CHROMOSOME BANDING OF GERMAN COCKROACH SPERMATOCYTES

by

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INTRODUCTION

The investigation of C-band morphology in Blattella germanica was begun as a logical extension of the long standing cytogenetics program on this insect (Ross and Cochran 1975). The use of chromosome banding techniques has become widespread in recent years, although the exact significance of the revealed structures is not completely clear. It was felt that the development of banding techniques would be useful for comparative studies with other cockroach species, analysis of translocation stocks, and investigations of meiosis in B. germanica.

The original intent of this investigation was to expand on studies of chiasma frequency that formed the basis for the author's M.S. thesis (Keil 1979). I hoped to make estimates of bivalent specific crossing-over and to explore the regulation of recombination by heterochromatin (Miklos and Nankivell 1976). However, the resolution of the best bands produced in meiosis proved to be inadequate for such studies. Therefore the investigations reported herein cover the establishment of a meiotic C-band protocol, identification of the individual bivalents through use of translocation stocks, comparative karyology of B. germanica and Blattella vaga, and investigations into the chemistry of the Romanowsky-type blood stains used in banding. The

latter set of experiments was undertaken to evaluate the high variability of the observed C-banding.

The manuscript is divided into three principal parts; a general literature review, C-banding of B. germanica and B. vaga meiocytes, and experiments on stain chemistry.

LITERATURE REVIEW

German Cockroach Cytogenetics

There is general agreement on chromosome number, sex determination, and general centromere position in *Blattella germanica* (Wasilieff 1907, Suomalainen 1946, Saitoh and Saitoh 1967, Cochran and Ross 1967,1969). Cohen and Roth (1970) confirmed the chromosome number to be 2n=23 in males and 2n=24 in females. Sex determination is of the XO type with heterogametic males. In male meiosis, the X chromosome is unpaired and heterochromatic and tends to separate precociously in the first division. Centromeres are metacentric to submetacentric in all mitotic chromosomes (Cohen and Roth 1970). The X chromosome is not distinguished as being especially heteropycnotic. The longest chromosome in the published photograph is distinctly submetacentric. Cochran and Ross (1969) found that only the longest chromosome (12) and the shortest (X) could be distinguished by length in early meiotic prophase I. The remaining ten chromosomes differ in length by a micron or less at pachytene and are difficult to distinguish by length alone.

An extensive formal genetics has been developed for this species (Ross and Cochran 1975). More than 20 stocks

of reciprocal translocation heterozygotes involving all chromosomes except number 2 and the X have been isolated. Thus it has been possible to identify linkage groups with their respective chromosomes. Unambiguous identification of the chromosomes has proven more difficult. Cochran (unpubl.) has been able to identify characteristic chromomere patterns which allow identification of the bivalents at pachytene. Position of meiotic centromeres has been very difficult to determine although the position of earliest diplotene opening gives some indication. Disjunction of translocation heterozygotes may have components under genetic control (Cochran 1976,1977). A knowledge of centromere position would greatly facilitate further studies along these lines. Finer details of chromosome morphology would be useful in determining the exact location of breakpoints.

The chromosome numbers of the Blattaria range from 2n=16 to 2n=80 with a mode of 38 (Cohen and Roth 1970). The chromosomes of species with higher diploid numbers tend to be smaller than those with lower diploid numbers (Cochran and Ross 1967). Species with higher chromosome numbers also appear to have a higher frequency of acrocentrics in their karyotypes. The family Blattellidae has diploid chromosome numbers ranging from 16 to 50 with obvious peaks at 24 to

26, 32 to 34, and 38 (Cohen and Roth 1970). B. germanica falls into the lowest group. Within this genus, reported chromosome numbers range from 24 to 30. The species with 2n=28 and 30 are undescribed and their generic affinities cannot be evaluated.

The widely divergent chromosome numbers in the cockroaches should make this group suitable for studies of chromosomal evolution. Unfortunately, the phylogenetic relationships hypothesized by McKittrick (1964) fail to establish a simple pattern of ancestral and derived groups based on chromosome number. The Blaberidae, an advanced group, tend toward high chromosome numbers as do the more primitive families, Polyphagidae and Cryptocercidae. However, chromosome numbers also tend to vary widely within genera. The possibility of recognizing an ancestral karyotype for the cockroaches is very slim (White 1976). Extreme karyotypes, deviations above or below the mode are assumed to be derived (White 1976). The occurrence of three peaks of chromosome number in the genus Blattella is difficult to interpret with this simplistic model.

There are few reported polymorphisms within species. Only the parthenogenetic-bisexual species pair, Pycnoscelus surinamensis and P. indicus, have been found to be polymorphic for chromosome number. The bisexual species P.

indicus has populations with 2n=36 and 38 in Hawaii (Roth and Cohen 1968). In Thailand the diploid number of this species is 34. The existence of hybrids between these chromosomal races and the mechanisms producing different chromosome numbers has not been adequately investigated. The parthenogenetic P. surinamensis is also polymorphic with chromosome numbers of 2n=34,35,37,39,53 and 54 (Cohen and Roth 1970). Because the chromosomes are invariably metacentric, White (1976) speculated that polysomy could account for the biotypes with greater than 34 chromosomes.

Spontaneous chromosomal rearrangements have been documented in two species. Chains and rings-of-four were found at premetaphase in inbred populations of Periplaneta americana and Blaberus dicoidalis (Lewis and John 1957, John and Lewis 1959). Pachytene configurations from B. discoidalis also contained quadrivalents with breakpoints in extensive blocks of centric heterochromatin (John and Lewis 1959). These multivalents were interpreted as evidence of translocation heterozygosity. Multiple translocations were found in the germ line of many individuals. The maintenance of this high level of translocation heterozygosity within a population is difficult to explain. Inversions, fusions, dissociations, extra heterochromatin, or supernumerary chromosomes have not been found in the cockroaches (White 1976).

It should be noted that the majority of cockroach species known karyologically have ranges outside the geographic areas of greatest cockroach diversity. Pest species in particular are heavily represented if only because they are so common. These species may not be representative of the majority of cockroaches in terms of karyotype as adaptation to life with man may involve extraordinary genetic constraints. As more collecting and comparative cytology is done with tropical cockroaches, the generalizations concerning the group's karyology may change significantly.

Chromosome Banding - C-Banding

The techniques known as C-banding evolved from *in situ* hybridization techniques used to localize satellite DNA sequences in mitotic chromosomes. Working independently, Pardue and Gall (1970), Arrighi and Hsu (1971), and Yunis et al. (1971) developed similar C-banding techniques for acetic acid:methanol fixed, air dried chromosome preparations. These original techniques utilized acid hydrolysis, treatment with a weak base, RNase digestion, a hot salt treatment, and Giemsa staining. Subsequently, a wide variety of modifications of this basic technique have been used on a variety of chromosomal material. Most investigators have completely eliminated the RNA digestion.

The concentration of the acid and base treatments, the timing, and the order of treatment is quite variable between laboratories. The original reports used 0.07N NaOH whereas most later investigators have switched to concentrated $Ba(OH)_2$. Apparently either the acid or base treatment can be omitted with certain material due to some redundancy in the effects (Hsu 1973). The hot salt treatment, usually 2 X SSC, (See Materials and Methods for composition) is retained in most recipes although very strong alkaline pretreatments have been shown to suffice with some material (Comings et al. 1973).

C-bands may also be produced by two completely different procedures. Klinger (1972) demonstrated the production of C-bands by allowing the trypsinization step of G-banding to go longer than recommended. Hubble et al. (1976) used warm formamide followed by hot salt to produce a C-band pattern in Cavia porcellus. The chemical mechanisms behind these procedures are not well understood.

As the C-banding procedure emerged from the in situ hybridization technique, it was originally thought that the mechanism of banding could be traced to DNA denaturation followed by differential renaturation of areas containing highly repetitive DNA sequences (Arrighi and Hsu 1971, Jones 1970, Yunis et al. 1971). The rationale, derived from

melting curves of satellite DNA, was that these highly repetitive sequences would renature more quickly than less repetitive sequences and be more available for staining. Subsequent investigations have shown differential renaturation rates to be an inadequate explanation of the C-banding phenomenon.

The basic C-band procedure (fixation;acid;alkali;hot salt) has been well characterized chemically and been shown to result in extensive removal of DNA and chromosomal proteins from treated chromosomes (Comings et al. 1973, Pathak and Arrighi 1973, Gendel and Posket 1978). Sumner et al. (1973) found that fixation in methanol:acetic acid removed large amounts of non-histone protein from metaphase chromosomes. Holmquist (1979) proposed that fixation primarily functions in hydrolyzing purine bases on DNA. Some precipitation of nuclear proteins undoubtably occurs.

The 0.2N HCl treatment has a dual function in preparing chromosomes for C-banding. Comings et al. (1973) found that this treatment removed 5.2-7.2% of extractable nuclear proteins. Comings and Avelino (1975) identified these proteins as primarily histones. They further discovered that the removal of histones did not alter the C-band pattern from chromosomes with histones intact. Gendel and Posket (1979) found that the standard C-banding treatment

extracted histones completely using the alkaline fast green staining method of Alfert and Geshwind (1953). Burkholder and Duczek (1980) found that the HCl treatment removed large quantities of all 5 histones and some non-histone proteins using SDS polyacrylamide slab gel electrophoresis. Residual quantities of H3 and H4 remained in the nuclei but these authors discounted the possiblity that the histones were responsible for band differentiation. However, Comings et al. (1977) were not able to associate any non-histone chromosomal protein with satellite DNA in a comparative study using Drosophila ezoana (no satellite DNA) and Drosophila virilis (40% satellite DNA). Heterochromatin content for these two species has not been determined.

The alkali treatment, 0.07N NaOH or conc. $Ba(OH)_2$, denatures chromosomal DNA that has been made labile by preceding fixation and acid treatments (Holmquist 1979). Comings et al. (1973) found that a large percentage of chromosomal DNA was extracted after the alkali treatment but their experimental conditions probably solubilized this DNA as quickly as it was extracted. Comings et al. (1973) found that the alkali treatment removed 19.2-29.9% of chromosomal proteins. After depurination in acid and the formation of aldehyde groups in the pentose sugars, alkali treatments result in chain breakage at the aldehyde sites (Jones et al.

1968, Holmquist 1979). Reduction of the aldehyde to an alcohol with sodium borohydride ($NaBH_4$) prevents chain breakage in alkali and prevents the formation of C-bands (Holmquist 1979).

The net effect of the fixation-acid-alkali treatments is the production of single stranded DNA fragments of variable size. Renaturation of C-band DNA occurs only slightly faster than the remaining chromosomal DNA, as measured by acridine orange fluorescence (Comings et al. 1973, Gendel and Posket 1978). Hot salt treatment for extended periods probably denatures the bulk of DNA again and solubilizes up to 60% of it (Comings et al. 1973). Holmquist (1979) suggested that the hot salt treatment converts lesions produced in earlier treatments into breaks in the phosphodiester backbone and solubilizes the fragments.

The complete C-band procedure results in the extraction of an average of 60% of the chromosomal DNA; the remainder is preferentially located in the C-band positive areas (Comings et al. 1973). The histone proteins are completely extracted (Comings and Avelino 1975). Non-histone proteins are extracted to variable degrees and probably account for the longitudinal differentiation of the chromosomes (Comings et al. 1973, Gendel and Posket 1979). The differential DNA

content of the chromosomes pretreated with the basic C-band procedure is not sufficient to explain the banding pattern because over-trypsination, which does not extract DNA appreciably, also produces C-bands (Comings et al. 1973, Klinger 1972). Microdensitometry of C-banded chromosomes stained with Feulgen reagent or Giemsa also indicates that differential DNA extraction cannot fully explain C-band patterns. Comings et al. (1973) discovered that both stains reacted at rates disproportionate to the amount of DNA present in comparisons of C-banded and untreated chromosomes.

The consensus of most investigations is that non-histone chromosomal proteins play an important role in the differentiation of chromosome bands (Comings et al. 1973, Gendel and Fosket 1979, Holmquist 1979). Gendel and Fosket (1979) demonstrated that although C-bands stained positively with Giemsa, protein was uniformly distributed along the chromosome as indicated by amido black staining. They completely hydrolyzed all unextracted DNA after C-banding pretreatments with trichloroacetic acid and found the remaining proteins in C-band areas had an increased affinity for Giemsa stain compared to those in non-C-band areas. Chromosomes treated in this manner continued to stain uniformly with amido black, indicating that proteins were

still uniformly distributed along the chromosome. The technique is not quanitative and some differential extraction of protein may have occurred. Matsukumo and Utakoji (1977) used dansyl chloride to demonstrate a unique protein associated with the centromeric heterochromatin of the mouse.

The question of whether the unextracted DNA in C-band regions protects non-histone chromosomal proteins associated with it from being solubilized or vice-a-versa remains to be settled. Comings et al. (1973) assumed that DNA sequence or base composition is unimportant because constitutive heterochromatin (see below) may be either A-T rich, G-C rich or mildly repetitive, or contain mixtures of sequences with variable base composition. Comings (1978) concluded that C-banding is the result of a complex interaction of DNA and chromosomal proteins. This assumes that heterochromatin is complexed with fundamentally different non-histone chromosomal proteins than euchromatin. In spite of the elegant and extensive work of these investigators, the fundamental mechanism producing C-bands (and every other form of chromosome banding) still remains a mystery.

The chemistry of the four C-banding pretreatments indicates that the effects of the individual treatments overlap and complement each other such that DNA is left only

in C-band regions in association with specialized non-histone proteins. However, the differential removal of DNA or protein remains to be accounted for. As the base composition of C-band region DNA is variable and the exact nature of the associated chromosomal proteins is unknown, band production is assumed to result from a complex interaction between DNA and non-histone proteins. Production of C-bands with agents that do not extract DNA indicates that non-histone chromosomal proteins are primarily responsible for C-band differentiation.

Stubblefield (1980) suggested that chromosome bands represent a subunit structure of chromosomes defined by replication time as well as banding. This borders on tautology as heterochromatin is partially defined as late replicating and is known to be localized in chromosome bands (John and Miklos 1979). The maintenance of the linear integrity of metaphase chromosomes in spite of extensive extraction of chromatin may be explained in part by studies of chromosome structure. The radial loop model of Marsden and Laemmli (1979) hypothesizes loops of chromatin extending radially from a chromosome core, much like a highly condensed lampbrush chromosome. C-banding procedures may differentially shear loops off unbanded regions (again mechanism unknown) leaving a core structure like those

visualized by Satya-Prakash (1980) and Kaiserman and Burkholder (1980) to maintain chromosomal integrity.

The visualization of C-bands requires staining of the pretreated chromosomes. Traditionally, Giemsa or related mixtures of thiazines and eosins have been used although Feulgen reagents also appear to work (Comings et al. 1973). Eosinated thiazine stains are primarily methylene blue, its demethylated oxidation products, and eosin Y (Lillie 1977). Sumner and Evans (1973) found that a methylene blue-eosin complex produced the best defined bands and that the tetramethyl thiazine (methylene blue) was a much more effective stain than the the incompletely methylated azures and thionin. In contrast, Comings (1975) found eosin to be unnecessary and that only the completely demethylated thionin was ineffective as a banding stain. Although methylene blue has a high affinity for double stranded DNA (Sumner and Evans 1973, Comings 1975), this dye apparently binds to non-histone proteins complexed with DNA in C-banded chromosomes (Gendel and Fosket 1979, Comings and Avelino 1975).

C-bands, Constitutive Heterochromatin, and Satellite DNA

Heitz (1933 and earlier papers) defined heterochromatin as chromosome regions that are condensed in interphase and prophase and that do not decondense after telophase. This

definition has remained functional for what has become termed constitutive heterochromatin. Another class of heterochtromatin, facultative, refers to reversibly inactivated chromosomes. The inactivation of one of two X chromosomes in female mammals or the entire paternal set of chromosomes in some coccids are the best examples (Lyon 1968). Satellite DNA refers to DNA that can be separated from the bulk of an organism's DNA by ultracentrifugation in a cesium chloride gradient (Yunis and Yasmineh 1971). Britten and Kohne (1968) established that these satellite DNAs are composed of short repetitive nucleotide sequences. Many satellite DNAs that have been sequenced apparently have repeating units that coincide with inter-nucleosome distances, 155-245 base pairs or multiples thereof (John and Miklos 1979). In situ hybridization of mouse satellite DNA localized these repetitive sequences in procentric areas identified as heterochromatic and C-band positive (Pardue and Gall 1970).

Evidence of this sort led Yunis and Yasmineh (1971) to assert that satellite DNA, repetitive DNA, heterochromatin, and C-bands are representative of the same phenomenon. Although there is a large degree of congruence among heterochromatin, satellite DNA, and C-bands, the relationship is not complete (John and Miklos 1979, Gosden

et al. 1975). In their review, John and Miklos found the exceptions to this correlation to be so common as to demand synthesis on a level completely removed from a cellular function for satellite DNA.

C-band areas and constitutive heterochromatin have become synonymous and appear to represent the same phenomenon. Areas defined as heterochromatic and C-band positive appear to lack structural genes (John and Miklos 1979). However, Hilliker (1976) has shown loci to be present in the centric heterochromatin of Drosophila melanogaster but at a highly reduced density.

