# AN EXAMINATION OF THE CHROMOSOMES OF SEVERAL PLANT SPECIES USING GIEMSA-BANDING TECHNIQUES

by

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ABSTRACT

AN EXAMINATION OF SEVERAL PLANT SPECIES USING GIEMSA-BANDING TECHNIQUES

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Five Giemsa-banding techniques were applied to Vicia faba, Hordeum vulgare (barley) and Lotus pedunculatus chromosomes. Barley contained two categories of Giemsa-differentiated heterochromatin, V. faba six. Chromosome pair six in L. pedunculatus appeared predominantly heterochromatic. All species had banded pericentromeric heterochromatin which was acid sensitive in barley and V. faba. Barley and L. pedunculatus had banded telomeres. Banding patterns in chromosome pairs were homologous except for pair seven in barley where a balanced translocation was noted. Telomeres of chromosome six in barley appeard associated during interphase. Interchromosomal connectives between telomeres in L. pedunculatus prophase chromosomes and between chromosomes in barley were noted. Barley chromosomes were polarized during interphase. Giemsa-positive heterochromatin may be involved in maintaining these nonrandom chromosomal associations. Giemsa-banding techniques differentiate heterochromatin probably due to the disruption of euchromatic chromosomal proteins by the pretreatments, which emphasize differences in heterochromatic and euchromatic coiling.

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UNE ETUDE DES CHROMOSOMES
DE PLUSIEURS ESPÈCES VEGETALES
FAISANT APPEL AUX TECHNIQUES DE
LA FORMATION DE BANDES PAR LE
GIEMSA

M.Sc. NIKKI EVERTS SHANKLAND Biologie

Cinq techniques faisant appel à la formation de bandes par le Giemsa furent appliquées aux chromosomes de Vicia faba, Hordeum vulgare (orge) et Lotus pedunculatus. L'orge contenait deux catégories d'hétérochromatine différenciable par le Giemsa, et V. faba, six. Chez L. pedunculatus, la paire chromosomique no. 6 semblait en majeure partie constituée d'hétérochromatine. Toutes les espèces possédaient des bandes d'hétérochromatine péricentromérique, laquelle se révélant sensible à l'acide chez l'orge et V. faba. L'orge et L. pedunculatus montraient des bandes d'hétérochromatine dans la portion télomérique de leurs chromosomes. Les chromosomes homologues présentaient un patron de bandes identique, sauf dans le cas de la paire no. 7 de l'orge où une translocation balancée fut remarquée. Chez l'orge, les télomères du chromosome no. 6 paraissaient associés au cours de l'interphase. Des liens chromosomiques furent notés entre les télomères des chromosomes prophasiques de L. pedunculatus, ainsi qu'entre les chromosomes de l'orge. Chez cette dernière espèce, au cours de l'interphase, les chromosomes étaient polarisés. L'hétérochromatine Giemsa-positive peut être impliquée dans le maintien

de ces associations habituelles entre les chromosomes de ces espéces. L'hétérochromatine est différenciée par le Giemsa probablement à cause du bris des protéines euchromatiques des chromosomes lors des prétraitements, ce qui a pour effet d'intensifier les différences de spiralisation entre hétérochromatine et euchromatine.

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> For from Him and through Him and To Him are all things. For all things originate with Him and come from Him; all things live through Him, and all things center in and tend to consummate and to end in Him. To Him be glory forever! Amen - so be it.
>
> Romans 11: 36(The Amplified Bible)

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## DEDICATION

This thesis is dedicated to the memory of James B. Bloom, my late grandfather, and Dr. Richard Skahen, late friend and mentor, both of whom, throughout my youth, nourished and stimulated my inquiring mind, teaching me how to think and reason and learn. Both of these men, each in his own way, loved the pursuit of knowledge and truth, and must bear much of the responsibility for setting me, albeit inadvertantly, on the path which led to this thesis.

## CLAIM TO ORIGINALITY

The author claims the following findings of this study to be original:

1.) The induction of Giemsa bands in the chromosomes of Hordeum vulgare and Lotus pedunculatus.

2.) The discovery in H. vulgare of a putative balanced translocation between the arms of a single chromosome seven.

3.) The possible heterochromatic nature of chromosome six in the L. pedunculatus complement.

4.) The characterization of pericentromeric heterochromatin in Vicia faba and Hordeum vulgare based on a comparison of the findings in this study with those of previous studies.

5.) The discovery of interchromosomal connectives between the telomeres of Lotus pedunculatus prophase chromosomes.

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# INTRODUCTION

Since 1968 when Caspersson and his colleagues published their first paper on the use of the fluorochrome quinacrine mustard to produce bands in the metaphase chromosomes of several plant and animal species, the field of chromosome banding has proliferated, almost explosively. The second, and technically more important stage was the discovery by Pardue and Gall (1970) and Arrighi et al. (1970) that the Giemsa stain preferentially colored areas of the chromosome rich in repetitive DNA after a pretreatment in sodium hydroxide followed by incubation in trisodium citrate solution at a neutral pH. The use of quinacrine mustard demands relatively sophisticated and expensive equipment and the fluorescence tends to fade rapidly. In contrast, the Giemsa technique uses standard laboratory reagents and equipment to elicite bands equivalent to those produced by quinacrine mustard. In addition, the Giemsa bands do not fade. Consequently, the Giemsa staining technique soon dominated the field.

To date many different chemicals with or without various pretreatments have been used to induce the nonrandom pattern of irregular staining along the metaphase chromosome termed "banding". Besides the more broadly used Giemsa and quinacrine mustard, postfixation banding has been produced using acridine orange (Castoldi et al., 1972), acetocarmine (Greilhuber, 1973), Feulgen (Rodman and Tahiliani, 1972), acetoorcein (Vosa, 1973b), and ethidium bromide (Unakul and Hsu, 1973). Bands produced by the prefixation treatment of human chromosomes with $^{3}$H-thymidine (Ganner and Evans, 1971), $^{3}$H-tryptophan (Djondjurov et al., 1972), tetracycline (Meisner et al., 1973) and actinomycin D (Shafer, 1973), provide insight

into the mechanism of the banding phenomenon: The most intriguing aspect of this phenomenon is the high degree of consistency between the banding patterns caused by agents of such wide diversity. Although the Giemsa staining technique has been used exclusively in this study, information on chromosome banding induced by other agents will be referred to where it provides some elucidation on the mechanisms of Giemsa-banding.

The bands reflect areas of constitutive heterochromatin and are, for the particular staining technique used, consistent morphological features of the chromosomes (Comings, 1972a). They have been used to resolve chromosome pairs (Sarma and Natarajan, 1973), and as markers in mapping breakpoints in balanced translocations (Döbel et al., 1973).

The bands also show promise of helping to elucidate some of the functions of heterochromatin (Stack and Clarke, 1973a). Constitutive heterochromatin has been implicated in the pairing of homologous chromosomes at meiosis and mitosis, and in the maintenance of the specific spatial arrangements of interphase chromatin observed in some plant species (Wagenaar, 1969).

Although most of the researchers working on the mechanisms and applications of banding have utilized mammalian chromosomes (Comings, 1972a), the last few years have seen the techniques successfully modified for plant material with some interesting positive results (Vosa and Marchi, 1973; Döbel et al., 1973; Vosa, 1973a; Marks and Schweizer 1974; Sarma and Natarajan, 1973).

In the plant species studied so far the amount and localization of constitutive heterochromatin as revealed by the bands varies both between individuals within a population (Marks and Schweizer, 1974; Vosa, 1973a)

and between species within a genus (Marks and Schweizer, 1974). Giemsa-banding techniques appear to be useful tools for resolving taxonomic relationships and studying chromosomal evolution in plants.

Although the chromosome banding patterns for many plant species have been determined, there are still many species whose chromosomes are as yet unbanded. Among the latter are two of economic and cytogenetic importance: Hordeum vulgare L., a cereal crop and standard laboratory organism for studying the effects of mutagens on chromosomal structure and behavior (Mohandas and Grant, 1972; Tomkins and Grant, 1972), and Lotus pedunculatus Cav. (Big Leaf Birdsfoot Trefoil) a putative ancestor of L. corniculatus L. (Birdsfoot Trefoil; Cheng and Grant, 1973), an important forage species. The present study was undertaken to determine if the heterochromatins of these two species would respond to the Giemsa-banding techniques.

Since the heterochromatin in Vicia faba had already been proven to be Giemsa positive by several different techniques (Schweizer, 1972; Döbel et al., 1973; Takehisa and Utsumi, 1973a), this species was used to test the success of a technique developed initially for banding human chromosomes on plant chromosomes. As well, the heterochromatins in V. faba showed some diversity in response to the techniques previously used, and therefore it seemed worthwhile to investigate this variability by comparing the response of these heterochromatins to different techniques.

# LITERATURE REVIEW

## The Metaphase Chromosome

At the light microscope level the metaphase chromosome appears to consist of two chromatids, joined at the centromere. When examined by electron microscopy it is apparent that the chromatids are fibrous in nature. This fibre is made up of DNA, RNA, and histone and nonhistone proteins (Comings, 1972a) and is referred to as the desoxyribonucleoprotein (DNP) fibre. X-ray diffraction studies on the DNP fibre reveal the presence of a regular tertiary structure which has been postulated by Pardon and Wilkins (1972) to be a "supercoil".

### DNA

Although proponents for polynemy still abound, most researchers agree that the DNA in each chromatid is in the form of a single, long, double helix (For a review of this problem see Comings, 1972a). Recent research indicates that the DNA in the chromosomes of Vicia faba (Bhattacharya and Sen, 1973), Drosophila (Kavenoff et al., 1974), and yeast (Petes et al., 1974) is uninemic.

### RNA

Much of the RNA attached to the DNP fibre is ribosomal RNA (rRNA), but there is a unique fraction of RNA associated with chromatin having a sedimentation coefficient of 45S (Ockey, 1973). The function of this high molecular weight ($4.0 \times 10^6$ D) chromosomal RNA is not known at present, but it has been found in Drosophila salivary gland chromosomes, HeLa, liver, and Chinese hamster chromatin (Ockey, 1973).

## Histone Proteins

The histones are a group of proteins characterized by their low molecular weights (10 - 20 x $10^3$ D) (Mahler and Cordes, 1966) and their high proportion (22%) of basic amino acids (Ockey, 1973) which gives them an isoelectric pH of 10 or higher (DuPraw, 1970).

The basic residues in the histones tend to be located at one end of the protein while the hydrophobic and acidic residues are located at the other (Comings, 1972a). Histones are bound to DNA by electrostatic attraction between the positive charges of the basic residues and the negative charges on the DNA's phosphates (DeLange and Smith, 1971). The α helical content of native nucleohistone is 40 - 60% (Louie et al., 1974); a figure which compares interestingly to the finding by Simpson (1972) that 30 - 55% of the histone in chromatin is not bound to DNA. As well, Simpson states that each histone protein contains firmly bound regions as well as detached regions. Areas containing a high concentration of like-charged residues usually don't form α helices due to the electrostatic repulsion between the like charges. It seems plausible then that the highly basic regions of the histones bind to the DNA and that those regions which are hydrophobic exist in an α helical state, are not bound to the DNA, and are free to interact with each other or with other proteins (Bradbury et al., 1975).

Histones were at first thought to control gene activation and repression since they inhibited the ability of DNA to be transcribed (Huang and Bonner, 1962). This role has been diminished recently by the observation that even after removal of practically all the histones, significant template restriction was retained in rat thymus chromatin (Spelsberg and

Hnilca, 1971). It seems evident now that the histones function in maintaining the structure of the DNP fibre, the formation of the metaphase chromosome, and possibly in the differential condensation of heterochromatin (Miller et al., 1971; Ruch and Rosselet, 1969).

The six common histone fractions are resolved on the basis of their terminal groups, presence or absence of cysteine and their behavior in gel electrophoresis (Ockey, 1973). The F1, F2a1, and F3 fractions are present in both animal and plant chromatin; the F2a2 and F2b fractions are found only in animal chromatin, and the plant Histone (PH) fraction is exclusive to plant chromatin (Ockey, 1973; Nadeau et al., 1974).

F1

The F1 histones are alanine rich and lysine rich with many basic amino acids (Comings, 1972a). When histones are removed from DNA by extraction with either weak acids or strong salts the F1 histones are the first to be removed (Ockey, 1973) indicating that they are the least tightly bound to the DNA (Stellwagen and Cole, 1969). They are also the most easily removed by proteolysis (Ockey, 1973).

Basically two lines of evidence indicate that the major function of the F1 histone is related to the condensation of the DNP fibre into the metaphase chromosome. 1) F1 histone is present in those unicellular organisms which have condensed chromosomes but is absent in yeast which, having a DNP fibre essentially equivalent to those of other eukaryotes (Gray et al., 1973), has no condensed chromosomes (Tonino and Rozijn, 1966). 2) F1 is rapidly phosphorylated at the $G_2$/M boundary and during prophase (Gurley et al., 1974; Bradbury et al., 1974) by a specific phosphokinase,

KII, whose activity predominates at $G_2$/M transition and whose natural *in vivo* substrate is F1 histone (Lake, 1973a; Lake and Salzman, 1973). The F1 phosphoform is maintained throughout mitosis (Lake 1973b) and dephosphorylated at M/$G_1$ (Lake *et al.*, 1972).

F2a2 and F2b

Both F2a2 and F2b are slightly lysine rich. Their absence in plant chromatin is balanced by the presence of the PH fraction which might mean that the function of F2a2 and F2b is taken over in plants by the plant histones (Nadeau *et al.*, 1974).

F2a1 and F3

F2a1 and F3 are arginine rich histones (Comings, 1972a). F3 is the only histone with appreciable amounts of cysteine (two residues per molecule) making it possible for it to form disulfide bridges. It is also the last fraction removed with weak acid and/or strong salt extractions (Ockey, 1973). These arginine rich histones are very important in the maintenance of the tertiary structure of the DNP fibre. DNA complexed with only F2a1, F3, and F2a2 histone fractions gives an X-ray diffraction pattern which indicates the presence of the tertiary structure; naked DNA and DNA complexed with F1 histone alone do not give this pattern (Pardon and Richards, 1972).

The rapid phosphorylation of F3 only at the $G_2$/M boundary and during prophase (Gurley *et al.*, 1974), and the proportionately greater number of disulfide bridges in F3 at metaphase than at interphase (DeLange and Smith, 1971; Sadgopal and Bonner, 1970) implicate the F3 histone fraction in the formation of the metaphase chromosome.

## Nonhistone Proteins

The nonhistone chromosomal proteins are a heterogeneous group including the phosphoproteins, the various enzymes related to the transcription, synthesis and repair of DNA and the regulatory proteins (Comings, 1972a; Ockey, 1973). Those isolated from pea chromatin range in molecular weight from 10,000 to 68,000 D (Lin et al., 1973). They are acidic and some contain cysteine as well as tryptophan, an amino acid which is absent in histone protein (Ockey, 1973). They are very difficult to extract and purify due to their tendency during extraction to aggregate with one another and to bind to histone proteins so that to date there is little known about their structure (Ockey, 1973).

The acidic proteins seem primarily to be involved in gene transcription and regulation (Ockey, 1973). The only nonhistone protein found so far to have a definite structural function are the phosphoproteins. There are more phosphoproteins in diffuse chromatin than in condensed chromatin and the phosphoproteins are able to alter template activity (Comings, 1972a). The key to their mode of action is found in the effect of phosvitin, a phosphoprotein, on the condensed chromatin of thymocyte nuclei. The phosphorylated form of the protein decondenses the chromatin by binding histones and thereby stripping them from the DNP fibre (Whitfield and Perris, 1968).

The nonhistone proteins may function as well in the condensation of the metaphase chromosome since the disulfide bridge content in this fraction is higher at metaphase than at interphase (Sadgopal and Bonner, 1970).

Perhaps the most compelling observation concerning the possible function of some of the nonhistone proteins is that made by Allfrey et al.

(1974) and Bekhor et al. (1974) that a specific fraction of acidic chromosomal protein is bound preferentially to highly repetitious DNA. A further insight, possibly bearing on the previous one, is that the distribution of tryptophan containing proteins along the metaphase chromosome is unequal (Djondjurov et al., 1972).

Structure of the DNP Fibre

There appear to be several orders of magnitude of the DNP fibre. Electron microscopy reveals fibres measuring 50 Å, 100 Å, 200 Å, and 300 - 500 Å (DuPraw, 1970; Comings, 1972a). Ris (1975) catalogues these fibres thusly: When the chromatin is in the form of the 200 Å fibre it is inactive; it can be unraveled by removing divalent cations with chelating agents to produce the 100 Å fibre. Treatment of this fibre with urea disrupts histone - histone hydrogen bonding, and results in a fibre 20 - 40 Å thick. Electron microscopy cannot as yet elucidate the problem of how the 20 - 40 Å fibre is arrayed in the 100 Å fibre because the necessary fixation procedures dehydrate the chromatin fibre causing the collapse of the tertiary structure in the 100 Å fibre (Ris, 1975).

Kornberg (1974) suggests that the DNP fibre, at its most basic level, is a repeating subunit of 200 DNA base pairs, the tetramer $(F2a1)_2(F3)_2$, and two molecules each of F2a2 and F2b associated as oligomers. Electron photomicrographs of stretched chromatin fibres show 60 - 80 Å spherical particles, "ν" bodies, connected to each other by a 15 Å filament (Olins and Olins, 1974). Woodcock et al. (1974) show that the filament is DNA and that the presence of the "ν" bodies depends on F3 and F2a1 histone. It seems possible that these "ν" bodies are structural representations of Kornberg's repeating unit.

The basic fibre coils to form a 100 Å fibre whose regular tertiary structure depends on the presence of F3, F2a1, and F2a2 histones (Pardon and Richards, 1972). This tertiary structure is maintained by histone - histone interactions (Ris, 1975). Gluteraldehyde fixation of chromatin leads to the formation of increasingly larger oligomers implying that the histones linearly overlap each other; the oligomers are made up of F2a2, F2a1, F2b and F3 histones (Chalkley and Hunter, 1975). Perhaps the histones in Kornberg's (1974) repeating subunits maintain the conformation of the 100 Å fibre by overlapping with the histones of adjacent subunits further along the filament.

