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Chromosome Banding and Heterochromatin in *Vicia faba*

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Summary. The distribution of bands in *Vicia faba* (broad bean) root-tip chromosomes as shown by acid treatment, quinacrine mustard fluorescence, SSC-Giemsa banding and orcein banding is documented. These bands coincide with the position of heterochromatin revealed by cold treatment. Heterochromatin in the large M chromosome is located in two areas: (a) around the centromere and (b) adjacent to the secondary constriction. Heterochromatin in the smaller, sub-telocentric S chromosomes is located in the intercalary and proximal areas of their long arms and in the short arm of two chromosomes. Most of the observed bands were not exclusive to one treatment but could be recognized in chromosomes prepared by several methods. The variable expression of particular chromosome segments with different banding techniques testifies to the existence of several classes of heterochromatin.

Key words: *Vicia faba* – Heterochromatin – Chromosome banding

Introduction

With the advent of new chromosome banding techniques over the past decade, differentially stained regions have been revealed in many animal and plant chromosomes, some of which were hitherto unknown (Caspersson et al. 1968, 1969a, b; Drets and Shaw 1971). This discovery has necessitated a careful reappraisal of that portion of the genome termed heterochromatin, a word normally used to designate those regions of the chromosome which stain differentially and are considered to possess certain distinctive properties as, for example, late replication, relative genetic inertness and allocycly (Lima-de-Faria 1969).

The present investigation was undertaken to:

(1) Verify the position of heterochromatin on the chromosomes of *Vicia faba* as defined by the classical technique of cold induction of negative H-segments.

(2) Characterise the similarities and differences of chromosome band distribution in this species using acid treatment, fluorescent banding, SSC-Giemsa banding and orcein banding.

Results from these two areas of study are compared in an attempt to further our understanding of heterochromatin in *V. faba*. This study concentrates on the study of the M chromosome which is more readily identified, especially when using harsh banding techniques, and its banding patterns more distinctive.

Materials and Methods

Vicia faba var. 'Coles Early Dwarf' beans were germinated in vermiculite. Lateral meristems from 10-day old seedlings were excised and the following method applied routinely unless otherwise stated: 0.05% colchicine for 3.5 h, fixation in freshly prepared 1:3 glacial acetic acid/methanol overnight, 10% aqueous pectinase for 5 h at 37°C. A squash preparation was then made and the coverslip removed by the liquid nitrogen technique.

Cold Treatment

8-day old seedlings were transferred to 4°C for 3-4 days prior to colchicine pretreatment and fixation. Fixed root-tips were then stained by the conventional Feulgen technique.

Acid Treatment

Following colchicine pretreatment and fixation either (a) 1N HCl-45% acetic acid (1:9) treatment for 15 min at 60°C and then Feulgen staining or (b) standard Feulgen staining but with prolonged maceration (30 min) in 45% acetic acid were used.

Quinacrine Mustard (QM) Fluorescence

The method of Caspersson et al. (1968) was applied following pectinase treatment.

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SSC-Giemsa Banding

Squash preparations were treated with 2 × SSC (pH 7.0) at 65°C for 20 h, rinsed in three changes of distilled water, air dried, then stained with Giemsa (2ml of Gurr's improved R66 diluted with 2ml of 0.1M Sorensen's buffer, pH 6.9) for 2 h.

Lacto-Aceto-Orcein Banding

The method of Kurita (1958) was applied. After colchicine pretreatment, root-tips were washed briefly in de-ionized water, treated with 1N HCl-45% acetic acid (1:9) for 15 min at 60°C, macerated in 2% orcein in glacial acetic acid/85% lactic acid (1:1) for 15 min, then squashed. Some root-tips were fixed overnight in 1:3 glacial acetic acid/methanol after colchicine treatment, then hydrolysed and stained as above.

Results

Cold Treatment Bands

Cold treatment induces negative bands varying in clarity. The M chromosome shows two bands designated M1/1 and M2/2, one on each side of the centromere (Figs. 1-3) as has been documented by others (La Cour 1951; McLeish 1953; Evans and Bigger 1961; Grant and Heslot 1966; Takehisa et al. 1976). Some of the S chromosomes show a prominent single mid-band on their long-arms (Fig. 3) and a band is often revealed in the short arm of two chromosomes. Shorter colchicine treatment resulted in a higher frequency of less contracted complements, which showed more negative bands.

