A Cytogenetic Study of Factors Affecting Sister Chromatid Differentiation in *Vicia faba* and *Hordeum vulgare*

by

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ABSTRACT

A study of the cytogenetic factors affecting sister chromatid exchange was undertaken due to difficulties encountered in obtaining sister chromatid differentiation in *Hordeum vulgare* L. (barley) and *Vicia faba* L. (broadbean). The following variables were studied: (1) temperature and duration of acid hydrolysis in the Feulgen procedure, (2) the use of an RNAse treatment and a trypsin digestion in the fluorescent-plus-Giemsa (FPG) technique, (3) the growth and treatment of *Vicia* seedlings, (4) the concentration and substitution of the base analogue 5-bromodeoxyuridine (BrdU), and (5) the duration of root tip fixation and cell wall maceration. Treatments such as the removal of seedling shoots or cotyledons, and the application of BrdU, led to a reduction in cell division in most plants. The staining protocol was modified to minimize these effects. Duration of fixation was found to significantly affect the quality of staining. A fixation time of seven hours was recommended. Good differential staining was obtained in *Vicia* and in barley by means of the Feulgen procedure, but not the FPG technique.

RESUME

Une étude des facteurs cytogénétiques affectant l'échange des chromatides soeurs, a été entreprise afin de pallier aux difficultés rencontrées lors de colorations différentielles des chromatides soeurs de l'Orge (Hordeum vulgare) et de la Fève (Vicia faba). L'étude a porté sur les facteurs suivants: la durée et la température à laquelle a lieu l'hydrolyse acide dans la méthode de Feulgen, la digestion par trypsine et ARNase dans la technique fluorescence-plus-Giemsa (FPG), la concentration et l'incorporation de la base analogue 5-bromodésoxyuridine (BrdU), la durée de la fixation des méristèmes apicaux radiculaires et la maçération de la paroi cellulaire.

Des traitements tels que l'excision de la tigelle ou des cotylédons ou l'application de BrdU ont conduit à une diminution du rythme de la division cellulaire. Conséquemment, le protocole de coloration a été modifié afin de minimiser ces effets. Il fut trouvé que la durée de fixation a un effet significatif sur la qualité de la coloration. Une durée de fixation de sept heures est recommandée.

De bonnes colorations différentielles ont été obtenues à la fois chez l'Orge et la Fève en utilisant la méthode de Feulgen mais aucune coloration satisfaisante n'a été obtenue avec la technique FPG.

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INTRODUCTION

The legume species, Vicia faba L., has been used by many workers in cytogenetic and mutagenic studies. Its advantages for use in mutagenesis have been cited by many individuals (Kihlman, 1971, 1975b; Grant et al., 1981; Constantin and Owens, 1982; Grant and Zura 1982; Ma, 1982; Uggla and Natarajan, 1982; Ma and Harris, 1985). Generally, it is the root tips and the rapidly dividing cells which make up the root meristem that are used in these studies. Among factors to which its popular use has been attributed are its small chromosome number ($2n=12$), chromosomes which are relatively large and easy to identify, and the fact that the cytology and physiology of this plant have been thoroughly investigated (Read, 1959; Chapman, 1983). The mitotic cycle in Vicia faba ranges between 18-22 h at 19 C (Kihlman, 1971). The methodology for germination is relatively simple and quick. Roots can be ready for treatment between 8-12 days after germination begins.

Sister Chromatid Exchange (SCE) is an event that takes place at the chromatid level, in which segments of double stranded DNA are exchanged between identical chromatids. These exchanges have been rendered visible in cytological studies by the unifilar or bilfilar incorporation of uridine and thymidine analogues into the chromosome such that the chromatid which has undergone replication during the previous S phase is differentially substituted with a base analogue before the dividing cells are fixed. Thus one of the chromatids stains differentially in relation to its sister chromatid (Wolff, 1977; Latt, 1981; Lambert, 1984).

A number of cytological techniques have been used to visualize SCE. From among these, two staining methods will be examined in this work. Both

methods take advantage of the differential properties of DNA which has been partially substituted with the halogenated nucleoside, 5-Bromo-2'-deoxyuridine (BrdU), a base analogue which substitutes for the normal nucleoside thymidine in a replicating DNA strand. When BrdU is incorporated during the first of two consecutive rounds of DNA synthesis, the chromosomes contain one unifilarly substituted and one bifilarly unsubstituted chromatid. This may be referred to as TT-TB substitution, where the letter T represents a thymidine-containing single strand of DNA, and the letter B represents the BrdU substituted single strand of DNA. Chromosomes which have replicated in the presence of BrdU for two cell cycles contain one unifilarly substituted and one bifilarly substituted chromatid. Likewise, this may be referred to as BB-BT substitution (Block, 1982).

The first of the two methods to be examined in this thesis is the Fluorescent-plus-Giemsa (FPG) technique. It is perhaps the one most widely reported in the literature and employs two different dyes, both which stain DNA; Giemsa, a mixture of methylene blue and its oxidation products, the azures and eosin Y (Sharma and Sharma, 1972) and the fluorescent dye "33258 Hoechst" (Perry and Wolff, 1974; Kihlman and Kronborg, 1975, Schubert et al., 1979, 1980; Grant et al., 1981; Cortes and Andersson, 1987). The second method which will be examined, the Feulgen procedure for sister chromatid differentiation, is based on a combination of extended acid hydrolysis of the chromatin and fuchsin staining (Vosa, 1981; Tempelaar et al., 1982).

While the biological mechanism by which SCEs are formed is not yet understood, SCE frequencies are used as a measure of S-independent chromosome damage, that is, chromosome damage that does not require DNA

synthesis for its manifestation. Vicia faba has been used as a test organism in a number of mutagenic studies in which SCE is the assay used as a indicator of chromosomal mutagenicity (Kihlman, 1975a; Kihlman and Sturelid, 1978; Andersson et al., 1981; Andersson, 1981; Andersson, 1985). The earliest evidence for chromosome damage in plants as a result of chemical exposure was in 1943, when Oehlkers showed that ethylurethane produced translocations in the meiotic cells of Oenothera and Antirrhinum (cited by Auerbach, 1976).

More recently a number of bioassays have been developed in higher plants to assess the chromosome damaging and/or mutagenic effects of chemical substances. Among the plants used in such bioassays are Allium cepa, Aribidopsis thaliana, Crepis capillaris, Glycine max, Hordeum vulgare, Tradescantia paludosa, Vicia faba, and Zea mays. Indigenous populations such as ferns have also been used to carry out in situ monitoring of environmental contamination (Klekowski, 1978).

A number of genetic end-points have been studied including chromosome alterations and aberrations in both meiotic and mitotically dividing cells. The general effect on chromosome segregation and mitotic function is also studied. A number of plant bioassays detect specific gene mutations at previously identified genetic loci. The advantages cited for the use of higher plants in bioassays for mutagenicity, are the morphological similarity of plant and animal chromosomes (Constantin and Owens, 1982), the fact that they appear to respond similarly to other eukaryotic organisms when exposed to mutagens (Constantin and Owens, 1982; Grant, 1982a; Grant and Zura, 1982; Dean, 1983), and that as bioassays, they are rapid and inexpensive and do not require extensive laboratory facilities (Shelby,

1980; Grant, 1982b; Ma, 1982). Finally, a number of genetic end-points are available. Among the disadvantages cited, are the lack of knowledge about the array of metabolic processes which are unique to plants, as well as the structural differences found between plant and animal cells such as the presence of a thick cell wall in the former (Kihlman, 1975b; Nilan, 1978; Dean, 1983).

In comparison to animal studies, the number of plant studies using SCE as a genetic endpoint are few, despite the fact that in mutagenic studies plant cells have shown similar response to their mammalian, insect and bacterial counterparts (Nilan, 1978; Grant, 1982a; Grant and Zura, 1982; Uggla and Natarajan, 1982; Constantin and Owens, 1982; Dean, 1983). However, the growing interest in the significance of plant metabolism in environmental mutagenesis, indicates that the role of plants in the development of mutagenicity bioassays is a growing one (Gentile et al., 1982; Uggla and Natarajan, 1982; Wildeman and Nazar, 1982; Plewa et al., 1983; Gentile et al., 1985; Takehisa, 1986).

The variations in the staining techniques for SCE in Vicia faba given in the literature are quite numerous (Kihlman and Kronborg, 1975; Scheid, 1976; Vosa 1976; Andersson, 1981; Grant et al., 1981; Tempelaar et al., 1982; Vosa, 1981; Cortes and Andersson, 1987;) and are thought by some to be an indication that technical problems have not yet been solved (Goto et al., 1978). Schvartzman and Cortes (1977) suggested that perhaps the reason the general mechanism of sister chromatid differentiation still remains obscure, is due in part to the number of modifications in the SCE techniques, in which each worker considers his own modification essential for obtaining good differential staining.

Among the problems encountered in obtaining SCE in plants noted by some authors, are (1) the poor incorporation of the base analogue BrdU by plant cells (Haut and Taylor, 1967; Kihlman and Kronborg, 1975; Vosa, 1976; Evans and Filion, 1980; Gonzalez-Gil and Navarrete, 1982; Uggla and Natarajan, 1982; Kihlman and Andersson, 1982, Cortes and Andersson, 1987), (2) the fact that low concentrations of BrdU (20-30 uM) yield poor sister chromatid differentiation (Evans and Filion, 1980), while higher concentrations (100-150 uM) yield a significantly lower proportion of metaphase cells per slide (Evans and Filion, 1980; Tempelaar et al., 1982; Kihlman and Andersson, 1984), (3) the difficulty in squashing the treated root tips and thus in obtaining good chromosome preparations due to the presence of the cell wall (Kihlman, 1975b; Vosa, 1976; Gonzalez-Gil and Navarrete, 1982; Kihlman and Andersson, 1982), (4) distortion of chromosome morphology, or loss of staining intensity due to the use of enzyme treatments whose purpose is to soften the cell wall (Kihlman, 1975b; Cortes et al., 1980; Tempelaar et al., 1982; Kihlman and Andersson, 1984), and (5) loss of differential staining due to the effect of impurities which may be present in these enzymes (Kihlman and Andersson, 1984). Kihlman and Kronborg (1975) noted that irregularities in the staining of plant chromosomes may be a problem with Giemsa, while other workers (Scheid, 1976; Scheid and Traupe, 1977) failed to obtain sister chromatid differentiation in Vicia faba without the prior proteolytic digestion of the chromosomes with trypsin. Finally, Kihlman and Andersson (1984) stated that the root meristem contains different types of cells, with mitotic cycles of different durations, and perhaps different sensitivities to chemical treatments.