The 100 Å "supercoil" then either undergoes coiling or else folds back on itself to produce the 200 Å fibre (Comings 1972a). DuPraw's (1970) photomicrographs of type A (100 Å) and type B (200 Å) fibres and his comparisons of the DNA packing ratios (mass/unit length) in these two fibres favor the view of the 200 Å fibre as a coiled supercoil.

Metaphase Contraction

Structurally the process of condensation into the metaphase chromosome alters the DNP fibre in two ways: 1) the fibre becomes even more coiled, as evidenced by the doubling of the DNA packing ratios, forming a 300 Å fibre (DuPraw, 1970), and 2) the fibre associates with itself to produce the shape of the chromatid.

The DNP fibre interacts with itself in longitudinal arrays or as a "whorl of folded, looping fibres" called chromomeres (Bahr et al., 1973). The classical chromomeres observable by light microscopy in the pachytene chromosomes of many plant and animal species appear as small

darkly staining beads which seem to be strung, semi-regularly, along the entire length of the chromosome. Their exact number and pattern of distribution along the chromosome is a shared feature of a particular chromosome pair. There is, however, a uniformity in the average distance between chromomeres of a specific size in many species of plants (Lima-de-Faria et al., 1959). It is thought that chromomeres are "constant expressions of the coiling system which accompanies chromosomal contraction" (Swanson et al., 1967). Their behavior during meiotic prophase matches that of the mitotic prophase chromomeres recorded by Bahr et al. (1973) in that as the chromomeres enlarge, indicating condensation of the fibre, the distance between two consecutive chromomeres decreases until at metaphase they are not resolvable (Lima-de-Faria, 1952). Further evidence aligning Bahr et al.'s (1973) electron-microscopic, mitotic chromomeres with the "classical" chromomere is the fact that chromomeres are present in the polytene chromosomes of dipteran species and these chromosomes develop from somatic chromatin.

The condensation process is dependent on chromosomal proteins. Actinomycin D competes with chromosomal proteins for binding sites on the DNA, and was shown to block the condensation of certain chromosomal regions (Arrighi and Hsu, 1965). The proteins which will become associated with chromosomes are synthesized in $G_2$ (Rao and Johnson, 1972) and if actinomycin D, which inhibits protein synthesis, is applied early enough in $G_2$ it will prevent entry into mitosis altogether (Arrighi and Hsu, 1965).

The metaphase fibre contains twice the amount of protein present in an interphase fibre while the DNA content remains the same. This

increase is due in part to nonhistone proteins, and probably reflects the presence of ribosomal protein as a result of the congregation of the ribosomes on the metaphase chromosomes (Ockey, 1973). There are also some acid insoluble proteins present in metaphase chromosomes that are absent in interphase chromatin (Sadgopal and Bonner, 1970).

The F1 and F3 histones as well as some nonhistone chromosomal proteins are probably involved in metaphase condensation. Both F1 and F3 histone fractions are phosphorylated at the $G_2$/M boundary (Gurley et al., 1974), the F1 phosphoform co-exists with the condensed metaphase chromosomes (Lake, 1973b) and is dephosphorylated as the metaphase chromosomes decondense (Lake et al., 1972). As well, the sulfur containing residues on both histone F3 and the cysteine-containing nonhistone proteins form more disulfide bridges in metaphase chromatin than in interphase chromatin (Sadgopal and Bonner, 1970).

How these alterations influence the chromatin can only be guessed at, however, the following hypothesis seems to fit the observed facts. It is highly probable that the interphase nucleus is highly ordered and that the chromatin fibres maintain specific, invariant spatial relationships within the interphase nucleus (Brasch and Setterfield, 1974; Comings, 1968; Feldman and Avivi, 1973; Wagenaar, 1969). This spatial orientation is probably stabilized by the attachment of the fibres to the nuclear membrane (Brasch and Setterfield, 1974; Avivi and Feldman, (1973). During prophase the fibres making up each chromosome begin to condense, due, perhaps, to the phosphorylation of their F1 and F3 histone fractions. At a particular ionic concentration, the phosphorylation of the two seryl residues at either end of the apolar segment in histone

F1 causes a large segment of histone F1 to detach from the DNA (Bradbury et al., 1975). This free portion could then interact with like regions in other F1 histones and in this way mediate mitotic condensation of the DNP fibre. The formation of disulfide bridges between the chromosomal proteins could stabilize the mitotic coil. The condensation process brings the fibre within each chromatid into closer contact to itself producing the shape of the metaphase chromosome. The intra-fibre spatial relationships during interphase would be the same as those in the metaphase chromosome except that in interphase the fibre is longer and hence the distances between the regions of the fibre would be greater.

## Heterochromatin

Heitz (1928) coined the term "heterochromatin" specifically to differentiate chromatin which stayed condensed in interphase from chromatin which condensed only during mitosis. The word itself means simply - different chromatin - and therein lies Heitz's foresight. The more that is learned about heterochromatin, the more heterogeneous a category is appears to be, such that Comings (1972a) in his review on The Structure and Function of Chromatin was prompted to say: "Heterochromatin is somewhat like human society - it is a complex subject and simple slogans are inadequate to characterize it." In fact so many sub-categories of heterochromatin have been established based on different behavioral, chemical, and structural criteria that we have almost come full circle in that there are very few general statements that can be made. Heitz's original structural definition still holds

and to it have been added two functional qualities, genetic inactivity and a timing of DNA replication that is different from the dominant chromatin species, termed euchromatin (Comings, 1972a).

Cytogenetically the most relevant of these is still Heitz's observation on heterochromatin's unique condensation properties. This condensation appears to be qualitatively different from that undergone by the DNP fibre as it enters mitosis. Prefixation exposure of plants to cold allows the euchromatin in metaphase chromosomes to contract but inhibits the heterochromatin from contracting (Boothroyd, 1953). This lack of contraction in cold reactive heterochromatin, or "negative heterochromatin" (-H), is due to protein starvation (Baumann, 1969, as quoted by Vosa, 1973c). Actinomycin D binds in the minor groove of DNA and competes for binding sites with polylysine (Seligy and Lurquin, 1973). The F1, or very lysine rich, histone appears to be linked with the condensation of the metaphase chromosomes (Bradbury et al., 1975). This condensation can be interfered with by actinomycin D (Arrighi and Hsu, 1965; Shafer, 1973). It is probable then that the F1 histone also binds in the minor groove. Combined with the above information, the study by Chalkley and Hunter (1975) indicates that the other histone fractions occupy the major groove. Sieger et al.'s (1971) observation that actinomycin D can bind to heterochromatin at interphase without influencing or being influenced by its degree of condensation implies that protein fractions binding in the major groove are involved in heterochromatic condensation. It is probable that some protein fractions are active in both heterochromatic condensation and metaphase condensation; the former depending on enzymatic modifications of different amino acid residues than the latter.

Those heterochromatins possessing unique DNAs owe their special tertiary structure primarily to the chemistry of their DNA. Much of the heterochromatin of the mouse is made up of satellite DNA, i.e., DNA which has a different base ratio to main-band DNA (Pardue and Gall, 1970). The DNAs in some heterochromatins are less methylated than euchromatic DNA (Comings, 1972b); and finally some heterochromatin consists of highly repetitious DNA (Arrighi et al., 1970). These qualitative differences in the DNA can influence the tertiary structure of the DNP fibre (Yunis and Yasmineh, 1971). Pardon and Richards (1972) found that poly dAT DNA will not form supercoils with a histone mixture although calf thymus DNAs will. This is related to Bram's (1971) finding that the pitch of the DNA helix in AT-rich (A + T/G + C ≥ 2) native DNAs is about 10% greater than that found in DNAs with moderate or low AT content. Bram postulated that "proteins which bind and operate on moderate AT-rich DNA might behave differently with very AT-rich DNA, and vice versa." Verification of this statement is seen in the preferential association of arginine rich histones with GC-rich regions (Clark and Felsenfeld, 1972) and lysine rich histones with AT-rich regions (Combard and Vendrely, 1970). Undoubtedly the pattern of base sequence in AT-rich repetitious DNA will also influence the secondary structure of DNA. A fraction of nonhistone chromosomal protein has been found which binds tightly to DNA and has a high affinity for highly repetitious DNA (Allfrey et al., 1974; Bekhor et al., 1974). Inactive chromatin fibres can be decondensed with urea which disrupts the hydrogen bonds between histones (Ris, 1975). There is much circumstantial evidence indicating that the specific protein - DNA and protein - protein interactions which are associated with and due to chemical differences along the DNA

contribute to heterochromatin's unique condensation.

## Constitutive and Facultative Heterochromatin

There are two subdivisions of heterochromatin, facultative and constitutive (Brown, 1966). Constitutive heterochromatin is a permanent characteristic of a particular length of DNP fibre; barring mutational events, it does not change from one cell generation to the next, or from cell to cell in the same individual (Yunis and Yasmineh, 1971). Facultative heterochromatin, however, is an impermanent state of a particular chromosome. The two classical examples of facultative heterochromatin are the one inactive X chromosome in mammalian females and the inactive paternal set of chromosomes in male mealy bugs. The apparent lack of facultative heterochromatin in plants might be related to the absence of the F2a2 and F2b histone fractions in plant chromatin (Nadeau et al., 1974).

## Localization of Heterochromatin in Plant Chromosomes

Classically, constitutive heterochromatin has been localized in plant chromosomes by the effects of cold treatment on mitotic metaphase chromosomes which reveals negative heterochromatin (-H), and by the examination of pachytene chromosomes which may contain positive heterochromatin of three general types: chromomeres, pericentromeric heterochromatin, and irregularly distributed blocks of densely staining chromatin called "knobs" (Stebbins, 1971).

## Heterogeneity of Heterochromatin

The heterogeneity of heterochromatin is evidenced in many studies.

Comparative electron microscopic studies of the -H fibres in Scilla sibirica and Fritillaria lanceolata show structural differences between them (LaCour and Wells, 1974). Merritt (1974) in his study of the genus Nicotiana picked out four sub-categories of heterochromatin in pachytene chromosomes depending on their size, shape and staining properties with propionocarmine. The same block of constitutive heterochromatin in different cell types takes on different forms due to the different chemical environments (Yunis and Yasmineh, 1971). Takehisa (1973) distinguishes two varieties of heterochromatin in chromosomes from Vicia faba root tips, while Rieger's (1973) study implies that Vicia faba heterochromatin is even more heterogeneous since six mutagens, each with slightly differing modes of action, caused aberration clustering in different heterochromatic regions. As well, the translocation of heterochromatin to a new chromosome can alter its "hot spot" characteristics. Some feature of the heterochromatin is affected by its immediate genomic environment.

One of the great breakthroughs in cytogenetics has been the discovery of a general staining procedure for constitutive heterochromatin in metaphase chromosomes.

## The Giemsa Bands

That the Giemsa bands are heterochromatic almost goes without saying since they react differently than most of the chromatin to the pretreatments and stains used. The bands also correspond to regions which have the following characteristics of heterochromatin: unique DNAs, late replication, and differential condensation.

The location of Giemsa bands have been correlated with regions containing satellite DNA in man and in mouse chromosomes (Sanchez and Yunis, 1974; Pardue and Gall, 1970), and also to AT-rich DNA in human and bat chromosomes (Schreck et al., 1973; Pathak et al., 1973b). The pericentromeric region of Vicia faba's M chromosome is banded with Giemsa (Schweizer, 1973; Takehisa and Utsumi, 1973a) and is probably AT-rich (Cionini, 1973).

In Vicia faba (Döbel et al., 1973), Rhoeo discolor (Natarajan and Natarajan, 1972), Scilla sibirica (Vosa, 1973a), human (Pearson, 1972) and bat (Pathak et al., 1973b) chromosomes the Giemsa bands correspond to areas that replicate their DNA later than the rest of the chromatin.

Gill and Kimber (1974) found that the Giemsa bands account for all of the knob and pericentromeric heterochromatin observed in the pachytene chromosomes of rye.

Heterochromatin is visualized in the interphase nuclei of dividing cells as chromocenters, that is, areas which are compact and condensed rather than diffuse (Swanson et al., 1967). The number and/or amount of chromatin involved in these chromocentres correlates positively with the number and/or amount of Giemsa-positive chromatin in the metaphase chromosomes of Trillium kamtschaticum (Utsumi and Takehisa, 1974), the mouse (Pardue and Gall, 1970), Allium cepa (Stack and Clarke, 1973a), and Tulipa (Filion, 1974).

The cold reactive -H segments of Vicia faba (Döbel et al., 1973), Scilla sibirica (Vosa, 1973a) Trillium grandiflorum (Schweizer, 1973), and Trillium kamtschaticum (Takehisa and Utsumi, 1973b) are equivalent

to Giemsa positive regions although the inverse situation does not hold; Giemsa may reveal noncold reactive heterochromatins as in Vicia faba (Takehisa and Utsumi, 1973a), Trillium kamtschaticum (Takehisa and Utsumi, 1973b), and Zea mays (Vosa and Marchi, 1972).

In Nicotiana otophora the Giemsa positive regions are equivalent to chromosomal regions that are naturally more contracted at prophase than the rest of the chromosome. Prefixation treatments with colchicine and 8-hydroxyquinoline emphasized this naturally occurring difference (Merritt and Burns,1974). The mechanism of inducing -H segments by exposure to cold or colcemid is apparently due to this same emphasis of a naturally occurring qualitative difference between the euchromatin and heterochromatin. The condensation of metaphase chromosomes seems to be superimposed on the condensation of constitutive heterochromatin.

Giemsa - DNA Interactions

Studies on the dye - DNA interactions show how the Giemsa dye reveals condensed versus less condensed regions of the chromosome.

The Giemsa stain is one of the Romanowsky stains; it is a mixture of the basic dye methylene blue, the oxidation products of methylene blue namely, Azures, A, B, C, and methylene violet (Bernthsen), and the acidic dye eosin Y (Gurr, 1965). Methylene blue and its oxidation products have a tricyclic nucleus structurally very similar to the tricyclic acridine nucleus of quinacrine (Modest and Sengupta, 1973). Since the first step in the binding of quinacrine to DNA is the intercalation of the tricyclic nucleus between the bases of the double helix (Caspersson et al., 1968; Selander and de la Chapelle, 1973; Modest and Sengupta, 1973), it is thought that methylene blue and its oxidants also intercalate into the

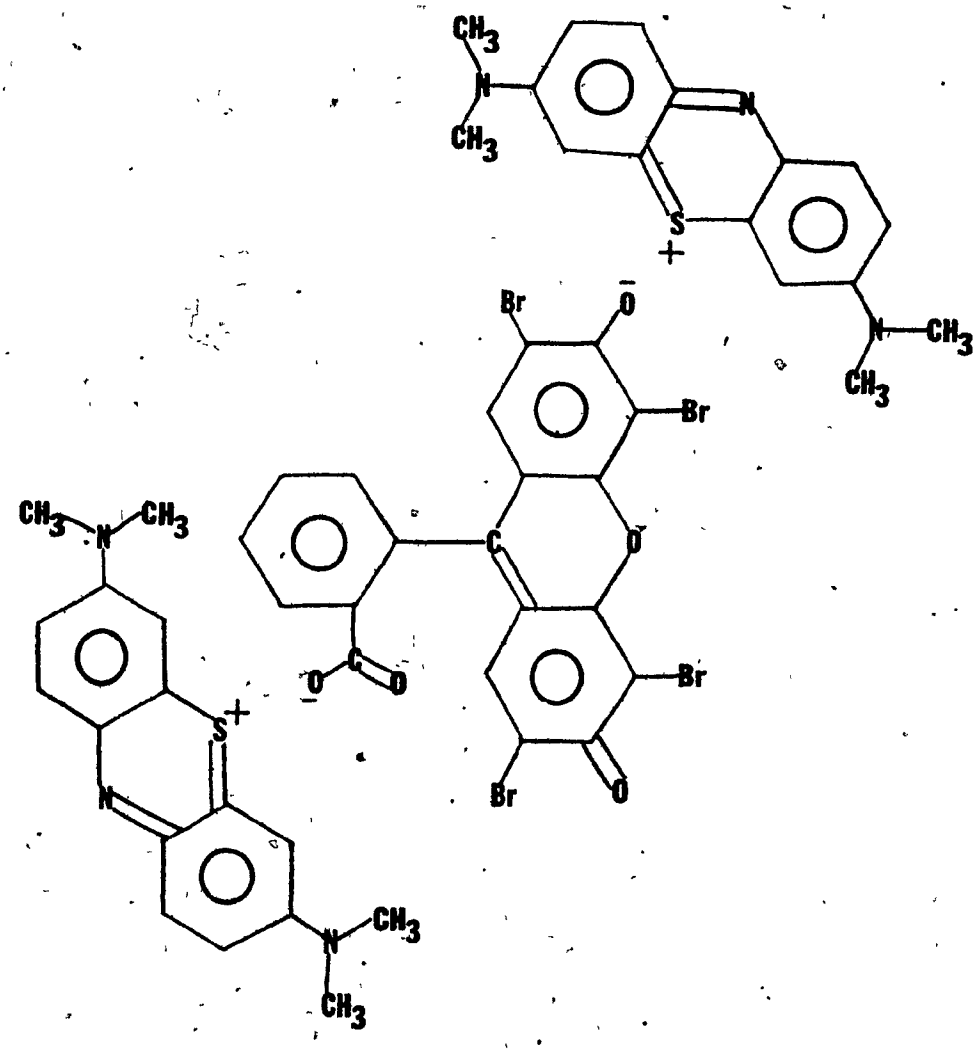
DNA helix (Meisner et al., 1974).

Sumner and Evans (1973) have shown that methylene blue is bound to the DNA ionically and, alone, will not produce bands. When stained with a mixture of methylene blue and eosin Y, chromosomes stain blue indicating that they have picked up methylene blue; banding occurs only after a magenta color starts to appear. This magenta dye compound is composed of a 2:1 molar ratio of methylene blue or possibly one of its oxidants, and eosin Y (Sumner and Evans, 1973; Meisner et al., 1974).