There is a general correlation between heterochromatin and satellite DNA distribution in spite of the exceptions. Peacock et al. (1977) documented the distribution of the six satellites of D. melanogaster. Two of the satellites appeared to occupy similar positions in the same blocks of non-centric heterochromatin, indicating that these two satellite DNAs are interspersed. Two other satellite DNAs hybridize predominantly to heterochromatin but also have a single euchromatic hybridization site each. The remaining two satellites are located in heterochromatin.

The problem may be one of resolution. To be visualized by the C-band technique, heterochromatin must necessarily be of a certain size or the band area will fail to

differentiate. *In situ* hybridization has a very high resolution and consequently may be able to detect regions with satellite DNAs much smaller than traditional heterochromatic blocks. Heterochromatin and satellite DNA may be exactly correlated but the differing levels of resolution of the techniques used to demonstrate them may interfere. Steffensen et al. (1981) confirmed the localization of two highly repetitive *D. melanogaster* satellites in large heterochromatic blocks but also showed dispersed locations that were previously unexpected. Discovery of structural gene loci within heterochromatic blocks by Hilliker (1976) also indicates that C-banding is a relatively crude technique for determining heterochromatin distribution. Small euchromatic areas within heterochromatic blocks are obscured by the staining reaction and small heterochromatic stretches in euchromatin are undetectable. There is not necessarily a correlation between the size of the heterochromatin block able to be stained after C-banding pretreatments and the actual size of such blocks.

Function of Heterochromatin

A number of functions have been ascribed to heterochromatin (and repetitive/satellite DNA). They have been reviewed by John and Miklos (1979). Briefly, they are as follows:

1. Stabilization of centromeres and telomeres: Extrapolating from the propensity of heterochromatin to be located in telomeric and centromeric areas, Yunis and Yasmineh (1971) and Holmquist and Dancis (1979) speculated that repetitive DNA may add structural integrity to these areas through the formation of secondary structures. Holmquist and Dancis (1979) viewed centromeric satellite DNA as functional kinetochore organizer genes responsible for microtubule polymerization.

2. Protection of vital euchromatin by forming a layer near the nuclear membrane in order to provide an absorbant target for incoming high energy particles and uv radiation (Hsu 1975).

3. Chromosome pairing: Yunis and Yasmineh (1971) and Peacock et al. (1977) suggested that repetitive DNA sequences in heterochromatin function to facilitate homologous chromosome recognition and pairing. This hypothesis suffers from the existence of organisms with chromosomes devoid of either satellite DNA or heterochromatin and exhibiting no pairing difficulties.

4. Regulation of recombination: Miklos and Nankivell (1976) found that sites of crossing-over were restricted

to euchromatin-heterochromatin borders and hypothesized that heterochromatin blocks could function in localizing chiasma formation.

5. Establishment of fertility barriers between species with radically different satellite sequences or locations (Yunis and Yasmineh 1971).

Hypothesized functions for heterochromatin range from cellular to population levels of organization. Yet none appears to account for the wide variations in satellite DNA composition and heterochromatin content observed among even closely related organisms. Some organisms are devoid of heterochromatin; e.g. Bufo arenarum, B. marinus, and Hyla arborea (Schmid 1978a), while others have appreciable quantities; over 60% of the genome of Drosophila nasutoides (Cordeiro et al. 1975). Until these variations are explained the question of function remains unanswered.

Chromosome Banding in Blattella germanica

INTRODUCTION

The cytogenetics of Blattella germanica has developed rapidly, especially with regard to the meiotic behavior of translocation heterozygotes (Cochran 1976,1977, Ross and Cochran 1981). These studies and others concerning meiosis (Keil and Ross unpubl.) have underscored the need to identify bivalents unambiguously during diplotene and prophase II and to locate the approximate position of the centromeres in translocation multivalents. The development of a C-banding procedure for meiotic chromosomes was undertaken for these purposes. The previous development of a substantial formal genetics for this species makes possible the correlation of linkage groups with elements of the banded karyotype through the use of translocation heterozygotes.

Chromosome banding techniques were developed in the late 1960's and early 1970's for use on mitotic mammalian chromosomes from cell culture (Caspersson et al. 1968, Arrighi and Hsu 1971, Sumner 1972). Because of the relatively high degree of condensation in meiotic bivalents, the C-banding technique was selected as the most likely to yield favorable results. Furthermore, the functional

significance of C-bands is much better understood than for G-banding and associated variants: Q, R, etc. C-bands represent areas of constitutive heterochromatin (Bostock and Sumner 1978). However, the correlation between the distribution of constitutive heterochromatin and repetitive DNA sequences is not complete. While it is certain that satellite DNA is associated with C-band regions, some C-bands do not appear to contain satellite DNA and some satellite DNAs do not hybridize to C-band regions (John and Miklos 1979).

C-banding techniques have been applied most successfully to synchronized mitotic metaphase chromosomes from *in vitro* systems. Few investigators have attempted C-banding in meiotic cells (Santos and Giraldez 1978, Naranjo and Lacadena 1980, Loidl 1979). Although the basic rationale is the same as for mitotic chromosomes, architectural/chemical differences of meiotic chromosomes make alterations of the basic procedure necessary.

The objectives of this study were: (1) establishment of a C-banding schedule for *B. germanica* meiotic chromosomes and determination of the characteristics of the C-banded karyotype, (2) correlation of bivalents identified through C-banding with known linkage groups from the formal genetics program and translocation stocks, and (3) comparison of the

B. germanica banded karyotype with that of a closely related species, *Blattella vaga*, to assess the evolutionary significance of heterochromatin distribution.

MATERIALS AND METHODS

The stocks of B. germanica used were as follows: VPI strain (wild type), T(8;9)/8;9 (Cochran and Ross 1974, Ross and Cochran 1977), T(9;10)/9;10 Pw (Ross and Cochran 1971), T(4;5;10)/4;5;10 (Cochran and Ross 1977) and T(3;12)/3;12 and T(7;12)/7;12 (Ross and Cochran 1975). In some cases, C-band morphology was intact in the translocation multivalent and identification could be made directly. Otherwise, identification was accomplished by elimination of the chromosomes known to be in the quadrivalent. B. vaga individuals were taken from a laboratory culture initiated in July, 1977, from animals collected near San Antonio, Texas.

Testes from third to fourth instar males were dissected into a drop of 15% acetic acid on a freshly cleaned slide. Adhering pieces of fat body and other debris were carefully removed. Testes were squashed under a coverslip using thumb pressure. The coverslip was removed immediately by freezing in liquid nitrogen. Dry ice did not produce satisfactory results; cells were disrupted extensively and frequently only debris could be seen microscopically. Preparations were fixed by placing them on a hot (65°C) slide warmer and flooding the slides with fixative, 3:1 methanol:acetic acid. Slides were aged 24 hours at 65°C and 3-5 days at room temperature.

The C-banding schedule was carried out in a series of Coplin jars as follows:

1. Slides were placed in 0.2N HCl for 10 min. (early attempts used 2N HCl, but produced bands with poorer resolution).
2. Acid was poured out and slides were rinsed three times with distilled water.
3. Slides were transferred to saturated $Ba(OH)_2$ for 30 min. and then washed in 70% ethanol (EtOH) and three changes of distilled water.
4. Slides were treated with 2 x SSC(0.3M NaCl and 0.03M Na citrate•$2H_2O$) at 61-65°C for 60 min. Initial pH of 2 x SSC was 7.20-7.25; solutions of higher pH did not produce favorable results.
5. Slides were rinsed in phosphate buffer,pH 6.8 (0.007M KH_2PO_4 and 0.014M $Na_2HPO_4 \cdot 12H_2O$).
6. Slides were stained in Leishman's stain (Sigma) diluted 1:15 in Gurr's phosphate buffer, pH 6.8 (Biomedical Specialties) for 120-180 min., depending on rate of staining. Leishman's stain was made from crystals by preparing a saturated methanolic solution (apx. 0.8%) at room temperature and diluting with 0.66 volumes of glycerol. Prepared stain was shielded from light.

7. Destaining was accomplished after the slides were air dried by dipping in 70% EtOH and 95% EtOH. The degree of staining was monitored and differed for each group of slides.

8. After air drying, coverslips were mounted with Permount.

The C-banded preparations were examined with bright field optics. Kodak technical pan film 2415, developed for high contrast with HC 110 developer, was used for photography.

RESULTS

Variations in the C-banding Protocol

Ageing of the slide preparations at room temperature was not a critical variable within the 3-5 day range. Less ageing time produced chromosomes with uniform staining but with little differentiation of C-bands. Increasing the ageing time produced "dirty" preparations with degraded cells and large amounts of debris. Early attempts to C-band *B. germanica* meiocytes utilized 2N HCl. When obvious bands were produced the chromosomes and associated bands had a fuzzy appearance that obscured the important details. Through examination of the slides after each step in the C-banding schedule with phase contrast microscopy, it was determined that the 2N HCl treatment was responsible for the poor resolution. Elimination of this step produced uniformly stained chromosomes. Reduction of the normality of the acid treatment to 0.2N produced good bands in most preparations and eliminated the problem.

Cells were sensitive to variations in the 2 X SSC treatment. Typically temperatures lower than 60°C did not induce banding. Temperatures above 65°C resulted in very weakly stained preparations although some cells did have discernable bands in telomeric regions (Fig. 1). These telomeric bands may be analogous to the T-bands of Dutrillaux (1973).

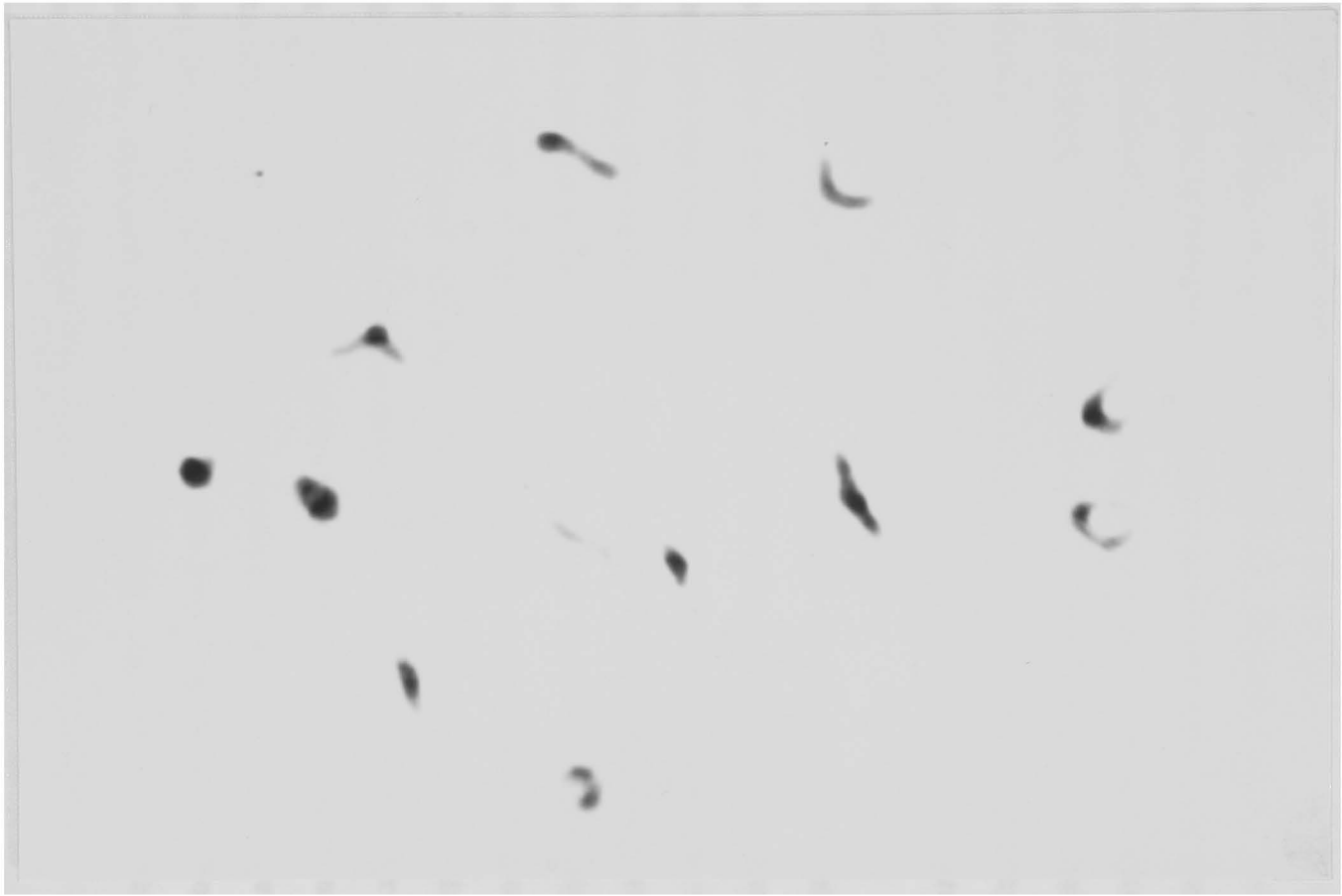


Figure 1. Spermatocyte at diakinesis that has been treated with 2 X SSC at 72¬ºC for 60 min. resulting in "T-banding". Staining of the bivalents is restricted to telomeric areas. Telomeric staining appears to correspond to chiasma position in many bivalents. Centromeric areas normally stained with the C-band procedure are unstained.

Initially Gurr's R66 Giemsa stain was used in these studies. The bands produced by this stain were never completely reproducible or of consistently high quality despite its wide recommendation. Accordingly, Leishman's stain was substituted (G.C. Webb, personal communication). The C-bands were of higher quality and more reproducible. The differences between Gurr's R66 Giemsa and Leishman's stains as well as experiments on with the ability of Leishman's stain to induce C-banding will be covered in another section in greater detail.

The C-band pattern produced by the above method was evident in both diplotene and prophase II chromosomes. Both metaphase I and II chromosomes failed to show a discernible banding pattern. The prophase II banding pattern was the best defined and was used as the standard for comparison. Bands were difficult to separate from chromomeres in pachytene and earlier configurations although distinct darkly staining areas could be visualized as early as zygotene. However, individual bivalents could not be traced. The staining intensity and the selective staining of C-bands varied widely from cell to cell on individual slides without apparent pattern. In spite of this variation, three distinct classes of chromatin could be distinguished by stain susceptibility. Very darkly staining

areas were restricted to procentric locations on a few chromosomes or to entire chromosomes. Lighter staining or gray regions occupied interstitial, telomeric, and centromeric areas. Negatively staining areas, presumably euchromatin, were also found in all three locations.

In prophase II, reciprocal translocation heterozygotes will produce six combinations of half bivalents resulting from various forms of disjunction. For this reason diplotene cells of the translocation stocks were used for chromosome identification. The most significant difference between diplotene and prophase II banding patterns was the relative size of the bands in respect to euchromatic regions. These differences apparently stem from differential rates of condensation between euchromatic and heterochromatic regions.

Translocations Involving Chromosome 9

T(8;9)/8;9 involves a chromosome with a complex banding pattern that tends to stain darkly overall, a lightly staining chromosome, and the two translocation products (Fig. 2). The lightly staining chromosome exhibits, at most, a light subterminal band. In this regard, the darkly staining area around the breakpoint is apparently an artifact of the pairing within the quadrivalent. Centromere position is very near the breakpoints in this interchange

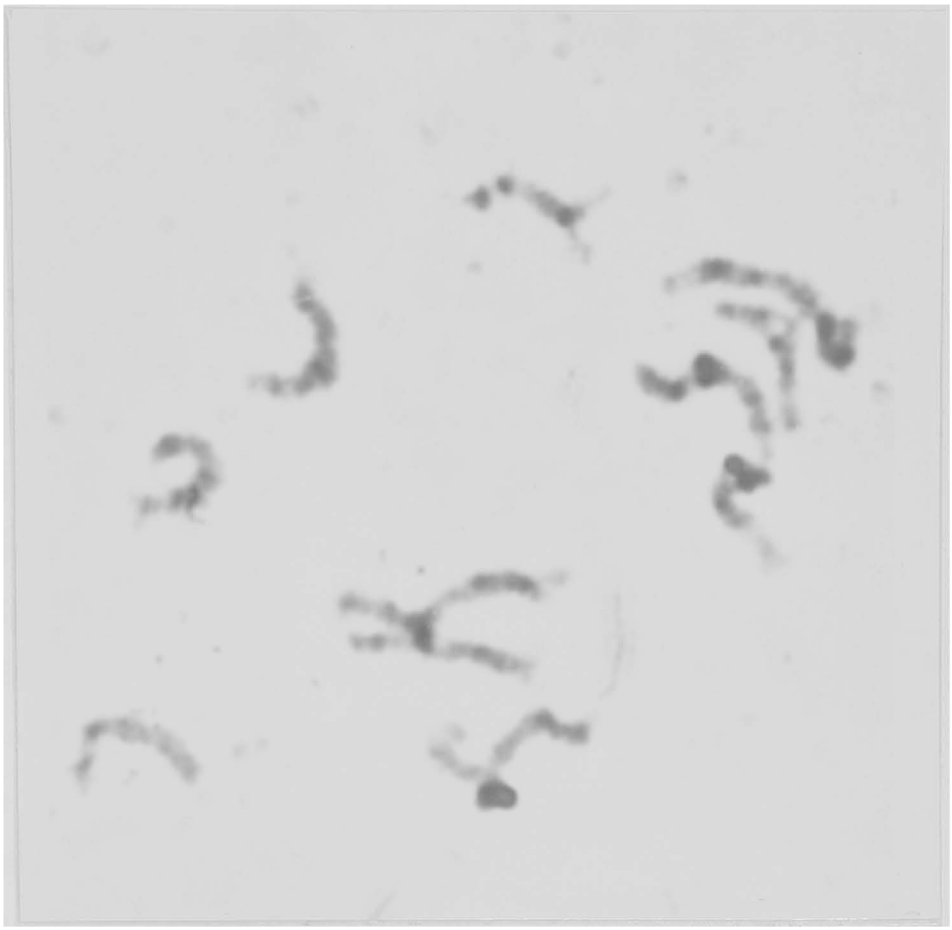


Figure 2a. C-banded early diplotene spermatocyte from a T(8;9)/8;9 male. The earliest diplotene opening is indicative of centromere position. Pairing within this quadrivalent is very tight.