In the original thesis proposal, I planned to use SCE as a genetic end

point to assay levels of DNA damage due to the application of a number of pesticides. However, while SCEs where obtained in Hordeum vulgare (barley) using the Feulgen procedure for SCE, it was impossible to obtain consistent results in replicate experiments. The various FPG techniques proved to be even less dependable. The techniques used and the problems encountered in the production of SCE in the initial work on this project indicated that a clear and careful study of a number of the steps involved in these procedures might shed interesting light on the inherent difficulties which were encountered in attempting to obtain sister-chromatid differentiation in plant cells. Thus, the present study was initiated in order to examine a number of the factors which might be related to the difficulties encountered in the Feulgen staining procedure and in the Fluorescence-Plus-Giemsa staining procedure for sister-chromatid differentiation in Vicia faba.

LITERATURE REVIEW

Early Studies of Sister Chromatid Exchange

According to Schvartzman et al. (1979b), SCE was inferred by McClintock as early as 1938 in her study of ring chromosome behavior in maize. Twenty years later SCE was detected for the first time by Taylor and workers in Vicia faba (1957) after he incorporated tritiated thymidine into the plant chromosomes and noted, "a chromatid might be labeled along only part of its length, but in every such instance the other (sister) chromatid was labeled in the segment lying opposite the unlabeled segment." In a later experiment with Bellevalia romana (Taylor, 1958), chromosomes were differentially labeled with tritiated thymidine and analyzed by means of autoradiography. By examining SCE frequencies in each of the four pairs of Bellevalia chromosomes, Taylor observed that the frequency of exchanges was nearly proportional to length. This work not only provided an important foundation for the study of the SCE phenomenon, but provided important insights into the structure and replication of eukaryotic chromosomes.

Subsequent to the cytogenetic studies carried out in ring chromosome behavior in maize by McClintock (1938) and others, the history of the investigations of SCE can be divided into an early period, when studies such as Taylor's were carried out by means of autoradiography, and the current period in which DNA is labeled with BrdU and various staining procedures are employed (Zakharov, 1982). Important questions, such as the significance of isolabelling, and the spontaneity of SCE were first raised in the early period. These questions and others, including the mechanism which causes the formation of SCEs, are still being discussed in the current period.

The current period began in 1972 when Zakharov and Egolina obtained chromatid differentiation in Chinese hamster ovary (CHO) cells using BrdU. Zakharov (1982) noted that an earlier worker (Huang, 1967) observed the "negatively heterochromatic appearance" of one sister chromatid in some chromosomes, in cells of Rattus natalensis, after treatment with BrdU. In their original work, Zakharov and Egolina (1972) showed that the cytological effect of BrdU treatment when followed by azure eosin staining, was the appearance of "unequal spiralization" of sister chromatids. They noted that the frequency of metaphases with differentiated chromosomes increased with increasing doses of base analogue. They also showed that the effect of double BrdU incorporation (BB-BT substitution) was an increased "delay of spiralization" in one of the two sister chromatids. Zakharov and Egolina attempted to explain the spiralization delay in one of a pair of sister chromatids (differential staining of sister chromatids), as either the result of transient inhibition of DNA synthesis, or the inhibition of protein synthesis which contributes to the condensation of the chromosomal DNA.

Following the study of Zakharov and Egolina, Latt (1973) used the base analogue 5-bromouracil and rendered human leukocyte chromosomes visible by means of "33258 Hoechst". With this fluorescent dye, Latt demonstrated that chromosome fluorescence decreased with increased BrdU substitution. Latt emphasized the effect of the incorporated BrdU alone and suggested that effects due to chromosome uncoiling or the influence of proteins on DNA uptake of "33258 Hoechst" were of secondary importance.

In 1974 a number of workers published results in which various methods were applied to the differential staining of sister chromatids. Kato

(1974b) used a method to detect SCE in CHO cells which involved the staining of BrdU labelled DNA with acridine orange, a fluorescent dye. Kato does not suggest a mechanism for the differential staining which he obtained.

Dutrillaux and workers (1974) studied the evolution of SCEs in successive cell generations, as well as the frequency of SCEs in human leukocytes by means of acridine orange fluorescence.

Ikushima and Wolff (1974) showed that the incorporation of either BrdU or 5-iodo-deoxyuridine (IUdR) during two rounds of DNA replication in CHO cells caused sister chromatid differentiation both in staining properties and chromosome morphology. These workers, using either azure A or Giemsa, noted that although treatment with base analogues may alter the DNA either physiochemically or structurally, the differential staining which they observed seemed to be related to the concentration of stain and the duration of staining, such that at high concentrations of stain or with prolonged staining, all chromatids were uniformly stained, regardless of differential substitution with base analogues.

Korenberg and Freedlender (1974) also used Giemsa to visualize sister chromatid differentiation and SCEs in CHO cells and in human peripheral lymphocytes in both singly and doubly BrdU substituted chromosomes. They noted that chromatids which are differentially substituted with BrdU show differential affinities for Giemsa stain after hot 1 M NaH_2PO_4 buffer treatment and suggested that the differential staining may reflect an underlying structural difference between chromatids, such as the removal of proteins.

Perhaps the most notable innovation among the techniques published during this period was introduced by Perry and Wolff in 1974. Working with

CHO cells, they combined the fluorescence of "33258 Hoechst" and the staining effect of Giemsa. The new technique was called fluorescent-plus-Giemsa or FPG. According to the authors this technique had all the advantages of the fluorescent techniques reported previously but with the added advantage of producing permanently stained slides.

Sister Chromatid Exchange in Plants

The FPG technique has subsequently been used by a great number of workers and a number of modifications have continued to be applied in plants. The first workers to do so were Kihlman and Kronborg (1975) who modified the FPG technique for use in Vicia faba root tip cells. The modifications employed by the authors include the following: 1) addition of fluorodeoxyuridine (FdU) to the BrdU solution to supress the cellular synthesis of thymidylic acid and thus stimulate the uptake of the base analogue, BrdU, by replicating DNA, 2) maceration of cell walls with the enzyme pectinase to obtain sufficient spreading of the metaphase chromosomes, 3) use of an RNAse treatment on cell squashes before staining, to clear cytoplasmic and nuclear RNA which stain with Giemsa and, therefore, may obscure chromosomes from observation, and 4) increased duration of Giemsa staining.

Using Vicia faba as the experimental material, Scheid (1976) obtained differential staining using either the fluorescent dye acridine orange or Hoechst 33258, followed by treatment with trypsin. Scheid noted that during observation with a fluorescence microscope the staining capacity of these dyes rapidly diminished during simultaneous treatment of chromosomes for extended periods of time with a trypsin solution. This work is in agreement

with earlier work carried out by Pathak and colleagues (1975) in which Giemsa staining was combined with a trypsin treatment to give differential staining in cultured mammalian cells. Pathak noted that continued treatment of the mammalian cells with trypsin obliterated sister chromatid differentiation. To explain this phenomenon, Scheid suggested that acridine orange, combined with visible light, induces single strand breaks in BrdU substituted DNA resulting in the destruction of this DNA upon digestion with trypsin. In his study, Scheid investigated the effect of UV light, known to induce single strand breaks in BrdU substituted DNA (Scheid, 1976), on the disintegration of bifilarly substituted chromatids during trypsin digestion of chromosomal proteins in the absence of fluorescent staining. The results of this work indicate that simultaneous treatment of BrdU substituted chromosomes with UV and trypsin is sufficient to cause the dislocation of broken particles of DNA in the bifilarly substituted chromatid, allowing sister segments differentiation as hypothesised. Scheid concluded that acridine orange plus visible light has essentially the same effect as UV light and thus causes differential staining to be visualized in the same fashion.

Scheid and Traupe (1977) followed this study with an investigation of the effects of cysteamine and potassium iodide in the same system. The authors hypothesised that substances such as cysteamine and potassium iodide, which have a reducing effect on BrdU radicles (Scheid and Traupe, 1977), might inhibit break formations in the sulphur bonding of DNA specific proteins, and in turn might reduce differential chromatid staining. Chromosomes were treated as before with trypsin and a photosensitive dye and either cysteamine or potassium iodide was added to the dye-trypsin solution.

Thus cysteamine or potassium iodide was present when the BrdU substituted DNA was irradiated with visible light. These authors found that differential staining was completely suppressed by cysteamine and reduced approximately three quarters by potassium iodide. They concluded that these results lend further support to the hypothesis that rapid dissolution of bifilarly substituted chromatids, as compared to unifilarly substituted chromatids, is due to single strand breaks induced by a photosensitive dye-visible light system.

Differential staining of sister chromatids with the FPG technique was obtained in Allium cepa chromosomes by Schvartzman and Cortes in 1977. In their paper a comparison is made between chromosomes which have undergone one round of replication in the presence of BrdU and those which have undergone two rounds. Several conclusions were reached in that study. The authors noted that differential staining is equally good whether chromatids are unifilarly or bifilarly substituted. With respect to fluorescent staining (Hoechst 33258), they stated that the minimum duration required is 0.5 h and that immediate exposure of slides thereafter to UV light improves differential staining significantly. They also showed that in cells which have undergone only one round of replication in BrdU, the mean value of SCEs per chromosome was one half the mean value of cells which were exposed to the same treatment for two consecutive cycles.

The FPG technique for SCE was applied to Secale cereale (rye) by Freibe in 1978 and to Zea mays by Chou and Weber in 1980. In the work of Chou and Weber, it was demonstrated that although supernumerary B chromosomes increased the total amount of intergenic recombination in maize by causing an increase in recombination, no significant increase in SCE

frequencies was seen in plants with B chromosomes. Thus the authors suggested that the two phenomena occur by different mechanisms.

