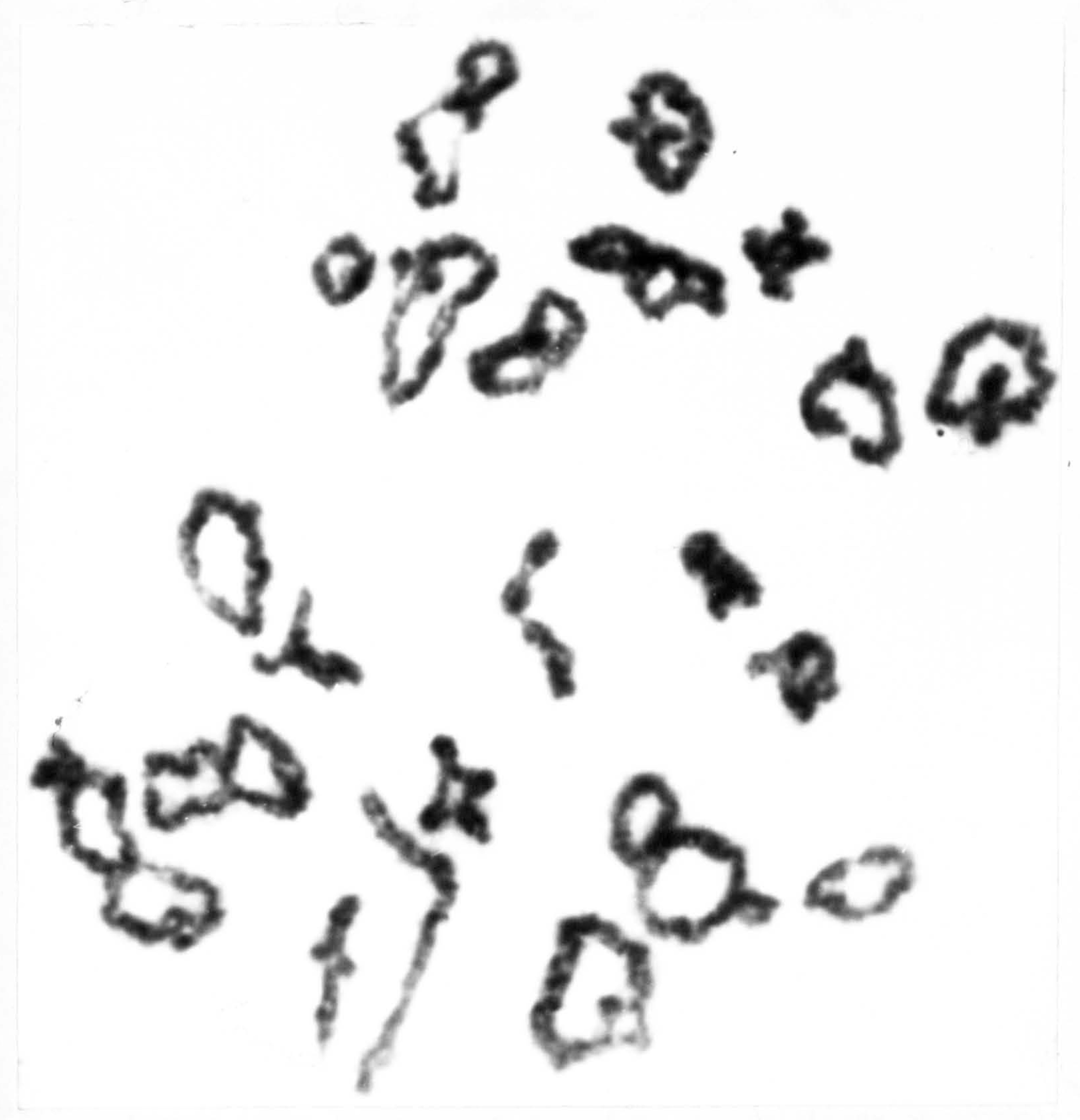


Figure 4.2

Primary spermatocyte from infertile male. Pachytene stage of meiotic division. Arrow indicates paramere structure bivalent 9. The chromomere patterns on the bivalents are well demonstrated with Leishman stain.

Figure 4.3

Second metaphase from infertile male. Arrows indicate the secondary constriction on chromosome 9.

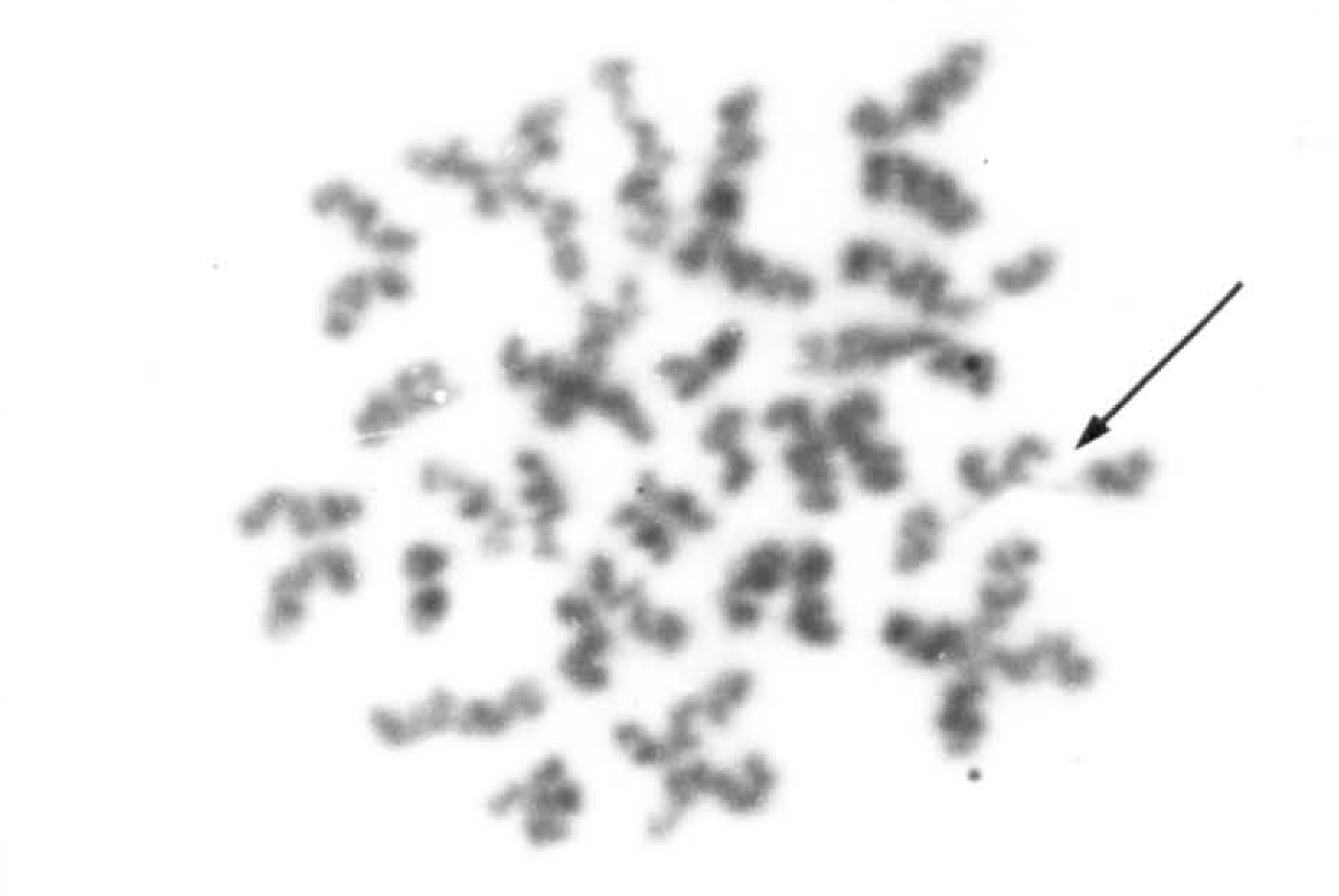
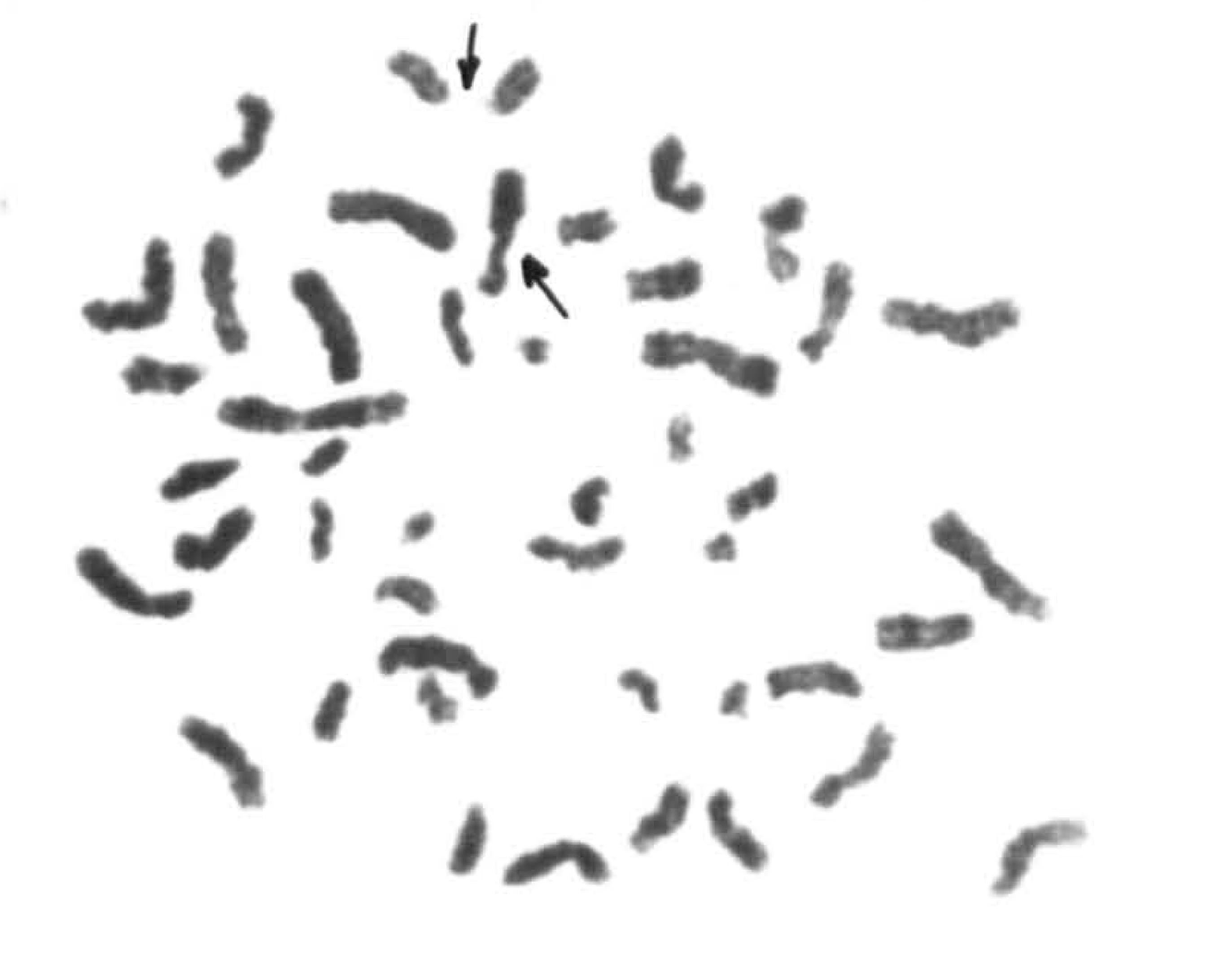


Figure 4.4

Spermatogonial metaphase from infertile male. Orcein stain. Arrows point to extended secondary constrictions on chromosomes 1 and 9. 46 chromosomes.



She generously allowed me access to her results.

Patients whose TB's were obtained after funding ceased, did not have plasma gonadotrophin evaluations. In these patients, routine laboratory 24 hour urinary gonadotrophin levels were recorded. Again, I was generously allowed access to the results.

CHAPTER 5

LABORATORY INVESTIGATIONS, OTHER THAN CYTOGENETICS

1. Introduction

2. Gonadotrophins

3. Histology

   A. Quantitative method

   B. Qualitative method

4. Correlations

1. INTRODUCTION

This Chapter reports the results of laboratory investigations, excluding cytogenetic studies, on the population of infertile males.

2. GONADOTROPHINS

Table 5.1 (see pages 71 and 72) records the plasma gonadotrophin levels for 42 of the 68 patients. Control plasma gonadotrophin levels were calculated from a series of normal men with values for FSH = 5.91 ± 2.34 iu/l (n = 51) and LH = 9.35 ± 2.46 iu/l (n = 25). Dr Lenton obtained these normal values from a group of men attending the vasectomy clinic. In patients whose gonadotrophins were measured from 24 hour urine samples, the results were categorised as high, low or normal.

The results are summarised in Table 5.2. No cases of low gonadotrophins were recorded. 12 cases (18%) were recorded with high FSH and LH values, 6 cases with high FSH values only and 13 cases with high LH values only. Thus 31 cases (46%) had an abnormally high gonadotrophin value affecting either or both gonadotrophins monitored. Facilities were not available for testosterone estimation.

Table 5.2 SUMMARY OF GONADOTROPHIN RESULTS

Results	No. of cases	% population
FSH + LH high	12	18
FSH high	6	9
LH high	13	19
Total abnormal gonadotrophins	31	46
FSH + LH normal	33	48
Not recorded	4	6
Total normal + abnormal gonadotrophins	68	100

TABLE 5.1

GONADOTROPHIN ESTIMATIONS OF 68 PATIENTS

WHO HAD TESTICULAR BIOPSIES TAKEN

Case No	Plasma gonadotrophins		Urinary gonadotrophins	
	FSH i.u/l	LH i.u/l	FSH	LH
6	-	-	N	N
7	-	-	N	N
8	-	-	N	N
9	5.6	12		
10	8.2	4.4		
11	*37	*22.4		
12	5.8	8.4		
13	*16.2	11.0		
14	-	-	N	N
15	5.6	*25		
16	2.5	4		
17	*12.2	*14.6		
18	*26	*31		
19	*14.8	9.2		
20	1.2	7.0		
21	1.9	9.8		
22	6.2	*21.5		
23	7.2	*16.0		
24	4.1	6.7		
25	*19.5	*35.0		
26	7.7	*15.5		
27	3.0	*16.0		
28	8.4	*19.5		
29	3.0	13.6		
30	8.7	*38.0		
31	10.5	12.8		
32	*14.5	*38.0		
33	5.6	*18.0		
34	*11.0	14.0		
35	5.0	10.0		
36	*30.5	*24.5		
37	7.8	13.0		
38	3.9	14.0		
39	5.7	14.2		
40	4.2	11.8		
41	6.8	*28.0		
43	-	-	N	N
44	8.3	14.0		
45	*26	*22		
46	*11.2	10.0		

Sample number	Plasma gonadotrophins		Urinary gonadotrophins	
	FSH i.u/l	LH i.u/l	FSH	LH
47	4.0	8.7	-	-
48	*14.2	*19.0	-	-
49	6.6	10.5	-	-
51	-	-	N	N
52	3.6	9.8	-	-
57	-	-	NK	NK
66	-	-	N	H
67	*14.0	9.8	-	-
68	5.8	8.7	-	-
69	-	-	N	N
76	-	-	NK	NK
79	-	-	N	N
80	-	-	NK	NK
82	4.0	6.7	-	-
88	Series discontinued		N	N
105			N	N
106			N	N
107			N	*H
116			N	*H
117			N	N
118			*H	N
121			N	*H
123			*H	*H
130			*H	*H
133			*N → H	*N → H
135			NK	NK
136			*H	*H
137			N	N

Normal range plasma gonadotrophins

FSH 5.91 ± 2.34; 1.23 - 10.59

LH 9.35 ± 2.46; 4.43 - 14.27

(95% confidence level)

*values outside normal range

Normal range urinary gonadotrophins

FSH 2 - 22

LH 4 - 45

*values outside normal range

NK = not known

3. HISTOLOGY

The histology of the testicular biopsies was studied by two methods.

A. Quantitative method

The testicular biopsy score count method described by Johnsen (1970) was used to obtain a quantitative value for each biopsy. This method scanned each biopsy and scored every intact tubule according to the presence of the most advanced stage of spermatogenesis noted in the tubule. It also took into account whether there were many or few of the most advanced stage noted in the tubule. A modification of Johnsen's original description was used, in that Stages 8, 9 and 10 diverged slightly from Johnsen's description. The tubules were scored from 1–10 according to the following criteria.

Stage 1 No cells in tubule.

Stage 2 Sertoli cells only present in tubule. Spermatogonia absent.

Stage 3 Spermatogonia only present in tubule. Spermatocytes absent.

Stage 4 Few spermatocytes (<5) only present in tubule.

Stage 5 Several to many spermatocytes only present. Spermatids and spermatozoa absent.

Stage 6 Few spermatids (<5) only present.

Stage 7 Several to many spermatids only present. Spermatozoa absent.

Stage 8 Spermatozoa present. Lumen obliterated. Germinal epithelium disorganised.

Stage 9 Spermatozoa present. Lumen obliterated. Germinal epithelium maintaining organised appearance.

Stage 10 Normal tubules. Spermatozoa present. Lumen open. Germinal epithelium with a regular organised appearance and thickness.

This description diverged from Johnsen's in the lack of distinction between tubules with many or few spermatozoa, and in the use of Stage 8 for obliterated lumen with disorganised germinal epithelium. This gave more weight to the extent of tubule blockage whereas Johnsen gave more weight to the presence of few or many spermatozoa. The testicular biopsy score count obtained in this manner can be examined in detail for the number of tubules at each stage. For comparative purposes a mean score, called the Johnsen Mean Score (JMS) was calculated. In order to calculate the JMS the number of tubules (x) recorded at each stage (Stage 1-10) was multiplied by the stage and the sum of all the multiplications was divided by the total number of tubules recorded :-

$$JMS = \frac{\Sigma(x \times 1 \ldots\ldots x \times 10)}{\Sigma x}$$

Table 5.3 (see pages 75 and 76) records the JMS for 59 of the 68 patients studied. Five biopsies could not be scored because of poor quality preparation. In 4 patients a biopsy was not processed for histology. Figure 5.1 shows the distribution of JMS for the 59 cases studied. The distribution is sharply truncated with no JMS above 8.9. The mean JMS for the population is 6.32 ± 2.08. Johnsen's JMS for normal males was 9.38 ± 0.24.

TABLE 5.3

HISTOLOGY : (a) Johnsen Mean Score (b) Qualitative Category

Sample No	JMS	Most advanced tube scored	Qualitative Score					
			NAD	B	RG	FAG	S + Other	Other + S
6	8.2	10			+			
7	7.1	9			+			
8	3.1	(10)					S+RG	
9	7.0	9			+			
10	6.2	8			+			
11	7.5	10			+			
12	7.4	10			+			
13	3.4	(10)					S+RG	
14	7.7	9		+				
15	4.0	5				+		+
16	6.2	8			+			+
17	7.8	10			+			+
18	3.9	5				+		
19	2.6	(8)					S+RG	
20	7.4	10			+			
21	7.8	10			+			+
22	8.1	10	+					
23	8.6	10	+					
24	7.6	9			+			
25	1.5	5				+		
26	6.5	9			+			
27	8.5	10	+					
28	5.1	8			+			+
29	6.6	10			+			+
30	not scoreable							
31	7.3	10			+			+
32	4.6	(10)					S+RG	
33	5.5	8			+			
34	5.8	10			+			+
35	7.2	9			+			
36	6.1	10			+			+
37	2.0	(4)					S+FAG	
38	not scoreable							

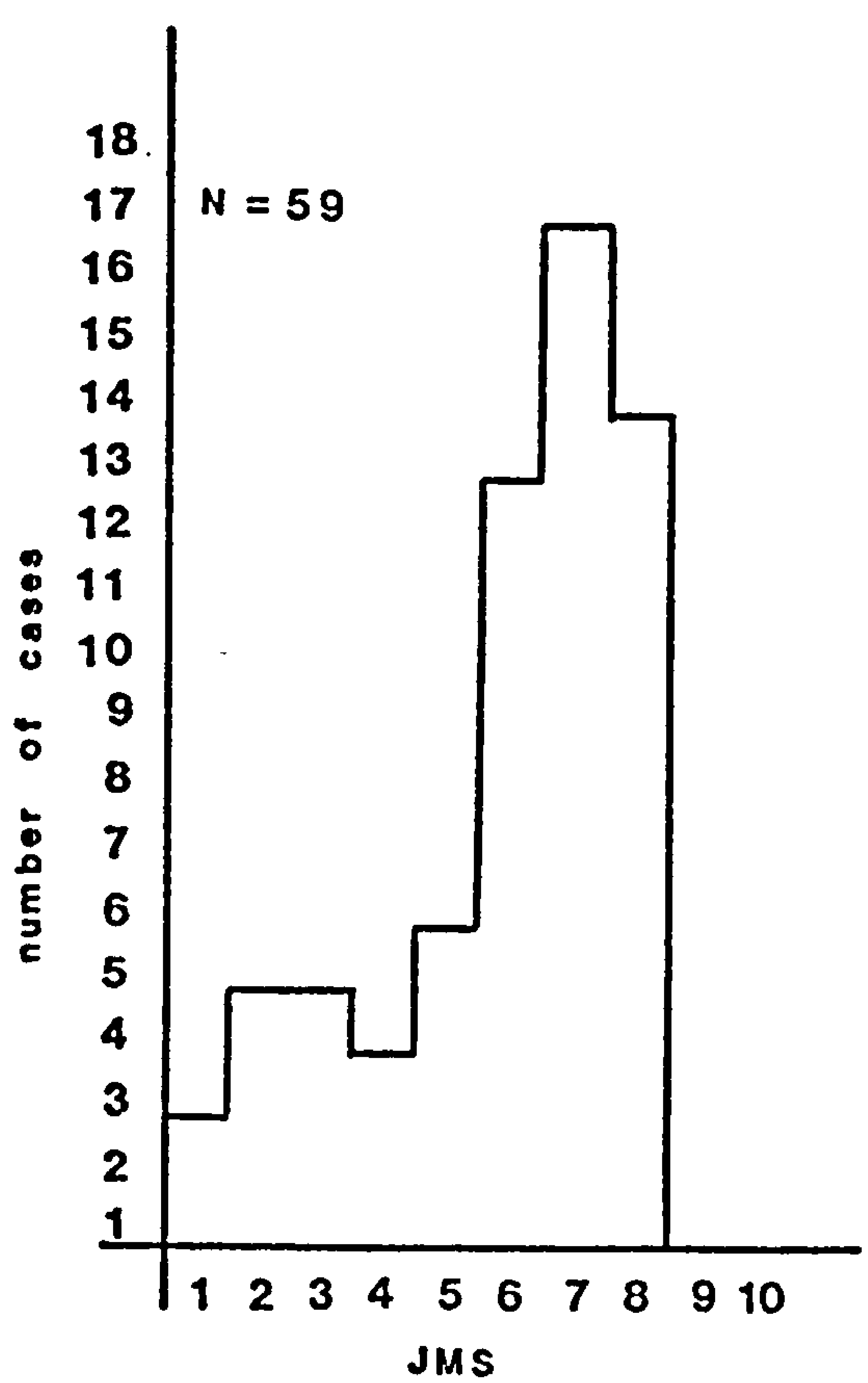
Sample No	JMS	Most advanced tube scored	Qualitative Score					
			NAD	B	RG	FAG	S + Other	Other + S
39	not scoreable							
40	not scoreable							
41	7.4	9		+				
43	6.7	10			+			
44	6.5	9			+			+
45	8.0	8		+				
46	6.6	8			+			
47	6.2	10			+			+
48	8.3	9		+				
49	7.6	9		+				
51	7.7	9			+			
52	7.2	10			+			
57	8.9	10	+					
66	—	—	—	—	—	—	—	—
67	8.5	10	+					
68	8.5	10	+					
69	6.7	10			+			+
76	2.0	3				+		
79	8.2	10			+			
80	8.3	10	+					
82	6.8	9			+			
88	not scoreable							
105	—	—	—	—	—	—	—	—
106	—	—	—	—	—	—	—	—
107	—	—	—	—	—	—	—	—
116	8.1	10	+					
117	5.0	6				+		
118	1.2	3				+		
121	8.1	10			+			
123	1.8	3				+		
130	2.0	3				+		
133	7.7	10			+			
135	8.7	10	+					
136	3.0	3				+		
137	5.4	10			+			+
		TOTAL	9	5	31	9	5	13

4 —— no histology : 5 ∿∿ not scoreable, poor section

most advanced tube scored in brackets = RG line of S/RG cases

Figure 5.1

Distribution of JMS for 59 cases



B. Qualitative method

The histology of the TB was reported in traditional pathology report language by the Pathologist and this was preferred by the Urologists to the JMS. In order to describe the state of the testicular epithelium to the Urologist, in terms other than those of the JMS, a series of germinal epithelial categories was instituted. These were as follows :-

NAD Apparently normal tubules with spermatozoa and with an open lumen and well organised germinal epithelium. The most advanced tubules scored at 10. The numbers of tubules at

	Stages less than 10 decreased rapidly with occasional tubules only, at Stages 6 to 4.
B	No tubules at Stage 10. Tubules present at Stages 9 or 8 as the most advanced Stage present and with a rapid reduction of numbers of tubules through Stages 6 to 4.
RG	Reduced spermatogenesis. All Stages up to Stage 10 may be present. The numbers of tubules at Stages 10 to 8 less than or equal to tubules at Stages 7 to 4.
FAG	Full arrest of spermatogenesis. No tubules beyond a particular Stage. Spermatids or spermatocytes absent. Spermatogonia present.
S	Sertoli cells only in all tubules.
S + other	Sertoli cells in m ajority of tubules. Occasional tubules with Scores up to 10.

These categories were used for a more descriptive evaluation than the JMS and were based on the number of tubules at each stage after counting, rather than as in the JMS Score itself. One further category was additional to those already described. This was "other category + Sertoli cells only tubules". In these cases occasional tubules with Sertoli only cells were noted. All categories assigned are noted in Table 5.3. Figure 5.2 compared the JMS with these categories and demonstrated that the JMS generally decreased with the decreasing state of the germinal epithelium. Cases categorised "RG" with the additional category " + occasional Sertoli only tubules" were not exclusively represented by the lowest "RG" category scores. Overall, the JMS Scores reflected the activity of the germinal epithelium as represented by the categorisation. However, the categorisation gave more information than the JMS about what is actually taking place in the germinal epithelium.

Figure 5.2

JMS versus histology categories

JMS

9·5 9·0 8·5 8·0 7·5 7·0 6·5 6·0 5·5 5·0 4·5 4·0 3·5 3·0 2·5 2·0 1·5 1·0 <1·0

Category	NAD	B	RG	FAG	S + other	RG+S
no. of cases	9	5	31	9	5	12

4. CORRELATIONS

In Figure 5.3 sperm counts were recorded against JMS. The results show a lack of correlation between the sperm count and the activity of the testicular epithelium as measured by the JMS. Johnsen (1970) reported a very high correlation between sperm count and JMS in his series which included 15% normal individuals with normal sperm counts. He commented that considerable variation of individual values occurred. In the present series, cases with $<20 \times 10^6$ sperm/ml had recorded JMS values of less than 6.0, whereas cases with $<5 \times 10^6$ sperm/ml had recorded JMS values of more than 8.0. These observations are very similar to those recorded by Johnsen when he considered individual values rather than the overall correlation of his population.

Figure 5.3

Sperm count versus JMS

JMS

9·5
9·0
8·5
8·0
7·5
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
1·5
1·0
<1·0

sperm count	AZOOSPERMIC	$<5 \times 10^{-6}$	$<10 \times 10^{-6}$	$<20 \times 10^{-6}$	$>20 \times 10^{-6}$
not scored	2	3	0	2	2
total	16	20	9	13	6

In the series as a whole, 16 patients were azoospermic. Nine of these had physical evidence of epididymal blockage or a previous history of testicular trauma and/or surgery. The histology category "B" did not correlate with patients who had physical evidence of epididymal blockage.

Table 5.4 records the results of the physical examination of the genitalia on 15 patients who had some type of abnormality together with other investigations on these patients. Eight patients had blocked epididymus; of these 5 were azoospermic and of these 5, one had "NAD" histology, 3 had "RG" histology and one was not scored. The other 3

patients with blocked epididymus consisted of two with very low sperm counts and "RG" on histology and one patient with a high sperm count (low motility) and "RG" on histology. Five patients had a previous history of testicular trauma; of these 3 were azoospermic and of these, one had a severe histological disturbance ("S/FAG"), one was "RG" and one could not be scored. The other two patients with a history of testicular trauma had very low sperm counts, one had "FAG" histology and the other could not be scored. Two patients had both physical evidence of blockage and a previous history of testicular trauma or surgery. One of these patients had a moderately low sperm count but unfortunately no histology sample was taken. In general, cases with distended epididymus tended to have relatively high JMS Scores together with very low sperm counts whereas a previous history of testicular trauma or surgery indicated both a relatively low JMS and low sperm counts.

Table 5.4 PHYSICAL EXAMINATION OF TESTIS + TESTICULAR HISTORY vs SPERM COUNT, JMS AND HISTOLOGY CATEGORY.

Case No.	Dist. Epid.	Prev. Test. Hist.	Absent Testis	Sperm Count	JMS	Hist. Cat.	Comments
6	+			0.0	8.2	RG	
7	+			0.0	7.1	RG	
10	+			1.9	6.2	RG	
16	+			0.0	6.2	RG	
21	+			101.0	7.8	RG	
22	+			0.0	8.1	NAD	
25		+	Right	1.5	4.5	FAG	
26	+			0.75	6.5	RG	
36		+	? Right	0.0	6.1	RG	
37		+		0.0	2.0	S + FAG	
40		+		0.0	-	-	
88		+	Atrophic	4.0	-	-	no score
105				0.0	-	-	no score
106	+(R)	+		13.5	-	-	no sample
136	+(L)	+	Atrophic	0.0	3.0	FAG	no sample

JMS and histology categories were investigated in cases with high gonadotrophin values. 31 cases had abnormally high gonadotrophin values. The JMS and histology categories for these cases are shown in Table 5.5. These results show that, out of 12 cases with high FSH and LH values, six had severely impaired histology categories ("FAG"/ "S + other"). Out of six cases with high FSH values, two had severely impaired histology categories. Out of 13 cases with high LH values, one case had a severely impaired histology category.

By taking the comparisons for the histology categories, the following information can be extracted from the data. Out of nine individuals with histology rated as "NAD", one had raised FSH and four had raised LH values. Of the 10 cases with "FAG" histology, seven had abnormal gonadotrophins; of these five had raised FSH and LH values, one had raised FSH values and one had raised LH values. Of the five cases with "Sertoli only cells + other tubules", one had raised FSH and LH values and two had raised FSH values. It is quite evident that not all cases with raised gonadotrophin values have severely impaired spermatogenesis. Neither do all cases with severely impaired spermatogenesis have raised gonadotrophin values. These results are in agreement with de Kretser et al (1972a) who found no correlation between serum FSH levels and the severity of oligospermia, when oligospermia was measured by a qualitative assessment of the testicular epithelium. Other authors (e.g. Rosen and Weintraub, 1971) have claimed a significant correlation between the activity of the testicular epithelium and gonadotrophin values. When de Kretser et al (1974) used quantitative evaluation of the testicular epithelium they were able to describe a significant correlation between serum FSH and the activity of the germinal epithelium. The conclusions which can be drawn from these conflicting results suggest that the correlation between raised

Table 5.5

COMPARING HIGH GONADOTROPHIN VALUES WITH JMS AND HISTOLOGY CATEGORY

Gonadotrophins	Case No.	JMS	Histology Category
FSH + LH high (12 cases)	11	7.5	RG
	17	7.8	RG
	18	3.9	FAG
	25	4.5	FAG
	32	4.6	S/RG
	36	6.1	RG
	45	8.0	B
	48	8.3	B
	123	1.8	FAG
	130	2.0	FAG
	133	7.7	RG
	136	3.0	FAG
FSH high (6 cases)	13	3.4	S/RG
	19	2.6	S/RG
	34	5.8	RG
	46	6.6	RG
	67	8.5	NAD
	118	1.2	FAG
LH high (13 cases)	15	4.0	FAG
	22	8.1	NAD
	23	8.6	NAD
	26	6.5	RG
	27	8.5	NAD
	28	5.1	RG
	30	—	not scoreable
	33	5.5	RG
	41	7.5	B
	66	—	no sample
	107	—	no sample
	116	8.1	NAD
	121	8.1	RG

gonadotrophins and testicular activity is not simple. Testicular activity needs to be described accurately, by a more sophisticated approach than a qualitative assessment, before the relationship can be understood fully.

CHAPTER 6

RESULTS OF CYTOGENETIC SCREENING OF THE INFERTILE SERIES

1. Results of mitotic karyotype analysis

2. Description of abnormal mitotic karyotypes

3. Analysis of gametic karyotype

   A. Introduction

   B. Normal meiosis

   C. Abnormal meiosis

   D. ? abnormal meiosis

   E. Absent meiosis

   F. Few figures

   G. Failed preparations

1. MITOTIC CHROMOSOME ANALYSIS

The results of mitotic chromosome analysis are recorded in Table 6.1

Out of the 68 patients investigated, blood samples were not received from 2 patients and 3 samples of blood failed to give preparations of good enough quality for analysis.

A normal 46,XY karyotype was found in 59 patients. Four of these had variant Y(Yq$^+$) chromosomes which were larger than the normal Y chromosome.

Four patients had abnormal karyotypes. Two of these patients had a similar autosomal translocation between chromosomes 13 and 14 with the resultant karyotype 45,XY,t(13q;14q). One patient had a pericentric inversion of chromosome 10. One patient had an unbalanced 46,XY,der(13),t(Y;13) karyotype, where the extra broken Y chromosome was translocated onto a chromosome 13.

During the period of the study 3 cases of XXY individuals were identified. These cases were suspected clinically and confirmed by chromosome analysis. Two other patients, presenting with suspected Klinefèlters syndrome, had normal 46,XY karyotypes. None of these 5 individuals were biopsied, so they are excluded from the series.

2. DESCRIPTION OF ABNORMAL MITOTIC KARYOTYPES

1. 45,XY,t(13;14)(13qter → cen → 14qter)

Cases T9 and T29 were heterozygous for an autosomal translocation between chromosomes 13 and 14. A partial karyotype from T9 is shown in Figure 6.1. There was no evidence for mosaicism in either individual. G-banding studies demonstrated that the entire q13 and q14 arms were

TABLE 6.1

MITOTIC KARYOTYPES FOR 68 INFERTILE PATIENTS

Case No	Abnormal somatic karyotype	Variant Y
6		
7		
8		
9	45,XY,t(13q;14q)	
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29	45,XY,t(13q;14q)	
30		
31		
32		Yq^+
33		
34	46,XY,der(13) t(Y;13)	
35		
36		
37		
38		

Case No	Abnormal somatic karyotype	Variant Y
39		
40		
41		
43		
44		
45		
46		
47		
48	Failed	
49		Yq^+
51	Failed	
52		
57		Yq^+
66		
67	Failed	
68		
69		
76		
79		
80		
82		Yq^+
88		
105		
106	No blood sample	
107	No blood sample	
116		
117		
118		
121	46XY,inv(10)	
123		
130		
133		
135		
136		
137		

present in the translocation product. C-banding studies showed no evidence for a double centromeric structure in the translocation product. Thus the translocation was probably formed by a type of centric fusion. Family studies were not pursued with these patients.

2. 46,XY,der(13)t(13;Y)(p11;q12)

Case T34 had a 46,XY karyotype in 50 metaphase cells studied. One chromosome 13 had prominent short arms well marked by the presence of a dark G-band (see Figure 6.2a). Staining with LAO demonstrated a chromosome with well marked short arms which was often difficult to distinguish from other members of the C group (Figure 6.2b). Studies of C-banded metaphases showed that the extra material was heterochromatic in nature, whilst the normal Y chromosome had a typical heterochromatic band on the distal q arm (Figure 6.2c). Q-staining with quinacrine dihydrochloride showed a brilliantly fluorescing band on the p arm of the abnormal 13 and a brilliantly fluorescing band on the distal q arm of the normal Y chromosome. In this preparation satellites were well developed on one chromosome 14 and on one chromosome 15. However, in the Q-stained preparations these satellites were not fluorescent to any marked degree. It was not possible to photograph the fluorescent pattern and fluorescence of buccal smears did not give a satisfactory result. The karyotype was interpreted from the staining investigations as the distal q arm segment of a broken extra Y chromosome translocated onto a chromosome 13 (Curtis, 1977). The break point on the extra Y chromosome was in the region

Yq 1.2 (Paris Conference, 1971*). No evidence was found for the survival of the remainder of the extra Y chromosome in any of the cells studied. Thus case 34 was a carrier of a Y/13 translocation in unbalanced form. The patient was unwilling for his parents to be approached for family studies.

Nielsen and Rasmussen (1976) together with their fellow workers estimated the incidence of Y/autosomal translocations in the general population to be approximately 1 in 2000. Nielsen and Rasmussen reviewed 26 reported cases of Y/autosomal translocations to which can be added the Y/22 case described by Chandley et al (1975). Within these 27 cases there was one other report of a Y/13 translocation (Gilgenkranz et al, 1973), but in this case the translocation was ($13q^+$; Yq^-). The specific karyotype in the present case 46,XY,der(13)t(13;Y) has not to my knowledge been previously described.

3. <u>46,XY,inv(10)(p11;q22)</u>

Case 121 had a 46,XY karyotype with an abnormal chromosome 10. The p arm of the abnormal chromosome 10 had an extra dark band present and the whole chromosome was markedly more metacentric than its normal homologue. A repeat blood culture confirmed the presence of the abnormal chromosome 10. Figure 6.3 demonstrates the abnormal chromosome together with its normal homologue. The break points are identified as p1.1 and q2.1 and there has been an inversion of the segment between the break points. The full karyotype description is given as follows :-

*Supplement (1975)

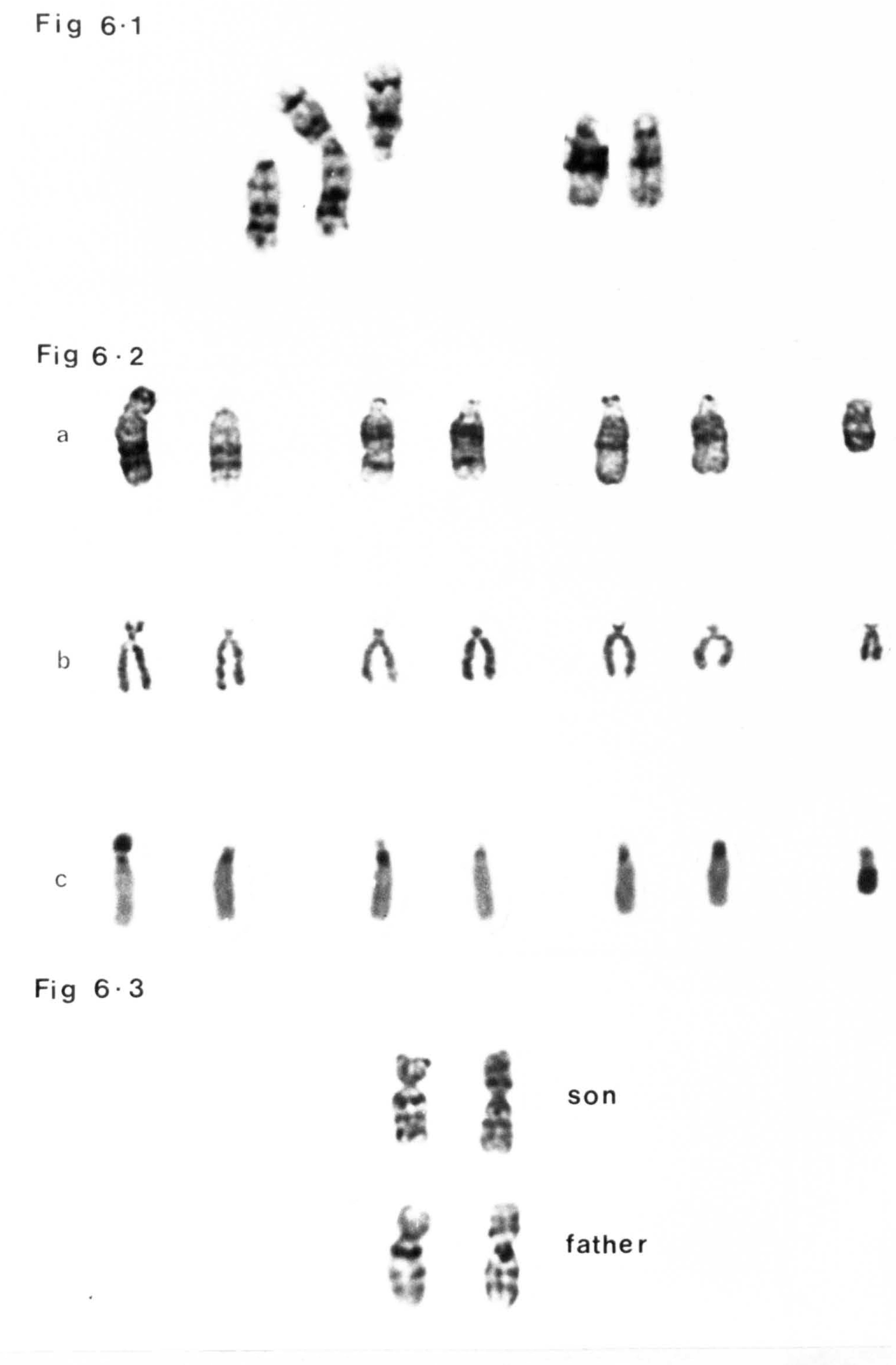


Fig 6·1
Fig 6·2
a
b
c
Fig 6·3
son
father

46,XY,inv(10)(pter→p11::q21→p11::q21→qter)

Chromsome analysis of the patient's parents demonstrated that the patient's father had an identical abnormal chromosome 10. Both case 121 and his father are therefore heterozygous for a pericentric inversion of chromosome 10.

3. ANALYSIS OF GAMETIC KARYOTYPE

A. Introduction

The analysis of chromosome division in material prepared for testicular biopsies has been discussed in Chapter 4 Section 7B. When reviewing the results of meiotic analysis it seemed clear that there was more than one way of describing meiosis as abnormal. For instance, diakenesis configuration may be abnormal or diakenesis figures may be absent altogether. Hendry et al (1976) considered this point and recorded their results under categories "normal meiosis, abnormal meiosis, ? abnormal meiosis, absent meiosis". Hendry et al gave few details of the categories they used. Therefore a similar scheme for categorising the results of analysis has been devised in which the categories are described as follows.

(i) "Normal meiosis"

Karyotypes described as MI,23,XY or MI,24,X,Y. The bivalents exhibited normal approximate bilateral symmetry. Large rings or chain like structures or more than one univalent absent. More than 7 cells analysed.

(ii) "Abnormal meiosis"

Karyotype other than MI,23,XY or MI,24,X,Y. Trivalents,

quadrivalents, several univalents or ring structures observed. Presence of obvious lack of bilateral symmetry recorded for one or more bivalents. Abnormal configuration and/or number noted in all cells studied.

(iii) "? abnormal meiosis"

Presence of similar abnormalities as in (ii). Abnormality suspected but not proven, often because the abnormality could not be detected in all cells studied.

(iv) "Absent meiosis"

Cells in diakenesis and MII not identified. Pachytene nuclei may or may not be present.

(v) "Too few figures for analysis"

A heterogeneous group. Less than 7 cells analysable, often accompanied by difficulty in analysis. Some cases, with rare cells at diakenesis, which were unequivocally normal with respect to number and configurations.

(vi) "Failed preparations"

Preparations for which analysis was impossible. Often the original biopsy was very small.

The results of gametic karyotype analysis on 68 infertile males are recorded in Table 6.2. Table 6.3 summarises these results.

TABLE 6.2

RESULTS OF GAMETIC KARYOTYPE ANALYSIS OF 68 INFERTILE PATIENTS

Case No	MEIOTIC KARYOTYPE				MEIOTIC CATEGORY						
	23, XY,	24, X,Y	Other	No. analysed	Normal	Abnormal	?Abnormal	Absent	Few figs	Failed	Other
6	4	2	22,X,Y(1)	7	+						
7	8	4		12	+						
8	3	0		3					NAD		
9	-	-	trivalent	-		+					
10	7	2		9	+						
11	-	-	achiasmate	-		+					
12	0	0		0						+	figures not analysable
13	0	0		0				+			pac. + occ.dia.
14	10	0	22,XY(1); 21,XY(2)	13	+						
15	1	0		1					+		dia.not analysable
16	5	1	22,XY,(2)	8	+						
17	10	1	24,XY,(1)	12	+						
18	0	0		0				+			occ. pac.
19	0	0		0				+			pac. absent
20	16	0	22,XY,(5); 24,XYI(2)	23	+						
21	12	3	22,XY,(1)	16	+						
22	-	-	? abnormal	-			+				
23	4	0		4					NAD		
24	3	4	22,XY(2); 21,XY,(2)	11	+						
25	0	0		0				+			pac. present
26	2	1	22,-XY(1); 23,-XY(2)	6					+		poor figures
27	5	6	22,XY(2); 23,X,Y(1)	14	+						
28	3	1		4					NAD		
29	-	-	trivalent	(11)		+					
30	0	0		0				+			pac. present
31	6	3	22,XY(1); 23/24(1)	11	+						
32	2	2		4					NAD		
33	0	0	22,XY(1)	1					+		pac. present
34	18	4	abnormal	(24)		+					
35	-	-	? abnormal	-			+				
36	4	2	22,XY,(2)	8	+						
37	0	0		0				+			pac. absent
38	2	4	MII,23(1)	7	+						
39	0	0		0				+			pac. present
40	0	0		0				+			pac. present
41	12	5	MII,23(1); 22-XY(1)	19	+						
43	12	0		12	+						

TABLE 6.3

SUMMARY OF RESULTS OF GAMETIC KARYOTYPE ANALYSIS

Meiotic category	No. of cases
Normal meiosis	26
Abnormal meiosis	4
? abnormal meiosis	3
Absent meiosis	14
Few figures	15
Failed preparations	6
TOTAL	68

B. Normal meiosis

26 cases were recorded with normal meiotic karyotypes. 288 cells in division were analysed from this "normal meiosis" group. The range of cells between individuals was 7–23 with an average per individual of 11.1 cells analysed. Two cells of the 288 were recorded as cells in MII division.

Of the 286 diakenesis cells analysed, 51 or 18% had an unassociated X and Y chromosome. 3 individuals had a greater number of cells with an X,Y complement than an XY complement. These 3 individuals, with 14 X,Y cells between them, accounted for 27% of all X,Y complement cells.

There was a high count of amodal cells. Out of the total 286 cells counted, 39 cells or 13% were amodal. 26 of these cells (66% of all amodal cells) had lost 1 bivalent; 9 of these cells (23% of all amodal cells) had lost 2 bivalents and 4 cells (10% of all amodal cells)

had an additional structure.

25 of the cases with "normal meiosis" had normal mitotic karyotypes. 1 case with "normal meiosis" had an abnormal mitotic karyotype.

CASE 121

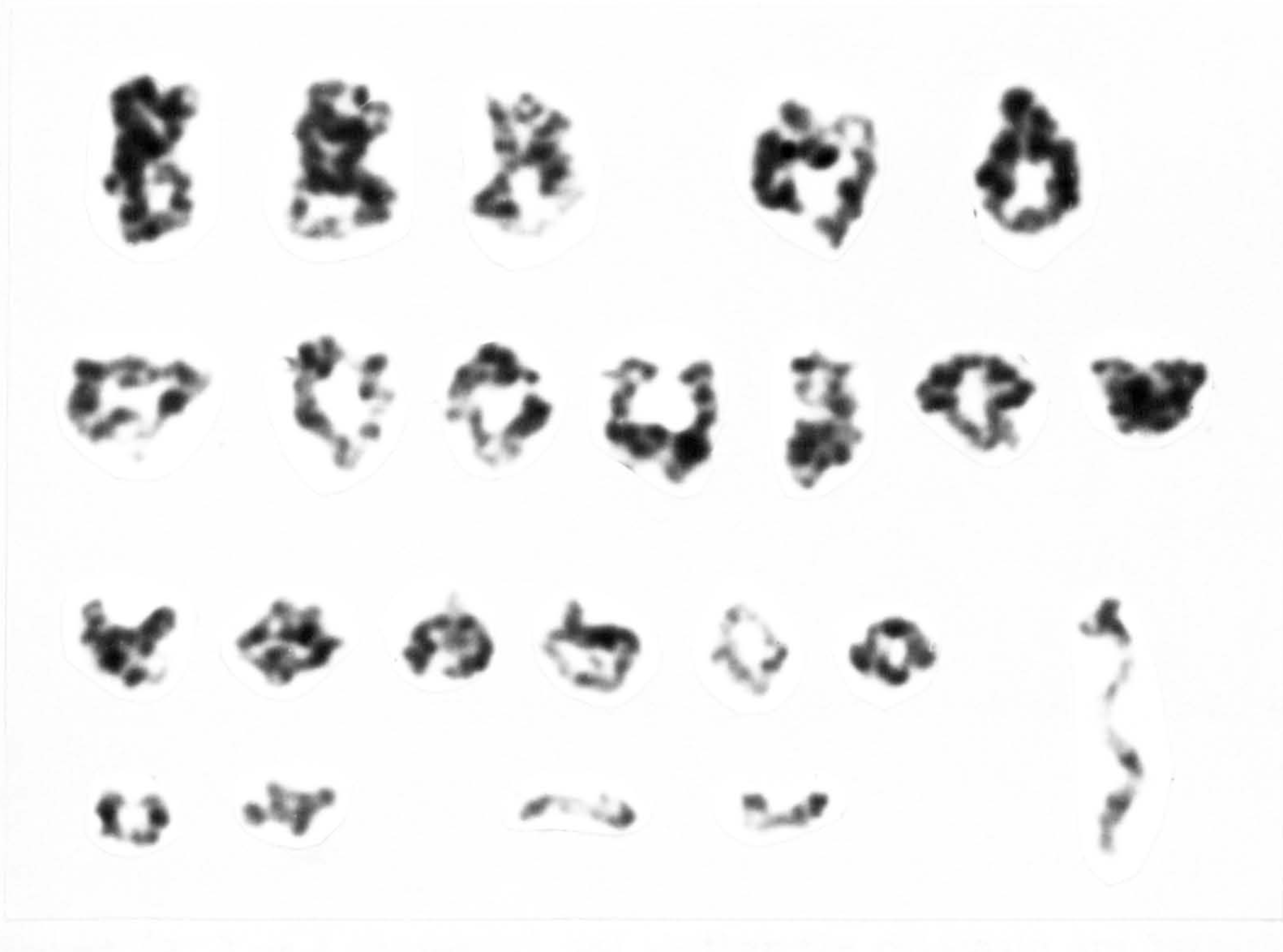
This individual had 46,XY,inv(10) karyotype which has been described in Section 2 of this Chapter.

Eight diakenesis figures were found in the meiotic preparations and the figures were all of good quality and analysable. Analysis recorded "normal meiosis" and one of the cells in shown in Figure 6.4. When the inversion was found in the mitotic karyotype the meiotic preparation was re-examined. No further analysable diakenesis cells could be found. The pachytene configurations were usually insufficiently spread for analysis and there was no evidence of failure to pair between segments of pachytene chromosomes or of the presence of inversion loops. Unfortunately C-banding failed to give a result, probably because the slides had aged too much to give a good C-band result.

The conclusion drawn from this case was that bivalent configuration showed no detectable abnormality. The patient inherited the variant chromosome from his father, therefore it was highly probable that the variant chromosome was present in the gonadal cells. The supposition was that pairing and segregation were not affected by the presence of the inversion and therefore could not be identified in diakenesis figures. De La Chapelle et al (1974) reported on pericentric inversions in chromosomes 9 and 10 in a number of families. They concluded that both abnormalities represent normally segregating common variants in the general population with a frequency which may be as high as 1%. The pericentric inversions in these cases, like T121, were all small inversions. In the case described by Dutrillaux (1973), there was

Figure 6.4

Diakenesis figure and karyotype from Case 121



a very large pericentric inversion which, in this family, gave rise to a child with an unbalanced karyotype of clinical significance.

C. Abnormal meiosis

Four cases were found with "abnormal meiosis".

CASE 9 and 29 MI,22,XY,III(13,13q14q,14)

Both cases have the same abnormal meiotic configuration envolving the production of a trivalent configuration due to a translocation between chromosome 13 and 14. The mitotic karyotype of both individuals demonstrated the same translocation, which has been described in Section 2 of this Chapter.

The details of analysis of diakenesis cells are as follows :-

	Case 9	Case 29
23,X,Y,III	0	2
22,XY,III	7	7
21,XY,III	2	1
20,XY,III	1	0
24,XY,III + I + I	-	1
polypoid cell		1
Total analysed	10	12

The trivalent was observed in all diakenesis cells studied, and an example from each case is shown in Figure 6.5. In these cases the configuration of the trivalent depends on the number of chiasmata present (2, 3 or 4 chiasmata) and whether the chiasmata are terminalised or not (Hultén and Lindsten, 1970). Examples of possible trivalent

configurations are shown in Figure 6.6 together with photographs of configurations observed and the number of each type scored for both individuals.

In Case 9 some cells were observed with complete breakdown of chiasmata. These cells showed the presence of many unpaired univalent structures and some multivalent ring-like structures, such a cell is shown in Figure 6.7. A total of 4 of these nuclei was observed whilst scanning for the 10 recorded diakenesis cells.