The magenta compound may be formed by salt linkage between the positively charged sulfurs in the methylene blue molecules and the carboxylic acid groups of the eosin Y (see Figure 1). The findings of Sumner and Evans (1973) suggest that eosin Y successfully competes with DNA for ionic binding sites on the methylene blue and that once the eosin Y - methylene blue compound is formed, it is held to the DNA by hydrogen bonds. Interaction with the DNA must somehow strengthen the bonds between eosin Y and methylene blue since once separated from the DNA the magenta compound resolves itself into its component molecules, but while attached to the DNA it is invulnerable to high salt concentrations (Sumner and Evans, 1973).

A corollary of this staining mechanism is that the two methylene blue molecules must be the appropriate distance apart in order for the eosin Y molecule to bind to both. This implies that the bands, after the pre-treatments, have more available DNA binding sites which are the correct distance apart than have the interbands. This spatial dependency for the formation of the magenta dye infers a difference in the degree of condensation between band and interband regions caused by the differential

Figure 1. Possible conformation of the 2 methylene blue: 1 eosin Y, magenta compound (Sumner and Evans, 1973). The chemical structures of methylene blue and eosin Y are taken from Gurr (1971).


CH3
N
CH3
N
S
+
N
CH3
CH3
Br
O
Br
C
O
O
C
O
Br
Br
O
CH3
N
CH3
S
+
N
N
CH3
CH3

sensitivity of these regions to pretreatment - induced decondensation.

The Induction of Bands

The reagents with which chromosomes are treated to produce Giemsa bands are chemically quite diverse yet the bands are highly consistent (Kato and Moriwaki, 1972). The only thing these reagents have in common is that they all affect proteins. Trypsin (Seabright, 1971) digests regions of chromosomal proteins which are not bound to the DNA (Simpson, 1972); urea (Shiraishi and Yosida, 1972) disrupts the H-bonds which maintain $\alpha$ helices in the histones and bind histones to each other (Mahler and Cordes, 1966); high salt concentrations (Comings et al., 1973) induce structural and interchain interactions in apolar sequences of histones (Bradbury et al., 1975); oxidation of disulfide bridges disrupts intra- and inter-protein connectives (Utakoji, 1978); alkali (Schnedl, 1971) could strip the acidic proteins from the DNA, and, by denaturing the DNA, disrupt DNA - protein linkages (Comings et al., 1973); actinomycin D competes with specific proteins for GC binding sites on the DNA (Shafer, 1973) and once bound alters the DNA configuration thereby influencing protein - DNA interactions (Müller and Crothers, 1968); $Ca^{++}$ and $Mg^{++}$ free saline (Dev et al., 1972) decondenses chromatin by altering the charge density along the DNA phosphate backbone and destabilizing DNA - protein bonds (Ris, 1975; Mahler and Cordes, 1966).

Altering the chromosomal proteins emphasizes the differences in condensation between euchromatin and heterochromatin. We can look at the metaphase chromosome as having been formed by at least one of two major levels of coiling, heterochromatic and mitotic; euchromatin has only the mitotic condensation, whereas heterochromatin might have only heterochrom-

atic coiling or both heterochromatic and mitotic coiling. Loosening of the condensation of only one level would produce a banding pattern after the chromosomes were stained in Giemsa.

When cold treated Trillium grandiflorum (Schweizer, 1973) and Trillium kamtschaticum (Takehisa and Utsumi, 1973b) chromosomes are pretreated and then stained with Giemsa, the less contracted -H segments still take up the stain more intensely than the euchromatic regions. If the cold treatment prevents heterochromatic regions from undergoing mitotic condensation without affecting the heterochromatic coiling of the DNP fibre, then pretreating the chromosomes with reagents which loosen only mitotic coiling will result in the positive Giemsa staining of heterochromatically-coiled chromosomal regions.

As the preceding implies, the fibres of Giemsa-positive regions are more intensely stained than the fibres of the interband regions (Ruzicka and Schwarzacher, 1974). The intense staining of banded regions is due to a higher concentration of dye in the fibres of the bands rather than a higher concentration of fibres in the bands.

The preferential loosening of one level of the DNP fibre's contraction probably depends on the relative vulnerability of the proteins maintaining the contraction, and this vulnerability could be due to structural or chemical factors. A higher order of condensation might afford the proteins associated with it protection from enzymatic attack. Fibres from inactive chromatin are 200 Å whereas active chromatin fibres measure 100 Å at interphase (Ris, 1975). The larger fibre size is due to a higher degree of compaction (DuPraw, 1970; Ris, 1975). Both fibres are subjected to the coiling of metaphase; for the 200 Å fibre this is a third

level of compaction, for the 100 Å fibre it is only a second level. Being less compacted could make the proteins of the euchromatic region, in general, more vulnerable to disruption by pretreatment, thereby loosening the condensation.

Trypsin, α chymotrypsin, pronase, protease (Dutrillaux, 1973), and collagenase (Trusler, 1975) have all been used as pretreatments to induce Giemsa bands and the patterns they reveal are consistent with each other, implying that substrate specificity is not a factor in enzymatic band production.

The F1 histone fraction is implicated in mitotic coiling and is also the first fraction to be digested by proteolytic enzymes (Ockey, 1973). These enzymes seem to loosen mitotic coiling by preferentially digesting F1 histone. If chromosomes are left in trypsin too long, the heterochromatic regions also lose their stainability (Comings et al., 1973; Burkholder, 1974), i.e., their condensation. It is likely then that the F1 histone is removed first because it is initially more accessible to attack than the proteins responsible for heterochromatic condensation.

Giemsa bands are also produced in human chromosomes with pretreatments which oxidize disulfide bridges (Utakoji, 1973) thereby loosening the DNP fibre's structure preferentially in the interband regions. Sumner (1974) found that the Giemsa bands were rich in disulfide bridges whereas interband regions were rich in sulphydryl groups. These findings infer that the disulfide bridges within the band regions are less vulnerable to attack by pretreatment agents.

## Mammalian Chromosome Banding

In mammals there appears to be two distinct levels of heterochromatin revealed by Giemsa banding techniques. These are the G- and R-bands which reveal intercalary heterochromatin and the C-bands which reveal centromeric heterochromatin (Comings, 1973).

### G- and R-Bands

Mild pretreatments or exposure to 90°C phosphate buffer prior to staining differentiate the intercalary heterochromatin. Structurally, these bands behave similarly to chromomeres in that generally a broad band in a metaphase chromosome is seen to consist of two to three narrower bands at prophase. This behavior is seen in human (Chen and Shaw, 1972), Muntjac (Patterson and Petricciani, 1973), and Rattus norvegicus (Unakul and Hsu, 1972) chromosomes. Also comparisons between the size of the G-bands and their distance from each other at metaphase in Chinese hamster (Kakati and Sinha, 1972), several species of Peromyscus (Pathak et al., 1973a), human (Pearson, 1972), Muntjac (Brown and Cohen, 1973) and Rattus norvegicus (Unakul and Hsu, 1972) chromosomes reveal an at least superficial uniformity. Taking their measurements from electron photomicrographs, Bahr et al. (1973) estimated that there are 2 major chromomeres to 1.07 G-bands and suggested that the G-bands are due to the "effects of various methods influencing and rearranging (the) basic organization" of the chromomeres. This would explain the strong correspondence of the G-bands and the chromomere pattern of pachytene bivalents found by Okada and Comings (1974) in the Chinese hamster.

Considerable evidence has accrued which implies that the G-bands

contain AT-rich DNA (Comings, 1973; Schreck et al., 1973; Miller et al., 1973; Pathak et al., 1973b; Shafer, 1973). G-bands are virtually equivalent to the fluorescent bands produced by quinacrine (Pearson, 1972). It is probable that the fluorescence of the Q-bands is due to interactions between AT-richness, a specific sequence and degree of base repetition, and the structure of the DNA helix (Weisblum and DeHaseth, 1972; Selander and de la Chapelle, 1973). Lysine-rich histones might be preferentially associated with G-bands by virtue of their association with AT-rich DNA (Combard and Vendrely, 1970).

C-Bands

C-bands are differentiated if the chromosomes are exposed for longer periods of time to the G-band inducing pretreatment reagents. While G-band treatments extract very little DNA or protein (not more than 9% of the former and 13% of the latter), C-band techniques often extract up to 58% of the DNA and 28% of the protein (Comings et al., 1973); the DNA is preferentially removed from non C-band areas (Alfi et al., 1973). The production of C-bands does not depend on DNA extraction since prolonged exposure to 0.0005% trypsin produces C-bands without extracting any appreciable amounts of DNA (Comings et al., 1973). However, another level of condensation is being affected by the C-band techniques; they often disrupt the interband DNP fibre at the level of the secondary structure of DNA while the G-band and R-band techniques decondense the coil at levels higher than this, preserving the DNA double helix. Perhaps the invulnerability of the C-bands is due more to the chemistry of their DNA and proteins than to the structural protection of these proteins.

The C-band heterochromatin seems to be even more condensed than the G-bands. The C-band located at the secondary constriction in the long arm of chromosome 9 of man does not stain positively after G-band techniques (Paris Conference, 1971). It is possible that this region is so contracted that the dye molecules cannot effectively penetrate it even after G-banding. Optimal magenta compound formation would depend on the relaxation of this region provided by the C-band pre-treatments.

Highly repetitious DNA has been localized in the C-bands of human (Sanchez and Yunis, 1974), Microtus agrestis (Arrighi et al., 1970), and Seba's fruit bat (Pathak et al., 1973b) chromosomes. The fraction of nonhistone protein associated with highly repetitious DNA (Allfrey et al., 1974; Bekhor et al., 1974) might be responsible for the coiling which gives the C-bands their relative invulnerability.

## Plant Chromosome Banding

Bands have been produced in plant chromosomes by some of the same techniques that have produced bands in animal chromosomes. By far the most frequently used technique for plant chromosome banding is the one based on the Barium-Saline-Giemsa (BSG) technique (barium hydroxide, 2XSSC incubation) used by Vosa and Marchi (1972). It is a modification of Sumner et al.'s (1971) C-banding technique for mammalian chromosomes and has been used successfully on rye (Sarma and Natarajan, 1973; Gill and Kimber, 1974; Hadlaczky and Koczka, 1974; Verma and Rees, 1974), Allium cepa, Ornithogalum virens (Stack et al., 1974), Scilla sibirica (Vosa, 1973a); six species of Anemone, Hepatica nobilis (Marks and Schweizer, 1974), and three Tulipa cultivars (Filion, 1974). A similar

technique using NaOH instead of a saturated solution of BaOH was used on Trillium kamtschaticum (Takehisa and Utsumi, 1973b), Rhoeo discolor (Natarajan and Natarajan, 1972), and Vicia faba (Takehisa and Utsumi, 1973a) chromosomes.

Most of the techniques used on plant chromosomes can be categorized as C-banding techniques although Döbel et al. (1973) used a G-banding technique to produce bands in Vicia faba and Schweizer's (1972) technique would best be qualified as "Intermediate"; he used overnight incubation at 60°C in 2XSSC, while C-bands were produced in mouse chromosomes with overnight incubation at 60°C in 6XSSC (Comings et al., 1973), and G-bands were produced in mammalian chromosomes by incubation for one hour at 60°C in 2XSSC (Sumner et al., 1971). Stack and Clarke (1973b) and Stack et al. (1974) used an R-banding technique to reveal pericentromeric heterochromatin in Ornithogalum virens, Plantago ovata and Allium cepa.

Whichever technique has been used, nothing really equivalent to G-bands has been observed in plant chromosomes although plant pachytene chromosomes have chromomeres. Possibly, as Natarajan and Natarajan (1972) have suggested, the rather extreme squash techniques necessitated in cytological preparations of plant cells, as a result of the presence of cell walls, destroy the more sensitive G-bands.

There is no consistent correlation between the Giemsa-induced bands and those produced by quinacrine in plant chromosomes. Vosa and Marchi (1972) found three types of heterochromatin differentiated by quinacrine and Giemsa staining: Giemsa positive, quinacrine reduced (Tulbaghia leucantha); Giemsa positive, quinacrine enhanced (Allium carinatum);

Giemsa positive, undifferentiated by quinacrine (Zea mays). The quenching of quinacrine fluorescence has been accredited to higher GC contents (Weisblum and DeHaseth, 1972) so that possibly in Tulbaghia leucantha the heterochromatin is GC-rich. The heterochromatin of Allium carinatum might then be relatively AT-rich, while in Zea mays the heterochromatin might have an AT/GC ratio identical to that of euchromatin. Cionini (1973) has shown in Vicia faba that the pericentromeric region of the M chromosome is AT-rich; there are three bands in this region which are Giemsa positive after certain pretreatments (Takehisa and Utsumi, 1973a) and quinacrine enhanced (Caspersson et al., 1969).

Extraction of all the histones using polystyrene sulfonate prior to quinacrine staining prevents the enhanced fluorescence of heterochromatin in human chromosomes (Kitchin, 1973). Overnight exposure of human chromosomes to 5 N HCl at 4°C extracts histones and also causes a loss of Giemsa-banding potential (Bobrow, 1974). Histones would seem to have an important role in band production in mammalian chromosomes. They may be more directly and specifically involved in the banding of plant chromosomes. In both Secale cereale and Vicia faba there is a category of HCl sensitive pericentromeric heterochromatin (see Tables I and II). The condensation of this region may be dependent on histones. In rye most of the interstitial heterochromatin is also HCl sensitive, whereas the knob heterochromatin and Nucleolar Organizing Region (NOR) chromatin are not. In Vicia faba the NOR depends on hot HCl treatment to be consistently differentiated. This might be another case of overly contracted chromatin where the removal of the histone fraction decondenses the chromatin enough to allow intense staining to occur.

TABLE I: The effect of hot HCl maceration on the presence of Giemsa-positive bands in *Secale cereale* after Barium-Saline-Giemsa technique

| Author | Hot HCl Maceration | BANDS | | | |
|---|---|---|---|---|---|
| | | Pericentromeric | Interstitial | Telomeric | NOR[1] |
| Sarma and Natarajan (1973); Verma and Rees (1973) | + | - | ± | + | + |
| Gill and Kimber (1974); Hadlaczdy and Koczka (1974) | - | + | + | + | + |

[1]Nucleolar Organizing Region

TABLE II: The effect of hot HCl on the presence of Giemsa-positive bands in *Vicia faba*

| Author | | Hot HCl | BANDS | | |
|---|---|---|---|---|---|
| | | | Pericentromeric | -H | NOR[1] |
| Takehisa and Utsumi (1973a) | a) NaOH<br>b) 6XSSC | - | + | + | ± |
| Döbel *et al.* (1973) | a) Urea<br>b) Sörenson's buffer | + | - | + | + |
| Matsui (1974) | a) 5% TCA, 90°C | + | - | - | + |

[1]Nucleolar Organizing Region

The hot HCl sensitivity of the pericentromeric heterochromatin from two quite divergent species might indicate that hot HCl sensitivity is a universal feature of plant pericentromeric heterochromatin.

# MATERIALS AND METHODS

## Hordeum vulgare

Seeds of barley (Hordeum vulgare L., cultivar Montcalm, obtained from the Department of Agronomy, Macdonald College) were placed on wetted filter paper in Petri dishes and allowed to germinate in the dark at room temperature (ca. 20°C). To aid in obtaining a sufficient number of condensed chromosomes a solution of 0.05% colchicine or 0.002 M 8-hydroxquinoline was added to the germinating seeds between two and four hours prior to harvesting the root tips. The root tips were fixed in Carnoy's solution (6 parts absolute ethanol: 3 parts chloroform: 1 part glacial acetic acid), or ethanol-acetic acid (3 parts 95% ethanol: 1 part glacial acetic acid), overnight. They were washed with distilled water and then macerated by one of the four techniques outlined in Table III. The root tips were stored in 70% ethanol in the refrigerator until used.

The meristematic region of the root tip was excised, placed in a drop of 45% acetic acid on a subbed slide and chopped up. A coverslip was placed on the preparation and the slide was gently heated over an alcohol lamp prior to squashing. The preparation was then thoroughly examined microscopically, and the co-ordinants of any appropriate metaphase spreads were recorded. The coverslips of well-prepared slides were removed by the quick freeze method of Conger and Fairchild (1953) with the following alterations: Cryokwik was sprayed on the coverslip before its removal, and after the alcohol washes the slides were allowed to air dry.

Five methods were used to induce banding:

1. Incubation in Sörenson's buffer prior to staining in Giemsa

TABLE III: Maceration techniques used on *Hordeum vulgare* and *Vicia faba*

| | |
|---|---|
| Acid | 6 hours (*Vicia faba*), 30 minutes (barley), 90% acetic acid, room temperature. |
| | Wash in distilled water. |
| Enzyme | 3 hours (*Vicia faba*), 1 hour (barley), 5% pectinase, 30°C. |
| | Wash in distilled water. |
| Acid-Enzyme I | 1 minute, 1 N HCl, room temperature. |
| | 12 minutes, 1 N HCl, 40°C. |
| | 1 minute, cold 1N HCl. |
| | Wash in distilled water. |
| | 60 minutes, 5% pectinase, 40°C. |
| | Wash in distilled water. |
| Acid-Enzyme II | 30-40 minutes, 70% acetic acid, room temperature. |
| | Wash in distilled water. |
| | 25-35 minutes, 5% pectinase, 35°C. |
| | 15-20 minutes, 5% cellulase, 35°C. |
| | Wash in distilled water. |

(Kato and Moriwaki, 1972).

2. Incubation in 2XSSC prior to staining in Giemsa (Schweizer, 1973).

3. Trypsinization prior to staining in Giemsa. (Wang et al., 1972).

4. Immersion in $KH_2PO_4$ followed by flooding with a mixture of Giemsa and trypsin in $KH_2PO_4$ (Sun et al., 1973).

5. Exposure to a saturated solution of BaOH followed by incubation in 2XSSC prior to staining in Giemsa (Sarma and Natarajan, 1973). The details of the pretreatments and staining procedures used are listed in Tables IV-VIII.

## Vicia faba

Seeds of Vicia faba, cultivar Broad Windsor Long Pod, obtained from Stokes Seeds and treated by this company with fungicide Arasan, were planted in soil and grown in the greenhouse. Root tips were taken from six-week-old plants and fixed immediately in ethanol-acetic acid or exposed to a 0.05% colchicine solution for two hours prior to fixation. After 18 to 24 hours in fixative, the root tips were washed in distilled water and macerated in either 70% acetic acid, or 5% pectinase (Table III). They were stored in 70% ethanol in a refrigerator until used.