Acid Treatment Bands

Acid treatment reveals three negative bands on the M chromosome, two corresponding in position to those revealed by cold treatment, the third (M2/1) arising on the M2 arm close to the centromere (Figs. 1, 4). After standard Feulgen staining, prolonged maceration (> 30 min) of root-tips in 45% acetic acid will also induce the appearance of band M1/1 and occasionally M2/1 and M2/2. These bands (M1/1, M2/1 and M2/2) are equivalent to bands 1, 2 and 3 respectively, of Takehisa (1973). The S chromosomes do not show negative banding except for an achromatic band which commonly appears on the short arm of two chromosomes.

Quinacrine Mustard Fluorescence

This technique, first introduced in *V. faba* by Caspersson et al. (1968), consistently reveals the M1/1 band which is much brighter than the rest, and also bands M2/1 and M2/2 (Figs. 1, 5). Occasionally an indistinct band (M2/3) is produced just distal to M2/2 (Fig. 6). A slight increase in fluorescence is often found close to the centromere on the long arms of some S chromosomes.

SSC-Giemsa Banding

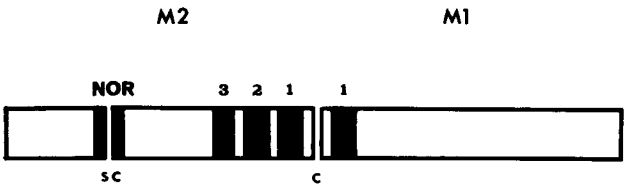
Four bands are located around the M chromosome centromeres (Figs. 1, 7). The M1/1 band is prominent, while on the M2 arm M2/1, M2/2 and M2/3 can be resolved. In most cases M2/2 and M2/3 merge to form one larger band (wider band in Fig. 7). The S chromosomes show a complex banding pattern. All display, to varying degrees, differential staining in the mid-region of their long arms and adjacent to and on the long arm side of their centromeres.

Lacto-Aceto-Orcein Banding

The LAO method produces distorted chromosomes. In clear spreads, however, a distinctive pattern of chromosome banding is seen on the M chromosome (Figs. 1, 8, 9). Consistent with acid treatment, M1/1, M2/1 and M2/2 occur as negative bands and, in contrast, a positively-stained band is present on either side of and directly adjacent to the secondary constriction. The length of the secondary constriction varies such that the flanking bands commonly merge and are mostly seen as one (the NOR band, Fig. 10). The rest of the chromatin is lightly stained.

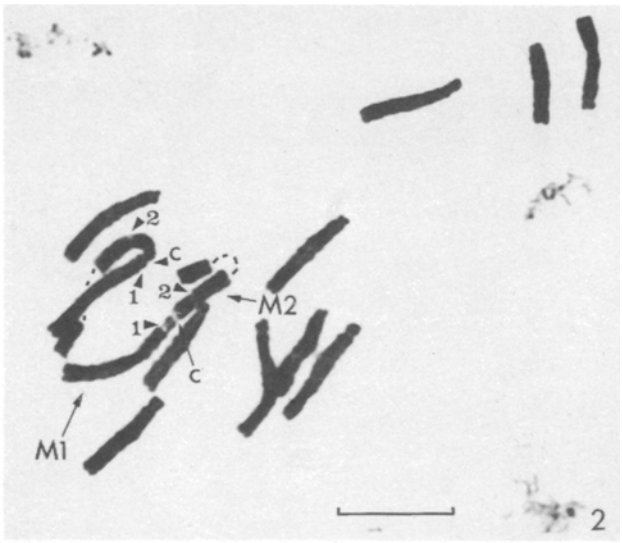
Phase contrast microscopy indicates that this banding pattern is produced prior to staining, i.e. after 60°C hydrolysis (Fig. 11). At this stage all chromosome arms are faint but the M chromosome has a distinct NOR band. Subsequent orcein staining reveals a general reduction in stain intensity compared to standard fixed orcein stained chromosomes (see below), while the NOR band is stained normally and hence is differentiated. Interphase nuclei show two chromocenters in contact with the nucleolus (Fig. 10).