Figure 6.8 shows the histology picture in these individuals. Sperm heads can be seen in both samples and the histology categories record reduced spermatogenesis.

There are rather few reports in the literature of meiotic studies on D/D translocation heterozygotes (Kjessler, 1966; Hultén and Lindsten, 1970; Chandley et al, 1972, 1976; Hendry et al, 1976). The structural abnormalities reported by all these authors were similar to the abnormal configurations reported in T9 and T29. The appearance of the histology in the two present cases was not markedly dissimilar from the cases reported by Hultén and Lindsten, 1970 and Chandley et al, 1972, 1976. In both Chandley's cases she reported a slightly lower mean chiasma count compared with control chiasma count values. Chiasma counts were not obtained on T9 and T29, but T9 had a number of cells in which there was apparently a complete breakdown of chiasmata.

The presence of translocations of the D/D type in familes does not necessarily impair fertility. Hamerton (1968) suggested that preferential orientation of the trivalent at meiosis could account for a low frequency of abnormal gametes being formed. Kjessler (1966) suggested that abnormal gametes may be selectively eliminated during spermiogenesis. Whatever the mechanism, the majority of families carrying a D/D translocation have not been diagnosed through infertility

investigations. Of the families which have been diagnosed through infertility clinics, some, but not all heterozygotes, suffer from increased abortion rates (Chandley et al, 1972). Family studies on T9 and T29 were not pursued therefore other heterozygotes were not identified, however, neither patient recalled any previous family history of infertility.

Figure 6.5

Primary spermatocytes from a) T29 and b) T9

a)

b)

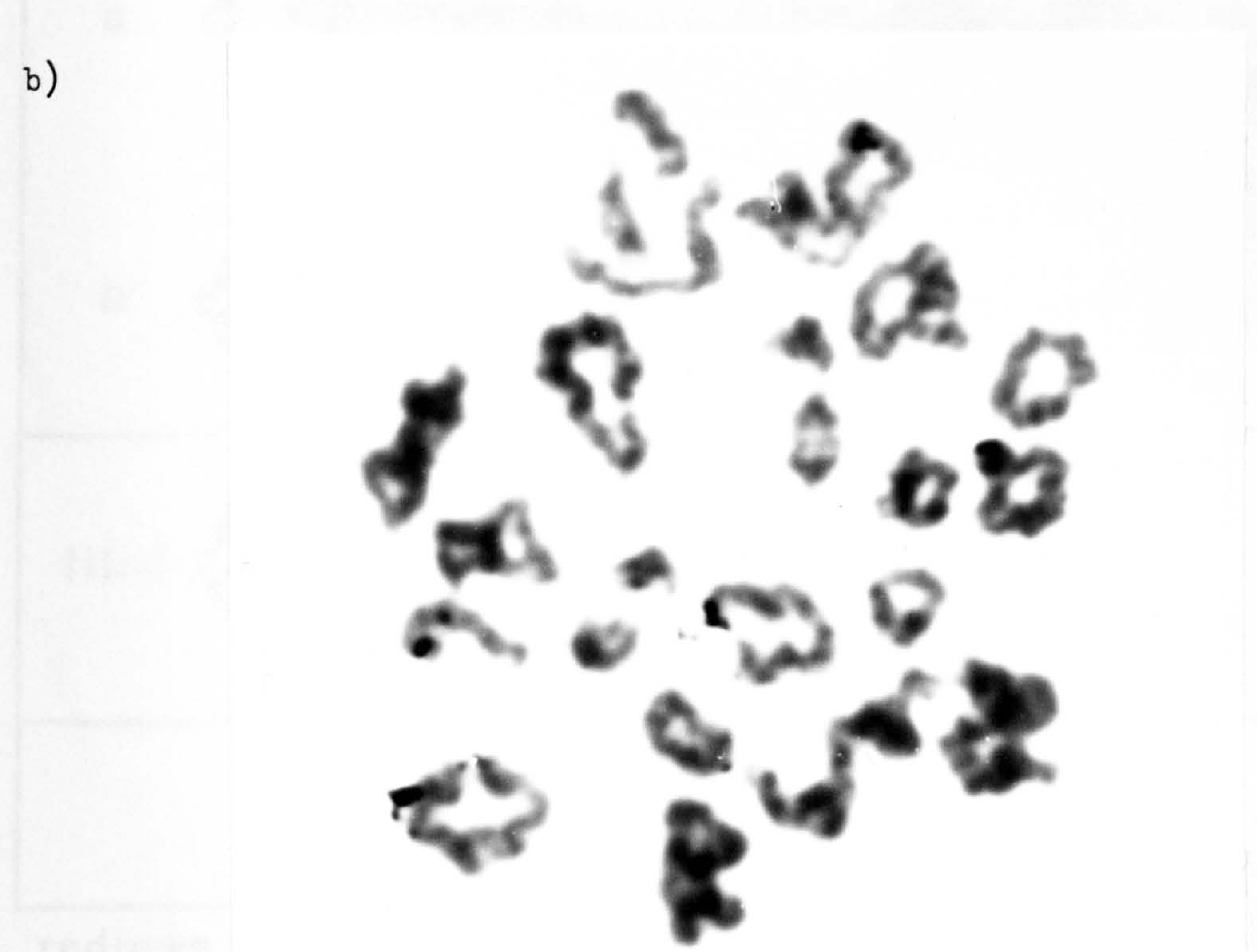


Figure 6.6

Diagram of possible trivalent configurations resulting from the presence of 2, 3 or 4 chiasmata. Examples of observed configurations are shown, together with the number of such configurations recorded in T9 and T29.

chiasmata configurations	example	T9	T29
III:2			
a		0	2
b		5	5
c		1	1
III:3			
a		2	3
b	not observed	0	0
III:4	not observed	0	0
	not recorded	2	1
	TOTAL	10	12

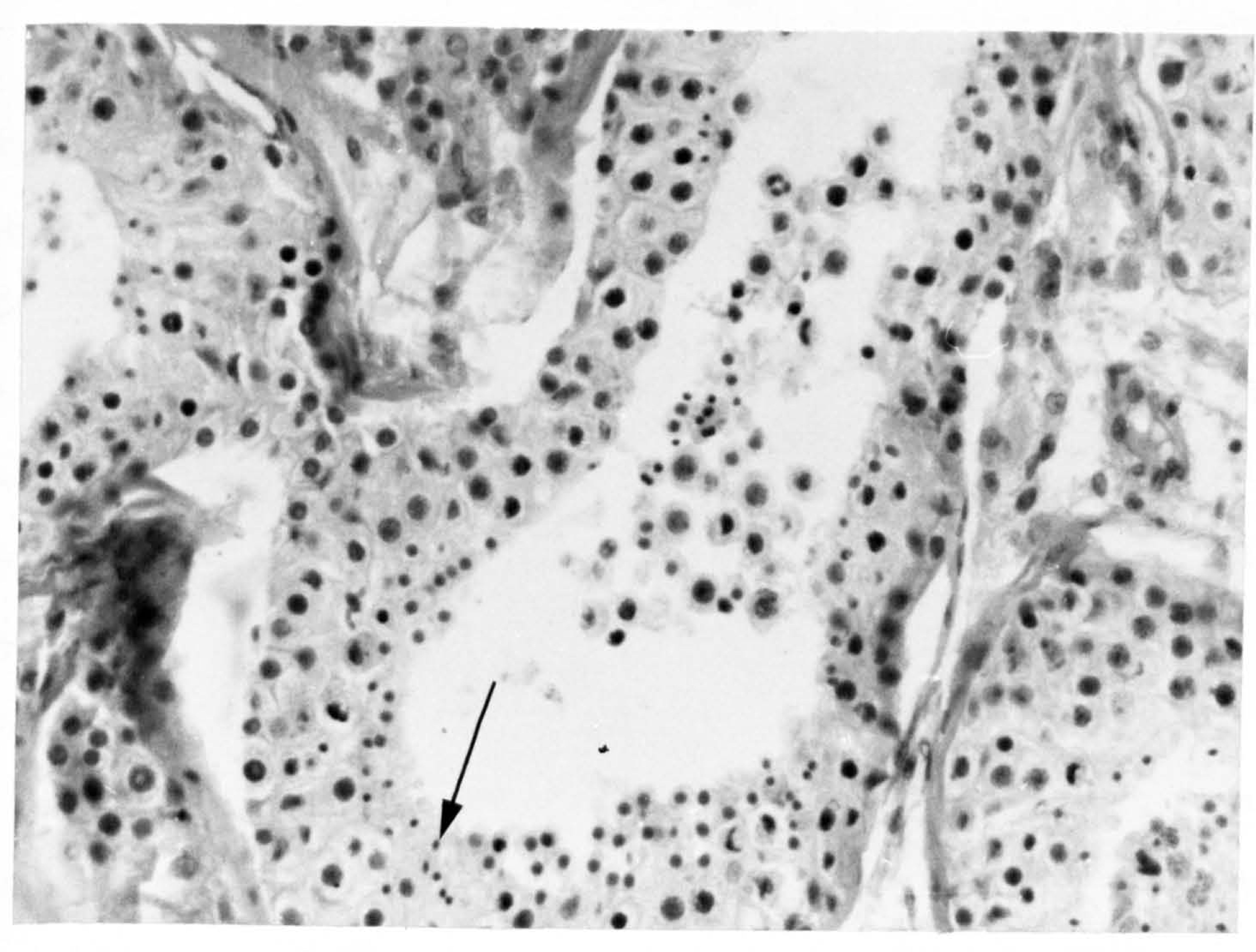
redrawn from Hulten 1974

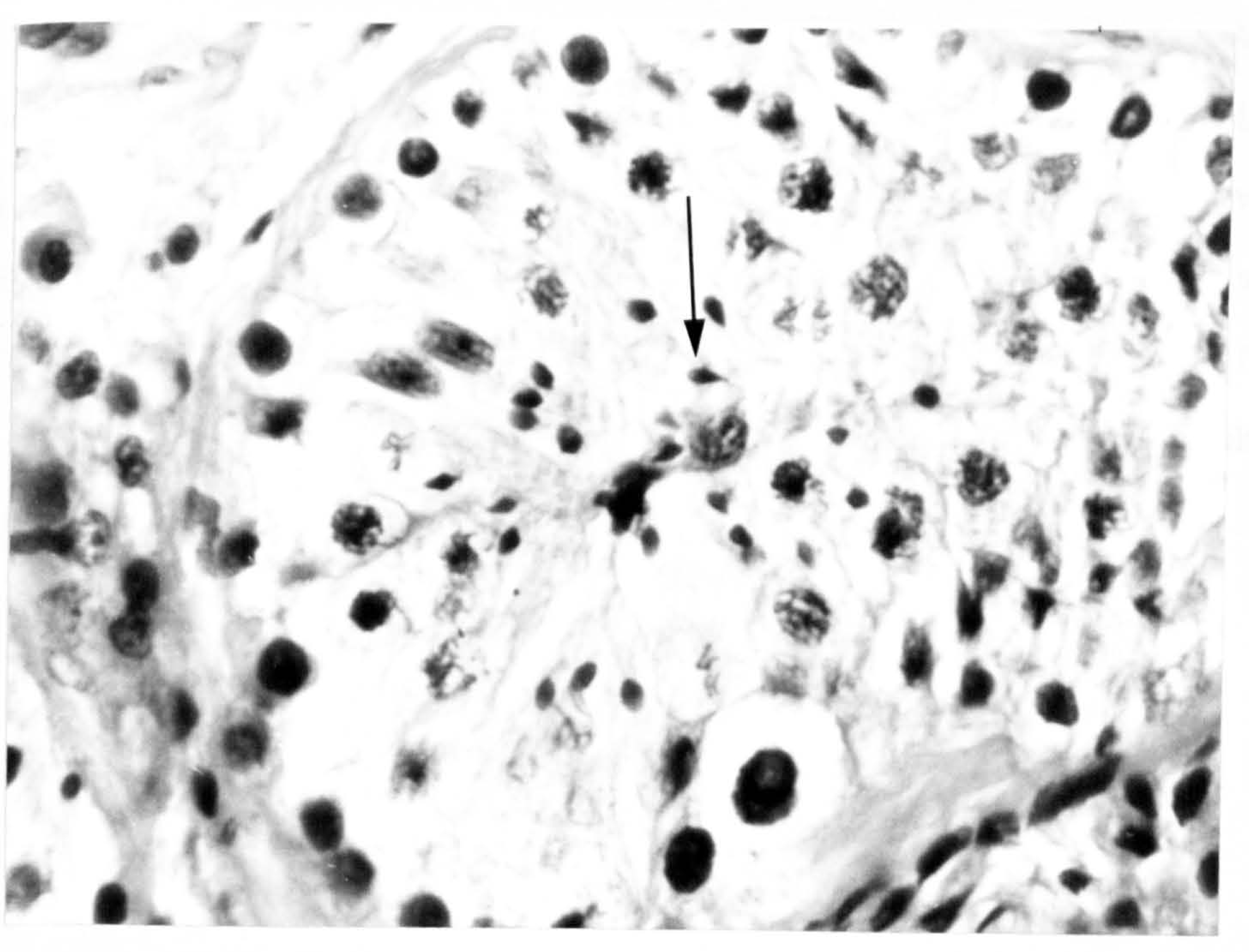
Figure 6.7

Achiasmate cell from T9

Figure 6.8

Histology from T9 and T29
(Arrows mark the presence of sperm heads)





<u>CASE 34</u> MI,23,XY,der(13),t(Y:13)

The mitotic karyotype of Case 34 has been described in Section 2 of this Chapter.

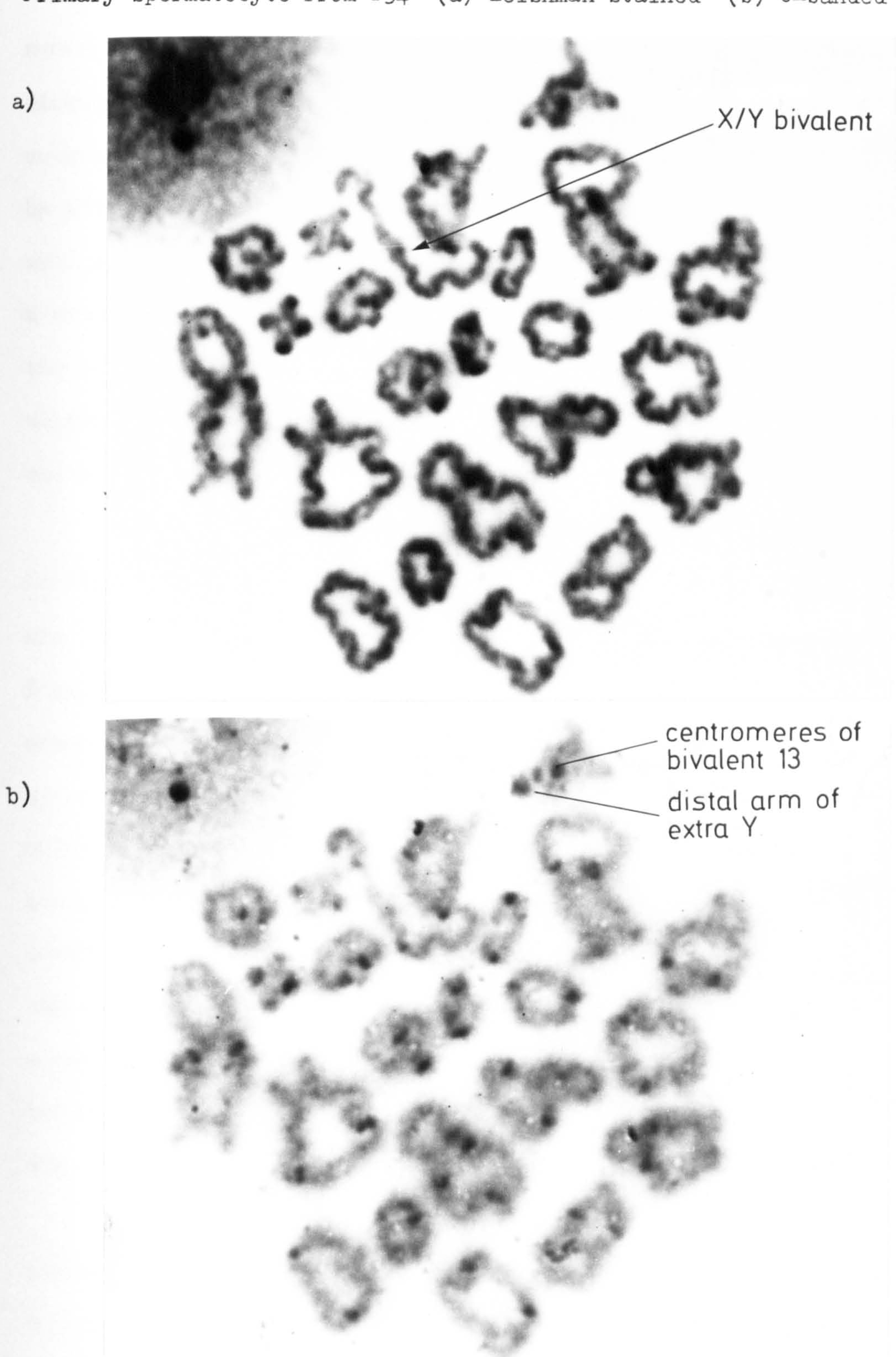
24 diakenesis cells were analysed with the following result, MI,23,XY, = 18; MI,24X,Y = 4; MI,21,XY = 2.

Two slides were treated with hot $Ba(OH)_2$ in order to study the C-banding pattern. One slide C-banded well and 7 cells were relocated and either rephotographed or the C-bands were marked on the original unbanded photographs with ink. In 2 cells the extra Y could be positively identifed, in 3 cells it could not be identified and in 2 cells the results were equivocal. One cell which clearly shows the presence of the translocated broken Y chromosome as a darkly staining spot on a D group bivalent is shown before and after C-banding in Figure 6.9.

In all the cells studied the XY bivalent was not seen to be in association with any other bivalent. This observation supported the view (Nielsen and Rasmussen, 1976) that the presence of an autosomal/Y translocation does not affect the segregation rate, carry an increased risk of abortion or stillbirths, or increase the risk of non-disjunction in the progeny of carriers. Chandley et al (1975) reported evidence from population studies which suggested that the frequency of sub-fertile males carrying extra Y chromosome material does not differ significantly from the frequency of individuals with extra Y chromosome material in the general population.

Figure 6.9

Primary spermatocyte from T34 (a) Leishman stained (b) C–banded



<u>CASE T11</u> Failure to maintain chiasmata

T11 had a normal 46,XY karyotype with no evidence for mosaicism.

The meiotic preparations showed an abundance of figures and numerous spermatogonial mitoses. It was noticeable that very few diakenesis figures could be analysed since many of the figures appeared unspread and individual bivalents appeared to be attached to each other by threads of chromatin material. Figures which were analysable were well spread and sharp in detail. Other figures were found which showed a breakdown of bivalent formation affecting from a few to nearly all of the bivalents. A count of 82 cells in diakenesis was made from four slides. Out of 82 cells, four cells (5%) were analysable and five cells (6%) were recognisable as achiasmate cells.

Figure 6.10 shows a normal MI,23,XY cell from this preparation. In Figure 6.11, there is a count of 24 possible structures, two univalents are indicated by arrows and the X and Y chromosomes are associated. Figure 6.12 shows structures with single centromeric chiasmata and several univalents. Figure 6.13 represents the type of cell recorded as achiasmate where there appears to have been complete breakdown of chiasmata. Large ring structures are seen which may represent large bivalents with precocious chiasmata terminalisation. Cross shaped structures with single central chiasmata and many univalents or fragments can also be seen. The cell is apparently polyploid and polyploidy was a noticeable feature of this preparation. This early case was not C-banded therefore no information regarding the position of the centromeres was recorded.

Four other authors have reported similar cases in the literature, Pearson et al (1970); Hultén et al (1970(2 cases); Dultrillaux and Guégen (1971); Hendry et al (1976). These cases show varying degrees of impairment from complete azoospermia with spermatogenic arrest at the primary spermatocyte level (Hultén et al, 1970) to the present

case with a JMS of 7.5 and histology categorised as "RG". Chandley et al (1976) reported two patients with a reduced autosomal chiasma count but did not consider that her cases showed as pronounced a defect as those reported in the literature. In this respect T11 had a proportion of cells which were not affected by chiasma failure.

Pearson et al (1970) provided experimental evidence that suggested that the failure was due to a reduced facility for repairing DNA breakage. More studies of the type described by Pearson are needed on individuals similar to T11 before the defect can be thoroughly understood.

Figure 6.10

Primary spermatocyte from T11; MI,23,XY,NAD

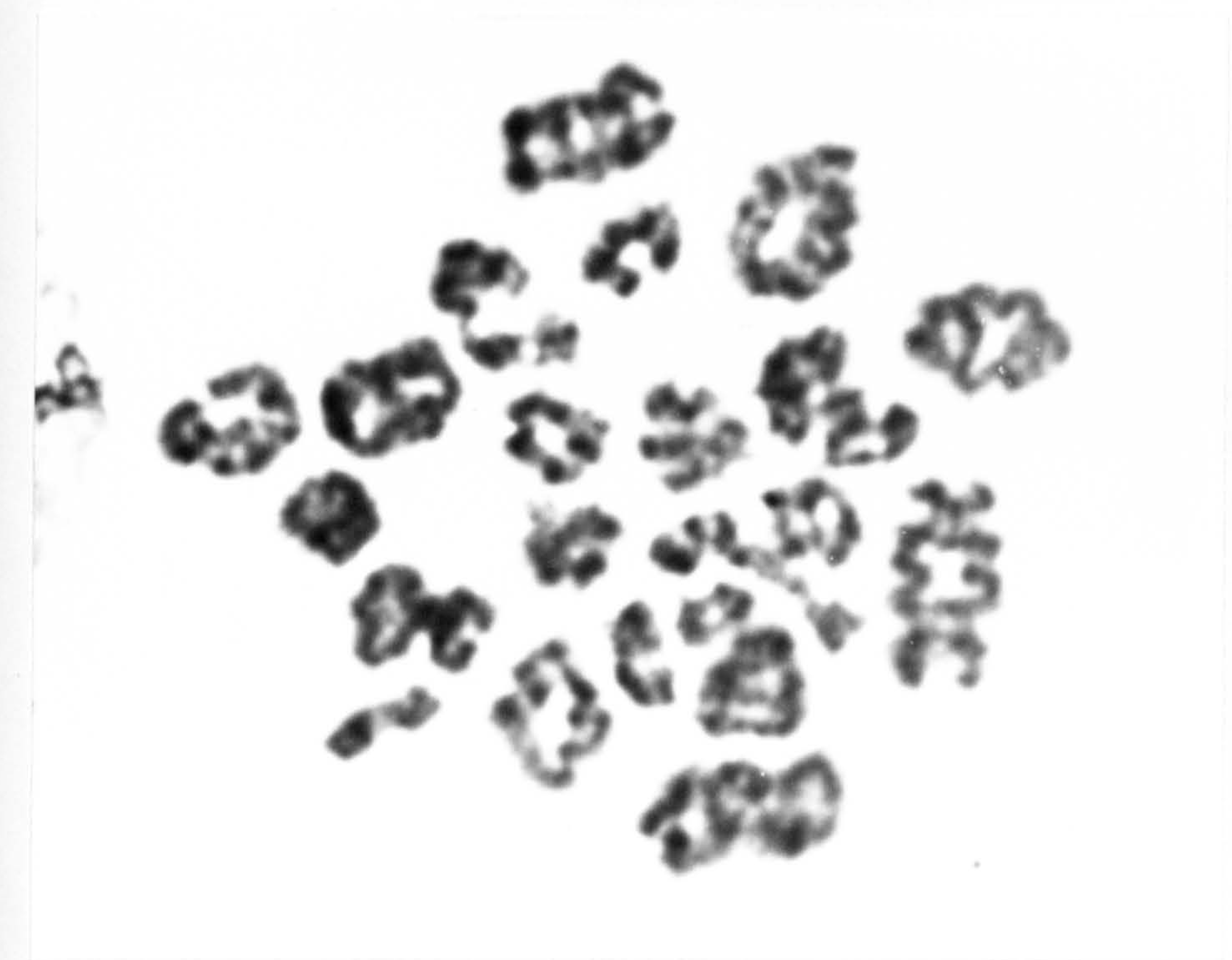


Figure 6.11

Primary spermatocyte MI,24,XY,I+I. Univalents indicated by arrows

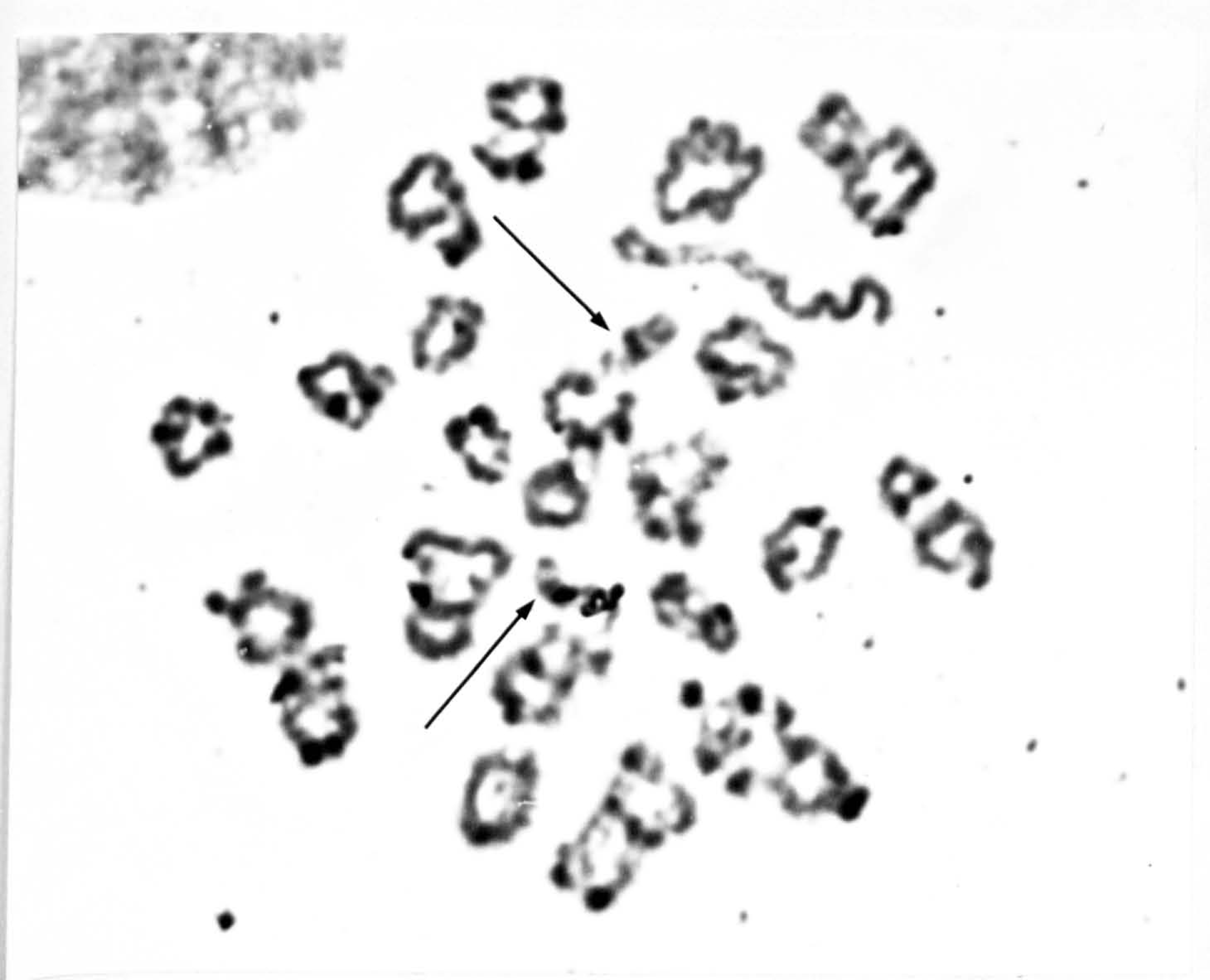


Figure 6.12

Primary spermatocyte from T11. A single centromeric chiasmata is indicated by a thick arrow. Univalents are indicated by thin arrows.

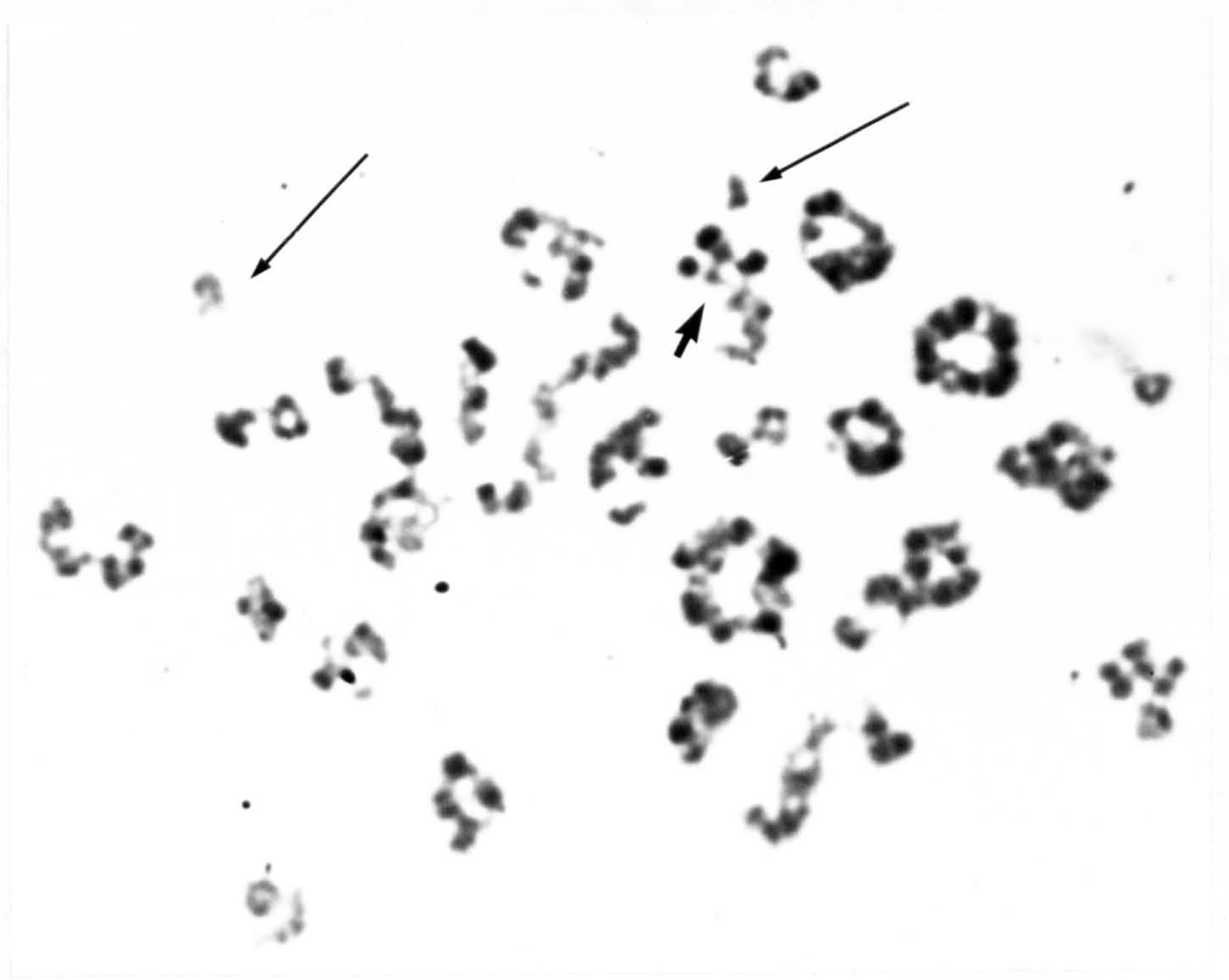
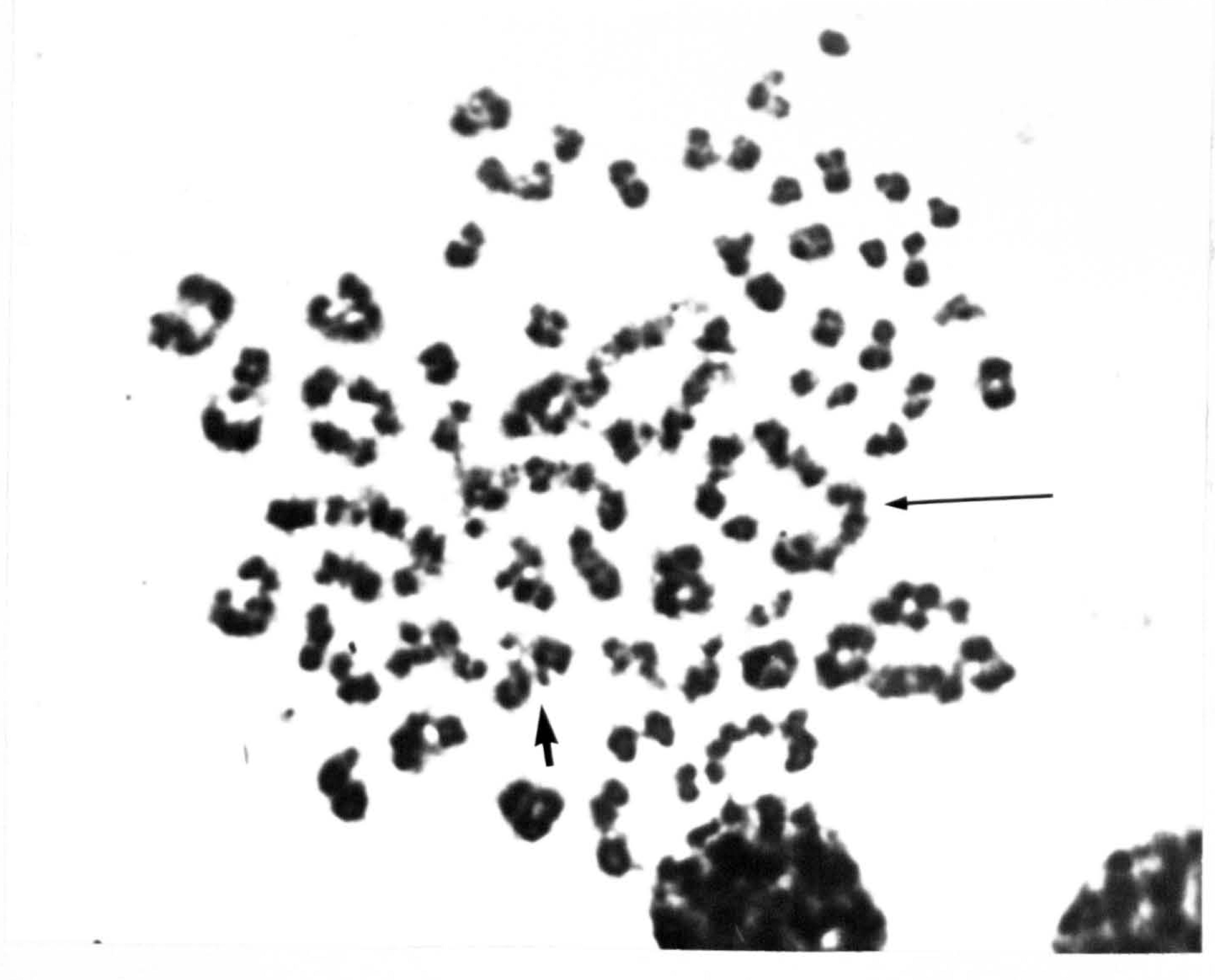


Figure 6.13

Achiasmate cell from T11. A large ring structure is indicated by a thin arrow. A structure with a single central chiasmata is indicated by a thick arrow.



D. "? Abnormal meiosis"

3 individuals were recorded with "? abnormal meiosis".

CASE 22

T22 had a normal 46XY karyotype with no evidence for mosaicism. Meiotic figures were relatively plentiful and the initial analysis gave an equivocal count of 22XY or 23XY as follows :-

23,XY	=	3	NAD
24,X,Y	=	1	
22,XY	=	4	loss of 1 bivalent
23,X,Y	=	2	

other abnormal or unanalysable figures = 6

The material was C-banded and the results are recorded in detail in Table 6.4. Figure 6.14 shows 1 normal figure (1/3) and a karyotype of a normal cell (4/11) with normal C-bands present. Figure 6.15 shows a cell (2/8) with a karyotype of MI,22,XY but with 46 C-bands present. An arrow indicates a possible quadrivalent structure consisting of 1 small ring with 3 C-bands and 1 larger ring with 1 C-band. In this karyotype an E bivalent was missing, indicating a possible translocation between an E and A group chromosome. Cell 1/1 (Figure 6.16) had a similar karyotype of MI,22,XY with a similar abnormal configuration to Cell 2/8. But C-banding failed and therefore confirmation of the possible translocation could not be obtained. Cell 1/5 (Figure 6.17) had a similar karyotype MI,22,XY but the abnormal configuration could not be identified. Figure 6.18 shows 2 cells (4/12 and 4/13) with abnormal C-bands. In these examples there is a single large C-band joining a double ring structure. The

TABLE 6.4

KARYOTYPE AND RESULTS OF C-BAND ANALYSIS OF T22

Slide and Cell No.	Karyotype	C-bands	Comments
Slide 1 Cell 1	22,XY	Failed	
2	19,XY	Failed	1 large ring structure
3	23,XY	Failed	
4	22,XY	Failed	
5	22,XY	Failed	
6	24	Failed	X,Y not identified
7	23,XY	Failed	
2 8	22,XY	Good	23 centromeres, 1 ? quadrivalent
9	?22,XY	Not developed	
3 10	23,X,Y	Not clear	
4 11	23,XY	Good	NAD
12	23,X,Y	Good	2 double rings with 1 central C-band
13	24,X,Y	Good	1 double ring with 1 central C-band
5 14	?23,XY or 22,XY	Developed	not informative
6 15	22,X,Y	Not clear	1 double ring
16	?23,XY	Not relocated	

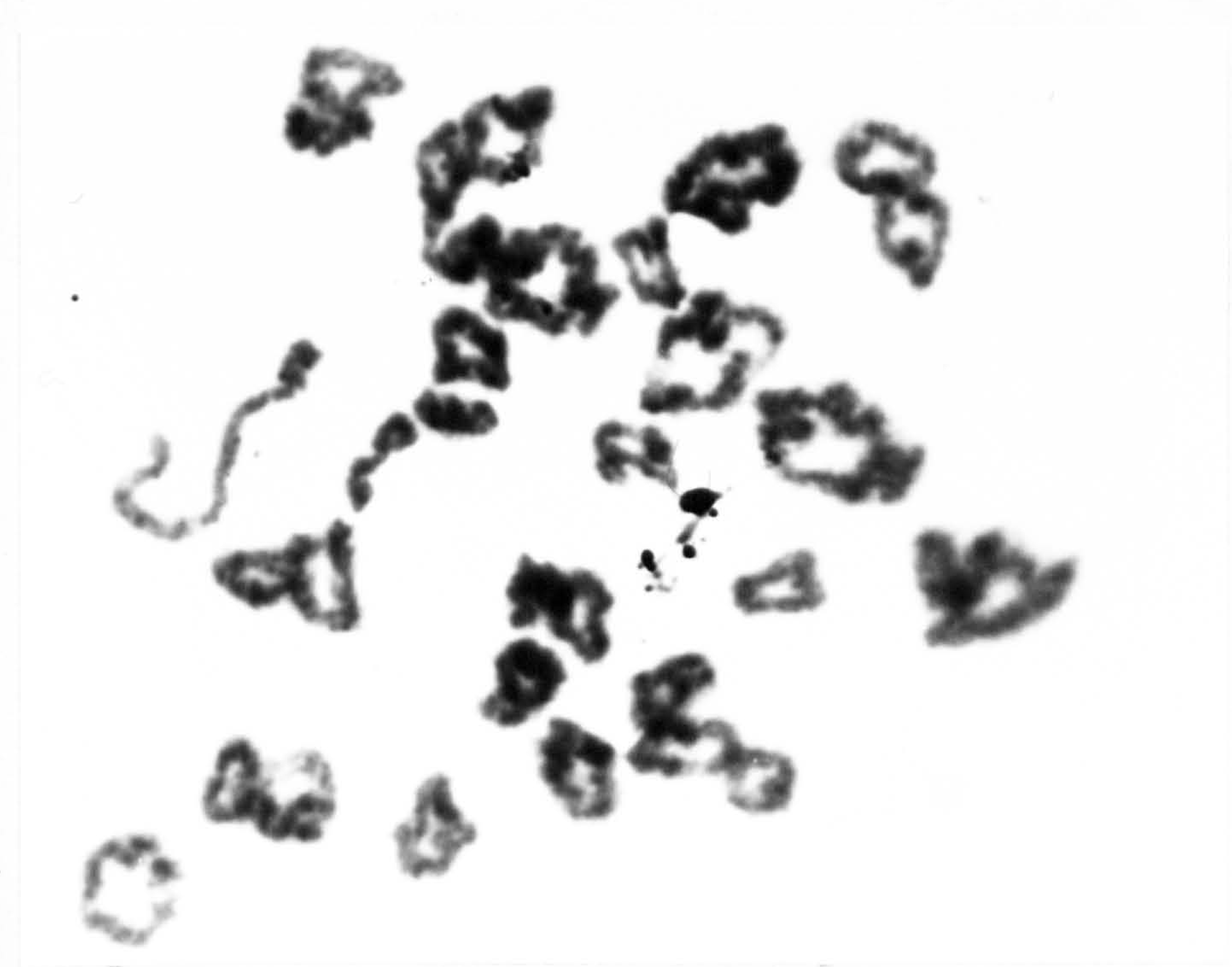
small size of the possible quadrivalents in these cells does not confirm the possibility that a translocation involving chromosomes of group A has occurred.

In this material the pachytene figures were particularly clear. Figure 6.19 shows 2 possible abnormal cross over points.

Case 22 was classified as "? abnormal". There certainly was a normal cell line present and some of the configurations were ambiguous. It was difficult to claim that this was a case of gonadal mosaicism when the abnormal figures differed between themselves. Neither was it possible to classify T22 as "normal meiosis".

Figure 6.14

a) Primary spermatocyte from T22 (1/3) MI,23,XY,NAD



b) Karyotype of 4/11 after C-banding; MI,23,XY,NAD

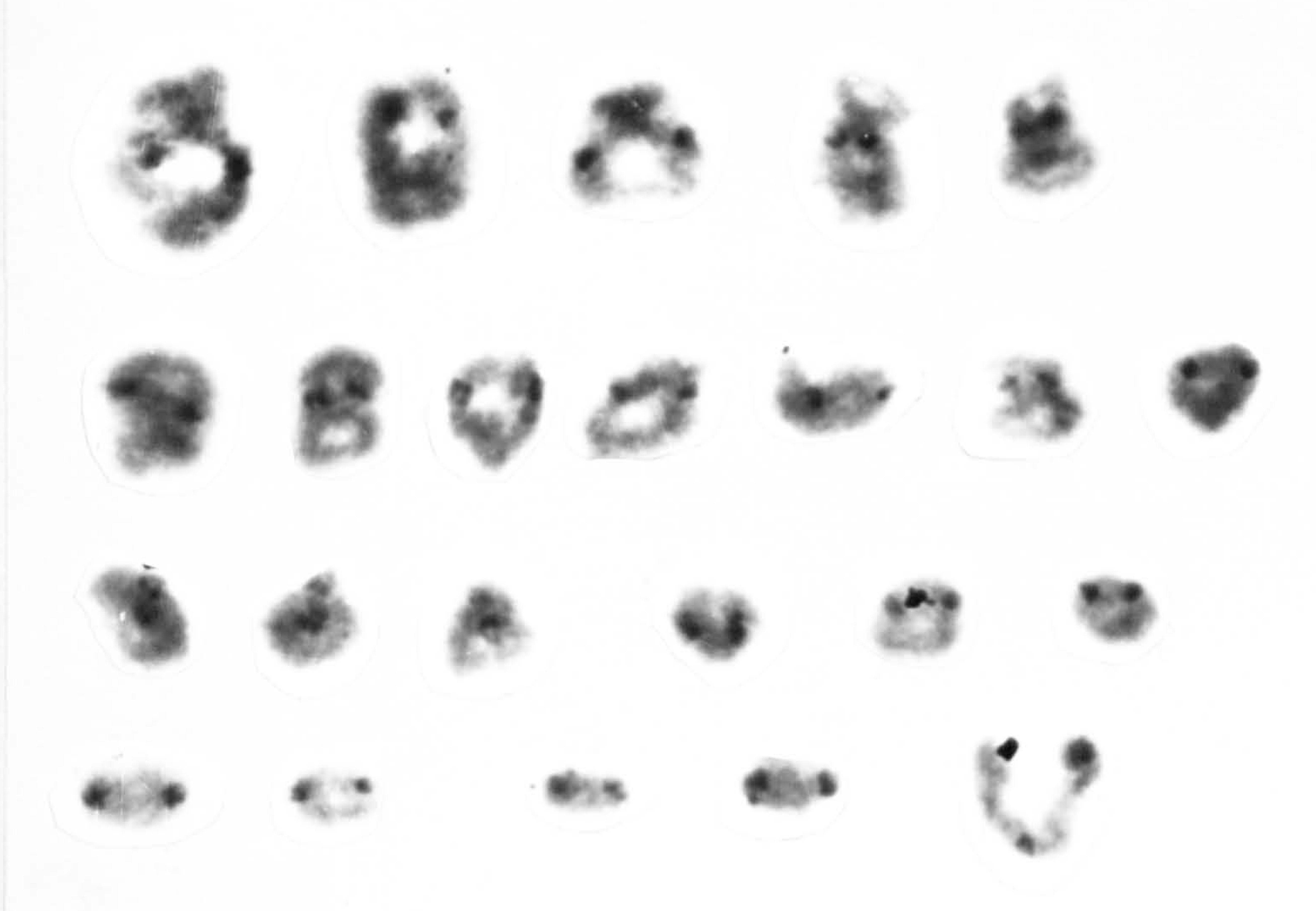


Figure 6.15

Diakenesis figure and karyotype of 2/8. C-bands are indicated on the photographs. An arrow indicates the possible quadrivalent structure.