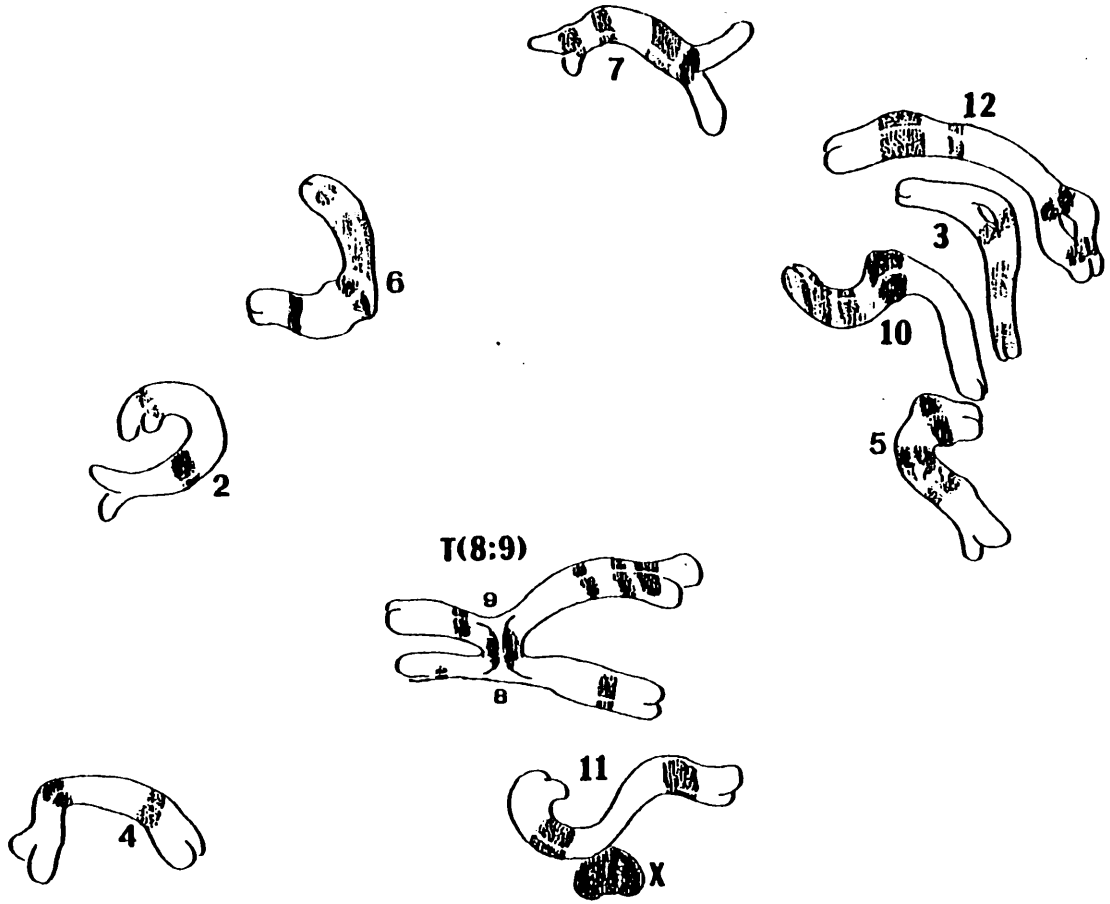


Figure 2b. Chromosome identification and C-band interpretation of T(8;9)/8;9 cell in Fig. 2a.

(Ross and Keil unpubl.). Most early diplotene C-banded translocation multivalents exhibit the dark band near the breakpoint but centromeric heterochromatin is also found in this region in most translocations. Chromosome 12 is easily recognized by length, it is the longest in the karyotype, and by its distinctive submetacentric centromeric C-band.

Through elimination of chromosomes 9 and 10 in the translocation quadrivalent of T(9;10)/9;10 Pw, chromosome 8 was identified as a free bivalent (Fig. 3). Chromosome 8 shows the overall light staining characteristic noted above. The banding pattern within the quadrivalent is not obvious but chromosome 9 can be distinguished from number 10 by length and similarity in banding pattern to the darkly staining member of the T(8;9)/8;9 quadrivalent. Chromosome 10 lies opposite chromosome 9 within the quadrivalent. It has one complexly banded dark arm and a strongly staining block of centromeric heterochromatin. Next to chromosome 12, 10 and 11 are the longest in the karyotype (Cochran and Ross 1969). From Figures 2 and 3, chromosome 11 can be distinguished by two lightly staining blocks of heterochromatin flanking the centromere.

Chromosome 12 Translocations

T(3;12)/3;12 confirms the identity of chromosome 12 on the basis of C-band morphology and inclusion in this interchange quadrivalent (Fig. 4). Chromosomes 11 and 10

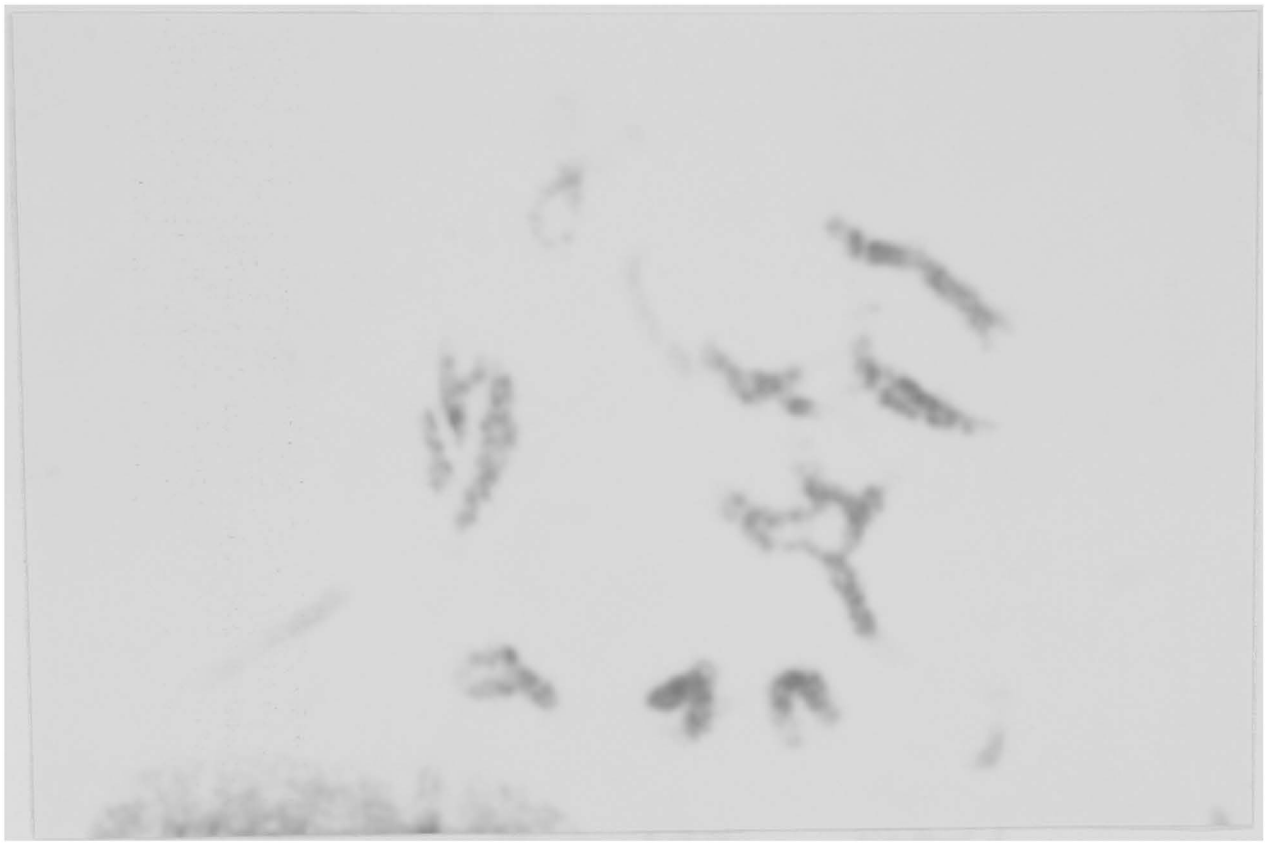


Figure 3a. C-banded early diplotene spermatocyte from a T(9;10)/9;10 male. Morphology of chromosome 10 is altered by inclusion in the quadrivalent; the prominent centromeric band in normal chromosomes is not evident. Pairing within the quadrivalent is typically not complete. Bivalents 4 and 6 are folded upon themselves in this preparation.

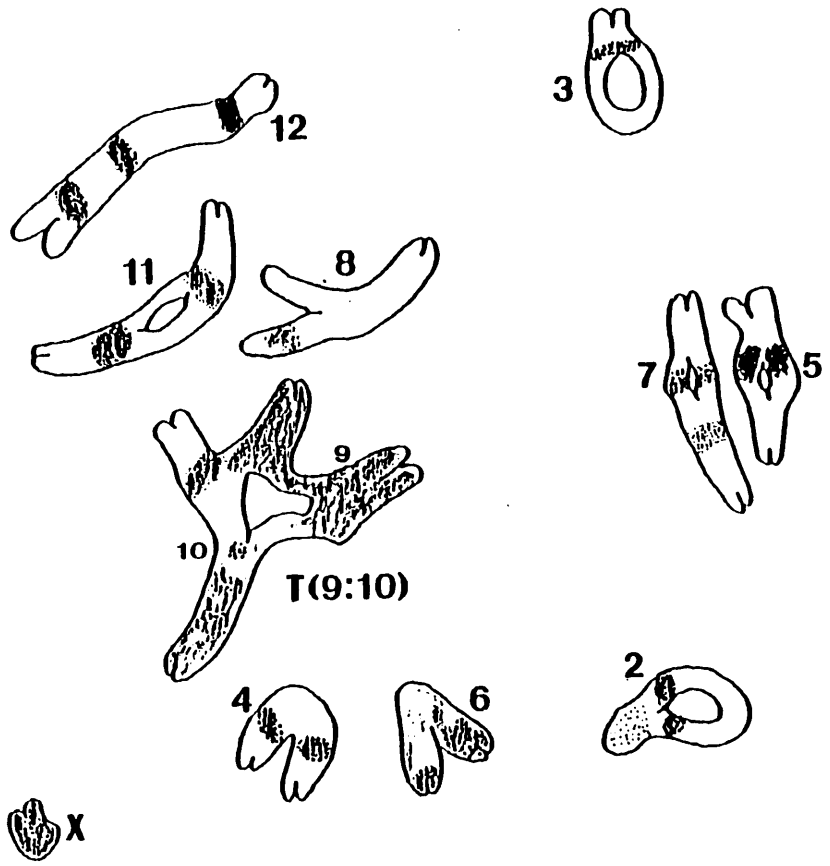


Figure 3b. Chromosome identification and C-band interpretation of T(9;10)/9;10 cell in Fig. 3a.

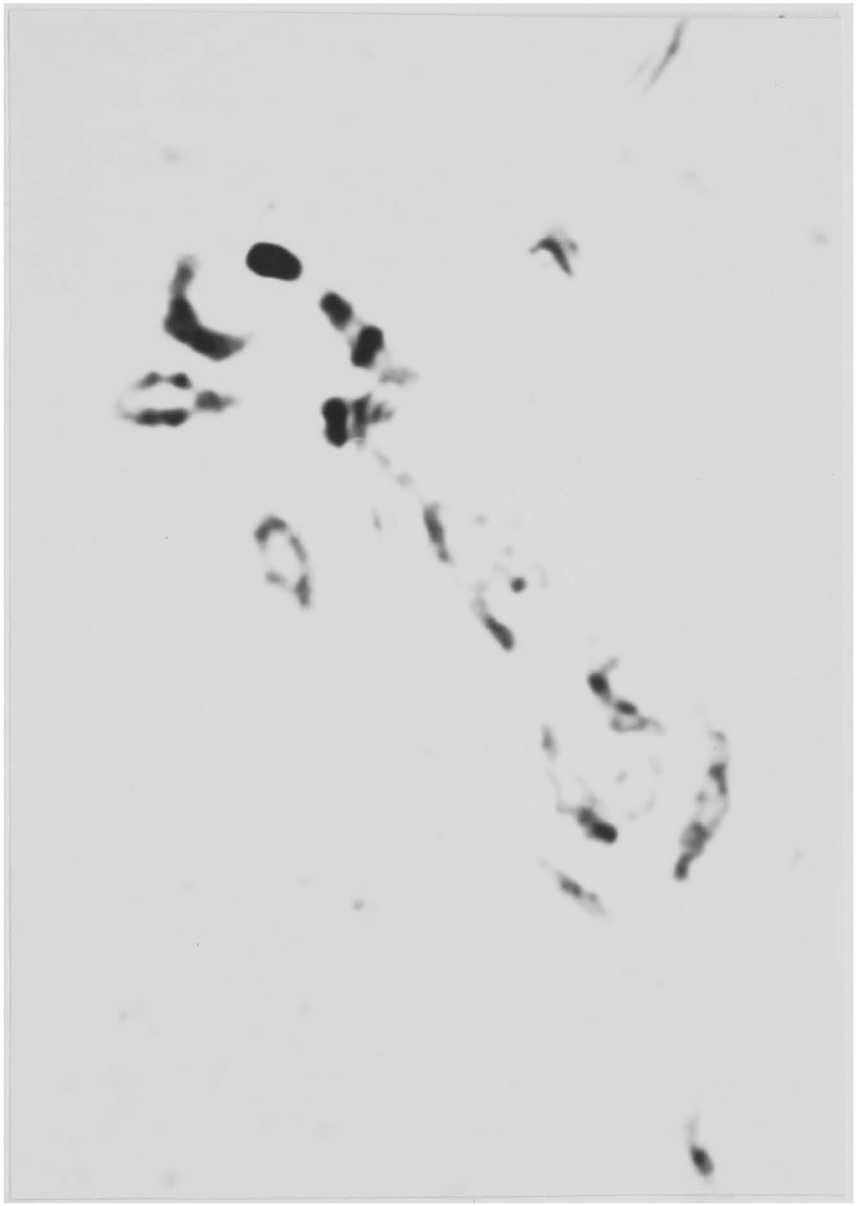


Figure 4a. C-banded diplotene spermatocyte from a T(3;12)/3;12 male. Chromosome 3 stains lightly in the quadrivalent and is difficult to discern.

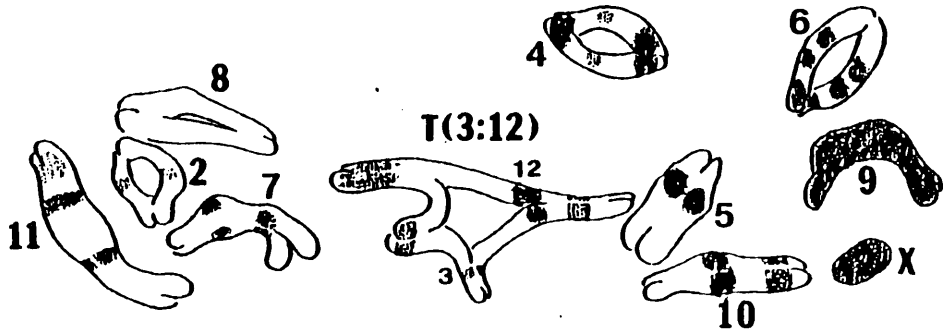


Figure 4b. Chromosome identification and C-band interpretation of T(3;12)/3;12 cell in Fig. 4a.

are clearly differentiated on the basis of length and C-band morphology. The different amounts of heterochromatin in chromosomes 8 and 9 are also quite evident. The morphology of the two smallest autosomes is similar although one appeares to stain less intensely than the other in most preparations. Chromosome 3 is the lighter staining of the two from its inclusion in the quadrivalent.