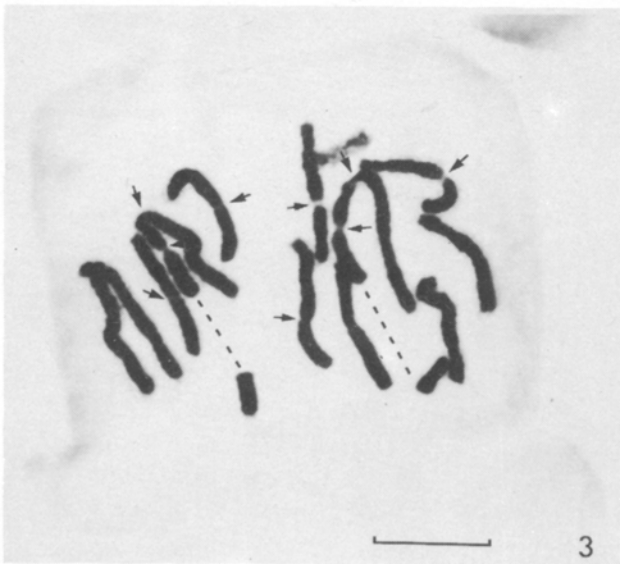
Standard fixed root-tips show the M chromosome with the three negative centromeric bands, more apparent as a result of the strong contrast with the now darkly-stained chromosome arms (Fig. 12). The NOR band is indistinguishable by phase contrast microscopy in preparations of these root-tips prior to staining. Interphase nuclei, unlike unfixed preparations, show chromocenters as in standard Feulgen preparations (i.e. up to 14 in number and varying in size).



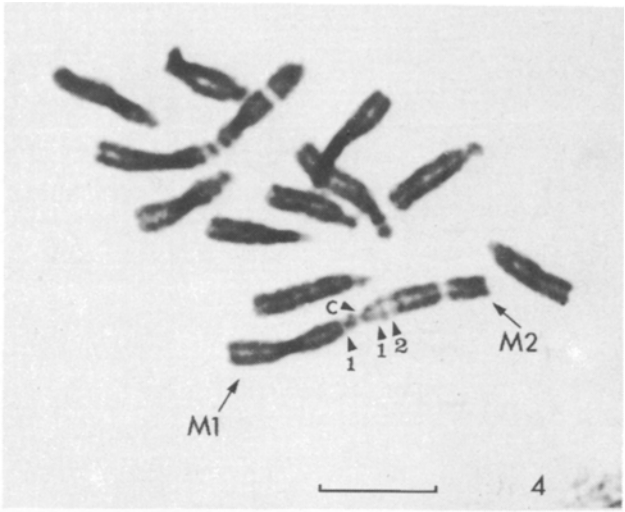
cold treatment	M1/1, M2/2
acid treatment	M1/1, M2/1,2
quinacrine mustard	M1/1, M2/1,2,3
ssc-Giemsa	M1/1, M2/1,2,3
orcein banding	M1/1, M2/1,2 NOR

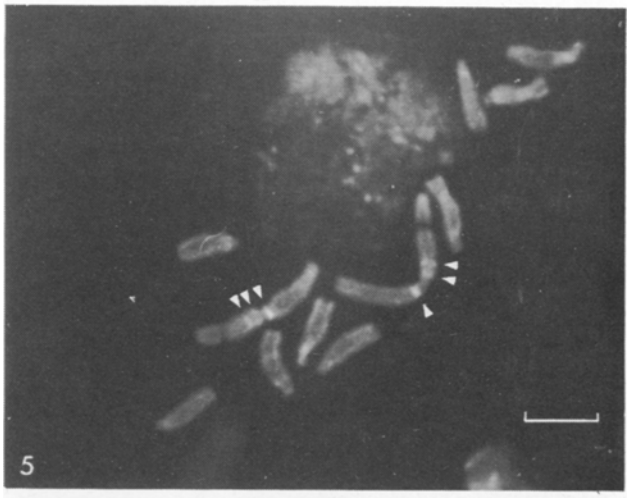
Fig. 1. Representation of the band pattern revealed on the M chromosome by different banding techniques