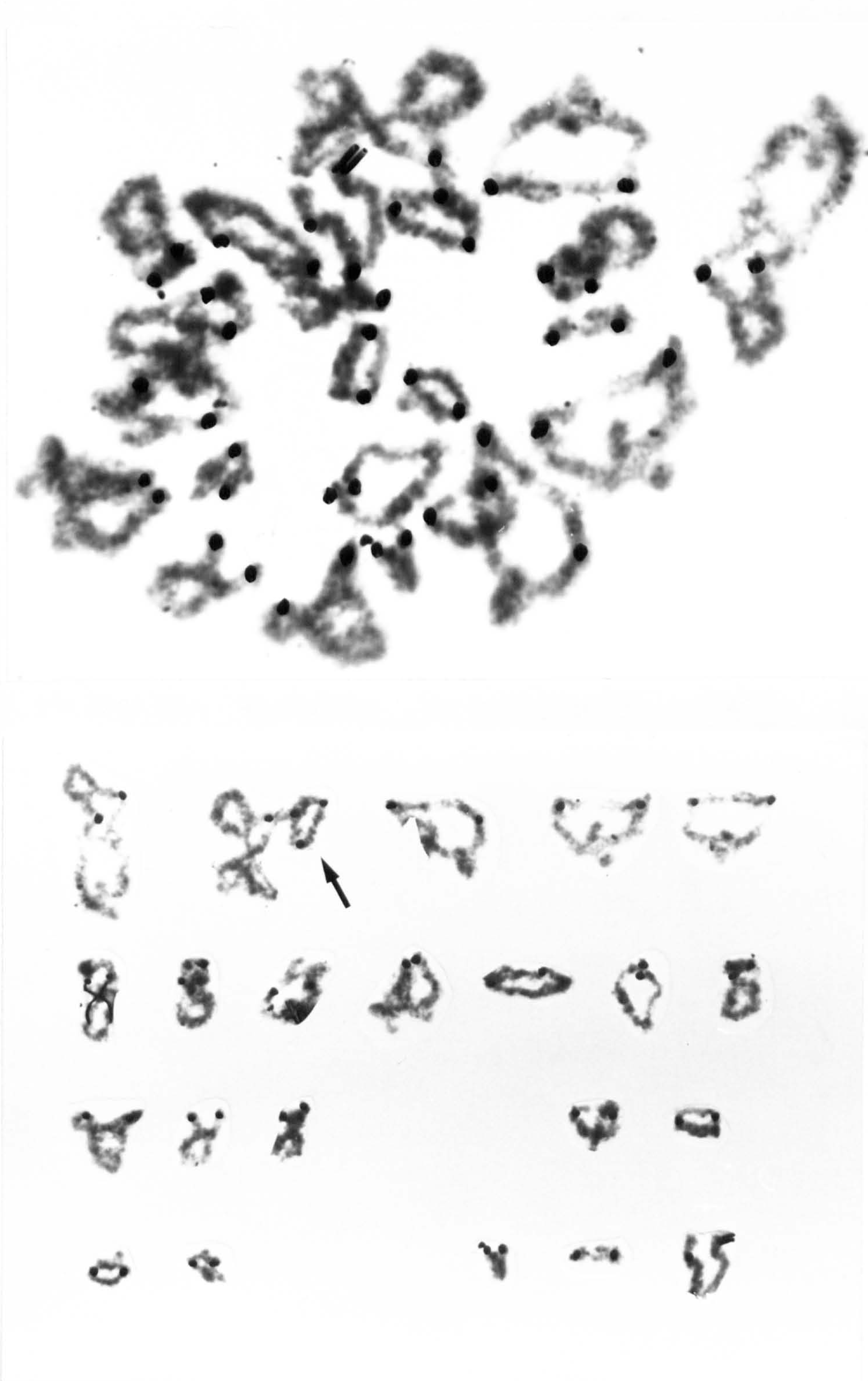


Figure 6.16

Cell 1/1 from T22; MI,22,XY,. The arrow indicates a possible quadrivalent

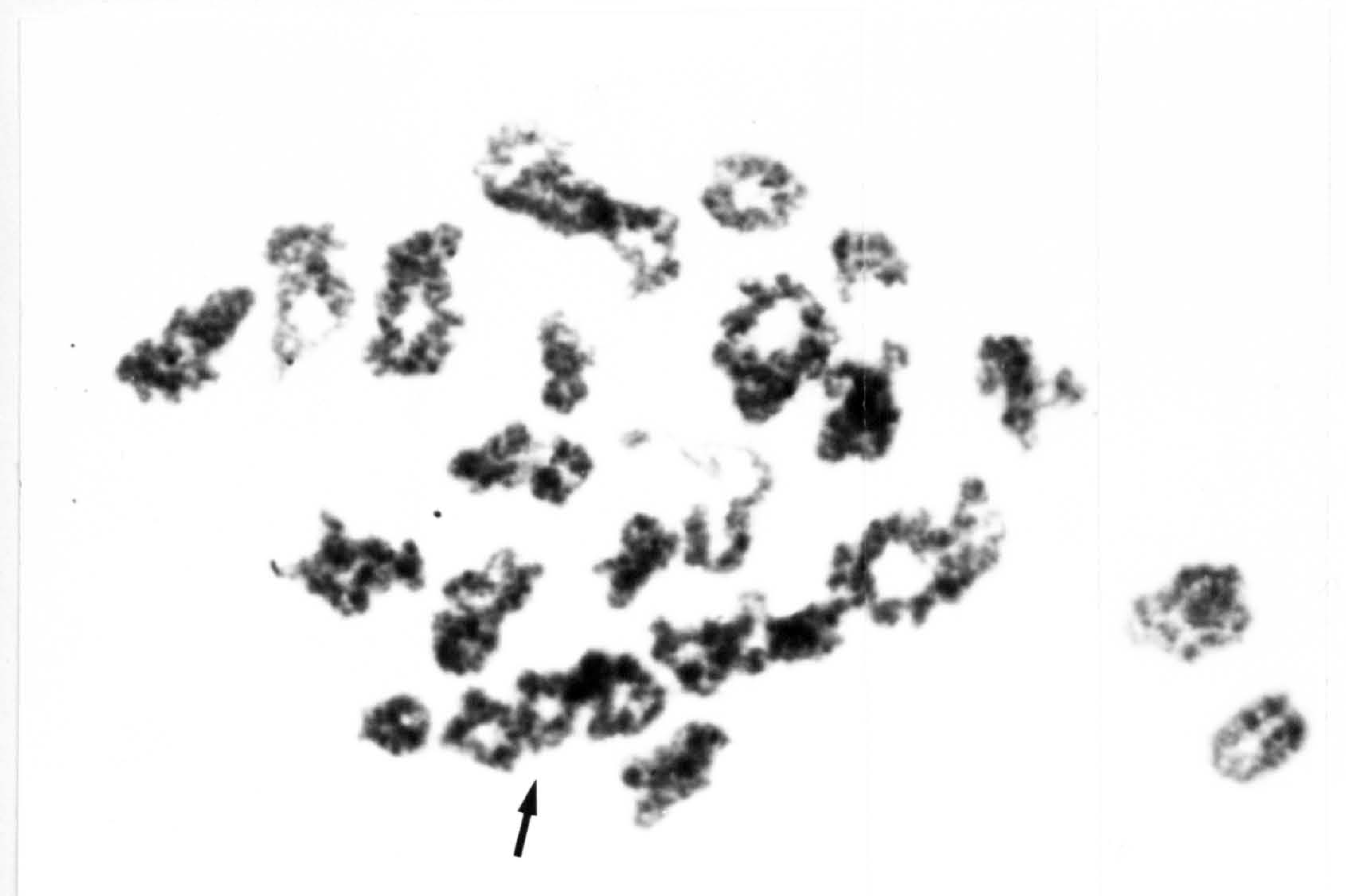


Figure 6.17

Cell 1/5 from T22; MI,22,XY,. The quadrivalent is absent

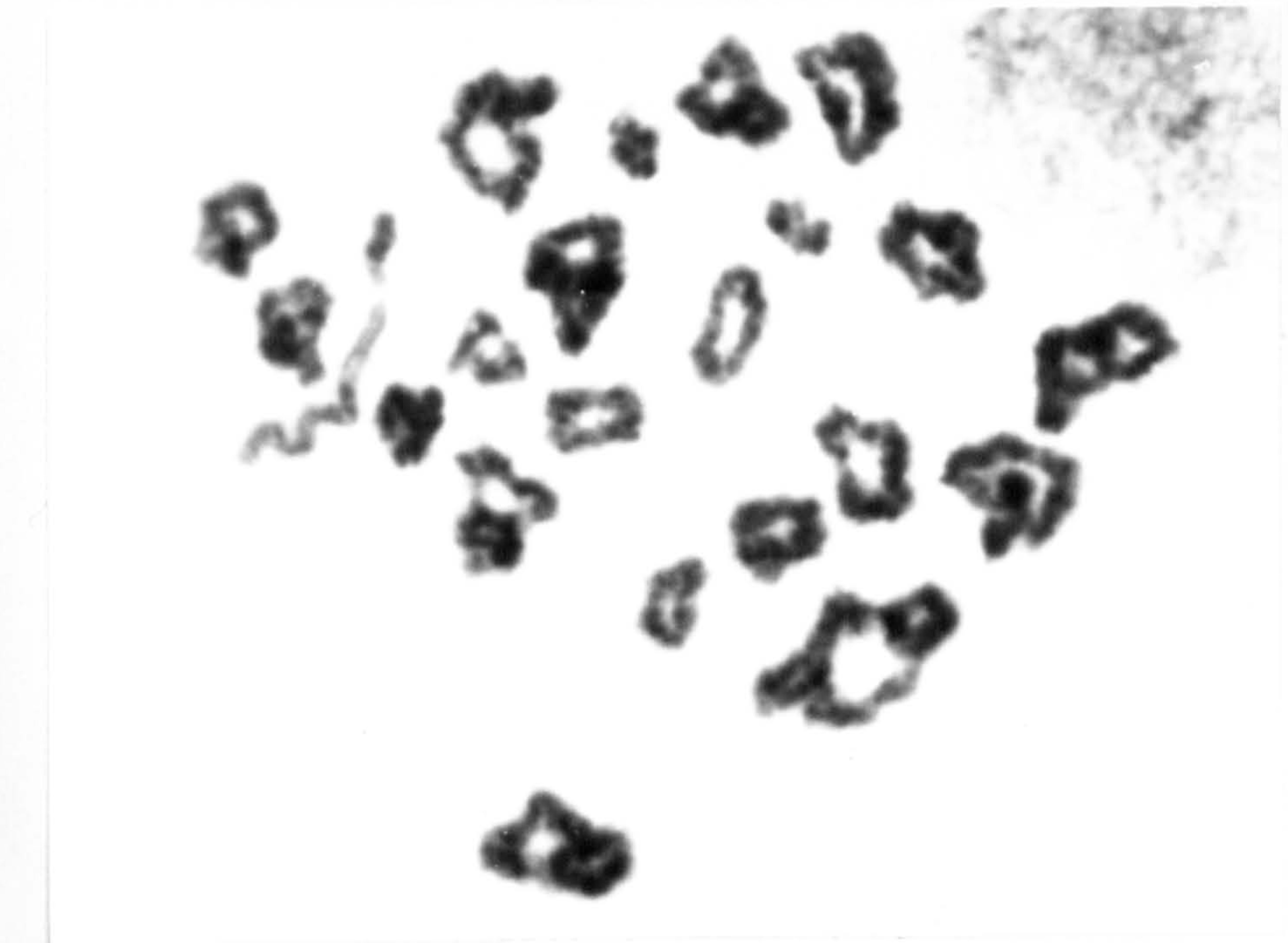
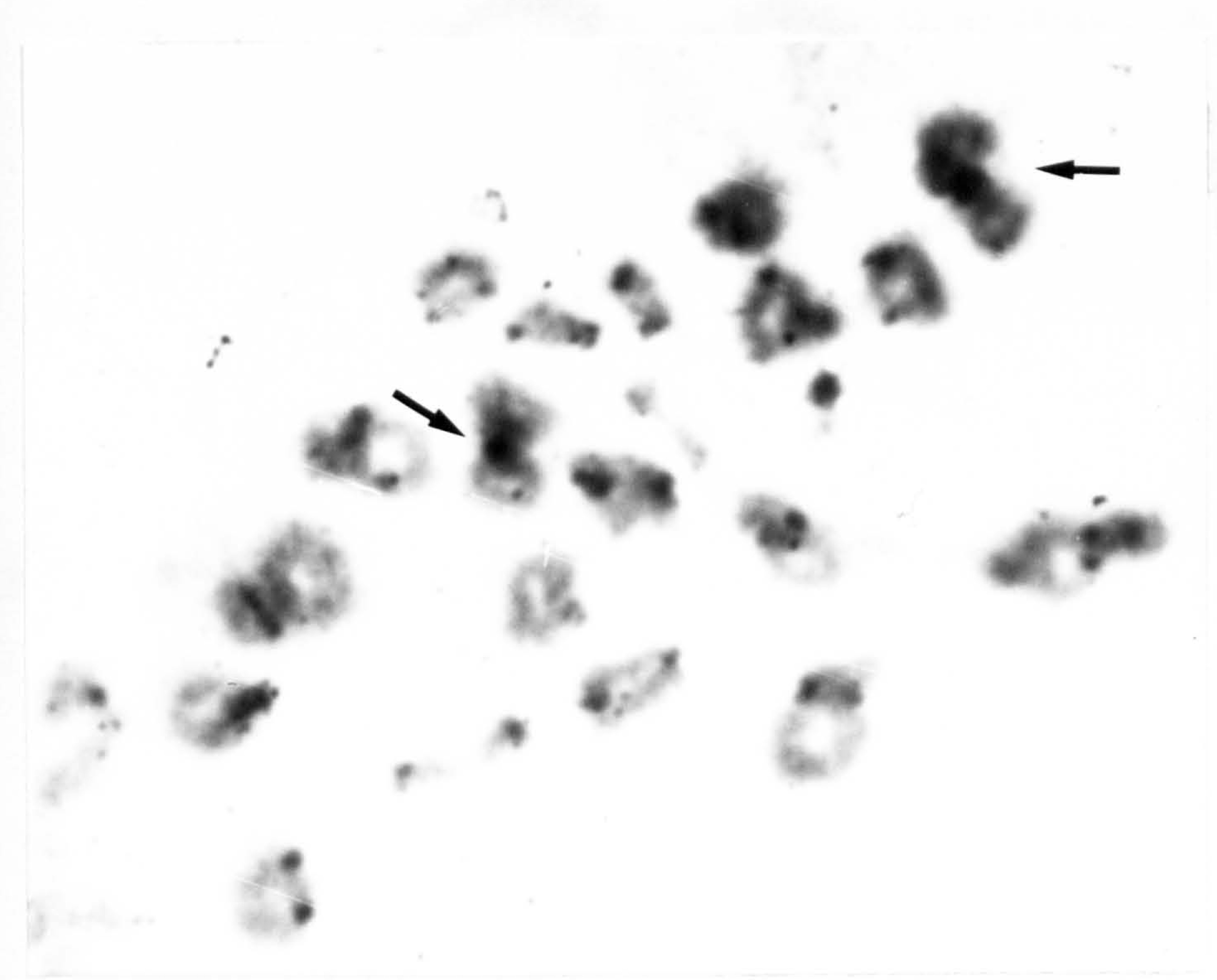


Figure 6.18

Two cells (4/12, 4/13) with abnormal C-bands. Arrows indicate bivalents with single large C-bands.



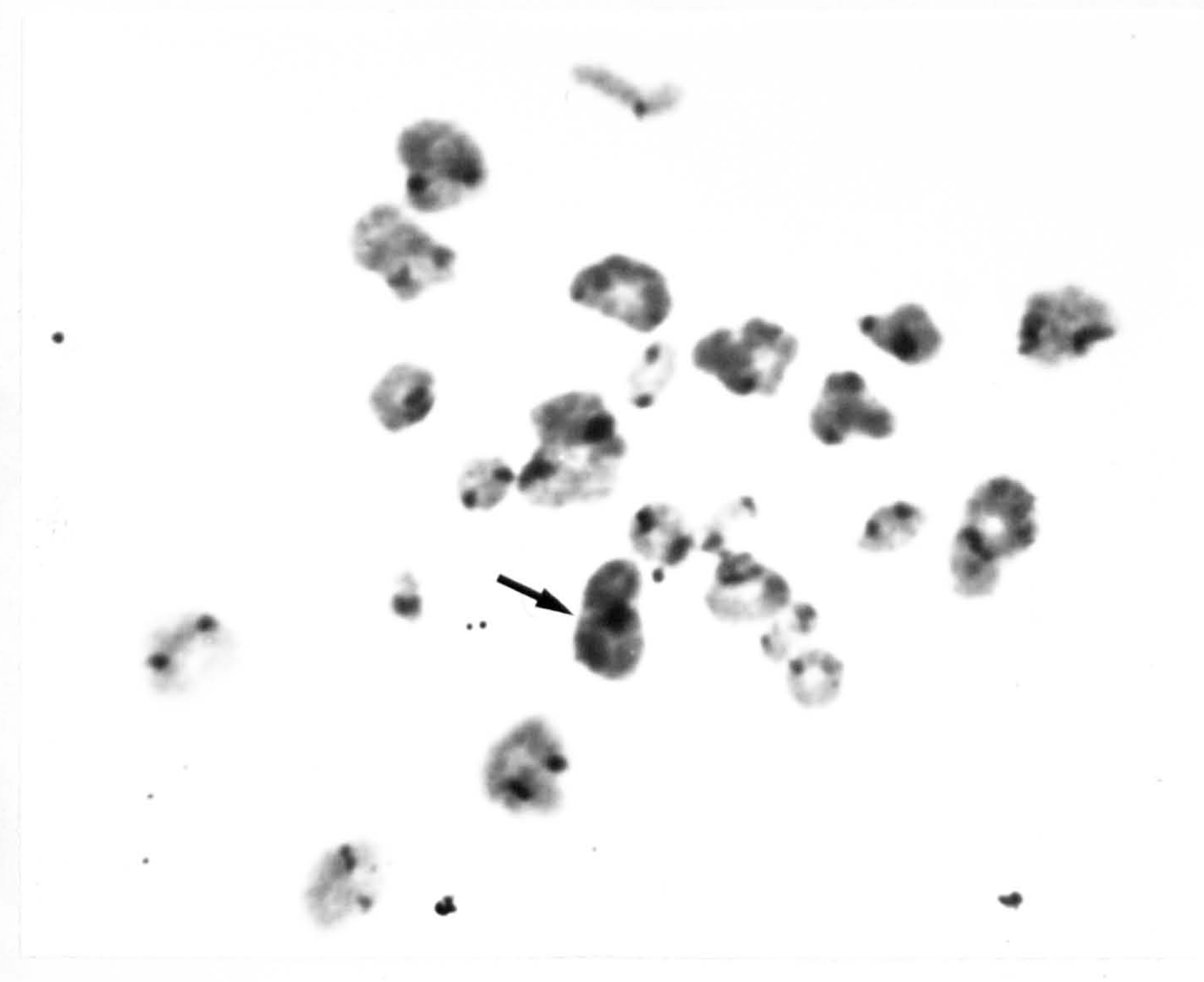
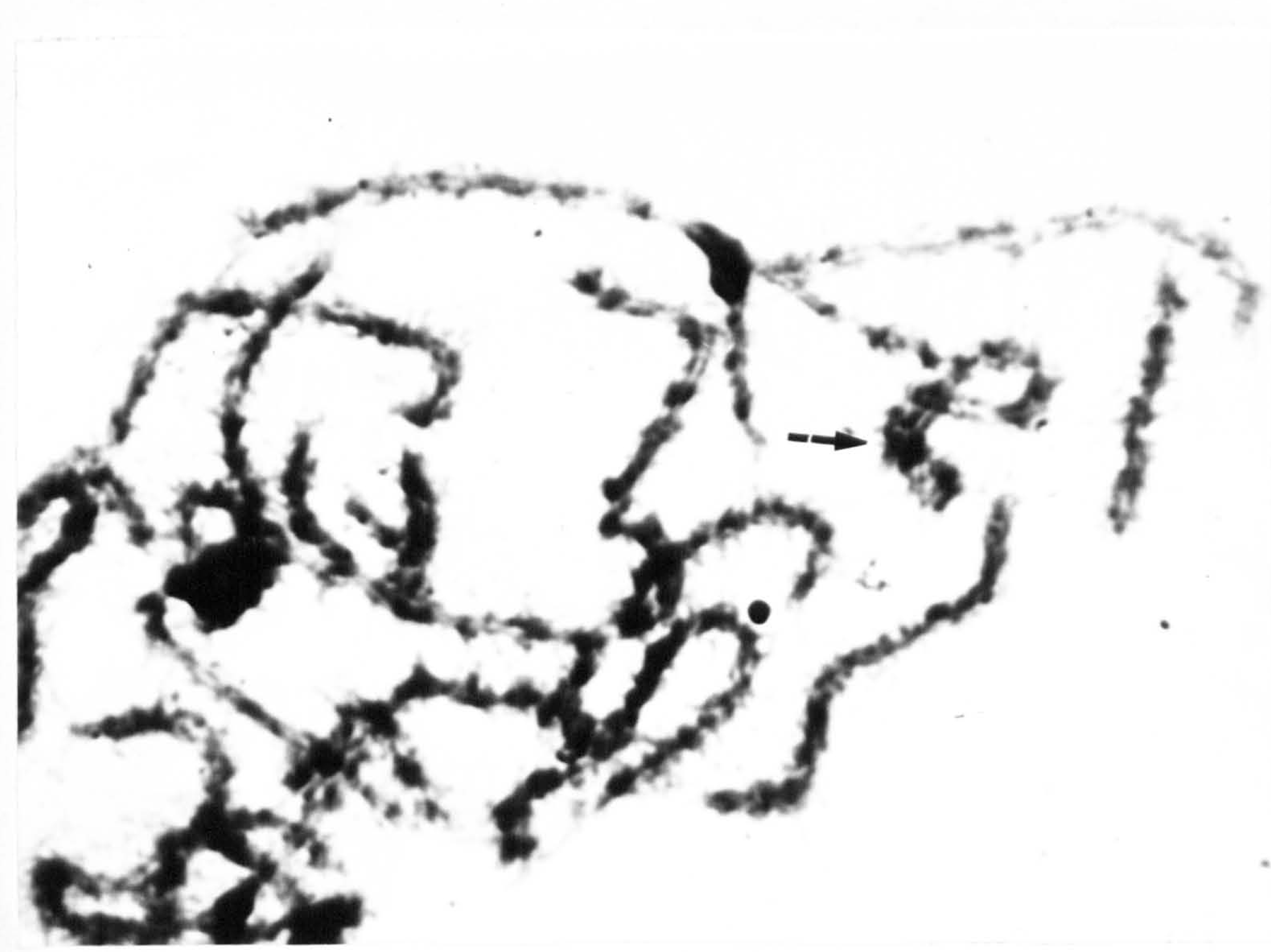


Figure 6.19

Pachytene figures from T22. Arrows indicate possible abnormal Configuration



CASE 35

Case 35 had a normal 46XY mitotic karyotype with no evidence for mosaicism. Meiotic cells were sparse; 4 figures in diakenesis were analysed and the presence of 2 chain structures in 3 out of 4 cells analysed was recorded. The material was C-banded in order to provide more information on the nature of the 2 chains. The C-bands have been marked on the photographs where they were observed.

The simplest explanation for the configurations observed is shown in Figure 6.20. In this karyotype the X and Y are separate and one D bivalent is located over a C bivalent. One bivalent has 2 C-bands but with the appearance of the D + G in association. Figure 6.21 is similar except that the X and Y are associated. Figure 6.22 has an unassociated Y chromosome, one D bivalent and 2 chain structures. One chain structure had 2 C-bands and could best be interpreted as a D bivalent. The other chain was very long and no C-bands could be located. The unassociated X was not identifiable but the chain could represent the X in association with the abnormal "D + G" configuration.

The interpretation of this karyotype was hampered by the limited material available for study. All karyotypes showed apparently normal G group bivalents. One possible explanation was that the abnormal configuration represented a deleted D chromosome with its normal homologue forming an abnormal bivalent. Alternatively the abnormal configuration has the appearance of a D + G translocation but in this case the normal D homologue cannot be identified in the karyotype.

Figure 6.20

Karyotype of T35; MI,24X,Y. C-bands are marked on the photograph. The abnormal D bivalent is indicated by an arrow.

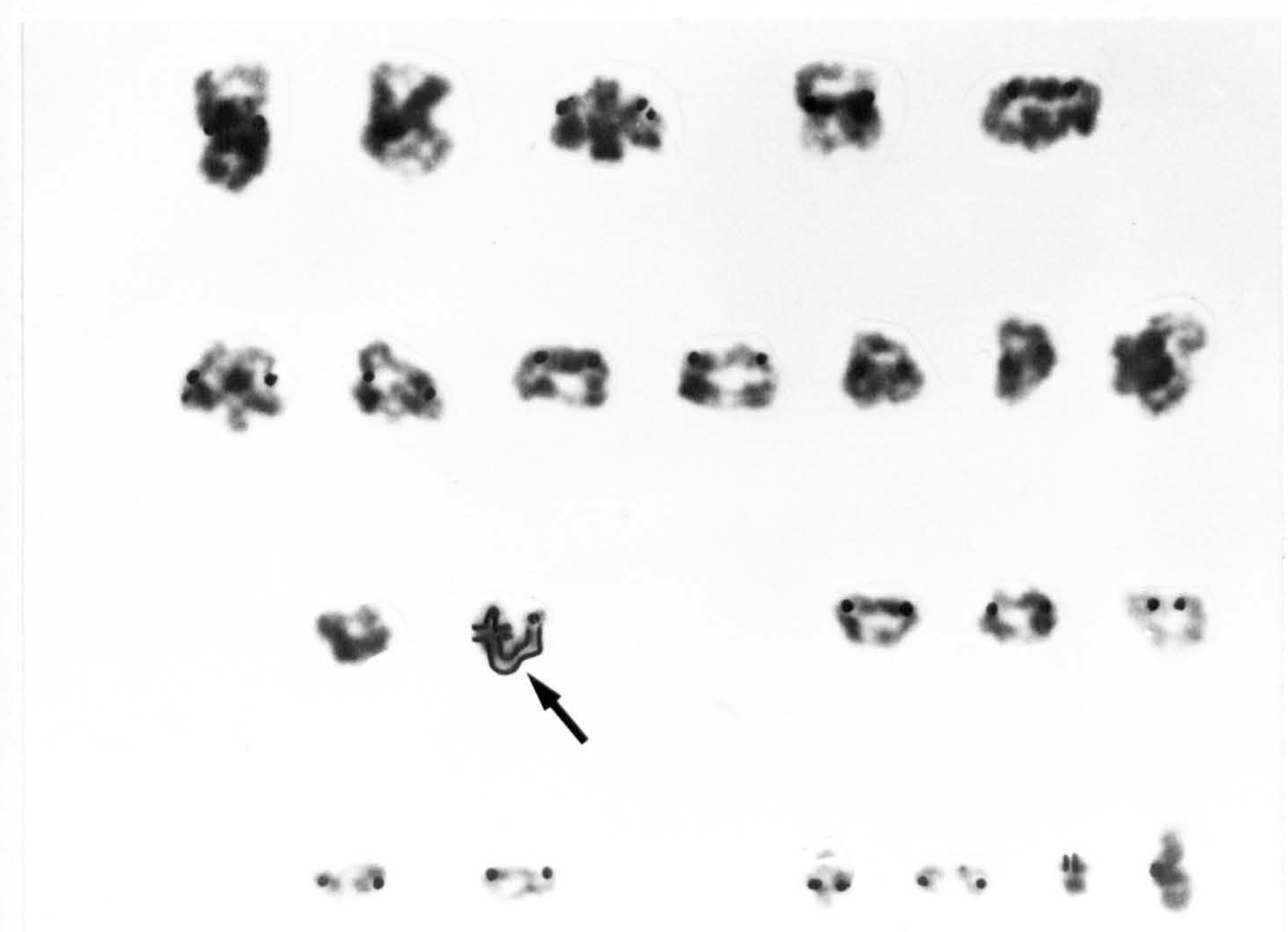


Figure 6.21

MI,23,XY. C-bands are marked on the photograph. The abnormal D bivalent is indicated by an arrow.

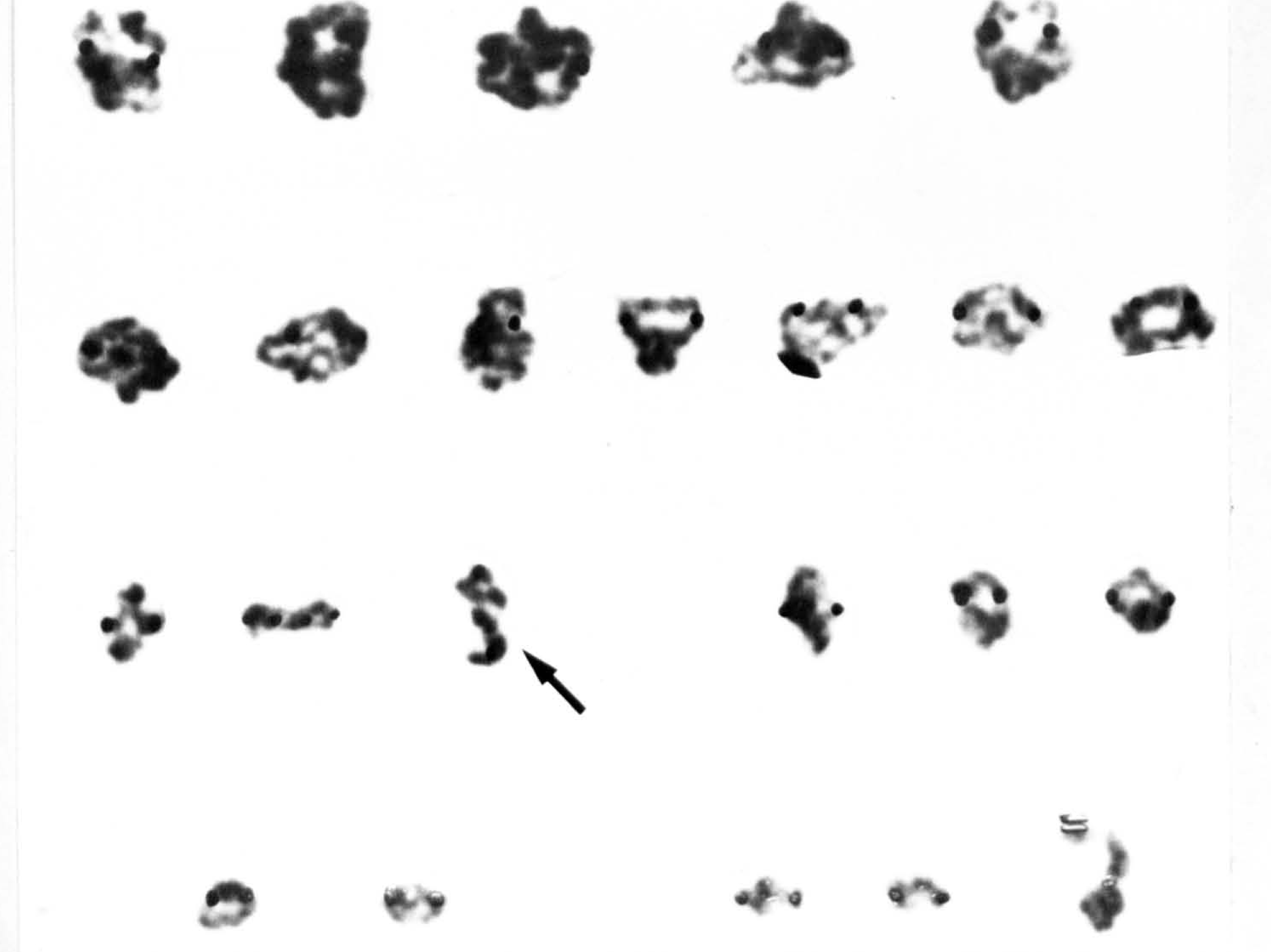
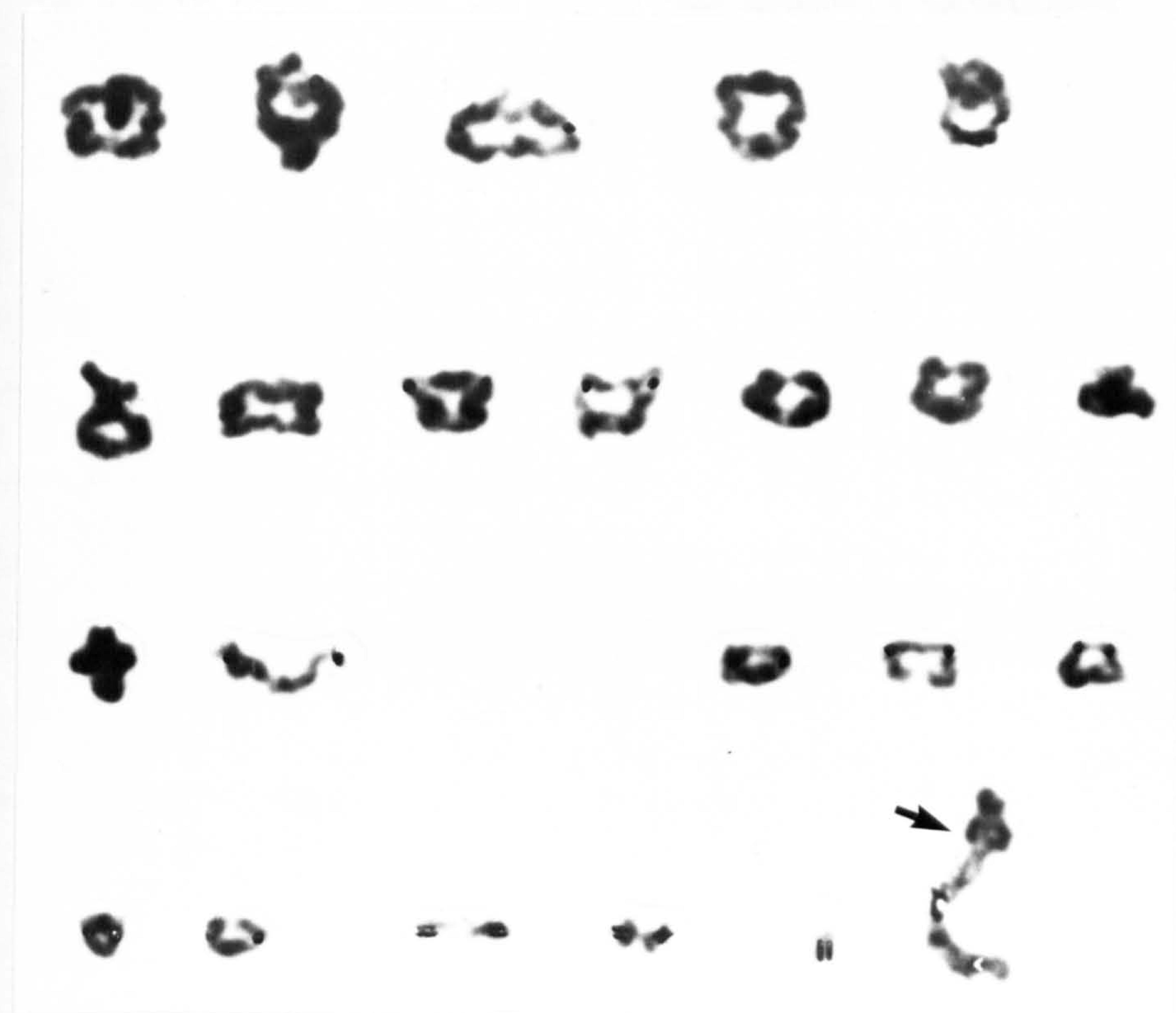


Figure 6.22

Karyotype of T35. Arrow markes chain association of X chromosome with abnormal D bivalent.



CASE 47

This case had a normal 46XY mitotic karyotype with no evidence for mosaicism or translocation. Long term fibroblast culture set up from a skin biopsy gave a normal 46XY karyotype.

Analysis of the meiotic chromosome configuration proved difficult to evaluate. Diakenesis figures were plentiful, analysis of 24 figures gave the following results (Table 6.5). The results show that 8 out of the 24 figures were apparently normal and one such cell is shown in Figure 6.23. 16 figures were abnormal and the abnormality was associated with the presence of univalents and difficulty in identifying the XY configuration. An abnormality of the XY chromosomes was thus suspected and an attempt was made to study the fluorescent banding pattern. Unfortunately no information could be gained using fluorescent techniques. Since fluorescence failed, the material was subjected to C-banding and some of the cells demonstrated C-band patterns. Figures 6.24a,b are 2 apparently normal cells after C-banding. Both cells show bivalents (marked by arrows) with more than 2 C-bands per bivalent. 6.24a has no Y C-band whereas 6.24b has a strong C-banded Y in association with the X chromosome. Both 6.24a and b show chain-like structures which could only be identified as bivalents after C-band staining. Figure 6.25 shows Cell No.8 (MI,22,-XY,I) before and after C-banding. In this cell the univalent has no C-band. One chain-like bivalent (arrowed) has 2 normal C-bands present. Figure 6.26 shows Cell No.6 (MI,22,-XY,+I+I). In this cell there are 2 univalents without C-bands (some bivalents are also without C-bands). In addition, there is a "panhandle" structure which has 3 C-bands. An XY configuration cannot be identified. In all the cells studied the C-banding analysis provided no evidence that the univalents could be extra Y chromosomes or that a translocation was consistently present.

TABLE 6.5

CASE 47

Cell No.	No. Autosomal Bivalents	XY	X+Y	Univalents	Total Structures
5	22	1			23
7	22	1			23
9	22	1			23
19	22	1			23
2	22		1+1		24
4	22		1+1		24
14	22		1+1		24
16	22		1+1		24
3	21	1		2	24
15	22	1		1	24
23	22	1		1	24
6	22			2	24
21	23			1	24
10	23	1			24
22	21	1		1	23
12	21	1		1	23
8	22			1	23
17	23				23
13	20			3	23
1	21	1			22
18	21	1			22
20	21	1			22
11	22				22
24	22				22

The simplest interpretation of this case was that it exhibited a failure to maintain chiasmata with the result that univalents and possible multivalents were recorded, there was an associated tendency to loss of XY association (4/8 NAD cells were X+Y configuration).

Figure 6.27 shows one recorded cell identified at MII

During the course of the investigation, this patient's wife became pregnant. The couple were offered and accepted foetal chromosome diagnosis. Cultured amnion cells were normal and the pregnancy continued to term with the delivery of a healthy child.

Figure 6.23 Primary spermatocyte from T24. MI,23,XY,NAD

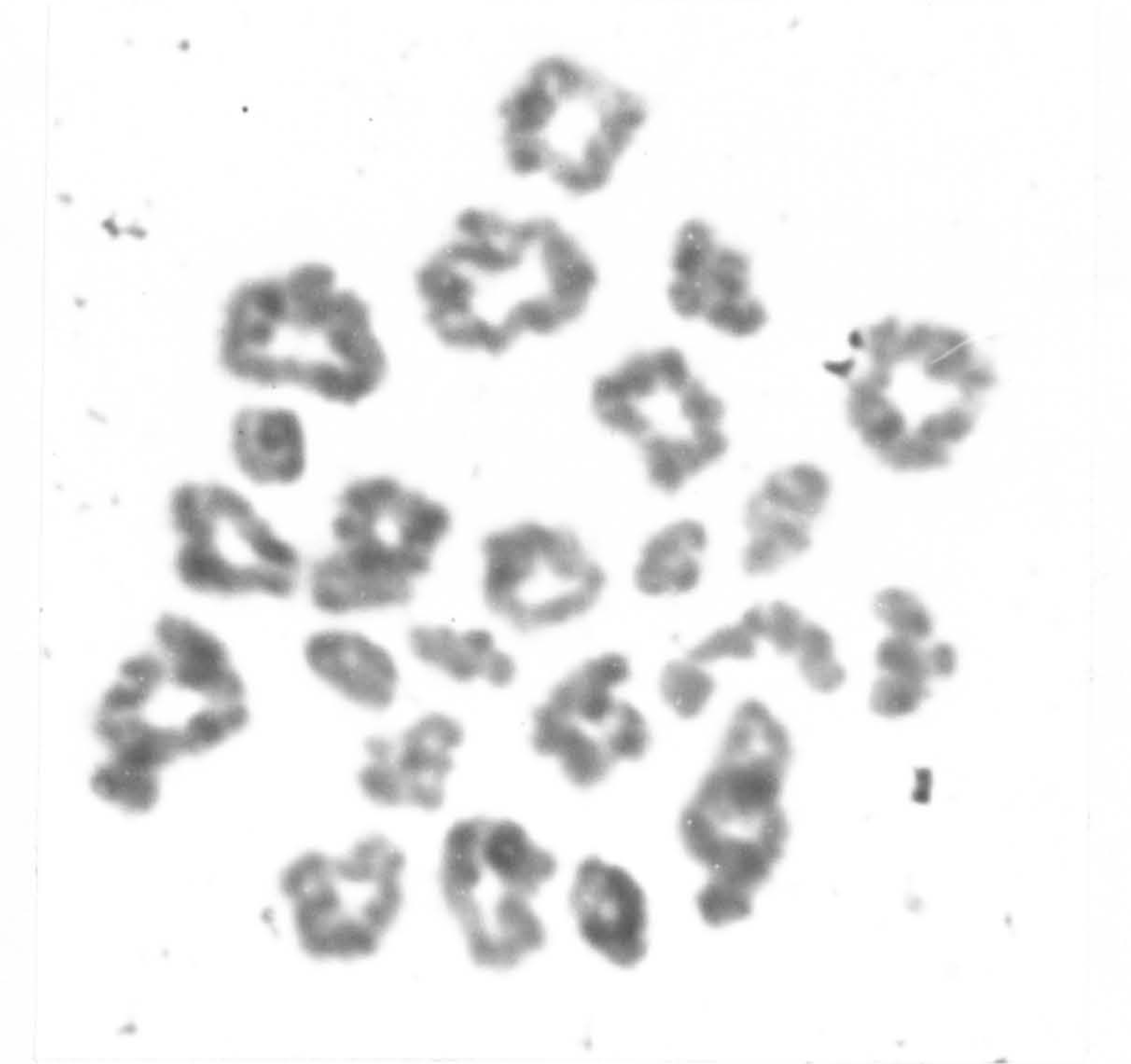


Figure 6.24

Two primary spermatocytes from T24. Arrows mark bivalents and chain-like structures with abnormal C-bands. C-bands marked on photographs.

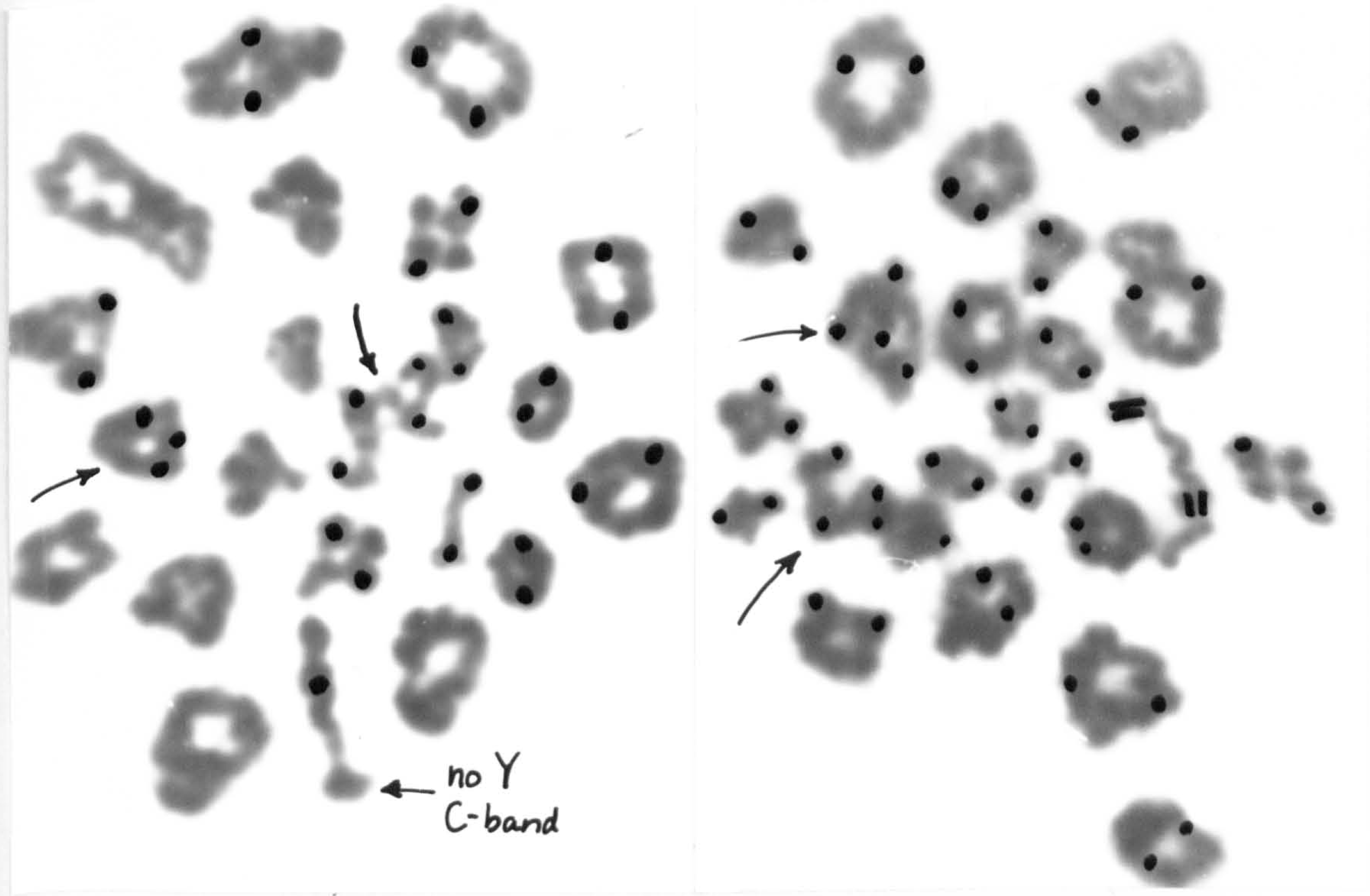


Figure 6.25

Cell No.8 (MI,22,-XY,I) before and after C-banding. Arrows mark the univalent and a chain-like structure identified in the unbanded figure.

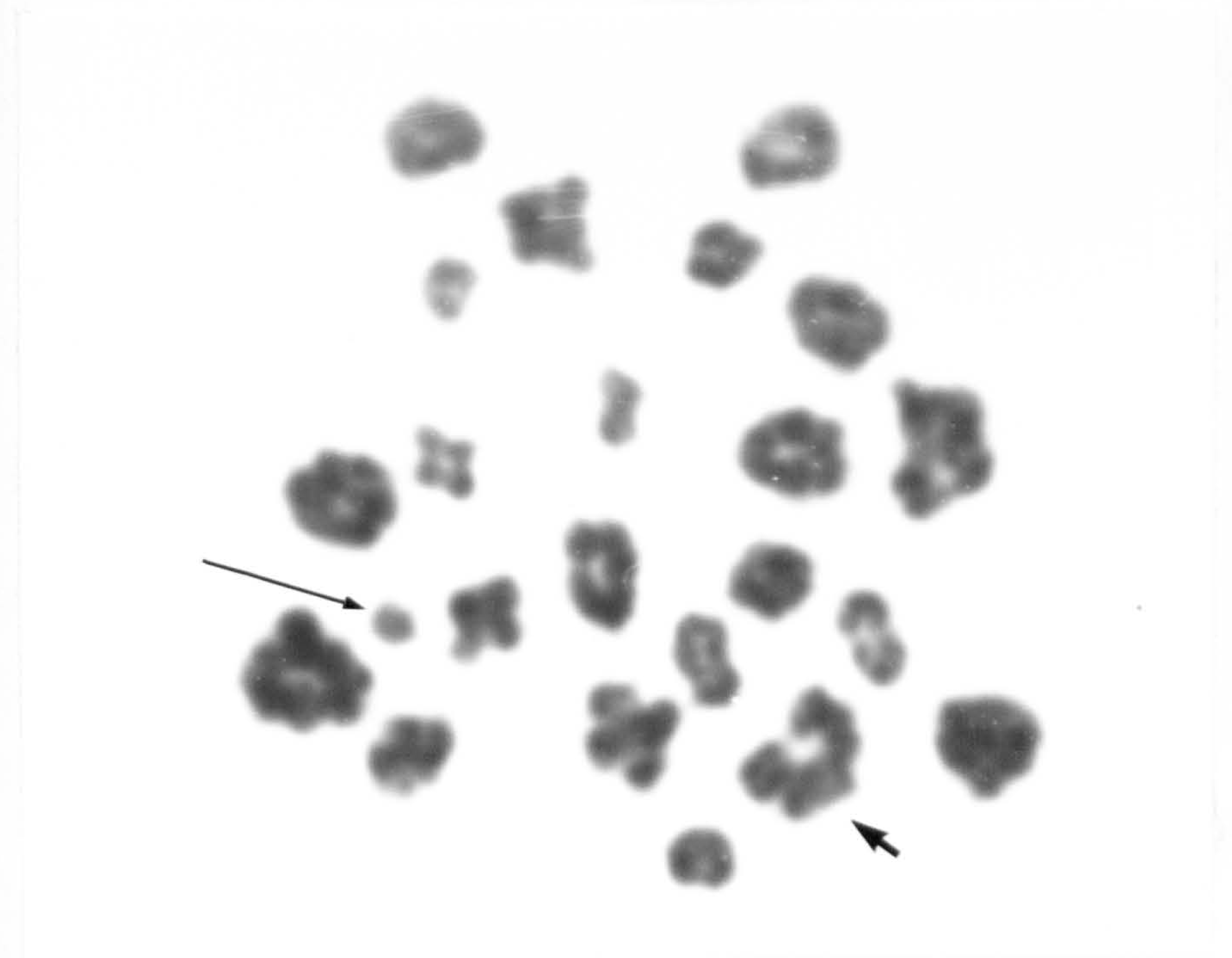


Figure 6.26

Cell 6 from T47. Univalents and an abnormal configuration marked by arrows. C-bands have been marked on the photograph.

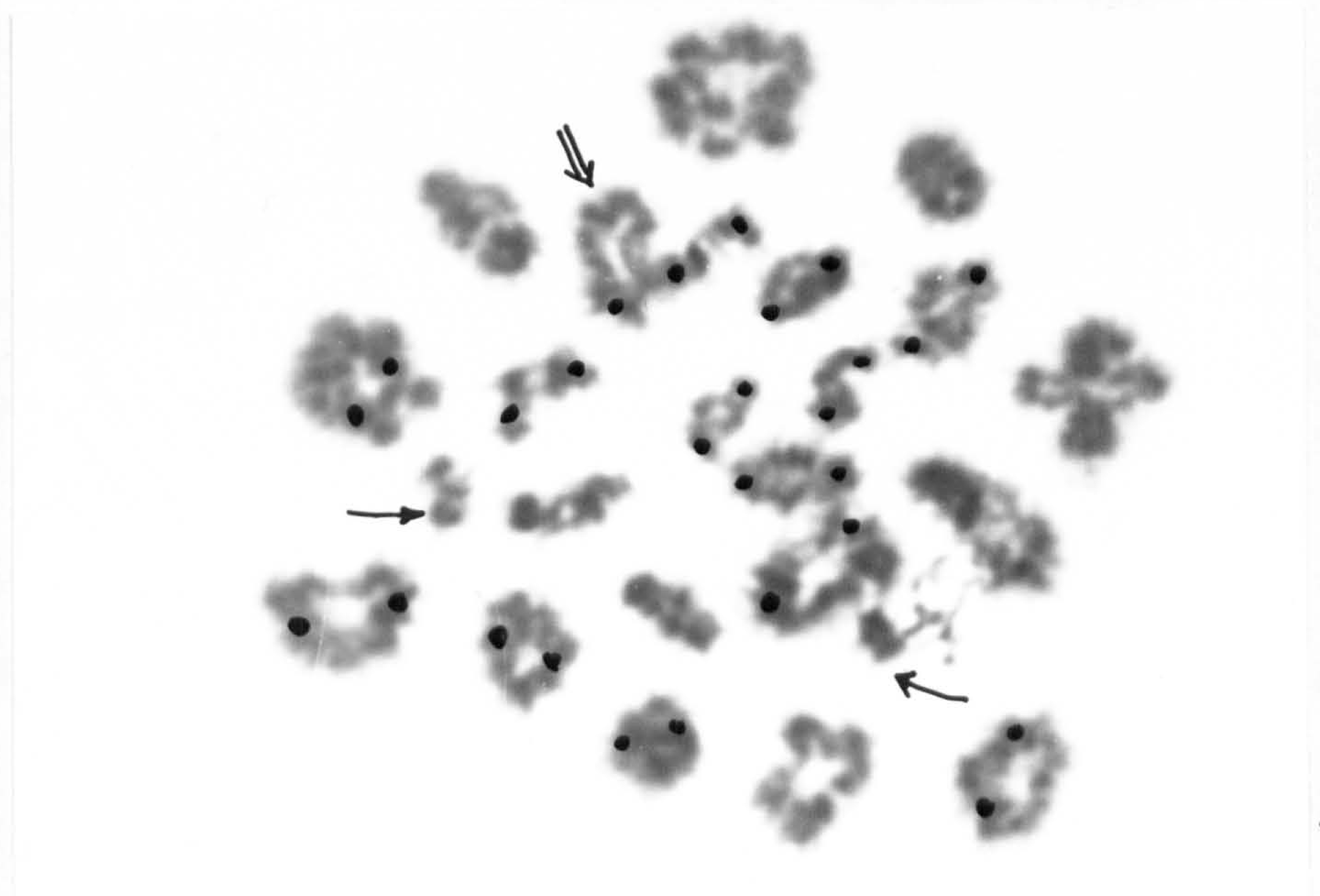
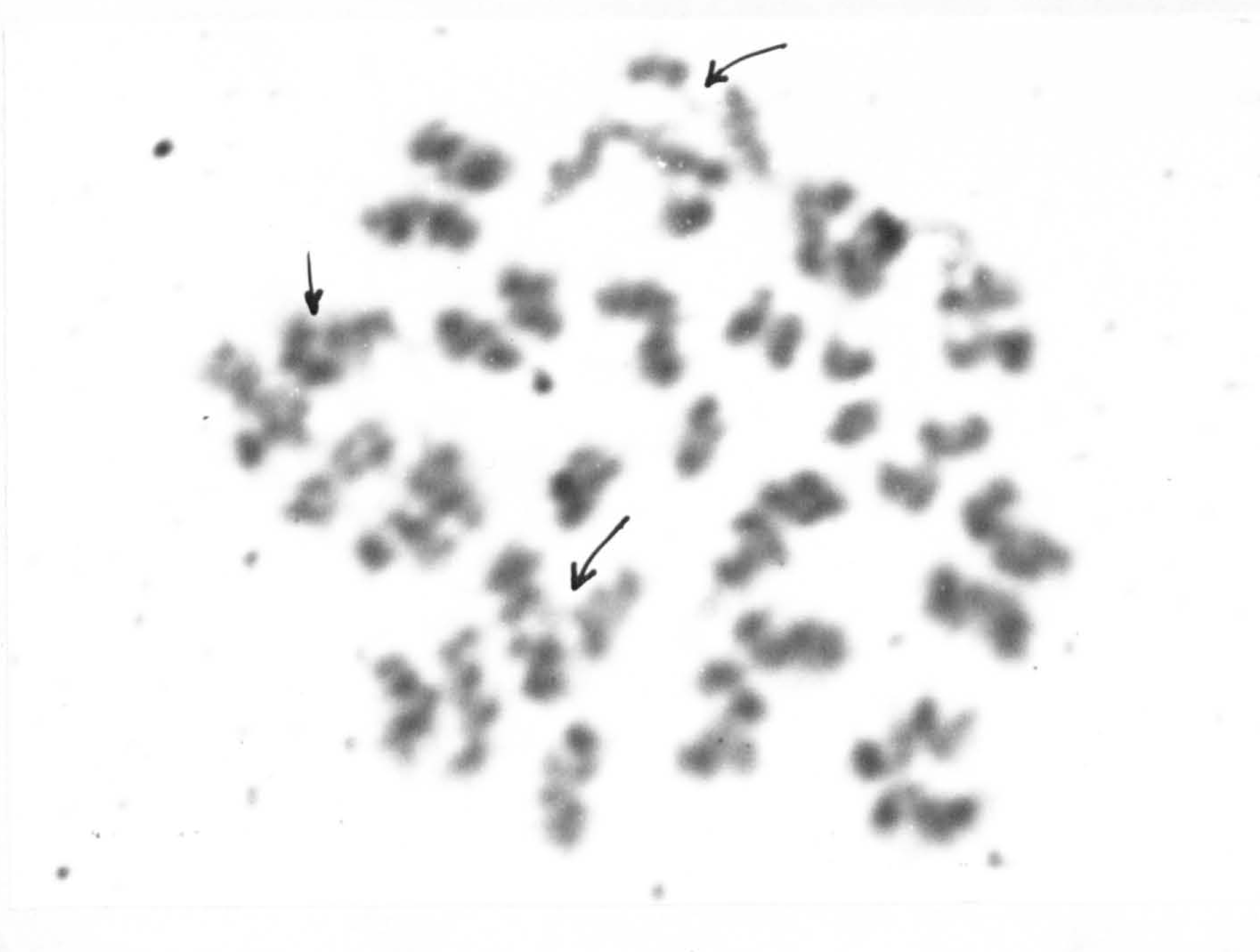


Figure 6.27

Cell in second metaphase



E. Absent meiosis

There were 14 cases classified as "absent meiosis". In 2 cases a single diakenesis figure was present together with abundant pachytene figures. Thus there was some possible overlap with cases categorised as "few figures".

In 3 cases pachytene figures were not observed. In a further 3 cases pachytene figures were present but rare. In 6 cases pachytene figures were abundant and were the only figures present.

In order to assess whether "absent meiosis" was a meaningful category, comparisons were made with the recorded histology categories in Table 6.6. The comparison shows that out of 11 cases which had histology categories available, 7 were recorded as "FAG". This represents 7 "FAG" cases out of a total of 9 "FAG" recorded in the population. Thus it seems probable that the category "absent meiosis" does record a spermatogenic event rather than assessing the quality of the preparation. In future the use of "absent meiosis" must be more strictly applied to exclude preparations where even one diakenesis figure is observed.

F. Few figures present

There were 15 cases recorded in the "few figures" category. Out of the 15 cases, 9 simply had very few figures present, and these figures showed no detectable abnormality (NAD). One of these cases had many polyploid cells present; the significance of noticeable polyploidy is not known. In 2 of the cases diakenesis figures were present but not analysable. In 4 cases there were very few figures present and abnormal counts or configurations could not be excluded.

These 15 cases are listed with their recorded histology categories in Table 6.7. The results demonstrated the heterogeneity of the "few

TABLE 6.6

CATEGORY "ABSENT MEIOSIS" COMPARED WITH HISTOLOGY CATEGORY

Case	Rare figs.	Pachytene	Histology Category	JMS
13	1 diak.	+	S + RG	3.4
18		Few	FAG	3.9
19		–	S + RG	2.6
25		+	FAG	4.5
30		+	No score	–
37		–	S + FAG	2.0
39		+	No score	–
40		+	No score	–
76		Few	FAG	2.0
118		+	FAG	1.2
123		Few	FAG	1.8
130		–	FAG	2.0
136	1 diak.	+	FAG	3.0
137		+	RG	5.4

TABLE 6.7

CATEGORY "FEW FIGURES" COMPARED WITH HISTOLOGY CATEGORY

Case	NAD figures	No. of figures	Histology Category	JMS
8	+	3	S + RG	3.1
15		1	FAG	4.0
23	+	4	NAD	8.6
26		6	RG	6.5
28	+	4	RG	5.1
32	+	4	S + RG	4.6
33		1	RG	5.5
44	+	6	RG	6.5
46	+	6	RG	6.6
48	+	4	B	8.3
67	+	5	NAD	8.5
80		2	NAD	8.3
82		1	RG	6.8
105	+	2	–	–
117		4	FAG	5.0

figures" category and suggested that this categorisation was descriptive of the technical quality of the preparation. This probably obscured any real correlation which might have existed between the presence of very few diakenesis figures and the activity of the germinal epithelium. It seems practically important to distinguish between cases with very few figures which were "NAD" and cases with figures which were amodal or unanalysable.

G. Failed

6 cases were recorded as "failed". In one of these the biopsy was extremely small and appeared damaged. In 3 cases figures were present but were unanalysable. These differed from the previous "few figures" cases (which seemed similar) because, in this category, the unanalysable figures present were plentiful. In 2 cases no reasons for failure were noted.