The C-band morphology of the T(7;12)/7;12 quadrivalent is exceptionally difficult to visualize. Even the normally distinctive pattern of chromosome 12 is difficult to discern. The quadrivalent has distorted pairing that characteristically produces a "frying pan" configuration (Fig. 5). Very faint interstitial C-bands can be seen in one arm of the chromosome 7 side of the quadrivalent. By elimination, chromosome 6 was identified as the bivalent with two sub-terminal to terminal heterochromatic bands.

T(4;5;10)/4;5;10

This is a progressive interchange in which chromosomes 4 and 5 are defined by their inclusion with chromosome 10 in the ring-of-six (Fig. 6). These two chromosomes were separated by length and C-band morphology. Chromosome 5 is characterized by a darkly staining metacentric centromere and one relatively darker staining arm. It resembles chromosome 10 in its morphology. Chromosome 4 stains very lightly and does not have a strong centromeric block of

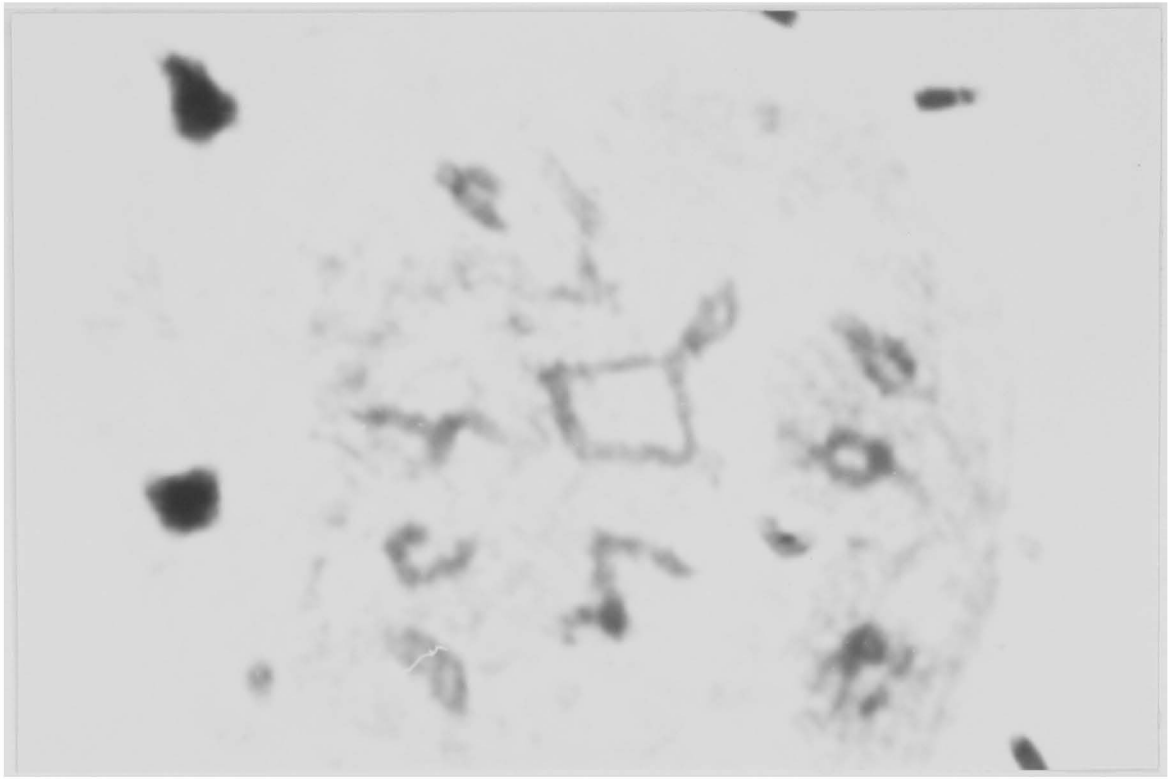


Figure 5a. C-banded diplotene spermatocyte from a T(7;12)/7;12 male. Staining is very light in the quadrivalent but the C-band pattern appears to be altered significantly.

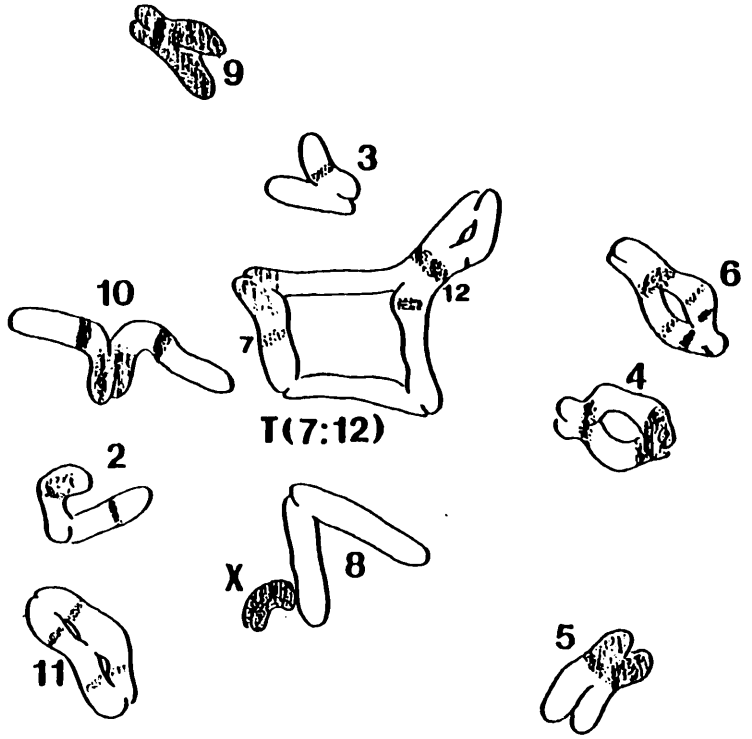


Figure 5b. Chromosome identification and C-band interpretation of T(7;12)/7;12 cell in Fig. 5a.

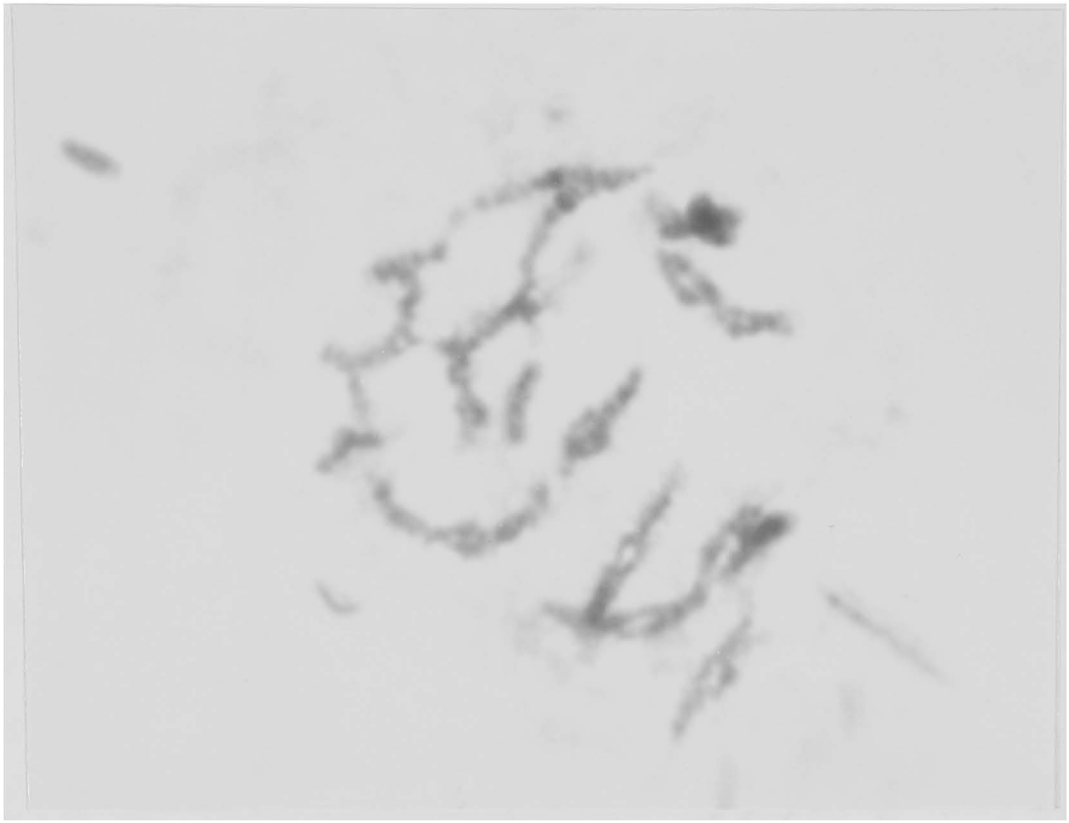


Figure 6a. C-banded early diplotene spermatocyte from a T(4;5;10)/4;5;10 male. Synapsis within the hexavalent is very loose. Points of earliest diplotene opening are indicative of centromere position.

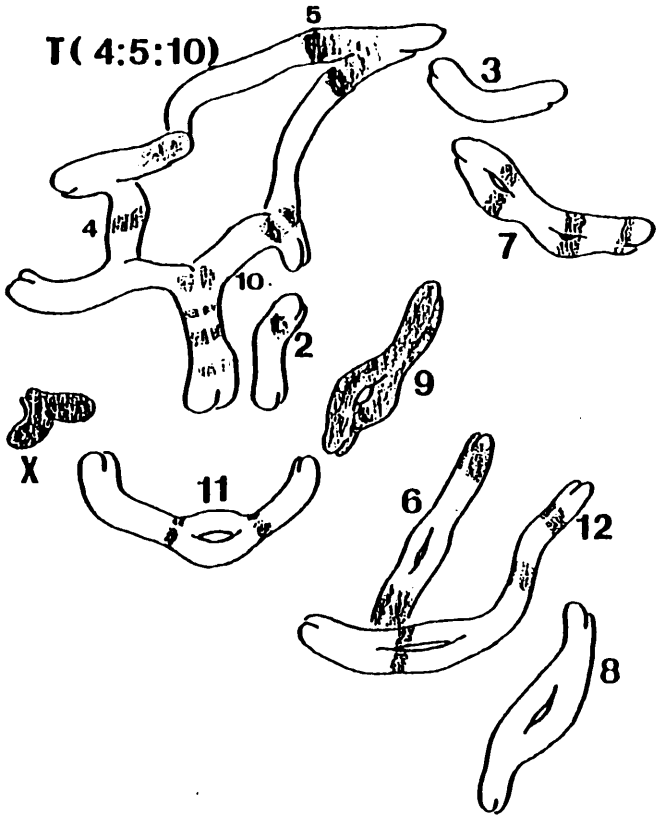


Figure 6b. Chromosome identification and C-band interpretation of T(4;5;10)/4;5;10 cell in Fig. 6a.

heterochromatin. Two light bands flanking the probable metacentric centromere are present. Chromosomes 12 and 11 are also evident in this preparation.

Wild Type

The differences between C-bands in diplotene and prophase II are illustrated in Figures 7 and 8. While bands are evident in the diplotene chromosomes, the greater condensation, lack of chiasmata and chromomeres, and perhaps different architecture of prophase II chromosomes results in much better definition of C-bands. The prophase II banding pattern closely approximates that of diplotene. The greater dispersion of the chromatin in diplotene allows visualization of a number of extra bands that merge in prophase II. Chromosome 9 in prophase II appears almost entirely heterochromatic but a more complex band structure is revealed in diplotene (Fig. 7). A sub-metacentric centromeric band, two bands in the long arm, and a single band in the short arm are evident. A similar comparison can be made with chromosome 12.

An idiogram with representative banded prophase II chromosomes is presented with measures of relative length; length measured in terms of chromosome 12 (Fig. 9). There is a relatively orderly progression from chromosome 12 to the X chromosome with one notable exception, chromosomes 10 and 11. Significantly, the relationship is reversed in

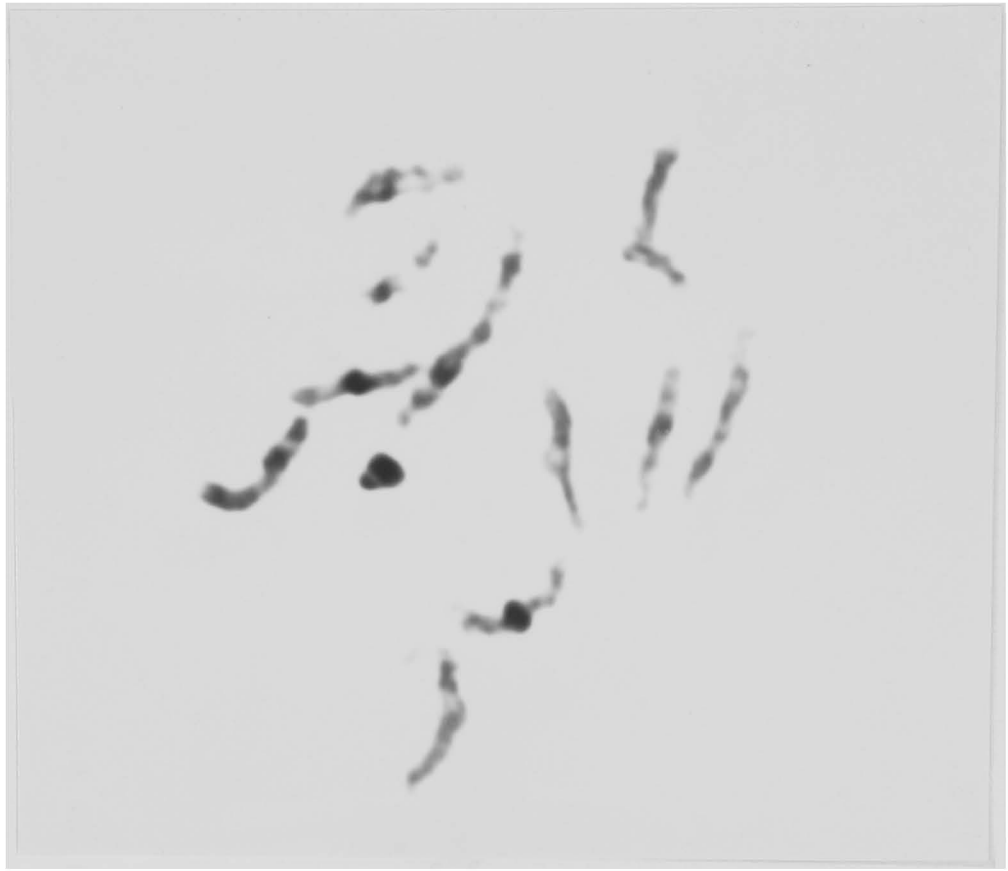


Figure 7a. C-banded early diplotene spermatocyte from a VPI wild type male. The C-banding pattern is confounded slightly by the presence of chromomeres. The points of earliest opening indicate centromere position.

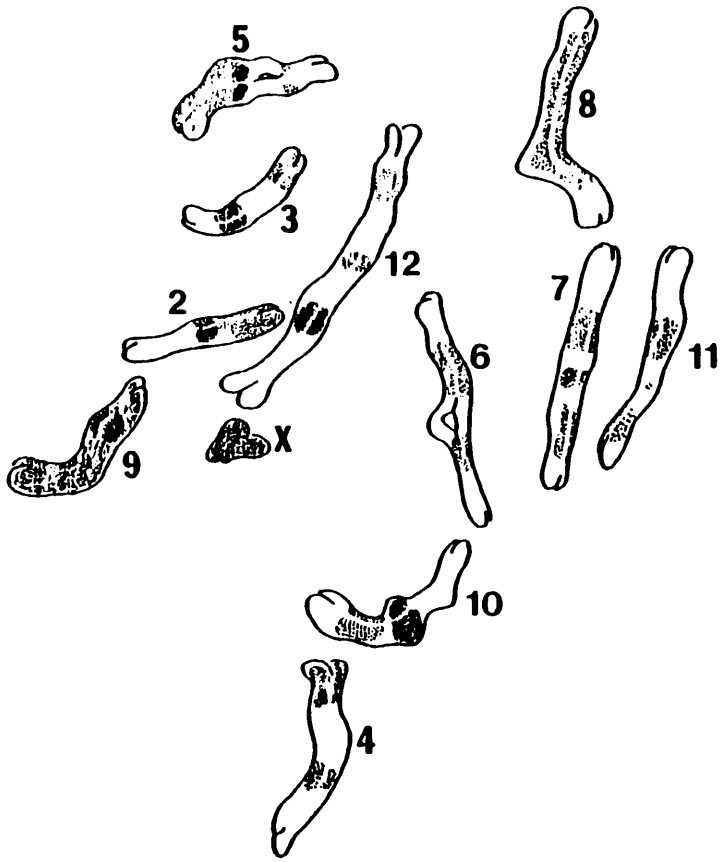


Figure 7b. Chromosome identification and C-band interpretation of VPI wild type diplotene cell in Fig. 7a.