The procedure for squashing the root tips and removing the coverslips was identical to that used on barley.

All V. faba preparations were stained according to the trypsin-Giemsa techniques detailed in Table IX.

TABLE IV: Banding techniques: *Hordeum vulgare* pretreatment with Sörenson's buffer[1]

| Expt. no. | Maceration | pH | STAINING | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Staining solution | (ml) | Concentration (%) | pH | Time (min) | Temperature (°C) |
| 1 | Acid-Enz. I | 6.8 | Sörenson's[2] | 20 | | 6.8 | 60 | 20 |
| | | | $H_2O$ | 30 | | | | |
| | | | Giemsa | 1 | 2 | | | |
| 2 | Acid-Enz. I | 6.95 | Sörenson's | 20 | | 6.95 | 30 | 37 |
| | | | $H_2O$ | 30 | | | | |
| | | | Giemsa | 1 | 2 | | | |
| 3 | Acid-Enz. I | 6.8 | Sörenson's | 40 | | 6.8 | 45 | 20 |
| | | | Giemsa | 1 | 2.5 | | | |

[1]Pretreatment time was 30 minutes, and pretreatment temperature was 37°C for all experiments.

[2]This staining solution was taken from Frey *et al.* (1972).

TABLE V: Banding techniques:
*Hordeum vulgare*
pretreatment with 2XSSC

| Expt. no. | Maceration | PRETREATMENT | | | STAINING | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | pH | Time (min) | Temperature (°C) | Staining solution | (ml) | Concentration (%) | pH | Time (min) | Temperature (°C) |
| 1 | Acid-Enz. I | 7.0 | 60 | 60 | Sörenson's[1] | 20 | | | 45 | 20 |
| | | | | | $H_2O$ | 30 | | | | |
| | | | | | Giemsa | 1 | 2 | 6.95 | | |

[1]This staining solution was taken from Frey *et al.* (1972).

TABLE VI: Banding techniques:
*Hordeum vulgare*
pretreatment with trypsin[1]

| Expt. no. | Maceration | PRETREATMENT | | | STAINING | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Reagent | pH | Time (min) | Staining solution | (ml) | Concentration (%) | pH | Time (min) | Temperature (°C) |
| 1 | Acid | Saline 85% 40 ml[2]<br>EDTA-2Na 0.08g<br>Trypsin, 5% 2 ml | 7.2 | 3 | Phosphate buffer[3]<br>Giemsa | 40<br>10 | 25 | 7.0 | 5 | 20 |
| 2 | Acid | Saline 85% 40 ml[2]<br>EDTA-2Na 0.08g<br>Trypsin, 5% 2 ml | 7.2 | 2 | Sörenson's[4]<br>$H_2O$<br>Giemsa | 20<br>30<br>1 | 2 | 6.8 | 60 | 20 |
| 3 | Acid-Enz. II | Sörenson's 40 ml<br>Trypsin, 5% 2 ml | 6.8 | 3 | Sörenson's<br>Giemsa | 40<br>10 | 25 | 6.8 | 5 | 20 |

[1]All pretreatment solutions had a final trypsin concentration of 0.25%; all preparations were incubated in the pretreatment solutions at 34°C.

[2]This pretreatment solution is a modification of Wang *et al.* (1972).

[3]This staining solution is taken from Ray & Hamerton (1973).

[4]This staining solution is taken from Frey *et al.* (1972).

TABLE VII: Banding techniques:
*Hordeum vulgare*
Trypsin-Giemsa[1]

| Expt. no. | Maceration | STAINING[2] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Staining solution | (ml) | Concentration (%) | pH | Time (min) | Temperature (°C) |
| 1 | Acid | $KH_2PO_4$, 0.025 M | 36.50 | | 6.8 | 10 | 20 |
| | | Methanol | 12.50 | | | | |
| | | Giemsa | 1.00 | 2 | | | |
| | | Trypsin, 0.1% | 0.25 | 0.0005 | | | |
| 2 | Acid | $KH_2PO_4$, 0.025 M | 36.50 | | 6.8 | 15 | 20 |
| | | Methanol | 12.50 | | | | |
| | | Giemsa | 1.00 | 2 | | | |
| | | Trypsin, 0.1% | 0.25 | 0.0005 | | | |
| 3 | Enzyme | $KH_2PO_4$, 0.025 M | 36.50 | | 6.8 | 15 | 20 |
| | | Methanol | 12.50 | | | | |
| | | Giemsa | 1.00 | 2 | | | |
| | | Trypsin, 0.1% | 0.25 | 0.0005 | | | |
| 4 | Enzyme | $KH_2PO_4$, 0.025 M | 26.50 | | 6.8 | 10 | 20 |
| | | Methanol | 12.50 | | | | |
| | | Giemsa | 1.00 | 2 | | | |
| | | Trypsin, 0.1% | 10.00 | 0.02 | | | |

[1]These pretreatment and staining techniques are based on the technique used by Sun *et al.* (1973).

[2]Before staining, all preparations were pretreated by incubating them in 0.025 M $KH_2PO_4$, pH 6.8, for 10 minutes, at 56°C.

TABLE VIII: Banding techniques:
*Hordeum vulgare*
Barium-Saline-Giemsa (BSG)[1]

| | PRETREATMENT | | | | STAINING | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Maceration | Reagent | pH | Time (min) | Temperature (°C) | Staining solution | (ml) | Concentration (%) | pH | Time (min) | Temperature (°C) |
| Acid-Enz. II | a) BaOH, sat. | - | 5 | 20 | Sörenson's | 40 | | 6.8 | 20 | 20 |
| | b) 2XSSC | - | 120 | 66 | Giemsa | 2 | 5.0 | | | |

[1]Technique of Sarma and Natarajan (1973).

TABLE IX: Banding technique: *Vicia faba* trypsin-Giemsa[1]

| Expt. no. | Maceration | STAINING[2] Staining solution | (ml) | Concentration (%) |
|---|---|---|---|---|
| 1 | Acid | $KH_2PO_4$, 0.025 M | 36.50 | |
| | | Methanol | 12.50 | |
| | | Giemsa | 1.00 | 2 |
| | | Trypsin, 0.1% | 0.25 | 0.0005 |
| 2 | Enzyme | $KH_2PO_4$, 0.025 M | 36.50 | |
| | | Methanol | 12.50 | |
| | | Giemsa | 1.00 | 2 |
| | | Trypsin, 0.1% | 0.25 | 0.0005 |
| 3 | Enzyme | $KH_2PO_4$, 0.025 M | 34.00 | |
| | | Methanol | 12.50 | |
| | | Giemsa | 1.00 | 2 |
| | | Trypsin, 0.1% | 2.50 | 0.005 |

[1]This pretreatment and staining technique is based on the technique used by Sun *et al.* (1973).

[2]All preparations were pretreated by incubating them in 0.025 M $KH_2PO_4$, pH 6.8, for 10 minutes at 56°C, and then were exposed to staining solutions adjusted to pH 6.8 for 10 minutes, at about 20°C.

## Lotus pedunculatus

Seeds from Lotus pedunculatus and the backcross between L. pedunculatus (female) and the primary trisomic (male) for chromosome number 5 (Chen and Grant, 1968) were germinated in Petri dishes and the seedlings planted in soil. Since a 16-hour day length causes flower formation which lowers the mitotic activity of the root tip meristems, the plants were kept in a growth chamber during the summer in which a 12-hour day length was maintained. In the fall the plants were transferred to a greenhouse.

After brief experimentation with various times of exposure to 0.002 M 8-hydroxyquinoline and 0.05% colchicine, a prefixation treatment using 0.05% colchicine for one hour was judged to give the greatest number of metaphase cells with optimum chromosome condensation and was used in all further experiments. After fixation in freshly prepared ethanol-acetic acid for 18-24 hours the root tips were washed in distilled water and any remaining soil was removed with dissection needles.

A modification of the maceration technique used by Gill and Kimber (1974) gave good cellular separations and virtually cytoplasm-free chromosome spreads: The root tips were left for 1 - 2 hours at room temperature in a solution of 5% pectinase and 1.25% cellulase to which three drops of 1 N HCl per 5 ml had been added. The root tips were then washed in distilled water and stored in 70% ethanol in the refrigerator until squashed. The squash technique used for L. pedunculatus was the same as that used for barley and Vicia faba.

To preserve the squashes from deterioration due to humidity, the slides were stored in an oven kept at 60°C until stained. Table X details the four Giemsa staining techniques tried on L. pedunculatus; three were

based on techniques successfully applied to plant chromosomes by Schweizer (1973), Döbel et al., (1973), and Verma and Rees (1974); one was a modiffcation of a technique used by Sun et al. (1973) on human chromosomes.

## General

Rehydrated Bacto-Trypsin from Difco Labs was used in concentrations ranging from 0.005% to 0.25%. All Giemsa solutions were made with G.T. Gurr's Improved R66 Giemsa Stain. Sörenson's buffer and 2XSSC were made according to the formulae of Frey et al. (1972) and Craig-Holmes and Shaw (1971), respectively.

After staining, all slides were washed in either distilled water alone or in a methanol rinse followed by distilled water and allowed to air dry. They were mounted in Euparal or immersion oil (Wang et al., 1972). All photomicrographs were taken with a Zeiss photomicroscope using either Kodak Photomicrography Monichrome Film SO-410 or Kodak Plus-X Pan Film.

TABLE X: Banding techniques:
*Lotus pedunculatus*

| Expt. no. | PRETREATMENT | | | | | STAINING[1] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Reagent | Concentration | pH | Time | Temperature (°C) | Staining solution | (ml) | Concentration (%) | pH | Time | References |
| 1 | 2XSSC | - | 7.0 | 24 h | 65 | Sörenson's | 50 | | 6.9 | 24 h | Schweizer (1973) |
| | | | | | | Giemsa | 1 | 2 | | | |
| 2 | a) Urea | 6 M | - | 30 min | 20 | Sörenson's | 50 | | 6.8 | 12 min | Döbel *et al.* (1973) |
| | b) Sörenson's | - | 7.2 | 5 min | 20 | Giemsa | 1 | 2 | | | |
| 3 | a) BaOH | Sat. | - | 5 min | 20 | Sörenson's | 50 | | 6.8 | 10 min | Verma and Rees (1974) |
| | b) 2XSSC | - | - | 1 h | 60 | Giemsa | 1 | 2 | | | |
| 4 | $KH_2PO_4$ | 0.025 M | 6.8 | 10 min | 56 | $KH_2PO_4$, 0.025 M | 34 | | 6.8 | 10 min | Sun *et al.* (1973) |
| | | | | | | Methanol | 12.5 | | | | |
| | | | | | | Giemsa | 1 | 2 | | | |
| | | | | | | Trypsin, 0.1% | 2.5 | 0.005 | | | |

[1]All staining was done at room termperature, ca. 20°C.

# RESULTS

## Effects of Maceration Procedure

When all other steps in the pretreatment and staining procedures were equalized, acid macerated preparations gave clearer chromosomal banding than did enzyme macerated material in both barley and Vicia faba root tips (Figures 2-5).

## Effects of the Degree of Condensation on Trypsinization

Chromosomes, at different stages of condensation, exposed under the same conditions to 0.25% trypsin for three minutes, are pictured in Figures 6-8. The prophase chromosomes were diffuse and their stainability was very reduced (Figure 6). The area around the centromeres and a few spots in the arms retained, relatively undiminished, their ability to take up the Giemsa stain. In the normal metaphase chromosomes (Figure 7), the chromatids had fused as had chromosomes touching each other. The general chromosomal structure was maintained; the satellites were clearly visible as was the outline of solitary chromosomes. C-metaphase chromosomes (Figure 8) retained even more of their structure; the chromatids of several chromosomes had not fused, and, although there were clusters of chromosomes, there was no fusion, and each chromosome was easily distinguishable.

## Effects of the Different Techniques Used on Vicia faba

Although the combination of Sun et al.'s (1973) technique with an acid maceration showed the most promising bands in Vicia faba chromosomes

Figures 2 to 5. Effects of the maceration procedure on trypsin-Giemsa-banding in Hordeum vulgare and Vicia faba chromosomes.

Figure 2. H. vulgare, acid maceration (Table VII, Expt. 2), X 1035

Figure 3. H. vulgare, enzyme maceration (Table VII, Expt. 3), X 1035

Figure 4. V. faba, acid maceration (Table IX, Expt. 1), X 790

Figure 5. V. faba, enzyme maceration (Table IX, Expt. 2), X 790

Figure 2

Figure 3

Figure 4

Figure 5

Figures 6 to 8. The effect of tryptic digestion on Hordeum vulgare chromosomes at three different stages of condensation, X 815.

All preparations were exposed to 0.25% trypsin for 3 minutes prior to staining in Giemsa.

Arrows indicate interstitial (large arrows) and pericentromeric (small arrows) Giemsa-positive heterochromatin.

Figure 6. Prophase

Figure 7. Metaphase

Figure 8. C-metaphase

Figure 6

Figure 7

Figure 8

(Figure 9), the best analyzable metaphase spread was the result of an enzyme maceration and Sun et al.'s (1973) technique (Figure 10). The bands appeared as bulges in the contour of the chromosomes as well as darkly staining regions. This is characteristic of Sun et al.'s (1973) technique, used originally on human chromosomes. With an acid maceration, the bulges disappeared and the bands became darker. Raising the concentration of trypsin in the enzyme macerated material from 0.0005% to 0.005% swelled the chromosomes; the bulges were no longer obvious and some of the bands became pale or disappeared altogether (Figure 11).

Karyograms of the two types of banding observed in V. faba in this study are compared with an idiogram of Döbel et al.'s (1973) banded V.faba chromosomes (Figure 12). The two major differences were in the reactions of the Nucleolus Organizer Region (NOR) on the M chromosome and the pericentromeric regions on the long arms of the S chromosomes and the M chromosomes. Döbel et al.'s (1973) technique banded the NORs and not the pericentromeric regions, while the trypsin-Giemsa technique (Table IX, Expt. 2) banded the pericentromeric regions and not the NORs. The pericentromeric regions in some of the chromosomes lost their bands with the higher trypain concentration used in Expt. 3 (Table IX).

Another difference was that negative bands appeared in the trypsin-Giemsa preparations. There were four categories of negative bands observed:

1. Substitutions. Some of the interstitial bands in Döbel et al.'s (1973) chromosomes showed up as negative bands in the trypsin-Giemsa techniques, notably the band in $S_3$ and $S_5$.

Figures 9 to 11. The effect of different maceration procedures and trypsin concentrations on the Giemsa-banding of Vicia faba chromosomes, X 790.

Figure 9. Acid maceration, 0.0005% trypsin (Table IX, Expt. 1)

Figure 10. Enzyme maceration, 0.0005% trypsin (Table IX, Expt. 2)

Figure 11. Enzyme maceration, 0.005% trypsin (Table IX, Expt. 3)

Figure 9

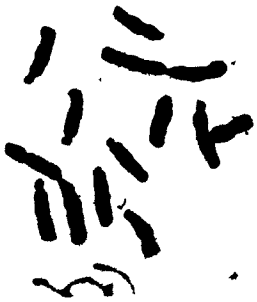


Figure 10

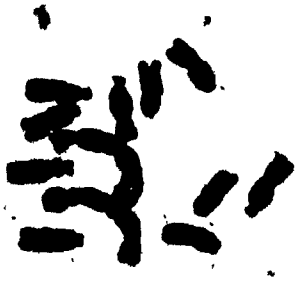


Figure 11

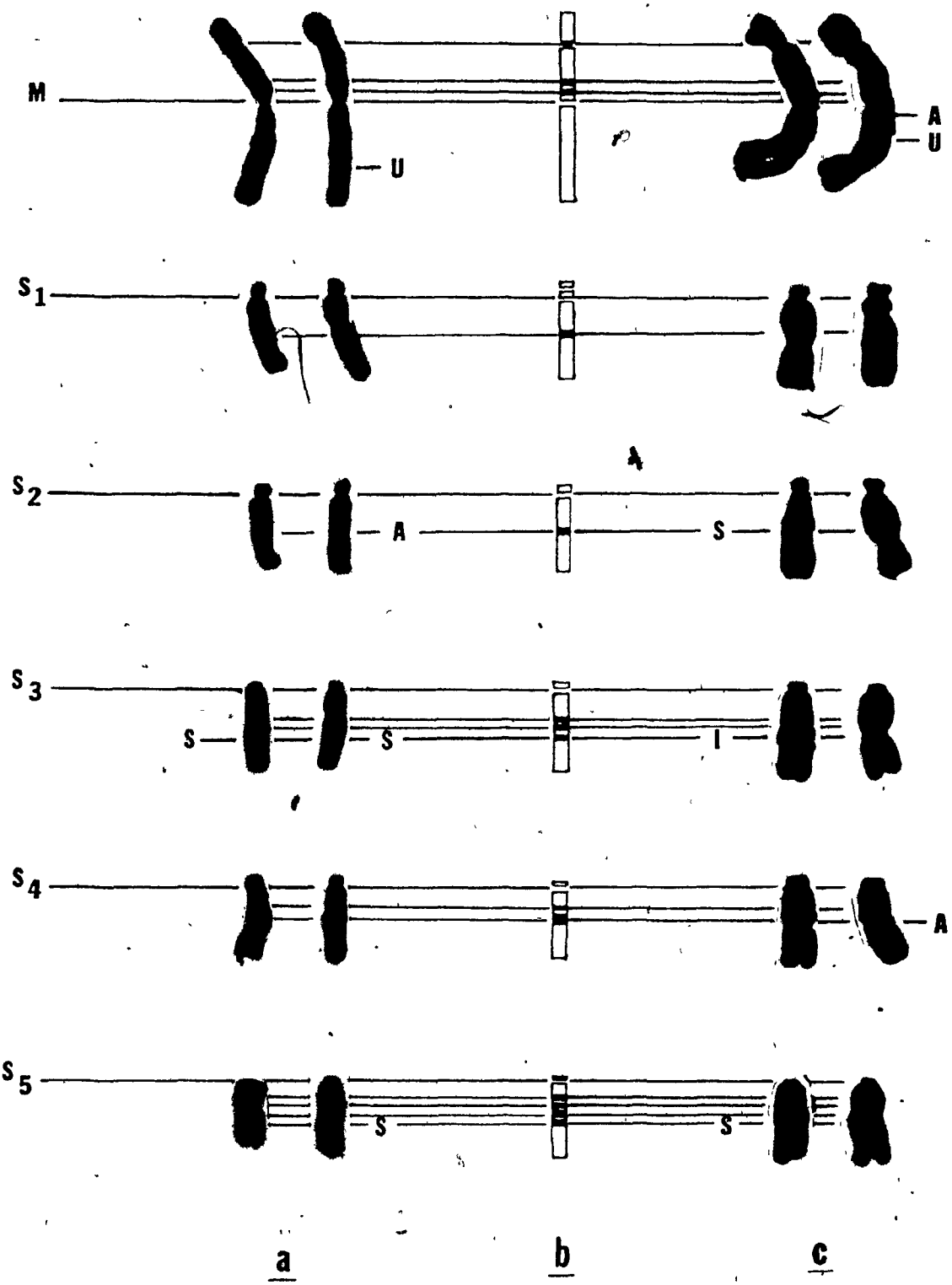
Figure 12. Comparison of the bands induced in Vicia faba chromosomes by three Giemsa-banding techniques, a and c (author's study), and b (Döbel et al., 1973), X 1400.