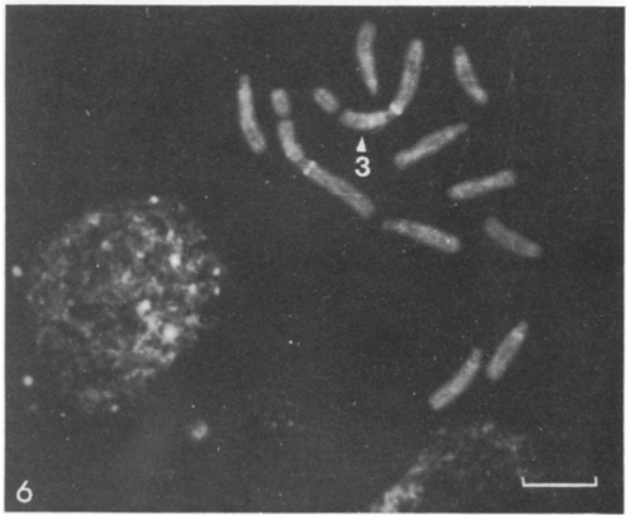




Figs. 2 and 3. Cold-treated, Feulgen-stained squashes. **2** Achromatic bands (M1/1 and M2/2), one on either side of each centromere (c). **3** Less contracted complement with many achromatic regions along the chromosome arms; identifiable bands arrowed. Bar = 10 μm

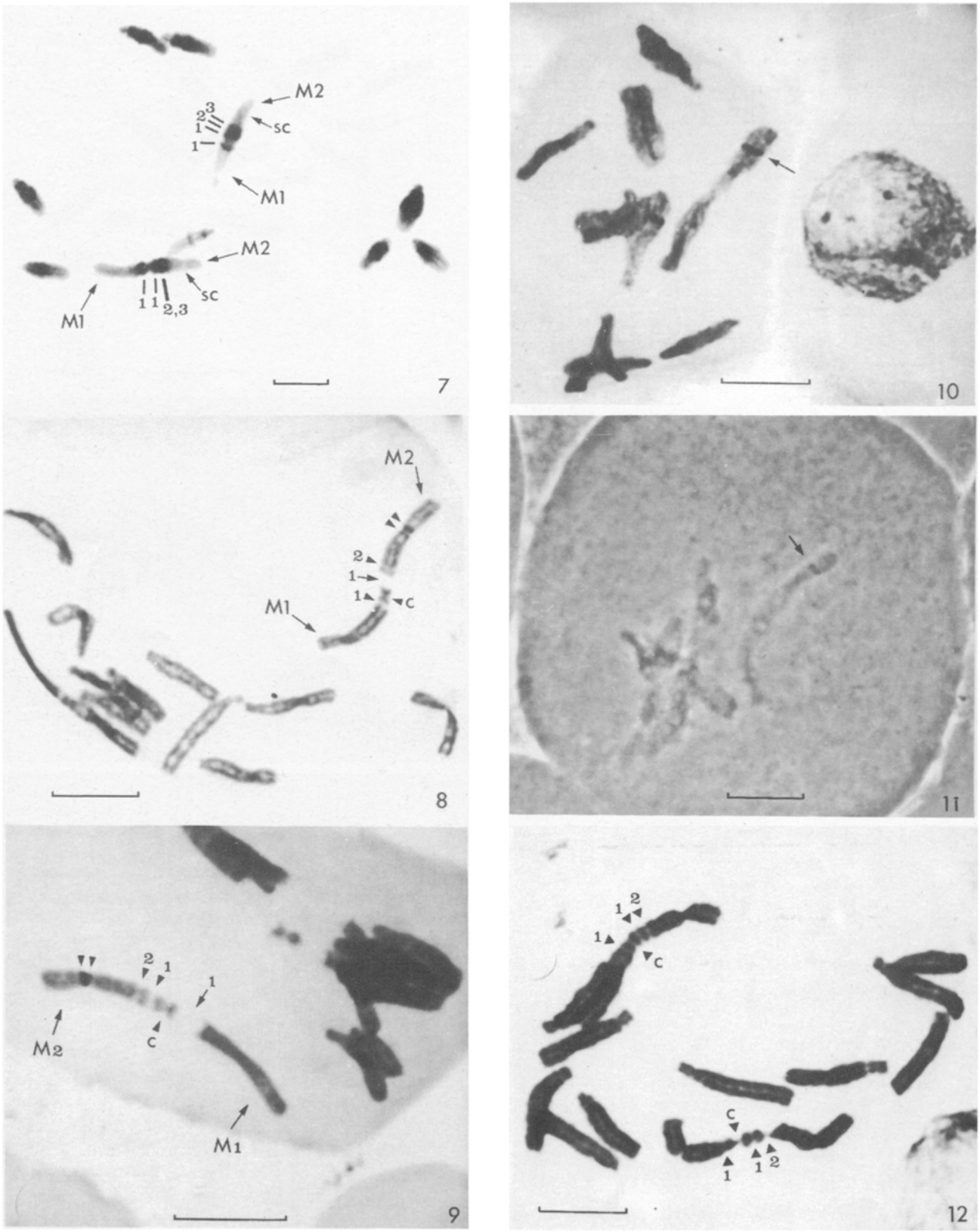






Figs. 4-6. Bar = 10 μm **4** Acid-treated, Feulgen-stained squash. Three achromatic bands (M1/1, M2/1, M2/2) neighbour the M centromere.