Schubert and workers applied the FPG technique to barley in 1980, using a modified version of the FPG technique of Kihlman and Kronberg (1975). This modification consisted primarily in increasing the BrdU treatment solution from a concentration of 100 uM to 500 uM. In the former study the frequency and the intrachromosomal distribution pattern of SCEs in barley chromosomes was reported. The authors divided the 14 chromosomes into 48 segments and found that the frequency of SCE in each of these 48 segments was proportional to length.

Evans and Filion (1980) used the BrdU-FPG staining technique for differential staining of sister chromatids to estimate the cell cycle time in Zebrina pendula. They used Vicia faba to verify their findings since its cell cycle had previously been well established.

In 1981 Grant and workers published a modified FPG technique for Vicia faba, which was similar to the technique of Kihlman and Kronborg (1975) except that instead of applying stains directly to root tip squashes, whole root tips were treated and maceration and squashing were carried out after staining. At about the same time, two workers independently published papers describing a Feulgen staining procedure for visualizing SCE in Vicia faba. In the classical Feulgen reaction it is thought that partial acid hydrolysis of the chromatin creates free aldehyde groups on the deoxyribose backbone of the DNA to which the fuchsin binds (Sharma and Sharma, 1972). In the case of Feulgen staining for SCE this hydrolysis reaction is prolonged to the point where the least substituted chromatid is more readily degraded by the acid treatment and thus stains more lightly (Vosa, 1981;

Tempelaar et al., 1982).

In 1983 Cortes and workers reported SCE in two additional Allium species, Allium ascalonicum and Allium sativum, using the BrdU-FPG staining method. Working with a third Allium species, Allium cepa, in which SCE had already been obtained, they attempted to analyze the relationships between DNA content, constitutive heterochromatin, and the frequency of SCEs in these three closely related species.

The next plant species in which SCE was reported was Tradescantia, by Grant and Goldstein (1983) using the Feulgen staining procedure for SCE published previously by Tempelaar et al. (1982). These workers reported that the crucial step for obtaining differential contrast by this technique was the duration of acid hydrolysis. In 1985 Andersson published a description of SCE obtained in Tradescantia paludosa by means of a modified FPG technique. Since the range of concentrations of 5-fluorodeoxyuridine (FdUrd) used in earlier staining prodedures in other plant material allowed only poor differentiation in Tradescantia (Andersson, 1985), this author studied the effect of various concentrations of FdUrd on the mitotic index of root tip cells in order to determine the concentration needed to suppress synthesis of thymidylic acid by the plant and still allow good sister chromatid differentiation. Andersson showed that mitotic activity in Tradescantia root tip cells treated with 5 uM FdUrd was restored to control levels with 0.5 uM BrdU, but that 100 uM BrdU was needed to obtain good sister chromatid differentiation. Andersson also reported that duration of Giemsa staining must be kept short, otherwise overstaining will result.

Other plant species for which SCE has been reported include Allium fistulosum (Suzuki et al., 1982 cited by Dolezel et al., 1986) and in five

species of Triticum (Guangyuan and Zili, 1983). SCE has also been reported in Crepis capillarus (Dimitrov, 1985).

BrdU-substituted DNA

A number of studies have been carried out investigating the effects of base analogue incorporation into chromosomal DNA. In particular BrdU incorporation has been shown to alter both the physical and chemical properties of chromatin.

In 1973, Gordon and colleagues reported that when chromatin containing ^{14}C-BrdU labelled DNA and ^{3}H-thymidine labelled BrdU unsubstituted DNA respectively, is subjected to thermal chromatography, that the elution of the BrdU substituted DNA is significantly delayed in comparison to the elution of unsubstituted DNA whereas, there is only a minor difference in the elution profiles of purified BrdU substituted and unsubstituted DNA. They also observed that the elution of purified BrdU substituted DNA is delayed if it is first mixed with either substituted or unsubstituted chromatin and that the magnitude of retardation was proportional to the ratio of chromatin proteins to the total amount of substituted DNA. They concluded that this delay is due to the altered interaction of BrdU substituted DNA with some component of chromatin, probably proteins. In a later publication, the work of Lin and Riggs (1972) is cited to support the hypothesis that BrdU substitution may affect the binding of certain regulatory proteins (David et al., 1974). Lin and Riggs had shown earlier that the lac repressor shows about 100 times greater affinity for BrdU substituted DNA than unsubstituted DNA.

Lapeyre and Bekhor (1974) described BrdU-induced alterations in the

properties and structure of chromatin. These altered properties include 1) increased thermostability, 2) increased acidic nature, 3) a reduction of primary binding sites for ethidium bromide, a DNA specific fluorescent dye, and 4) increased condensation or supercoiling. The authors suggested that alterations in specific non-histone protein-DNA binding interactions may account for the increased acidic nature of BrdU-substituted chromatin as well as the changes in chromatin condensation.

Gordon and workers (1976) measured relative affinities of chromosomal proteins for both unsubstituted and BrdU-substituted DNA using thermal chromatography on hydroxylapatite, as in their previous study (David et al., 1974), as well as selective retention on nitrocellulose filters. In the former case, histones appeared to be the dominant protein component which selectively retarded BrdU-substituted DNA on hydroxylapatite, while in the latter case, it is the non-histone proteins which caused the greatest selective retention of BrdU-substituted DNA to nitrocellulose filters. These authors concluded that BrdU-substituted DNA binds selectively with certain chromosomal proteins relative to unsubstituted DNA. Because this selective binding can be shown by nitrocellulose filtration, which does not involve heating, the authors suggested that the same phenomenon exists within living cells. It is also suggested that the opposing results obtained from thermal chromatography and nitrocellulose filtration experiments, with regard to the class of proteins which preferentially bind to BrdU-substituted DNA, are due either to heat denaturation of non-histone proteins in the former case or perhaps because histone and non-histone proteins are differentially bound to the hydroxylapatite.

Staining Mechanisms of some DNA Specific Dyes

Other studies have centered on investigations into the staining properties as well as the mechanisms of staining, of differentially substituted DNA. Some of these mechanisms, such as the Feulgen procedure for SCEs (Vosa, 1981; Tempelaar et al., 1982), as well as the FPG staining with trypsin (Scheid, 1976; Scheid and Traupe, 1977), have been discussed. Additional studies regarding the mechanisms of Giemsa staining, the FPG technique, and the fluorescent dye 33258 Hoechst, will be discussed in this section.

Giemsa:

Giemsa is a dye which is specific both to DNA and ribonucleic acid (RNA). It was suggested by Meisner et al. (1973) that the staining reaction of Giemsa probably involves thiazine intercalation of the DNA and eosin interaction with protein. Meisner noted that chromosome banding pretreatments alter the conformation of chromatin in such a way that chromosomes, which in the absence of such pretreatments would normally be uniformly stained, take on a banded appearance in which pale and dark staining regions are formed. Thus according to Meisner, it is the disruption of DNA-protein associations through protein loss, physiochemical modifications, or conformational changes which result in the decreased staining of some portion of the chromosome.

Comings (1975a) carried out an investigation into the optical properties of the different components of Giemsa. Giemsa is made up of a mixture of the thiazine dyes; methylene blue, and azure A, B or C, and eosin Y. The thiazine dyes, which have a similar chemical structure, were shown

by Comings to be strongly metachromatic. He suggested that in the presence of DNA, low concentrations of methylene blue bind in an intercalative fashion. At higher concentrations, mythylene blue shifts to a different type of binding called side stacking. These changes in dye-DNA binding are associated with characteristic shifts in the spectral absorption curves of this dye in the presence and absence of DNA. Comings noted that eosin does not bind to DNA. Unlike Meisner et al. (1973), who proposed protein interactions as the probable mode of action for the eosin component of Giemsa, Comings suggested that eosin is not a necessary component for banding.

Takayama and Sakanishi (1977) and Sakanishi and Takayama (1978) obtained differential staining with Giemsa in CHO cells. They found that trypsin and hot acid pretreatment, either perchloric, hydrochloric acid, or a weak acidic salt solution of NaH_2PO_4, enhanced Giemsa staining and resulted in darkly staining bifilarly substituted chromatids and lighter staining unifilarly substituted chromatids. This is the reverse of the type of differential staining which is normally obtained when Giemsa staining is combined with a fluorescent dye, such as in the FPG technique. These authors pointed out that in the various FPG techniques, it is the bifilarly substituted chromatid which is more sensitive to the photolytic disruption of the DNA caused by treatment with photoreactive dyes and light, and therefore stains less intensely, while in their experiments the bifilarly substituted DNA seemed more resistant to acid treatment than the unifilarly substituted one and, therefore, stains more darkly. Takayama and Sakanishi (1977) suggested that hot acid treatment combined with trypsin digestion results in the preferential extraction of DNA and probably some proteins,

from the unifilarly substituted chromatid, resulting in loss of staining intensity by Giemsa.

Takayama and Sakanishi (1979) again obtained preferential staining of the bifilarly substituted chromatid using Giemsa. In this experiment, chromosomes were stained directly in a NaH_2PO_4-Giemsa solution without pretreatment of any kind. Here the authors ruled out the differential loss of DNA between sister chromatids as the cause of differential staining, since subsequent Feulgen staining of the same preparations gave rise to sister chromatids which stained with equal intensity. Differential staining, in contrast, was observed after Feulgen staining in their previous experiments (Sakanishi and Takayama, 1978). Thus they concluded that, although the mechanism of such staining is not clear, differential chromatid staining is probably due to differential DNA-protein interactions between differentially substituted chromatids.

Burkholder (1979) also obtained differential Giemsa staining in which the BrdU-substituted chromatid was either more darkly or more lightly stained than its unsubstituted sister chromatid when cells were pretreated with low or high pH NaH_2PO_4, respectively. He commented that such findings indicate that the nature of the pretreatment plays a major role in determining the staining effect. Burkholder explained the reciprocal nature of these two types of staining by two different mechanisms. Autoradiographic studies showed that the pretreatment of chromosome preparations with $Na\ H_2PO_4$ at high pH, resulted in the extraction of a significant amount of the BrdU-substituted DNA, while DNA from the non-substituted strand was extracted to a much lesser extent. The mechanism proposed in this case was that preferential photolysis of the BrdU strand

and subsequent treatment with NaH_2PO_4 at high pH resulted in preferential extraction of this DNA and hence reduced staining. Thus Burkholder felt that protein alterations are unlikely to play an important role in this staining mechanism.