CHAPTER 7

TESTIS IN LONG TERM CULTURE

1. Introduction

2. Investigating the method

   A. Cell suspension culture

   B. Whole tubule culture

   C. Results of whole tubule culture

3. Establishing in vitro differentiation

   A. Labelling with tritiated thymidine

   B. Results

   C. Discussion

4. Conclusions

1. INTRODUCTION

One method of studying a differentiating system such as the spermatogenic cycle, is to maintain the system in culture. If this can be achieved, then observations can be made and changes with time documented. It is valuable to use a radioactive tracer in this type of work since known points in the cycle can then be followed. Before such documentation could begin I needed to establish that testicular tissue could be maintained and would continue to differentiate in vitro.

Several authors (Kodani, 1962; Yao and Race, 1964; Lima de Faria et al, 1968; Steinberger et al, 1970; Matte and Sasaki, 1971; Dutrillaux, 1971; Chandley and Kofman-Alfaro, 1971; Ghatnekar et al, 1974) have reported methods that would maintain differentiation of the human testicular epithelium in culture. These authors sought to maintain in vitro differentiation for three main reasons.

1. To increase the number of cells in meiotic division thereby improving the quality and confidence of meiotic chromosome analysis.
2. In order to study parts of the spermatogenic cycle.
3. To examine the effects of changing the chemical constituents of the culture medium with the hope of understanding the biochemical control of the spermatogenic cycle.

Thus Dutrillaux (1971) reported that after 48 hours in culture meiotic figures (principally diakenesis figures) increased in quantity. Lima de Faria and co-workers (1968) and Chandley and Kofman-Alfaro (1971) studied the sequence of meiotic division; their work made use of autoradiography to follow cell progress through meiosis. Steinberger and co-workers (1970) investigated the biochemical pathways of hormones

involved in the maintenance of spermatogenesis. Recently the earlier work of Chandley and Kofman-Alfaro (1971) has been elegantly expanded by Hotta, Chandley and Stern (1977) to study the biochemistry of meiotic division in the male mouse.

Between them, these authors described a variety of recipes for culture media. In addition, the ways in which cultures were initiated varied between authors. Thus Dutrillaux (1971) and Chandley and Kofman-Alfaro (1971) used cell suspensions which were maintained for relatively short periods of time in relation to the length of the spermatogenic cycle. In contrast, Steinberger et al (1970) kept small pieces of testis in culture for relatively long periods of time. Still further variation was described when these workers reported methods of preparing material for analysis. Thus Chandley and Kofman-Alfaro (1971) used cell suspensions, Steinberger et al (1970) prepared histology sections and Lima de Faria et al (1968) used squash techniques.

The results of these authors work described no clear method of achieving in vitro differentiation. Therefore, as a result of this review, I decided to re-investigate methods of maintaining in vitro differentiation. The small size of the biopsy available for experimental work necessitated considering alternative methods of preparing the material for analysis.

2. INVESTIGATING THE METHOD

A. Cell suspension culture

(i) Methods

Cell suspensions were sterilely prepared by teasing cells out of the tubules using the modified method of Evans et al (1964) (Chapter 4 Section 3A2). Other cell

suspensions were prepared by trypsin digestion of the tubules. Both types of cell suspension were set up in carrel flasks in a sterile medium consisting of :-

75% Eagles Minimum Essential Medium (Wellcome Reagents Ltd)
20%. Foetal Calf Serum (Flow Laboratories)
5% Tryptose Phosphate Broth (Difco Laboratories)

Carrel flasks were gassed with CO_2 before incubation at 37°C.

(ii) Results and Comments

Both types of preparation yielded the occasional figure in diakenesis and spermatogonial mitosis when harvested after 24 hours and 48 hours incubation. These results did not confirm the results reported by Dutrillaux (1971) who found an increased frequency of figures after 48 hours in culture. Trial additions of colcemid to these incubated cultures made no determinable difference to the results of culturing suspensions of testicular cells.

Some of the cell suspensions prepared by trypsin digestion were kept in tissue culture for up to 6 weeks. In these preparations settling of the cell suspensions occurred after about 12 hours but cell adhesion and growth was very slow. After about 1 week there appeared to be some fibroblast-like cell growth in adhered cells. Unadhered cells, harvested after 1 week, showed mainly degenerative nuclei. The fibroblas-like cells were maintained in tissue culture for up to 6 weeks and were treated

and changed like ordinary fibroblast cultures. There was still very little growth and attempts to harvest figures were never successful. Steinberger and Steinberger (1966a) reported obtaining similar fibroblast-like cell growth from cell suspension cultures maintained for several weeks.

The extremely small amount of tissue available for making the cell suspensions and the very low yield of recoverable cells after 24 hours incubation was a major problem with this experiment. This part of the investigation was not pursued further.

One positive piece of information gained from this experiment was the suggestion that meiosis continued after the biopsy was excised. Some previous workes have emphasised the need for speed after excision in order to catch cells in diakenesis, the implication being that meiosis is halted by excision. In these experiments, occasional cells in diakensis after 24 hours and 48 hours in culture suggested that division was not necessarily halted by excision.

B. Whole tubule culture

(i) Preliminary trials

The second type of culture technique investigated was the maintenance of whole tubules in culture.

Initially tubules were gently separated and cut into approximately 2 cm lengths. The tubules were then incubated in carrel flasks containing culture medium (as previously described) for periods of 24 hours - 7 days. This technique was apparently never successful, figures were not recovered after this treatment and the cells which were recovered

usually showed degenerative changes.

Subsequently it was decided to place small pieces of of the biopsy in culture. Similar treatments of the testicular biopsy were described by Steinberger et al (1970) and by Ghatnekar et al (1974). These methods could not be duplicated in Sheffield but modifications were made which permitted the basic essentials of the two techniques to be maintained.

(ii) <u>Methods for whole tubule culture</u>

1. A testicular biopsy was placed sterilely in medium and transported as rapidly as possible to the laboratory.

2. The medium consisted of :-

   10% Foetal Calf Serum (Flow Laboratories)

   90% Eagles Minimum Essential Medium (Wellcome Reagents Limited)

   This was enriched with :-

   1 mM Sodium pyruvate (Sigma Chemical Company)

   0.1 mM Minimum Essential Amino Acids (Gibco Bio-cult)

   4 mM glutamine (Gibco Bio-cult)

   This complete testis medium (CTM) was used for transport and incubation of the biopsy.

3. The biopsy was sterilely cut into small pieces approximately "grain of rice" size.

4. The small pieces of biopsy were placed on a sterile micropore filter paper which was supported on a stainless steel mesh. The steel meshes were bent such that the sample was a few millimetres above the floor of the receptacle.

5. A sterile disposable plastic petri dish (5 cm in diameter) was used as the culture vessel. The lid of the petri dish was ribbed which allowed circulation of sterile 5% CO_2/95% air.
6. CTM was added to the petri dish in sufficient quantity to leave the tubule samples at the medium/gas interface.
7. Biopsies were incubated in a gas incubator maintained at 31°C. CTM was changed every third day.

Micropore filters were used because they were easily sterilised. When the filters were taken out of their holders, they emerged flat. The rubber gasket was retained since it helped to keep the biopsy specimen on the micropore filter when the petri dish had to be moved during experiments. Despite scrupulous sterile precautions, contamination was a problem. It was difficult to envisage improving sterility without purchasing equipment specially designed for this type of culture work.

Two unusually large biopsy specimens were received and these provided the opportunity to attempt long term culture of tubules. Details relevant to these samples are recorded in Table 7.1. It was ironical that one of these biopsies had an abnormal meiotic chromosome complement.

TABLE 7.1

DETAILS OF 2 CASES WHICH PROVIDED BIOPSIES FOR

THE LONG TERM CULTURE EXPERIMENT

Case No.	Clinical reason for biopsy	Mitotic chromosome complement	Meiotic chromosome complement	Histology
T34	Infertility	46XY,der(13) t(Y:13)	MI 23XY MI 24X,Y extra deleted Y identified	50% tubules ⟶ spermhead stage 38% maturation arrest 12% tubules, Sertoli only
T36	Infertility	46XY,NAD	MI 23,XY, MI 24,X,Y	35% tubules ⟶ sparse spermhead stage 48.5% tubules, arrest late spermatid 5.0% tubules, Sertoli only

C. Results

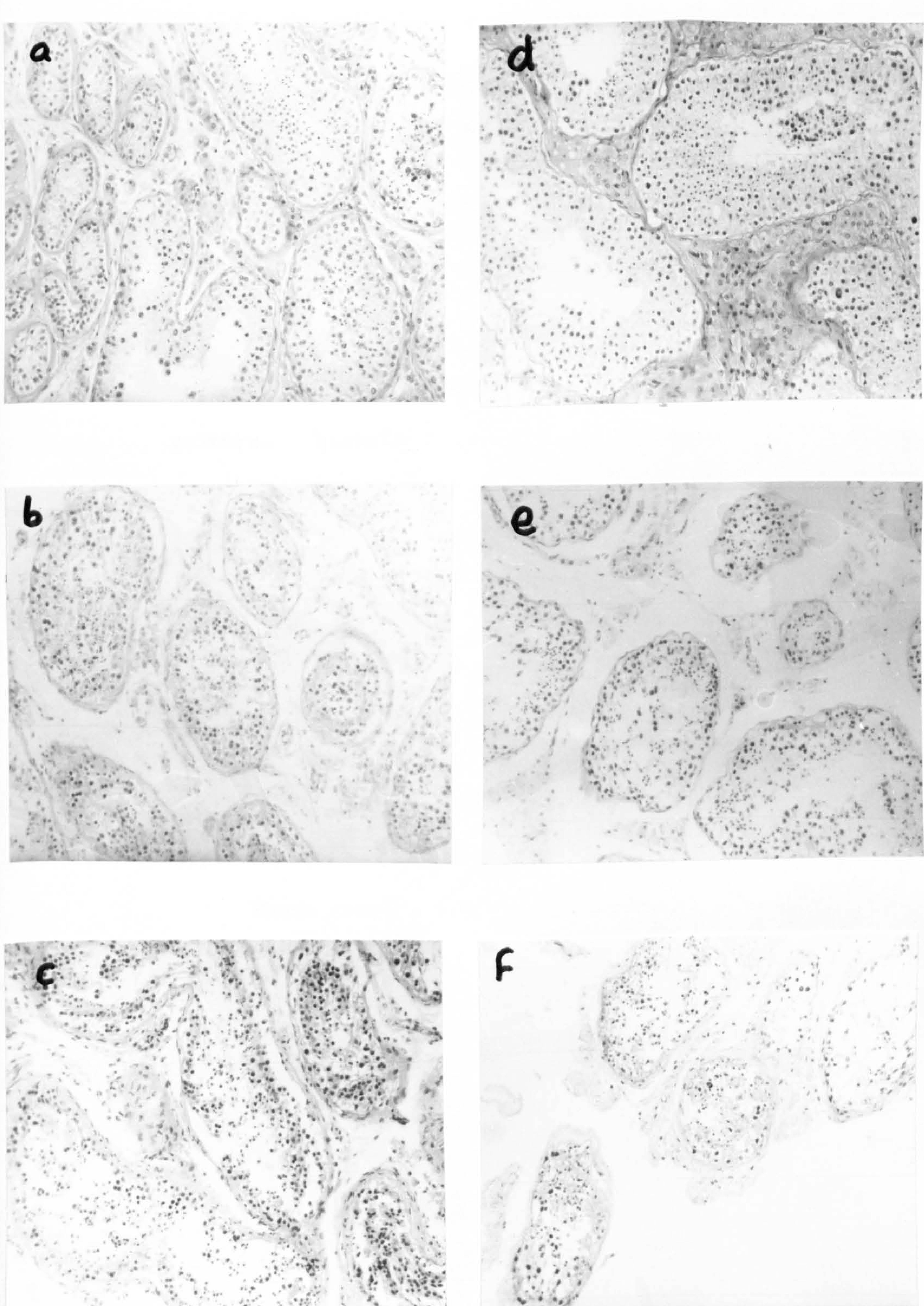
(i) T34 Three samples were harvested at 0 hours, 24 hours and 5 days for histology. The histological appearance is shown in Figure 7.1a,b,c. After 5 days in culture, spermatogenesis was well preserved and the architecture of the tubules was intact. This was an encouraging result. An attempt to harvest meiotic figures from the 5 day sample was disappointing. The modified method of Evans et al (1964) was used. However, the sample was extremely small which would have made success unlikely even with a fresh

Figure 7.1

T34 and T36. Histology after long term culture

T34, a) 0 hours b) 24 hours c) 5 days

T36, d) 0 hours e) 3 days f) 10 days



biopsy specimen. At this point in the experimental work, I decided to use the modified method of Meredith (1969) for harvesting meiotic figures from whole tubules. This decision proved an extremely important step and was used in all subsequent experimental work. The advantages and disadvantages of Meredith's method have been discussed in Chapter 4, Section 3C. In general these results from sample T34 appeared promising.

(ii) T36 T36 was a very large biopsy which provided the opportunity for harvesting at intervals up to 21 days in culture. Histology sections were prepared after 0 hours, 3 days and 10 days in culture and are shown in Figure 7.1d, e,f. At day 10, the tubules were intact with their architecture preserved and spermatogenesis present through to sperm head stage. Table 7.2 records the types of cells observed at each time in culture harvested. Figures 7.2 and 7.3 show spermatogonial mitoses and diakenesis figures present at representative time intervals throughout this experiment.

These results recorded the presence of spermatogonial mitoses and diakenesis figures after 21 days in culture, at which time the experiment was terminated. This suggested that the activity of the germinal epithelium was being maintained since spermatogonial mitoses were observed after 21 days in culture. It also suggested that meiotic division was being maintained since diakenesis figures were observed after 21 days in culture. In addition, the results recorded an apparent burst in diakenesis production covering

the 10–14 days period in culture. It was noted that many of the figures were polyploid in character, but it was not known whether this was a characteristic of T36 or whether it was an in vitro culture characteristic.

TABLE 7.2

TYPES OF MEIOTIC FIGURES OBSERVED IN T36
DURING LONG TERM CULTURE EXPERIMENTS

T36	0 hrs. 36/1	30 hrs. 36/2	3 days 36/3	10 days 36/4	11 days 36/5	12 days 36/6	14 days 36/7	21 days 36/8
Spermatogonial mitoses		3		22				6
Pachytene	lots	lots	lots	lots	11	2	1	2
Diakenesis		2	3	12	11	5	2	3
MII							1	
Polyploid diakenesis						1	5	
Unclassifiable figures								5

All these results may be explained by the maintenance of cells in culture without in vitro differentiation. Therefore further experimental work was planned to investigate whether the observed diakenesis figures were the result of in vitro differentiation or whether they represented the maintenance of figures present when the cultures were initiated.

Figure 7.2

Figures observed in T36 after long term culture. a) spermatogonial mitosis; 30 hours. b) diakenesis; 30 hours. c,d) diakenesis ? polyploid; 10 days. e) diakenesis; 14 days. f) ?M_{II}; 21 days. All photographed at x 100 magnification.

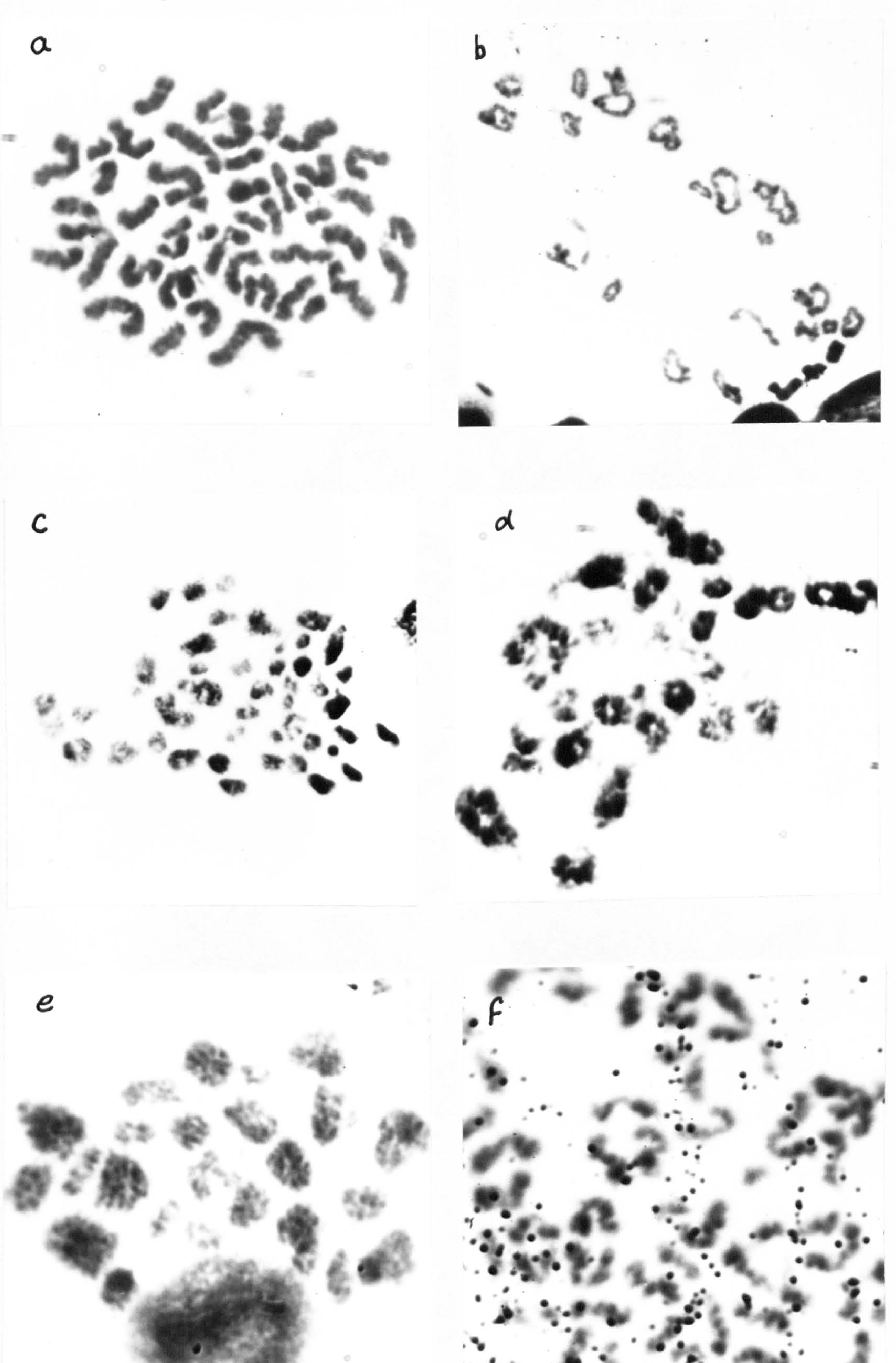
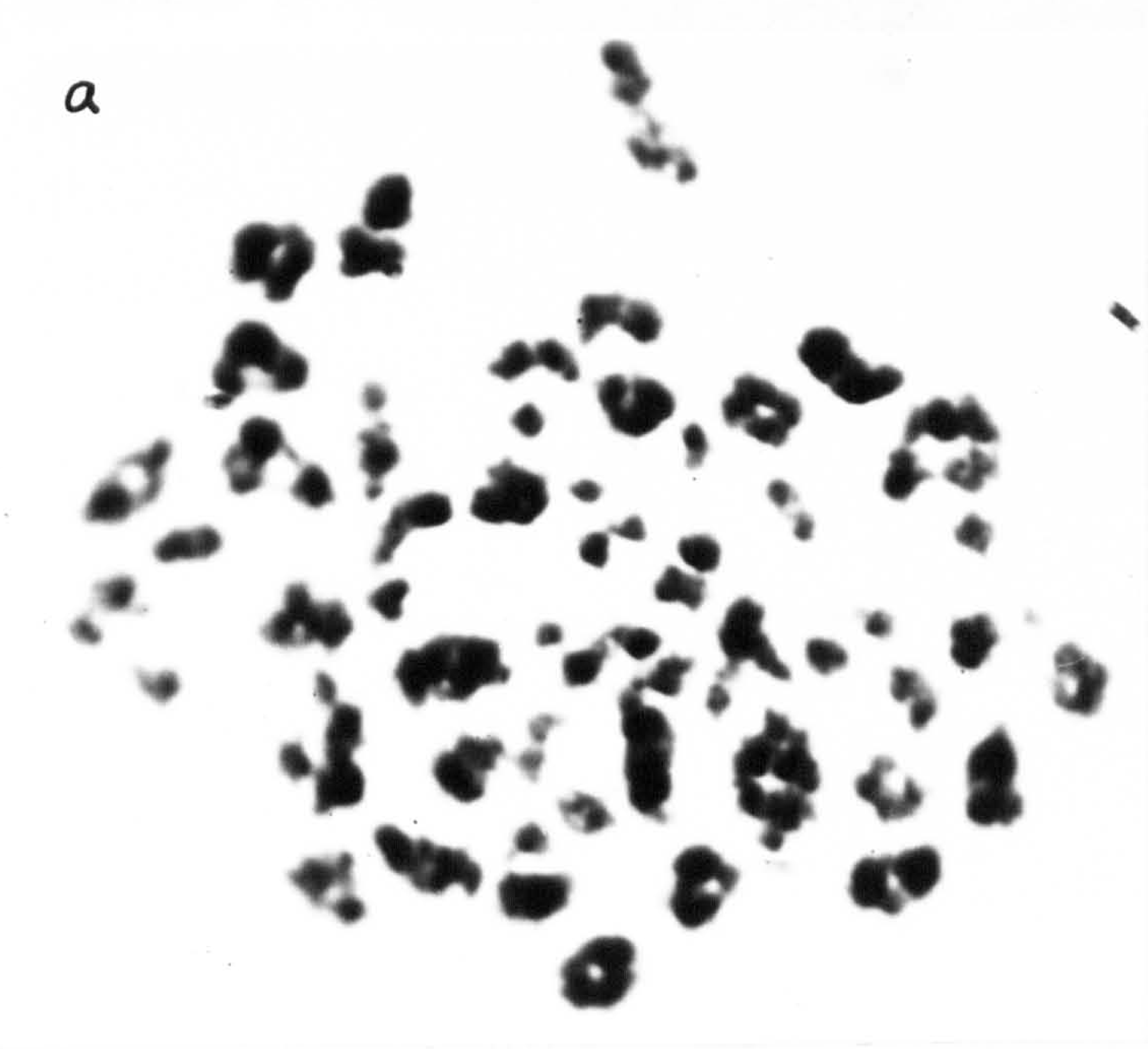
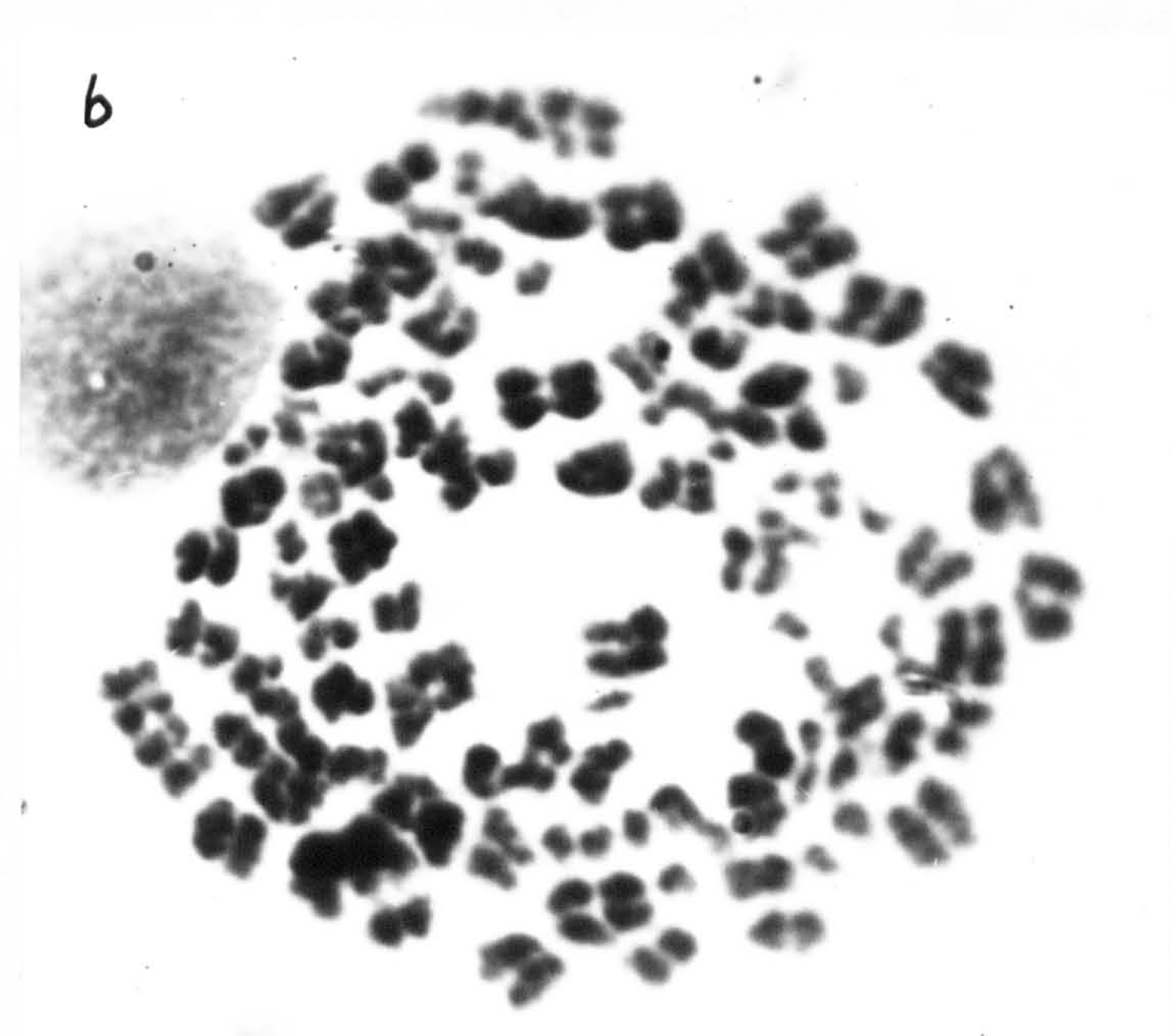


Figure 7.3

Polyploid figures from T36. Photographed at x 100 magnification. a) polyploid diakenesis; 10 days. b) polyploid spermatogonial mitosis; 14 days.





3. ESTABLISHING IN VITRO DIFFERENTIATION

A. Labelling with tritiated thymidine

In vitro differentiation of diakenesis figures was tested using tritiated thymidine (H^3Tdr). If H^3Tdr was added to actively differentiating tubules then S phase spermatogonia and S phase spermatocytes would take up label. If the most advanced type of labelled cell was recorded at time intervals following H^3Tdr innoculation, then, when differentiation had occurred, progressively later labelled stages of division could be recorded after suitable times in culture.

Choice of a suitable concentration of H^3Tdr to be added to the culture depended on a number of interrelated factors.

1. Need to use a short period of labelling in order not to obscure subsequent labelling patterns in a cell population that has more than 1 cell type capable of taking up label.
2. Premeiotic S phase is known to be relatively long, therefore a brief period of contact with label will result in sparsely labelled cells.
3. Need to be able to identify labelled cells from background label, which is a technical hazard of autoradiography.
4. It is useful to be able to identify labelled cells without having to resort to stripping off the film before identification can be made with some degree of confidence.
5. It is useful to avoid very long autoradiographic exposure times which are tedious when waiting to analyse results in order to carry out further experiments and which tend to maximise background label.

Testicular biopsies from 2 apparently normal individuals were set up in culture as described in Section B(ii) of this Chapter. Details of the patients are recorded in Table 7.3. Part of this experiment tested 2 different concentrations and incubation times with H^3Tdr.

TABLE 7.3

DETAILS OF PATIENTS WHO PROVIDED BIOPSIES FOR TESTING IN VITRO DIFFERENTIATION

Case No.	Clinical reason for biopsy	Mitotic chromosome complement	Meiotic chromosome complement	Histology	Fertility
NT11	Investigation of testicular lump	46XY	MI,23,XY MI,24,X,Y	Not available	Not known
MT14	reanastomisation of vas following earlier vasectomy	46XY	MI 23,XY MI 24,X,Y	Not available	Proven

Incubation with H^3Tdr was carried out as follows :-

1. Tubule samples were set up in culture as described in Section B(ii) of this Chapter.
2. H^3Tdr, specific activity 5ci/mmol was used in this and all succeeding experimental work. The final concentration added to the cultures in TCM was :-
   a) NT11; 0.5μc/ml for 1 hour at 31°C
   b) MT14; 5μc/ml for 2 hours at 31°C

3. After incubation, CTM containing H^3Tdr was removed and the samples washed in 3 rinses of CTM.
4. The samples were reincubated in fresh CTM at 31°C for the duration of the experiment.
5. CTM was changed every 3rd day throughout the experiment.
6. Methods of harvesting, slide preparation and autoradiography have been described in Chapter 4, Sections 3A, 3B and 6.

Harvests on the two samples were spread over a period of 10–21 days in culture in order to obtain more information over the critical time of the diakenesis surge observed in the previous experiment. Control harvests of NT11/1 and MT14/1 were obtained directly after exposure to H^3Tdr. Other harvests were made at the following time intervals, NT11/2 – 24 hours; NT11/3 – 10 days; NT11/4 – 12 days; NT11/5 – 13 days; NT11/6 – 14 days; MT14/2 – 15 days; MT14/3 – 16 days; MT14/4 – 17 days; MT14/5 – 19 days and MT14/6 – 21 days.

B. Results

Table 7.4 records the presence of cells at different stages of meiotic division, of cells in spermatogonial division and of one non-dividing cell type present at each time interval sampled. The numbers of these cell types present have been recorded qualitatively. Diakenesis figures were usually rare with no more than 10 figures per slide in a good preparation. In contrast pachytene figures were plentiful. These parameters were kept in mind whilst making qualitative assessments. Figures were recorded as + if they required searching for, ++ if figures were easily identified and as +++ if figures were plentiful.

TABLE 7.4

TYPES OF CELLS OBSERVED DURING LONG TERM CULTURE

Sample No. and time after addition of H^3Tdr	Spermatogonial Mitosis	Pre-Pachytene	Pachytene	Diakenesis	MII	Degenerating ? Pachytene	Large pale nuclei
11/1 0 hours	+	0	+++ (17.25%)	+	+		+ (13.75%)
14/1 0 hours	0	0	++ (26.8%)	0	0		++ (23.6)
11/2 24 hours	++	+	+++	+	+		+
11/3 10 days	+	+	++	+++	0	+	+
11/4 12 days	+	+	+	++	0	++	++
11/5 13 days	+	+	+	++	0	++	++
11/6 14 days		NT11 INFECTED: CULTURE TERMINATED					
14/2 15 days	+	++ (6.7%)	+	+	0		+++ (75%)
14/3 16 days		POOR PREPARATIONS					
14/4 17 days	+	++	0	+	0		+++
14/5 19 days	+	+	0	+	0		+++
14/6 21 days	0	?	0	0	0		+++

\+ figures have to be searched for. Counts made on how many available.

++ figures easily seen. Counts made on 25 cells.

+++ plenty of figures. Counts made on 50 cells.

Figures in parenthesis. Cell category expressed as a percentage of total cell population

These results show a surge in diakenesis figures around day 10; diakenesis figures can still be found up to day 19. Pachytene figures show a decrease in frequency which starts around day 10 and continues until, by day 15 and 16 they are rare. The prepachytene figures, in contrast, increase in frequency around day 15. Spermatogonial mitoses are found throughout time in culture. The large pale nuclei (the non-dividing cell type) increased in frequency as time in culture increased, until these large nuclei dominated the sample. The data recorded in Table 7.4 indicates that the two samples used in this experiment differed in cell content at the beginning of the experiment. Thus NT11 was an apparently normal sample of testis whereas Sample MT14 had fewer figures present, including fewer pachytene figures and a relatively higher frequency of large pale nuclei. Finally at day 10 very large degenerating nuclei were observed. These nuclei had some appearance of "old" pachytene nuclei. Their numbers increased in Sample NT11 with increasing time in culture.

Table 7.5 records both the most advanced type of labelled cell present and the proportion of labelled to unlabelled cells counted. Labelled diakenesis figures appeared in culture at day 10 and persisted through to day 17. Labelled early pachytene figures were present at day 10 and persisted in culture until pachytene figures were no longer identifiable. Prepachytene figures were found labelled at 24 hours in culture and persisted until the end of the experiment. Prepachytene figures became more frequent as time in culture increased. By day 15 and 17 there were large heavily labelled nuclei present but identification could not be certain without stripping the slide. Other similarly large but unlabelled nuclei were identified as prepachytene nuclei; therefore the large labelled nuclei were also cautiously

TABLE 7.5

RECORDED NUMBERS OF LABELLED TO UNLABELLED CELLS

OBSERVED AT VARIOUS TIMES IN CULTURE

Background label in all samples <5 grains per large pale nucleus equivalent area.

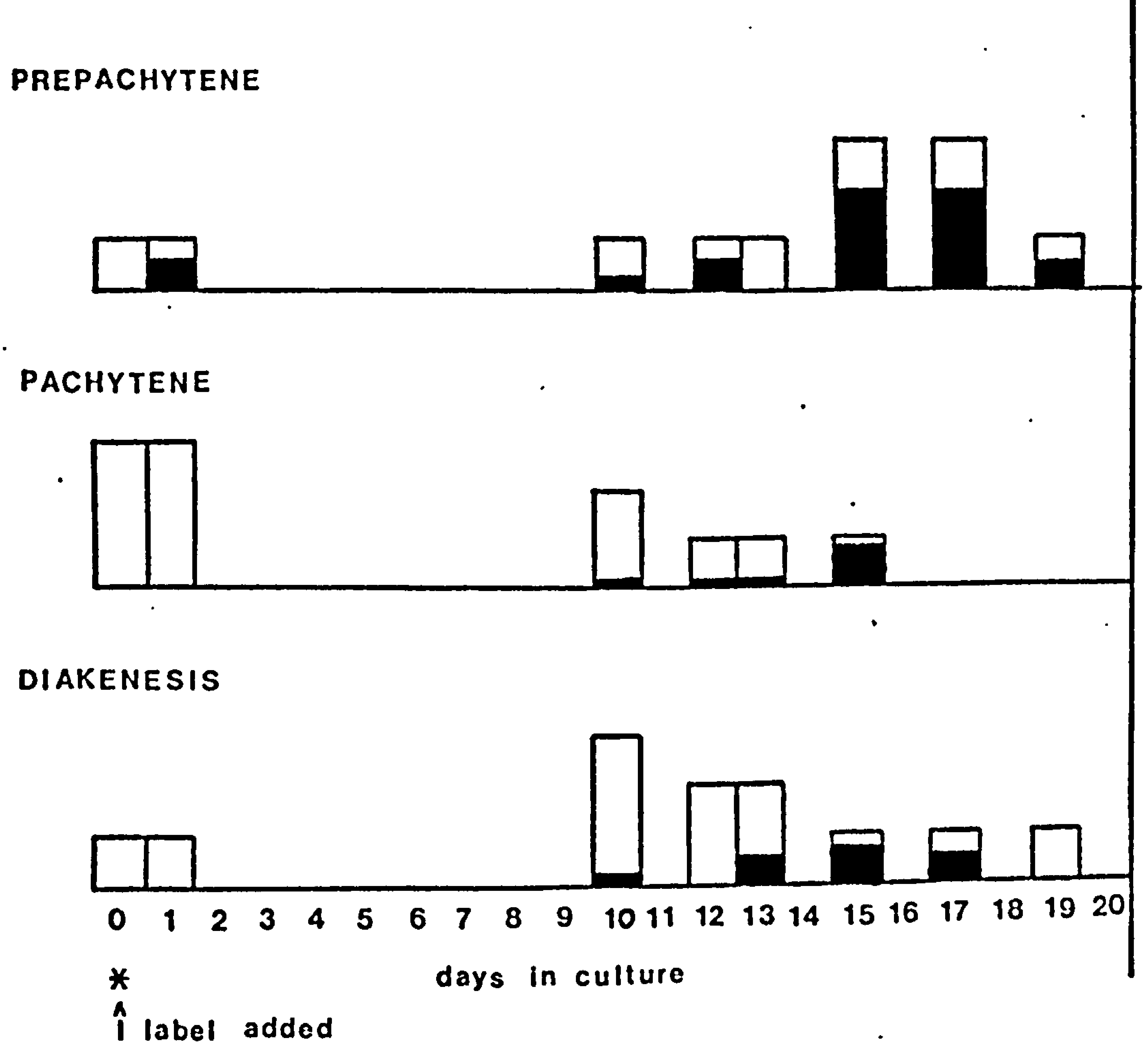
Sparse label = S = < 5 grains; light label = l = >5<20 grains;
Labelled nuclei = L = >20grains; heavy label = h = >>20 grains;
Labelled cells = β cells.

Harvest time	Spermatogonial mitosis	Prepachytene	Early Pachytene	Middle Pachytene	Late Pachytene	Diakenesis
11/1 (0 hrs)						
all cells	2	0	100 (all pachytene)			0
β cells	0	0	0			0
11/2 (24 hrs)						
all cells	20	17	50	50	50	3
β cells	1	9L+2h	0	0	0	0
11/3 (10days)						
all cells	9	9	25	25	25	50
β cells	3L	2L	1s+5L	2s	0	1s+4l
11/4 (12days)						
all cells	10	19	25	10	11	50
β cells	1L	4l+3L+1h	1L	0	1L	0
11/5 (13days)						
all cells	5	0	14	6	13	50
β cells	1s+1L	0	2l+1L	0	1l+3L	1s+5l+3L
14/2 (15days)						
all cells	5	16	2	0	4	4
β cells	3L	5L+many h	2L	0	3L	3L
14/4 (17days)						
all cells	1	many	0	0	0	10
β cells	1L	many h	0	0	0	5L
14/5 (19days)						
all cells	2	Present	0	0	0	1
β cells	0	Labelled	0	0	0	0

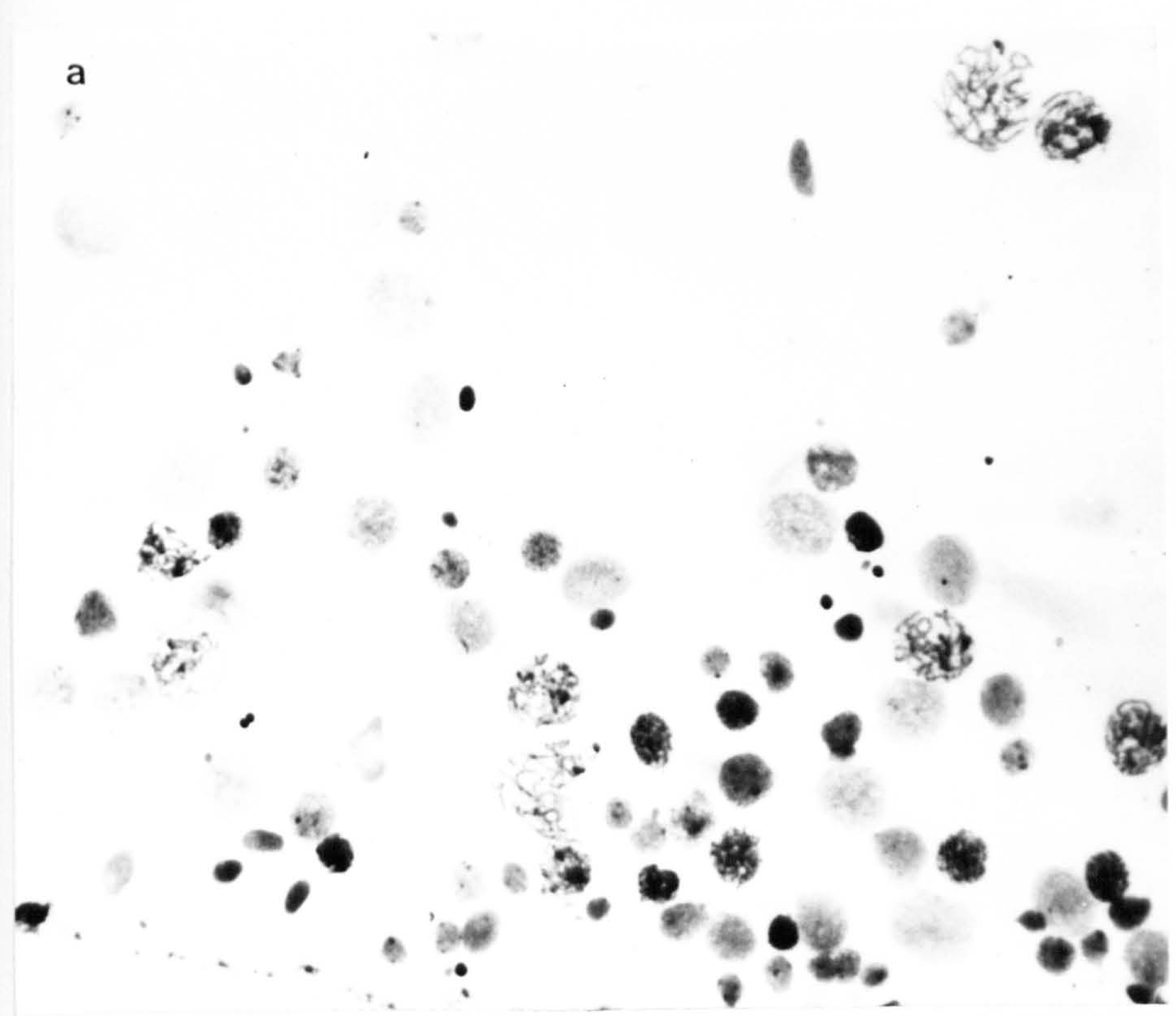
identified as prepachytene nuclei. Labelled spermatogonial mitoses were present in culture up to day 17. Figure 7.4 describes the appearance of labelled pre-pachytene, pachytene and diakenesis figures with time in culture. The blocks in the diagram represent the relative frequency of each category recorded in Table 7.4 as +, ++ and +++. The shaded area of each block represents the proportion of labelled nuclei in each category for each time interval. Zero hours in this diagram is the addition of H^3Tdr to the culture.

Figures 7.5 to 7.10 give some examples of the appearance of labelled nuclei after selected time intervals in culture.

Figure 7.4 : Appearance of label in cells in meiotic division at intervals after exposure to H^3Tdr



T11/2. 24 hours in culture: photographed at magnification indicated.
a) low power view of cell types (x10). b) labelled prepachytene cell (x100).



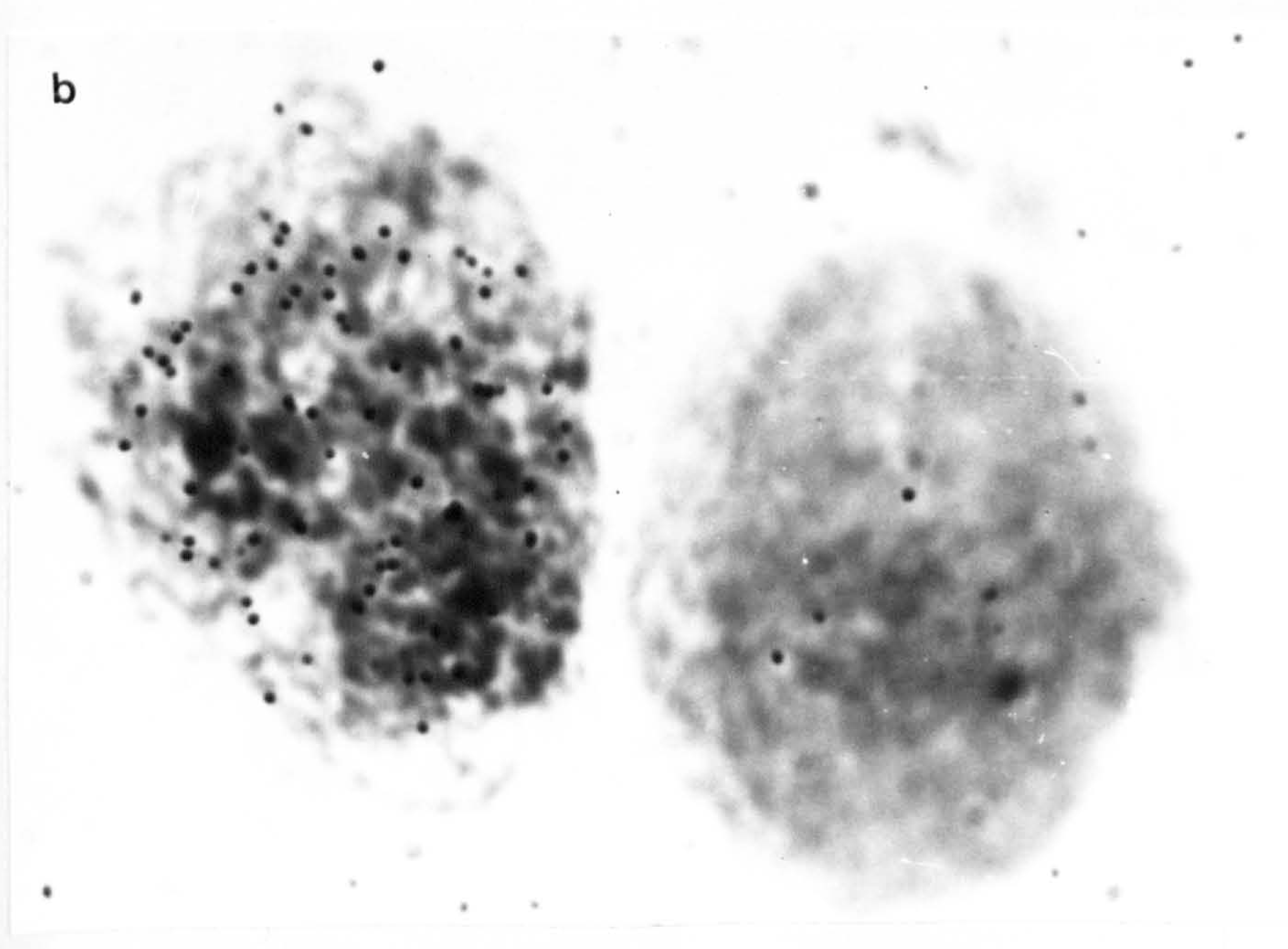
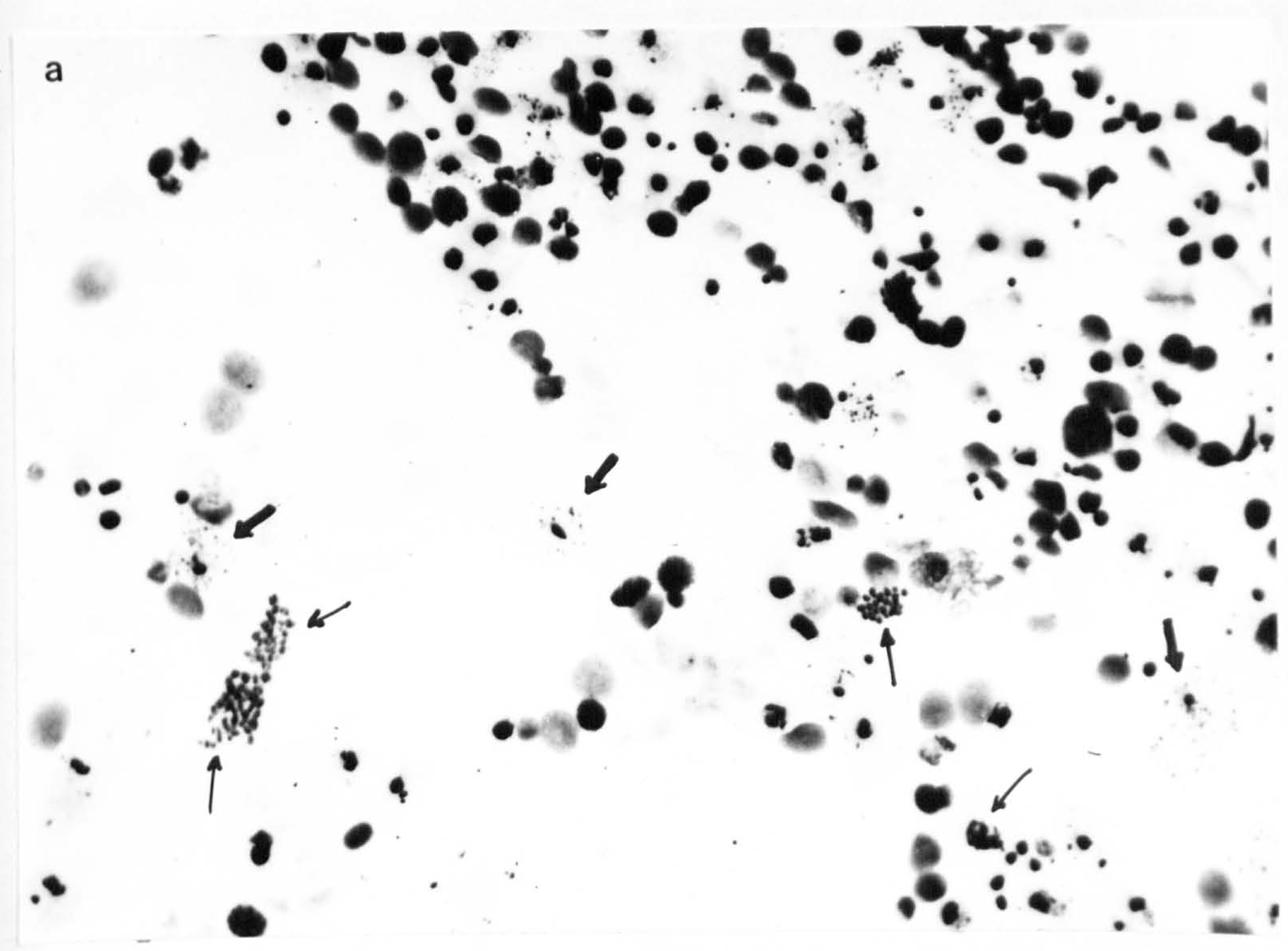


Figure 7.6

T11/3. 10 days in tissue culture: photographed at magnification indicated. a) low power plan showing diakenesis "surge". Arrows indicate diakenesis figures (some are ployploid); wide arrows indicate degenerating pachytene cells (x10). b) labelled prepachytene nucleus (x100). c) labelled early pachytene nucleus, arrow indicates sex vessicle (x100).