S - "Substitutions," I - "Interchangeables," A - "Adjacents," U - "Uniques." See the text for an explanation of these categories.

a. Enzyme maceration, trypsin-Giemsa technique (Sun et al, 1973); 0.0005% trypsin (this study: Table IX, Expt. 2).

b. Hot HCl maceration, incubation in 6 M urea, 30 minutes, followed by incubation in Sörenson's buffer, pH 7.2 for 5 minutes, followed by staining in Giemsa. Idiogram taken from Döbel et al. (1973)

c. Enzyme maceration, trypsin-Giemsa technique (Sun et al., 1973); 0.005% trypsin (this study: Table IX, Expt. 3)


M
U
A
U
S1
S2
A
A
S
S3
S
S
I
S4
A
S5
S
S
a
b
c

2. Interchangeables. Negative bands and dark bands appeared to be interchangeable in some instances; on the $S_3$ chromosome in the trypsin-Giemsa preparation (Figure 12, c) one chromatid had a negative band where the other had a positive band.

3. Adjacents. Negative bands often appeared adjacent to darker bands as in $S_2$, $S_4$, and the long arm of M.

4. Uniques. These were negative bands in the long arm of the M chromosome that did not correlate with any bands produced by Döbel et al.'s (1973) technique.

Effects of the Different Techniques Used on Lotus pedunculatus

Of the four banding techniques applied to Lotus pedunculatus chromosomes (Table X), the only one which produced clear bands in metaphase chromosomes was Schweizer's (1973) (Figure 13). The other three techniques produced uniformly stained metaphase chromosomes (Figures 14-16). The chromosomes in the banded spread were more contracted than those in the other three preparations.

In the preparation stained according to Schweizer (1973), the distribution of bands and the morphology of the chromosomes allowed an exact visual pairing of the chromosomes without the necessity of making detailed measurements (Figure 17). One of the two smallest pairs stained intensely along its entire length. At prophase this pair was already fully condensed whereas the other chromosomes were not (Figure 18).

One of the larger prophase chromosomes, 3 (Figure 18), showed large areas of pericentromeric heterochromatin and smaller areas of telomeric heterochromatin; a pattern which was repeated in L. pedunculatus chromosome 3

Figures 13 to 16. Effects of different techniques on Giemsa-banding in Lotus pedunculatus chromosomes, X 840.

Figure 13. Table X, Expt. 1 (Schweizer, 1973)

Figure 14. Table X, Expt. 4 (Sun et al., 1973)

Figure 15. Table X, Expt. 3 (Verma and Rees, 1974)

Figure 16. Table X, Expt. 2 (Döbel et al., 1973)

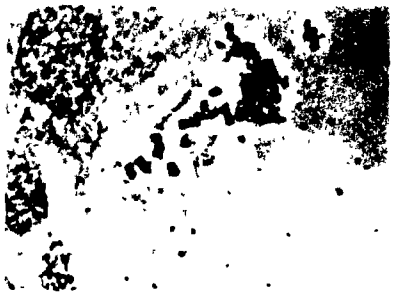


Figure 13

Figure 14

Figure 15

Figure 16

Figure 17. Lotus pedunculatus metaphase chromosomes; Table X, Expt. 1 (Schweizer, 1973), X 1792.
Numbers indicate chromosome pairs.
Arrow is pointing at a faint band in the short arm of the larger satellite chromosome.

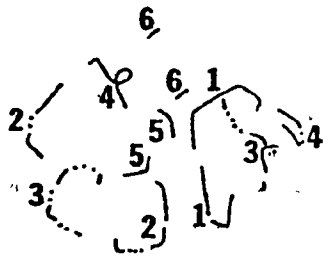
Figure 18a. L. pedunculatus prophase chromosomes; Table X, Expt. 2 (Dobel et al., 1973), X 840.
Arrows indicate interchromosomal connectives.

Figure 18b. A line interpretation of the chromosomes in Figure 18a.
Numbers indicate chromosome pairs.

Figure 19 to 20. L. pedunculatus trisomics (Chen and Grant, 1968); unpretreated and unstained, phase contrast, X 840.
Arrow in Figure 19 indicates chromosome 2 which has a block of heterochromatin in the short arm.

Figure 17

a




b

Figure 18

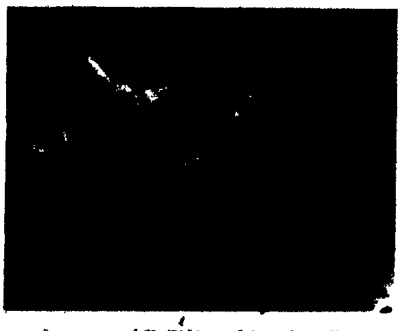


Figure 19

Figure 20

stained according to Schweizer's (1973) technique (Figure 17).

## Effects of the Different Techniques Used on *Hordeum Vulgare*

Two levels of comparison were used to analyse the barley results: Within Treatments and Between Treatments.

### Within Treatments

Pretreatment with Sörenson' s Buffer: Table IV

The major variable here was the time of immersion in the Giemsa stain. The staining time which produced optimum chromosome banding was 30 minutes (Figure 21). Although bands were discernible in metaphase chromosomes stained for 45 (Figure 22), and 60 minutes (Figure 23), there was little distinction in the intensity of the uptake of the stain between banded and nonbanded areas. Prophase chromosomes stained for longer than the optimum time and the optimum time gave no evidence of banding (Figures 24 and 25, respectively). The prophase chromosome structure in these preparations was distinct and clearly showed that proximal areas were more condensed than the distal ones.

Pretreatment with Trypsin: Table VI

Comparison of chromosome banding techniques resulting from Expts. 1 and 3 showed some significant differences in quality and degree. In Expt. 1, the chromatids fused, the bands stained darkly, and the interbands remained unstained (Figure 26). In Expt. 3, the chromatids did not fuse and although banding was present there was less difference in the intensity of stain between the bands and interbands (Figure 27). Both preparations were exposed to 0.25% trypsin for three minutes and then stained for five minutes in 25% Giemsa.

Figures 21 to 25. Effects of Sörenson's buffer on banding in Hordeum vulgare chromosomes; all preparations pretreated in Sörenson's for 30 minutes.

Figures 21 and 25. Metaphase and prophase chromosomes, respectively; 30 minutes in Giemsa (Table IV, Expt. 2); arrows indicate satellite chromosomes, X 1035

Figures 22 and 24. Metaphase, X 1035, and prophase, X 815, chromosomes, respectively; 45 minutes in Giemsa (Table IV, Expt. 3)

Figure 23. Metaphase chromosomes; 60 minutes in Giemsa (Table IV, Expt. 1), X 1035

Figure 21

Figure 22

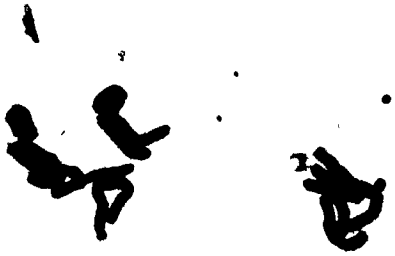


Figure 23

Figure 24

Figure 25

Figures 26 to 28. Effects of pretreatment with 0.25% trypsin on banding in Hordeum vulgare chromosomes, X 1035.

Figure 26. Three minute exposure to trypsin solution containing EDTA in 85% saline (Table VI, Expt. 1)

Figure 27. Three minute exposure to trypsin solution consisting of Sörenson's buffer (Table VI, Expt. 3); arrows indicate satellite chromosomes

Figure 28. Two minute exposure to trypsin solution containing EDTA in 85% saline (Table VI, Expt. 2)

Figure 26

Figure 27

Figure 28

The maceration techniques were different, but both included a 30 minute exposure to 70% acetic acid (Table III).

The differences between these two techniques were in the preparation and pH of both the trypsin solutions and Giemsa solutions. The difference in pH between the solutions in Expts. 1 and 3 was only 0.4 for the trypsin solutions, and 0.2 for the staining solutions. The staining solution in Expt. 1 was made from Gurr's buffer tablets (L654), while the staining solution used in Expt. 3 was diluted Sörenson's buffer.

The major difference in the make up of the trypsin solutions was the presence of the chelating agent (ethylenediamine tetracetic acid (EDTA) in Expt. 1 and its absence in Expt. 3. The sodium ion concentrations were also a source of variance; the concentration in the trypsin solution from Expt. 1 was about 14 M whereas the concentration in the solution from Expt. 3 was maximally 0.2 M.

Treatment of chromosomes for two minutes with the high salt, EDTA, trypsin solution (Figure 28) produced fusion of the chromatids and banding, but the distinction between interband and banded areas was not as vivid as in those chromosomes treated for three minutes with the same trypsin solution. The staining technique for the former was different and entailed a 60 minute incubation in 2% Giemsa rather than the five minute incubation in 25% Giemsa used in the latter technique. This may have had some effect on the relative stain intensities of band and interband regions.

Trypsin-Giemsa: Table VII

Experiments 2 and 3 are identical except for the maceration procedures used. When acid was used, bands were produced; when 5% pectinase was used,

no bands were produced (Figures 29 and 30). The only banding observed when an enzyme maceration was used was after the concentration of trypsin had been increased 40 fold (Expt. 4; Figure 31).

In Expts. 1 and 2, the effect of the time of exposure to the trypsin-Giemsa staining solution can be seen (Figures 32, 33, 29). There is some difficulty in comparing the effect of these two treatments since the chromosomes exposed to the trypsin-Giemsa for 15 minutes (Table VII, Expt. 2; Figure 29) were more condensed than the chromosomes exposed for 10 minutes (Table VII, Expt. 1; Figures 32 and 33). A comparison of the Total Chromosome Length (TCL) in the two karyotypes (Figures 29 and 33) showed that the chromosomes in Figure 29 were 18% shorter than the chromosomes in Figure 33. Therefore, the increase in width of almost 100% in the chromosomes treated for 15 minutes with the trypsin-Giemsa solution (Figure 29) cannot wholly be accounted for by condensation and must be due in part to the longer time of exposure to trypsin.

The telomeres were more obviously stained and the pericentromeric regions less obviously stained in the less condensed preparation exposed for 10 minutes (Figure 33) than in the more condensed preparation exposed for 15 minutes (Figure 29). Both preparations had about the same number of countable bands.

Between Treatments

All chromosomes in the barley karyotype were either metacentric or submetacentric, and the shortest chromosome was 77% of the length of the longest. This symmetry made the resolution of chromosome pairs rather difficult, especially in incomplete metaphase spreads. As mentioned in

Figures 29 to 33. Effects of the trypsin-Giemsa technique (Sun et al., 1973) on Hordeum vulgare chromosomes, X 1035.

Figure 29. Acid maceration, 0.0005% trypsin, 15 minutes (Table VII, Expt. 2); arrows indicate satellite chromosomes

Figure 30. Enzyme maceration, 0.0005% trypsin, 15 minutes (Table VII, Expt. 3)

Figure 31. Enzyme maceration, 0.02% trypsin, 10 minutes (Table VII, Expt. 4)

Figures 32 and 33. Acid maceration, 0.0005% trypsin, 10 minutes ( Table VII, Expt. 1)

Figure 29

Figure 30

Figure 31

Figure 32

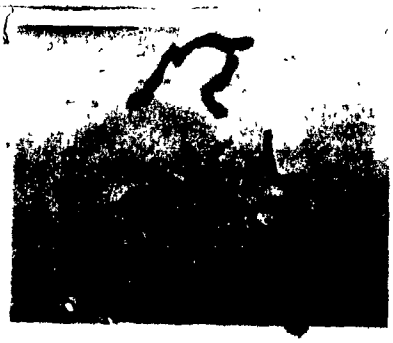


Figure 33

the MATERIALS AND METHODS section, to facilitate pretreatment and staining it was necessary to flatten and spread out the chromosomes which sometimes caused a distortion of the chromosomes (Figure 21) and incomplete metaphase complements (Figure 34). Barley chromosomes also seemed to have an unsettling propensity to stickiness in these experiments making identification of chromosome pairs even more difficult. The only easily distinguishable chromosomes in the barley karyotype were the two pairs of satellited chromosomes, and it was relatively easy to tell them apart: the larger one was submetacentric and had the shorter satellite, while the smaller one was metacentric and had the longer satellite (Figure 35). Therefore, the different banding techniques were compared on the basis of the number of bands present on the two pairs of satellite chromosomes (Figures 21, 34, 36, 37, 27, 29). The technique which elicited the most bands with the highest degree of contrast between banded and nonbanded areas with the least destruction of chromosome structure was determined the best. The results of this comparison are presented in Table XI and suggest that the pretreatments with 2XSSC (Figure 34) or trypsin (Figure 27) gave the best banding.

The Chromosome Bands

Several observations on the pattern of chromosome banding and on the nature of the bands themselves can be made. The bands were characterized in these preparations as being intense or faint. The five different techniques used on barley chromosomes produced at least three different patterns (see Table XI).

The trypsin, trypsin-Giemsa, and 2XSSC techniques (Figures 27, 29, 34

Figures 34 and 36. Effects of 2XSSC on banding in Hordeum vulgare chromosomes (Table V ), X 1035.

Figure 35. A diagram of the two satellite chromosomes of H. vulgare taken from the U. S. Department of Agriculture, Agricultural Handbook No. 33[illegible] (1968); r. l. - relative length.

Figure 37. Effects of the Barium-Saline-Giemsa technique (Sarma and Natarajan, 1973) on banding in H. vulgare chromosomes (Table VII), X 1035.

Arrows indicate satellite chromosomes.

Figure 34

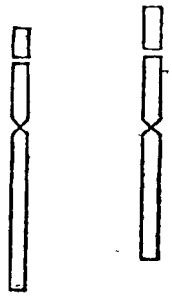



Figure 35

Figure 36

Figure 37

TABLE XI: Banding of the satellite chromosomes in Hordeum vulgare

| Table | Technique | BAND LOCALES | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Chromosome 3 | | | | Chromosome 5 | | | |
| | | Pericentromeric | Long arm | NOR[1] | Telomere | Pericentromeric | Long arm | NOR[1] | Telomere |
| II | Sörenson's buffer see Figure 21 | + | - | - | - | + | - | - | - |
| III | 2XSSC see Figure 31 | + | + - | + | + - | + | + | + | + - |
| IV | Trypsin see Figure 27 | + | + | + | + - | + | ? | + | + |
| V | Trypsin-Giemsa see Figure 29 | + | + | - + | - + | + | + | + - | + - |
| VI | BSG[2] see Figure 37 | - + | + | + | + | - | + | + | - |

[1]Nucleolar Organizing Region.

[2]Barium-hydroxide-Saline-Giemsa

and 36) produced maximum banding. The pericentromeric bands were uniformly faint. The trypsin and trypsin-Giemsa techniques elicited banding patterns that differed from each other only in degree: if a band was faint in a trypsin preparation it was even fainter in a trypsin-Giemsa preparation. The quality of the interstitial bands was the only major difference between the 2XSSC treated preparation and the two trypsin treated preparations; it was very faint in the former and intense in the latter. The preparations pretreated in Sörenson's buffer (Figure 21) gave no banding at the NOR, the telomeres of the satellite chromosomes or interstitial locales. However, it did produce pericentromeric bands.

The inverse situation was observable in the barium hydroxide treated preparation (Figure 37); the NOR, and the telomeres of the satellite chromosomes and interstitial areas were banded whereas the pericentromeric regions were not.