In contrast, the same author found that treatment of chromosomes with NaH_2PO_4 at low pH did not result in a significant loss of radioactively labelled DNA, from either the substituted or the unsubstituted chromatid, therefore suggesting that the mechanism of staining is not dependent on DNA loss from either chromatid. In this case, the autoradiographic studies showed that labelled DNA in the unsubstituted strand was more dispersed than in the substituted strand. Burkholder hypothesised that the dispersion of DNA in the unsubstituted strand results in a chromatid which stains less intensly with Giemsa than its sister.

Goto and workers (1978) noted that there are probably two main categories of the Giemsa method for sister chromatid differentiation. The first uses the thermal stability and resistance to hot acid extraction of BrdU substituted DNA such as in the reverse differential staining obtained in the work discussed above (Takayama and Sakanishi, 1977; Sakanishi and Takayama, 1978; Burkholder, 1979; Takayama and Sakanishi, 1979). The second uses the UV sensitivity of heavily BrdU labeled DNA such as the results obtained by most workers using the Fluorescent-plus Giemsa technique. This latter suggestion is in agreement with Burkholder's findings.

In experiments using [^{3}H]-BrdU labeled DNA, Webber and coworkers (1981) found that following pretreatment with UV-light and hot salt treatment, there was a loss of about two thirds of the [^{3}H]-BrdU from Giemsa stained BB substituted chromosomes, while there was a loss of only one third

of the [^{3}H]-BrdU label from Giemsa stained TB substituted chromosomes. These authors point out that the UV-light and hot salt treatments act in collaboration, since neither treatment affected loss of [^{3}H]-BrdU when administered alone.

These authors also pointed out that for all methods, good sister chromatid differentiation can be obtained without DNA loss, such as when chromosomes are stained with 33258 Hoechst in conjunction with a light pretreatment (Wolff and Perry, 1974; Goto et al., 1975), or when alkaline Giemsa is used to obtain reverse differential staining (Takayama and Sakanishi, 1979).

33258 Hoechst:

Since 33258 Hoechst was introduced by Hilwig and Gropp in 1972, the use of this compound has become popular in chromosome studies. This is not only because of its specificity for DNA but also because of its relatively bright and long lasting fluorescence (Das et al., 1979). Although it is not clear by what mechanism 33258 binds to chromatin, intercalation is ruled out by most authors (Latt and Wohlleb, 1975; Comings, 1975b).

Latt (1973) noted that the fluorescence efficiency of 33258 Hoechst bound to the polynucleotide poly(dA-BrdU) is less than one fifth of that of the dye bound to poly(dA-dT). In cytological observations of differentially stained human metaphase chromosomes substituted with [^{3}H]-BrdU, Latt reported that autoradiographic estimation indicated that the dull chromatid regions had incorporated about twice as much [^{3}H]-BrdU as brightly fluorescing regions.

In a study undertaken by Comings (1975b), some properties of the

Hoechst-DNA interaction were examined. Comings argued that the fluorescent dye binds by an attachment to the outside of the DNA double helix by interacting with base pairs within the DNA.

Gonzalez-Gil and Navarrete (1982) noted that when acid hydrolysis of DNA was carried out with HCl before staining with 33258 Hoechst, no differential staining was obtained. These authors attributed this loss of staining to the partial depurination of DNA induced by HCl treatment and considered that the fluorochrome is no longer able to associate with the altered DNA.

It has also been suggested that 33258 Hoechst has a greater affinity for AT rich DNA than for GC rich DNA (Latt and Wohlleb, 1975; Comings, 1975b), and alternatively, that chromosome condensation may play an important role (Gatti et al., 1976; Latt and Wohlleb, 1975; Das et al., 1979). In the latter case, Das and colleagues suggested that dye binding, and thus dye fluorescence may be affected in some way by the DNA-protein ratio, since they noted that fluorescence of 33258 Hoechst was quenched following the removal of proteins from the chromosomes.

Similarly, the fluorescence of 33258 Hoechst is reduced in BrdU substituted DNA (Latt, 1973). DNA bound proteins, which may show an affinity for BrdU substituted DNA, are thought to be capable of competing with the dye, and so reduce the fluorescence of BrdU substituted DNA (Latt and Wohlleb, 1975)

On the other hand, it has been suggested that BrdU substituted DNA undergoes rapid hydrolysis in the presence of a photosensitive dye, such as 33258 Hoechst and strong light, and that the role of 33258 Hoechst is to sensitize and enhance the photolysis of BrdU labeled DNA (Sugiyama et al.,

1976; Goto et al., 1978). While the exact mechanism of this photolysis is not understood, Sugiyama and workers (1976) suggested that light activated dyes situated near the strong magnetic field of the halogen atoms of the halogenated base analogue, may act as an alkali, causing chain breaks and disorganization of the chromatid DNA. The role of the halogenated base analogue is apparently an important factor, since no degradation has been demonstrated when 33258 Hoechst stained unlabeled DNA is exposed to UV light (Goto et al., 1978).

Fluorescent-plus-Giemsa:

While the exact mechanism of differential staining of sister chromatids obtained by the fluorescent-plus-Giemsa technique is not known, there is considerable agreement among workers in this area as to the probable mechanisms involved.

There are three essential steps in the Hoechst-Giemsa method of the FPG technique. They are (1) treatment of chromosomes with 33258 Hoechst followed by (2) exposure to strong light and then (3) Giemsa staining. In the first two steps, the combination of fluorochrome staining and exposure to strong light is thought by most authors to cause the photolysis of BrdU substituted DNA. As a result of this photolysis, there is a preferential loss of DNA from the BrdU substituted strand. This alteration in the structure of the DNA thereby prevents further binding of azure dyes (i.e. Giemsa) to the DNA, resulting in the differential staining which is observed in the FPG technique (Goto et al., 1975; Sugiyama et al., 1976; Ockey, 1980; Gonzalez-Gil and Navarrete, 1982; Jan et al., 1984).

Evidence supporting photolysis of BrdU substituted DNA by fluorochrome

dyes and light, and its subsequent removal from the chromatin, as the predominant cause of differential staining, are as follows:

BrdU containing chromatids, which have been stained with 33258 Hoechst and thus fluoresce dully, lose their ability to bind Giemsa when exposed to strong light. This would indicate that light activated chemical reactions alter the chromatid components in an essential way (Goto et al., 1975).

BrdU substituted chromatids which are treated with photoreactive dyes and light, and subsequently stained with Feulgen, give rise to reduced Feulgen staining, indicating that DNA photolysis, is the predominant cause of differential staining (Goto et al., 1975)

Using quantitative autoradiography, Ockey (1980) analyzed CHO cells labeled with either [^{3}H]thymidine or [^{3}H]BrdU and showed that only [^{3}H]BrdU label was removed from metaphase chromosomes after FPG staining. [^{3}H]BrdU is differentially removed from BB labeled chromatids when compared with TB labeled chromatids. Furthermore, this author showed that if the UV step is omitted from the FPG technique, no loss of label and no harlequin staining occurs.

Citing the work of Hutchinson (1973), several workers noted that if UV light debrominates BrdU substituted DNA and the resulting free radicle produces a single strand nick on the 5' side of the debrominated base as Hutchison suggests, then when both strands carry BrdU, double strand breaks may be produced (Webber et al., 1981; Gonzalez-Gil and Navarrete, 1982). Like others they agree that light exposure of the BrdU substituted DNA in the presence of a photosensitive dye, probably increases the frequency of the double-stranded nicks, and that the combined effects of 33258 Hoechst staining, light exposure, and hot saline (a treatment which is employed

prior to Giemsa staining in most FPG protocols), apparently act synergistically to facilitate the degradation and washing out of DNA from the chromatin.

Proposed Mechanisms of Sister Chromatid Exchange Formation

Although SCE has been used to study a number of chromosomal phenomena such as chromosome structure, chromosome damage, the DNA repair deficiency syndrome, as well as being employed as an indicator of quantitative genetic damage in mutagenicity and carcinogenicity bioassays (Carrano et al., 1978), the actual mechanism of SCE formation has yet to be clarified. Sasaki (1982) notes that current knowledge regarding the mechanism of SCE formation is highly speculative and he states that it remains one of the most significant unanswered questions in research concerning mammalian DNA metabolism. The mechanism has nevertheless been studied by a number of workers, who have used the differential properties of bases analogue substituted DNA as well as the widely varying nature of SCE induction in a number of in vitro and in vivo systems, in an attempt to further clarify our present understanding of this phenomenon.

A number of workers agree that a single model cannot adequately describe the formation of SCEs, given the wide variety of treatments and conditions under which this genetic endpoint can be induced (Kato, 1977a; Latt, 1981; Schvartzman and Tice, 1982; Shafer, 1982). Kato (1977a) suggested, based on data showing strikingly different responses of SCE formation to X-irradiation and UV light, that the mechanism by which these two events might arise are most likely not identical. For example, it has been shown that while X-irradiation causes an increase in SCE, the frequency

reaches a saturation level at very low doses, while SCE frequencies induced by UV irradiation treatment do not reach a saturation level until much higher doses are administered (Kato, 1974c). Additionally, it has been demonstrated that some chemically induced SCE (ie., 4-nitroquinoline 1-oxide [4NQO], and Mitomycin C [MMC]), as well as UV light induced SCE, seem to be evolved through (a) process(es) sensitive to postreatment with caffeine, while other SCE inducing chemicals such as proflavin, show no such sensitivity (Kato, 1974c). These results led Kato to suggest that any model concerning the mechanism of SCE formation by UV light, 4NQO or MMC should involve a caffeine sensitive step. Stetka (1982) suggested the existence of more than a single mechanism for the formation of SCE since a variety of lesions are thought by some authors to be related to the SCE event. Others (Evans, 1977) suggested that since SCE may be induced by a wide variety of mutagens, they may likely be the result of a response on the part of the cell's replicating machinery to a variety of different lesions in the DNA.