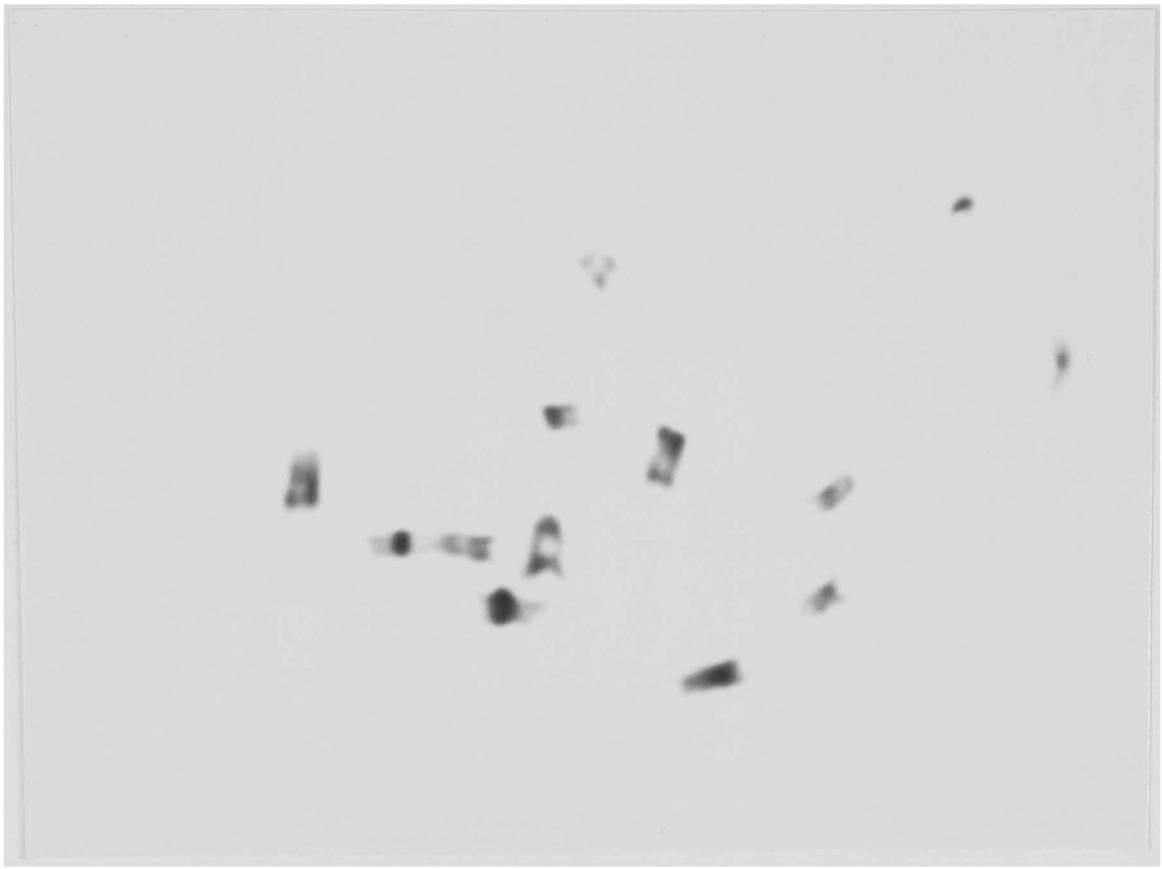


Figure 8a. C-banded prophase II spermatocyte of a VPI wild type male. Chromosome 8 stained so lightly so as not to appear on the photograph. Its position was determined from the negative.

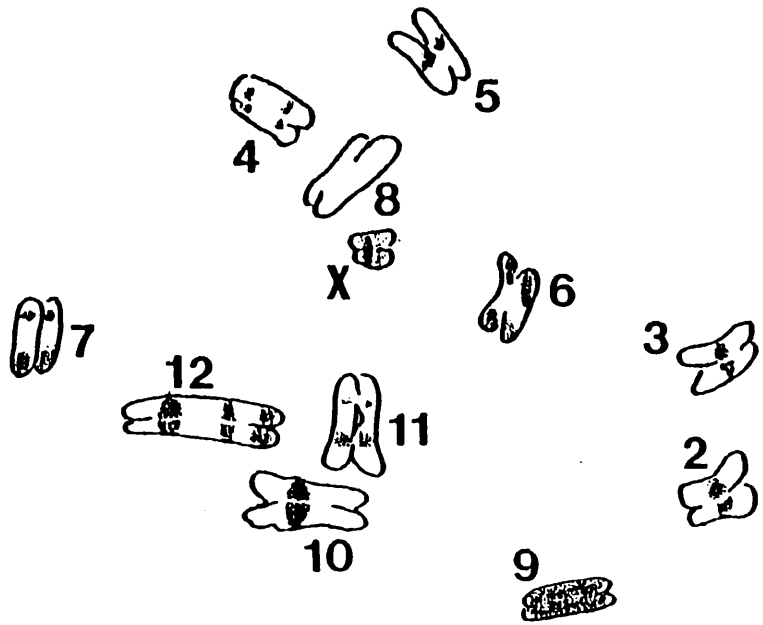


Figure 8b. Chromosome identification and C-band interpretation of VPI wild type prophase II cell in Fig. 8a.

diplotene, chromosome 11=0.75 and 10=0.67 units of chromosome 12. These variations are probably the result of varying amounts and distribution of heterochromatin in these two chromosomes. Overall, there is more C-band material in chromosome 10 than in chromosome 11 (Fig. 9). Heterochromatin is prematurely condensed at diplotene by definition. Therefore, most further contraction occurs in euchromatin. Bivalents that are more euchromatic condensed relatively more in moving from diplotene to prophase II than those that are heterochromatic. There is other evidence for this relationship in comparison of chromosome 8, relatively euchromatic, to chromosome 9, relatively heterochromatic. At diplotene, the mean relative lengths for chromosomes 8 and 9 are 0.67 and 0.58 units respectively. In prophase II, the mean relative lengths are similar for these two chromosomes; 0.58 units for chromosome 8 and 0.61 units for chromosome 9. Schollmayer et al. (1981) has recently documented a similar process in human mitotic prophase chromosomes using acridine orange R-banded chromosomes synchronized by thymidine deprivation.

Blattella vaga

B. vaga and B. germanica are similar in terms of chromosome number, centromere position, and number of chromosome arms. The phenotypes of the two species are also quite similar; the major differentiation being a somewhat

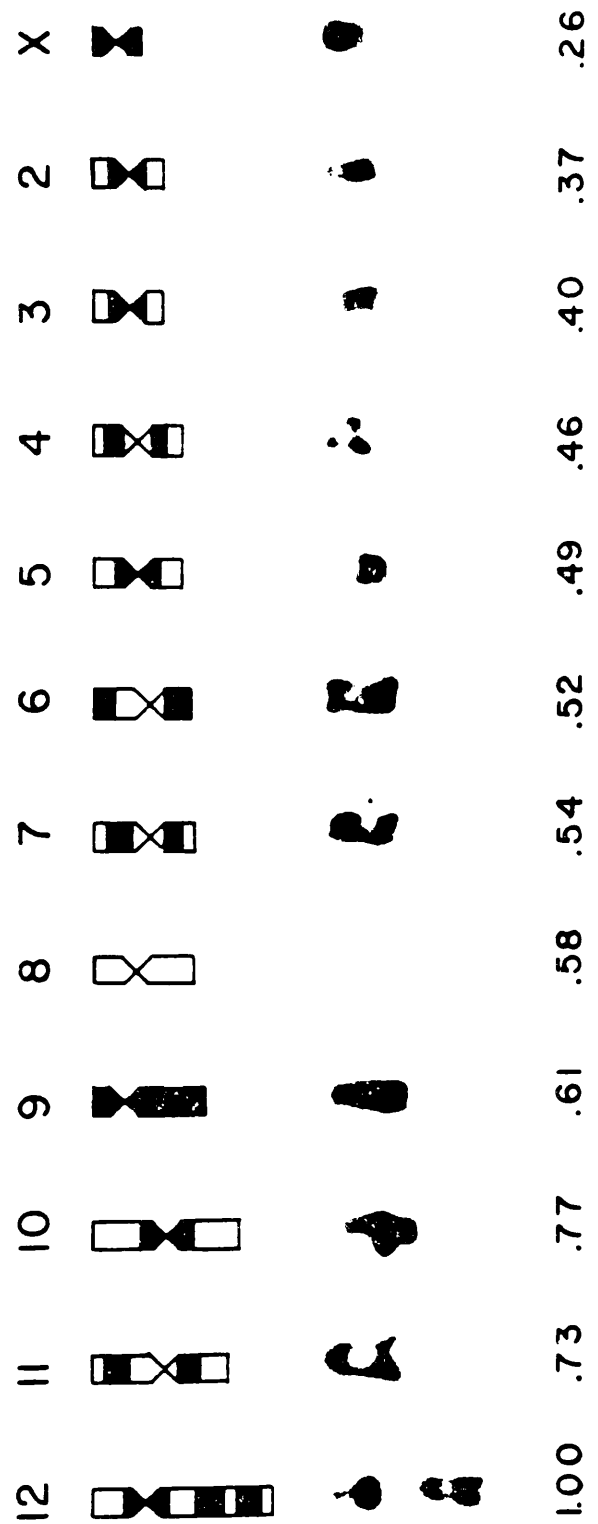


Figure 9. Prophase II C-banded idiogram of Blattella germanica with representative photographs of the chromosomes. Constrictions indicate centromere position as determined from diplotene figures. Chromosome lengths are expressed in terms of the length of chromosome 12 (n=8).

smaller size in B. vaga and variations in body color and pattern. Interspecific hybrids between B. vaga and B. germanica could not be produced despite a number of reciprocal mating trials. The B. vaga prophase II karyotype is numbered by length and apparent homeology to the B. germanica karyotype (Fig. 10). The relative length measures decrease in a regular manner from the largest chromosome (12) to the smallest (2). Chromosomes 9, 8, and 7 are very similar in length. Slight measurement errors account for the differences in relative length.

The differences in the karyotypes of B. vaga and B. germanica can be summarized as follows. The B. vaga X chromosome is much larger and apparently just as heterochromatic as that of B. germanica. There are apparent additions of C-band positive material to chromosomes 11 and 8 in B. vaga. The addition to chromosome 11 is in the centromeric area, while in 8 an additional C-band is located near the end of one arm. In B. germanica, the two smallest autosomes, chromosomes 2 and 3, are similar in size and morphology. In contrast, one small autosome is present in B. vaga. The second smallest chromosome (3) in this species is much longer than the presumed homeolog in B. germanica and has a C-band in each arm in an interstitial position. In general, there appears to be more heterochromatin in the B. vaga karyotype as exemplified by more C-bands and heavier

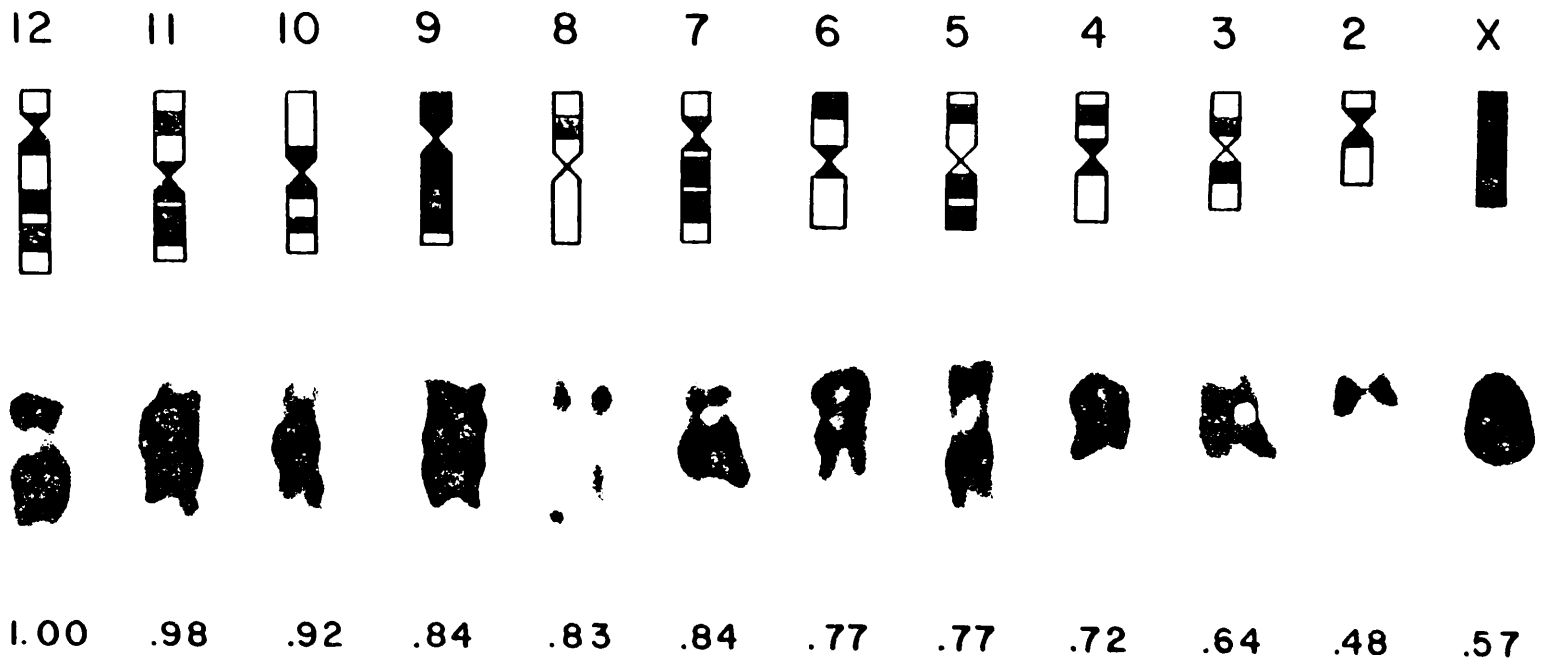


Figure 10. Prophase II C-banded idiogram of Blattella vaga with representative photographs of the chromosomes. Constrictions indicate centromere position as determined from diplotene figures. Chromosome lengths are expressed in terms of the length of chromosome 12 (n=8).

staining. The chromosomes of B. vaga are larger in an absolute sense than those of B. germanica and larger in terms of relative length of the longest chromosome in the karyotypes. Chromosome 12 in both species is of similar length. The additional heterochromatin may account for these differences. Nevertheless, the banding pattern of chromosomes 12, 10, 9, and 2 is conserved between these two species. The mid-range chromosomes are repatterned to a large extent. No obvious homologies are apparent.

DISCUSSION

The C-banding treatments reported in the literature are variable. Sumner (1972), in one of the first published protocols, did not utilize an acid treatment but only warm (30°C) $Ba(OH)_2$. Arrighi and Hsu (1971) used NaOH with similar results. Gallagher et al. (1973) used saturated $Ba(OH)_2$ followed by one to two hours in 65°C 2 X SSC. The HCl pretreatment was added by Pathak and Arrighi (1973). None of the less severe procedures produced C-bands in B. germanica spermatocytes. This suggests that the chromosomal proteins in Blattella may have a somewhat different composition; perhaps more basic non-histone proteins are involved in packing chromatin into the meiotic chromosomes. It should be noted that other investigators have successfully banded meiotic bivalents with little or no modification of the C-banding protocol used for mitotic chromosomes (Webb 1976, Hagele 1979, John and King 1977).

Hsu and Arrighi (1971) speculated that variable band intensity represented variations in the amount of highly repetitive DNA present in the band area. However, their supposition was based on the mistaken notion that C-banding involves preferential renaturation of DNA. Gatti et al. (1976), Pimpinelli et al. (1976), Holmquist (1975a) and others have documented the variable repetitive DNA content

in C-band positive heterochromatin. It is probable that the variation in the staining intensity of the C-bands in B. germanica reflects the relative amounts of highly repetitive DNA in the chromosome. It might be possible to quantify this variation using sequence-specific fluorochrome staining techniques.

La Cour (1978) found two distinct classes of C-band positive heterochromatin in Fritillaria, essentially centric and non-centric, that were distinguished by intensity of the bands. Holmquist (1975b) reported a similarly highly structured distribution of two types of heterochromatin in Drosophila virilis. Bedo (1980) was able to visualize gray areas in the sex chromosomes of Lucilia cuprina by modulating his C-band procedure. The difficulties in obtaining good C-band preparations from B. germanica may be related to the large amounts of "gray" heterochromatin in the karyotype. The optimum between staining only the dark bands on a few chromosomes and not denaturing the chromosomes sufficiently may be very narrow and difficult to achieve consistently.

In a number of preparations bands were resolved in diplotene bivalents that were not present in prophase II (See Figs. 7 and 8). In all likelihood these darkly staining areas represent chromomeres that do not contain constitutive heterochromatin. John and King (1977a,b)

reported a similar case in which a large distal heteropycnotic block visualized in meiosis using conventional staining techniques was not in evidence in C-banded preparations of mitosis and meiosis. Chromomeres and G-bands represent equivalent classes of chromatin (Okada and Comings 1974) but the relationship between C-bands and chromomeres is unclear.