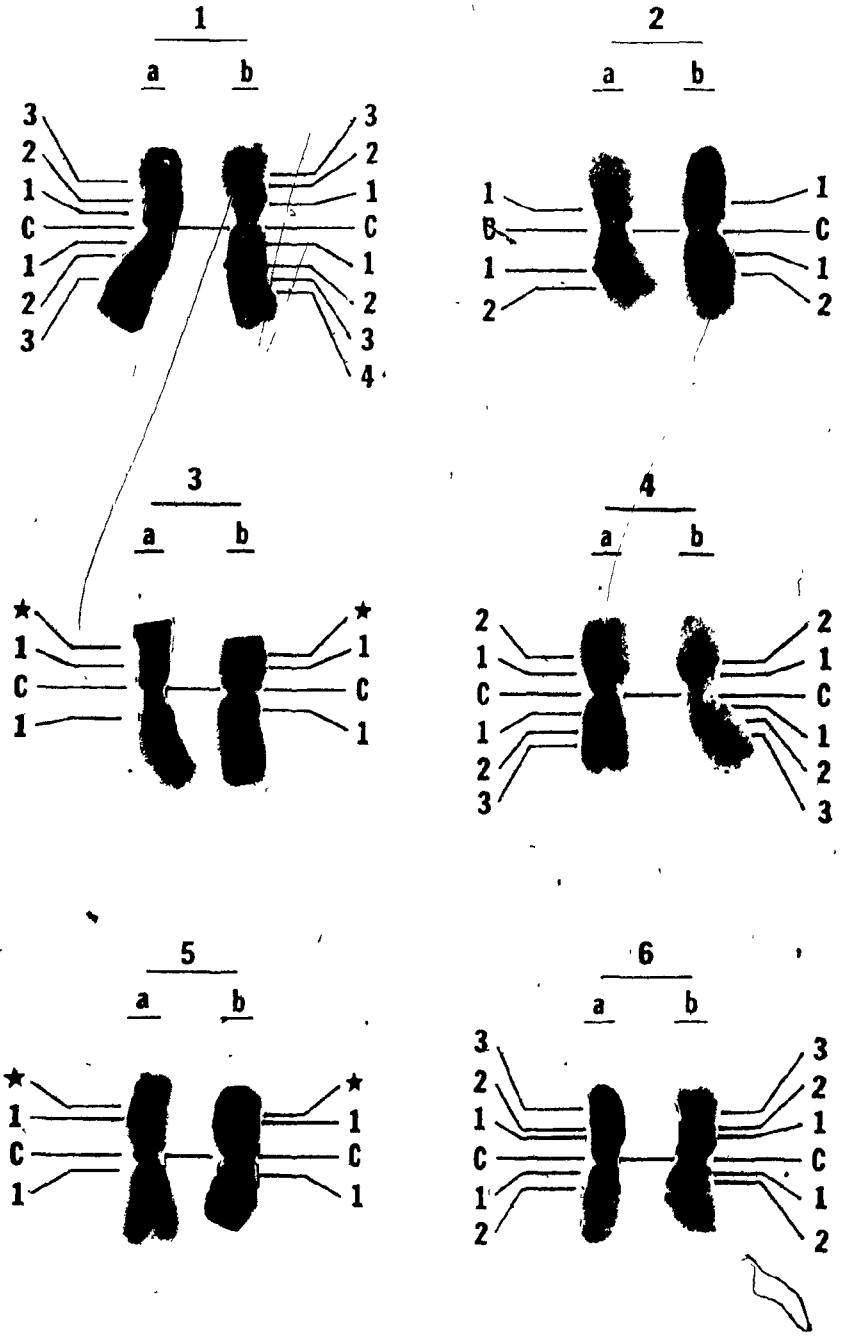
## The Barley Banded Chromosome Karyotype

The banding patterns of a barley karyotype were determined as follows. A well spread barley chromosome complement was isolated, treated with trypsin-Giemsa, then measurements of each chromosome were taken and the centromeric index calculated for each chromosome. Photomicrographs of one metaphase (Figure 29) were distributed to seven people who were asked to independently pair the homologous chromosomes. Pairing was based on similarities of size, shape, and banding patterns. There were three pairs of chromosomes which five people agreed on (pairs 4, 6 and 7; Figure 38), and one pair which four people agreed on (pair 1; Figure 38). The pairing of the last six chromosomes was accomplished by comparing the relative

Figure 38. The banded karyogram of Hordeum vulgare (n = 7).

Bands are numbered from the proximal to the distal part of each arm; the distance from the centromere to each band is recorded in Table XIV.

C - centromere, ★ - Nucleolar Organizing Region.


1
a
b
3
2
1
C
1
2
3
3
2
1
C
1
2
3
4
2
a
b
1
B
1
2
1
C
1
2
3
4
a
b
2
1
C
1
2
3
2
1
C
1
2
3
5
a
b
★
1
C
1
★
1
C
1
6
a
b
3
2
1
C
1
2
3
2
1
C
1
2
7
a
b
C
1
2
3
1
C
1
3

lengths and centromeric indices of each possible karyotype with the same values for barley published in the U.S. Department of Agriculture, Agricultural Handbook No. 338 (1968). The final karyotype arrived at had an average deviation from the handbook measurements of 0.05 per measurement (see Table XII).

An idiogram (Figure 39) of this metaphase was constructed, the units of which were based on relative lengths of the chromosomes. The bands were localized on the idiogram by measuring the distance in millimeters from the centromere to the centre of the band and converting this value into units of relative length according to the formula in Table XIII. The letters "a" and "b" refer to each chromosome in a pair and correspond to the "a" and "b" in Figure 38. The intense bands, except for one pair, were consistent between two homologues; a band might be intense in one chromosome and faint in the other but it would always be present in both if it was intense in one.

"Ring" Chromosomes

A pair of chromosomes forming two rings was present in three of the less contracted metaphase spreads (Figures 40, 41, and 42). The rings did not appear in preparations in which the chromosomes were more highly condensed (Figure 27). There were suggestions of a ring in a prophase squash (Figure 24) where two chromosomes were bent over onto themselves, one at 1200 hours, the other at 0700 hours. However, there did not seem to be any actual attachments between the two arms of either chromosome, while there was some sort of fusion in the other rings. The rings were seen only in trypsin treated preparations which suggests that the trypsin was responsible for their formation.

TABLE XII: Statistics on the *Hordeum vulgare* chromosome complement

| Chromosome number: | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A[1] | B[2] | A | B | A | B | A | B | A | B | A | B | A | B |
| Relative length: | 1.00 | 1.00 | 0.96 | 0.92 | 0.92 | 0.90 | 0.89 | 0.87 | 0.88 | 0.86 | 0.87 | 0.84 | 0.77 | 0.72 |
| Short arm/long arm: | 0.75 | 0.79 | 0.86 | 0.86 | 0.60 | 0.71 | 0.92 | 0.99 | 0.94 | 0.95 | 0.77 | 0.95 | 0.73 | 0.69 |

[1]"A" measurements were taken from U.S. Department of Agriculture, Agricultural Handbook No. 338 (1968).

[2]"B" measurements were taken from the barley karyogram in Figure 38.

TABLE XIII: Band location on *Hordeum vulgare* chromosomes

| Chromosome | Region | Band no. | a[1] | | | | | b[1] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $x_1$ | $x_2$ | c.l. | r.l | $\frac{x_1 x_2}{2} \times \text{r.l.}^2$ / c.l | $x_1$ | $x_2$ | c.l. | r.l | $\frac{x_1 x_2}{2} \times \text{r.l.}^2$ / c.l. |
| 1 | long arm | 1 | 3.00 | 2.75 | 22.50 | 1.00 | 0.13 | 2.00 | - | 22.75 | 1.00 | 0.09 |
| | | 2 | 5.00 | 4.75 | 22.50 | 1.00 | 0.22 | 5.25 | - | 22.75 | 1.00 | 0.23 |
| | | 3 | 8.00 | 8.25 | 22.50 | 1.00 | 0.36 | 7.25 | 7.00 | 22.75 | 1.00 | 0.31 |
| | | 4 | - | - | 22.50 | 1.00 | - | 9.25 | 9.75 | 22.75 | 1.00 | 0.42 |
| | short arm | 1 | 2.50 | 2.75 | 22.50 | 1.00 | 0.12 | - | 2.00 | 22.75 | 1.00 | 0.09 |
| | | 2 | 4.25 | 4.50 | 22.50 | 1.00 | 0.19 | 3.50 | 3.50 | 22.75 | 1.00 | 0.15 |
| | | 3 | 6.00 | 6.75 | 22.50 | 1.00 | 0.28 | 5.75 | 7.25 | 22.75 | 1.00 | 0.29 |
| | centromere | | 2.00 | 2.50 | 22.50 | 1.00 | 0.09 | 2.00 | - | 22.75 | 1.00 | 0.09 |
| 2 | long arm | 1 | 5.00 | 3.00 | 20.50 | 0.92 | 0.18 | 3.50 | 3.50 | 21.25 | 0.92 | 0.15 |
| | | 2 | 8.50 | 6.75 | 20.50 | 0.92 | 0.34 | 6.00 | 5.75 | 21.25 | 0.92 | 0.25 |
| | short arm | 1 | 2.50 | - | 20.50 | 0.92 | 0.13 | 4.00 | - | 21.25 | 0.92 | 0.17 |
| | centromere | | 3.50 | 3.25 | 20.50 | 0.92 | 0.17 | 2.50 | 3.00 | 21.25 | 0.92 | 0.12 |
| 3 | long arm | 1 | 3.00 | 3.00 | 21.75 | 0.90 | 0.12 | 3.00 | 2.25 | 18.75 | 0.90 | 0.13 |
| | short arm | 1 | 4.50 | 3.75 | 21.75 | 0.90 | 0.17 | 3.75 | 3.00 | 18.75 | 0.90 | 0.16 |
| | NOR[3] | | 6.25 | - | 21.75 | 0.90 | 0.22 | 4.75 | - | 18.75 | 0.90 | 0.23 |
| | centromere | S. | 2.00 | - | 21.75 | 0.90 | 0.08 | 1.50 | - | 18.75 | 0.90 | 0.07 |
| | | L. | 2.25 | - | 21.75 | 0.90 | 0.09 | 1.50 | - | 18.75 | 0.90 | 0.07 |
| 4 | long arm | 1 | 2.25 | - | 19.00 | 0.87 | 0.10 | 3.75 | 2.75 | 20.25 | 0.87 | 0.14 |
| | | 2 | 4.75 | - | 19.00 | 0.87 | 0.22 | 4.50 | 5.00 | 20.25 | 0.87 | 0.20 |
| | | 3 | 6.75 | 7.00 | 19.00 | 0.87 | 0.32 | 8.00 | 8.75 | 20.25 | 0.87 | 0.35 |
| | short arm | 1 | 3.00 | 2.75 | 19.00 | 0.87 | 0.13 | 2.25 | 2.75 | 20.25 | 0.87 | 0.11 |
| | | 2 | 5.00 | 5.00 | 19.00 | 0.87 | 0.23 | 4.75 | 3.75 | 20.25 | 0.87 | 0.18 |
| | centromere | | 2.75 | 3.00 | 19.00 | 0.87 | 0.13 | 2.50 | - | 20.25 | 0.87 | 0.11 |

TABLE XIII continued

TABLE XIII continued

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | long arm | 1 | 2.25 | 2.75 | 20.75 | 0.86 | 0.10 | 2.00 | - | 20.25 | 0.86 | 0.09 |
| | short arm | 1 | 5.00 | 5.00 | 20.75 | 0.86 | 0.21 | 4.75 | - | 20.25 | 0.86 | 0.20 |
| | NOR[3] | | 6.75 | 7.00 | 20.75 | 0.86 | 0.29 | 6.00 | 6.50 | 20.25 | 0.86 | 0.27 |
| | centromere | | 2.50 | - | 20.75 | 0.86 | 0.10 | 2.25 | - | 20.25 | 0.86 | 0.10 |
| 6 | long arm | 1 | 2.50 | 2.00 | 19.50 | 0.84 | 0.01 | 2.00 | - | 18.50 | 0.84 | 0.09 |
| | | 2 | 4.25 | 3.75 | 19.50 | 0.84 | 0.17 | 3.00 | - | 18.50 | 0.84 | 0.13 |
| | short arm | 1 | 2.75 | 2.25 | 19.50 | 0.84 | 0.11 | 3.00 | 2.75 | 18.50 | 0.84 | 0.13 |
| | | 2 | 3.00 | - | 19.50 | 0.84 | 0.13 | 3.50 | - | 18.50 | 0.84 | 0.16 |
| | | 3 | 6.50 | - | 19.50 | 0.84 | 0.28 | 6.25 | 6.00 | 18.50 | 0.84 | 0.28 |
| | centromere | | 2.00 | - | 19.50 | 0.84 | 0.09 | 2.00 | 1.75 | 18.50 | 0.84 | 0.09 |
| 7 | long arm | 1 | 3.00 | 2.75 | 16.00 | 0.72 | 0.15 | 4.00 | 3.50 | 17.00 | 0.72 | 0.16 |
| | | 2 | 5.00 | - | 16.00 | 0.72 | 0.23 | - | - | 17.00 | 0.72 | - |
| | | 3 | 7.25 | 6.50 | 16.00 | 0.72 | 0.31 | 7.00 | 7.50 | 17.00 | 0.72 | 0.31 |
| | short arm | 1 | - | - | 16.00 | 0.72 | - | 5.00 | 5.00 | 17.00 | 0.72 | 0.21 |
| | centromere | | 3.00 | 2.25 | 16.00 | 0.72 | 0.12 | 2.00 | - | 17.00 | 0.72 | 0.09 |

[1]a and b represent the two chromosomes in a homologous pair and correspond to a and b in Figure 38.

[2]$x_1$ = distance from centromere in mm, chromatid 1.

$x_2$ = distance from centromere in mm, chromatid 2.

c.l. = length of chromosome in mm.

r.l. = relative length = c.l./c.l. of the longest chromosome.

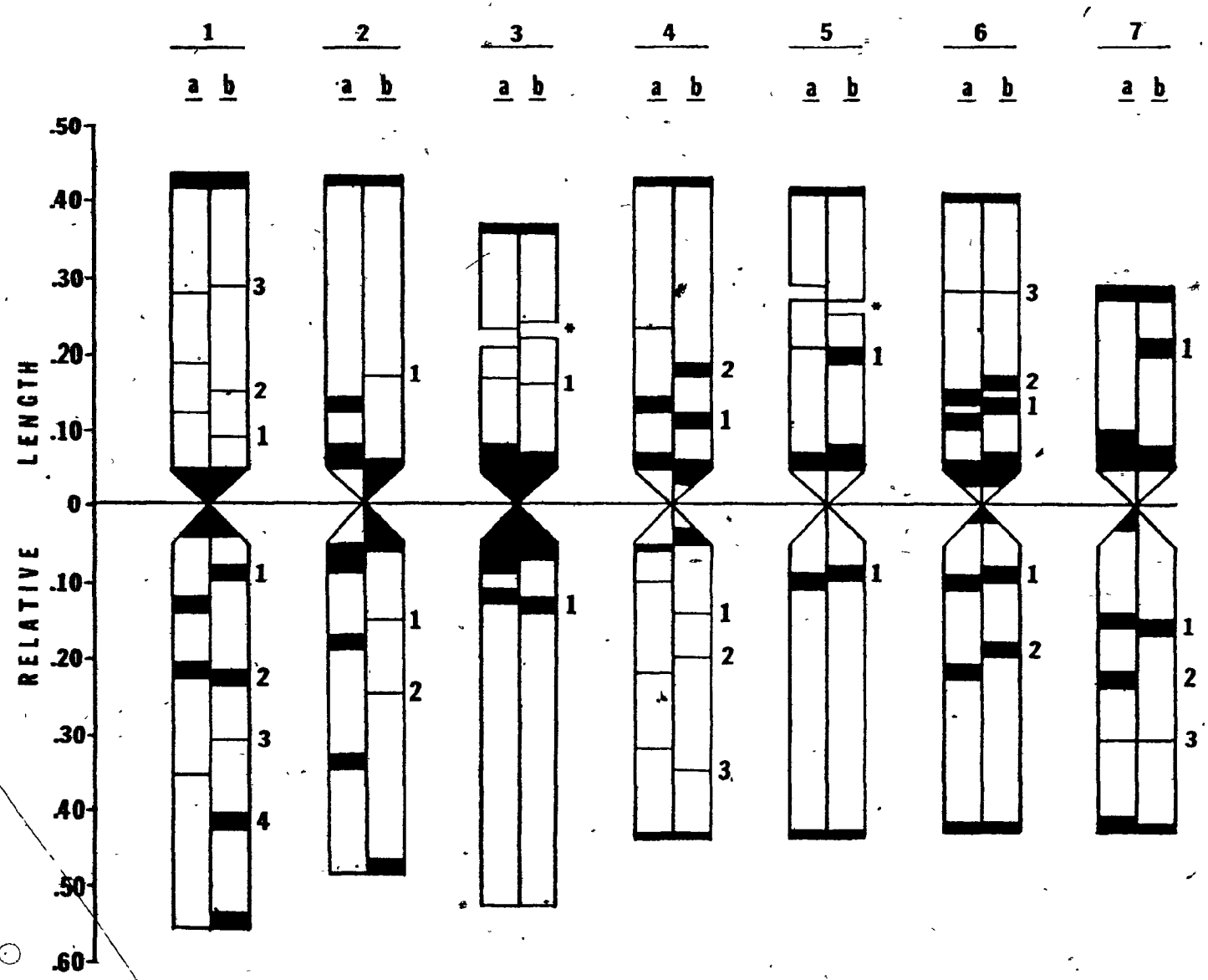
$\frac{(x_1+x_2)}{2} \times \frac{}{c.l.}$ r.l. = Idiogram value in terms of relative length.

[3]NOR = Nucleolar Organizer Region.

Figure 39. A banded idiogram for Hordeum vulgare chromosomes.

Location of the bands was determined independently for each chromosome, "a" and "b," in a pair; see Table XIV and Figure 38.

* - Nucleolar Organizing Region.