Since SCE is generally regarded as the reciprocal exchange between two identical DNA molecules, or sister chromatids (Bender et al., 1974; Kato, 1974a; Perry and Evans, 1975; Sasaki, 1977; Kato, 1979; Cleaver, 1981; Danford, 1983; Speit et al., 1984; Latt et al., 1984; Sorsa, 1984; Anon, 1985b), mechanisms describing such an event share a common requirement for a single strand break in one chromatid, with a simultaneous or subsequent break, at the same position in the corresponding sister chromatid (Kato, 1977a; Dillehay et al., 1983).

The role of BrdU in SCE formation

Perry (1980) wrote that the base analogue BrdU is a known mutagen which causes base-pairing errors, resulting mainly in the transition of A-T pairs to G-C pairs. It also causes chromosome aberrations, increases radiosensitivity, alters the binding affinity of proteins and has several other effects that are not known to be related to its substitution into DNA.

Wolff and Perry (1975) reported findings early on, in which chromosomes that had been bifilarly substituted with BrdU, and submitted to colchicine treatment to induce the formation of endoreduplicated cells, had an approximate ratio of 2:1 single (SCE induced in the first S phase [S_1]) and twin (SCE induced in S_2) chromatid exchanges in such cells, implying that an approximately equal number of exchanges occurred in the first and second cell cycles. Shiraishai et al. (1982, 1983) reconfirmed Wolff's findings using normal, endoreduplicated BrdU-substituted human lymphocyte cells.

Perry and Evans (1975) noted that the incorporation of BrdU into DNA enhances its sensitivity to damage by both X-rays and ultraviolet light. In CHO cells they noted a linear dose-dependent increase in SCE frequencies when cells were treated with X-rays in S and in G_1, but not in G_2, suggesting that DNA replication is required between SCE induction and SCE formation. The linear dose response of SCE induction to X-rays, as well as to other SCE inducing substances, suggests a single hit mechanism for this event (Carrano and Thompson, 1982; Shafer, 1984). In studies using CHO cells, Dillehay et al. (1983) suggested that a considerable fraction of the SCE observed at higher BrdU levels (0.5 uM) are a response to the BrdU. Newly synthesized DNA was studied using alkaline elution, a technique which

provides information regarding the distribution of single-strand size in a population of labelled DNA molecules. According to the authors, alkaline elution profiles showed that mature BrdU-substituted DNA contain strand breaks and/or alkali labile sites not detected in normal DNA, and suggested these lesions may induce SCE when DNA is replicated.

Schvartzman et al., (1979b) showed that visible light illumination was able to increase SCE frequencies in BrdU substituted chromosomes while visible light illumination has no apparent effect in native DNA.

Mazrimas and Stetka (1978) noted that BrdU substitution can cause an increase in SCE frequency even in the absence of light. They believed that SCE frequencies were directly related to the percentage of BrdU substitution for thymidine (dThd) after two cell cycles. They also noted that the relationship between the degree of BrdU substitution for dThd and SCEs induced by this analogue is clearly linear and is independent of prior BrdU exposure. Thus they concluded that BrdU incorporation into nascent DNA results in SCE formation at, or soon after the time of replication and is not related to the BrdU substitution of the replicating DNA. At about the same time, Kihlman and workers (1978) reported that they found no marked differences between the frequencies of SCE in cells in which the DNA had been unifilarly substituted and those in which it had been bifilarly substituted.

The findings of Schvartzman and colleagues (1979a) are in sharp contrast to those of earlier workers. They showed that the yield of SCEs per cell cycle varies according to the number of replicated rounds completed by Allium cepa meristematic cells in the presence of BrdU. Experimental material was exposed to one, two or three rounds of BrdU and then to

unlabelled thymidine the next one or two cell cycles. Schvartzman and workers found that the SCE frequencies were lowest in cells which had undergone only one round of DNA replication in the presence of BrdU and highest in cells which had undergone three rounds of substitution. Findings of Davidson and workers (1980) also contradict the earlier work of Mazrimas and Stetka (1978) described above. In their experiments these authors found that the increase in SCEs due to raising the BrdU concentration at a fixed level of BrdU substitution (ie., at a fixed ratio of 3:2 BrdU:dThd) in CHO cultured cells accounted for most of this increment. These authors suggested that it is the concentration of BrdU in the medium rather than the amount of BrdU incorporated into the DNA which is the major factor determining the induction of SCE frequency. Furthermore, when experiments were carried out in which cells were treated with a fixed concentration of 12 uM BrdU while the level of BrdU was increased by decreasing the BrdU:dThd ratio, this increase reportedly had only a small effect on the SCE frequency. Thus, these authors concluded that the level of BrdU substitution does not play a major role in determining BrdU induced SCE frequencies.

DuFrain and Garrand (1981) conducted experiments in which the three thymidine analogues, BrdU, CldU and IrdUrd, were substituted into the DNA of human lymphocytes in cell culture. The authors predicted that when substituted at various levels, these analogues should cause different degrees of change in the molecular conformation of DNA since the atomic radii for these three halogens are 1.80. 1.95 and 2.15 A, respectively. These authors found that the incorporation of these halogenated analogues dictates a SCE frequency that is dependent both on the halogen used and the

level of incorporation. Although DuFrain and Garrand charactarize their work as far from conclusive, they speculated that conformational alterations of the DNA probably play a major role in the molecular mechanism of SCE formation.

Heartlein and workers (1983) noted that the presence of CldU in replicating DNA induces SCE frequencies five times that of BrdU, and that this induction is largely due to the nature of halogen substitution, rather than any difference in the amount of base analogue substituted. The extent of BrdU and CldU substitution was estimated by percent substitution in DNA of BrdU and CldU by means of radioactive HPLC.

O'Neill et al. (1983, 1984) suggested that the induction of SCEs is dependent only on the replication of analogue substituted DNA. In these studies, cultured CHO cells were grown in the presence of BrdU or CldU for seven days to obtain bifilarly substituted DNA in both of the sister chromatids. Analogue incorporation was followed by two rounds of replication in the presence of dThd alone. The SCE frequencies in these cells were approximately twice those found in cells which had undergone only two rounds of replication in base analogues. Such cells contain exactly one half the amount of substituted DNA at the time of DNA replication during the S phase previous to cell harvest. Furthermore, the authors claim that SCE frequencies in cells grown in one cell cycle of BrdU or CldU, followed by a second cell cycle in dThd, are very similar to the SCE frequencies found when both replications were carried out in a base analogue, further supporting the suggestion that it is the replication of the substituted DNA rather than the presence of analogue at the time of replication which affects the SCE frequency. Similar results have been reported by Suzuki and

Yosida (1983).

Stetka and Spahn (1984) attempted to resolve the issue of whether SCE induction is caused by the replication of BrdU substituted DNA templates or by BrdU incorporation into nascent DNA. When cells were exposed to 10 uM BrdU during S_1 and S_2 and subsequently exposed to several different concentrations of BrdU during S_3, the observed SCE frequencies were unaffected by changes in the BrdU concentration during S_3. On the other hand, when the BrdU incorporation during S_1/S_2 was varied over the same concentration range as that in the S_3 of the above experiment, and then held constant at 10 uM in the S_3, S_3 SCE frequencies did increase as a function of BrdU concentration, and the correlation between S_3 frequency and concentration of BrdU in S_1/S_2 was highly significant. Therefore the authors concluded that incorporated BrdU that is serving as a template during a subsequent S phase, induces SCEs, while the act of incorporation of BrdU into nascent DNA does not.

DuFrain (1984) drew similar conclusions, both with regard to the effects of halogen substitution, as well as the role of analogue substituted templates in the formation of SCEs. In a study in which six different halogenated pyrimidines were substituted into the DNA of cultured human lymphocytes, he noted a trend toward higher SCE frequencies as the halogen size deviated from the normal methyl group of thymidine. This author suggested that the incorporated halogenated pyrimidine catalyzes deformation of the DNA strand and that when this deformation is in the template strand, that alterations in the specificity of recognition and binding of DNA polymerase may lead to SCE formation.

In light of all the seemingly contradictory results, Schvartzman and

Tice (1982) had earlier suggested the BrdU likely induces SCE through at least two different mechanisms. One mechanism may be mediated by the direct incorporation of a base analogue, while the other mechanism would be mediated by some other factor, as suggested by studies which show a relationship between SCE frequency and the degree of analogue substitution into DNA, or later studies which suggested a relationship between SCE frequency and BrdU levels in the DNA template strand.

Hetero- and euchromatin and the formation of SCE

Several authors (Carrano and Wolff, 1975; Bostock and Christie, 1976; Kato, 1979) carried out studies showing that the distribution of SCE frequencies in the euchromatin of several mammalian species was proportional to its metaphase length, while SCE localized in heterochromatic regions were fewer than expected. Additionally, many SCEs were detected at hetero-euchromatin junctions in these studies. Kato (1979) reasoned that suppression of SCE in heterochromatin probably occurs due to tertiary structure rather than its repetitive nucleotide sequence, since SCE would probably be facilitated by the presence of repetitive DNA sequences considering that pairing of homologous strands is involved. Instead he suggested that repetitive DNA such as that found in heterochromatin, which is reported to have a unique distribution of DNA associated proteins, may be less susceptible to cleavage by nucleases. He further suggested that the configuration of DNA-protein complexes in the hetero-/euchromatic junctions might be in a "locally uncoiled" state, thus facilitating the formation of SCEs in this region. Cortes (1980) agreed that these regions are highly sensitive to the occurrence of SCE because they might be transitional

regions which may remain uncoiled at times due to differential association with nucleoproteins when compared with other DNA regions.

In studies of the two large M chromosomes of Vicia faba, which contain seven heterochromatic segments that can be distinguished by chromosome banding techniques, Vosa (1976) found that while overall, SCE frequency is proportional to length in this chromosome pair, the long arm has fewer SCEs than expected in the largest heterochromatic band. Vosa suggested that in Vicia, these different frequencies of SCE in heterochromatic regions may be related to discordant DNA synthesis. Other workers have reported non-random distribution of SCEs in the chromosomes of Allium cepa (Schvartzman and Cortes, 1977; Cortes, 1980; Cortes et al., 1985).