Extra bands can also be resolved within certain translocation multivalents at diplotene. Chromosomes 8 and 4 both had a lightly staining centric band in the translocations T(8;9)/8;9 and T(4;5;10)/4;5;10, respectively. Chromosome 8 was C-band negative at prophase II but in diplotene occasionally had a very light interstitial band in one arm. In normal cells, chromosome 4 was characterized by two terminal C-bands, one in each arm. Baimai (1977) and Keyl (1965) both reported similar heterochromatization of selected areas in chromosomes involved in structural rearrangements.

The C-band negative chromosome (number 8) frequently stained so lightly in prophase II that it did not appear in photographic prints. Unless undetectably small stretches of repetitive DNA, heterochromatin, are dispersed throughout the length of the chromosome, the apparent absence of heterochromatin calls into question the mechanical or genetic roles associated with such chromatin. At prophase

II, both chromosome 9 and the X chromosome were completely heterochromatic, yet through the formal genetics of *Blattella germanica* it is known that there is transcribed chromatin in both linkage groups (Ross and Cochran 1975). The euchromatic sequences are evidently intercalated between the large heterochromatic blocks. Hilliker (1976) has documented the existence of seven loci in the 2L arm and six loci in the 2R arm heterochromatin of *Drosophila melanogaster* using deficiency mapping. The loci known to be on chromosome 9 of *B. germanica* are very tightly linked, the total map distance being only 4.4 units (Ross and Cochran 1975). Very early diplotene preparations did show apparently euchromatic areas in chromosome 9. It may be significant that chromosome 9 appears to have an increased tendency to participate in translocations (Ross and Keil unpubl.). The preferential location of breakpoints in heterochromatin is well documented (John and Miklos 1979, Yoon and Richardson 1976).

The banding patterns of the *B. germanica* and *B. vaga* karyotypes are unique in the diversity of patterns represented. Schmid (1978a,b) reported five species of Amphibia that had no discernible C-band staining, indicating that there was no constitutive heterochromatin in the genome. John and King (1977b) discovered a number of whole arm C-band polymorphisms in the grasshopper, *Cryptobothrus*

chrysophorus. The various combinations of interstitial and terminal C-bands in a variety of grasshoppers has been well documented (King and John 1980). In contrast, Blattella, does not exhibit any noticeable chromosomal polymorphism, yet has completly heteropycnotic bivalents, C-band negative bivalents, and the standard interstitial, procentric, and terminally banded chromosomes. It was surprising that no C-band polymorphism was found.

The differences in heterochromatin distribution between B. germanica and B. vaga are unusually great for two congeneric species. While the addition and deletion of specific bands has occurred in a number of chromosomes, the bands in B. vaga are generally larger. From the data at hand, it is not certain which of these species gained or lost heterochromatin. Bedo (1980) suggested that gains in heterochromatin content may be more common than losses. The greater length of B. vaga chromosomes and increased heterochromatin content argue that a gain has occurred in this species either as a result of speciation or through events unrelated to speciation. The differences in chromosome lengths appear to be due primarily to the amplification of C-band areas. The smaller size of B. germanica chromosomes makes the transformation of heterochromatin to euchromatin (King 1980) unlikely. Complex chromosomal rearrangements such as deletions

followed by Robertsonian fusions, fissions, and translocations probably have not played a role in the differentiation of these two species since the chromosome number, centromere positions, and general C-band morphology have remained intact.

One of the striking aspects of this comparison is that certain chromosomes have conserved a particular pattern of heterochromatin distribution with little internal adjustment. Another group of chromosomes has undergone extensive structural rearrangement in heterochromatin without gain or loss of chromosome arms and without alteration of centromere position.

There are three peaks in the distribution of haploid chromosome number in the Blattellinae, at n=19 and 20, 16 and 17, and 12 and 13 (Cohen and Roth 1970). The chromosome numbers of the genus Blattella fall within the last peak. The abundant interstitial C-bands may represent relic centromeres from chromosomes that have fused to produce the current karyotype (Hsu et al. 1975). The more primitive cockroaches, the Polyphagidae and Cryptocercidae, have chromosome numbers in the range of n=20 to 32 (Cohen and Roth 1970). Most of their chromosomes are sub-metacentrics and acrocentrics which are more easily involved in fusions. The small arms of the acrocentrics may be deleted at less genetic risk. The differential stain susceptibility of some

C-band positive areas may be due to the variable integration of these relic centromeres into the genome.

Degradation of Romanowsky Stains Used in Chromosome Banding

INTRODUCTION

Experience has shown that the C-banding procedure developed for B. germanica spermatocytes produces variable results even though the pretreatments had been apparently optimized. Chromosome staining was probably the faulty step. Dean et al. (1977) indicated that Romanowsky-type blood stains such as Giemsa and Leishman's degrade appreciably in methanolic solutions by oxidative demethylation of the thiazine components. Comings (1975) and Sumner and Evans (1973) have both documented the importance of the thiazine-DNA interaction in the induction of chromosome bands. Neither of these reports attempted to link banding failures to specific degradation products in the stain mixture. Analysis of these stains by thin layer chromatography (TLC) (Marshall and Lewis 1974a, Loach 1971) is a much better method for detecting contaminants at low concentrations than either spectrophotometry or paper chromatography (Persijn 1960), allowing for a much finer analysis of the problem.

The Romanowsky blood stains used in chromosome banding (Leishman's and Giemsa) are composed of methylene blue,

related thiazines, and eosins. As methylene blue is probably a poor histological stain by itself, commercial preparations are generally polychromed or oxidized to produce a progression of azures and methylene violet (Lillie 1977). While these polychromed methylene blue eosinates are excellent histological and blood film stains, they are not necessarily as effective for production of chromosome bands.

Through the study of stain chemistry it was hoped that inferences could be made on the nature of the stain-chromatin interaction, especially the degree of methylation of the thiazine compounds. Comings (1975) demonstrated that at least one methyl group is necessary for staining, using DNA-gelatin films. Sumner and Evans (1973) found a more complex situation using similar techniques and chromosome analysis. First, these investigators found eosin to be essential for the production of bands. Comings (1975) disputed this, claiming that the magenta color of chromatin Sumner and Evans attributed to a eosin-methylene blue complex was a result of metachromasia. This metachromatic effect is due to polymerization of the thiazines at high concentrations in aqueous solutions (Rabinowitch and Epstein 1941). Secondly, Sumner and Evans (1973) found that less than fully methylated thiazines were unsatisfactory for producing G-bands on chromosomes.

The object of the present experiments was to identify the cause of banding failures due to components of the stain, to optimize storage of stain stock solutions, and to infer the banding process itself from the chemistry of the staining solutions.

MATERIALS AND METHODS

Standard Dye Solutions

The dyes used in this study and their sources are as follows: methylene blue and eosin Y from Fisher; azure II from Matheson, Coleman, and Bell; ethyl eosin and sodium fluorescein (uranin) from Allied Chemical; and azure A, azure B (bromide), azure C, thionin, methylene violet, and Leishman's stain from Sigma. Stock solutions were made up to 0.5% w/v in methanol with correction for the stated dye content on the label. The fluorescein stock solution was 0.5% in distilled water as this compound was not sufficiently soluble in methanol (Lillie 1977). All stock solutions were stored in the refrigerator in tightly stoppered flasks and the thiazines were wrapped in aluminum foil to retard photodegradation. Dye content, spectral characteristics, and major dye impurities of these compounds are listed in Table 1.

Stock solutions of Leishman's and Gurr's R66 Giemsa (Searle Diagnostics) were also utilized. The Giemsa was purchased as a stock solution; saturated methanolic solution with glycerol (1:1) added to inhibit oxidation. Leishman's was made up from crystals by preparing a saturated methanolic solution, approximately 0.5 grams of dry stain in 50ml of methanol. The solution was stirred at room

Table 1.

Characteristics of Dye Standards and Staining Solutions

Compound	Supplier	Dye Content	Absorbance Maxima	Impurities (TLC)
Xanthenes				
Fluorescein Na	Allied	—	484	None
Ethyl Eosin	Allied	89%	522	Eosin Y
Eosin Y	Fisher	90%	517	Tribromofluorescein
Thiazines				
Thionin	Sigma	87%	599	Nona;a
Azure C	Sigma	50%	609 - 610	Azures A & B, M.B. ?
Azure A	Sigma	85%	633	Azures B & C, M.B.
Azure B	Sigma	90%	651	Azure A, M.B.
Methylene Blue	Fisher	88%	660	Azure B
Methylene Violet	Sigma	80%	610	Azure C
Azure II	M,C,&B.	--	642	Methylene Blue, ?
Staining Solutions				
R66 Giemsa	Searle	--	610 & 517	Azures A, B, & C ?;c
Leishman's	Sigma	--	660 & 517	Azures A & B; c

a; Thionin spot was very large, purity was uncertain.
b; Methylene violet was heavily contaminated with azure C, its absorbance maximum is 610um, a 580um peak was also present and was probably methylene violet.
c; Eosin Y components always contained tribromofluorescein.
Note; M.B. indicated methylene blue and ? indicates an unknown compound.

temperature for one hour. Two volumes of glycerol for every three volumes of methanol (33ml for 50ml of methanol) were added and the solution stirred for another hour. Prepared Giemsa stain was stored in brown glass and Leishman's was stored in aluminum foil wrapped flasks at room temperature.

Absorption Spectra

A Perkin-Elmer 124 double beam spectrophotometer with an automatic strip chart recorder was used to determine absorption spectra for the dye standards and chromosome staining solutions. One cm cuvettes were used for all measurements. Stock solutions were diluted with distilled water for spectral determinations. The final concentrations of the working solutions ranged from 0.1% to 0.5% of the stock solution in water. The reference cell was filled with water and an appropriate concentration of methanol.

Thin Layer Chromatography

Two thin layer chromatography (TLC) systems were tested for their ability to separate the components of the chromosome stains and the dye standards. Loach (1971) recommended triple development of 250um silica gel plates with a 95% ethanol-chloroform-acetic acid (85:10:5) solvent system. This method was time consuming and gave poor separation of the xanthene components of the Romanowsky

stains. Consequently, the TLC system of Marshall and Lewis (1974a) was adopted. The solvent system was as follows: 1-n-butanol, 12 volumes; 1% w/v aqueous $AlCl_3$, 5 volumes; and 2% v/v formic acid, 2 volumes. After vigorous shaking in a separatory funnel, the bottom, aqueous layer was discarded. The butanol layer was used to develop the chromatograms.

Silica gel plates, 250um thick, were prepared by mixing 25gr of silica gel G (Sigma Chemical) with 55ml of distilled water in a Waring blender. The slurry was spread on five 20cm X 20cm glass plates with a plate spreader (Brinkmann) and allowed to air dry. Samples were applied with disposable 2ul capillary tubes (Microcaps) in 8mm bands, 2-4ul per band. The chromatography tank was lined with Whatman 3M chromatography paper and wrapped with aluminum foil to exclude light. The liners were saturated with solvent prior to TLC plate development. Plates were developed for about 4 hours at ambient temperatures.

After the developed plates had dried, the thiazines were visualized in normal light. The xanthenes were viewed under ultraviolet light as these compounds fluoresce in a characteristic manner. Rf's were very reproducible. The developed plates were photographed with both Kodak High Speed Ektachrome and 2415 Technical Pan (B&W) films with a yellow filter - Y(K2) to establish a permanent record.

Purification of Azure B Standard

Preliminary analysis showed that the standards, azure A and azure B (bromide), could not be adequately distinguished by TLC because they were heavily contaminated with each other and with other oxidation products. Pure azure B was prepared to establish Rf values for these standards. Lohr et al. (1975) developed a purification technique based on large bore column chromatography. The solvent extraction system of Marshall and Lewis (1976) was chosen, however, as it was less expensive and technically not as demanding. My application of their procedure was as follows.

1. A 2% w/v aqueous solution of methylene blue was prepared and its pH adjusted to 11.3 with 1M NaOH. At high pH the oxidation of methylene blue to azure B is favored.

2. The solution was transferred to a separatory funnel and an equal volume (12.5ml) of carbon tetrachloride was added with vigorous shaking. Azure B is more soluble in CCl_4 than water at this pH and methylene blue is insoluble in CCl_4.

3. Eight sequential extractions of the of the aqueous layer with CCl_4 were performed. The extracts were collected through several layers of filter paper that

were replaced after each extraction to limit any aqueous contamination. The 6th, 7th, and 8th extracts were allowed to stand ten minutes to enhance azure B extraction from the aqueous layer.

4. Azure B was extracted into aqueous solution by shaking the pooled CCl_4 extracts with 50ml of 4.5×10^{-4} M hydrobromic acid (HBr). This procedure was repeated ten times. Azure B was never fully extracted from the CCl but this phase became very lightly colored. Gelatinous crystals formed in the aqueous layer during the extractions.

5. The aqueous HBr extracts were pooled and 12.5ml of concentrated (9.0N) HBR was added. The solution was allowed to crystallize overnight in the refrigerator after the solution had cooled two hours in the freezer.

6. Gelatinous blue-black azure B crystals were filtered from the solution and dried *in vacuo* for twelve hours.

7. The purity of of azure B during the various stages of extraction was monitored by TLC.

Stain Degradation Experiment

Seventy-five ml of 0.5% w/v Leishman's stain in methanol was prepared and divided into three equal parts.

One aliquot served as a control and was characterized by chromatography and spectrophotometry. The control was measured for volume, stoppered tightly, wrapped in aluminum foil, and stored in a refrigerator. The second and third aliquots were treated similarly but one was wrapped in aluminum foil and placed in a constant temperature cabinet at 36-38°C. The third aliquot was left unwrapped in a Pyrex flask and placed under a fluorescent lamp. Ambient temperature near the light degradation experiment was 26°C. After 50 days, the volume of the three samples was measured and the samples were analyzed by spectrophotometry and TLC.

An additional 25ml sample of 0.5% Leishman's stain was stored in a tightly stoppered, aluminum-foil wrapped flask that had been purged with nitrogen. After 25 days in a refrigerator, this sample was analyzed in a similar manner to the previous samples.

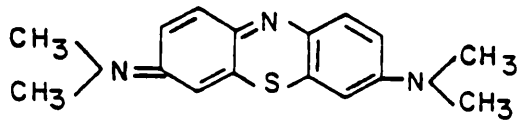
Cytology

The cytological methods followed those outlined in the previous section on chromosome banding of *B. germanica* spermatocytes.

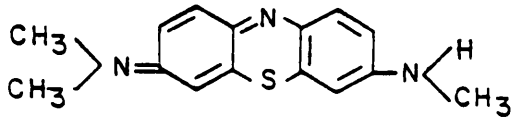
RESULTS

Dye Standards

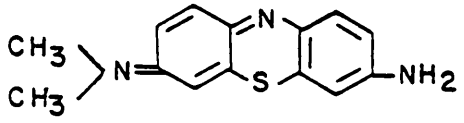
The Romanowsky-type blood stains used for chromosome banding are composed of methylene blue, its oxidation products, and two eosin components. The structural formulas (Lillie 1977) and absorption spectra for the major compounds are shown in Figures 11 and 12, respectively. Methylene blue is completely methylated, with the remainder of the series, azure B, azure A, azure C and thionin, being progressively less methylated. The absorbance spectra show a similar pattern; methylene blue's absorbance maximum is at the longest wave length (660um) and thionin's is at the shortest (599um). The azures have peak absorbances at appropriately intermediate wave lengths. The thiazine spectra (Fig. 12) contain a more or less conspicuous shoulder at characteristic wave lengths. The height of this shoulder is in part related to the degree of contamination by other members of the series as determined by TLC (Fig. 13). Another component of this shoulder is generated through the metachromatic effect of polymerization (Rabinowitch and Epstein 1941). Thionin was relatively pure, it may have contained trace amounts of azure C (Fig. 13) and had the lowest absorbance peak of the series. Its shoulder, however, was pronounced and on the ultraviolet side of the peak. Similarly, methylene blue was relatively pure,



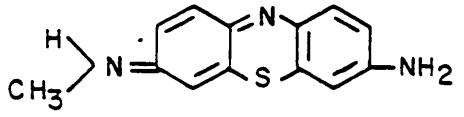
Methylene Blue



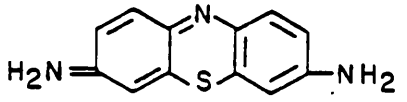
Azure B



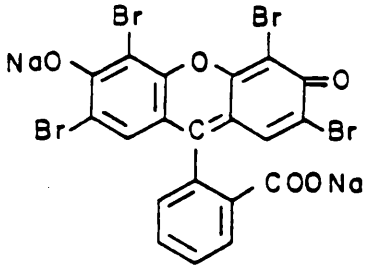
Azure A



Azure C



Thionin



Eosin Y

Figure 11. Structural formulae of the major components of Romanowskytype blood stains used in chromosome banding.