1
2
3
4
5
6
7
a b
RELATIVE LENGTH
.50
.40
.30
.20
.10
0
.10
.20
.30
.40
.50
.60

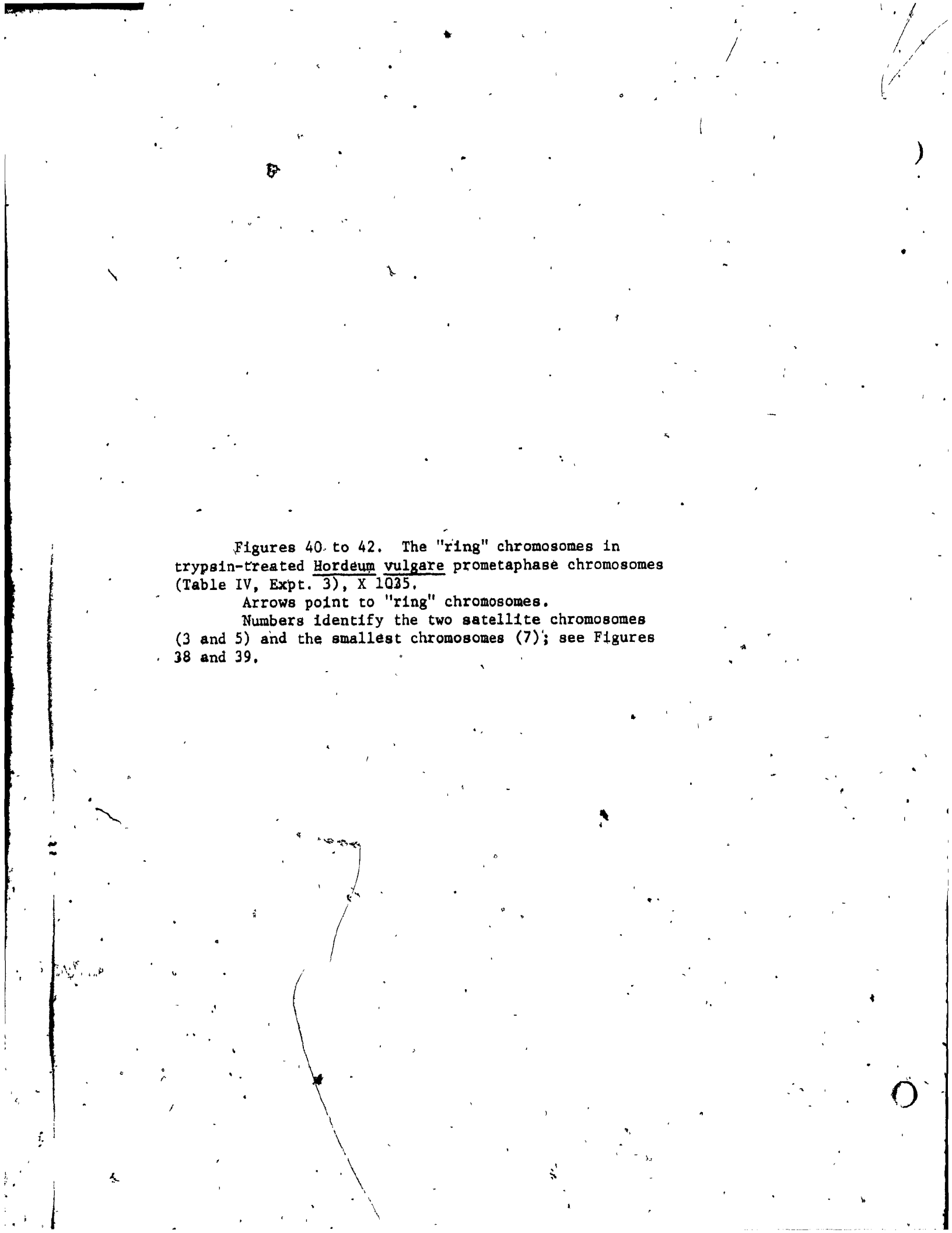
The formation of rings was not an arbitrary phenomenon. As the following evidence suggests, the rings were formed from the same chromosome pair in all three preparations. In the complements which had two obvious rings (Figures 40 and 42), both rings were identical in length. The five rings observed from the three different preparations were practically the same length, measuring from 14.0 - 14.5 mm. As well, they shared a similar banding pattern - two dark bands in the long arm, one large or two smaller bands in the shorter arm, and a centromere which stained less intensely than the interstitial bands. Measurements taken from Figure 41 suggest that the rings were formed from the chromosomes of pair six; the long satellite chromosome was 19.5 mm, the short satellite chromosome was 15 mm, the ring chromosome was 14.5 mm, and the shortest chromosome was 11 mm. The banding pattern also corresponded closest to that represented in the idiogram (Figure 39) for chromosome six.

Pair Seven

The last pair was heteromorphic; "a" had no band on the short arm but had two intense bands on the long arm, while "b" had an intense band on the short arm and only one intense band on the long arm (Figures 38 and 39).

## Order Within The Interphase Nucleus

Photomicrographs of prophase nuclei (Figures 43 and 44) showed a distinct polarization of the chromosomes; centromeres were all adjacent to each other at one pole and the telomeres extended towards the opposite pole. Arms from the same chromosome were close to each other (Figure 43). In Figure 45 two telophase nuclei and a prophase nucleus share the same



Figures 40 to 42. The "ring" chromosomes in trypsin-treated Hordeum vulgare prometaphase chromosomes (Table IV, Expt. 3), X 1035.

Arrows point to "ring" chromosomes.

Numbers identify the two satellite chromosomes (3 and 5) and the smallest chromosomes (7); see Figures 38 and 39.

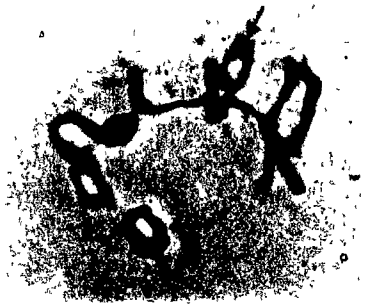


Figure 40

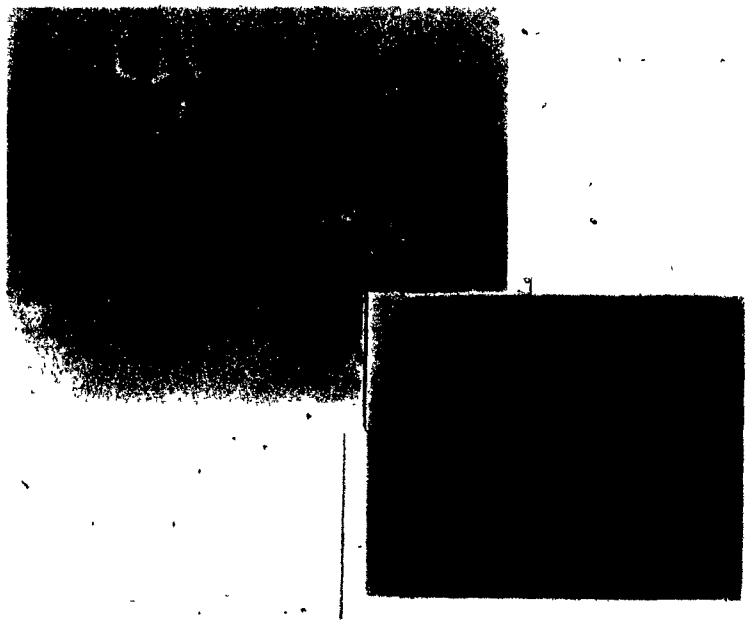


Figure 41

Figure 42

Figures 43 to 46: Nonrandom chromosomal arrangements in Hordeum vulgare, X 815.

Figure 43. Late prophase chromosomes, polar view (Table II, Expt. 1)

Figure 44. Early prophase chromosomes, side view (Table VI, Expt.1)

Figure 45. Late prophase and anaphase cells showing chromosomal polarization, Feulgen stained

Figure 46. Metaphase chromosomes (Table V, Expt. 4)

Arrows indicate interchromosomal connectives.

Figure 43

Figure 44

Figure 45

Figure 46

polarization of chromosomes and arrangement of chromosome arms. This consistency is most easily explained by assuming that the chromosomes remained polarized throughout interphase.

Although the same prefixation treatments and fixatives were used on Vicia faba, Lotus pedunculatus, and Hordeum vulgare, only the metaphase chromosomes of the latter were noticeably sticky. In two complements of barley chromosomes a distinct connection was observed between the telomere of one chromatid and an interstitial region of a chromatid from another chromosome (Figure 43, 0900 hours, and Figure 46, 1000 hours).

Connectives were also seen between the prophase chromosomes of Lotus pedunculatus (Figure 18) although no metaphase attachments were observed and the condensed chromosomes were not sticky. Most of the attachments were end-to-end.

# DISCUSSION

## Maceration

Comparisons between acid-macerated and enzyme-macerated, Giemsa-banded Vicia faba and Hordeum vulgare (barley) chromosome preparations substantiate Schwiezer's (1973) contention that an acid pretreatment facilitates chromosome banding in some plant species (see Figures 2-5). A hot HCl maceration will render the pericentromeric heterochromatin unbanded in both rye (Sarma and Natarajan, 1973) and V. faba chromosomes (Döbel et al., 1973) while in V. faba a rigorous acid pretreatment preferentially induces the Nuclear Organizing Region (NOR) to band (Matsui, 1974). Clearly an acid-sensitive chromosomal constituent is playing a very important role in the Giemsa-banding of plant species.

If Hordeum vulgare chromosomes are macerated in acid the concentration of trypsin needed to induce bands is much lower (compare Figures 29, 30 and 31). The acid maceration must therefore be affecting chromosomal proteins by hydrolyzing them and/or by removing some of the acid-soluble proteins, e.g. the histones.

## Pretreatments

Despite the equivalency of trypsin concentrations and exposure times in Experiments 1 and 3, Table VI, there is a rather large difference in the quality of banding between the barley chromosomes in these experiments (see Figures 26 and 27). This difference is most probably due to the presence of the chelating agent, ethylenediamine tetraacetic acid (EDTA), in the high saline solution of trypsin in Expt. 1, although the small differences in pH and composition of the staining solution might play a

minor role. The decondensation of the DNP fibre caused by the removal of divalent cations (Ris, 1975; Huberman and Attardi, 1966; Olins and Olins, 1972) must be preferentially occurring in the nonband regions since $Ca^{++}$ and $Mg^{++}$ free saline induces Giemsa bands (Dev et al., 1972). This $Ca^{++}$- and $Mg^{++}$-free-saline-induced decondensation would make the chromosomal proteins in the interband regions more vulnerable to tryptic digestion, decondensing the fibre even further, and thereby explaining the exaggerated interband loss of stainability observed in the chromosome preparations from Expt. 1, Table VI (see Figure 26).

## Condensation

As has been previously explained, the mechanism of staining depends on the correct spatial relationship between bound methylene blue molecules along the DNA helix (Sumner and Evans, 1973). The degree of coiling of the DNP fibre will influence this parameter. It was postulated in the LITERATURE REVIEW that a higher degree of coiling protects the associated proteins from disruption by band inducing agents thereby preserving the condensation and hence the stainability of the chromatin. This is illustrated in Figures 6-8 where the effect of trypsin on three different stages of mitotic condensation is seen. The more condensed the barley chromosomes are at the time of trypsin pretreatment, the more their stainability and structural detail are preserved. This implies that the condensed state protects the chromosomal proteins from trypsin digestion.

Proteolysis removes the F1 histone fraction first (Ockey, 1973); it is this fraction that is thought to be involved in mitotic condensation (Gurley et al., 1974; Lake, 1973b; Lake et al., 1972). Perhaps it is

this fraction that is both responsible for the mitotic condensation and relatively protected by it once the chromosomes have fully condensed.

The constitutive heterochromatin in the bands maintains its stainability throughout the condensation process indicating that it is qualitatively different from the euchromatin (Figures 6-8). These areas are somehow resistant to trypsinization, either because they are more highly contracted to begin with or because their proteins are relatively resistant to trypsinization. Trypsinization of rabbit liver chromatin digests all but a small fraction of nonhistone chromosomal protein comprising about 2.5% of the total native chromosomal protein (Simpson, 1972). Unless barley chromatin contains this fraction in large enough quantities to cover all its constitutive heterochromatin, the former explanation seems the most reasonable.

This differential condensation of heterochromatin at prophase is seen also in at least one of the Lotus pedunculatus chromosomes (Figure 18a) where the pericentromeric and telomeric heterochromatin is already well condensed while the euchromatin is still uncondensed.

## Heterochromatin

### Lotus pedunculatus

Although Döbel et al.'s (1973) pretreatment (Table X, Expt. 2) did not elicit bands in Lotus pedunculatus chromosomes at metaphase (Figure 16), regions which stain more intensely with Giemsa were clearly visible in at least one of the prophase chromosomes pretreated in the same way (Figure 18a). The location of the bands in prophase chromosome 3

(Figure 18a) is identical to the location of the Giemsa-bands in metaphase chromosome 3 (Figure 17) successfully pretreated with Schweizer's (1973) technique (Table X, Expt. 1). In all of the banded chromosomes there are distinct pericentromeric bands and less distinct telomeric bands. Also, one of the larger satellite chromosomes in an unstained, unpretreated L. pedunculatus cell has a block of heterochromatin in the proximal region of its short arm (Figure 19) which is Giemsa-banded in the preparation treated according to Schweizer (1973) (Figure 17). The congruity of what is possibly a naturally occurring heterochromatic condensation visualized in prophase and in an unstained, unpretreated metaphase chromosome with the Giemsa bands in metaphase chromosomes adds verification to the generally held belief that the bands reflect areas of heterochromatin, and that heterochromatic condensation is qualitatively different from mitotic condensation. Merritt and Burns (1974) pressed home this idea in their study on bands in Nicotiana otophora where they were able to see bands in unfixed, unpretreated, unstained prophase chromosomes. Their contention was that the prefixation exposure to the super-contracting action of C-mitotic agents and postfixation pretreatments emphasized naturally occurring differences in the condensation of euchromatin and heterochromatin in prophase chromosomes.

It is perhaps significant that the banded metaphase chromosomes (Expt. 1, Table X; Figure 13) were more contracted than those pretreated according to the other three techniques (Expts. 2-4, Table X; Figures 14-16). The extra contraction in the chromosomes which were by chance treated with Schweizer's (1973) technique might have been a determining factor in whether or not bands were induced in L. pedunculatus metaphase

chromosomes. If this is the case, the other three procedures cannot be totally ruled out as potentially successful band inducers.

As has been noted in the RESULTS, the two smallest chromosomes in the L. pedunculatus genome stain intensely along their entire lengths at C-metaphase (Figure 17) and are prematurely fully condensed at prophase (Figure 18a and b). This behavior intimates that these two chromosomes are heterochromatic. A practical application of this finding would be in the easy identification of the four smallest chromosomes in the L. pedunculatus karyotype. Their closeness in size and morphology has made resolution of the extra small chromosome in certain L. pedunculatus trisomics (Chen and Grant, 1968) difficult (Figures 19 and 20). By facilitating the identification of these chromosomes and by providing new morphological features the Giemsa-banding techniques could further cytogenetic studies in the genus Lotus as they have done in Anemone (Marks and Schweizer, 1974), Scilla sibirica (Vosa, 1973a) Tulipa (Filion, 1974) and Triticale (Darvey and Gustafson, 1975; Sarma and Natarajan, 1973).

Hordeum vulgare

Two types of constitutive heterochromatin were resolvable in barley with the band-inducing techniques used in this study. The first type was found in the pericentromeric regions and the second type was found in the interstitial and satellite regions (see Table XIV). While trypsin (Figure 27), trypsin-Giemsa (Figure 29), and 2XSSC (Figure 34) stained both types, the satellite and interstial bands were lighter than the pericentromeric bands. Pretreatment with Sörenson's buffer (Figure 21) resolved only the pericentromeric heterochromatin of the satellite chromosome, and barium hydroxide (Figure 37) brought out only the interstitial

TABLE XIV: Giemsa-staining properties of heterochromatin in Hordeum vulgare satellite chromosomes

| Technique | Pericentromeric | Interstitial | Satellite |
|---|---|---|---|
| 1. Trypsin Trypsin-Giemsa 2XSSC | intense | faint | faint |
| 2. Sörenson's buffer | intense | - | - |
| 3. Barium hydroxide | - | intense | intense |

and satellite regions (Table XI).

From my interpretation of the literature, the banding behavior of pericentromeric heterochromatin appears to be different from the rest of the heterochromatin in Secale cereale (Gill and Kimber, 1974; Sarma and Natarajan, 1973), Vicia faba (Döbel et al., 1973; Takehisa and Utsumi, 1973a), and Allium cepa and Ornithogalum virens (Stack et al., 1974). This study demonstrates that the same is true for the pericentromeric heterochromatin of Hordeum vulgare (see Table XIV).

The macerations and pretreatments which combined to either produce or prevent pericentromeric banding are listed in Table XV. Although the information comes from work done on four different species, all of the species tabulated are Monocots: Hordeum vulgare and Secale cereale are both from the Graminae while Allium cepa and Ornithogalum virens are from the Liliaceae. The pericentromeric heterochromatin in all four species shares a consistent response to the macerations and pretreatments applied (compare lines 3, 4, and 9, and lines 7 and 8, Table XV). Therefore, it seems safe to make the following generalizations about pericentromeric heterochromatin at least in Monocots:

1. A strong, or hot, acid maceration does not prevent pericentromeric banding if it is followed by a pretreatment at a neutral pH (lines 5-8, Table XV).

2. Pericentromeric heterochromatin is sensitive to barium hydroxide (possibly to any alkaline pretreatment?) after a hot, or strong, acid maceration (line 1-4, Table XV). Although the Barium hydroxide-Saline-Giemsa (BSG) technique involves incubation in 2XSSC as well as in barium hydroxide, incubation in 2XSSC alone after a strong acid maceration does

TABLE XV: Dependency of pericentromeric banding on pretreatments and macerations

| Source | Material | Maceration | Pretreatment | pH | pericentromeric bands | other bands |
|---|---|---|---|---|---|---|
| 1. Gill and Kimber, 1974 | Secale cereale | mild acid/ enzyme | a) BaOH<br>b) 2XSSC | alkaline<br>neutral | + | + |
| 2. Hadlaczky and Koczka, 1974 | S. cereale | enzyme | a) BaOH<br>b) 2XSSC | alkaline<br>neutral | + | + |
| 3. Sarma and Natarajan, 1974 | S. cereale | hot acid | a) BaOH<br>b) 2XSSC | alkaline<br>neutral | - | ± |
| 4. This study, Table VIII; Fig.37 | Hordeum vulgare | strong acid/ enzyme | a) BaOH<br>b) 2XSSC | alkaline<br>neutral | - | + |
| 5. This study, Table V; Fig.34 | H. vulgare | hot acid/ enzyme | a) 2XSSC | neutral | + | + |
| 6. This study, Table VI, Expt. 3; Fig.27 | H. vulgare | strong acid/ enzyme | a) 0.25% trypsin | neutral | + | + |
| 7. This study, Table IV, Expt. 2; Fig.21 | H. vulgare | hot acid/ enzyme | a) Sorenson's phosphate buffer | neutral | + | + |
| 8. Stack et al., 1974 | Allium cepa, Ornithogalum virens | acid | a) Phosphate buffer | neutral | + | - |
| 9. Stack and Clarke, 1973a | A. cepa | acid | a) BaOH<br>b) 2XSSC | alkaline<br>neutral | - | + |

not inhibit the banding of pericentromeric heterochromatin (lines 4 and 5, Table XV).

3. Pericentromeric heterochromatin is preferentially stained after pretreatment in phosphate buffer at a neutral pH (lines 7 and 8, Table XV).