Schubert et al. (1979) found that the inter- and intra-chromosomal distribution of SCE was length proportional after mutagenic treatment and FPG staining of Vicia faba chromosomes. In a second study, Schubert and workers (1980) found that SCE frequencies in Hordeum vulgare were also proportional to length and were moreover, independent of heterochromatin content. Gatti et al. (1979) studied the distribution of SCEs in the chromosomes of Drosophilia melanogaster. SCEs are distributed in female chromosomes in proportion to length. The heterochromatin of the Y chromosome of the male has a significant excess of SCEs. Within chromosomes, SCEs are preferentially localized in heterochromatin. At the junction between hetero- and euchromatin, these chromosomes exhibit high SCE frequencies. Hoo and Parslow (1979) found that in cultured human peripheral lymphocytes SCEs occurred approximately three times more frequently in euchromatic segments, than in heterchromatic segments. According to the authors, this preferential involvement of euchromatin in SCE formation may

be due to the more condensed and rigid nature of heterochromatin which might prevent rotation of the sister chromatid, should breakage occur in this region, thus reducing SCE formation in such regions. Ambros and Schweizer (1983) reported a three to elevenfold increase of SCE's at the euchromatin/heterochromatin borders and at the centromere regions of Ornithogalum longibracteatum, while noting that the distribution of SCE's within euchromatin and heterochromatin was nearly the same.

Dimitrov (1987) made a distinction between hetero-/euchromatic junctions and junctions between early and late replicating regions. He estimated a high incidence of SCE at both types of junctions in dividing cells of Crepis capillaris. The non-random distribution of SCEs in this organism was also due to lower than expected frequencies of SCE in heterochromatin. Gamma-ray induced SCE distribution is similar to the spontaneous distribution. Dimitrov suggested that the non-random distribution of SCEs might be due to differences in the time of replication of individual chromosome regions, which he noted is in agreement with data showing that SCEs are formed during DNA replication.

DNA lesions associated with SCE formation

Tice (1984) pointed out that SCE per se do not indicate the specific nature of the lesion responsible for its induction. In general, correlations have been made between specific types of DNA damage, or DNA lesions, and SCE because agents which are known to cause characteristic DNA damage, also induce SCE (Speit et al., 1984). For example, it has been noted that beta particles emitted from ^{3}H-thymidine, X-rays, UV-light and chemical mutagens, which damage DNA, can cause increased SCE frequencies

(Wolff, 1977). Carrano and Moore (1982) noted that different types of DNA lesions exhibit different relative efficiencies for producing SCEs. According to these authors, UV-light, mono- and bifunctional alkylating agents and reactive forms of the larger aromatic hydrocarbons are substances which form covalent adducts or otherwise distort the DNA bases and are efficient inducers of SCE. On the other hand, those agents capable of directly breaking the DNA, such as ionizing radiation and heavy metals, while efficient inducers of chromosome aberrations, are not efficient inducers of SCEs. Other agents implicated in the formation of SCE have been cross-linking agents (Shafer, 1977), intercalating agents, and some large DNA binding agents (Shafer, 1984).

The fact that most DNA damaging agents cause a wide range of molecular DNA alterations, has made the study of the relationship between any single DNA lesion and SCE induction difficult (Littlefield et al., 1981; Fujiwara et al., 1984; Speit et al., 1984) and has led some researchers to propose that a wide variety of lesions may lead directly or indirectly to the induction of SCE (Carrano and Thompson, 1982; Littlefield et al., 1981). Wolff (1982) suggests that uncertainty about which lesions actually give rise to SCE formation is a reflection of the lack of knowledge about the mechanism of its formation. Some authors have suggested that because the formation of SCEs seem to take place during the S phase of the cell cycle, one might conlude that the lesions responsible for SCE production are those which persist in the DNA until the S phase, or in other words, those which remain unexcised or unrepaird in the DNA after cell treatment with DNA damaging agents (Perry, 1980; Schvartzmann and Gutierrez, 1980; Wolff, 1982).

Cyclobutane pyrimidine dimers, and specifically thymidine dimers are the primary lesions induced by UV-irradiation (Perry, 1980) and are thought by some to be responsible for most of the biological effects observed after UV-irradiation of DNA (Natarajan et al., 1981). The fact that UV-light is an efficient inducer of SCE has led some authors to link UV-light induced pyrimidine dimers to the production of SCE (Kato, 1973; Reynolds et al., 1979; Ishizaki et al., 1980).

However, Wolff (1977) showed that in experiments comparing the responses of repair deficient and normal Chinese hamster cells that sensitivity to SCE formation was not related in a simple way to the amount of repair. Thus, he suggested that SCE formation is not directly related to the formation of thymidine dimers or other lesions which are presumably removed by replication repair. It is Wolff's belief that minor photoproducts, or alkylation products that are not measured either by excision or post replication repair, seem to be responsible for SCE formation. Natarajan et al. (1981) noted that other unknown, minor, UV-induced DNA adducts, which may be difficult to measure and which may also be repaired by photoreactivating enzymes, would be equally as likely to lead to SCE formation, as thymidine dimers.

Single-strand breaks have also been implicated in the formation of SCE. Evans (1977) noted that since visible light treatment of BrdU substituted DNA results in both single-stranded breaks and an increased SCE frequency, that single-strand breaks present during replication are probably responsible for most of the SCE's observed under these conditions. However, Speit and co-workers (1984) argued that since DNA-breaking agents such as X-rays are not good inducers of SCE, it can be assumed that single-strand

breaks do not represent a major pathway for their production. They have also pointed out that while SCE formation must involve well defined and orderly breaking and rejoining of DNA strands, neither directly induced single or double strand breaks meet these conditions.

Mytomycin C, a cross-linking agent, is known as a potent inducer of SCE and has been linked to SCE formation (Shafer, 1977, 1984; Fujiwara et al., 1984), however, evidence contradicting this conclusion has also been reported and is discussed later in this review (p. 43).

More recently, Hirsch and Cai (1988) note a correlation between what they call aphidicolin induced fragile sites in human peripheral blood lymphocytes, and the occurrence of SCEs at these sites, suggesting that common fragile sites are hot spots for SCE formation.

DNA replication and the formation of SCE

Many authors have proposed involvement of the DNA replicating machinery in the formation of SCEs. Latt (1981; Latt et al., 1984) pointed out that several properties of replicating DNA make it a candidate for involvement in the SCE phenomenon, including the proximity of sister DNA strands at the time of replication, discontinuities in newly synthesized fragments, and the ligation of interreplicon gaps. Indeed, Wolff et al., 1974) demonstrated in cultured Chinese hamster cells, that when cells underwent treatment with UV irradiation at different stages of the cell cycle, SCE induction can occur at any stage, but the cell must undergo DNA replication before the exchange is formed. Kato (1974a), on the other hand, using cells from the same organism and examining only the largest pair of chromosomes in the genome, found that the formation of SCE was enhanced only

when visible illumination was carried out during the S phase. In the same study, a parallel between DNA synthetic activity and SCE frequency was noted in the X chromosome which consists of a short arm that replicates in the earlier part of the S phase and a long arm which replicates in the later part of the S phase. He observed that SCEs induced by visible illumination were only observed in the short arm in the early part of S and in the long arm in the later part of S, thus further linking the formation of SCEs to DNA synthetic activity. Kato suggested that such a concurrence could be due either to direct involvement of the replication fork in the recombination process, or perhaps to some geometrical constraint which might favour the interaction of two broken ends. In later papers, Kato (1977a,b) examined and compared the frequency of fluorescent light induced SCE in unifilarly and bifilarly BrdU substituted chromosomes, respectively. He predicted two possibilities for the outcome of these experiments and noted the implications of each. Since trifilarly substituted chromosomes would bear single-strand breaks in three out of four DNA strands, the first possibility was that if SCE was initiated simply at the site of a single-strand break by means of a mechanism similar to that of meiotic recombination and without a requirement for DNA synthesis, then the number of SCEs induced in trifilarly substituted chromosomes should be twice that induced in unifilarly substituted chromosomes (not three times, since replicating cells begin to utilize the de novo synthesized thymidine pool and salvage nucleotide pools at about equal extent during the second replication cyle). Thus, the number of breaks which would be induced by fluoresent light illumination in the unifilarly substituted chromosome would be about the same as the sum of the number of breaks induced in the two newly formed strands in the trifilarly

substituted chromosome.

The second possibility predicts that if SCE formation is restricted to (the) DNA replication point(s), then the SCE frequency would be the same for both types of chromosomes, because exchange would be a function of the number of DNA replication forks and not the number of breaks induced in the chromosome by fluorescent light illumination.

The results showed the SCE frequency in the bifilarly substituted chromosome was only slightly higher than in the unifilarly substituted chromosome but that this difference was not statistically significant indicating that the majority of SCEs are induced as a function of replicating analogue substituted DNA. Kato suggested that at least two different mechanisms are involved in the induction of SCEs. The first model, which would be responsible for by far the large majority of SCEs induced under these conditions, requires a single-strand break in the BrdU substituted template strand either in the pre-replicational DNA region or directly at the replicating point. The molecular process of this pathway is unknown. A second mechanism, whose contribution, while minimal in comparison to the first, would require two staggered or juxtaposed single strand breaks in a BrdU substituted chromosome. Double-stranded exchange between DNA strands of like polarity would follow and is proposed to be similar to that of meiotic recombination (Holliday, 1964, cited by Kato, 1977b) or gene conversion (Whitehouse, 1963, cited by Kato 1977b) and would occur independently of DNA replication.

Kato linked DNA replication to SCE induction in another study in which he investigated the effect of temperature on the incidence of SCE, in an attempt to interpret the mechanism of spontaneous SCE as opposed to the

mechanism of SCE which is induced by various DNA damaging agents. Kato assumed that if SCE arises spontaneously, then either the cause of SCE or the mechanism by which it is carried out would be affected by temperature. Increase in temperature in the experiment not only led to an increase in SCE frequency but also brought about an elongation of the S phase which Kato considered to be due to a disturbance in the process of DNA replication. Kato showed that the frequency of SCE could be plotted on a regression line as a function of temperature, and that this regression line showed an obvious break at 39 C, indicating that the effect of temperature was not simply to accelerate SCE formation but to increase the frequency with relation to temperature. Marked elongation of the S phase also began above this temperature, thus Kato concluded that these results not only suggest the involvement of DNA replication in SCE formation but also, that the break in the regression line of temperature versus SCE frequency implies that at least two biochemical processes may be involved in SCE formation, one of which enhances SCE formation when the temperature rises above 39 C. Further investigation resulted in the discovery that an increase in SCE frequency beyond the control level was detected only when temperature treatment was carried out during S phase, reinforcing Kato's earlier assertions closely linking SCE formation with DNA replication.