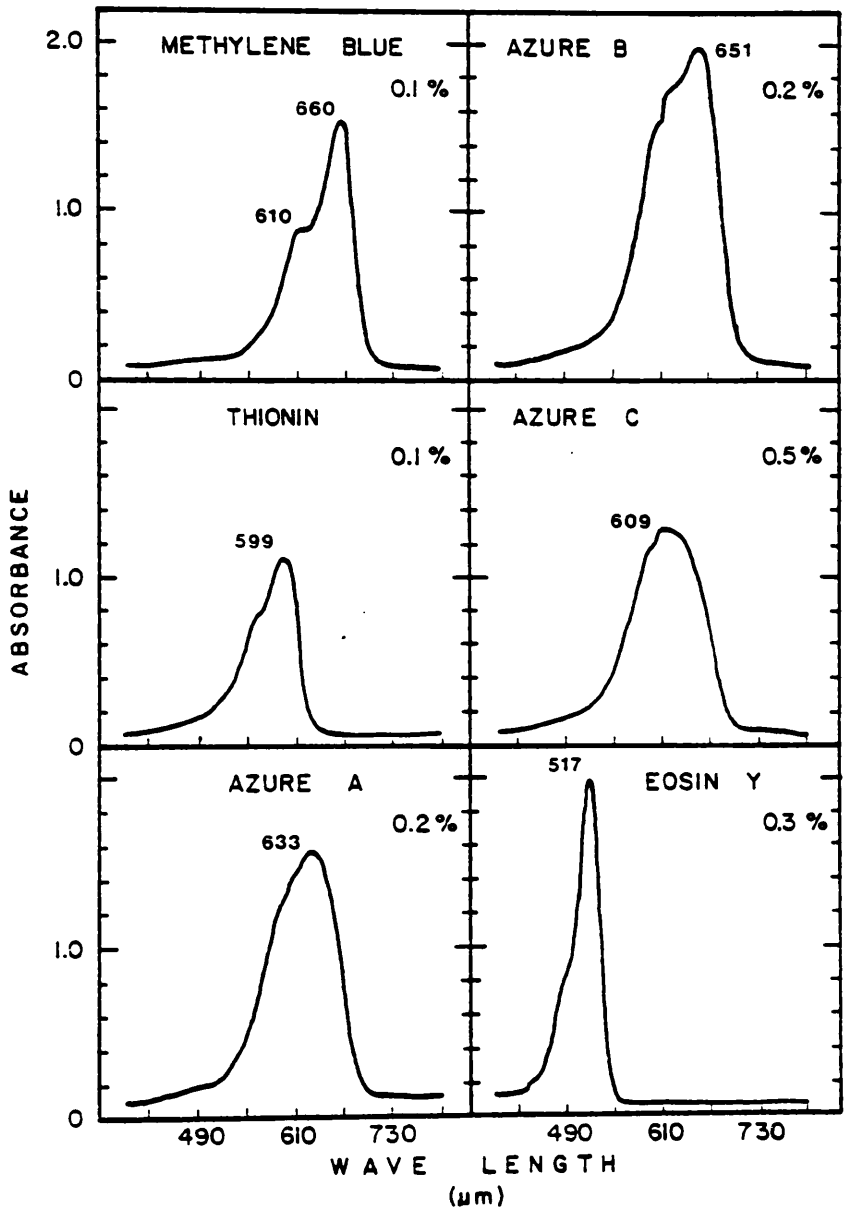


Figure 12. Absorbance spectra of the major components of Romanowsky-type blood stains used in chromosome banding. Concentration is expressed as a percentage of 0.5% w/v methanol stock solution.

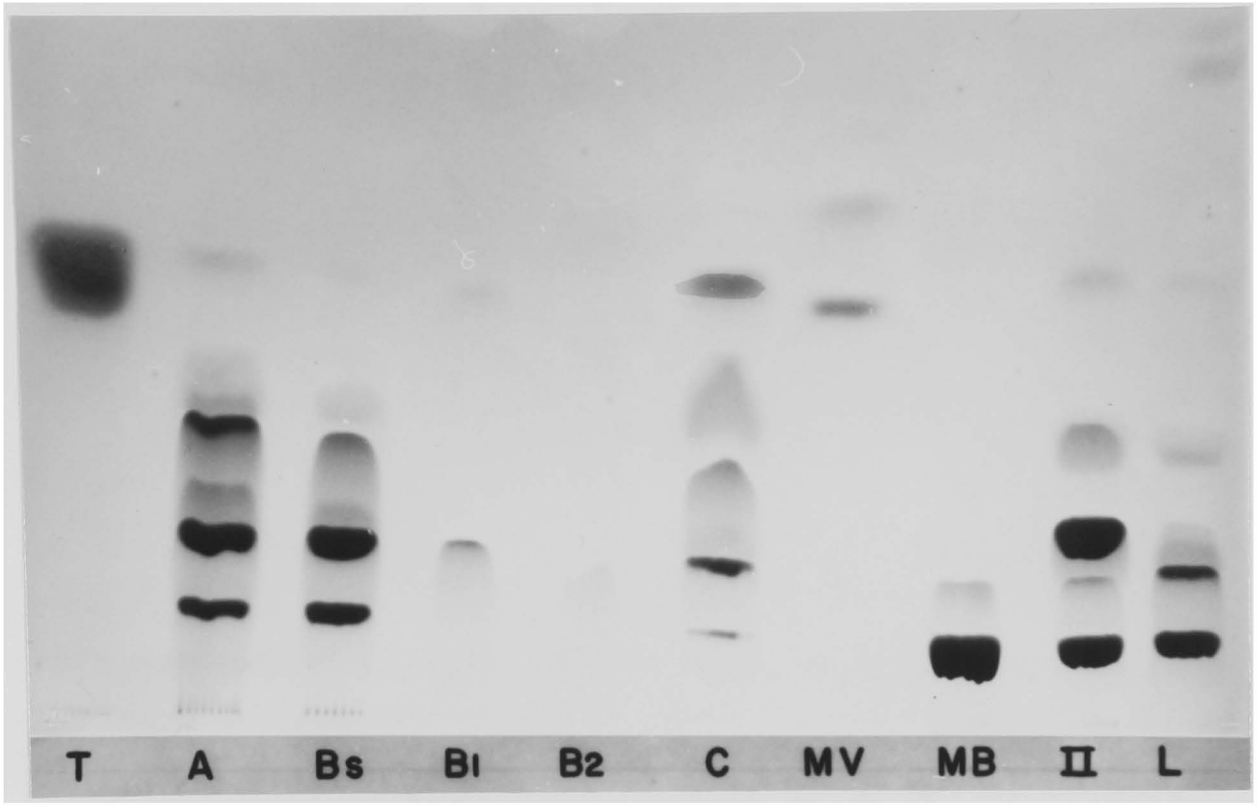


Figure 13. Thin layer chromatogram of thiazine standards and freshly prepared Leishman's stain. The abbreviations are as follows; T-thionin, A-azure A, Bs-azure B standard, B1-azure B purified (carbon tetrachloride extract), B2-azure B purified (aqueous extract), C-azure C, MV-methylene violet, MB-methylene blue, II-azure II, and L-Leishman's stain (fresh). Table 2 on the following page contains the Rf values of the spots.

Table 2.

Rf Values and Spot Identification of Chromatogram of Thiazine Dye Standards Figure 13.

Lane*	T	A	Bs	B1	B2	C	MV	MB	11	L
Methylene Blue		0.12	0.12			0.12		0.11	0.11	0.11
Azure B		0.21	0.21	0.21	0.20	0.20		0.19	0.18	0.18
		0.24	0.24						0.25	0.25
Azure A		0.32	0.32			0.30				0.32
			0.33							
		0.38				0.41			0.39	
Thionin	0.45									
Azure C	0.54	0.50		0.48		0.50	0.50		0.52	0.52
							0.60			
Eosin Y										0.81
										0.85

*Abbreviations for the lanes of the chromatogram are the same as for Figure 13 on the preceding page.

contaminated with only a trace of azure B, yet it had a pronounced shoulder at 610um. The main peaks of these two compounds were sharp and narrow in contrast to the broad peaks of the more impure standards. The shape of the absorbance spectra of the thiazines is determined by three factors; absorbance by the pure monomer, absorbance by possible polymers, and absorbance by contaminants.

Samples of azure B purified by solvent extraction revealed a small amount of azure C in the carbon tetrachloride fraction (Fig. 13). The HBr aqueous extracts were pure by TLC analysis. The Rf values (Table 2) of these pure samples establish azure B, Rf=0.20, as being slower migrating than azure A, Rf=0.32, and thereby establish the identity of both in the dye standards.

Eosin Y produced an absorbance spectrum with a sharp peak at 517um with a slight shoulder near the base on the ultraviolet side (Fig. 12). Thin layer chromatography revealed that the standard contained two spots, one greenish-yellow, Rf=0.77, and the other orange fluorescing, Rf=0.84, under ultraviolet light (Fig. 14 and Table 3). Lillie (1977) indicated that common contaminants of eosin Y were fluorescein and its bromination products, especially tribromofluorescein. Eosin Y is tetrabromofluorescein. The fluorescein standard had a sightly greater mobility, Rf=0.91, than either of the components of the eosin Y

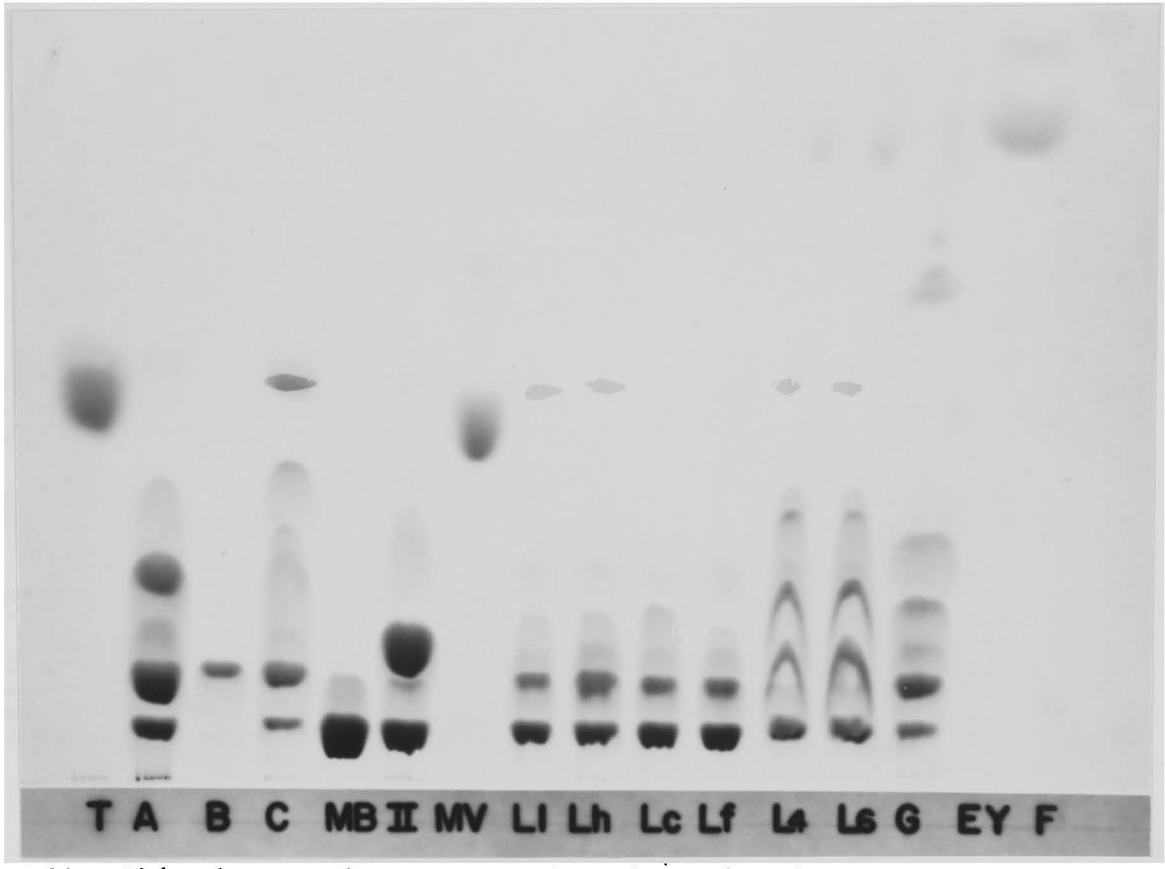


Figure 14. Thin layer chromatography of aged Leishman's and Geimsa stains with appropriate standards. Abbreviations are as follows; T-thionin, A-azure A, B-azure B (purified), C-azure C, MB-methylene blue, II-azure II, MV-methylene violet, L1-Leishman's stain (light degraded), Lh-Leishman's stain (heat degraded), Lc-Leishman's stain (aged control), Lf-Leishman's stain (freshly prepared), L4-prepared Leishman's stain (aged 266 days), L6-prepared Leishman's stain (aged 20 days), G-Gurr's R66 Geimsa, EY-eosin Y and, F-fluorescein. Table 3 on the following page contains the Rf values of the spots.

Table 3.

Rf Values and Spot Identification of Chromatogram of
Degraded Romanowsky Stains and Dye Standards
Figure 14.

Lane*	T	A	B	C	MB	II	MV	L1	Lh	Lf	L4	L6	G	EY
Meth. Blue		0.07		0.07	0.07	0.07		0.07	0.07	0.07	0.07	0.07	0.07	
Azure B		0.13	0.13	0.13	0.13	0.13		0.13	0.13	0.13	0.13	0.13	0.13	
								0.16	0.16	0.16	0.16	0.16	0.16	
		0.18				0.18		0.20	0.20	0.20	0.19		0.20	
Azure A		0.25		0.25				0.25	0.25	0.25	0.25	0.25	0.25	
				0.28		0.29							0.28	
		0.34	0.34								0.33	0.33		
Thionin	0.40													
Azure C	0.47	0.46		0.46			0.45	0.47	0.47	0.47	0.47	0.47	0.45	
Meth. Viol							0.59						0.59	
Eosin Y								0.77	0.77	0.77	0.77	0.77	0.77	0.77
								0.84	0.84	0.84	0.84	0.84	0.84	0.84

*Abbreviations for the lanes of the chromatogram are the same as for Figure 14 on the preceding page.

standard and fluoresced a brilliant yellow-green under ultraviolet light. As tribromofluorescein standards are not commonly available, the additional compound in the eosin Y standard was assigned this identification tentatively. The mobility of the unknown, Rf=0.84, and its fluoresence (orange) match those reported for tribromofluorescein (Lillie 1977, Marshall and Lewis 1974b). Eosin Y standards and eosin components of prepared stains were not observed to degrade in any of the following experiments.

Composition of Gurr's R66 Giemsa

The absorbance spectrum of this Giemsa showed two peaks at 517um and 610um (Fig. 15). The eosin peak (517) corresponds to that of the eosin Y standard. The thiazine peak (610) was broad and corresponded to the absorbance maximum of azure C. Thin layer chromatography confirmed that other thiazines were present, including methylene blue, azures A and B, methylene violet, and at least three unknown thiazines, Rf's=0.16,0.20, and 0.28 (Fig. 14). In theory, methylene blue should have been the primary thiazine in this stain (Lillie 1977), but, judging from the absorbance spectrum and TLC analysis, azures C and B were the primary thiazines. The relatively low concentration of eosin Y in this mixture was also notable.

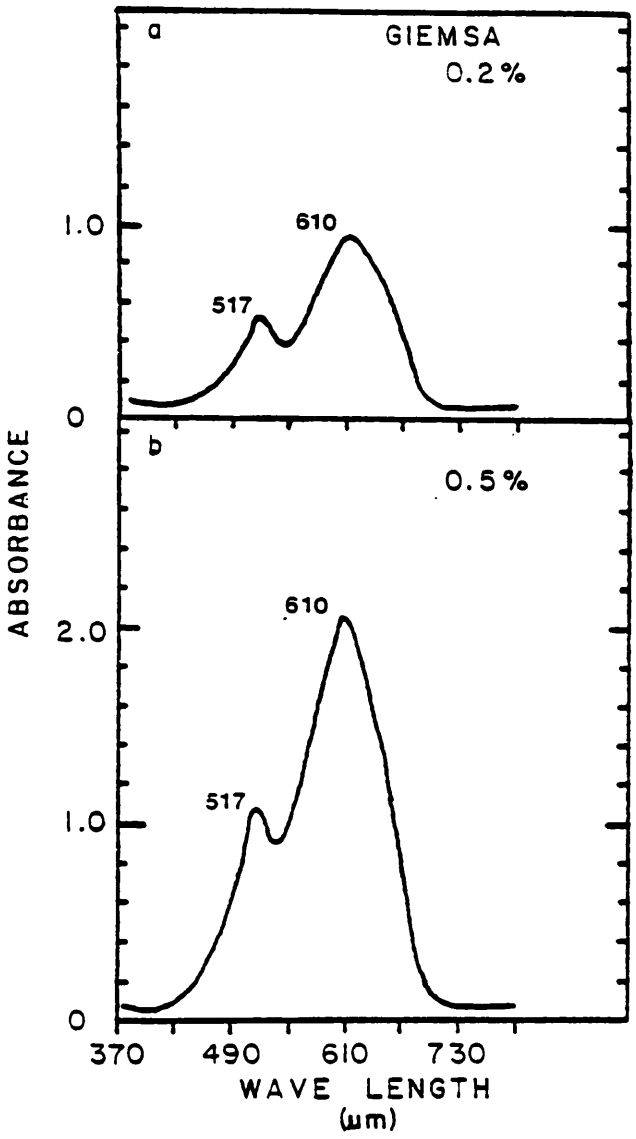


Figure 15. Absorbance spectra of Gurr's R66 Geimsa at two concentrations. Absorbance spectra were as indicated. Concentration refers to an aqueous solution of prepared stock solution.