Vicia faba

There are at least three categories of chromatin within the V. faba genome: chromatin stainable only by Döbel et al.'s (1973) technique (e.g., the Nucleolar Organizing Region), heterochromatin stainable only by the trypsin-Giemsa techniques (e.g., the pericentromeric regions on the long arms of the M and S chromosomes) and heterochromatin differentiated by both techniques (e.g., most of the interstitial bands). See Figure 12.

Pericentromeric Heterochromatin

The pericentromeric regions on the long arms of the S and M chromosomes show enhanced fluorescence with quinacrine (Caspersson et al., 1969) and do not bind $^{3}$H-actinomycin D ($^{3}$H-AMD) as readily as do the rest of the chromosomal regions (Cionini, 1973). Enhanced quinacrine fluorescence in some instances reflects regions of AT-richness (Selander and de la Chapelle, 1973). This would appear to be the case in the pericentromeric regions of V. faba chromosomes since $^{3}$H-AMD, which binds adjacent to GC base pairs (Müller and Crothers, 1968), binds less frequently to these regions of enhanced quinacrine fluorescence.

As well as being hot HCl sensitive (see Table II), the pericentromeric bands appear to be trypsin sensitive since those chromosomes treated with the higher trypsin concentration lacked distinctive pericentromeric bands

(Figure 12a and c). It has been noted elsewhere that histones are vulnerable to HCl extraction (Bobrow, 1974) and that the F1 histone fraction is the first fraction affected by trypsinization (Ockey, 1973) and is believed to bind preferentially, if not exclusively, to AT-rich DNA (Combard and Vendrely, 1970). Circumstantially then, there are indications that the F1 histone fraction might be specifically responsible for the condensation of pericentromeric heterochromatin in V. faba.

The Nucleolar Organizing Region (NOR)

The NOR is an enigmatic region. Although differentially condensed and containing highly repetitious DNA, it is not made up of constitutive heterochromatin since its DNA is actively transcribing rRNA (Ritossa and Spiegelman, 1965). However, since it does share with heterochromatin the property of unique condensation (Matsui, 1974) its staining behavior sets it apart from the rest of the euchromatin and may provide insights into the preferential banding of heterochromatin.

The NOR does not show enhanced fluorescence with quinacrine (Caspersson et al., 1969). Without an acidic maceration it stains negatively (Figures 10 and 11; Takehisa and Utsumi, 1973a). The NOR actually needs a strong acid treatment to be differentiated positively, has a specific acidic protein associated with it, and contracts more extremely in response to C-mitotic agents than does any other chromosomal region (Matsui, 1974). Since the contractility of chromatin is due to proteins and RNA (Hoskins, 1968), the specific acidic protein fraction and the high concentration of rRNA associated with the NOR are probably responsible for the NOR's unique contractility.

The NOR of one M chromosome binds $^3$H-AMD rapidly while the NOR of the other M chromosome doesn't bind it at all (Cionini, 1973). Possibly the NOR $^3$H-AMD binding pattern is reflecting a region of chromatin with a very high GC content which, in one of the NORs, is so tightly contracted or whose binding sites are so covered by protein that the $^3$H-AMD cannot bind. Since V. faba's NORs behaved consistently in response to the Giemsa-banding pretreatments used in this study and Döbel et al.'s (1973) study (see Figure 12), the latter possibility seems the likeliest as a difference in condensation should be picked up by these techniques. The size and number of nucleoli can vary within the cells of a single organism and seem to be dependent on the metabolic requirements of the cell (DuPraw, 1970). Therefore, the differential $^3$H-AMD uptake shown by V. faba's NORs might be reflecting the differential activity of these two regions. If this were the case, the inactive NOR would be a candidate for plant facultative heterochromatin. A testable prediction of this hypothesis is that the nucleoli in some V. faba cells should be associated with only one NOR.

The Interstitial Bands

The interstitial heterochromatin showed considerable heterogeneity when stained with trypsin-Giemsa and after treatment by the procedure of Döbel et al. (1973) (see Figure 12). Based on their reactions to these techniques, four categories of interstitial heterochromatin were resolved which stained less intensely than the surrounding chromatin in response to at least one technique (see RESULTS). The variability seen in these "negative bands" is, perhaps, due to the chemical heterogeneity seen in the sensitivity of different heterochromatic regions in Vicia faba

chromosomes to specific mutagens (Rieger, 1973).

The lack of dye uptake in the negative bands could be due to extremely tight condensation preventing the binding of methylene blue dye molecules or an extreme decondensation preventing the eosin Y linkage step (see LITERATURE REVIEW). The former seems to be the better explanation since, in the $S_3$ chromosome of V. faba, the most distal band is positive after the Döbel et al. (1973) technique, positive or negative after treatment with 0.005% trypsin-Giemsa ("Interchangeable") and negative after treatment with 0.0005% trypsin-Giemsa ("Substitution") (Figure 12,b, c, and a, respectively). Clearly, the milder the treatment the more probable that overly condensed chromatin will remain condensed and thus be unstainable. Those bands that are occasionally negative, such as the "Interchangeables" and the "Substitutions", could be assumed to be more tightly condensed than those heterochromatic regions which stain positively all of the time.

One of the two "Unique" negative bands in the M chromosome of V. faba corresponds to a GC-rich, cold reactive band (Cionini, 1973). Since the NORs in both trypsin-Giemsa treated M chromosome pairs are also negative staining, and probably GC-rich, it is possible that the GC-richness confers a higher degree of condensation due either its lower helical pitch (Bram, 1971) or to the association of a particular protein, such as the arginine-rich histone (Clark and Felsenfeld, 1972) or a specific acidic protein (Matsui, 1974).

Some of the negative bands occur immediately adjacent to positive bands ("Adjacents", Figure 12). This is reminiscent of Vosa's (1973d) finding that many of the quinacrine enhanced bands in Allium flavum had

neighboring bands showing reduced fluorescence. If some of the Giemsa negative bands are indicative of a higher GC content as the reduced fluorescence bands are thought to be (Selander and de la Chapelle, 1973), then the same phenomenon may be occurring in both A. flavum and V. faba. The significance of this is difficult to assess.

## Order Within the Interphase Nucleus

### Hordeum vulgare

Kumar and Natarajan (1966) proposed a model for the arrangement of chromosome strands in barley interphase nuclei: the chromosome strands are highly polarized; the arms radiate from the poles at a small angle, and the strands are located in definite, recurrent sites. An examination of Figures 43-45 give visual verification to this model. The chromosomes are obviously polarized with the centromeres at one pole and the telomeres at another. The simplest explanation of the chromosomes' polarization at both anaphase and prophase (Figure 45) is that this polarization is maintained throughout interphase.

Nonrandom arrangements of the chromosomes at interphase have been observed in other plant species. The chromosomes of Crepis capillaris and Aegilops squarrosa (Kitani, 1963), and Ornithogalum virens (Ashley and Wagenaar, 1974) are polarized, while wheat chromosomes occupy defined nonrandom positions within the interphase nucleus (Feldman and Avivi, 1973b).

The chromosomes of C. capillaris (Wagenaar, 1969) and O. virens (Ashley and Wagenaar, 1974) show interchromosomal connectives between their telomeres and display telomeric heterochromatin (Schweizer, 1972; Stack et al., 1974, respectively). Although no consistent telomeric attachments have

as yet been found in barley, this species does possess telomeric heterochromatin (Figures 38, 39 and 44).

Heneen and Nichols (1972) suggested in their study of Muntjac chromosomes that the nonrandom organization of the nucleus might depend on the association of specific heterochromatic regions. The heterochromatic telomeres of Allium cepa do in fact fuse during interphase (Stack and Clarke, 1973a) and there is some evidence that the telomeres in the chromosomes of several other plant species not only fuse to each other during interphase, but attach as well to the nuclear membrane (Sved, 1966). Association of telomeric heterochromatins might be a factor in the organization of the barley nucleus.

The nonrandom organization of interphase chromosomes in wheat depends on tubulin, the microtubular protein which is attached to the centromere. During metaphase the microtubules connect the centromeres to the spindle fibres; during interphase they connect the centromeres to the nuclear membrane (Avivi and Feldman, 1973). Wheat has no telomeric heterochromatin, but does have some pericentromeric heterochromatin (Sarma and Natarajan, 1973) which might function in strengthening the centromeric region.

Barley contains both telomeric and centromeric heterochromatin (Figures 38, 39 and 44). The maintenance of polarization of barley chromosomes throughout interphase could be due to both the attachments of its telomeric heterochromatin to the nuclear membrane at one pole and the binding of the centromeres by means of the microtubules to the nuclear membrane at the opposite pole.

The ring chromosomes in barley formed from chromosome six appear only in trypsin-treated prometaphase cells (Figures 40-42). Trypsin radically

disrupts the chromatin fibre (Simpson, 1972), fusing the chromatids (Wang et al., 1972), and over exposure results in a loss of stainability (Burkholder, 1974). If chromatids tend to fuse, then chromosome arms or ends lying sufficiently close to each other would fuse as well after pretreatment with trypsin. It seems likely then that the rings were formed when the ends of a chromosome lay very close to each other in the spread; treatment with trypsin fused the ends creating a ring. Since only the telomeres fused they must have been closer to each other than were the arms, and/or contain heterochromatin having more of a propensity to fuse with itself after trypsinization than the rest of the chromatin. Both possibilities imply that the telomeres of chromosome six are closely associated and possibly fused during interphase.

The shortest chromosome pair in barley is heteromorphic for its banding pattern (Figure 39). The distance between the centromere and Band 1 in the short arm of "b" and Band 2 in the long arm of "a" is almost the same. The simplest explanation is that a balanced translocation between the arms of a single chromosome seven, involving four breaks, has occurred, resulting in the transposition of the second band in the long arm to the short arm (Figure 39). This indirectly verifies Kumar and Natarajan's (1966) model of the spatial arrangement of the barley chromosome arms. If the two arms of a chromosome seven were consistently lying next to each other, as the model predicts, the likelihood of four breakage events in these two arms giving rise to the observed chromosomal rearrangement is greater than it would be if the arms were arrayed randomly in the interphase nucleus.

Interchromosomal connectives were seen in many preparations and one type of connective was found in two quite differently treated metaphase

spreads (see Figures 43 and 46). These interchromosomal connectives might be related to the observation by Fedak and Helgason (1970) that homologues of somatic barley metaphase chromosomes in ordinary squash preparations are closer to each other than expected if the distance between them were dependent solely on random forces. This close association of homologues in barley during interphase terminates before prophase except in colchicine treated cells (Yoshida and Yamaguchi, 1973) which might explain the lingering presence of interchromosomal connectives in the prophase and metaphase chromosomes of this study.

Lotus pedunculatus

Connectives were also seen between the telomeres of prophase chromosomes in Lotus pedunculatus (Figure 18a). The telomeres are heterochromatic which suggests that there may be some consistent relationship in plant chromosomes between heterochromatin and interchromosomal connections.

## SUMMARY

The Giemsa-banding techniques differentiate between heterochromatin and euchromatin in metaphase chromosomes and have many uses in the field of plant and animal cytogenetics. The bands on the chromosomes provide markers which facilitate karyotyping, genetic mapping, and the resolution of inter- and intraspecific chromosomal variation. The Giemsa-banding techniques have also become useful tools in the investigation of chromosome structure and the role of heterochromatin. In this study, as will be detailed below, various procedures using the Giemsa stain were carried out on Hordeum vulgare L., Lotus pedunculatus Cav. and Vicia faba L.

1. An analysis of the literature indicates that the mechanism by which the Giemsa stain bands the heterochromatic regions in plant and animal chromosomes depends on the preferential decondensation of the desoxyribonucleoprotein (DNP) fibre in the euchromatic regions of the metaphase chromosome. The decondensation is caused by the disruption of chromosomal proteins which are relatively more vulnerable to the pretreatments in euchromatic regions. This may be caused by either a lower degree of coiling in these regions or the type of proteins associated with the euchromatin. This study showed that ethylenediamine tetraacetic acid facilitated the induction of bands by trypsin, probably because the removal of $Ca^{++}$ and $Mg^{++}$ ions decondenses the DNP fibre making the proteins in the euchromatin even more vulnerable to tryptic digestion. Conversely, it was noted that the more condensed the chromosomes, the less vulnerable the euchromatic regions were to tryptic digestion. There was an observable difference between the coiling of the heterochromatic regions and the mitotic coiling of euchromatic regions since even at the most decondensed

stage the heterochromatin maintained its stainability while the euchromatic regions had lost both stainability and chromosomal structure.

2. The histone proteins appear to be involved in the induction of banding patterns in plant chromosomes. An acidic maceration gave higher quality bands in H. vulgare and V. faba chromosomes although, in some instances, it prevented the banding of pericentromeric heterochromatin in these two species. That the acid maceration was influencing the bands through its effect on the protein constituent of the DNP fibre was apparent from the synergistic effect of trypsin and an acidic maceration combined; when acid was used, the concentration of trypsin needed to induce bands in H. vulgare chromosomes was lowered 40 fold.

3. Lotus pedunculatus

a. Four different Giemsa-banding techniques were tried on L. pedunculatus chromosomes but the only one which elicited bands in metaphase chromosomes was Schweizer's (1973) technique which entailed incubation in trisodium citrate (2XSSC). The chromosomes in the successfully banded metaphase complement were more contracted than those in the complements which showed no banding when pretreated by the other techniques. It is possible then, that L. pedunculatus chromosomes need to be super-contracted before they will band satisfactorily.

b. The banding pattern was homologous for each pair of L. pedunculatus chromosomes. In addition, all the chromosomes had intense pericentromeric bands and telomeric bands which were fainter than the pericentromeric bands. Chromosome 1 had a faint interstitial band in its long arm. Chromosome 2 had a faint band in the proximal region of its short arm which appeared as well in a chromosome 2 from an unstained, unpretreated

preparation. The telomeric and centromeric bands in chromosome 3 corresponded to areas which were more condensed in a prophase chromosome 3 than the rest of the chromosome. Chromosome 6 was probably heterochromatic since it took up the Giemsa dye intensely over its entire length and was fully condensed when the other chromosomes were not.

4. Hordeum vulgare (barley)

a. The response of barley's heterochromatin to five banding techniques revealed two major categories of Giemsa-positive heterochromatin: 1.) pericentromeric heterochromatin and 2.) interstitial, telomeric and peri-Nucleolar Organizing Region heterochromatin. The trypsin-Giemsa, trypsin and 2XSSC techniques produced bands in both types. Pretreatment with Sörenson's buffer only revealed the first category of heterochromatin while the Barium hydroxide-Saline-Giemsa technique banded only the second category. Trypsin-based Giemsa-banding techniques and incubation in 2XSSC appeared to give the broadest range of bands in barley.

b. A banded barley idiogram was assembled from a trypsin-Giemsa treated chromosome preparation. The banding pattern for each chromosome pair was homologous except for pair seven. Barley chromosomes had Giemsa-positive pericentromeric, telomeric and interstitial heterochromatin.

c. Unlike the rest of the Giemsa-positive heterochromatin, pericentromeric heterochromatin in several Monocot species, including H. vulgare, would not band if an acidic maceration was followed by an alkaline pretreatment or another acid pretreatment although it would band if the pretreatment were neutral. Pericentromeric heterochromatin was preferentially differentiated by phosphate buffer pretreatment irregardless of the maceration technique used.

5. The pericentromeric heterochromatin in Vicia faba was both acid sensitive and trypsin sensitive. This suggests that histone proteins were involved in the maintenance of condensation in V. faba's pericentromeric heterochromatin.

6. Vicia faba

a. Vicia faba chromosomes were stained according to the trypsin-Giemsa techniques using two different concentrations of trypsin. The bands in these karyotypes were compared with bands elicited in V. faba chromosomes by Döbel et al. (1973). The six categories of heterochromatin which were resolved depending on their response to these techniques were: pericentromeric heterochromatin which was positive with trypsin-Giemsa but undifferentiated in Döbel et al. (1973), the interstitial regions which stained positively with both techniques, and four groups of interstitial heterochromatin which in some instances stained less intensely than the euchromatin and were termed "negative bands".

b. The four negative bands in V. faba were categorized as follows: "Substitutions," interstitial bands staining positively in Döbel et al. (1973) and negatively with the trypsin-Giemsa techniques; "Interchangeables,": positive in Döbel et al. (1973), and negative or positive with the trypsin-Giemsa technique; "Adjacents," negative bands which appeared next to positive bands in trypsin-Giemsa treated preparations; "Uniques," heterochromatic regions which were undifferentiated in Döbel et al. (1973) and negative with the trypsin-Giemaa techniques. One of the "Unique" bands corresponded to a cold-reactive, GC-rich region. The lack of stain uptake in these heterochromatic regions might have been the result of the DNP fibre being so tightly coiled that there was not enough room for

the dye molecules.

c. The Nucleolor Organizing Region of V. faba also stained negatively with the techniques used in this study possibly because it is more highly condensed than the rest of the euchromatin. It may also have been GC-rich indicating that possibly GC-richness and highly condensed chromatin are correlated in V. faba chromatin.

7. Order within the interphase nucleus.

a. The telomeres and centromeres of barley anaphase and prophase chromosomes were polarized; the telomeres were all located at one pole, the centromeres at the other. This polarization probably persists during interphase. The centromeres and telomeres might have maintained this spatial arrangement during interphase by attachments to each other and to the nuclear membrane. Barley chromosomes contained Giemsa-positive pericentromeric and telomeric heterochromatin so that telomeric and pericentromeric heterochromatin might have played a role in these functions.

b. Interchromosomal connectives were observed between barley chromosomes and between Lotus pedunculatus prophase chromosomes. In L. pedunculatus these connectives were primarily telomeric; possibly the telomeric heterochromatin observed with Giemsa-banding is involved in the formation of these connectives.

c. Barley's chromosome 6 tended to form rings in trypsin-treated prometaphase chromosomes which suggested that there might have been some nonrandom interactions between the two telomeres of this chromosome during interphase.

d. An analysis of the barley banded karyotype revealed that all but the last pair of chromosomes were homologous for their banding patterns.

The simplest explanation of the heteromorphism of chromosome pair seven's banding pattern is that it involved a balanced translocation between the arms of a single chromosome.

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