Kato suggested that the replication process per se probably does not initiate SCE, since unless single-strand breaks in the parental strand are present, the free end of the discontinuously growing nascent strand of the same polarity would not be available for interaction. Rather, in the process of replication, when strand discontinuities arise in the nascent strands at the replication point, they would then be free to recombine in

some way with a break in the parental DNA strand near, or at, the replicating region. Kato suggested that such strand breaks may be caused by endonucleases involved in excision repair, and he speculated that spontaneous SCE may arise by the repair of spontaneous damage, occurring normally in mammalian cells. With respect to temperature induced increase in SCE frequencies, Kato suggested that in normal cells, spontaneous damage is repaired quickly and perhaps for this reason only temperature shock administered in the S phase results in an increased incidence of SCE.

Hori (1983) used 5-azacytidine (5-azaC), a cytidine analogue and a potent inducer of SCE, which when incorporated into DNA inhibits DNA methylation leading to hypomethylation, to study the relationship between replication of differentially methylated DNA strands and SCE induction. He found that 5-azaC substitution during a single cell cycle was sufficient for maximum SCE induction by this analogue and suggested that SCE induction occurs during the replication of either hemi-methylated or demethylated 5-azaC substituted DNA. Hori hypothesized that hypomethylation of DNA, may alter chromatin structure and result in a retardation in the rate of DNA replication, which in turn might induce SCE formation.

Cleaver (1981) noted that in a variety of human and Chinese hamster cell lines, the baseline SCE frequencies increase as a function of the average replicating unit (replicon). Baseline SCE frequencies were plotted against the average replicon size for a variety of cell types. Cells with larger replicons were observed to have larger baseline SCE frequencies suggesting that a larger replicon has a greater tendency to give rise to errors which result in exchanges during replication. Thus, according to the author, SCE formation might be explained in the following way. Because of

the multiple orders of coiling involved in the packing of eukaryotic DNA, newly replicated DNA remains in an entwined form, which may be subsequently unravelled to produce separate daughter helices by the action of topoisomerases as well as other enzymes involved in the unwinding process. Cells with small replicons, in which the DNA replication origins are located more closely together, presumably present a less demanding task for enzymes such as topoisomerases, which unravel replicated DNA, than cells whose replication origins are located farther apart. In organisms with smaller replicons there would be less opportunity for error induced SCE which might occur at tension spots created by the unwinding process. Thus, such errors would induce SCE formation behind the replication fork, as opposed to inducing SCE formation at or in the path of the replication fork as some authors suggest.

Dillehay et al. (1983) studied SCE induction in a normal Chinese hamster cell line (AA8) and a mutant line derived from it (EM9) in which the baseline SCE frequencies were increased 12-fold and ethylmethanesulfonate (EMS) induced SCE frequencies were increased 7-fold in the mutant line. Additionally, the mutant (EM9) expressed defective DNA strand-break repair after exposure to alkylating agents or ionizing radiation. As discussed on page 27, these workers studied newly synthesized DNA using alkaline elution, to detect whether or not any unusual aspects of DNA replication could be associated with the conditions that produced high SCE frequencies in the mutant cell line. By studying the behavior of nascent DNA strands the authors hoped to investigate the basis of high SCE frequency that had been observed in the CHO mutant strain EM9. Elution patterns indicated a delay in the replication of BrdU-substituted templates two times greater in EM9

than in AA8. This replication delay is thought by authors to be genetically linked to SCE frequency since the delay was decreased in a revertant strain that had a low SCE frequency. High SCE frequencies in EM9 cells are thought by the authors to be the result of two interacting factors. First, the mutant cells may either have more lesions in the template strand or the replication machinery may take longer to bypass the lesions which have been induced, thus explaining the replication delay in such cells. Secondly, since the EM9 mutant is defective in DNA strand break repair it contains longer-lived strand breaks than normal cells, which may facilitate the exchange process. Authors noted that the results of this work are consistent with those who propose replication blocks as a prerequisite for SCE formation (Shafer, 1977; Ishii and Bender, 1980; Painter, 1980; 1982).

The replication bypass model to explain the induction of SCEs, was first formulated by Shafer in 1977 as a mechanism which allows replication to continue past a crosslink in the DNA template strand. The model was criticized on theoretical grounds by Stetka (1979) and refuted in part by the experimental results of Carrano et al. (1979). The latter workers workers compared the SCE inducing capablilities of mitomycin C (MMC), a potent inducer of DNA cross-links, with that of its monofunctional derivative, decarbamoyl mitomycin C (DCMMC), which lacks cross-linking activity. DCMMC was found to have an even greater SCE inducing capability than MMC, thus it was concluded that DNA interstrand cross-links cannot be the major lesion responsible for SCE induction. Stetka (1979) suggested that, at best, the replication bypass model should be limited to SCEs induced specifically by cross-linking agents, as it does not account for all the available data regarding SCE induction.

Shafer (1982, 1984) defended the model on several grounds, while proposing alternative mechanisms which may account for the data of Carrano et al. (1979) and others. Shafer suggested that as normal replication progresses in damaged DNA it will be interrupted by a variety of DNA lesions. This may bring about the induction of parental-strand incisions by repair endonucleases, which may result in parental-strand transfers, ending in SCE formation. Shafer noted that SCE may arise by a number of different mechanisms, much in the same way different lesions give rise to a variety of repair pathways. The most likely steps in a repair pathway to affect SCE formation would be either an endonuclease incision in one DNA strand or the pre-incision binding of an endonuclease at the incision cite. Thus Shafer broadened the possible applications of his original model by proposing a series of related mechanisms, linked to DNA lesions other than DNA cross-links, or to repair intermediates which disrupt normal DNA replication. Therefore, according to Shafer's model, SCE probably occurs when incised parental DNA strands located at, or near, an unrepaired or partially repaired lesion site, are exchanged in order to allow DNA replication to continue past the lesion.

Painter (1980, 1982) proposed a model for SCE formation based on the physical constraints of DNA replication at the molecular level. He stated that during DNA replication, supercoiled subunits of the chromosome are physically separated from each other by RNA and/or protein. He suggested that the DNA at junctions between replicating and supercoiled DNA is susceptible to double-strand break formation. Furthermore, he supposed that any damage, such as chemical damage, which may slow down or stop DNA replication, may cause the DNA in these junctions to remain unreplicated for

longer than normal periods of time additionally increasing the probablility of double-stranded breaks in this region. It is these double-stranded breaks, according to Painter, which give rise to SCE by providing the opportunity for the daughter strands to become available for interaction and thus exchange with the DNA in the unreplicated adjacent cluster. This model, according to Painter is consistent with data showing that SCE are a linear function of dose, thus, even though it requires two double-stranded molecules to participate (i.e. a double-stranded break), it conforms to single-hit kinetics.

Another replication model for SCE was proposed by Ishii and Bender (1980) which the authors call the "replication detour" model. In their experiments the authors examined the effects of a number of inhibitors of DNA synthesis on spontaneous as well as UV induced SCE frequencies. The results show that both types of SCE frequencies were influenced significantly by only some of these inhibitors. The authors suggested that chain elongation is possibly the sensitive step involved in both types of SCE. Two models are proposed to explain the formation of spontaneous and induced SCE respectively. In the former case, it is suggested that a nick in either of the template DNA strands of actively replicating DNA, would give rise to single-stranded ends which could sometimes rejoin with the newly synthesized daughter strand of the same polarity, resulting in the formation of a SCE. For UV induced SCE the mechanism is the same, a nick in the template strand resulting from the presence of a thymidine dimer in the DNA.

DNA repair and the formation of SCE

Since DNA damaging agents increase SCE frequency, this suggests the possibility that SCE may be a reflection of a basic DNA repair process (Sasaki, 1977, 1982). There are a number of known mechanisms for the repair of DNA. Single-strand damage may be corrected either by photoreactivation or excision repair. Unrepaired damage which passes through replication may leave a gap in the newly synthesized strand opposite to the damaged strand. Such gaps may be corrected by recombinational exchange between single-strand portions in the daughter duplexes. This is referred to as post-replication repair, and involves the provision of a template for either gap filling or excision of the original DNA damage (Shafer, 1977).

In 1973, Kato pointed out that since caffeine is a known inhibitor of post-replication repair, the finding that caffeine reduced SCE frequency in UV-induced cells, suggests a correlation between UV-induced SCE and post-replication repair, or some process closely related to it. However, Gatti and workers (1980) showed that in mei-41 mutant cells, which are defective in a caffeine sensitive pathway of post-replication repair, SCE levels were normal. These authors concluded that the formation of SCE is not a manifestation of post-replication repair. Shiraishi et al. (1982) reported findings that suggest double-strand rather that single-strand exchange is involved in SCE formation. Since post-replication repair of DNA damage involves the exchange of single strands of DNA, Shiraishi et al. (1982) stated that a relationship between SCE and post-replication repair seems doubtful. Kato (1977a) had previously suggested that if DNA strand breakage is involved in SCE formation then DNA repair mechanisms, which can induce strand breaks by endonucleolytic action at the site of DNA damage, or ligate

breaks which are induced by other means, might be implicated in SCE formation. If this were the case, then according to Kato, the pathway of SCE induction may depend on both where and how the initial DNA strand breaks are introduced and which repair mechanism is functioning at a given lesion.

Bender et al. (1974) noted that recombination repair like SCE is stimulated by UV-irradiation and inhibited by caffeine, thus suggesting a recombinational repair origin for some SCE events. On the other hand, Ishii and Bender (1980) expressed serious doubts concerning models which implicate either recombinational DNA repair or replicative bypass repair through branch migration, in the formation of SCE for two reasons. First, neither of these repair mechanisms has been well established in mammalian cells, and secondly, they remain as candidates for a role in SCE induction, not because any direct evidence suggests that they play a role but rather, because they have not been disproven.