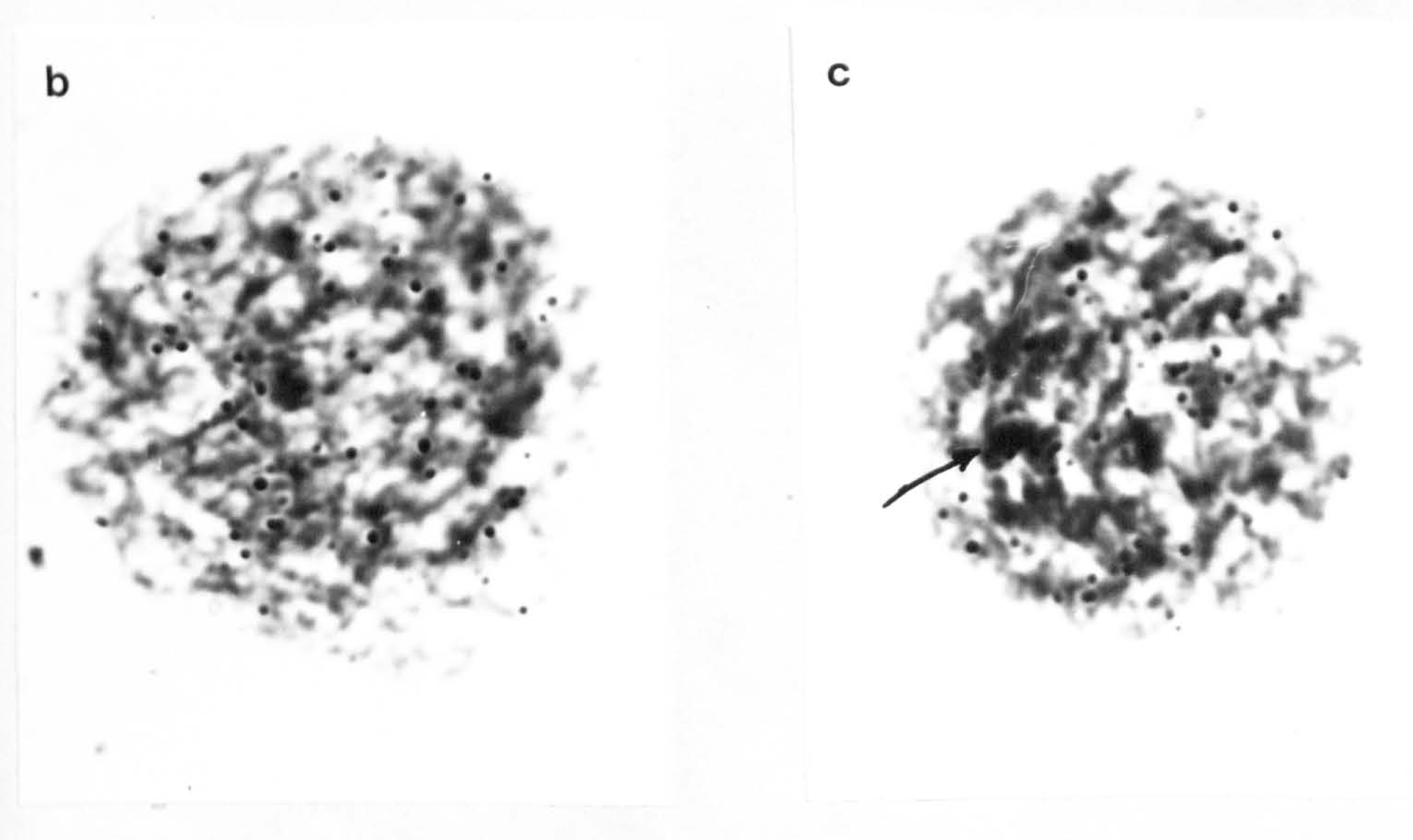
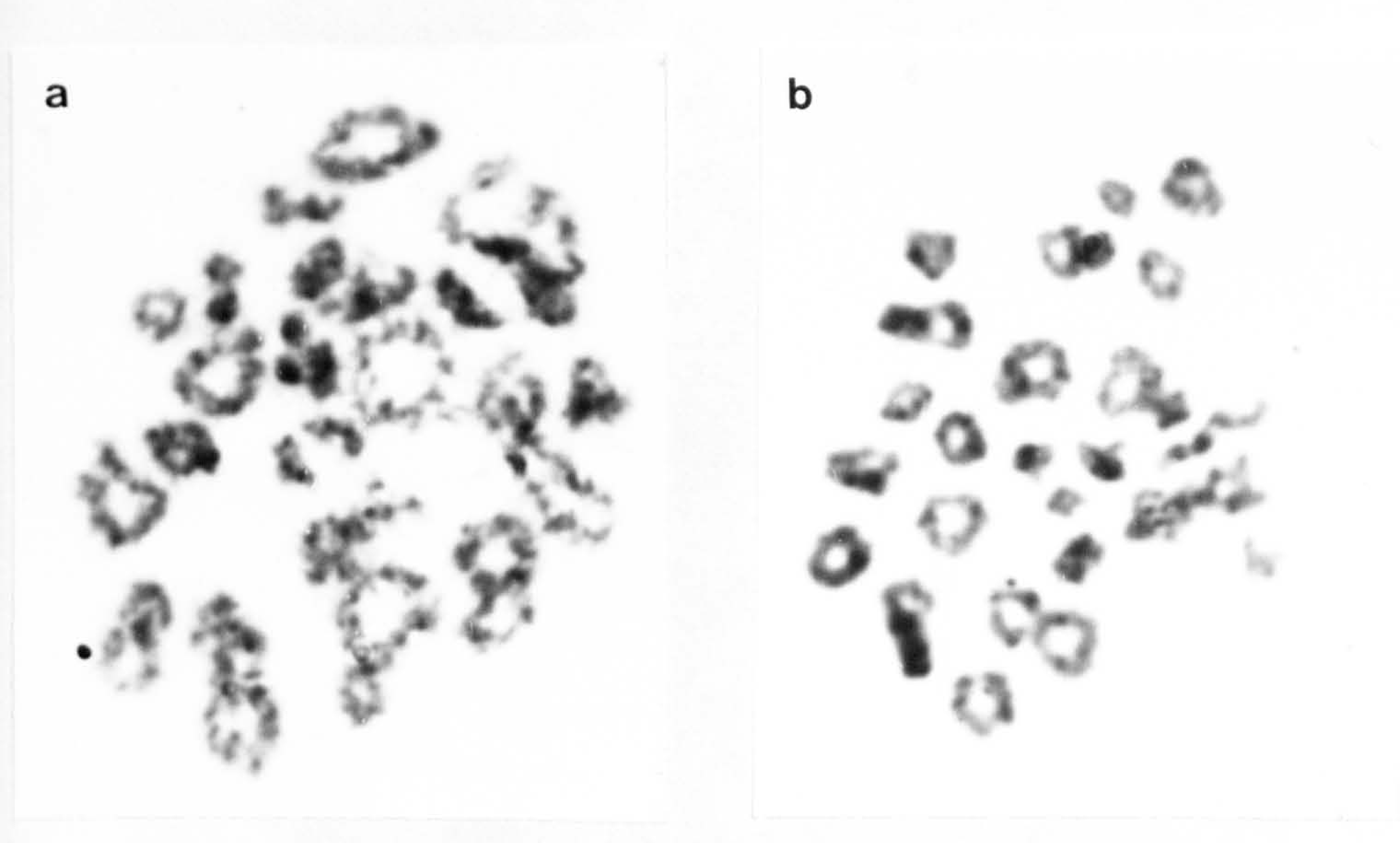


Figure 7.7

T11/3. 10 days in culture: photographed at x100. a,b) unlabelled diakenesis figures. c) labelled diakenesis figure. d) labelled spermatogonial mitosis.



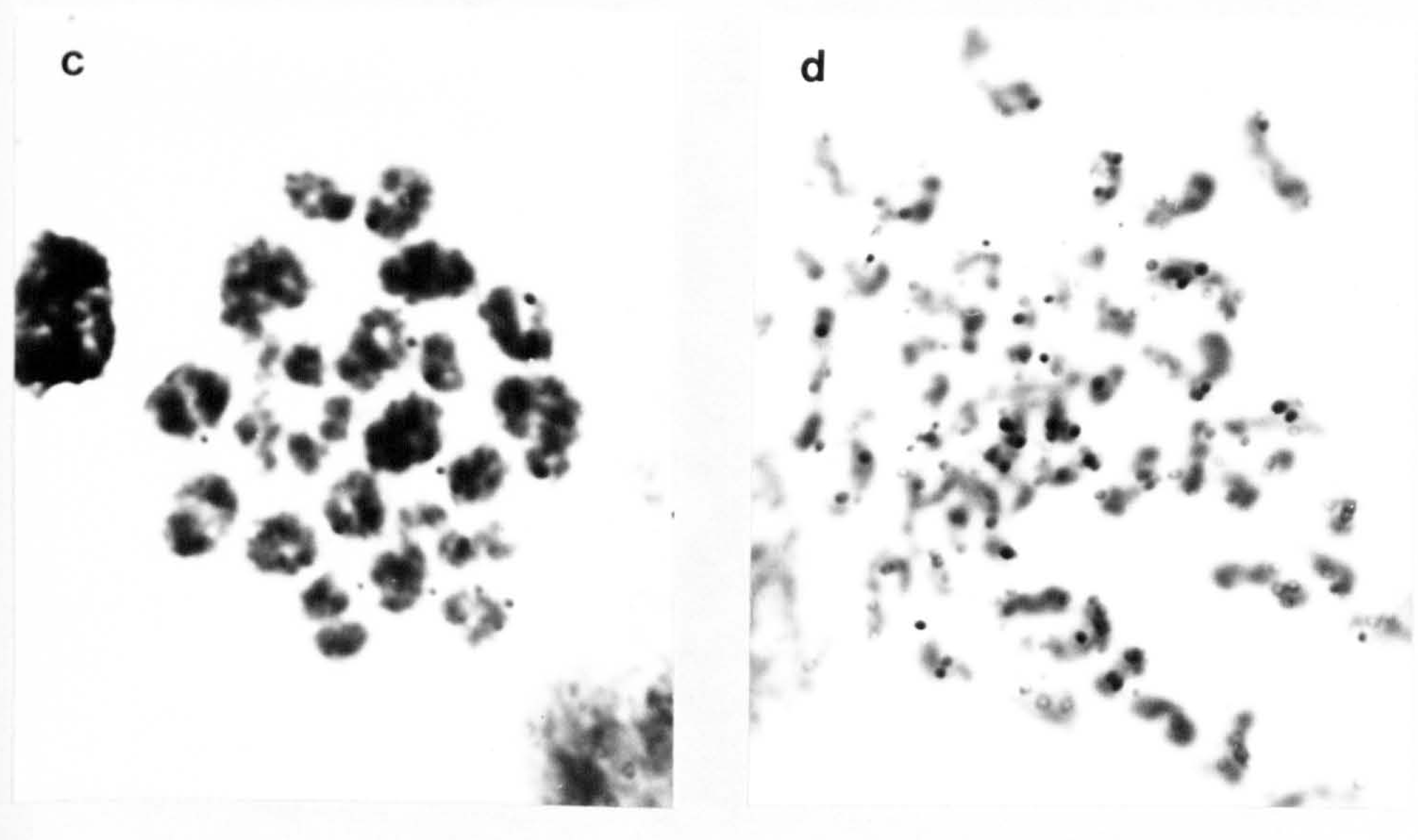
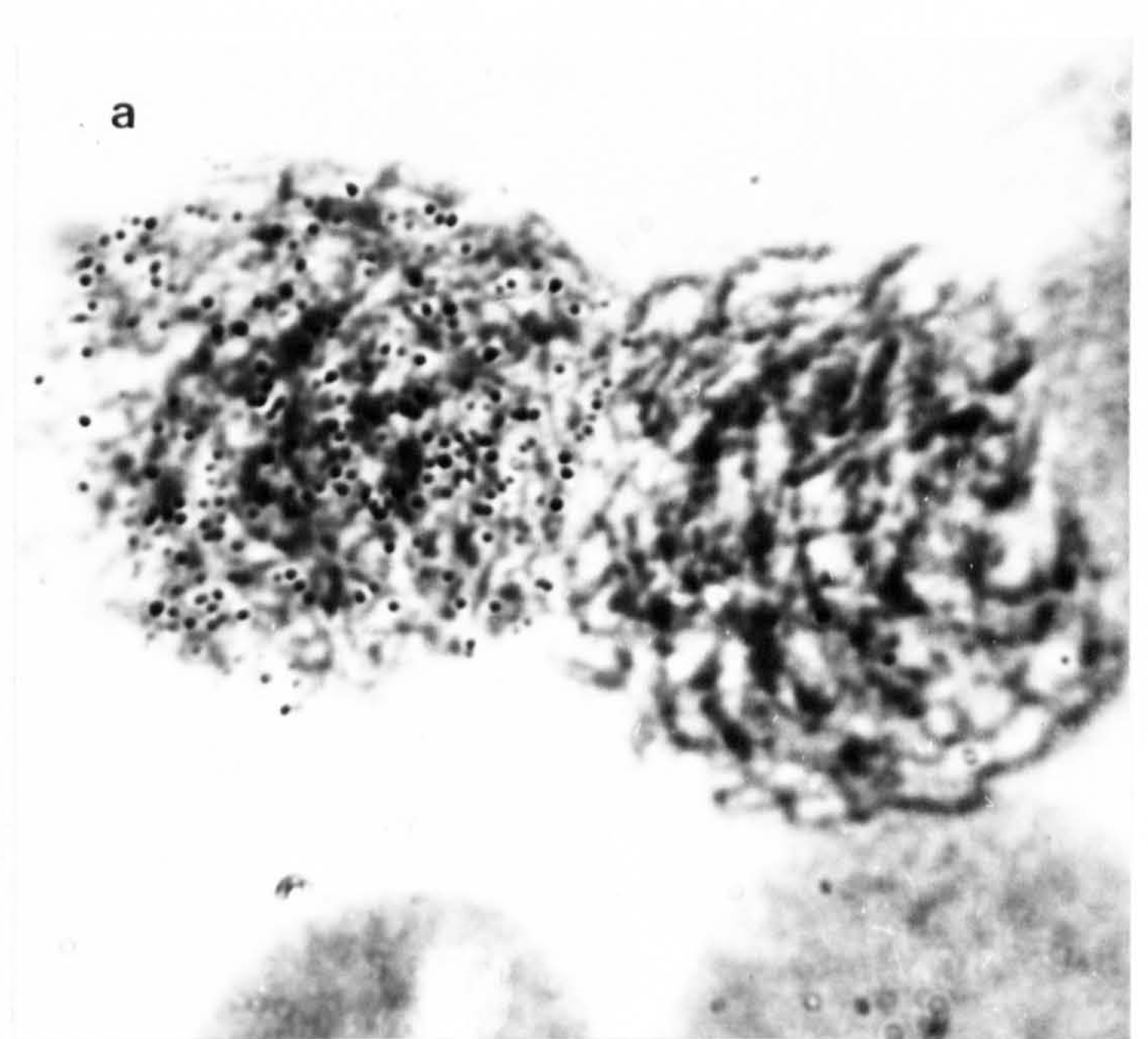


Figure 7.8

T11/4. 12 days in culture: photographed at x100. a) labelled prepachytene nucleus adjacent to unlabelled pachytene nucleus. b) labelled pachytene nucleus.



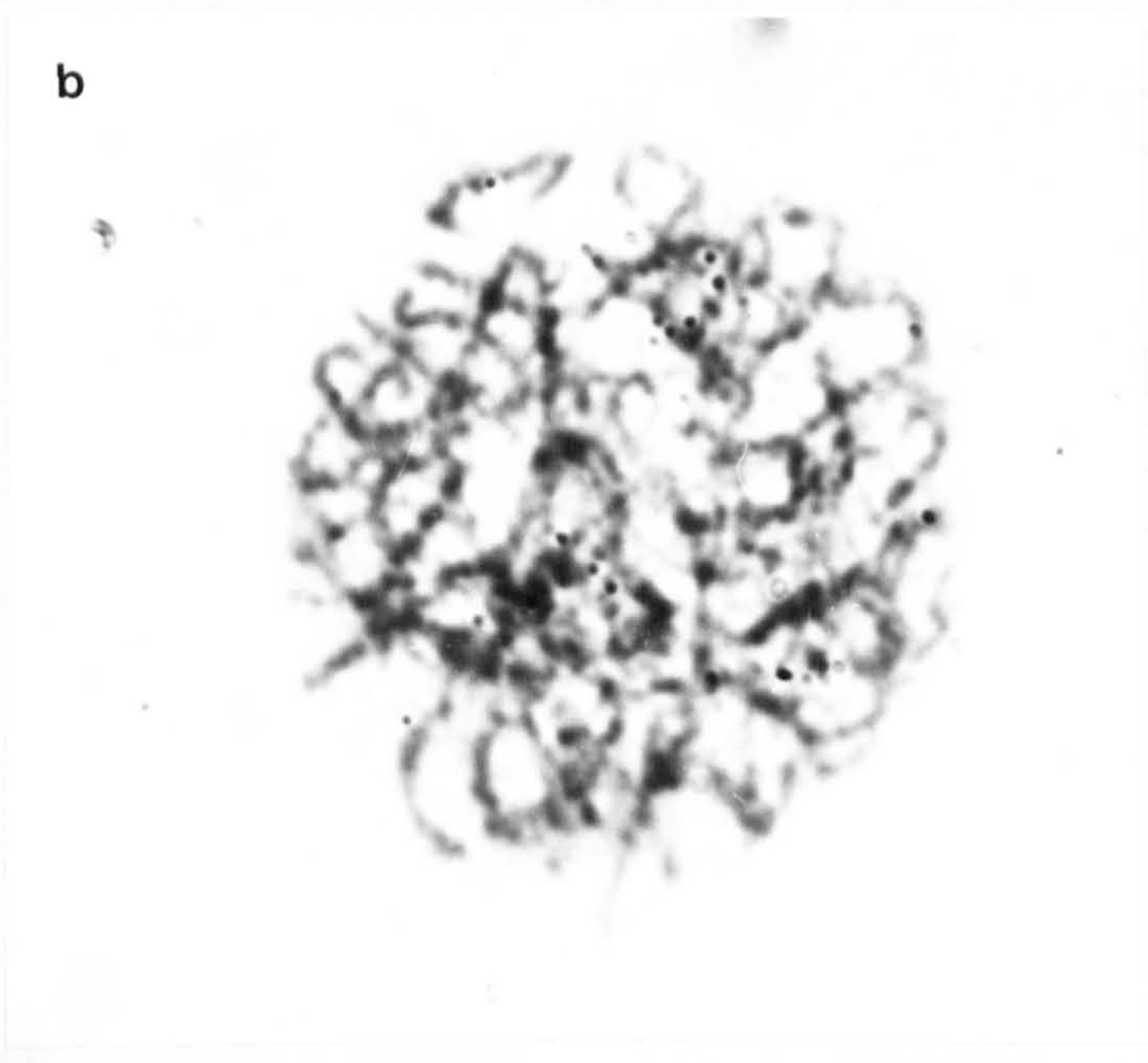
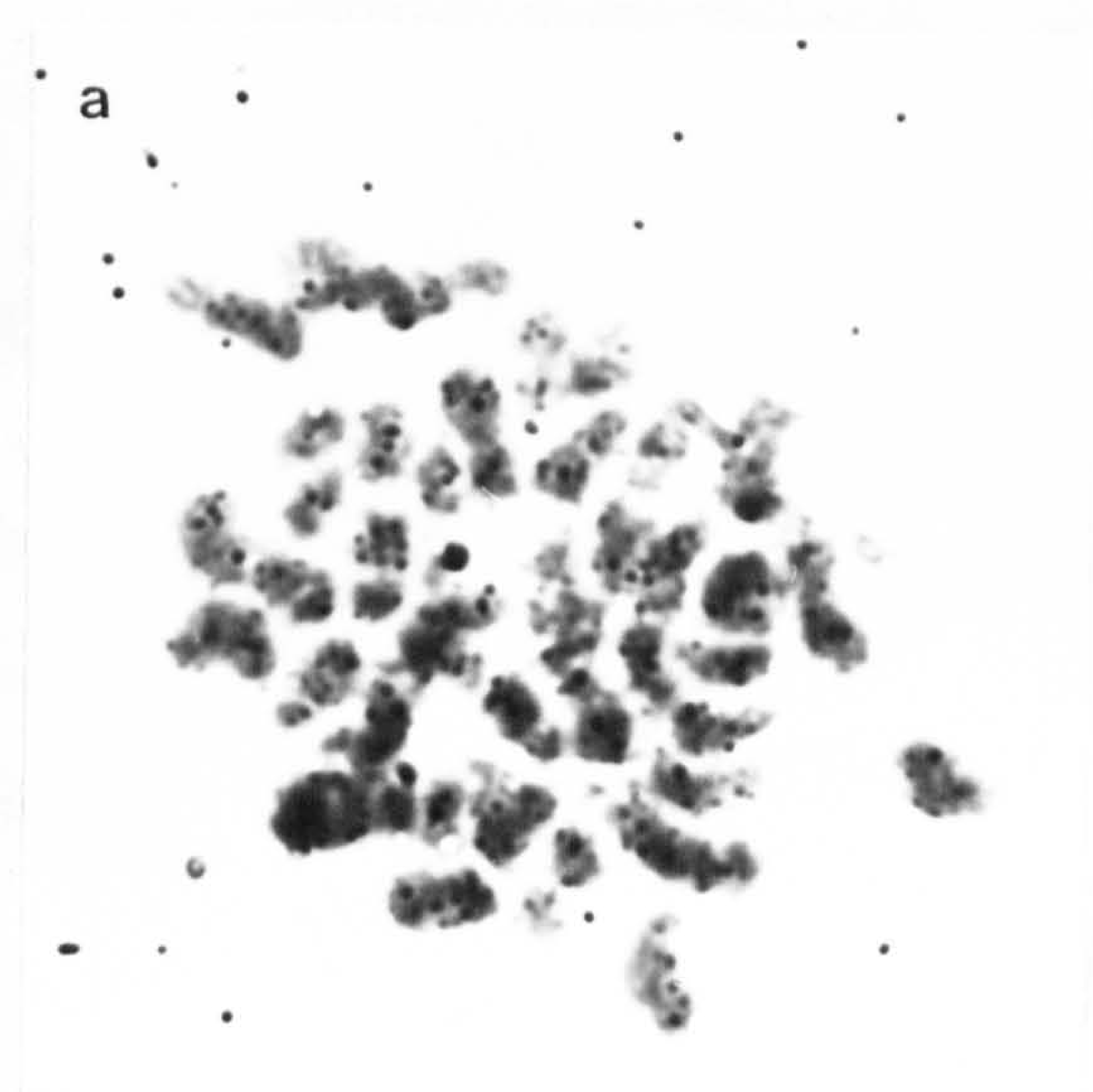


Figure 7.9

T14/2. 15 days in culture: photographed at x100. a) labelled spermatogonial mitosis. b,c) labelled diakenesis figures.



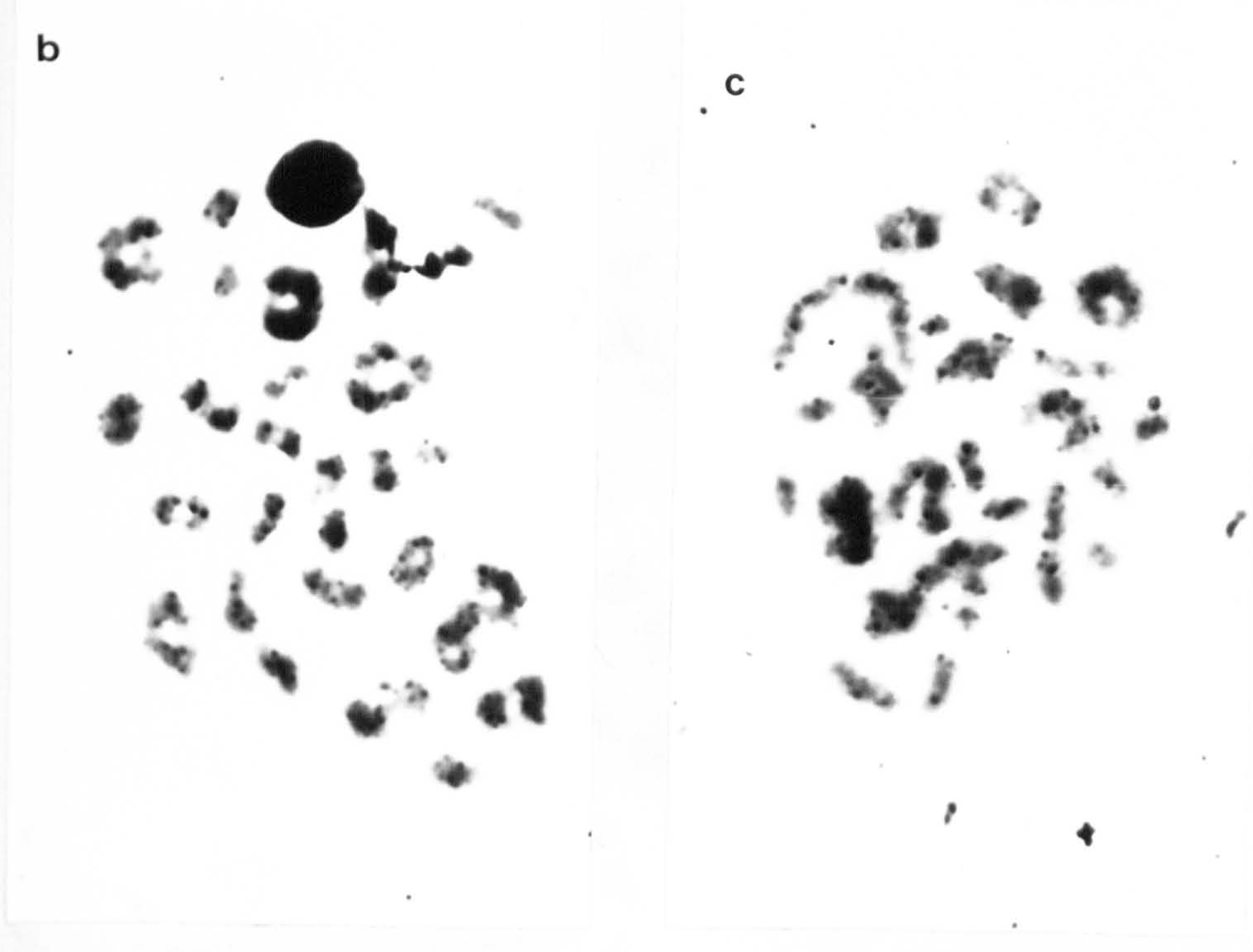
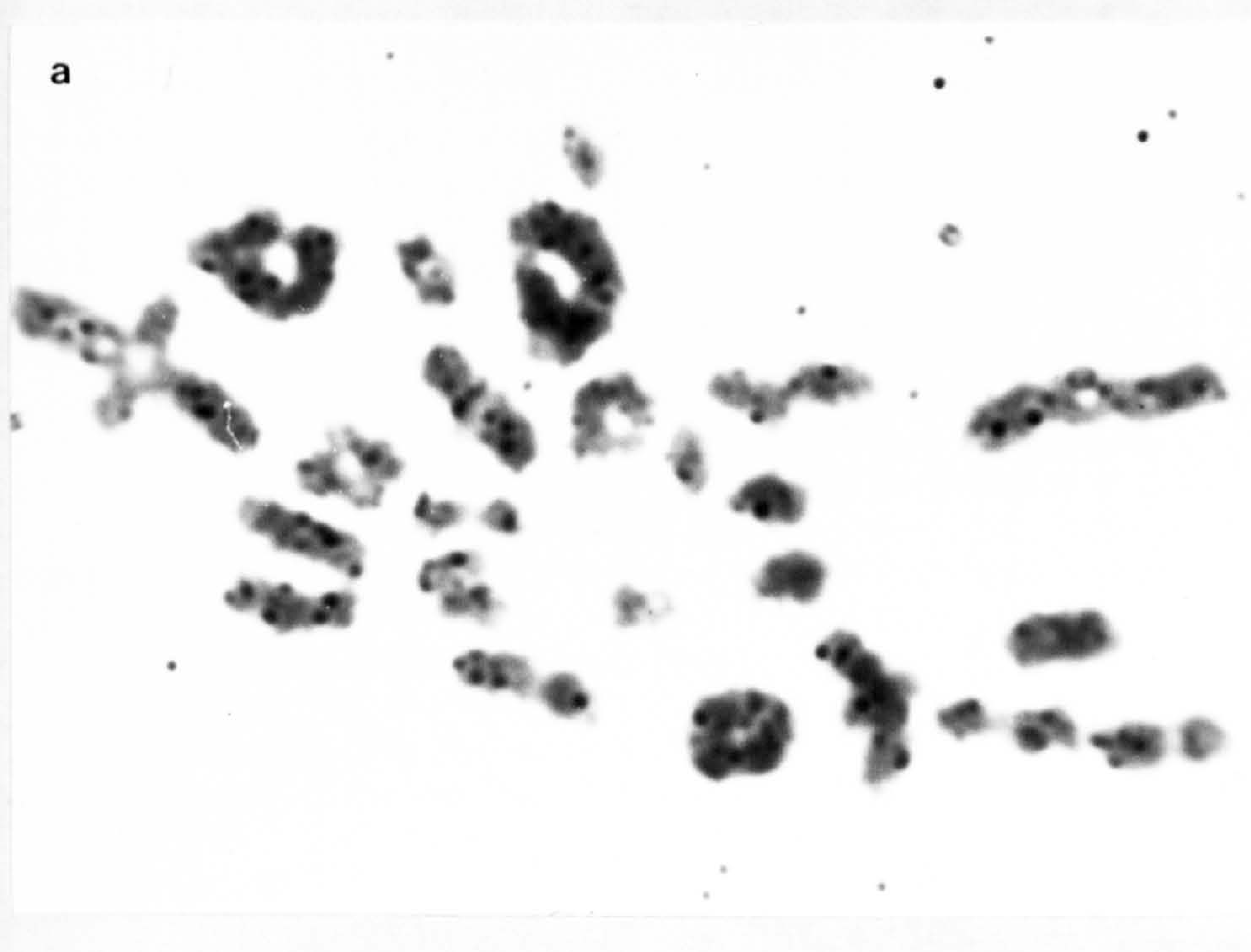


Figure 7.10

T14/4. 17 days in culture: photographed at x100. a) labelled diakenesis figure. b) labelled spermatogonial mitosis.



C. DISCUSSION

The purpose of this experiment was to test whether cells in long term culture continued to differentiate or whether the presence of cells at advanced stages of meiotic division after some days in culture was due to the maintenance of cells without differentiation at the stage they had entered the experiment. H^3Tdr was incorporated into the primary spermatocyte during S phase of meiotic division. A further minor amount of H^3Tdr incorporation might occur in the primary spermatocyte during chiasmata formation (Stern and Hotta, 1969; Chandley and Kofman-Alfaro, 1971) and this will be referred to as R (repair) synthesis.

Diakenesis figures were not recorded in the control preparations. Diakenesis figures recorded after 24 hours in culture were not labelled, therefore cells in diakenesis at or near to H^3Tdr pulsing were unable to incorporate H^3Tdr. If the labelled diakenesis figures observed in culture at day 10 after H^3Tdr pulsing had been present at culture initiation and had simply been maintained in culture they would not be labelled. The label seen in the figures at day 10 and at later times must have been incorporated during S phase or R synthesis. This supports the view that in vitro differentiation takes place. A supposition which covers the observed frequency of labelled figures is as follows. The surge of largely unlabelled figures at day 10 could be the result of development of cells largely in pachytene when H^3Tdr was administered. The few day 10 labelled diakenesis figures observed might have taken up H^3Tdr during R synthesis. The surge of figures continued but there was essentially a gap of 3 days before labelled diakenesis figures were again recorded at day 13. These labelled nuclei might have taken up H^3Tdr during S phase.

Pachytene figures were unlabelled in the controls and in the

24 hour sample. Some labelled pachytene figures were recorded after 10 days in culture. The label seen in these figures must have been incorporated during S phase or R synthesis. Unlike the diakenesis labelling results, these results did not provide any evidence relating to whether uptake was during R synthesis or S phase. The increasing frequency of degenerating "? pachytene" nuclei first observed at day 10 suggested that cells were partially blocked to further differentiation at this point. However, the results suggested that 24 hours after H^3Tdr administration was too short a time interval for recording the appearance of R synthesis in differentiating pachytene nuclei.

Label in prepachytene nuclei was present after 24 hours in culture but was not identified in the control samples. Heavily labelled nuclei, identified as prepachytene nuclei, were recorded in day 10 cultures and these nuclei increased in frequency around day 15. Label seen in these nuclei must have been incorporated during S phase or R synthesis. These results supported the supposition that R synthesis occurred very early in the meiotic division process and essentially before nuclei could be recognised in meiotic division. 24 hours after uptake of H^3Tdr, nuclei which were recognisable as in meiotic division (prepachytene nuclei) could be identified as labelled nuclei. The time span sampled did not permit separation of S phase and R synthesis. The increasing numbers of labelled prepachytene nuclei in the later samples suggested that these nuclei were unable to differentiate further.

4. CONCLUSIONS

The conclusions drawn from this experimental work were as follows :-

1. H^3Tdr had been incorporated during S phase or R synthesis.

2. Cells incorporating H^3Tdr had continued to differentiate under the conditions of the culturing method.

3. Differentiation had continued in culture up to and including the diakenesis stage of meiosis.

4. A surge of diakenesis figures was identified after 10 days in culture.

5. A suitable concentration of H^3Tdr to give optimum labelling patterns was tested and found satisfactory.

I did not observe fibroblast outgrowths from any of the biopsies cultured under these conditions.

CHAPTER 8

ANALYSIS OF THE MIXED CELL POPULATION DERIVED FROM THE DIFFERENTIATING TESTICULAR EPITHELIUM

1. Introduction

2. Classifying the mixed cell population
   - A. Subjective classification based on size and texture
   - B. Subjecting cell categorisation to measurement
   - C. Conclusions

3. Variation in the frequency of cell categories in the mixed cell population
   - A. Introduction
   - B. Analysis of cell category frequency within the individual
   - C. Analysis of variation in cell category frequency within the individual, due to time in culture
   - D. Analysis of variation in cell category frequency between individuals
   - E. Analysis of variation in cell category frequency taking differences between individuals and time in culture into account

4. Conclusions

1. INTRODUCTION

The observer, who examines preparations made from testicular biopsies, is confronted by a rich variety of cell types which vary both in size and in nuclear detail. These cells are a mixed cell population which is derived from the differentiating testicular epithelium and which represents the different cellular stages of the spermatogenic cycle. These cell types include the generations of spermatogonia which have received so little attention from previous workers. It was essential, therefore, to attempt some classification of recognisable cell types.

Mixed cell populations can be prepared by modifications of the methods of Meredith (1969) or Evans et al (1964). The two types of preparation differed in three respects. In Meredith type preparations,

1. there was a wide range of nuclear size differences,
2. there appeared to be better preservation of nuclear morphology,
3. the cell sample represented all the cells from one section of intact tubule.

The first two differences can be related to differences in fixation since, in general, fixation plays a major role in the preservation of structural detail in fixed cells. The third difference is a feature of the different approach to handling and preparing the material.

Thus Meredith's method was the preferred method for studying the mixed cell populations, harvesting material grown in vitro and preparing chromosomes for meiotic analysis.

Whilst reference is made to cell populations and cell categories, it is in fact the nuclei of cells which are visible and which are described throughout this work.

2. CLASSIFYING THE MIXED CELL POPULATION

The problem at this stage was to find some way of establishing, by the most objective means possible, a classification of the visually distinctive cell types present in the mixed cell population. One difference existed which was due to nuclear size. When numerical data are available it is possible (although by no means simple) to establish divisions on statistical criteria. A further difference existed which was due to differences in nuclear structure. This latter feature, in conjunction with staining intensity, will be referred to as texture. Texture together with size represented the overall visual impact of the nucleus. In these mixed cell populations the most distinctive feature was texture. Pattern recognition is a currently active research field but in practice no unbiassed instrumental method is available for analysing features such as texture. It was therefore decided to establish a subjective classification based on a combination of texture and relative size and subsequently to check this for reproducibility.

A. Subjective classification based on size and texture

Many preparations were studied visually before starting this part of the work. The subjective classification of cell types was based on photographs taken of cell populations from six individuals who were thought to have tubules undergoing more or less normal spermatogenesis. A list of these individuals is given in Table 8.1, together with reasons for biopsy, sperm counts and Pathologists report. The seven categories of cell types selected as distinctive are shown in Figure 8.1, together with a brief description of texture and size typical for each category. In Figure 8.1 examples have been selected from two individuals to give some idea of the variation encountered in each category. There were a few other cell types seen which did not fit these seven categories and these were collected together in a miscellaneous group H.

TABLE 8.1

DETAILS OF SIX INDIVIDUALS SELECTED FOR CELL CATEGORISATION

Case No.	Reason for Biopsy	Sperm Count	Pathology report	Sample times
NT11	Investigation of testicular lump	NK	NK	0 hrs 24 hrs 10 days
T51	Infertility Varicocoele repair	11.5×10^6/ml	Spermatogenesis reduced Desquamation of precursors into lumen	0 hrs 24 hrs
VI	Orchidectomy	NK	Spermatozoa present Scant epithelium	0 hrs 96 hrs 7 days
T105	Infertility Blocked epididymus	Azoospermic	NK	0 hrs 0 hrs
T133	Infertility Varicocoele repair	13.5×10^6/ml	Spermatozoa reduced Maturation arrest	0 hrs 24 hrs 48 hrs 72 hrs
T80	Infertility	17.0×10^6/ml	NAD Lumen blocked	0 hrs 0 hrs 24 hrs 48 hrs

1. Category A was a very clearly defined group of large nuclei with pale overall staining and no distinctive texture.
2. Category B nuclei were clearly defined by their distinctive texture and large size. The texture included an intense dark staining area which was the sex vesicle. Both textures and size varied widely in this group but the smallest B nucleus was always one of the largest nuclei in the cell population.
3. Category C nuclei were smaller than Category A nuclei and had intensley staining chromatin condensations over the nucleus. Some of the condensations were peripheral.

Example of nucleus	Size	Texture	Category
	Large	Overall pale No distinctive texture	A
	Large	Overall Moderate to dark Distinctive texture present	B
	Medium	Overall pale No distinctive texture Dark staining spots present	C
	Medium	Overall moderate to dark Distinctive texture present	D
	Medium to Small	Overall dark Distinctive texture present	E
	Small	Overall pale No distinctive texture Dark staining spots present	F
	Small to Tiny	Overall dark No distinctive texture	G

4. <u>Category D</u> nuclei were medium sized and moderately darkly stained. The texture in these nuclei was important and can best be described as "resembling a brain". The texture was not grainy or fibrous and the intensity of stain was usually evenly distributed.

5. <u>Category E</u> nuclei were sometimes difficult to identify and in most samples these nuclei were sparse in numbers. The staining was intense and the structure was distinctly chromosome-like.

6. <u>Category F</u> nuclei closely resembled Category C nuclei except that they were smaller. Sometimes the chromatin bodies appeared larger and more regular and more solidly formed than in Category C nuclei.

7. <u>Category G</u> nuclei were small to tiny nuclei with an intensely stained appearance. Sometimes G nuclei were extremely regular, other times this was not so.

B. <u>Subjecting cell categorisation to measurement</u>

Having established cell categorisation subjectively some check for objectivity was obviously essential. It has already been pointed out that texture cannot be quantified but one component of the analysis involved size and this was certainly amenable to investigation.

(i) <u>Method</u>

There are a number of hazards in attempting this type of cell measurement. First, sample preparation involved a prefixation swelling treatment. Thus samples of tubules from different individuals were subjected to independant swelling regimes and it was not possible to be sufficiently accurate with swelling time measurement to assure

homogeneity of swelling. Also, different tubule samples will probably respond differently to swelling however well controlled the actual time of treatment. Secondly, preparation of cell samples from intact fixed tubules required the digestion of tubules with 60% acetic acid. The time of digestion and the time the dispersed cells remain in 60% acetic acid before samples were sequentially put onto the slide affected cell size. Both events were difficult to control with sufficient accuracy for cell measurement experiments. In order to alleviate, as much as possible, the considerable component of variation in size due to technical preparation of samples, photographs were taken of samples of 400 adjacent cells. Each sample of 400 cells was taken from one circle of cells on one slide from each individual investigated. Cells were photographed on a Leitz Orthomat-W automatic camera system using a x54 oil immersion objective. All photographic prints were enlarged at a standard lens height of 24 cm giving enlargement of x4.53 under standard development conditions. The individual photographs were reconstructed as a mosaic panoramic view of the cell sample with a total enlargement of x approx. 3,000. It was thought that nuclear area would be a better measure of size than some single "diameter" measurement. Consequently the product of the maximum diameter and a line at right angles to it was taken and expressed as millimetres squared. This was the area of the smallest enclosing rectangle of the nucleus which was directly proportional to nuclear area. 400 nuclei in each cell sample studied were assigned to one of the cell categories A-H. The 400 nuclei were then measured and the measurements recorded against the designated cell category. Means and standard deviations for each cell category within each sample were calculated. The data are recorded in Table 8.2 together with times at which samples were harvested. Samples, throughout

TABLE 8.2

MEANS AND STANDARD DEVIATIONS FOR MEASUREMENTS OF CATEGORISED NUCLEI

Sample No.		CELL CATEGORY							Hours in culture
		A	B	C	D	E	F	G	
11/1	x̄	587	616	286	303	248	137	103	0 hours
	sd	268	169	105	127	81	40	36	
11/2	x̄	923	1018	376	512	331	204	117	24 hours
	sd	302	399	115	154	106	70	42	
11/3	x̄	667	684	266	424	285	159	106	10 days
	sd	270	200	62	156	110	41	50	
51/1	x̄	1021	710	354	361	278	147	87	0 hours
	sd	345	342	147	132	92	39	21	
51/2	x̄	855	781	385	410	364	175	76	24 hours
	sd	346	288	146	115	129	55	31	
VI-1/2	x̄	774	859	305	360	335	142	101	0 hours
	sd	291	361	104	97	98	29	35	
VI-5/2	x̄	497	466	204	492	221	102	69	96 hours
	sd	219	157	63	210	71	32	31	
VI-6/2	x̄	928	793	316	438	300	105	98	7 days
	sd	417	332	148	127	104	39	43	
105/2-1	x̄	976	959	391	481	322	194	115	0 hours
	sd	350	380	118	176	161	91	26	
105/3-1	x̄	1001	912	373	389	384	177	113	0 hours
	sd	371	355	121	147	142	29	27	
133/1	x̄	440	518	219	267	244	108	74	0 hours
	sd	162	141	62	90	94	30	38	
133/2	x̄	571	502	243	262	230	110	95	24 hours
	sd	305	191	66	82	111	28	44	
133/3	x̄	561	526	237	264	too small sample	115	80	48 hours
	sd	190	200	69	80		35	47	
133/4	x̄	453	439	227	256	"	104	65	72 hours
	sd	210	149	65	60	"	29	25	
80I/1	x̄	828	1055	366	420	399	174	125	0 hours
	sd	334	322	146	124	173	55	64	
80I/2	x̄	573	620	238	332	299	120	77	24 hours
	sd	213	261	69	89	113	30	41	
80I/3	x̄	443	451	232	263	229	106	97	48 hours
	sd	140	136	73	82	72	30	40	
80II/1	x̄	959	1104	372	448	413	171	117	0 hours
	sd	321	345	121	158	190	43	40	

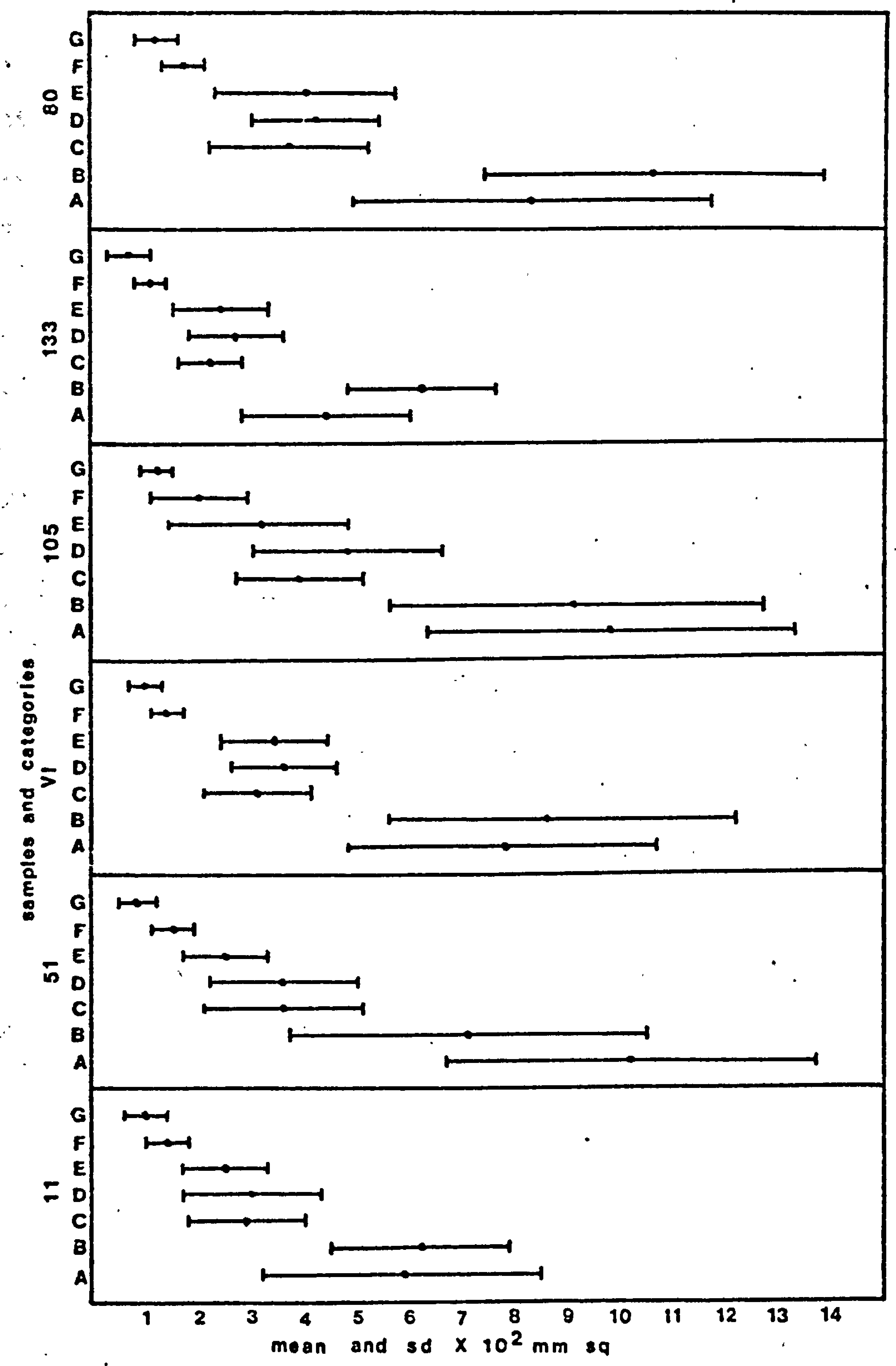
this section of work, were from individuals listed in Table 8.1.

(ii) Results and discussion

Figure 8.2 shows the mean and standard deviation for each cell category from six individuals at 0 hours in culture. Throughout this work (unless otherwise stated) 0 hours refers to initiation of cultures following incubation with H^3Tdr. Category A and B are not distinguishable from each other on size alone but their distinctively different textures make them identifiable as two distinct categories. Categories C, D and E similarly are not separate on size but they are texturally distinctive. Categories C and F are texturally similar. Their size range in 4 out of the 6 individuals chosen is distinctly separate which suggests that placing them in two separate categories rather than in one category with a very wide size range is valid. Category E cells were few in number in most samples studied and this category was one of the hardest to define. Their size range was distinct from Category B cells. Category E cells appeared denser and more intensely stained than Category D cells from which they could not be distinguished on size alone. Finally, Category G cells were always the smallest cells in the sample. They probably represented two cell types distinguishable (though not so distinguished in this analysis) into smaller more regular and more intensely staining cells and relatively larger, less regular and less intensely staining cells.

Figure 8.2

Mean and standard deviation for each cell category from 6 individuals at 0 hours in culture



C. Conclusions

The visual cell categorisation appears valid. Cell measurement data confirms that the subjectively selected cell categories represent distinct cell types present at different stages during spermatogenesis. Thus the various elements of the mixed cell population can be separated and catalogued. The data recorded in Table 8.2 demonstrate that it is impossible to make between sample comparisons on an absolute size basis. This is probably due to differential swelling during slide preparation.

The dynamic nature of the differentiating spermatogenic cell population has not been forgotten whilst analysing cell categorisation. The wide variation in size within each cell category represented by the large standard deviation probably represents the process of growth in spermatogenic cells.

3. VARIATION IN THE FREQUENCY OF CELL CATEGORIES IN THE MIXED CELL POPULATION

A. Introduction

The cell categorisation analysis recorded in Section 2B also gave the number of nuclei assigned to each cell category. This data provided an estimate of the frequency of each cell category in each sample.

Table 8.3 records the numbers of nuclei assigned to each cell category in each sample studied and the times at which these samples were harvested. The individuals studied are those previously listed in Table 8.1.

The results presented in Table 8.3 show that cell categories vary in frequency both between categories within individuals and within

TABLE 8.3

NUMBER OF CELLS IN EACH CATEGORY

Sample No.	CELL CATEGORY								Total No. nuclei	Time in culture
	A	B	C	D	E	F	G	H		
11/1	55	69	57	96	21	78	24	0	400	0 hours
11/2	104	59	60	50	35	45	25	22	400	24 hours
11/3	137	26	28	76	13	35	29	56	400	10 days
51/1	59	88	76	56	38	51	12	20	400	0 hours
51/2	52	114	72	70	26	51	10	5	400	24 hours
VI/1-2	98	122	94	16	9	25	10	26	400	0 hours
VI/5-2	77	41	36	31	34	61	84	36	400	96 hours
VI/6-2	106	86	37	33	33	40	42	23	400	7 days
105/2-1	81	90	107	62	19	29	9	3	400	0 hours
105/3-1	94	88	95	60	25	26	11	1	400	0 hours
133/1	68	53	70	75	17	73	28	16	400	0 hours
133/2	70	56	58	51	24	103	25	13	400	24 hours
133/3	73	74	55	33	10	87	37	31	400	48 hours
133/4	132	63	33	31	10	74	18	39	400	72 hours
80I/1	58	98	75	71	18	35	25	20	400	0 hours
80I/2	75	81	94	22	9	56	30	33	400	24 hours
80I/3	83	73	43	59	24	63	20	35	400	48 hours
80II/1	45	100	101	74	8	35	10	27	400	0 hours

categories between individuals. Variation is also encountered as a result of time in culture. The subsequent wections analysed components of this variability.

B. Analysis of variation in cell category frequency within the individual

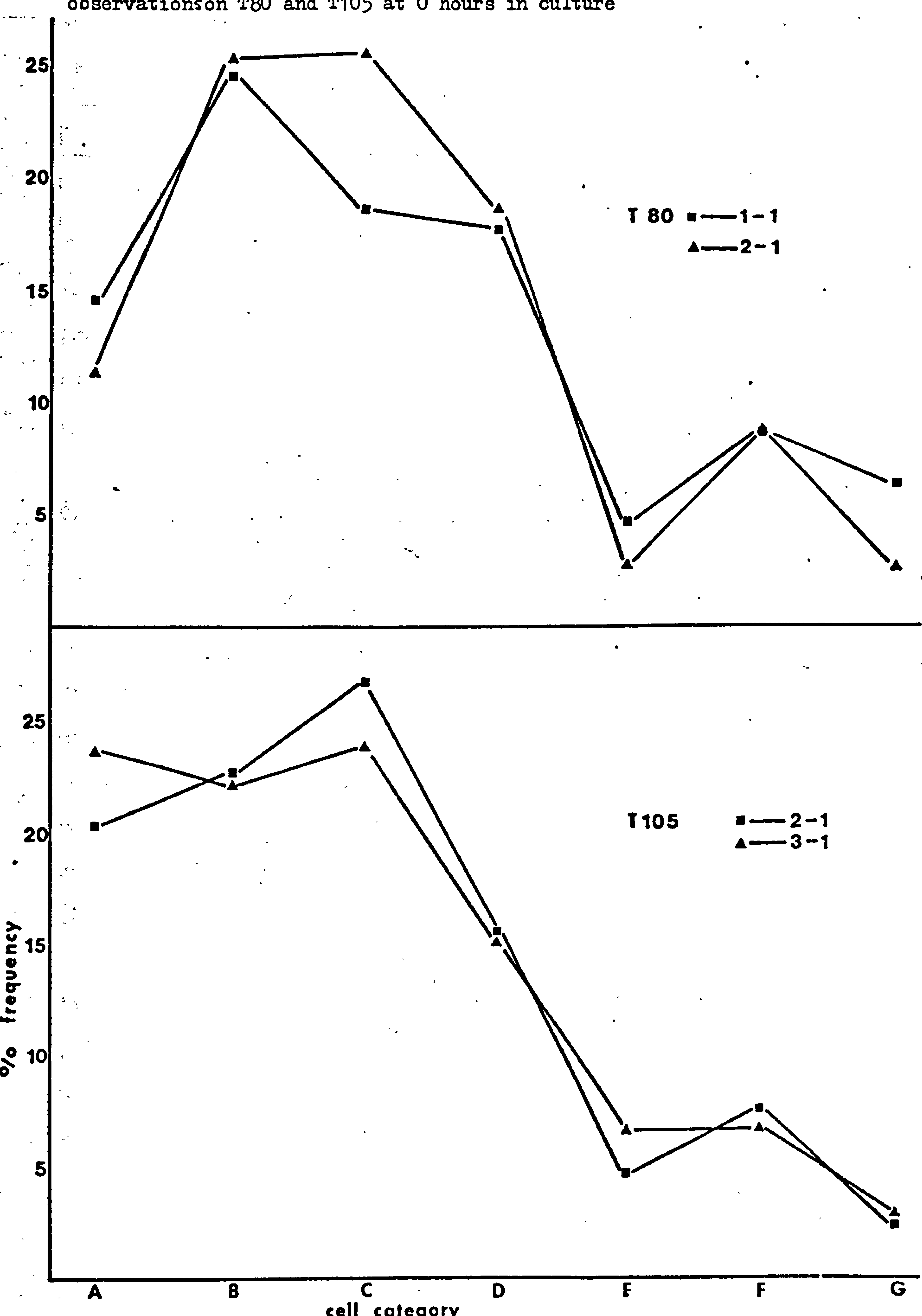
As noted above there are variations in frequency between cell categories within individuals. Therefore the question which must first be resolved is "are these frequencies reproducible?". Or, in other words, "is the sample of 400 nuclei a reliable estimate of the diversity of the mixed cell population within each individual?"

Within the individuals studied there are replicate samples from two individuals, T105/2-1 and T105/3-1, T80I/1 and T80II/1. Both represent two different samples of the testicular biopsy harvested as controls.

It would have been possible to replicate any of these scores by making a second count of 400 nuclei of any of the samples studied. The labour involved in this type of analysis expedited the particular choice of replicates since data for these samples had already been analysed. In fact, they are more rigorous replicates than counting a second set of 400 nuclei from the same preparation as the first set, since they come from a different sample of tubules. Figure 8.3 is a graph showing the percentage frequency for replicate observations on these two individuals. The graphs show good replication of frequency data within individuals. Category H has been omitted from the graphs since it will be remembered that this category is a collection of miscellaneous unclassifiable nuclei. Figure 8.3 also shows that there is a difference between these individuals in the frequencies recorded for particular cell categories. This replicate data was tested using 2xn contingency X_2 for heterogeneity between replicates. The actual numbers of nuclei were used in the statistical calculations. Categories E and G were excluded from the calculations from both sets of data since

Figure 8.3

Percentage frequency of cell categories recorded for replicate observationson T80 and T105 at 0 hours in culture



there were too few numbers of nuclei recorded in these categories in one or other of the sets of data. The results were as follows :-

Samples tested	X_2	df	p	Significance
105/2 and 105/3	1.848	4	>70%	non-significant
80/1 and 80/2	5.099	4	30-20%	non-significant

Thus there was no significant difference between replicate observations from the two individuals tested. The frequency data was therefore reproducible and accepted as a good estimate of the mixed cell population from which the sample was taken.

C. Analysis of variation in cell category frequency, within the individual, due to time in culture

Having established reproducibility of frequency data it was possible to proceed to the question of whether time in tissue culture affected the frequency of cell categories. Table 8.3 records the numbers of nuclei within each cell category for T133 for harvests made at 0 hours, 24 hours, 48 hours and 72 hours in tissue culture. Statistical analysis of the 4 samples at 4 times of harvest for T133 using r x c contingency X_2 showed only that there was a large amount of variation present in the data (X_2 for 18df = 99.582; p = <0.1% - highly significant).