This Giemsa was ineffective in producing C-bands in B. germanica spermatocytes. Only the most demonstrative bands on chromosomes 10 and 12 were ever stained. In most preparations the chromosomes were stained uniformly with a characteristic light blue hue. The cytoplasm was generally unstained.

Composition and Degradation of Leishman's Stain

The absorbance spectrum of Leishman's stain prepared fresh for chromosome staining in glycerol and methanol showed two peaks, eosin Y at 517um and methylene blue at 660um (Fig. 16a). Both peaks had the characteristics of the dye standards although the 610um shoulder of methylene blue was slightly higher than that of the standard. Since polychromed methylene blue is used in Leishman's stain (Lillie 1977), it was not surprising that TLC revealed azures A, B, and azure C in small amounts (Fig. 13). One batch of this stain (#4) was used for chromosome banding, and its quality was measured spectrophotometrically over time. Initially this batch gave consistently high quality C-bands. The slides were deeply stained after one hour in the buffered staining solution. The cytoplasm was stained a pink-red color. The chromatin was clearly differentiated into three types by the stain; darkly stained magenta areas (classic C-bands), lightly staining magenta areas, and areas

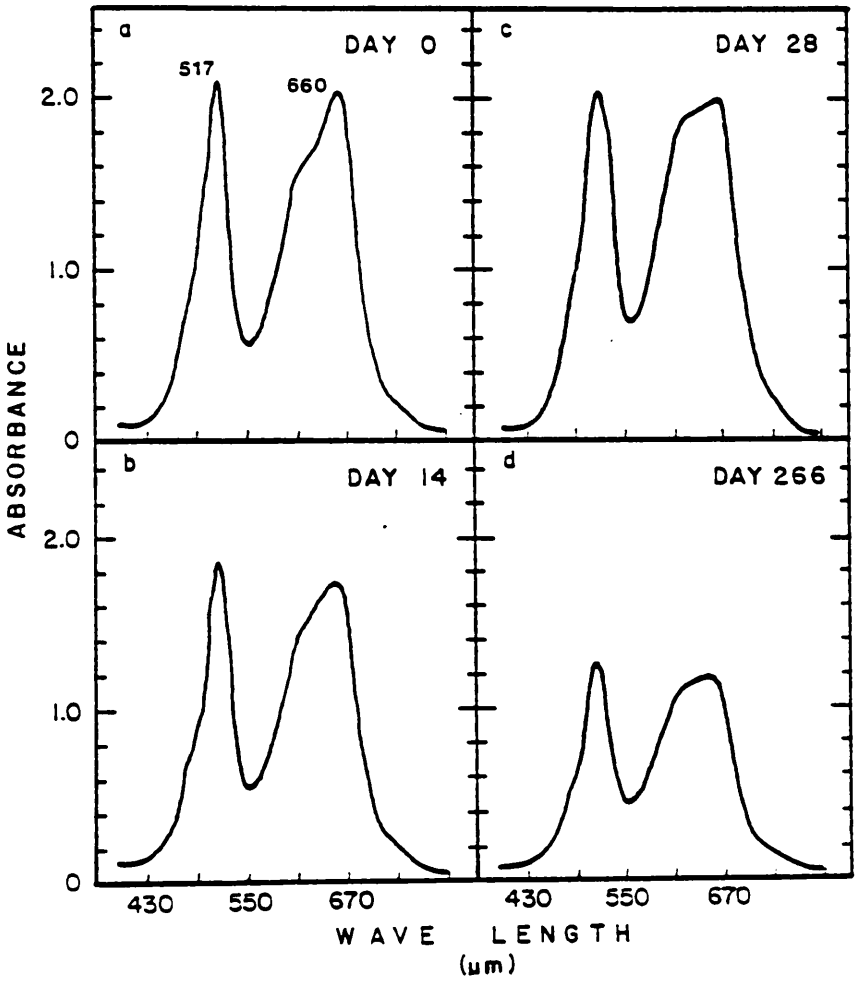


Figure 16. Absorbance spectra of Leishman's stain prepared for chromosome banding, batch #4 (see text), at intervals after preparation. Storage was at room temperature in shielded flasks.

that stained light blue if at all (euchromatin). After 12 days, the chromosome staining ability of this batch of stain declined rapidly. The first indication of banding failure was the absence of the pink-red staining of the cytoplasm. As chromosome bands become less discernible, the color shifted from magenta to blue. When it became obvious that this batch was no longer adequate (17 days), another absorbance spectrum was determined (Fig. 16b). The rounded appearance of the thiazine peak and the relatively increased height of the shoulder was most striking. The continued degradation of this batch at room temperature was followed spectrally for 266 days. Selected spectra are presented in Fig. 16c & d. Quanitative comparisons of the optical densities of these spectra are difficult to interpret and may be meaningless as the absorbance spectra of the thiazines do not follow Beer's Law, presumably because of the monomer-polymer effect (Comings 1975, Rabinowitch and Epstein 1941). Qualitatively, however, the shape of the thiazine spectra changed significantly. Although the maximum absorbance did not shift from 660um, the curve became less sharp and more rounded toward the ultraviolet.

Thin layer chromatography of batch #4 at 266 days and a batch (#6) 20 days old revealed that methylene blue was still a primary thiazine component although the azures were

all better represented than in fresh stain (Fig. 14). Both batches contained a thiazine compound, Rf=0.16, with mobility intermediate to azure A, Rf=0.25, and azure B, Rf=0.13, that was only present in trace amounts in fresh stain. The azure C spot in the aged Leishman's stain was also prominent. The mobilities of the more mobile components of the aged Leishman's batches were altered somewhat by increases in concentration of glycerol in the stain due to evaporation of methanol. Dilution with methanol and heavier loading of the TLC plate did not prevent this. This experiment cannot entirely rule out polymerization of methylene blue as the cause of spectral changes and banding failure.

Experimental Degradation of Leishman's Stain

As the previous experiment did not control the concentration/volume of the staining solution and because the glycerol component of the prepared stain interfered with TLC, a more controlled experiment was undertaken. The absorbance spectrum of the entire 75ml batch was typical for freshly prepared Leishman's stain (Fig. 17a). After ageing 50 days in an aluminum foil wrapped, tightly stoppered flask the control retained its original volume (25ml), yet the absorbance spectrum showed a marked decrease in the acuity of the methylene blue peak (Fig. 17b). The aliquots exposed to fluorescent light and to 36-38°C temperatures for 50 days

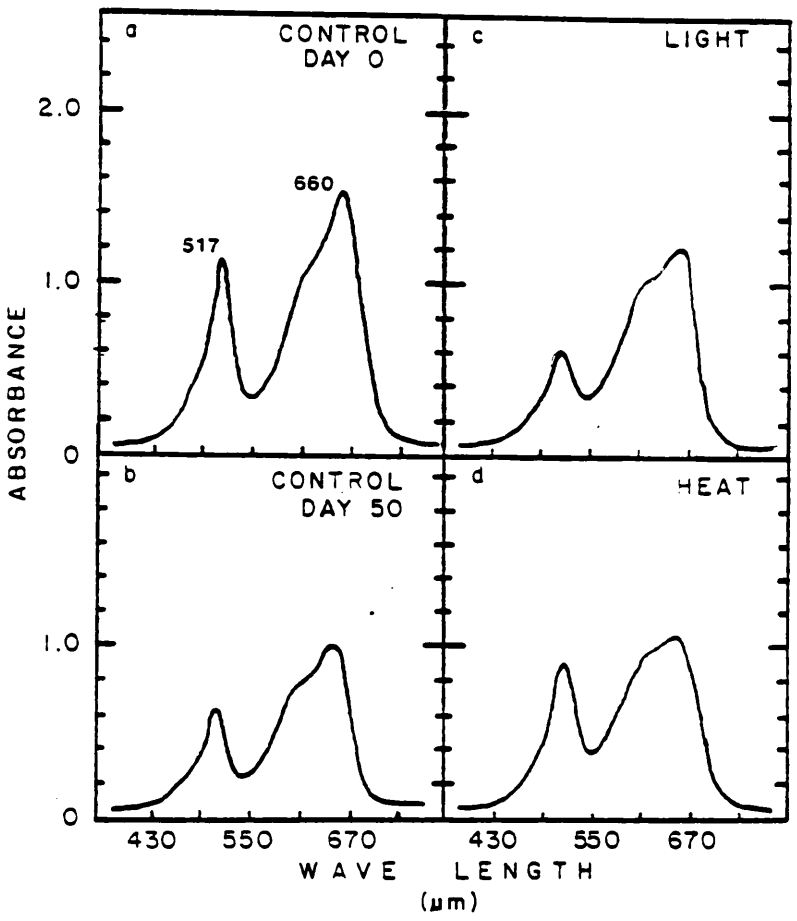


Figure 17. Absorbance spectra of 0.5% w/v methanolic Leishman's stain experimentally degraded. Absorbance maxima were as indicated. Spectra of light and heat degraded samples were taken after 50 days.

also showed spectral changes after their volumes were corrected to 25ml. The heat-degraded aliquot had lost 4.3ml and the light-degraded aliquot lost 1.6ml of methanol. Both aliquots' absorbance spectra contained substantially rounded thiazine peaks indicating that methylene blue had been extensively changed. This effect was more pronounced in the heat-degraded sample, which contained a much heavier azure B spot than the light-degraded aliquot as analyzed by TLC (Fig. 14). Both the light- and heat-degraded samples showed obvious azure C spots, in contrast to the aged control and the freshly prepared Leishman's stain. All three aged samples had quanities of the unknown, Rf=0.16, which migrated at a rate between azure A and B. This compound was absent from the freshly prepared stain.

The absorbance spectra also contained irregularities in the eosin Y peak, 517um (Fig. 17). Both the aged control and the light-degraded samples had eosin Y peaks of reduced magnitude. This effect was not apparent on the TLC plates as reduced spot size or in reduced fluorescence of either tribromofluorescein or eosin Y. The cause of these changes is enigmatic.

A sample of methanolic Leishman's stain stored under refrigeration in a wrapped flask purged with nitrogen did not show spectral or chemical changes when analyzed after 25 days.

DISCUSSION

The chemistry of Romanowsky-type blood stains is complex in that the thiazine components present in any given batch are highly variable. The production of these stains through "polychroming" or the variable oxidation of methylene blue is in part responsible for the variation. Stain components also degrade through further oxidation during storage and shipping (Marshall and Lewis 1974b, Dean et al. 1977). Additional complications are added by a metachromatic effect due to the formation of polymers of the dye molecules in aqueous solutions (Rabinowitch and Epstein 1941).

The failure of Gurr's R66 Giemsa to produce C-bands is easily understood in light of information concerning its composition. Sumner and Evans (1973) and Comings (1975) both identified methylene blue as the primary thiazine in the production of chromosome bands. The particular batch of Giemsa used in the present study appeared to contain very low concentrations of this dye; low enough that the absorbance spectrum was significantly altered. Analysis by TLC confirmed this by indicating the relatively low concentration of methylene blue present. The lack of spectral shift at a lower concentration indicated that metachromatic effects were not important in determining the shape of the absorbance curve.

All experiments in which the various thiazines have been tested for the production of chromosome bands have used G-banding as the standard of measurement (Sumner and Evans 1973, Lober et al. 1973, Comings 1975). Nevertheless, inferences on the mechanism of C-banding can be made from these studies because DNA-dye binding is also an important variable in the process. The various pretreatments simply make more or less DNA available to the dye. Lober et al. and Comings both agree that eosin is unimportant to the banding process. Sumner and Evans found that a 1:1 ratio by weight of methylene blue and eosin Y provides optimal staining conditions. Further, they hypothesized a mechanism of intercalation of two molecules of methylene blue and one molecule of eosin Y with DNA to produce a magenta colored band. Comings (1975) refuted this by calling attention to metachromatic effects and the chromotrophic character of the gelatin used as a substrate for calf thymus chromatin in their study. However, their cytological evidence is irrefutable. Staining solutions that did not contain eosin Y were not effective in chromosome banding. In the current study, slide preparations that did not exhibit eosin staining of the cytoplasm were the first indication of the failure of C-banding. The color of the chromosomes also changed from magenta to blue as C-banding failed in accordance with Sumner and Evans's (1973) results.

TLC analysis indicated that changes in absorbance spectra and failure to produce C-bands cytologically were correlated with degradation of methylene blue to azure A, azure C, and an unknown thiazine. Azure A and azure C increased in both batches of Leishman's stain (#4 and #6) when banding failed. Comings (1975) found that only the completely demethylated thionin was ineffective in binding to DNA-gelatin films and in producing G-bands. He obtained positive results with all four of the methylated thiazines although he did not illustrate this with figures. Furthermore, when these studies were conducted, the extreme contamination of these stains was unknown and unexpected. His positive banding and binding results may have been due to batch variations in the stains utilized. Sumner and Evans (1973), using thiazine-eosin Y mixtures, found methylene blue and azure B to be effective chromosome banding compounds and azure A to be ineffective.

In the current study, the presence of increased quantities of azure C and A in ineffective stains indicates that symmetry of methylation may be important in determining the ability of a thiazine dye to produce C-bands. Both azure A and azure C are methylated on only one side of the molecule. Presumably, a thiazine with a single methyl group on each side would be an effective chromosome banding stain. The obvious cytological tests with eosinated and uneosinated

purified stains have not been attempted.

Sumner and Evans (1973) also noted that excessive amounts of eosin Y in methylene blue staining solutions (greater that 1:1) results in a loss of banding ability. As methylene blue in Leishman's stain degrades, the relative concentration of eosin Y increases because the latter compound is photo- and thermally-stable in methanol (Dean et al. 1977). The nature of the eosin Y - thiazine interaction is uncertain but there may be an optimal equilibrium with eosin Y and methylene blue interacting in solution in the same 1:2 ratio that is hypothesized for DNA. At low concentrations, the available methylene blue would be quickly bound by eosin Y and made unavailable for binding to DNA. Eosin Y itself has no DNA binding ability (Comings 1975). Since azure A and C bind to DNA (Comings 1975), but do not produce banding, these compounds would account for a greater proportion of the stain binding to chromatin. If they do not complex with eosin Y, this may explain the characteristic blue staining associated with degraded Leishman's and Giemsa.

We have begun to make fresh Leishman's stain just before processing slides for C-banding to avoid stain degradation. The results have become much more reliable.

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CHROMOSOME BANDING IN GERMAN COCKROACH SPERMATOCYTES

by

Clifford Bennett Keil

(ABSTRACT)

A chromosome banding protocol for Blattella germanica spermatocytes was developed to reveal locations of constitutive heterochromatin (C-bands). Normality of the acid treatment used to denature basic (histone) proteins, temperature of the salt solution used to extract DNA from unbanded regions, and the components of the Romanowsky-type stain proved to be critical components in the C-banding process. The thiazine component of a standard Giemsa stain had an absorbance maximum altered from that of methylene blue, the putative major thiazine in this stain and was ineffective in producing C-bands. Two samples of Leishman's stain were examined chromatographically, spectrophotometrically, and for their C-banding ability as they aged. Banding failure was accompanied by a rounding of the thiazine spectral peak and the appearance of unknowns with chromatographic mobility intermediate to azures A and B, and an apparent increase in azure C content. Experimental thermal and photo-degradation of Leishman's stain showed similar alterations of the thiazine components. C-banded prophase II and diplotene karyotypes revealed a highly heteromorphic pattern of C-band distribution. Blocks of constitutive heterochromatin were not soley associated

with centromeres but occurred interstitially and terminally also. One chromosome was C-band negative, number 8, while two others, 9 and the X, were almost completely heterochromatic. Differential rates of condensation from prophase I to prophase II for euchromatic and heterochromatic regions were documented. The karyotype of B. germanica contained many gray bands that may indicate euchromatin interspersed with heterochromatin. Translocation heterozygote stocks were used to correlate the banded karyotype with linkage groups. Translocation multivalents frequently contained C-bands not resolved in wild type chromosomes. A C-banded prophase II karyotype of a closely related species, Blattella vaga, was prepared to assess the variability of heterochromatin distribution. The basic banding pattern was preserved in four of the twelve chromosomes although the bands were larger in this species. Two chromosomes, 11 and 8, had a single additional C-band in each. The B. vaga X chromosome was about twice as large as that in B. germanica. The mid-sized chromosomes were extensively repatterned. Overall, B. vaga chromosomes were longer than those of B. germanica. Increased heterochromatin content appeared to be the cause of the greater length.