5 and 6 Quinacrine mustard fluorescent squashes. **5** Three bands (M1/1, M2/1, M2/2) neighbour the centromere on each M chromosome. **6** A third M2 band (M2/3) is on the chromatids of one M chromosome



Figs. 7 - 12. Bar = 10 μm 7 SSC-Giemsa squash. Four bands (M1/1, M2/1, M2/2, M2/3) neighbour the centromere on each M chromosome. sc = secondary constriction.

8-11. Lacto-Aceto-Orcein banded squashes. **8** Positive bands flank the secondary constriction of the upper M chromosome. Two negative bands (M1/1, M2/2) neighbour the centromere (c) with a break at M2/1; centromere position is judged from cold treatment and acid treatment studies. **9** Two bands are positioned at the secondary constriction and two negative bands neighbour the centromere (c) with a break at the M1/1 region. **10** Common observation of the flanking secondary constriction bands as one band, the NOR band (arrow). Interphase nucleus shows two chromocenters associated with the nucleolus. **11** Unstained hydrolysed squash. NOR band is arrowed (phase contrast microscopy). **12** Squash of standard fixed root-tip treated with LAO method. Achromatic bands are arrowed on the M chromosome. c = centromere

Discussion

A central question in the present investigation is whether the bands shown by acid treatment, quinacrine mustard fluorescence, SSC-Giemsa banding and orcein banding, demonstrate the presence of heterochromatin. The following discussion sets the criteria for detecting heterochromatin and questions the heterochromatic character of chromosome bands shown by the various banding techniques in the present study.

Methods for detecting heterochromatin include the following:

(1) direct visualization by staining. Those chromosome regions which demonstrate heteropycnosis (differential condensation) are judged to be heterochromatic. These regions are seen as chromocenters in interphase nuclei or differentially condensed areas in prophase chromosomes. Ohno et al. (1957) clearly recognized heterochromatin in the proximal regions of mouse prophase chromosomes by this method. (2) use of cold treatment; seen as negatively-stained cross-bands in metaphase chromosomes. (3) use of autoradiography to detect late DNA-replicating regions.

Heterochromatin is known to possess other attributes, such as relative genetic inertness, low or high recombination, it exerts position effects and it can show ectopic pairing. Not unexpectedly, there are exceptions in behaviour to some of the above features (Lima-de-Faria 1969). It is upon a combination of these criteria, however, that a chromosome band may be judged as being heterochromatic.

Bands M1/1 and M2/2 are revealed by cold treatment and are seen by acid treatment, QM fluorescence, SSC-Giemsa banding and orcein banding. The remaining M centromeric bands (M2/1 and M2/3) are not revealed by cold treatment but M2/1 is seen by all the other methods whereas M2/3 is seen only by the quinacrine mustard and SSC-Giemsa banding techniques. That the last two bands are not shown by cold treatment may reflect on the inadequacy of this technique for defining all heterochromatin. This would seem true especially for band M2/1. Also, in the *V. faba* M chromosome, acid treatment bands, QM bands, Giemsa bands (as shown by the regime used in this study) and orcein bands, are all localized within the late-replicating region (Woodard et al. 1961; Evans 1964) except for the NOR band. Therefore, given that there are differences of band expression within this limited region with specialized techniques, all the M centromeric bands revealed (M1/1, M2/1, M2/2 and M2/3) are judged to represent the position of heterochromatin.

The NOR band is one of the classical examples of nucleolus-associated heterochromatin described by Heitz (1928, 1929) and later studied by Caspersson and Schultz (Caspersson 1950). This is seen convincingly in Figure 10 where the two chromocenters of the nucleus associated with the nucleolus represent the NOR heterochromatin from the two M chromosomes. NOR heterochromatin in *V. faba* differs from the rest of the heterochromatin in not replicating late (Burger and Scheuermann 1974), and being located on either side of the secondary constriction it would escape detection by cold treatment.