In order to examine in more detail the variation in frequency due to time in tissue culture, the analysis was divided into comparisons between paired samples relative to time in tissue culture. The method of analysis used was the 2xn contingency X_2 test using actual numbers of nuclei observed and excluding categories with recorded numbers of nuclei of ten or less.

The results of these tests are recorded in Table 8.4.

Figure 8.4 is a diagram representing these results. The paired observations are represented by lines joining the two samples studied,

TABLE 8.4

2xn CONTINGENCY X_2 TEST OF PAIRED OBSERVATIONS AFTER VARIOUS TIMES IN CULTURE

Paired Samples	χ^2	df	P	Significance
133/1 - 133/2 (0 hours - 24 hours)	12.275	6	10-5%	*
133/1 - 133/3 (0 hours - 48 hours)	24.169	5	<0.1%	***
133/1 - 133/4 (0 hours - 72 hours)	102.413	5	<0.1%	***
133/2 - 133/3 (24 hours - 48 hours)	10.140	5	10-5%	*
133/3 - 133/4 (48 hours - 72 hours)	30.953	5	<0.1%	***
133/2 - 133/4 (24 hours - 72 hours)	36.887	5	<0.1%	***

Asterisks used to denote levels of significance throughout tables

*	borderline significance
**	significant
***	highly significant

the figure above the line is the value of X_2 and the figure under the line is the probability. This diagram helps to demonstrate that there is least change between 0 hours and 24 hours and between 24 hours and 48 hours. There is most change between 48 and 72 hours. Taking samples separated by more than 24 hours in tissue culture shows that there is a high level of heterogeneity between samples.

This analysis demonstrates that cell category frequency changes

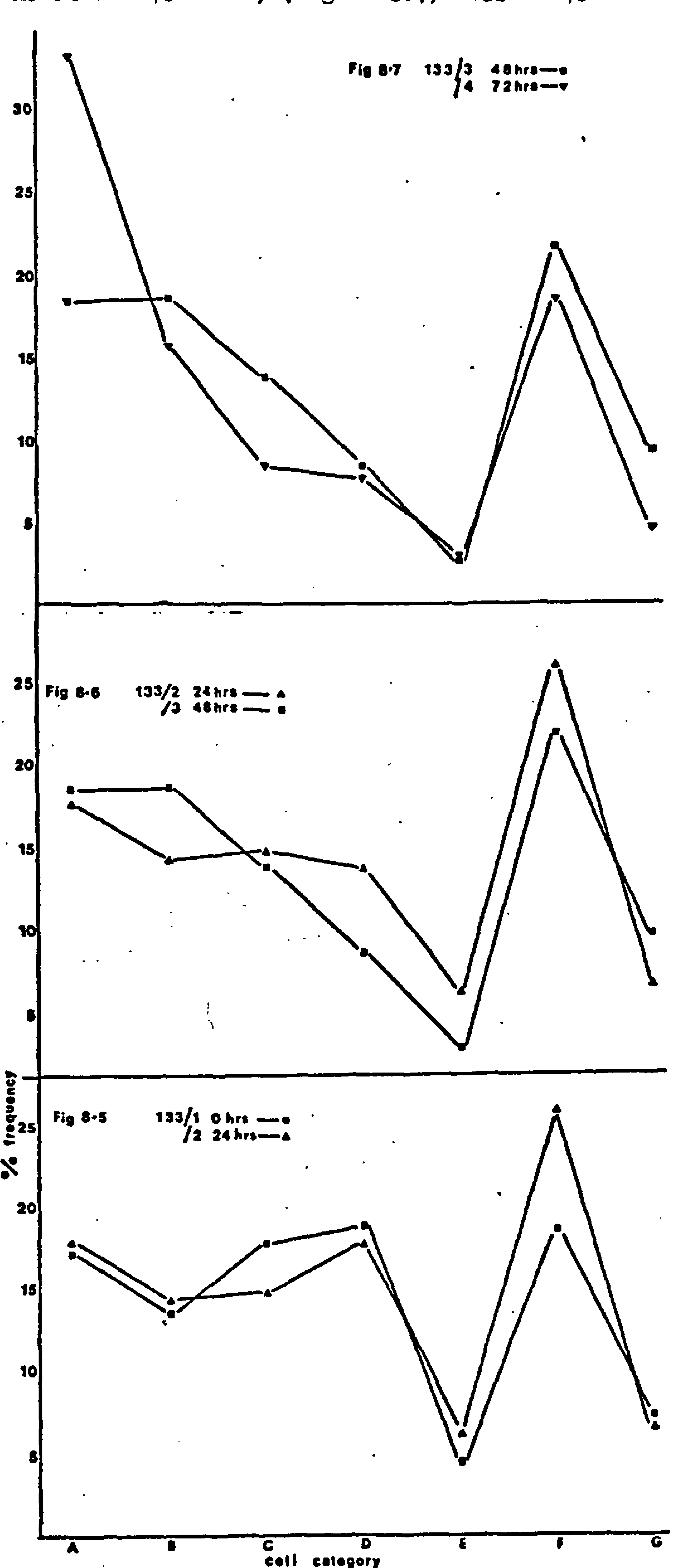
Figure 8.4

Summary of X_2 between paired observations, T133

T133 samples	1 2 3 4
Paired observations. χ_2 probability	12 10 - 5% 10 10 - 5% 31 <0.1% 24 <0.1% 36 <0.1% 102 <0.1%

with time in tissue culture. However, as yet nothing is known about how the variability is distributed. It might be confined to particular categories or it might be an overall change affecting all categories more or less equally. Figures 8.5, 8.6 and 8.7 are graphs of the percentage frequency of each cell category between the paired observations separated by 24 hours in tissue culture, i.e. between 133/1 and 2, 133/2 and 3, and 133/3 and 4. The graphs suggest that some categories vary more than others. In order to test this statistically, each paired observation was treated in detail. The numerical differences between each category in a paired observation were found and marked

Figures 8.5, 8.6, 8.7: % frequency of cell categories between (Figure 8.5) T133 at 0 hours and 24 hours; (Figure 8.6)T133 at 24 hours and 48 hours; (Figure 8.7) T133 at 48 hours and 72 hours



from highest to lowest. Cumulative X_2 tests were then performed by subtracting categories in decreasing order of difference until X_2 was non-significant and the samples were homogeneous over the remaining categories. Thus for paired observations between 133/1-2 the results were as follows :-

Categories tested	X_2	df	p	Significance
All categories	12.275	6	10- 5%	borderline
All categories -F	5.960	5	50-30%	non-significant

Within the paired observation 133/1-2 the category which contributed significantly to the overall variation was Category F.

Between paired observations 133/2-3, X_2 became non-significant when either of the equally differing Categories B or D were removed. Categories B and D together were significantly heterogeneous. Therefore between samples 133/2-3 the categories which contributed most with equal weight to the overall variation were B and D. Between paired observations 133/3-4, Category E was excluded due to too few observations. X_2 became non-significant with the subtraction of Category A, therefore Category A contributed most variation to the heterogeneity between samples 133/3-4. Figure 8.8 describes these results. The actual changes in frequency which occurred within categories which varied significantly were those between 133/1-2, F increased in numbers; between 133/2-3, D decreased whilst B increased in numbers and between 133/3-4, A increased in numbers.

Three other individuals had data recording sequential harvests during the early stages of long term culture. The results which were obtained from these individuals together with T133 are listed in Figure 8.9.

Figure 8.8

Analysis of categories contributing most variation to the between sample differences within T133

133/1	133/2	133/3	133/4
0 hrs	24 hrs	48 hrs	72 hrs

all cats, 10-5% (133/1 – 133/2)

all cats, -F, 50-30% (133/1 – 133/2)

all cats, 10-5% (133/2 – 133/3)

all cats, -D/B, 10-20% (133/2 – 133/3)

all cats, <0.1% (133/3 – 133/4)

all cats, -A, >20% (133/3 – 133/4)

F increases (133/1 – 133/2)

D drops
B increases (133/2 – 133/3)

A increases (133/3 – 133/4)

Figure 8.9

Analysis of categories contributing most variation to the between samples with time variation for individuals T51, T133, T11 and T80

Paired Observations	A	B	C	D	E	F	G	Significance and Varying categories
T51/1 /2 O - 24 hours								not significant
T133/1 /2 O - 24 hours						*		borderline sig. F F increase
T133/2 /3 24 - 48 hours		*		*				borderline sig. B=D B increase D decrease
T133/3 /4 48 - 72 hours	*							highly sig. A A increase
T11/1 /2 O - 24 hours	*			*		*		highly sig. A>D>F A increase D+F decrease
T80I/1 I/2 O - 24 hours				*		*		highly sig. D>F D increase F increase
T80I/2 I/3 24 - 48 hours			*	*				highly sig. C>D C decrease D increase

* Categories contributing significantly to heterogeneity between samples

These results demonstrated the extremely variable nature of this material. There was one individual, T51, with homogeneous frequencies between 0-24 hours in culture. Perhaps this event represented no growth and/or death in culture. All other individuals showed some change. Categories D and F seemed to be important indicators of change during culture. Category F was variable in 3 out of 4 of the 0-24 hours comparisons, but was not significantly variable except at 0-24 hours. In two samples Category F increased in frequency, in one sample it decreased in frequency. Category D was significantly variable in four out of seven comparisons but variability was not restricted to any one time span.

In this Section it has been possible to describe in detail the changes which occurred with time in culture in 4 individuals. The results indicate a large amount of variation due to differences between individuals which may mask significant changes with time in culture.

D. Analysis of variation in cell category frequency between individuals

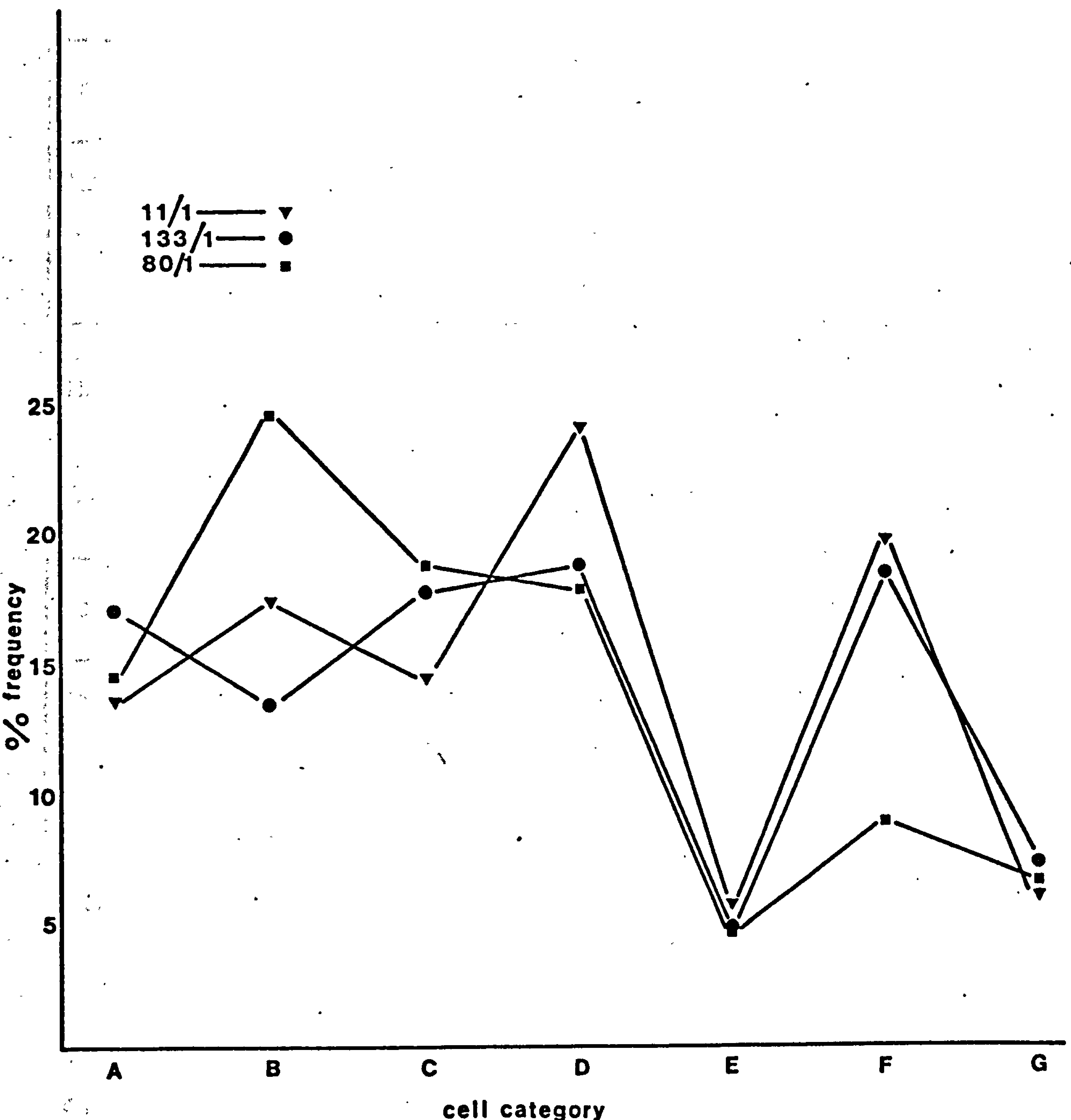
Analysis of the data thus far has indicated that considerable variation exists between individuals. Inspection of the data suggested that some individuals were more alike and some were less alike. This assumption could be tested using the 2xn contingency test between paired individuals and using data obtained from samples harvested at 0 hours. Two individuals, T133 and T11 were homogeneous at all categories tested.

Thus X_2 for 6df = 7.953, p = 30-20%, non-significant.

Figure 8.10 is a graph which represents the frequencies for T133, T11 and T80. T80 obviously differs only slightly from T133 and T11. Therefore categories which contributed significantly to the variation

Figure 8.10

Variation in % cell category frequency between individuals T133, T11 and T80 at 0 hours in culture



were sought using the method of progressively excluding categories with the largest num erical difference outlined in Section C. When T80 was compared with T133 the following results were obtained :-

Sample	Categories tested	df	X_2	p	Significance
T80/T133	All categories	6	20.034	<0.1%	Highly significant
	-B Category	5	10.796	10-5%	Borderline significant
	-B-F Category	4	1.034	90-80%	Not significant

Thus between T80 and T133 the difference is significant at B>F categories.

When T80 was compared with T11 the following results were obtained :-

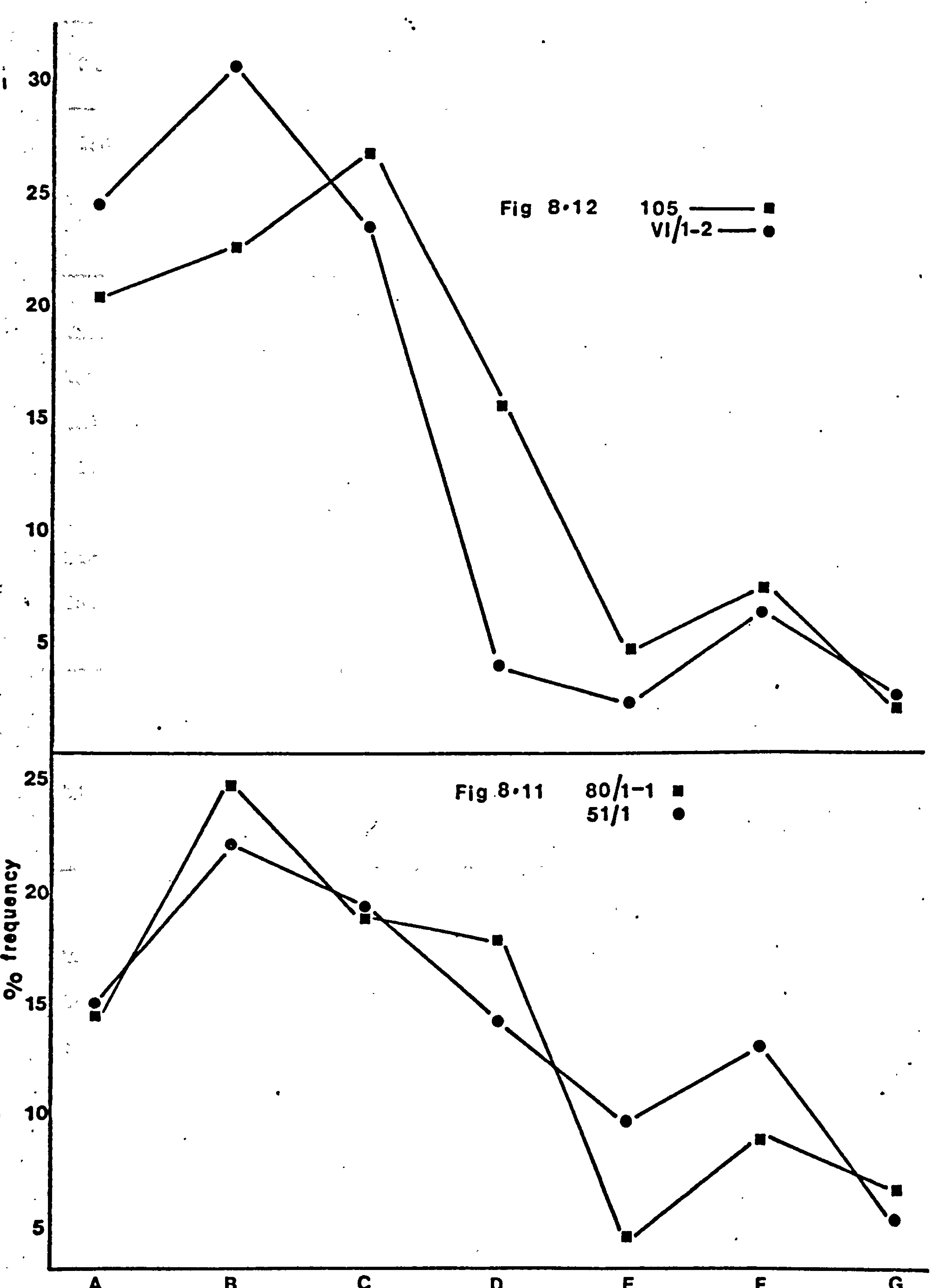
Sample	Categories tested	df	X_2	p	Significance
T80/T11	All categories	6	27.43	<0.1%	Highly significant
	-F Category	5	10.783	10-5%	Borderline significant
	-F-D Category	4	6.456	20-10%	Not significant

Thus between T80 and T11 the difference is significant at F>D categories.

In summary, these results record that T11 and T133 are homogeneous, whereas T80 differs from T11 and T133 at Category F and from T11 at Category D and from T133 at Category B.

Figure 8.11 is a graph which shows that T80 and T51 have a similar profile with respect to the frequency of cell categories. When T80 was compared statistically with T51 the following results were obtained :-

Figures 8.11, 8.12: Variation in % cell category frequency between individuals (Figure 8.11) T80 and T51, and (Figure 8.12) T105 and Expt. VI at 0 hours in culture



Sample	Categories tested	df	X_2	p	Significance
T80/T51	All categories	6	10.011	<1%	Highly significant
	-E Category	5	9.308	10-5%	Borderline significant
	-E-F Category	4	4.811	50-30%	Not significant

Thus between T80 and T51 the difference was significant at E>F categories. T80 shared similarities with T11 and T133 but T51 differed from both T11 and T133 at more than three categories and thus cannot be said to be similar to either.

Two final individuals T105 and TVI remained. These two individuals have a similar profile (Figure 8.12). When compared statistically the following results were obtained :-

Sample	Categories tested	df	X_2	p	Significance
T105/TVI	All categories	6	37.69	<0.1%	Highly significant
	-D-B Category	4	6.209	10-20%	Not significant

Thus between T105 and TVI the difference is significant at D>B categories. Both T105 and TVI differed from all other individuals at more than three categories.

Figure 8.13

Comparison of cell category frequency between individuals

INDIVIDUALS TESTED	A	B	C	D	E	F	G
T51 / T80					*	*	
T11 / T80				*		*	
T80 / T133		*				*	
T11 / T133							
T105 / TV1		*		*			

* Categories contributing significantly to heterogeneity between individuals

The overall results of analysing possible relationships between these 6 individuals are shown in Figure 8.13. Categories which differ significantly are indicated with an *.

E. Analysis of variation in cell category frequency taking differences between individuals and time in culture into account

The preceeding sections give a picture of changes in frequency due to differences between individuals and due to time in culture as separate components of variation. As a result of the previous analysis the data can be reviewed in an attempt to understand what happens to the frequencies as a result of growth in culture.

T11 and T133 were homogeneous with respect to cell category frequency at culture initiation, 0 hours. After 24 hours they became heterogeneous with significant variation at Category F.

Sample	Categories tested	df	X_2	p	Signficance
T11/T133 (24 hrs)	All categories	6	31.444	<0.1%	Highly significant
	-F Category	5	4.956	50-60%	Not signficant

Therefore the divergence between these individuals after 24 hours in culture can be located as changes in frequency at Category F. From Section C we can recall that changes after 24 hours in culture in T133 were located in Category F and Category F also varied, together with A

and D, in T11 at 24 hours culture.

In Section D similarities and differences were noted between T80 and the homogeneous samples T11 and T133 at 0 hours. After 24 hours in culture the variation in category frequencies between T80 and T11 was large and indeed, Category F showed least variation. After 24 hours in culture the variation in category frequencies between T80 and T133 was also large but in this example Category F was amongst the significantly varying categories.

Similarly the variation between T80 and T51 after 24 hours in culture became very large and Category F was not amongst the most significantly variant categories.

There was insufficient data to test variation between cell category frequency after 24 hours in culture.

The conclusions drawn from this section are as follows :-

1. After 24 hours in culture the homogeneous samples T11 and T133 remain more alike than any other samples studied.
2. After 24 hours in culture all heterogeneous samples showed large variations in cell category frequency with respect to each other and with respect to T11 and T133.
3. Evidence that between individual variation in cell category frequency is maintained during culture is scanty and based only on evidence from the initially homogeneous pair.
4. There is no evidence that cell category frequency variation between individuals is reduced as a result of time in culture.

4. CONCLUSIONS

The conclusions drawn from this Chapter are as follows :-

1. Cells of the testicular epithelium can be classified into categories based on texture and size.
2. Replicate counts of cell category frequency show that cell category frequency is a reliable estimate of the cell types present in any individual.
3. Cell category frequency varies from individual to individual.
4. Cell category frequency varies within the individual with time in tissue culture.

CHAPTER 9

TRACING THE UPTAKE OF H^3TDR IN SPERMATOGONIAL CELLS

1. Introduction

2. Methods

   A. Selection of material for analysis

   B. Methods of analysis

3. Results

   A. T133

      (i) T133, 0 hours in culture

      (ii) T133, 24 - 72 hours in culture

   B. T80, 0 - 48 hours in culture

   C. T11

      (i) T11, 0 - 24 hours in culture

      (ii) T11, 10 days in culture

4. Discussion

   A. Difficulties in equating this work with the results reported by other workers

   B. A review of the information obtained related to each cell category

5. Conclusions

   Sequence of spermatogenesis. Model I.

1. INTRODUCTION

In Chapter 7, H^3Tdr was used to locate the most advanced labelled cell type present in the mixed cell population. This cell type was the primary spermatocyte which had taken up H^3Tdr during the meiotic S phase. In addition H^3Tdr uptake must have occurred during the mitotic S phases of the spermatogonial generations. In Chapter 8 a method was devised for categorising cell types present in the mixed cell population. Some of these cell types must represent cells of the spermatogonial generations. It should be possible to identify the spermatogonial cells by categorising all labelled nuclei in the mixed cell population. Once the spermatogonial cells have been identified, it should be possible to identify changes in these cells with time in culture and hence to record the progression of spermatogonial cells during the early proliferative phase of spermatogenesis.

There are three reasons for attempting this analysis.

1. No one, to my knowledge, has reported studying the early part of the cycle using autoradiography in this way.
2. The sequence of cell categories within the cycle could be documented. This would provide an independant assessment of Clermont's human stem renewal theory and could clarify which cell type is in fact the stem cell.
3. Almost nothing is known about lesions of the spermatogenic cycle in infertile men prior to meiotic division. This type of analysis could identify lesions occurring early in the cycle.

2. METHODS

A. Selection of material for analysis

The individuals, samples and preparations which were available for analysis have been described in Table 8.1. The cell frequency studies showed that T11 and T133 were homogeneous at all cell categories tested at 0 hours. T80 differed from T11 and T133 at one shared and one different category. T51 differed from T80 at 2 categories but was clearly different from T11 and T133. On this basis these 4 individuals were selected for analysis; T105 and TVI were excluded because they exhibited clear differences from the other individuals available.

All autoradiographs prepared from these individuals were exposed for 25 days; the handling and development of autoradiographs was kept as constant as possible. After autoradiography T51 had to be excluded from the analysis because the autoradiographs were technically unsatisfactory. T11 which had been used as an early labelling experiment to test dosage (Chapter 7 Section 3A) had lightly labelled nuclei. The labelled nuclei could be distinguished from nuclei with background label, Table 9.1. However, the overall lightness of labelling made identification of labelled nuclei difficult. T80 and T133 were treated with the higher dosage (Chapter 7 Section 3A). These more heavily labelled nuclei were easier to identify but required stripping for accurate categorisation. Background label in T133 and T80 remained comparable with the T11 sample.

This discussion of the selection of samples for study, highlights some of the difficulties encountered. The samples available for experimental work were usually very small and opportunity for replicate work or subjecting samples to a range of conditions was limited. In

TABLE 9.1

GRAIN COUNT OVER 1000 NUCLEI, T11, 0 HOURS IN CULTURE

No. of grains	0,	1,	2,	3,	4,	5-10,	11-20,	21-30,	31-40,	41-50,	51-60,	61-80,	>80
No. of nuclei	881,	53,	18,	5,	5,	1,	3,	1,	9,	3,	6,	9	6

addition, the known variability between individuals made between individual comparisons hazardous. Thus when T11 and T133 were found to be homogeneous for cell category frequency, T11 was included in the analysis despite the difference in H^3Tdr treatment between the two samples.

B. Method of analysis

The samples analysed were obtained from T11, T80 and T133. The data obtained from categorising and measuring a sample of 400 nuclei from each individual at each time interval in culture was available as a control for the labelled studies. This data was recorded in detail in Table 8.2. All labelled nuclei within each sample of 400 nuclei were photographed using a x100 oil immersion lens. The sampling of labelled nuclei was then extended beyond the limits of the 400 unlabelled nuclei until 50 labelled nuclei had been photographed. Some slides were stripped for relocation of nuclei at this point. Subsequently these samples of 50 labelled nuclei were found to be rather small for analysis. Therefore, the whole cell sample, circumscribed by the ring of cells deposited during slide making, was scanned for labelled nuclei. Some of these samples provided around 150 labelled nuclei for photography. Subsequently the slides

were stripped and the nuclei relocated for categorisation. Some were rephotographed for illustrative and comparative purposes.

All labelled nuclei were measured from photographs in the manner described for unlabelled nuclei in Chapter 8. After categorisation the means and standard deviations of labelled nuclei, within any single category, could be calculated. In order to compare the means and standard deviations with the unlabelled nuclei a conversion had to be made since the unlabelled nuclei were photographed using a x54 lens system.

This conversion was achieved by multiplying by a constant of 3.4 (microscope lens ratio^2 = $\left[\frac{100}{54}\right]^2$ Means and standard deviations for the unlabelled data were then referred to as the converted mean and standard deviation for any cell category under comparison. Comparison between the means and standard deviation of labelled and unlabelled population of cells within any category under inspection were tested using a students t-test.

3. RESULTS

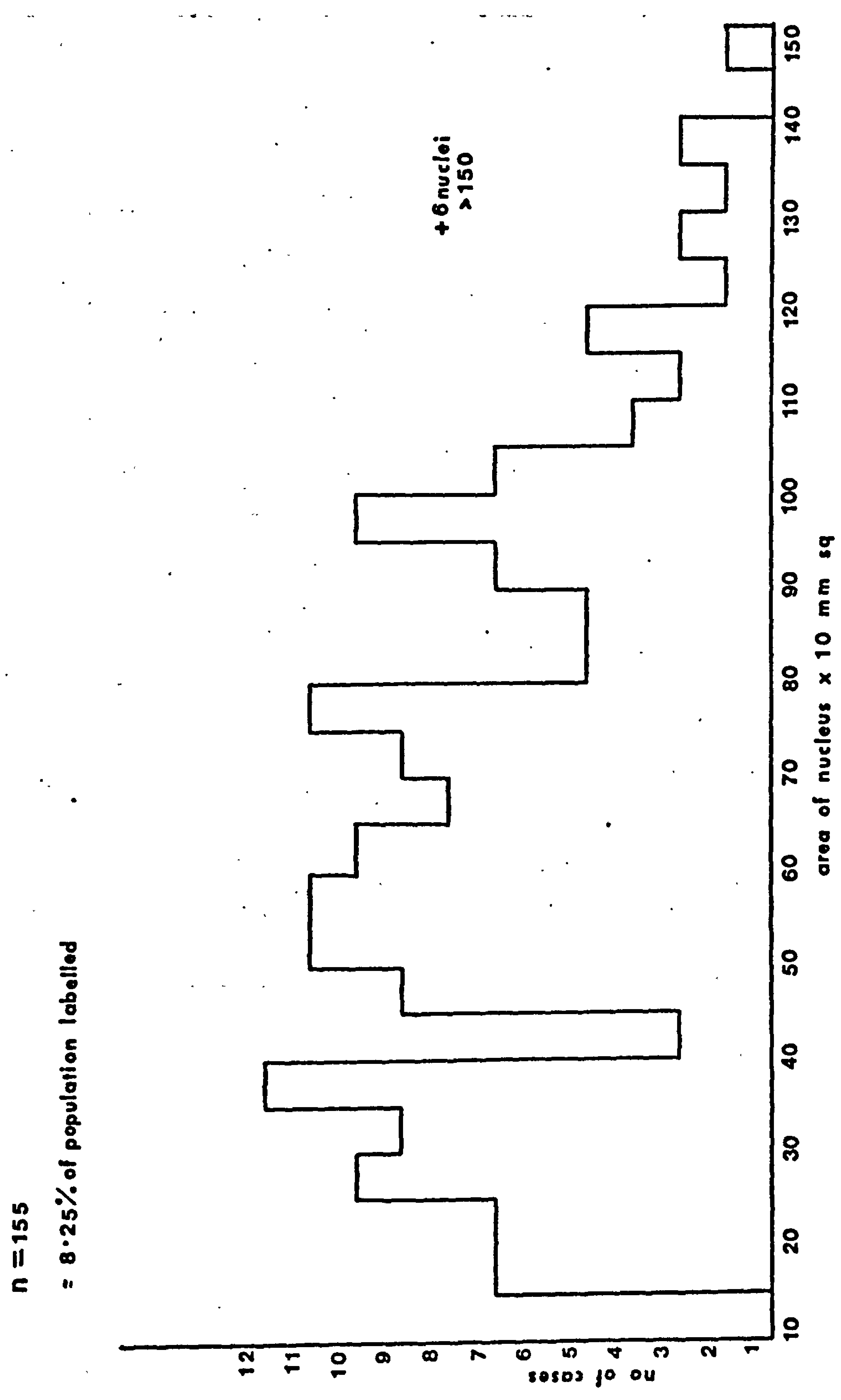
A. Sample T133

(i) 133/1 at 0 hours in culture

Figure 9.1 is a histogram constructed from measurements of all recorded labelled nuclei in this sample. There appeared to be three main peaks which might correspond to preliminary observations indicating the presence of 3 types of labelled nuclei. This observation could be tested after the nuclei were stripped and labelled. Out of the 155 labelled nuclei 123 were relocated and categorised as A, D or G nuclei. No other categories of nuclei were represented within the labelled population in this sample. The presence of only 3 categories

Figure 9.1

Sample 133/1, 0 hours in culture. Histogram of measurements of all recorded labelled uncategorised nuclei



of labelled nuclei confirmed the earlier observations prior to stripping and categorisation. In Figure 9.2 histograms have been constructed from measurements of nuclei sorted into A, D, G categories. These histograms showed that when the textures of the nuclei were taken into account the population of labelled nuclei consisted of three distinct populations of nuclei separable on a combination of size and texture. The means and standard deviations of the three labelled cell populations (A, D, G) are shown in Figure 9.2 as bars. These parameters can be compared with the converted mean and standard deviation of the unlabelled control populations of category A, D, G, which are also shown as bars on Figure 9.2. In Figure 9.2 the labelled and unlabelled G populations appear similar in size. But both A and D labelled populations, on the whole, appear smaller than the unlabelled control A and D populations. Differences between the labelled and unlabelled populations were tested using students t-test, with the following results :-

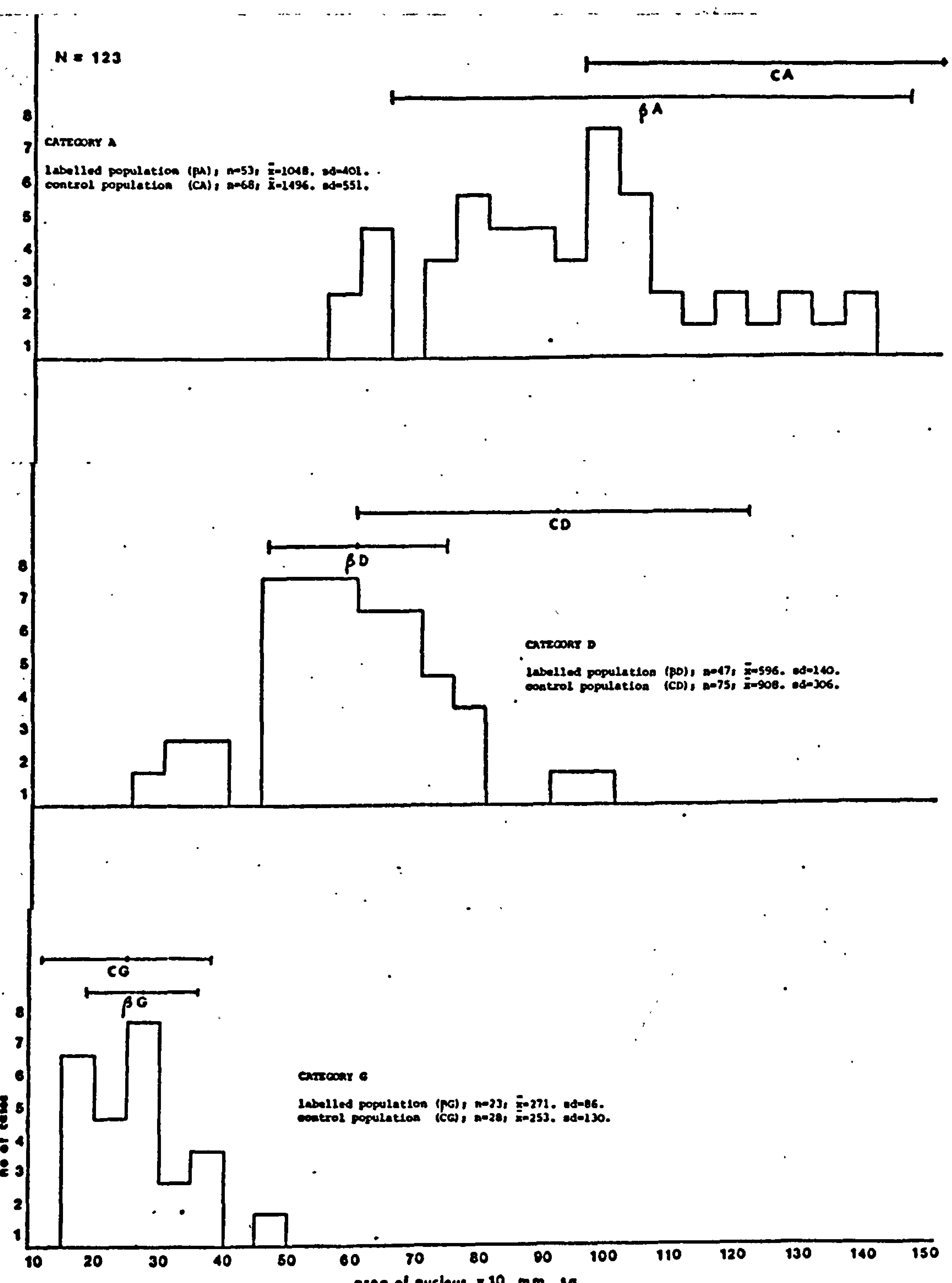
t-test between labelled and unlabelled populations within categories for T133/1

Category	t-test	df	Probability	Significance
A	5.170	119	<0.1%	Highly significant
D	7.632	120	<0.1%	Highly significant
G	0.600	49	50 - 60%	Not signficant

Statistically the labelled populations of A and D nuclei are highly significantly different from their unlabelled controls. Since they were texturally identical with the controls, it did not seem likely that they had been wrongly categorised. Thus the difference presented

Figure 9.2

Sample 133/1, 0 hours in culture. Histograms of measurements of labelled, categorised nuclei.



by labelled A and D nuclei seemed to be concentrated in their smaller size in comparison to unlabelled A and D nuclei. Labelled and unlabelled populations of Category G nuclei were not signficantly different from each other. The results obtained from analysis of T133/1 have been presented in detail.

(ii) Sample T133, 24–72 hours in culture

The rest of the analysis followed a similar pattern to 133/1 but the results are presented in a more concise form as histograms, tables and summaries. Measurements of labelled nuclei from Sample T133 after varying times in culture are shown in the following histograms; T133/2 at 24 hours in culture, Figure 9.3; T133/3 at 48 hours in culture, Figure 9.4; T133/4 at 72 hours in culture, Figure 9.5. Table 9.2 gives the results from t-tests between the labelled and unlabelled control populations in these samples (including T133/1 at 0 hours in culture).

The results obtained from T133 from 0 hours through to 72 hours can be summed up briefly as follows :-

1. There are three types of labelled nuclei in all 4 samples of T133. The three labelled types are identical texturally with Category A, D, G nuclei in the unlabelled control population studied.
2. At 0 hours labelled G nuclei are identical in size to the unlabelled population of G nuclei. During 72 hours in culture, labelled G nuclei decrease in numbers, often appear to be degenerating and increase in size compared with their controls.
3. At 0 hours in culture, labelled A and D nuclei are distinctly smaller in size compared with their controls. During 72 hours

Figure 9.3

T133/2, 24 hours in culture. Histogram of measurements of all labelled, uncategorised nuclei recorded.

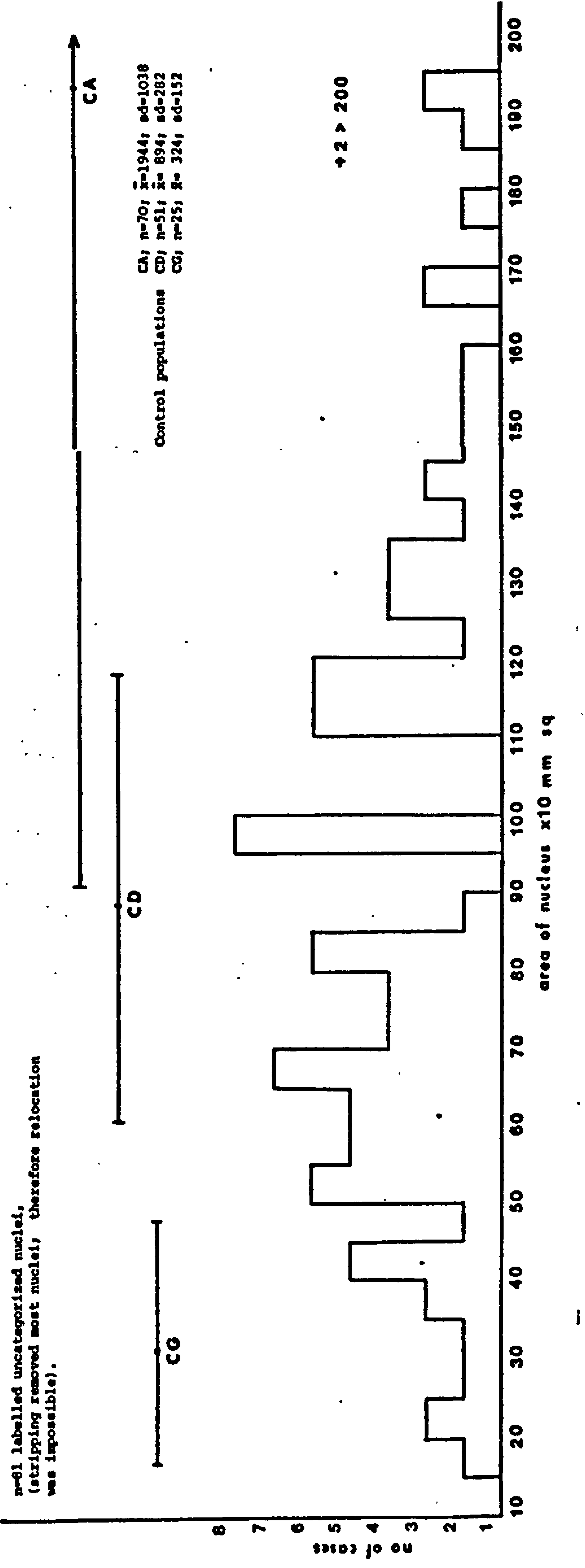


Figure 9.4

T133/3, 48 hours in culture. Histogram of measurements of labelled, categorised nuclei.

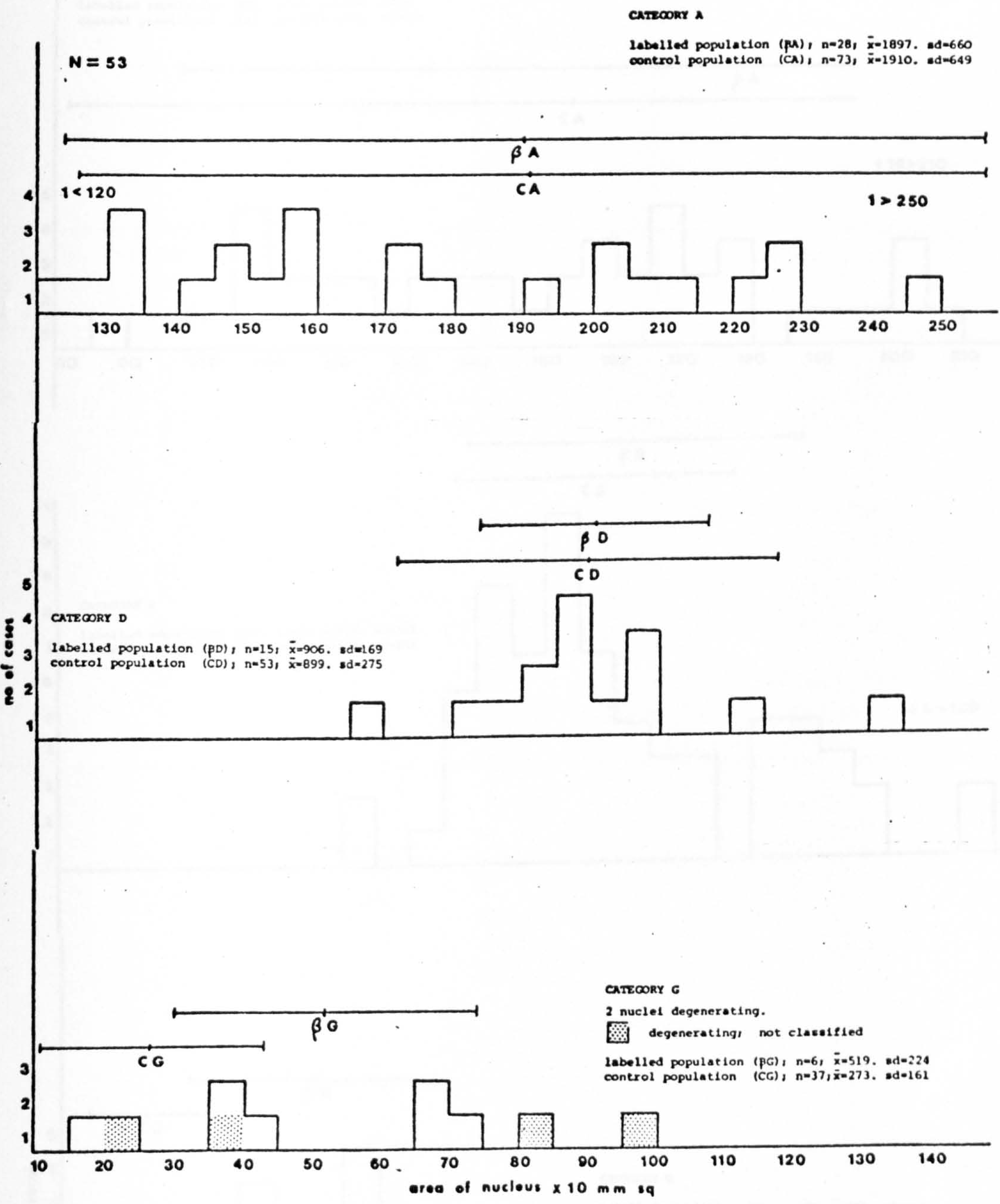
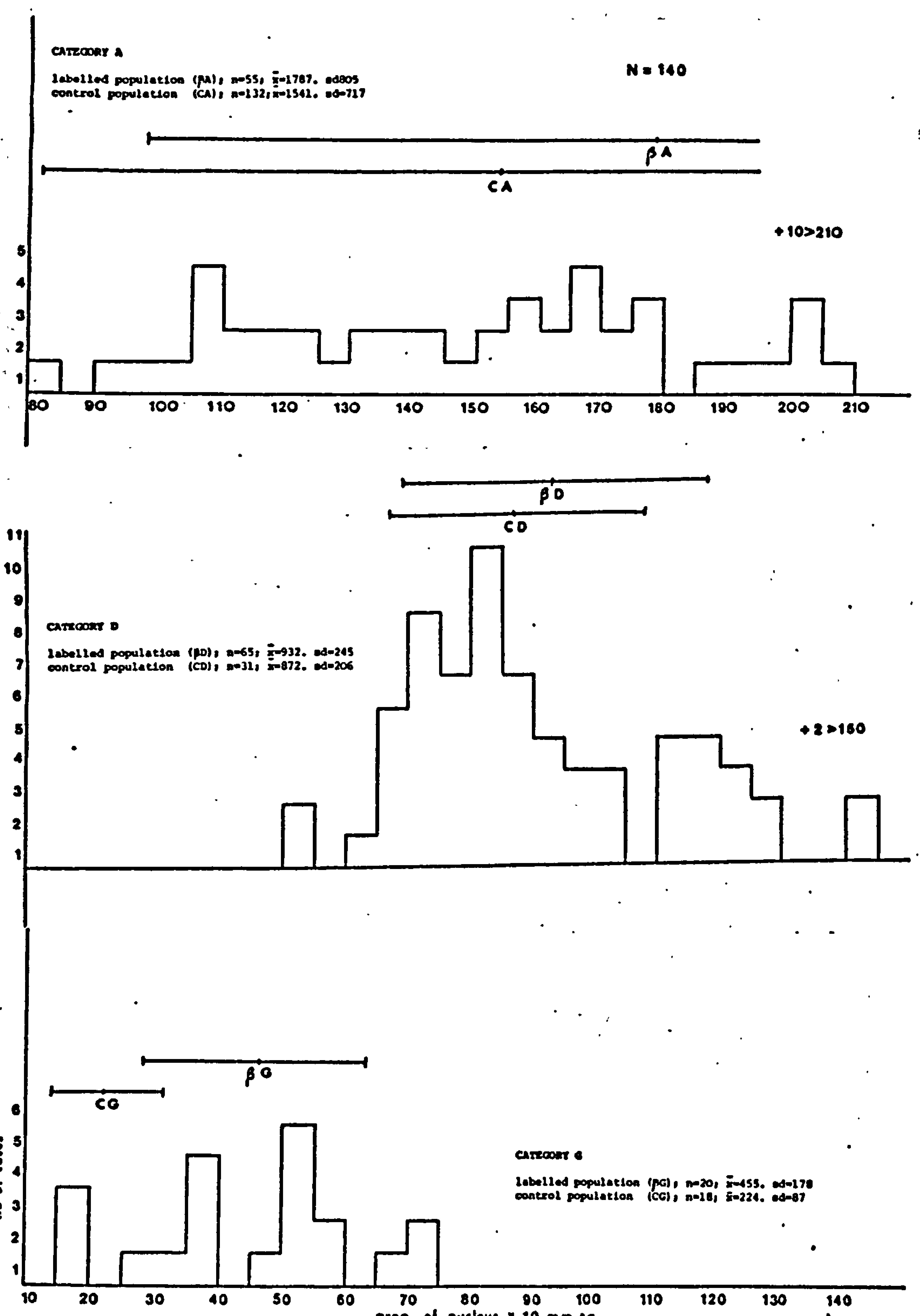


Figure 9.5

T133/4, 72 hours in culture. Histogram of measurements of labelled, categorised nuclei.



t-TEST BETWEEN LABELLED AND UNLABELLED POPULATIONS WITHIN CATEGORIES FOR SAMPLE T133

Sample Hrs. in culture	Cats tested	t test	df	P	Significance	Size difference Labelled versus control
133/1 0 hrs.	A	5.170	119	<0.1%	***	labelled smaller than control
	D	7.632	120	<0.1%	***	labelled smaller than control
	G	0.600	49	60-50%	not sig.	labelled ≡ control
133/2 24 hrs						No information
133/3 48 hrs	A	0.097	99	>90%	not sig.	labelled ≡ control
	D	0.100	46	>90%	not sig.	labelled ≡ control
	G	2.584	41	2%-1%	**	labelled larger than control
133/4 72 hrs.	A	1.958	185	>5%	*	labelled ± larger than control
	D	1.254	94	30-2%	not sig.	labelled ≡ control
	G	5.159	36	<0.1%	***	labelled larger than control

in culture A and D nuclei increase in size until labelled A nuclei are more or less larger than their controls and labelled D nuclei are identical in size to their controls.

4. There is no evidence for any other labelled cell type during 0-48 hours in culture. At 72 hours there was evidence for labelling in early prophase of meiotic division.

B. Sample T80

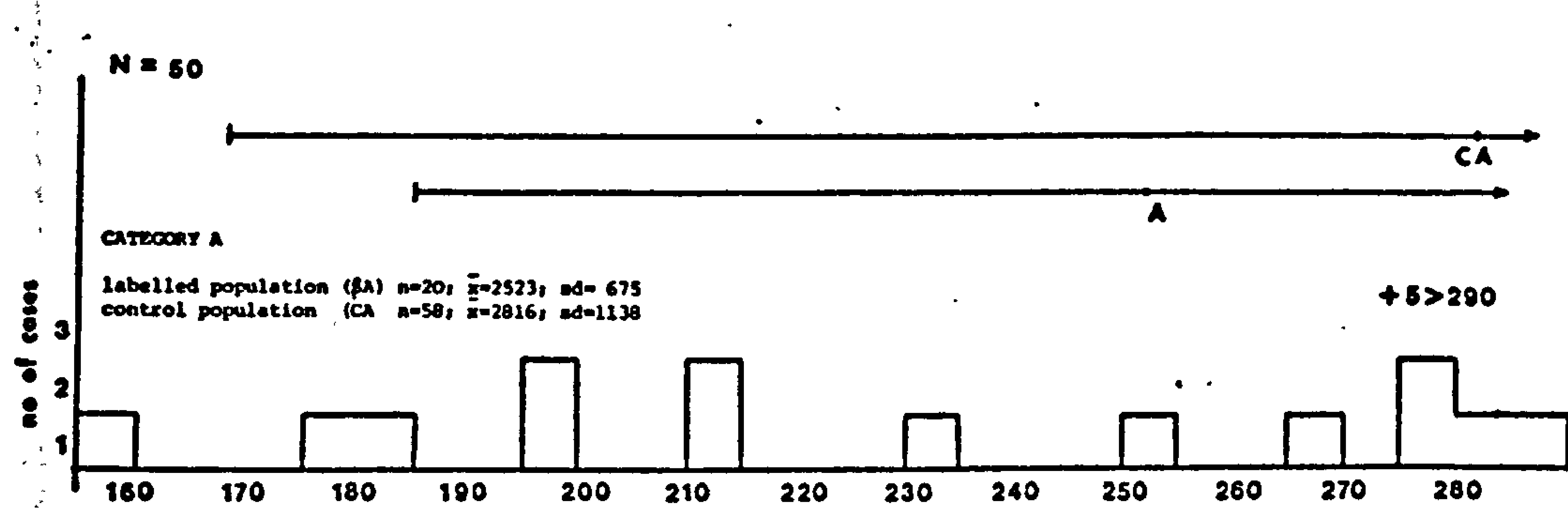
Measurements of labelled nuclei from Sample T80 after varying times in culture are presented in the following histograms; T80 I-1 at 0 hours in culture, Figure 9.6; T80 I-2 at 24 hours in culture, Figure 9.7 and T80 I-3 at 48 hours in culture, Figure 9.8. Table 9.3 gives the results from t-tests between the labelled and unlabelled control populations in these samples.