The significance of the Lacto-Aceto-Orcein banding technique is that it reveals two classes of heterochromatin simultaneously on the large *V. faba* M chromosome. NOR heterochromatin stains positively and centromeric heterochromatin stains negatively. NOR band chromatin is resistant to acid hydrolysis, whereas centromeric band chromatin is sensitive, which implies some structural difference between the two regions elicited by this technique. Furthermore, it is because chromosomal material has been removed that the structural differences are manifest rather than a qualitative change in these two regions alone. Hydrolysis of the nucleic acids by the strong acids involved would undoubtedly have an important effect on chromosome architecture but it is difficult to envisage how this could lead to specific banding. An alternative proposition is that LAO banding is related to protein complexes and their binding to DNA, with the possible removal of a histone-type protein. Histones play an important role in maintaining chromosome structure (Bonner and Garrard 1974) and their removal may account for the distortion witnessed by this technique without standard fixation. Should any proteins remain, one might expect the NOR band to be constructed of acidic proteins or of a histone-type protein resistant to the acid procedure. Mattingly (1963) interestingly discovered lysine-rich areas on perinucleolar chromatin in *V. faba*. She deduced that because the nuclear histones are known to have greater stability than the residual proteins and because a major constituent of the histone proteins is lysine, there is a possibility that the lysine fraction is a part of the histone moiety in this portion of the genome. If it is part of the NOR band then this lysine-rich protein must be of the acid resistant kind and be bound in a very tight manner.

If differential banding is achieved by the removal of a histone-type protein this has implications as regards the structure of Giemsa bands. The SSC-Giemsa banding regime reveals Giemsa bands on the *V. faba* M chromosome in regions which coincide with the negative centromeric bands seen by the LAO banding technique. This would suggest that Giemsa bands as seen by the SSC-Giemsa technique are composed of a basic histone-type protein.

The negatively-stained band seen on the small arm of one or two S chromosomes after acid treatment is also seen after cold treatment. And in most S chromosomes there appears to be a correlation between the position of discrete Giemsa bands in the intercalary regions of their long arms and the location of cold bands, which in turn correspond to the location of late-replicating DNA (Woodard et al. 1961; Evans 1964). Those bands seen in the S chromosomes by acid treatment, QM fluorescence and Giemsa banding are also judged to represent the position of heterochromatin.

No differential banding was seen in the S chromosomes by the LAO technique or in their long arms after fixed

acid treatment. Whereas the NOR band is resistant to these methods and M centromeric band chromatin very sensitive, S chromosome heterochromatin (in the intercalary regions of the long arms) is indistinguishable from all the remaining chromatin and tolerably resistant to acid hydrolysis enough to contribute towards chromosome recognition. That S chromosome heterochromatin should behave in this fashion complicates speculation on its properties. Being heterochromatin one might expect it to behave like M centromeric band chromatin. This suggestion is based on cold treatment and late-labelling observations of heterochromatin expression and behaviour in *V. faba*. One can only reflect that whereas M centromeric band chromatin and NOR band chromatin possess different properties as manifest by the LAO method, S chromosome heterochromatin has, with respect to this treatment, similar properties to euchromatin.

All the chromosome bands revealed in this study by acid treatment, quinacrine mustard fluorescence, Giemsa banding and orcein banding are judged to represent the position of heterochromatin in *Vicia faba* chromosomes. Studies of kangaroo rat chromosomes (Bostock and Christie 1974) and *Trillium* and *Cypripedium* chromosomes (Yamasaki 1971), however, indicate that there may be several different states or degrees of heterochromatin. Greilhuber (1975) also addresses the question of heterochromatin heterogeneity in *V. faba* chromosomes. The evidence of the present work supports this view. Bands M1/1 and M2/2 may represent the extreme case of heterochromatin shown by nearly all techniques, whereas M2/1, M2/3 and the NOR band represent different classes of heterochromatin revealed only by specialized techniques. Intercalary bands on the S chromosomes are shown to varying degrees by various banding techniques. In summary, most of the observed bands were not exclusive to one treatment but could be recognized in chromosomes prepared by several methods. The variable expression of different bands with particular banding techniques testifies to certain architectural differences between banded regions, which imply the existence of several classes of heterochromatin.

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