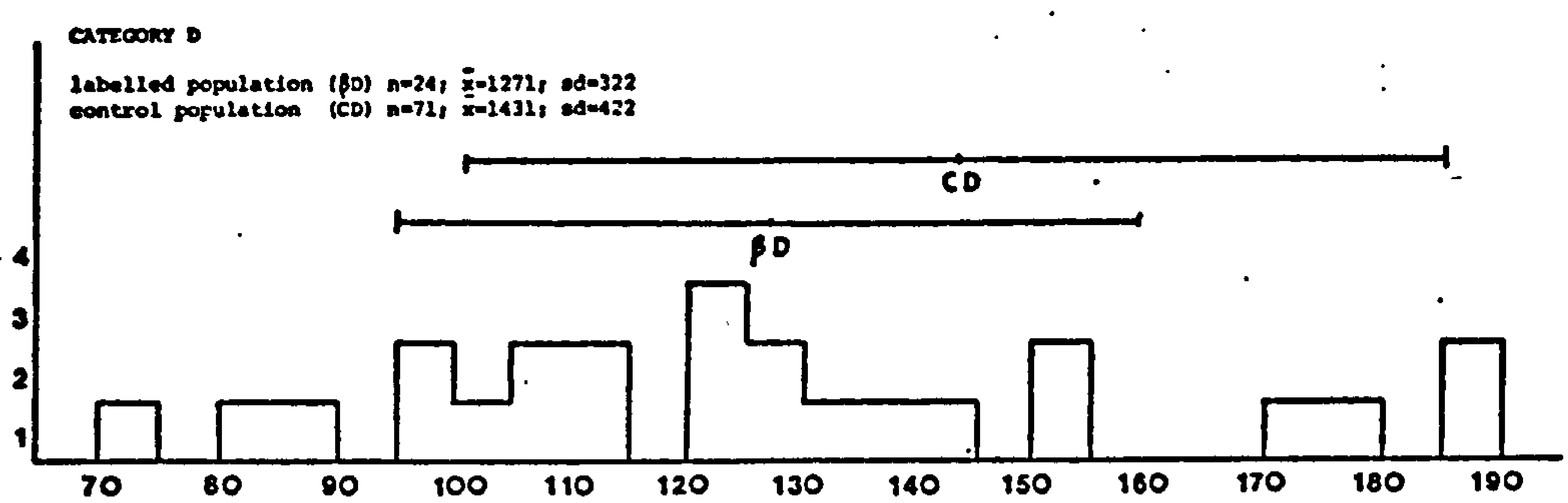
The results obtained from T80 from 0 hours - 48 hours can be summarised as follows :-

1. There are 3 major types of labelled nuclei in all three samples from T80. The three labelled types are identical texturally with categories A, D and G in the control population.
2. At 0 hours in culture labelled G nuclei are significantly larger in size than the controls and this size distinction is maintained through 48 hours in culture. Labelled Category G nuclei show considerable degeneration with time in culture.
3. At 0 hours Category A and D labelled nuclei are more or less similar in size to control A and D nuclei. By 48 hours in culture both labelled categories were smaller in size than their controls.
4. These results are in contrast to the results obtained from T133.

Figure 9.6

T80 I-1, 0 hours in culture. Histogram of measurements of labelled, categorised nuclei.





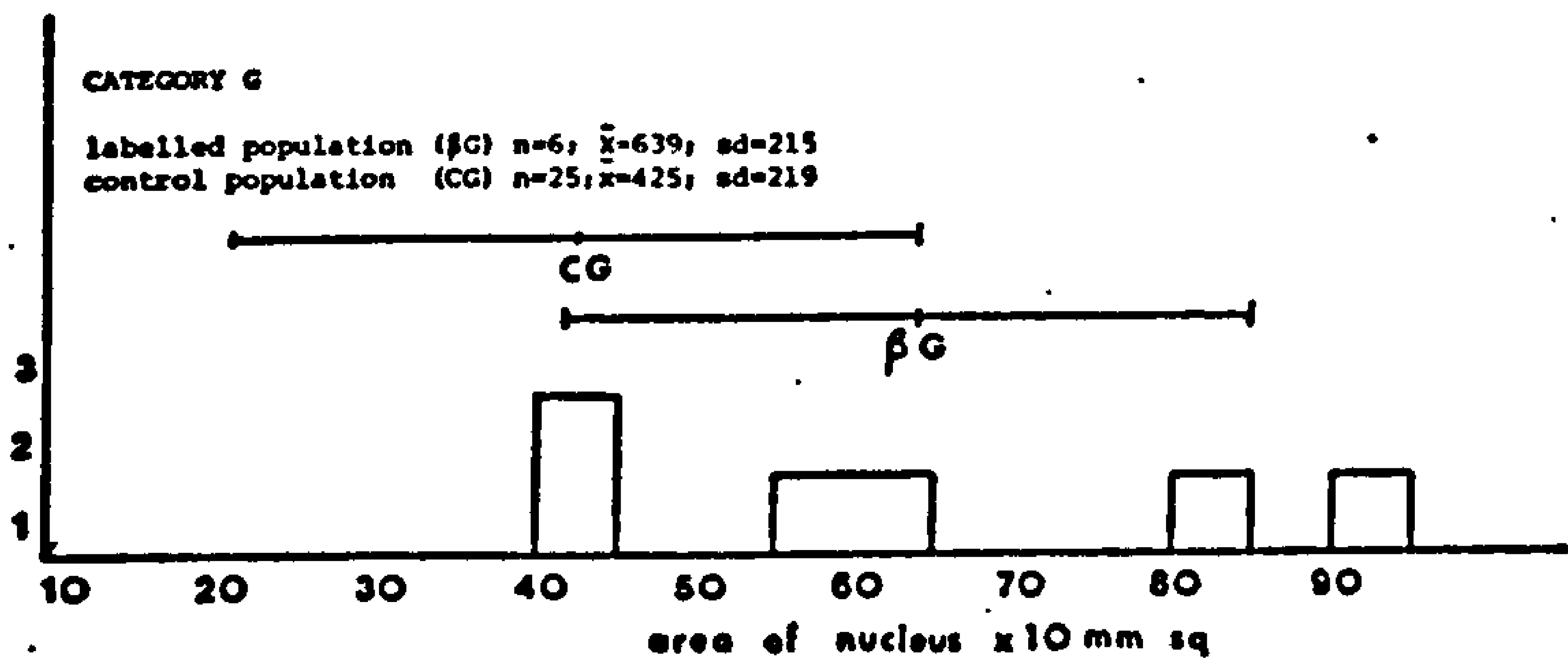


Figure 9.7

T80 I-2, 24 hours in culture. Histogram of measurements of labelled, categorised nuclei.

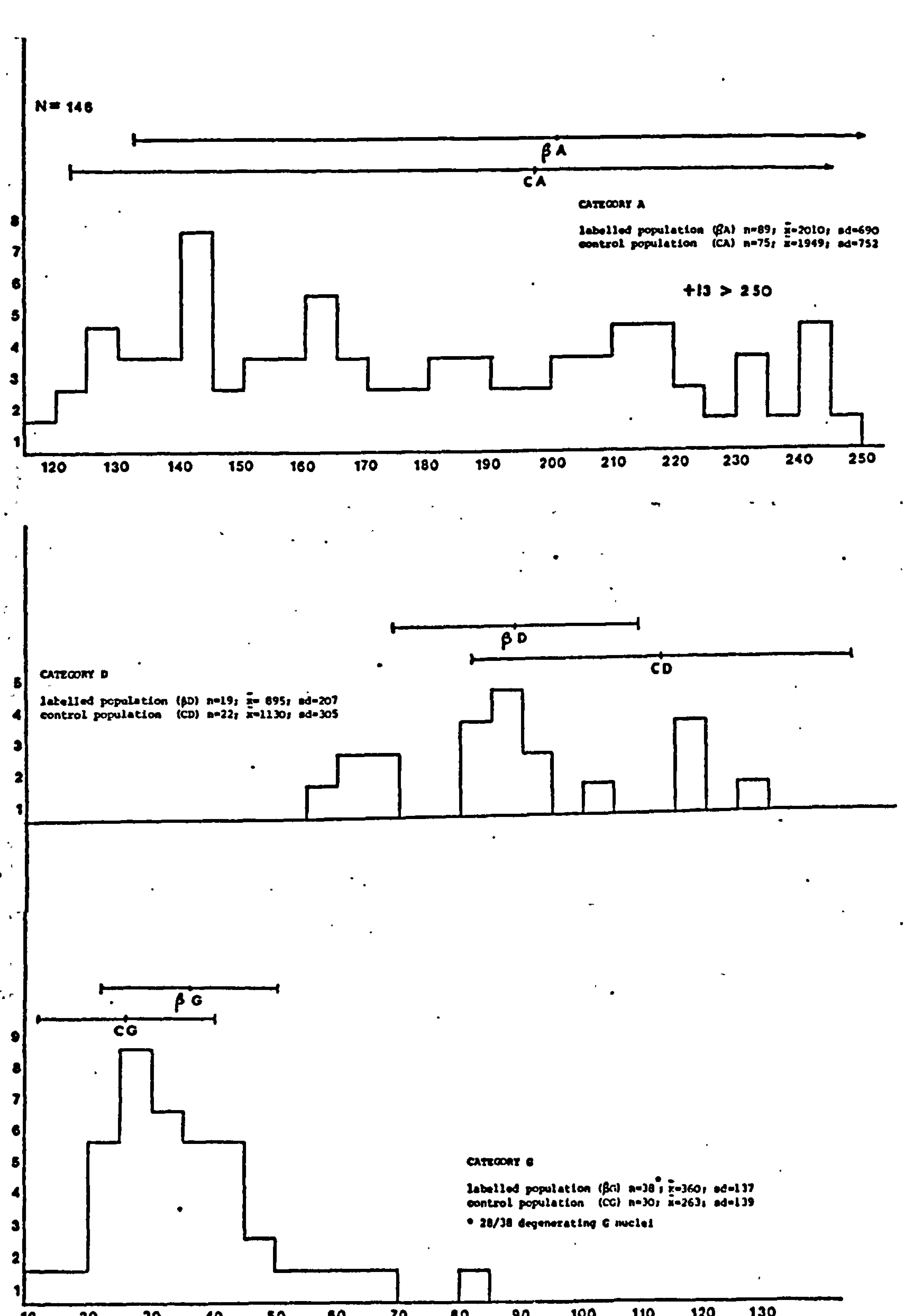


Figure 9.8

T80 I-3, 48 hours in culture. Histogram of measurements of labelled, categorised nuclei.

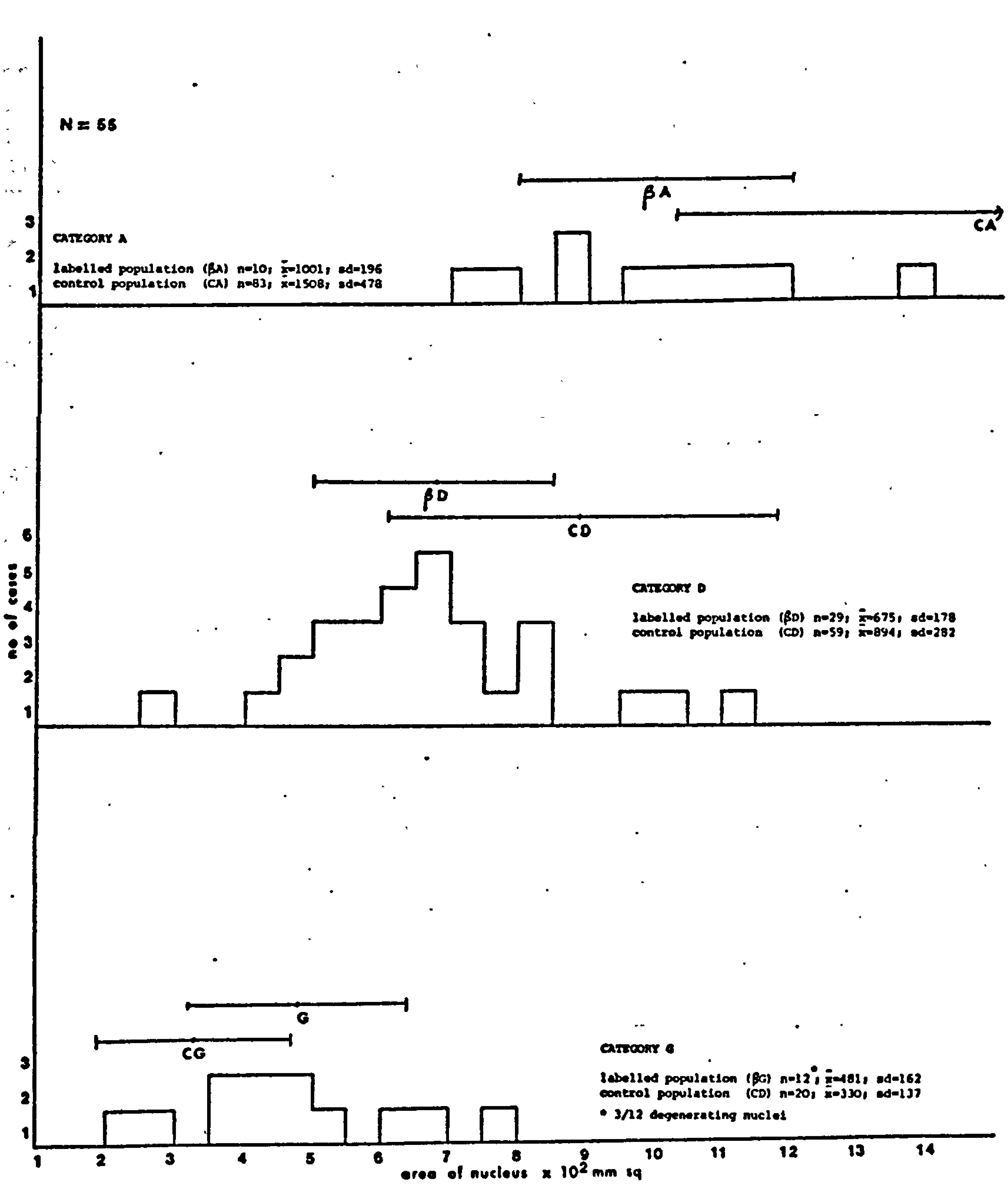


TABLE 9.3

t-TEST BETWEEN LABELLED AND UNLABELLED POPULATIONS
WITHIN CATEGORIES FOR SAMPLE T80

Samples Hrs. in culture	Cats. tested	t test	df	P	Signi-ficance	Size difference Labelled versus control
80I-1	A	1.381	76	20-10%	not sig	labelled ≒ control
0 hrs.	D	1.939	93	10-5%	*	labelled ± smaller than control
	G	2.176	29	5-2%	**	labelled larger than control
80I-2	A	0.547	162	<90%	not sig	labelled ≡ control
-24 hrs.	D	2.919	39	1-0.1%	**	labelled smaller than control
	G	2.868	66	1-0.1%	**	labelled larger than control
80I-3	A	6.247	91	<0.1%	***	labelled smaller than control
48 hrs.	D	4.429	86	<0.1%	***	labelled smaller than control
	G	2.700	30	1%	**	labelled larger than control

5. Occasional labelled nuclei identified as Category C nuclei were identified after 24 hours in culture.

C. Sample T11

(i) 0-24 hours in culture

Measurements of labelled nuclei from Sample T11 at 0-24 hours in culture are presented in the following histograms; T11/1 at 0 hours in culture, Figure 9.9; T11/2 at 24 hours in culture, Figure 9.10. Table 9.4 gives the results from t-tests between the labelled and unlabelled control populations in these samples. The results can be summed up briefly as follows :-

1. The three major types of labelled nuclei are present in the samples. These three types of labelled nuclei are texturally identical with categories A, D, G in the control population.
2. The size relationships between labelled and unlabelled populations are the same as those recorded for T133 after similar times in culture.
3. Category G nuclei degenerate rapidly after culture initiation.
4. One labelled Category C nucleus was observed after 24 hours in culture.

(ii) Sample T11, 10 days in culture

In this sample labelled cell categorisation was difficult. This had not been a problem with the analysis of the unlabelled sample. The difference was probably due to the higher magnification of the nuclei and the consequent finer and sharper focus on nuclear texture.

A total of 140 labelled nuclei were measured and sorted into the following categories.

Figure 9.9

T11/1, 0 hours in culture. Histogram of measurements of labelled, categorised nuclei.

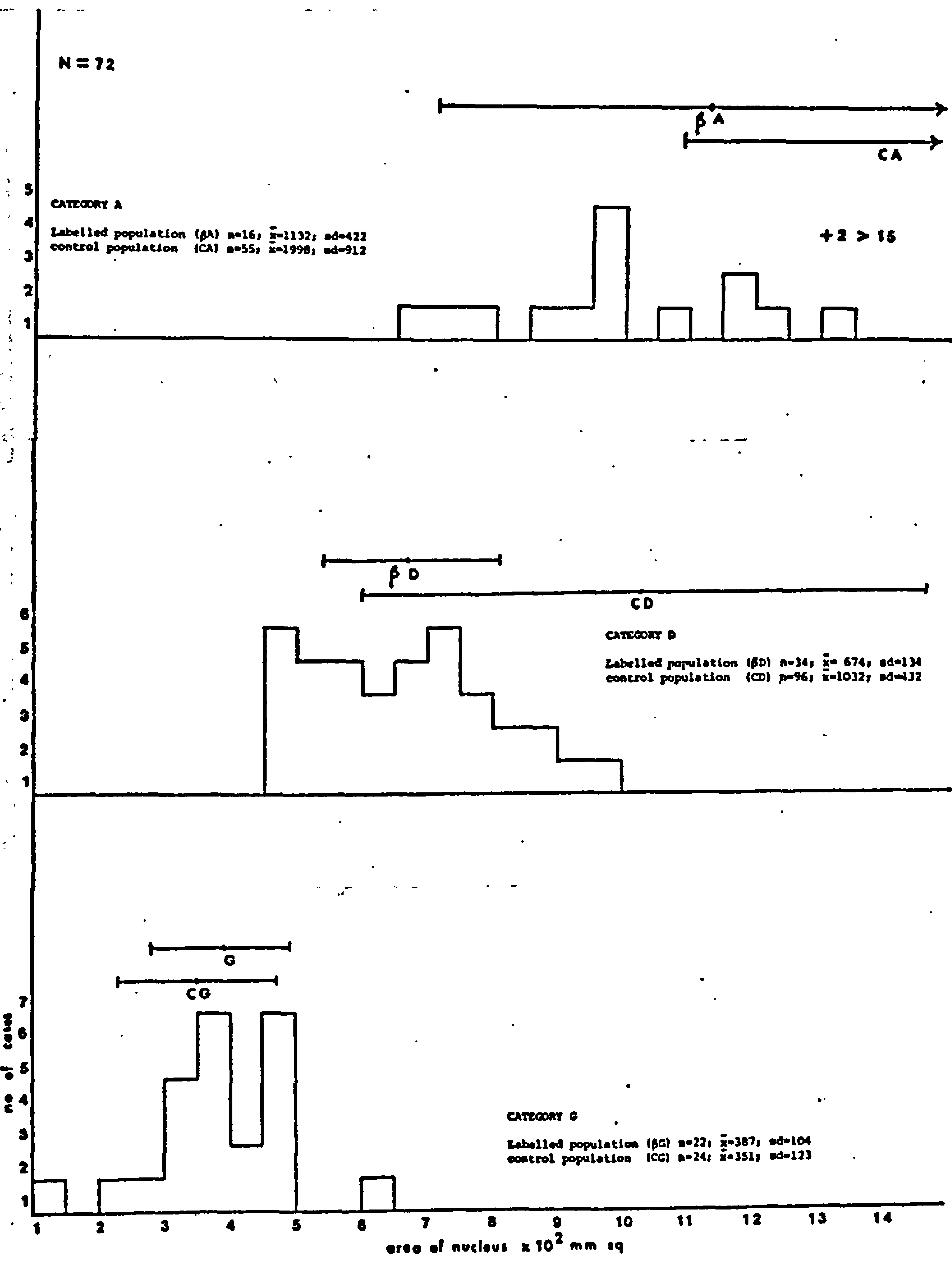


Figure 9.10

T11/2, 24 hours in culture. Histogram of measurements of labelled, categorised nuclei.

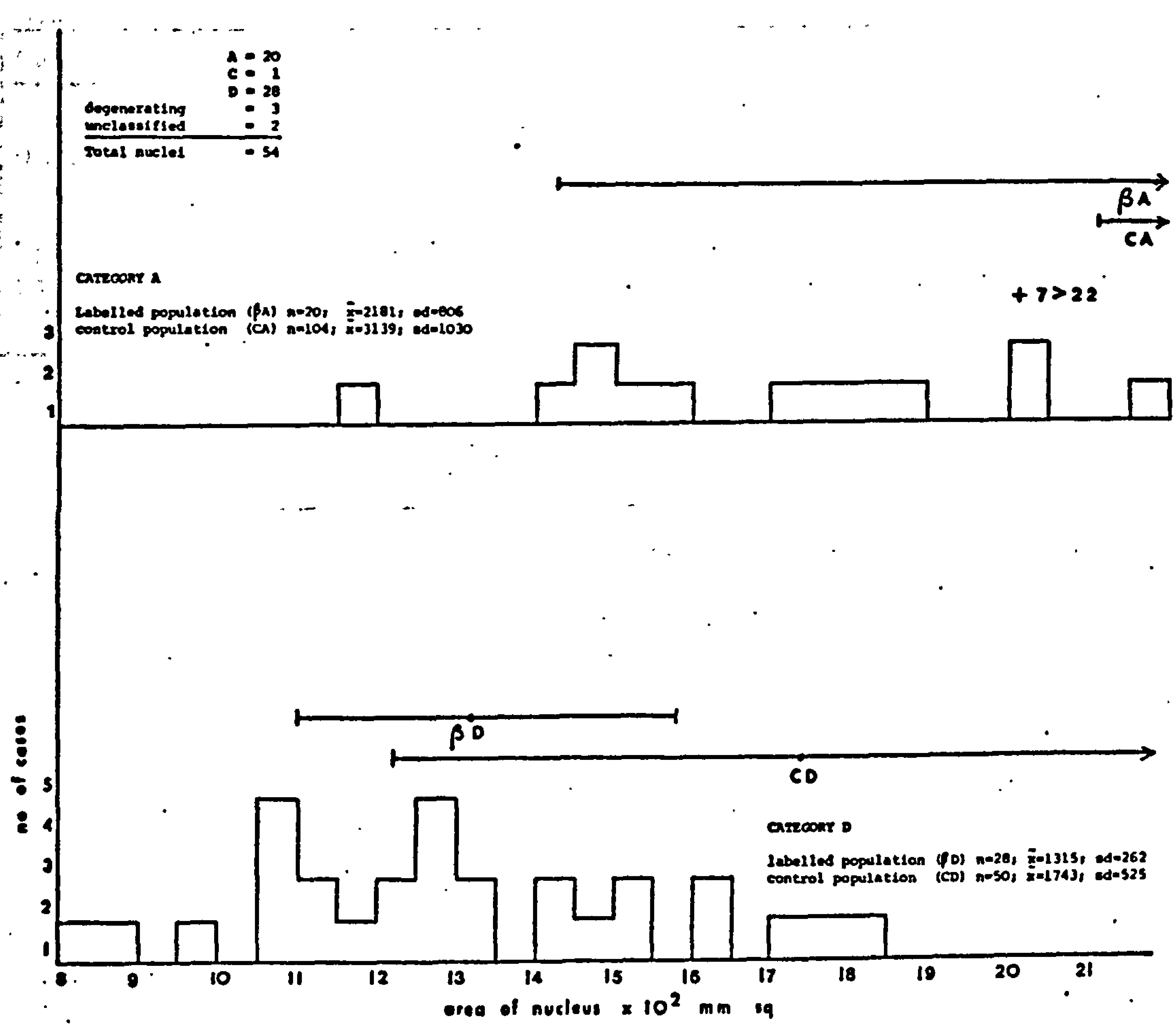


TABLE 9.4

t-TEST BETWEEN LABELLED AND UNLABELLED POPULATIONS WITHIN CATEGORIES FOR SAMPLE T11

Samples Hrs. in Culture	Cats. tested	t test	df	P	Signi-ficance	Size difference Labelled versus control
T11 0 hrs	A	5.343	69	<0.1%	***	labelled smaller than control
	D	7.294	130	<0.1%	***	labelled smaller than control
	G	1.092	44	30.20%	not sig	labelled ≡ control
T11 24 hrs	A	4.640	122	<0.1%	***	labelled smaller than control
	D	4.791	76	<0.1%	***	labelled smaller than control
	G	too few to test				

Type I	Large pale staining and usually untextured nuclei (Equivalent to Category A)	= 8 nuclei
Type II	Large pale nuclei. Texture with threads of chromatin, occasional evidence of sex vesicle	= 18 nuclei
Type III	Middle nuclei (Equivalent to Category D)	
	(a) Large, pale staining, coarse chromatin texture	= 40 nuclei
	(b) Middle, dark staining, regular chromatin texture	= 42 nuclei
	(c) Small, dark staining, rather less chromatin texture that III(b)	= 23 nuclei
Type IV	Small nuclei (Equivalent to Category G) Small, dark staining and without chromatin structure	= 2 nuclei
	Unclassified nuclei	= 7 nuclei
	Total	140 nuclei

Examples of these categories are shown in Figure 9.11. The original categories were still present but diversification of the cell types had occurred. Category A nuclei were equivalent to Type I nuclei. Category G nuclei, which had virtually disappeared from the population, were recorded as Type IV nuclei. Category D nuclei had diversified in texture and size so that three distinct groups could be identified, these were recorded as Type IIIa,b,c. Type II nuclei had not been identified in the earlier categorisation.

Measurements of labelled nuclei from Sample T11 at 10 days are shown in Figure 9.12. Table 9.5 gives the results from t-tests between the labelled and unlabelled control populations in these samples.

Figure 9.11 Sample 11/3, 10 days in culture

(a) labelled Type IIIc nucleus; (b) labelled Type IIIb nucleus, similar to Category D nucleus; (c) labelled Type IIIa nucleus; (d) same nucleus as (c) after stripping, adjacent Type I (Category A) nucleus; (e) labelled Type II nucleus, large, pale staining, chromatin threads present; (f) same as (e) after stripping; arrow marks probable sex vesicle.

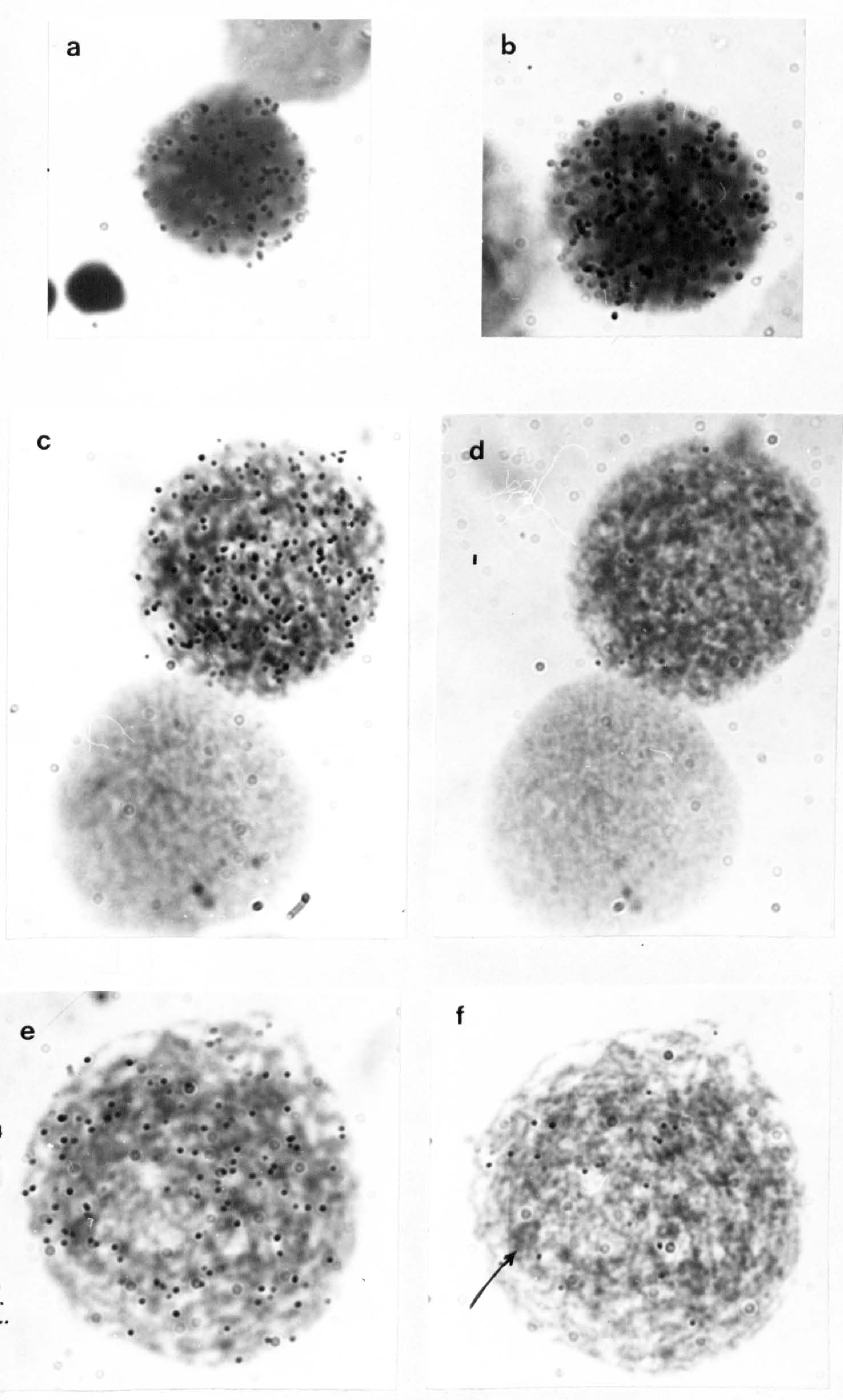


Figure 9.12

Sample 11/3, 10 days in culture. Histogram of measurements of labelled, categorised nuclei.

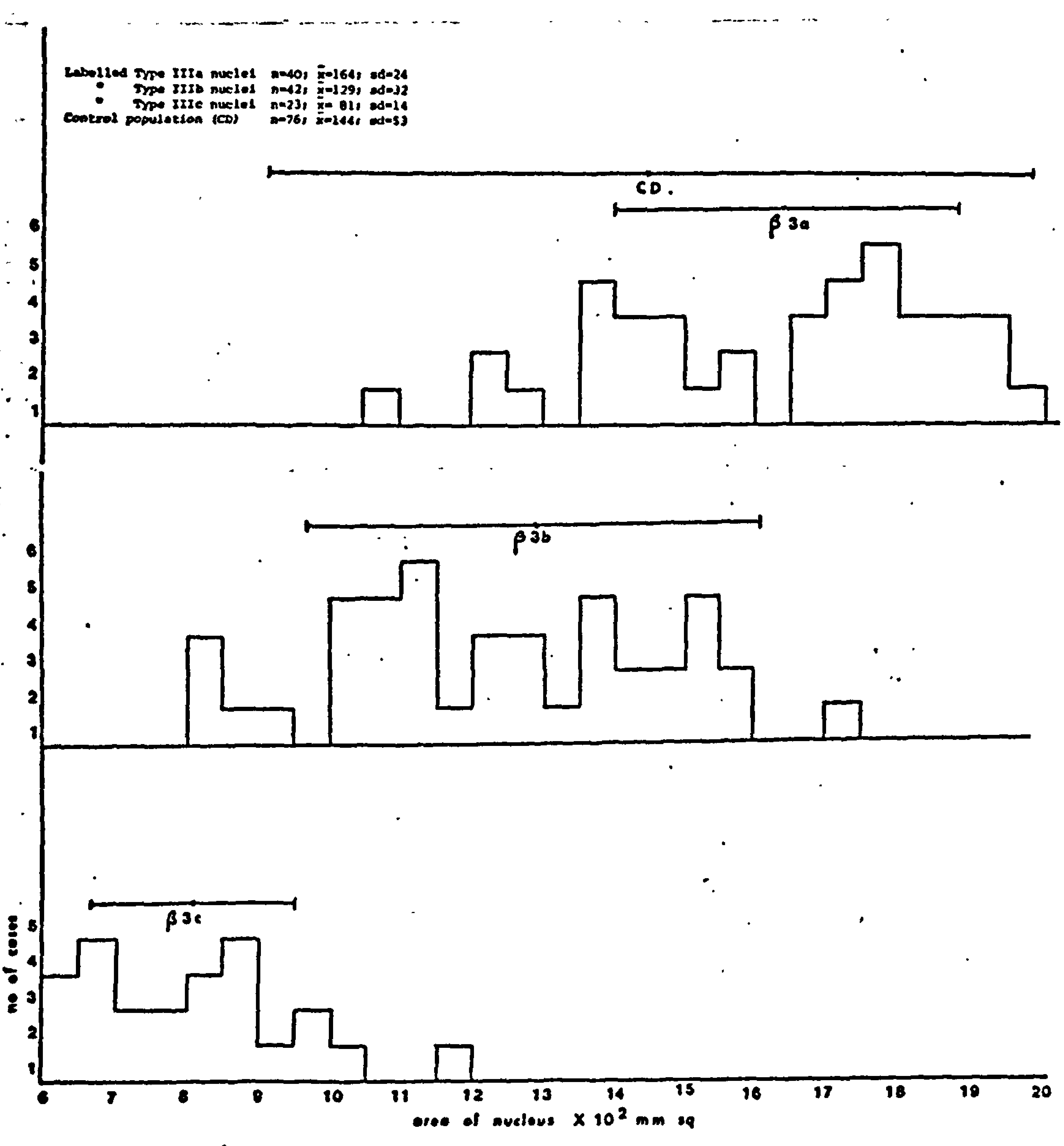


TABLE 9.5

T11, 10 DAYS IN CULTURE

t-TEST BETWEEN LABELLED AND CONTROL POPULATIONS

Control Cats. tested	Labelled Cats. tested	t test	df	P	Signi-ficance	Size difference labelled versus control
D	IIIa	2.728	114	1-0.1%	**	labelled larger than control
D	IIIb	1.951	116	10-5%	*	labelled $\equiv$ control
D	IIIc	9.292	97	<0.1%	***	labelled smaller than control
G	IIIc	10.363	47	<0.1%	***	labelled larger than control

These results can be summarised briefly as follows :-

1. Category D nuclei are now distinguishable as three types on texture and size.
2. Type IIIb is equivalent in size and texture to Category D nuclei.
3. Type IIIa are larger in size and texturally different from classic category D nuclei.
4. Type IIIc represent a group of small nuclei which are texturally similar to category D nuclei.
5. Type IIIc nuclei are significantly larger than control Category G nuclei.
6. In day 10 cultures a new type of nucleus Type II could be recognised.

These results were interpreted as demonstrating growth in culture of Category D nuclei to give Type II nuclei. These type II nuclei were thought to represent the primary spermatocyte which under culture conditions were present in large enough numbers to be distinguished from Category D nuclei. The presence of chromatin threads and the presence of a sex vesicle in some of these nuclei was regarded as supportive evidence for this supposition.

4. DISCUSSION

A. Difficulties in equating this work with the results reported by other authors

The results of analysis clearly identified 3 cell categories which were in S phase when H^3Tdr was added to the culture. In addition, progress of these labelled categories could be followed for up to 10 days in culture. The information from this analysis can be used to order cell categories within the sequence of spermatogenesis, but the difficulty in doing this lies directly with previous descriptions of the spermatogenic cycle.

If we take Clermont's work on stem cell renewal as a model of spermatogenesis, Clermont (1966b) described the following sequence from histology sections :-

Ad ⟶ Ap ⟶ B ⟶ R (resting primary spermatocytes = S phase primary spermatocytes in this work)

Clermont therefore described 3 generations of spermatogonia and one generation of the primary spermatocytes which would give 4 labelled cell types. Roosen-Runge and Barlow (1953) described early primary

spermatocytes as very small with a characteristically poorly defined nuclear membrane. Lima de Faria (1968) identified small labelled nuclei at Leptotene/Zygotene and thought that presence of label was proof of repair synthesis taking place in these nuclei. Chandley and Kofman-Alfaro (1971) thought that Lima de Faria's labelled Leptotene/Zygotene nuclei were in fact S phase primary spermatocytes. It would appear that Chandley and Kofman-Alfaro equated the relative size of the primary spermatocyte to other spermatogonial generations in man with evidence from mouse. In mouse Kofman-Alfaro and Chandley (1970) described resting primary spermatocytes as being smaller and more densely staining than preceeding Type B spermatogonia. In general, in mouse, the primitive or stem cell Type A nuclei are the largest spermatogonial generation and size decreases with progress through spermatogenesis.

None of these authors attempted to analyse uptake of label into the spermatogonial cells, in man, directly. Indeed, Chandley and Kofman-Alfaro (1971) say "the spermatogonial cells cannot be identified". Clermont's autoradiographic work (Heller and Clermont, 1963) was designed to examine the length of time over which spermatogenesis (including spermiogenesis) occurred. He discusses the point at which spermatogenesis can be said to be initiated; concentrating on the tracing of the most advanced type of labelled nucleus present in his sections. He makes little or no comment concerning the uptake of label into the spermatogonia.

The problem of equating cell categories with cell types described by other workers remains. In this respect Clermont used different fixation methods from the present work and he repeatedly states that his methods are critical for the accurate identifcation of his cell types in histological sections.

One way of tackling the difficulties outlined in the preceeding paragraphs is to discuss the results of cell categorisation followed by autoradiography described in the present work. This takes the form of reviewing all the information obtained for each cell category in turn. Particular discrepancies can then be discussed as they become relevant.

B. A review of the information obtained related to each cell category

Category B nuclei

This category clearly represented nuclei at the beginning of meiotic division, i.e. from early pachytene to late pachytene and probably included unspread figures in early diplotene. A sex vesicle was usually distinctive in the early pachytene nuclei. These nuclei were not labelled in the early stages of culture. After 72 hours in culture occasional early pachytene nuclei were labelled; this observation was not followed further. In the long term culture experiments tracing in vitro differentiation, labelled pachytene nuclei were identified.

Category E nuclei

These were nuclei with distinct chromosome structure therefore this category included some diplotene figures, all diakenesis and MII figures and any figures in mitotic division where chromosome structure could be recognised. This heterogeneous group, which was too small in numbers to figure in the analysis, thus included occasional cells of the spermatogonial generations. Labelling was not seen in these nuclei in the early labelling experiments but in the long term experiments both spermatogonial mitoses and diakenesis figures were labelled.

Category F nuclei

These nuclei were never observed with label. They were thought to be spermatids which were post-meiotic division and prior to spermiogenesis. Nuclei in spermiogenesis including spermatozoa were excluded from categorisation. In fact, such nuclei were rare in these mixed cell populations from infertile individuals.

Category C nuclei

These nuclei were similar in texture to Category F nuclei; from this point of view they could have represented an early spermatid stage which decreased in size towards Category F nuclei as spermiogeneis proceeded. However, Category C and F nuclei, in most samples studied, were distinctly separate populations when size was taken into account. When the accounts of early workers such as Branca (1924) were examined, Category C nuclei showed a close resemblance to nuclei between MI and MII division. This interlude between the two stages of division is reported as transitory. Therefore observations should record rather few nuclei at this stage whereas observations actually recorded rather large numbers of these nuclei. This incongruence suggested that alternative places in the cycle should be considered. One alternative view was that Category C nuclei were one of the spermatogonial generations. Some support for this view was found in Clermont's description of his Ap and B type spermatogonia. In this respect Category A nuclei resembled Clermont's Ap nuclei and Category C nuclei resembled Clermont's B type. This simple view had to be discarded when no evidence was found for the presence of labelled C nuclei at 0 hours in culture. Rare labelled C nuclei were observed after 24 hours in culture. A possible reason for recording rare C type nuclei was C type textures represented a different phase in the cycle from the S phase

textures which could be identified due to the presence of label taken up at 0 hours. If this were so, larger numbers of labelled C type nuclei ought to have been observed after time in culture, but only rare labelled C nuclei were ever observed. At this stage of the analysis Category C nuclei could not be clearly placed within the sequence.

Category D nuclei

At 0 hours these nuclei were heavily labelled and were clearly part of the premeiotic cycle. At 0 hours in culture labelled members of this category were relatively smaller than unlabelled D nuclei. With time in culture the labelled nuclei increased in size until they were similar in size to unlabelled D nuclei. This was interpreted as representing growth in culture. Texturally these nuclei resembled Clermont's R nuclei. In the 10 day cultures, evidence was recorded for the development of D nuclei into early pachytene figures. However, there was also evidence in these cultures that not all D nuclei were involved in this progression. A population of labelled D nuclei, which remained similar to the D population at 0 hours, could be observed and could be differentiated on size criteria from the larger meiotically committed nuclei. It thus seemed probable that Category D represented at least one spermatogonial generation in addition to S phase spermatocytes. These two cell types could not be distinguished under normal conditions (0 hours in culture). When artificial conditions of long term culture operated, discrimination between the two cell types was possible.

Category G nuclei

At 0 hours these nuclei were heavily labelled and were clearly

part of the premeiotic cycle. As they were the smallest labelled nuclei recorded they apparently fitted the description of S phase spermatocytes supplied by workers such as Roosen-Runge and Barlow (1953), Lima de Faria et al (1968), Chandley and Kofman-Alfaro (1971). This was clearly at variance with designating Category D nuclei (which fitted Clermont's description) as S phase spermatocytes. However, it was possible that D nulcei were the last spermatogonial generation and G nuclei were in fact S phase spermatocytes. The observations recording D nuclei differentiating into S phase spermatocytes provided one piece of evidence against designating G nuclei as S phase spermatocytes. Further evidence against this supposition was the fact that labelled G nuclei showed a marked degeneration after 24 hours in culture. Despite degeneration, in vitro differentiation was established. It was thus thought unlikely that G nuclei were S phase spermatocytes. In the two concordant samples T133 and T11, at 0 hours in culture, labelled G nuclei were similar in size to unlabelled G nuclei. After time in culture, taking degeneration into account, labelled G nuclei increased in size until they were somewhat larger than unlabelled G nuclei. This was interpreted as representing growth in culture. Thus it would seem likely that G nuclei were a spermatogonial cell type proceeding D nuclei.

Texturally these nuclei were similar to Clermont's description of his Ad type suggesting that they represented the stem cell type.

Category A nuclei

These nuclei were moderately labelled at 0 hours in culture. Descriptively they fitted Clermont's Ap type and a lot of effort was put into considering them as a spermatogonial generation. This view seemed to be substantiated by their labelling pattern. At 0 hours in

culture labelled A nuclei were smaller in size than unlabelled A nuclei. By 72 hours the labelled nuclei had increased in size until they were similar to unlabelled A nuclei.

One difficulty in describing A nuclei as spermatogonia was the relative size difference between A and G nuclei. If A nuclei were equated with Clermont's Ap type and G nuclei with Ad then the small stem cell type spermatogonia gave rise to large spermatogonia which divided to give smaller D nuclei. As an alternative proposition Steinberger et al (1970) suggested that Ap was the stem cell type rather than Ad. They based their hypothesis on the fact that, with increasing time in tissue culture, Clermont's ratio of 1Ad:1Ap changed to the situation where Ap had become predominant. Certainly the frequency of Category A nuclei increased with time in tissue culture to become the predominant cell type. However, to invoke this system would mean that Category A nuclei would divide to give G nuclei and presumably more A type nuclei as part of the stem cell renewal cycle. This situation, which involves some sort of differential mitosis, seems as unlikely as the reverse situation if G nuclei were designated stem cell type.

An alternative explanation was that Category A nuclei were not spermatogonia and that Category G nuclei were the stem cell type both renewing themselves and giving rise to further spermatogonial generations ultimately designated D nuclei.

5. <u>CONCLUSIONS</u> <u>Sequence of spermatogenesis. Model I</u>

To sum up, the proposed model for the sequence of cell categories within the cycle of spermatogenesis could be described as follows :-

Category A ——— ? ———> Category G ———> Category D ———>
Category D', S phase spermatocyte ———> Category B ———>
Category E ———> Category F. Category C not known.

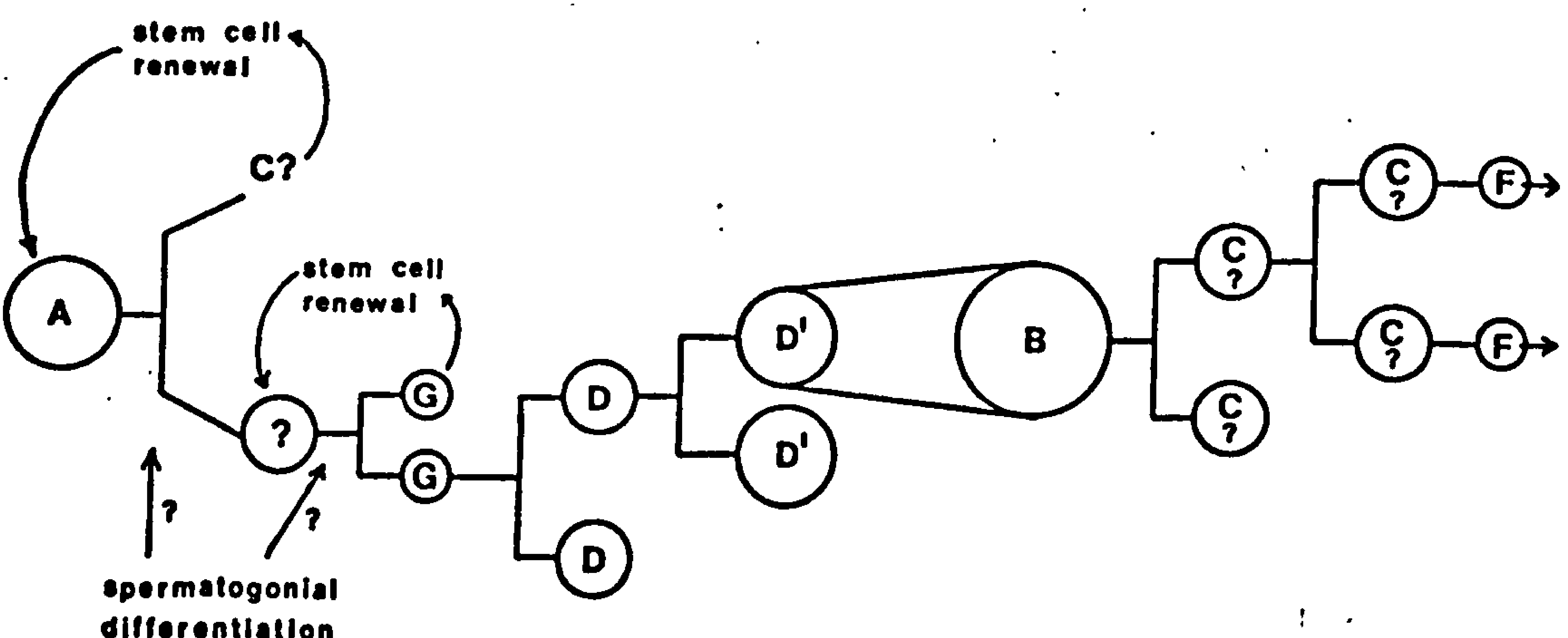
Figure 9.13 represents this scheme. The figure includes the possibility that either A or G could be the stem cell type and includes possible positions of Category C.

The results of this work in relation to other authors work can be summed up as follows :-

1. It was possible to identify spermatogonial cell types. There were at least 2 types of spermatogonia present in the testicular epithelium which could be separated on testure and size in these preparations.
2. It is very difficult to equate these cell types with those described by other workers. In part this was due to differences in material preparation.
3. In this human material S phase primary spermatocytes were larger in size relative to other cell types than previous authors had described.

Figure 9.13

Sequence of spermatogenesis. Model I nuclei shown in S phase



CHAPTER 10

ANALYSIS OF CELL CATEGORIES RECORDED FROM INFERTILE INDIVIDUALS

1. Introduction

2. Results

3. Data analysis
   - A. The sequence of cell categories in the spermatogenic cycle, Model II
   - B. Sertoli cells only and Category A cells
   - C. Correlation and regression analysis
   - D. Testing models of spermatogenesis
   - E. Category A as an indicator of testicular epithelial function
   - F. A practical method of detecting lesions of spermatogenesis

4. Conclusions

1. INTRODUCTION

In this Chapter the frequencies of cell categories obtained for the series of infertile males listed in Chapter 3 are recorded. It was hoped that this data would describe events within the spermatogenic cycle which could then be related to each individual's infertility.

Out of the total population of 68 infertile cases, only 37 cases were scored. Many of the earlier preparations had deteriorated badly and in some cases whole tubule preparations had not been made. 5 cases could not be included in the category analysis since they apparently contained different categories of nuclei, the implications of this will be discussed later.

2. RESULTS

The results of scoring the frequency of cell categories in 37 infertile individuals are listed in Table 10.1 together with histology categories and JMS values. It was apparent that extreme variability existed between individuals in the frequencies reocrded. For instance the frequency of Category A ranged between 19% and 86%; this category demonstrated the greatest range of scores.

This data was accumulated in order to look for points of lesion in the cycle. One way of attempting this would be to examine the numerical relationships between the cell categories. An initial attempt to analyse these numerical relationships met with little success. This was largely because the sequence of cell categories within the cycle was still uncertain. Inspection of the data suggested that the sequence of cell categories could be clarified from the recorded frequencies. This proved to be the case and subsequently an analysis of the numerical relationships could be attempted. The next section describes the analysis of the cell category frequency data listed in Table 10.1.

TABLE 10.1

FREQUENCY OF CELL CATEGORIES, HISTOLOGY CATEGORIES

AND JMS FOR INFERTILE POPULATION

Case No	% frequency cell category								JMS	Histology Category
	A	B	C	D	E	F	G	H		
20	42.25	21.25	10.0	17.5	2.0	3.0	3.0	1.0	7.4	RG
21	46.5	30.25	11.0	4.0	1.5	2.75	3.0	1.0	7.8	RG + S tubes
22	no tube preparations								8.1	NAD
23	47.5	20.25	17.75	6.25	3.0	1.75	2.0	2.0	8.6	NAD
24	30.75	21.0	14.0	5.5	7.25	13.0	7.0	1.5	7.6	RG
25	different categories								4.5	FAG
26	36.5	27.75	13.25	9.25	0	4.5	6.25	2.5	6.5	RG
27	49.75	20.75	9.0	6.0	3.5	4.75	3.25	3.0	8.5	NAD
28	59.25	15.0	10.25	5.75	2.5	1.25	3.25	2.75	5.1	RG + S tubes
29	43.5	31.5	7.75	5.75	3.75	3.5	3.5	0.75	6.6	RG + S tubes
30	47.25	14.0	13.5	7.25	6.0	5.75	3.5	2.75	no score	-
31	30.5	23.0	15.0	11.25	2.5	9.5	5.25	3.0	7.3	RG + S tubes
32	48.5	17.0	10.75	4.5	1.0	6.75	8.5	3.0	4.6	S/RG
33	56.75	17.75	6.25	3.5	0.5	5.25	10.0	0	5.5	RG
34	no tube preparations								5.8	RG + S tubes
35	44.25	25.5	12.25	8.0	1.5	4.5	3.5	0.5	7.2	RG
36	24.5	34.75	10.0	13.5	2.0	8.0	4.75	2.5	6.1	RG + S tubes
37	no tube preparations								2.0	S/FAG
38	30.75	31.25	16.5	6.75	0.25	8.75	3.75	2.0	no score	-
39	no tube preparations								no score	-
40	no tube preparations								no score	-
41	36.75	25.25	15.0	6.75	0	7.75	3.5	5.0	7.4	B
43	no tube preparations								6.7	RG
44	no tube preparations								6.5	RG + S tubes
45	26.5	29.0	19.25	7.25	1.5	10.25	3.25	3.0	8.0	B
46	56.0	17.25	8.0	2.5	1.0	5.25	8.75	1.25	6.6	RG
47	different categories								6.2	RG + S tubes

TABLE 10.1 continued

Case No	% frequency cell category								JMS	Histology Category
	A	B	C	D	E	F	G	H		
48	38.25	20.75	15.75	5.5	4.25	10.75	2.5	2.2	8.3	B
49	53.75	18.5	10.75	3.5	3.25	3.5	4.75	2.0	7.6	B
51	19.25	30.5	18.25	11.5	6.0	9.75	2.75	2.0	7.7	RG
52	too poor score								7.2	RG
57	28.75	37.0	14.0	9.25	1.5	2.75	6.75	0	8.9	NAD
66	not counted								no sample	-
67	40.0	26.75	15.25	8.25	0.25	2.75	6.75	0	8.5	NAD
68	30.75	27.75	17.0	9.75	0	8.25	6.5	0	8.5	NAD
69	22.75	20.75	31.25	7.25	1.5	9.0	4.75	2.75	6.7	RG + S tubes
76	85.75	5.0	0	3.5	0.5	0	4.25	1.0	2.0	FAG
79	27.0	25.75	30.75	5.5	1.25	3.75	6.0	0	8.2	RG
80	18.75	25.25	24.0	6.25	1.25	15.25	6.75	2.5	8.3	NAD
82	56.5	13.0	6.75	6.0	0	0.5	14.75	2.0	6.8	RG
88	30.75	12.5	21.75	3.0	2.0	22.75	4.75	2.5	no score	-
105	29.75	28.25	17.75	8.5	3.0	8.25	3.0	1.5	no sample	-
106	not counted								no sample	-
107	not counted								no sample	-
116	33.75	22.75	16.25	11.25	1.5	9.5	4.25	0.75	8.1	NAD
117	46.5	22.5	6.5	11.5	4.5	2.0	5.75	1.25	5.0	FAG
118	different categories								1.2	FAG
121	no tube preparations								8.1	RG
123	different categories								1.8	FAG
130	different categories								2.0	FAG
133	21.25	31.25	17.75	11.75	4.0	8.5	4.5	1.0	7.7	RG
135	29.25	26.75	19.75	7.5	2.25	11.0	2.75	0	8.7	NAD
136	68.5	11.0	6.0	2.75	1.0	0.25	9.25	1.25	3.0	FAG
137	48.0	10.0	14.25	11.5	1.75	4.0	7.0	3.0	5.0	RG + S tubes

3. DATA ANALYSIS

A. The sequence of cell categories within the spermatogenic cycle. Model II.

In Case 76, Categories C and F were absent, the histology was recorded as "FAG", the JMS was 2.0 and the most advanced tubule stage recorded was at Stage III. When the mixed cell population was scored, cells were assigned to the cell categories without difficulty. The implication of the absence of Categories C and F was that they represented events in the normal sequence of the cell cycle which occurred, in this case, after the arrest of spermatogenesis. The most advanced categories identified in this preparation were cells in pachytene, recorded as Category B and two cells in diakenesis, recorded under Category E. Categories C and F must therefore occur after MI division has taken place.

Reasons have been given for assigning Category F to the post-meiotic spermatid stage. Category C might be assigned to the stage between MI and MII division or to a stage representing an early form of the spermatid. The presence of occasional labelled C nuclei after 24 hours in culture suggested a preference for the stage between MI and MII division since such cells could have picked up label during R synthesis. Direct confirmation for this assignment could be obtained by observing labelled C nuclei appearing between 72 hours and 10 days in culture in some numbers. Unfortunately such material was not available for study. In addition information from later harvested cultures indicated that differentiation did not proceed beyond diakenesis. So although it seemed unlikely that direct confirmation for the position of Category C in the sequence could be obtained, it was clear that Category C did not constitute one of the spermatogonial generations.

Indirect confirmation for the assignment of Category C was sought by examination of the cell categories recorded for other cases with histology categorised as "FAG".

Within the series there are 9 such cases (including T76); two other cases had been scored for cell category frequency, four cases were described as "different categories" and two cases had not been scored as they were early preparations which had deteriorated. All nine cases were reviewed and the observations recorded are shown in Table 10.2. T117 and T136 have all categories present although C and

TABLE 10.2

CASES RECORDED AS FAG ON HISTOLOGY

FAG Case No.	JMS	Most advanced tube scored	% Frequency cell category presence or absence							
			A	B	C	D	E	F	G	H
117	5.0	6	46.5	22.5	6.5	11.5	4.0	2.0	5.75	1.25
15	4.0	5	+	+	-	+	-	-	+	-
18	3.9	5	+	+	-	+	-	-	+	-
25	4.5	5	+	few	-	few	-	-	+	-
136	3.0	3	68.5	11.0	6.0	2.75	1.0	0.25	9.25	1.25
76	2.0	3	85.75	5.0	-	3.5	0.5	-	4.25	1.0
118	1.2	3	+	+	-	+	-	-	+	-
123	1.8	3	+	+	-	+	-	-	+	-
130	2.0	3	+	-	-	+	-	-	+	-

F have low frequencies. All other samples have C and F unrecorded. Case 130 had Category B unrecorded. T123, had nuclei which were large, palely staining and had a thread-like chromatin texture rather than a grainy texture. These could be Leptotene/Zygotene nuclei and their presence in large numbers might represent a block at this stage.

In all these samples Category A nuclei were present as the majority of the cell sample. Category B, D, G, when recorded, were present in very small numbers. In T25, B and D nuclei were rare but observed.

It was noticeable that the JMS and the most advanced tubule stage scored did not correspond exactly with the cell categories recorded, although they certainly reflected the overall quality of the testicular epithelium. In general within the "FAG" group, the cell category data indicated more mature activity of the germinal epithelium than the histology sections.

B. Sertoli cells only and Category A

Following re-examination of cell categories in the "FAG" histology category, cases assigned to Sertoli cell only category ("S and other") were re-examined. It is important to recall that this histology category was assigned when the majority of tubules were S cells only but other types of tubule were also present. Table 10.3 records information following re-examination of these cases. T32 had approximately 1/3 tubules as Stage X and all cell categories were present in the mixed cell population. In the other two samples (T8 and T13) in which cell categorisation was possible, Categories C and F were absent. This was consistent with placing Category C during or after meiotic division. During re-examination a further interesting observation was recorded (see Table 10.3). The 5 individuals could be separated into 2 groups on three criteria.

TABLE 10.3

CASES RECORDED AS "S and OTHER" ON HISTOLOGY

Case No.	JMS	No. of tubes scored 1 2 3 4 5 6 7 8 9 10	Cell categories A B C D E F G	Distribution of S tubes and other tubes
8	3.1	0 42 0 0 4 0 3 2 2 1	+ + - + - - +	small patches ± N tubes. S tubes, spindle cells
13	3.4	0 36 1 0 4 0 0 7 1 1	+ few - - - - +	small patches ± N tubes. S tubes, spindle cells
19	2.6	0 50 0 4 0 0 0 2 0 0	no preparations	Small patches ± N tubes. S tubes ± spindle cells
32	4.6	0 33 0 0 0 0 0 2 0 15	49% 17% 11% 5% 1% 7% 9%	Mixed S tubes + others. S tubes, round cells
37	2.0	11 28 3 8 0 0 0 0 0 0	No preparations	Mixed S tubes + others. S tubes, round cells

1. Samples 8, 13, 19 : cells lining S tubules were oblong shaped with distinct spindle shaped nuclei and a prominent darkly staining nucleolus.

   Samples 32, 37 : cells lining S tubules were round cells with round, palely staining nuclei and a darker staining nucleolus.

2. Samples T8, T13, T19 : the distribution of more or less normal tubules was in patches or in a single area.

   Samples T32, T37 : the distribution of more or less normal tubules was mixed with S only tubules.

3. Samples T8, T13, (no T19) : many spindle shaped nuclei with dark staining nucleoli were noted in the mixed cell population.

   Sample T32, (no T37) : spindle shaped nuclei were not recorded in the mixed cell population.

On this basis, T8, T13, T19 are clearly different from T32, T37. One interpretation of this separation is that T32 and T37 have been misclassified and their proper classification is either RG/T32 or FAG/T37.

In the normal histology sections which Clermont (1963) and Steinberger et al (1970) used to illustrate their work, Sertoli cells were marked as round cells with pale cytoplasm and darkly staining nuclei. Such cells could be identified in the histology sections of the infertile series in general. In addition these cell types were present in large numbers in T32 and T37 when they were recorded as Sertoli cells and they were the cell type present with greater than average frequency in a number of other individuals.

Finally the "RG + S" group, which consisted of 12 cases with a low score of Sertoli cell only tubules, was re-examined for the presence of round or spindle shaped Sertoli cells. 5 cases had round type Sertoli cells lining the tubules and were thus comparable with T32 and T37. In the other 7 cases some tubules were unequivocally lined with round type Sertoli cells. But, in other tubules in each case, Sertoli cells were so numerous that they became close packed and then appeared elongated. These tubules were not exactly the same as tubules lined by spindle shaped cells observed in T8, T13, and T19. The next step was to reconsider the data derived from the mixed cell population. The only recorded observations of spindle shaped Sertoli cells came from T8 and T13 where they were obvious. Spindle shaped cells had not been observed in the original cell populations derived from the normal tubules on which categorisation was based. However, Category A nuclei were observed in the mixed cell population. They were highly variable in frequency with particularly high frequencies recorded in cases assigned to the "FAG" histology group. The frequency of

Category A nuclei increased with time in tissue culture and they represent a dividing cell population. Category A nuclei identified from the mixed cell population appear to correspond to the round type Sertoli cells identified in the histology sections.

It would thus appear that there are 2 types of "Sertoli cells" present in the testicular epithelium and these can be recognised in both the mixed cell populations and in histology sections. It is not known whether they are mutually exclusive nor is their relationship to the Sertoli cells described by other workers clear. It is possible to speculate that round type cells which are present in normal testes are an invasive type. They increase in numbers either because their increase limits spermatogonial production or because they replace the independently diminishing production of spermatogonia. Spindle shaped Sertoli cells might represent the situation where the testicular epithelium has never become active. The problem with this suggestion is that it is based entirely on retrospective analysis. It does however, confirm the view that Category A nuclei are not part of the spermatogonial generations.

After completing this part of the analysis a literature search identified one other report of two types of Sertoli cells existing in Man. Johnsen (1969) described two types of Sertoli cells which could be differentiated on the basis of a 5 dye staining process. He reported the presence of both types of Sertoli cells in different ratios in Sertoli cell only cases, Klinefelters, normal individuals, prepubertal testes and in an unspecified case of hypospermatogenesis. His 2 cell types were observed together both in spindle shaped and round celled Sertoli cell only tubules.

C. Correlation and regression analysis

Cell category frequencies, which represent estimations of cell numbers present at any one stage of the cycle, show considerable variation between individuals. However, when ratios between paired cell categories were considered (see Table 10.8) there was some suggestion that these ratios showed consistent trends throughout the population. Such population trends can be tested for, using correlation analysis. The presence of a highly significant positive correlation would suggest that the ratio between two categories was nearly constant throughout the population.

Table 10.4 lists correlation coefficients between all possible paired categories. The value r^2 is an estimate of the percentage of the total variability which is accounted for by the correlation between the two variables. Thus 60% of the total variability between A and C is accounted for by the correlation whereas only 7.8% of the total variability between A and G is accounted for by the correlation leaving the rest due to other sources of variation.

Figure 10.1 gives the correlation coefficients for the probable combinations which describe the succession of events in the spermatogenic cycle. These results show that the highly significant relationships are A with B, C and F (negative) and C with F (positive).

These results are difficult to interpret since the frequency figures are normalised data representing a closed array series. This means that as one category increases in numbers, other categories must decrease in numbers. The increasing class can be referred to as a dilution factor. The presence of a dilution factor implies that the other categories will retain their positions with respect to each other whether these positions represent meaningful ratios or not.

The most striking feature of the correlation analysis is the

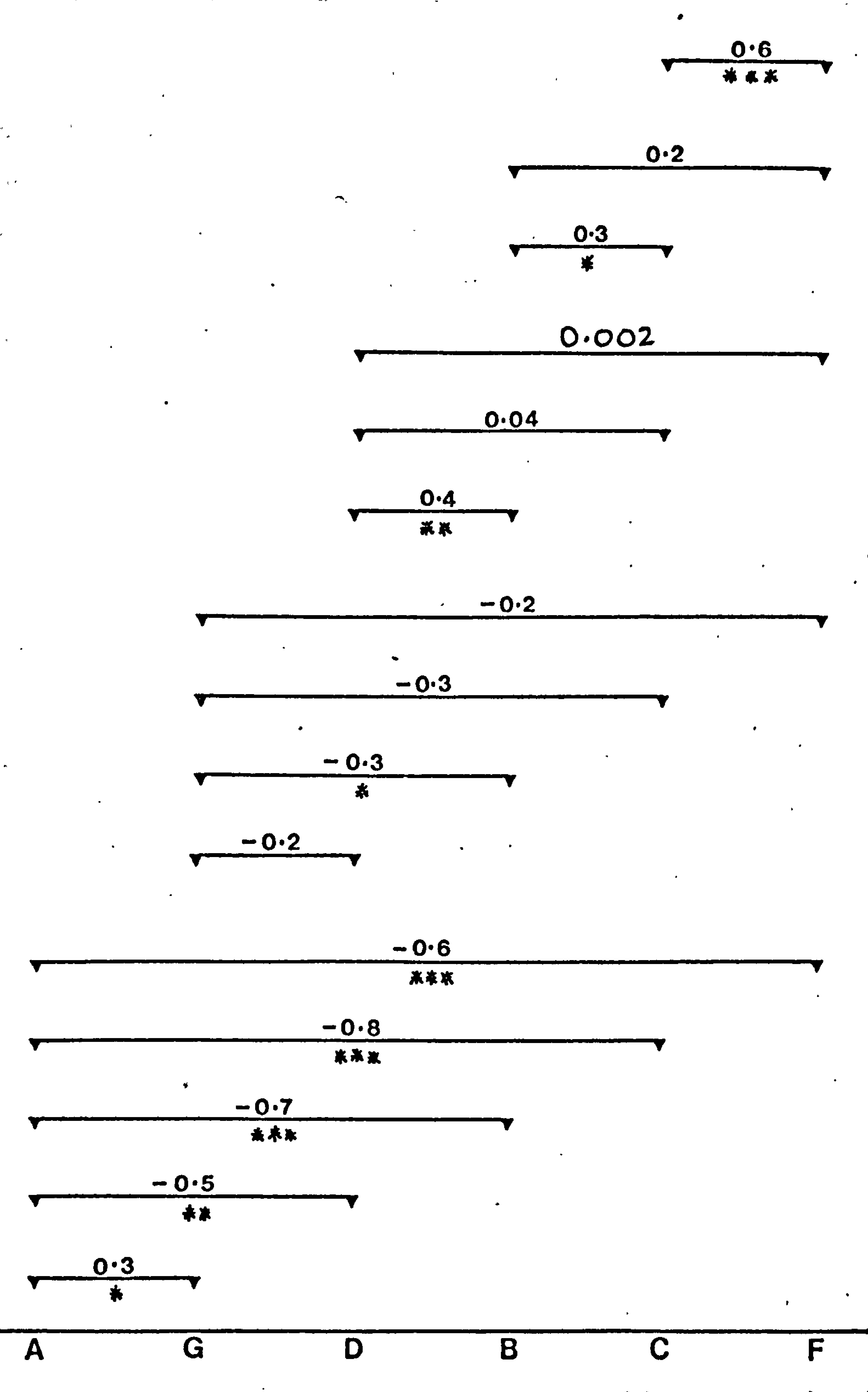
TABLE 10.4

ALL POSSIBLE COMBINATIONS OF CORRELATIONS

Categories	r (df=35)	Significance	r^2
A-B	-0.723	***	0.523
A-C	-0.776	***	0.602
A-D	-0.50	**	0.250
A-F	-0.646	***	0.417
A-G	0.279	*	0.078
B-C	0.330	*	0.109
B-D	0.412	**	0.170
B-F	0.163	non sig	
B-G	-0.327	*	0.107
C-D	0.044	non sig	
C-F	0.567	***	0.321
C-G	-0.262	non sig	
D-F	0.002	non sig	
D-G	-0.241	non sig	
F-G	-0.197	non sig	

Figure 10.1

Correlations for combinations within sequence of spermatogenic cycle



highly significant negative correlation between Category A and Categories B, C and F which says that as Category A increases in frequency the frequencies in the other categories decrease. Category A is in fact acting as a dilution factor. In this system the fact that Category A arithmetically behaves as a dilution factor can be interpreted in terms of the spermatogenic system. Thus increasing numbers of Category A nuclei were recorded as the germinal epithelium became increasingly inactive (see high frequency of Category A nuclei in cases recorded as "FAG").

Therefore the frequency of Category A may be interpreted as measuring the potential activity of the germinal epithelium.

When the relationships between the other categories were considered, significant positive correlations were found between D and B and C and F, thus indicating the presence of constant ratios in these categories. But, because of the presence of the dilution factor, the analysis fails to differentiate between confirmation of the expected trends and the maintenance of a random relationship independent of biological prediction. However, in a proliferative system such as spermatogenesis, the numbers of cells should increase in some type of regular progression due to division events occurring throughout the cycle.

Having discussed the uncertainties of using frequency data to establish ratios between categories, weight was given to the likelihood (based on the biological predictions) that such ratios existed within the frequency data. Linear regression analysis was used to derive these relationships between categories where the slope and standard error of the slope expressed the ratio and the error attendant on the ratio in quantitative terms.

The results of the regression analysis are given in Table 10.5. Since the correlation between G and D was not significant, this regression

was not tested. The results give a numerical basis for the ratios between categories which were approximately,

10D : 9B

5B : 1C

5C : 2F

In the case of the ratio between B/C, the correlation coefficient had been of borderline significance. Testing with linear regression analysis gave a variance ratio which was non-significant. This result suggested that the relationship was not strong enough to be meaningful and in this respect the standard error was noticeably huge. Thus the doubtful significance of the correlation was confirmed by linear regression analysis.

Overall these results suggest that there are relationships between categories but there is a large amount of variation present in the data probably due to differences between individuals.

TABLE 10.5

REGRESSION ANALYSIS BETWEEN SIGNIFICANTLY CORRELATED CATEGORIES

Between categories	Slope	Standard error of slope	VR	Probability	Significance
D/B	0.901	0.290	11.825	1–0.1%	**
B/C	0.193	0.148	1.694	>20%	non sig.
C/F	0.400	0.112	12.736	0.1%	***

D. Testing models of spermatogenesis

Having established some apparently significant ratios between cell categories it is useful to consider possible models for cell division which would provide "predicted" ratios. In all these cases, it is assumed that each cell stage occupies an equal proportion of the total spermatogenic cycle time (this assumption is discussed further below). The predicted ratios can be derived as follows :-

1(a) If G divised once to give 2D, the ratio is 1:2, which is equivalent to a slope of 2.

(b) If G divides once to give 2 more G followed by a division to give 4D, the ratio is 3:4, which is equivalent to a slope of 1.33.

2(a) If D divides once to give 2B, the ratio is 1:2, which is equivalent to a slope of 2.

(b) If D divides once to give 2 S phase D type nuclei and these nuclei then grow to become B nuclei, the ratio is 1D:2D (S phase primary spermatocytes : 2B = 3:2, which is equivalent to a slope of 0.66.

3(a) If C is inter MI and MII division, then MI division of B gives 2C, the ratio is 1:2, which is equivalent to a slope of 2.

(b) If C is an early spermatid stage then B divides twice during meiosis to give 4C, the ratio is 1:4, which is equivalent to a slope of 4.

4(a) If C is as in 3(a), then 1C divides at MII division to give 2F, the ratio is 1:2, which is equivalent to a slope of 2.

(b) If C is as in 3(b), then there is no division between C and F and C = F.

The theoretical relationships expressed as a slope can be compared with the calculated slope derived by linear regression analysis from

observed frequencies using Students t test. The results are recorded in Table 10.6. The only ratio where the t test is not highly significant is D:B = 3:2. So in this one case we can say that the observed ratio between D and B is not significantly different from the expected ratio of 3D:2B. This fits the analysis of changes in D spermatogonia through to S phase spermatocytes through to early pachytene nuclei described from the analysis of the autoradiographic patterns of D nuclei in Chapter 9.

TABLE 10.6

t TEST BETWEEN OBSERVED RATIOS (REGRESSION ANALYSIS TABLE 10.4) AND PREDICTED RATIOS

Between categories	Expected ratios tested	t test (35df)	P	Sig.
D/B	1:2 (2a)	3.781	<0.1%	***
	3:2 (2b)	0.807	40%	not sig.
B/C	1:2 (3a)	12.158	<0.1%	***
	1:4 (3b)	25.618	<0.1%	***
C/F	1:2 (4a)	14.260	<0.1%	***
	1:4 (4b)	32.085	<0.1%	***

In an attempt to minimise the effect of between individual variation, regression analysis and tests of observed to expected ratios were analysed for the 12 cases categorised as "NAD" or "B" on histology. The results are recorded in Table 10.7. The results are not very different from testing the whole population except that this data fails

to discriminate between 1:2 or 3:2 ratios in the D/B relationship.

TABLE 10.7

REGRESSION ANALYSIS AND t TESTS BETWEEN OBSERVED AND EXPECTED RATIOS ON 12 CASES CATEGORISED "NAD" OR "B" ON HISTOLOGY

Categories	Slope	Standard Error	VR	P	t test	Expected ratio	P.	Sig.
D/B	1.347	0.623	4.671	5%	1.045 1.102	1:2 3:2	30% 30-20%	not sig. not sig.
B/C	0.195	0.242	0.652	>20%	-			
C/F	0.731	0.242	9.060	1%	5.223	1:2	<0.1%	***

Clermont's model proposed that the spermatogonial stem cell passed through 3 successive divisions before giving rise to spermatocytes. His ratios, which were based on the number of divisions occurring at each stage, were as follows :-

1 Ad : 1 Ap : 2B = 4 Pl (resting primary spermatocytes)

In this work I have not been able to identify 3 spermatogonial divisions although the data and observations do not preclude their presence. In addition correlation between Clermont's cell types and cell categories was not possible. The conclusions which were drawn from applying the results of correlation and regression analysis to models of spermatogenesis are as follows. Relationships do exist between categories but in general they do not correspond to ratios dependant solely on division events as suggested by Clermont and other workers. Indeed

the ratios between cell categories should be affected by two factors.

1. The number of cell divisions which take place before one cell stage is transformed to a subsequent cell stage.
2. The duration which each cell stage occupies as a proportion of the total cell cycle time.

If the frequency data could be accounted for by ratios simply dependant on division events, then each cell stage would have to occupy a similar proportion of cell cycle time. This assumption does not seem to have been mentioned by Clermont or his associates. In fact, the pachytene stage of division is a known example of a cell stage which occupies a proportionally long period of cycle time. In this present programme of experiments the duration of the various cell stages was not investigated. It would therefore seem likely that a fuller analysis of the ratios reported between cell categories must await determination of the duration of the cell stages represented by the cell categories.

E. Category A as an indicator of testicular epithelial function

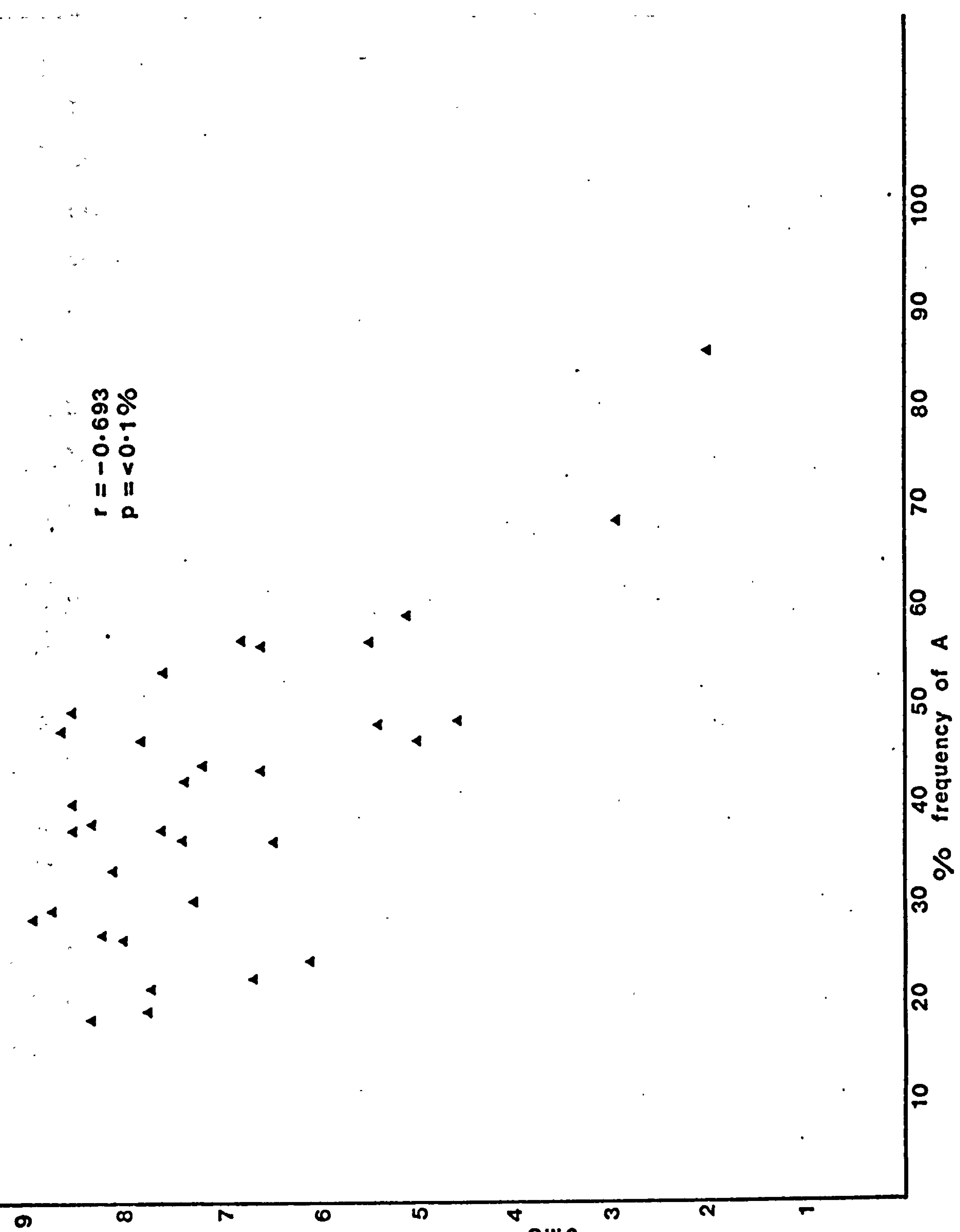
I have already indicated that the frequency of Category A nuclei may be an important marker of the activity of the testicular epithelium. Category A shows a high negative correlation with all other categories except Category G. It is possible to examine the assumption that it marks activity by testing the correlation of Category A with JMS, since the JMS is another numerical method of scoring testicular activity. A plot of the correlation is shown in Figure 10.2. The correlation between the two variables is highly significant,

$$r = -0.693,\ 33df,\ p < 0.1\%$$

indicating that as the frequency of A increases the JMS falls.

Figure 10.2

Correlation between frequency of Category A nuclei and JMS



In Table 10.4 (Section C), the highest correlation in the series A versus other categories is that between A and C where

$r = -0.776$, 35df, $p < 0.1\%$, highly significant

This ratio appears to be another useful indicator of epithelial activity since is spans nuclei, which are pre-spermatogonial, with C nuclei, which are post-spermatogonial. When the A/C ratio is plotted against the JMS the correlation is again high,

$r = -0.686$, 32df, $p < 0.1\%$, highly significant

Thus it would seem that the frequency of Category A nuclei can be used as an indicator of the activity of the testicular epithelium. In addition, an overall measure of activity and productivity (excluding spermiogenesis) can be demonstrated by calculating the A/C ratio.

F. A practical method of detecting lesions of spermatogenesis

Reasons have been presented for believing that between cell category frequency ratios represent a combination of factors rather than a single factor. Thus the frequencies may represent the product of the division event and the proportional duration of any particular cell type.

In this section of work I have tried to devise a method whereby I can subjectively select individuals whose cell frequencies lie outside the average for the population. In order to do this the ratios between categories in each individual have been calculated taking the sequence in order. Thus the ratios calculated were A/G, G/D, B/C, C/F, with the first category in each pair being given unit value. Then the mean and standard deviation for each ratio between paired categories was calculated for the infertile population. The results are shown in Table 10.8. These means and standard deviations gave an average value for each ratio within the population. The calculation gave equal weight to each individual which, without information from normal fertile individuals, seemed appropriate.

TABLE 10.8

RATIOS BETWEEN CATEGORIES FOR INFERTILE POPULATION

Case No.	RATIOS BETWEEN CATEGORIES				
	A:G	G:D	D:B	B:C	C:F
20	1:0.07	1:5.8	1:1.2	1:0.47	1:0.3
21	1:0.06	1:1.3	1:7.5	1:0.36	1:0.25
23	1:0.04	1:3.1	1:3.2	1:0.87	1:0.10
24	1:0.23	1:0.7	1:3.8	1:0.67	1:0.93
26	1:0.17	1:1.5	1:3.0	1:0.48	1:0.34
27	1:0.07	1:1.8	1:3.5	1:0.43	1:0.53
28	1:0.05	1:1.8	1:2.6	1:0.68	1:0.12
29	1:0.08	1:1.6	1:5.5	1:0.25	1:0.45
30	1:0.07	1:2.1	1:1.9	1:0.96	1:0.43
31	1:0.17	1:2.1	1:2.0	1:0.65	1:0.63
32	1:0.17	1:0.5	1:3.8	1:0.63	1:0.63
33	1:0.19	1:0.4	1:5.1	1:0.35	1:0.84
35	1:0.08	1:2.2	1:3.2	1:0.48	1:0.37
36	1:0.19	1:2.8	1:2.6	1:0.29	1:0.80
38	1:0.12	1:1.8	1:4.6	1:0.53	1:0.53
41	1:0.09	1:1.9	1:3.7	1:0.59	1:0.52
45	1:0.12	1:2.3	1:4.0	1:0.66	1:0.53
46	1:0.16	1:0.3	1:7.1	1:0.45	1:0.66
48	1:0.06	1:2.2	1:3.8	1:0.76	1:0.68
49	1:0.09	1:0.7	1:5.3	1:0.58	1:0.33
51	1:0.14	1:4.2	1:2.6	1:0.60	1:0.53
57	1:0.23	1:1.4	1:4.0	1:0.38	1:0.20
67	1:0.17	1:1.2	1:3.2	1:0.57	1:0.18
68	1:0.21	1:1.5	1:2.8	1:0.61	1:0.49
69	1:0.21	1:1.5	1:2.9	1:1.5	1:0.29
76	1:0.05	1:0.8	1:1.4	0	0
79	1:0.22	1:0.9	1:4.7	1:1.2	1:0.12
80	1:0.36	1:0.9	1:4.0	1:0.95	1:0.64
82	1:0.26	1:0.4	1:2.2	1:0.52	1:0.07
88	1:0.15	1:0.6	1:4.2	1:1.74	1:1.04
105	1:0.10	1:2.8	1:3.3	1:0.63	1:0.46
116	1:0.13	1:2.6	1:2.0	1:0.71	1:0.58
117	1:0.12	1:2.0	1:2.0	1:0.29	1:0.31
133	1:0.21	1:2.6	1:2.6	1:0.57	1:0.48
135	1:0.09	1:2.7	1:3.6	1:0.74	1:0.56
136	1:0.14	1:0.3	1:4.0	1:0.55	1:0.04
137	1:0.15	1:1.6	1:0.8	1:1.4	1:0.26
n	37	37	37	36	36
$\bar{x}$	0.14	1.75	3.45	0.67	0.45
sd	0.07	1.13	1.45	0.34	0.24

In Table 10.9, 33 cases with complete sets of data, including JMS, were ranked for increasing frequency of Category A. Then deviations from the average ratios (calculated from the $\bar{x} \pm 1$ standard deviation) were recorded against each individual. For instance an increase or decrease in the proportional duration of any stage will respectively increase or decrease the frequency recorded for that category. Similarly restriction on entry to or exit from the category will respectively decrease or increase the frequency of the category. Some of these situations are illustrated in Figure 10.3 together with deviations in the ratios. A sequential analysis of ratios might have indicated which event was most likely to have occurred. In this analysis, paired ratios were used since they were thought to eliminate all but the first affected ratio, provided that only one category in the system was affected.

The following information can be seen in Table 10.9 :-

1. Out of 12 individuals without ratio deviations, 10 were in the lower half of Category A frequency ranking.
2. In general, ratio deviations in Categories C and F (representing the end of spermatogenesis) tended to be associated with low frequencies of Category A.
3. In general, ratio deviations within the spermatogonial generations tended to be associated with high frequencies of Category A.
4. Out of 8 cases with histology rated as "NAD", 5 were ranked in the lower half of Category A and of these, 4 had no ratio deviations. The other 3 cases showed increased frequencies of Category A and ratio deviations indicating disturbances of spermatogenesis which histology categorisation had failed to record.

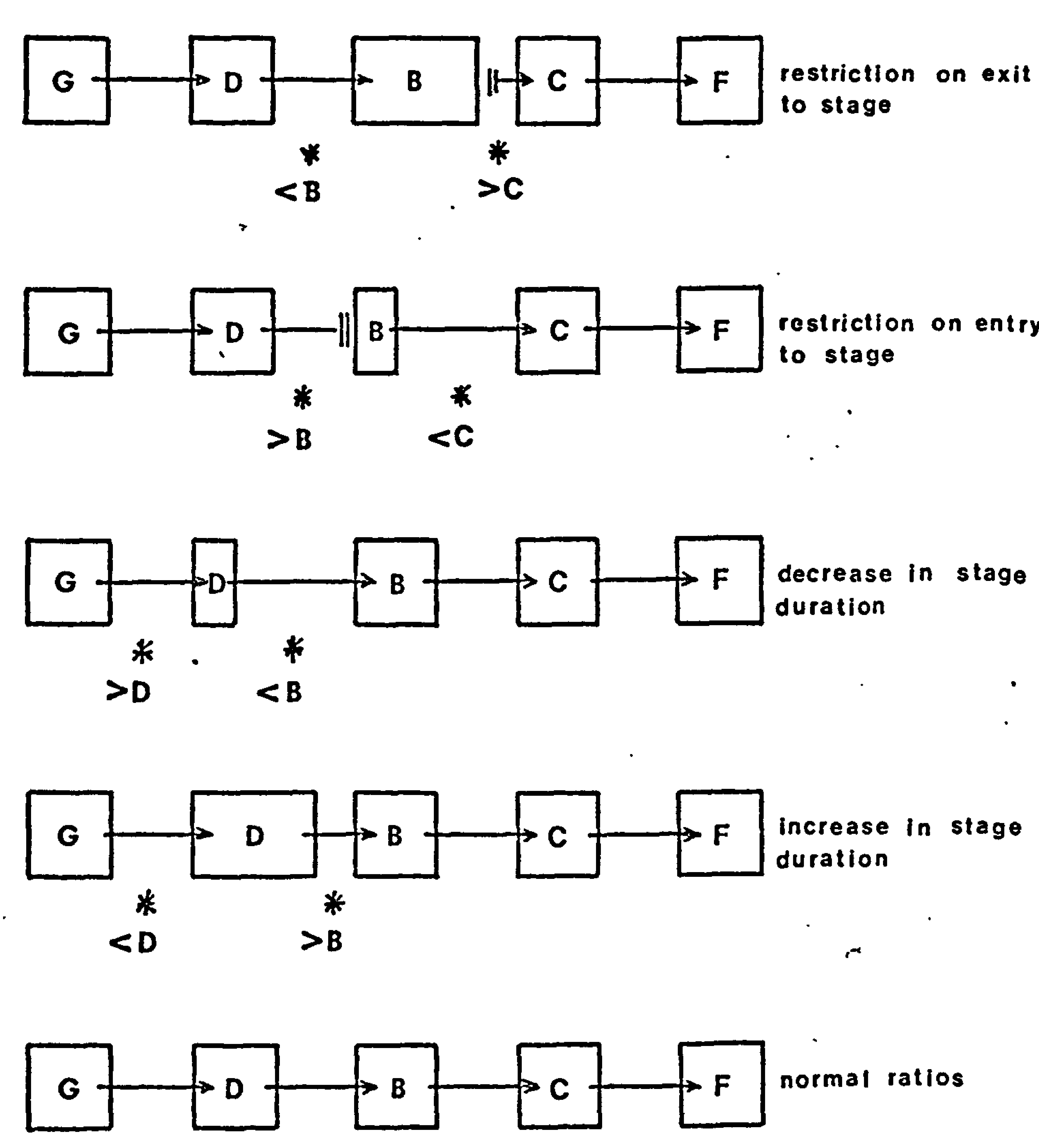
TABLE 10.9

33 INFERTILE CASES, RANKED FOR INCREASING FREQUENCY OF CATEGORY A NUCLEI, SHOWING DEVIATIONS FROM AVERAGE POPULATION RATIOS

Cases Ranked Category A	histology category	No abn. ratios	F +/-	C +/-	Both F & C	B +/-	D +/-	Both D & B
130	FAG							
76	FAG							
136	FAG						-	
28	RG+S		-					
82	RG						(-F)-	
46	RG							*
33	RG							*
49	B					+		
27	NAD	*						
137	RG+S					(+C)-		
23	NAD						+(-F)	
117	FAG			-				
21	RG+S					+		
35	RG	*						
29	RG+S					+(C)		
20	RG							*
67	NAD		-					
48	B	*						
41	B	*						
26	RG	*						
116	NAD	*						
31	RG+S	*						
24	RG		+					
68	NAD	*						
135	NAD	*						
57	NAD		-					
79	RG				*			
45	B	*						
36	RG+S				*			
69	RG+S			+				
133	RG	*						
51	RG						+	
80	NAD	*						

Figure 10.3

Illustration of some situations causing deviations to average ratios



5. The group with histology rated as "RG" were scattered throughout Category A ranking.

T51 had a ratio deviation at G/D which points to a lesion in early spermatogonial proliferation; whereas T24 had a ratio deviation suggestive of a post-meiotic lesion. The JMS scores were 7.6 and 7.7 respectively.

T133 and T35 were without ratio deviations but differed on Category A ranking. In these cases the JMS scores confirmed their different epithelial activity.

To summarise, Category A was a reliable indication of the activity of the testicular epithelium and deviations from average ratios indicated possible points of lesion within the cycle. It is important to notice that the ratio deviations point to the recognition of lesions during the early proliferative part of the cycle.

4. CONCLUSIONS

In this Chapter the cell category frequencies recorded from the series of infertile males have been analysed in detail.

Initially the sequence of cell categories within the spermatogenic cycle was clarified. The considerable variation encountered in the frequency of Category A nuclei was investigated and shown to be significantly correlated with other measures of the activity of the germinal epithelium. Analysis of the relationships between cell categories suggested that it was unlikely that these relationships were simply due to cell division taking place. Finally an attempt was made to use the cell frequency data from the infertile population in order to describe points of lesion in the spermatogenic cycle of an individual.

CHAPTER 11

DISCUSSION

1. Experiments designed to study in vitro differentiation

2. Experiments designed to study the mixed cell population

3. Summary

1. EXPERIMENTS DESIGNED TO STUDY IN VITRO DIFFERENTIATION

Conditions under which in vitro differentiation could be successfully maintained have been described. Essentially they consisted of incubating small pieces of biopsy at 31°C at a 5% CO_2/95% air gas interface with standard Eagles minimum essential medium enriched with sodium pyruvate, glutamine and minimum essential amino acids. These conditions were similar to those described by Steinberger et al (1970) and Ghatnekar et al (1974). They take account of the high (4mM) concentration of glutamine which Steinberger and Steinberger (1966b) recommended for successfully achieving in vitro differentiation. This method of growing whole tubules in culture is particularly suitable for the small amount of material obtained at testicular biopsy.

Under these culture conditions differentiation, traced using H^3Tdr, proceeded through to the diakenesis stage of meiotic division for 17 days in culture. Steinberger et al (1970) observed differentiation through to the late pachytene stage for 3 weeks in culture and Ghatnekar et al (1974) observed differentiation through to Telophase II after 14 days in culture.

The culture conditions used by Ghatnekar included addition of coconut milk, fructose and gonadotrophins to the culture medium. In one out of five cases which Ghatnekar studied, they observed Telophase II after 14 days culture in the sample with added high concentrations of FSH + LH. Prophase II nuclei were identified in 2 cases after 14 days in culture. In one of these cases Prophase II was identified only in the control, whereas in the other case Prophase II was identified in the control and in the samples with added low and middle concentrations of FSH + LH. The variable state of the germinal epithelium in the

cases studied by Ghatnekar et al could account for the variability of results. However, Steinberger et al (1970) found no improvement in differentiation after the addition of various concentrations of testosterone or gonadotrophins.

Whilst differentiation, in the present work, could not be observed beyond diakenesis some viability of the tissue was retained up to 21 days in culture. By this time there was a marked increase in Category A nuclei. Steinberger et al (1970) recorded an increase in Ap:Ad ratios from 1 to 4.6 with increasing time in culture. In contrast to Ghatnekar et al, I was not able to identify cells in the later stages of division either in the control preparations or in samples grown in culture. The use of a swelling solution during preparation of material for analysis may have hampered identification of these later division stages.

Matte and Sasaki (1971) using cell suspensions obtained from a whole testis, were able to observe differentiation through to early spermatid stage after 32 days in culture. Under these conditions the germinal cells grow in suspension. The Steinbergers (1966a) grew rat germinal cells under similar conditions. They interpreted their results as demonstrating maintenance of cells without differentiation but did not attempt labelling studies. Matte and Sasaki used a very high concentration of H^3Tdr for a long incubation time and were able to record the progression of labelled cells with time in tissue culture. Thus they claimed that differentiation had been accomplished. Their experimental conditions were not suitable for use with the small amount of tissue obtained at testicular biopsy. Matte and Sasaki observed degenerating pachytene nuclei after 14 days in culture. They regarded this degeneration as a critical block in meiotic differentiation. Degenerating pachytene nuclei were observed in my work after 10 days

in culture. The degenerating nuclei were present in sufficient numbers to suggest that differentiation may be partially blocked at this stage. Thus my work agrees with these findings of Matte and Sasaki.

There is general agreement between workers that the time taken from H^3Tdr uptake in pre-meiotic S phase until labelled pachytene nuclei can be observed, is about 2-3 weeks. Lima de Faria et al (1968) estimated that labelled pachytene nuclei appeared in culture 14 days after H^3Tdr uptake. These workers regarded this observation as confirmation of Heller and Clermont's (1964) work. In a later paper by the same workers (Ghatnekar et al, 1974) they were able to identify later stages of meiosis. They then revised their estimate so that from H^3Tdr uptake to the completion of meiosis took 14 days. Their results are reported as "main cell stages labelled after 14 days in culture" and they give no information on labelled cells after alternative periods in culture.

My work used a method of material preparation which gave good diakenesis figures. This proved unexpectedly advantageous in the work on sequential labelling studies since diakenesis figures were clearly identifiable and present briefly during meiosis. Thus labelled diakenesis figures were observed after 10 days culture and then after a gap at 13 days culture. Superimposed on this labelling sequence was observation of a burst of diakenesis figures which occurred between days 10 and 14. The labelling sequence suggested that the two peaks of labelled figures represented H^3Tdr uptake at pre-meiotic S phase and R synthesis. Other authors have attempted to identify S phase and R synthesis after brief incubation following H^3Tdr uptake. Thus Lima de Faria (1968) identified heavily labelled Leptotene and Leptotene/Zygotene cells after incubation following H^3Tdr exposure. Chandley and Kofman-Alfaro (1971), working with UV irradiated material in both man and mouse, described a low level of possible R synthesis. They suggested that Lima de Faria's labelled

Leptotene/Zygotene were in fact S phase pre-meiotic cells. Matte and Sasaki (1971) reported labelled Leptotene/Zygotene appearing in culture after 15 hours incubation. I did not observe labelled prepachytene nuclei until 24 hours in culture and the labelling patterns of pachytene nuclei were not informative about the timing of R and S synthesis.

Future work, using clearly identifiable diakenesis figures as indicators of growth could be planned. The period 4-10 days in culture (omitted from the present programme) could be studied, information on the surge of figures (observed in 2 further cultures recently) checked and the bimodal appearance of labelled diakenesis figures confirmed. Then observations of labelled nuclei could be linked with timing of S and R synthesis with greater confidence.

2. EXPERIMENTS DESIGNED TO STUDY THE MIXED CELL POPULATION

Analysis of the mixed cell population resulted in the cell types present being assigned to the sequence of spermatogenesis. The difficulties encountered in equating cell categories with cell types described by other workers have been discussed in the text and will not be repeated here. Future work could be directed towards devising a method whereby direct comparisons between sections prepared for histology and mixed cell populations would be possible. One outcome of this type of approach would be to study histology sections from material grown in culture, when the progress of labelled cell types in situ could be followed.

One important result of the mixed cell population studies was the designation of some of the cell categories as spermatogonial cell types. The progress of the spermatogonial cells was then followed in vitro and changes with time in the spermatogonial generations were recorded.

The results presented are promising but as yet inconclusive. Studies by other authors on differentiation during spermatogonial proliferation are not available for comparison.

The frequency studies, which developed from cell categorisation, were used to study models of spermatogenesis. Significant relationships were found to exist between sequential cell categories. These relationships could not be explained simply as changes in cell frequency due to the occurrence of division events as Clermont (1966b) postulated.

At least two events would affect cell frequency, i.e. the number of cell division events and the proportional duration of each cell stage; combinations of more than one factor produce rate effects. In recent years the study of rate effects in cell populations has been pursued under the general title of cell kinetics. Many authors have published work on cell kinetics and have used autoradiographic techniques to describe rate effects present in different cell systems. Cleaver (1967) collected together the available information on the incorporation of H^3Tdr into mitotically dividing cell populations. More recently Steel (1977) has collected and reviewed information related to the kinetics of tumerous cells. Both authors discuss the problem of determining rate effects in a variety of types of cell systems.

In his chapter on Population Kinetics in animal tissues, Cleaver describes exponentially growing populations and steady-state populations as two examples of many types of cell population which may exist. He notes that different types of populations will be determined by the relative importance of three parameters: the rate of cell gain from other populations, the rate of cell loss to other populations and the rate of cell division within the population. Spermatogenesis is a steady state system where the stem cells are constantly dividing to maintain a constant cell population and where cells are lost from

the system as a result of sperm production. In between these two events cells enter one stage, divide and exit to the next stage. This later event fits Cleaver's description of a "dividing transit system". In a later section of the same chapter, Cleaver describes the kinetics of a system apparently identical to spermatogenesis although spermatogenesis is not mentioned. He notes that it is necessary to know the transit times, i.e. the duration of each stage, before cell divisions can be estimated. In my present work no information on the duration of the stages was obtained. Therefore it seems that until some estimation of this parameter can be obtained, the division events per se are not calculatable.

Recently attempts have been made to calculate the duration of the meiotic stages by cytofluorometry (Libbus and Schuetz, 1978). This technique depends on detecting quantitative differences of DNA content in diploid, haploid and tetraploid cells. Intrinsically this method is unable to differentiate between the duration of several diploid spermatogonial cell stages.

What could be calculated from the frequency data was the overall rate effect ratios between cell categories which would be dependant on both cell division and the proportional duration of each cell stage to the total cycle time. In this instance it is surprising that data derived from a heterogeneous group of infertile males should provide highly significant estimations of ratios between cell categories. This suggests that the ratios are remarkably constant throughout the population. The implication of these results is that the process of spermatogonial proliferation and meiotic division is not easily disrupted.

The results of analysing the frequency of Category A nuclei in the infertile population suggests that this category alone plays a significant part in affecting the activity of the testicular epithelium.

Figure 11.1 shows the percentage frequencies of the infertile population ranked for increasing frequency of Category A nuclei. The results from this normalised data show how increasing frequencies of Category A nuclei reduce the proliferative potential of the cycle, even though the cycle may subsequently function relatively normally. Although the proportion of Category A varies widely through the population, the remaining categories are present in very similar proportions.

In this work, the use of material from infertile individuals can be seen as a limiting factor. Certainly it was thought that further testing of models of spermatogenesis against data derived from an abnormal population would not differentiate between lack of fit due to a wrong model or lack of fit due to abnormal cell systems. However, it is interesting to speculate whether the effect of increasing frequencies of Category A nuclei could have been deduced from material from normally functioning testicular epithelia.

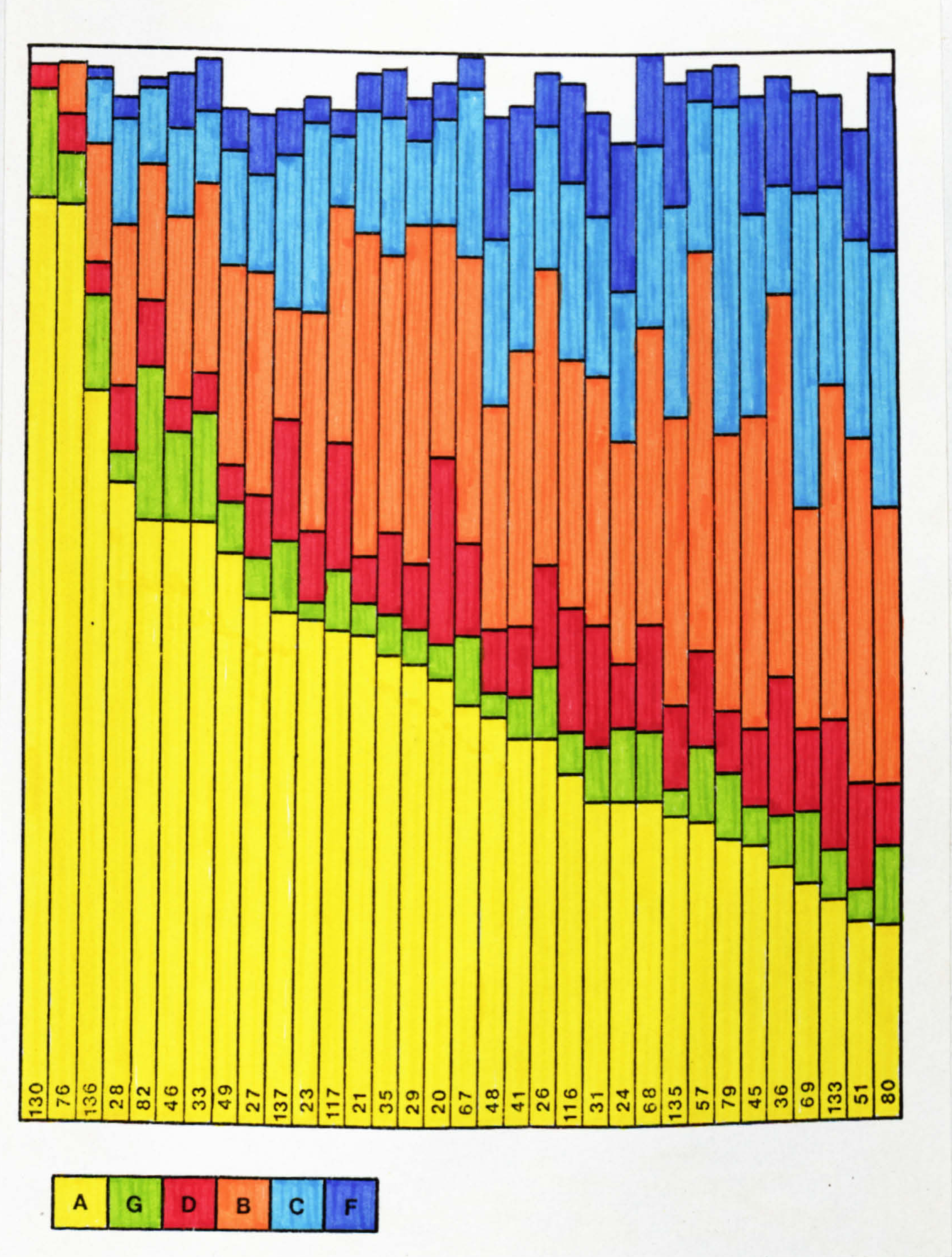
In practical terms the frequency data analysis on infertile individuals provides a parameter for measuring testicular activity (Category A frequencies either alone or as a ratio with Category C nuclei) and a means of locating specific lesions in the spermatogenic cycle. In this respect the identification work on frequencies of the spermatogonial cell types may in future extend our ability to document early lesions in the cycle.

Successful treatment of infertility must surely depend on which part of the cycle is impaired, therefore a more rigorous analysis of centres of impairment, as suggested by this type of cell category analysis, may prove useful to the clinician. Certainly no single method of traditional assessment of the status of the infertile testis provides a clear picture of testicular function.

When considering future developments of this work, it seems

Figure 11.1

Percentage frequency of cell categories ranked for increasing frequency of Category A nuclei



clear that there are two major areas open to investigation. Firstly, testicular differentiation can be maintained in culture and the spermatogonial stages can be recognised and traced. This implies that, spermatogenesis as a cell differentiating system, can be observed and recorded. If the duration of the cell stages can be estimated, then the kinetics of the system can be elaborated further. Secondly, the implications of increasing frequencies of Category A nuclei and the correlation of these nuclei with traditional Sertoli cell types needs further elaboration. Furthermore the correlation of clinical infertility with the status of the testicular epithelium in the infertile male is still in its infancy.

3. SUMMARY OF RESULTS

1. Study of the mixed cell population provided a method of examining the spermatogenic cycle as a process.
2. Categorisation of cell types and their assignment to the sequence of spermatogenesis meant that disturbances of the cycle could be accurately located.
3. The frequency of Category A nuclei provide a measure of the activity of the testicular epithelium.
4. Category G and D are spermatogonial cell stages prior to meiotic division. Thus the frequency of these cell types records the activity of the proliferative stage of the cycle.
5. The implication of studying cell frequencies is that they record a type of rate effect due to a combination of events rather than simply recording the number of division events which have occurred.
6. Cell frequency analysis can be practically linked with screening for chromosome abnormality. Some abnormal chromosome complements are described.

7. No single traditional method of assessing the status of the infertile testis gives a clear diagnostic answer to the question "Why is this patient infertile and how can he be treated?"
8. Analysis of the spermatogenic cycle using cell category frequency data can pinpoint disturbances in the cycle. In the future, this information may be of use to the clinician when he decides on treatment strategy.

Hind thought :-

"Time is natures way of preventing everything happening all at once."

Modern Chinese Proverb

APPENDIX 1 MALE INFERTILITY

NAME: NO:

AGE: OCCUPATION:

AGE AT MARRIAGE:

HISTORY:

Duration of Infertility: .... years

Contraception used (if any):............................

Duration:............

Previous Conceptions (All partners): ....

Libido: Normal Abnormal

Age at First Intercourse: ....

Frequency of Intercourse: .... weekly

Coital Difficulties: YES NO

Details: ....

....

Testicular Operations / injuries / infections: YES NO

Specify ....

....

Herniorrhaphies: YES NO

Details ....

....

Previous Specific Treatment: YES NO

Details: ....

....

Age onset of Puberty: ....

Frequency of Shaving: Daily ....

Other than daily (Specify: ....

Previous Medical History

Venereal Disease	YES	NO	
Specify			
....			
Mumps	YES	NO	AGE
(mumps) orchitis	YES	NO	
Bells Palsy	YES	NO	
Mono nucleosis	YES	NO	
Herpes Labialis	YES	NO	
Herpes Genitalis	YES	NO	
Infectious Hepatitis	YES	NO	
Measles	YES	NO	
Chicken Pox (varicella)	YES	NO	
Rubella	YES	NO	

OTHER INFECTIONS ....

....

Comments: ....

....

FAMILY HISTORY:

No. in Sibship (e.g. 3/4 = third child in sibship of 4): ....

Family History of Infertility: YES NO

Specify ....

Family History of Other Diseases: YES NO

Specify ....

Age of Mother at Patient's Birth: ....

Age of Father at Patient's Birth: ....

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