Wolff et al. (1975) attempted to test whether known DNA repair processes are involved in the formation of SCEs. Various human cell lines were employed. Untransformed and transformed SV40 cell lines from normal patients as well as those from excision-repair defective, and post-replication repair defective Xeroderma pigmentosum patients and from patients with other hereditary diseases were used. No significant differences were seen in the yield of SCEs between Xeroderma Pigmentosum cells and normal cells. Neither the amount of excision-repair nor the presence of defective post-replication repair appeared to affect SCE yields. A similar conclusion was reached by Wolff (1978) who showed that a number of Chinese hamster cell lines with different excision repair capacities, showed the same yield of SCE following UV-irradiation.

However, DeWeerd-Kastelein and colleagues (1977) reported that four out of five excision deficient Xeroderma Pigmentosum cell lines irradiated with UV-light showed significantly higher SCE frequencies than control cells, suggesting that unrepaired damage is involved in SCE production. And Shafer (1982) argued that studies such as that of DeWeerd-Kastelein et al., coupled with the evidence that SCE induction requires the occurrence of both DNA damage and DNA replication, suggested that unexcised UV-induced damage plays an important role in the high SCE response of some Xeroderma Pigmentosum cells to UV treatment.

Painter (1980), who proposed the replicon as the site of SCE formation, suggested that the only role which DNA repair might play in SCE formation would be to reduce the amount of damage present in the DNA before replication. In fact, Schvartzman and Gutierrez (1980) showed that a given damaging treatment, such as irradiation with visible light, yields different numbers of SCEs depending on the period of the cell cycle in which the damaging treatment is given. Using BrdU substituted chromosomes of Allium cepa L., these authors found that the effectiveness of visible light irradiation in inducing SCE increases the closer it is administered in the beginning of S phase. The highest increase over the baseline frequency, coincides with irradiation in early S phase. SCE induction decreased rapidly as cells progress through the S phase and is zero when treatment is given during G_2. These authors suggested that this differential effectiveness of visible light irradiation may be due to the period of time available for the cell to carry out cell repair after DNA damage occurs and before the onset of DNA replication. High frequencies of SCE would thus be directly related to the number of unrepaired DNA lesions remaining in the

DNA at the time of replication.

SCE as a bioassay for mutagenicity and carcinogenicity testing

Latt (1974) carried out the first in vitro bioassay using SCE induction as an indication of chromosome damage by Mitomycin C in human lymphocytes. Since then a number of studies using the SCE test as a bioassay for induction of chromosomal damage have been carried out in higher plants. All of these studies have employed the base anologue substitution of chromosomes with BrdU and the FPG staining technique, usually a modification of the original technique of Kihlman and Kronborg (1975). Such studies in higher plants use the rapidly dividing cells of the root meristem.

The effects of chemical treatment on SCE frequencies in Vicia faba have been studied by several authors. The chemicals include thiotepa, caffeine, 8-ethoxy caffeine (Kihlman, 1975a), methyl methanesulphonate, ethyl methanesulphonate, N-methyl-N'-nitrosoguanidine, quinicrine mustard, thiotepa phosphine sulfide (Kihlman and Sturelid, 1978), ethanol (Schubert et al., 1979), mitomycin C, maleic hydrazide (Kihlman and Sturelid, 1978; Schubert et al., 1979), X-rays (Andersson et al., 1981), bleomycin, streptomycin (Kihlman and Sturelid, 1978; Andersson, 1981) and paint thinner (Gomez-Arroyo and Castillo-Ruiz, 1985).

In Allium cepa the effects of the fungicide vinclozolin (Escalza et al., 1983), the base analogues BrdU (Gutierrez et al., 1983) and FdU (Escalza et al., 1985), as well as the effects of caffeine (Cortes and Hazen, 1984) and pyronin Y (Cortes and Hazen, 1984, Armas-Portela et al., 1985) have been studied. The physical effects of visible light (Schvartzman

et al., 1979b), temperature (Gutierrez et al., 1981), oxygen dependency (Gutierrez and Lopez-Saez, 1982) and green light (Armas-Portela et al., 1985) have also been examined in Allium cepa. The effect of chemical treatment with thiotepa on SCE frequency has been studied in Tradescantia paludosa (Andersson, 1985).

In these studies, with the exceptions of 8-ethoxy caffeine, vinclozolin, and bleomycin, all chemical substances strongly induced increased SCEs with increasing dose. X-rays had little effect on SCE frequencies. Culture temperature was found to be inversely proportional to SCE induction, and increased as a function of oxygen tension. Both FdU and BrdU were efficient inducers of SCE in plant bioassays.

Many authors now regard the SCE bioassay as a valuable tool for the detection of mutagenic and carcinogenic activity of chromosome damaging agents (Miller, 1978; Abe and Sasaki, 1982; Carrano and Moore, 1982; Lambert et al., 1982; Latt et al., 1984; Shafer, 1984). SCE, thought to represent a direct interaction of genotoxic materials with DNA (Danford, 1983; Speit et al., 1984; Tice, 1984; Anon, 1985b), can be induced by a variety of agents which damage DNA, while DNA is thought to be the primary target of physical or chemical mutagens (Wolff, 1981). Moreover, many mutagenic agents are known to induce SCEs (Abe and Sasaki, 1982; Carrano and Moore, 1982; Carrano and Thompson, 1982; Sorsa, 1984). Takehisa (1982) suggested that in some cases the SCE test is more sensitive than the Ames test in detecting known carcinogens and mutagens such as urethane diethylstilbestrol and saccharin. Latt (1981) noted that about 80% of carcinogenic chemicals studied, are known to produce SCEs either in vitro or in vivo. In cultured Chinese hamster cells, treated with four different mutagens, each known to cause a

different kind of DNA lesion, a linear relationship was observed between SCE induction and induced gene mutation (Carrano et al., 1978; Carrano and Thompson, 1982). Genetic transformation has also been linked to SCE induction (Carrano and Thompson, 1982; Popescu and DiPaolo, 1984). It has been suggested that SCE may be valuable as an indicator of damage which is compatible with subsequent cell survival, whereas chromosome breakage assays are not.

Advantages of the SCE technique over other cytogenetic bioassays are its increased sensitivity as an indicator of chromosomal damage (Wolff, 1977; Perry, 1980; Shafer, 1982; Carrano and Thompson, 1982; Fujiwara et al., 1984; Latt et al., 1984; Sorsa, 1984; Tice, 1984; Gomez-Arroyo and Castillo-Ruiz, 1985), since the effects of many DNA damaging agents can be detected at much lower levels than those which are needed to induce chromosomal aberrations (Perry and Evans, 1975; Solomon and Bobrow, 1975; Carrano et al., 1978; Wolff, 1981; Takehisa, 1982; Deknudt, 1984). Other advantages of the SCE assay which have been cited in the literature include its rapid methodology and analysis (Perry and Evans, 1975; Carrano et al., 1978; Lambert et al., 1982; Shafer, 1982; Danford, 1983; Anon, 1985b), the fact that scoring is simple and reproducible (Solomon and Bobrow, 1975; Wolff, 1977, 1978; Anon, 1985b) and that few cells need to be scored in order to give rise to statistically significant increases in SCE frequencies (Solomon and Bobrow, 1975; Wolff, 1978; Perry 1980; Deknudt, 1984). Additionally, this assay can be used as a means for <u>in situ</u> detection of environmental or occupational genotoxic contaminants (Perry and Evans, 1975; Wolff, 1981; Lambert et al., 1982; Sorsa, 1984; Watanabe and Endo, 1984). Takehisa (1982) noted that another advantage of the SCE test over tests

which use micro-organisms to assay for mutagenicity and carcinogenicity such as the Ames test, is its use of eukaryotic cells. He suggested that in vivo mammalian assays can more accurately illustrate damage to mammalian systems than bacterial cells.

However, SCE induction alone is not generally accepted as sufficient evidence for the classification of an agent as a mutagen (Hansteen, 1982; Danford, 1983; Tice, 1984; Anon, 1985b). A recent W.H.O. publication (Anon, 1985a) on the use of SCE assays, suggested that the role of this bioassay in the detection of carcinogens has yet to be defined, while some authors clearly feel that as a bioassay for mutagenicity it is presently overrated. It has been pointed out, for example, that some mutagens such as ionizing radiation (Solomon and Bobrow, 1975), ethidium bromide, cytosine arabinoside, chloramphenicol, cyclohexamide (Gebhart, 1982) and clastogens in general (Danford, 1983; Anon, 1985b; Sorsa, 1984) have little effect on SCE induction, while bleomycin and maleic hydrazide did not yield a clear dose-dependent response (Gebhart, 1982). Solomon and Bobrow (1975) suggested that the failure of some mutagenic agents to induce SCE may limit the practical usefulness of SCE as a mutagenic bioassay. Perry (1980) noted that pyrene, which nearly doubles SCE frequency in CHO cells is a well documented non-carcinogen. Gebhart (1982) suggested that the large number of false negative results demonstrate the limits of the test, as well as the large numbers of weakly positive results which are difficult to interperet when these same substances have yielded negative results in other mutagenicity assays. Carrano and Thompson (1982) warned that although single gene mutation and SCE induction are linearly correlated for some agents, this does not imply that all agents will demonstrate such a

relation, nor that the same lesions produce both endpoints. It could be that the cellular processing of different, or like lesions, lead to these endpoints, thus not every SCE inducing agent would be mutagenic and visa versa. Some authors pointed out that the ratio of induced SCEs to gene mutations (Carrano and Moore, 1982) and carcinogens (Erskine et al., 1984) varies widely for both chemical and physical agents. According to Wolff (1981) the SCE to mutation ratio may differ with species, tissue type and dose rate.

Some authors felt that because the mechanism of SCE formation as well as the biological significance of this endpoint are poorly understood, this represented an important disadvantage to using it widely as a test for mutagenicity (Gebhart, 1982; Shafer, 1982). Others pointed out that since the genetic significance of SCE is not known, then its significance in terms of mutagenicity and carcinogenicity can not be determined (Shafer, 1982; Danford, 1983).

It has been suggested that <u>in vivo</u> studies of human exposure to potentially hazardous environmental or occupational materials, which show a positive correlation between exposure and SCE induction, should always be considered as an adverse sign of exposure, since they probably indicate DNA damage and a qualitatively increased risk of mutagenesis and carcinogenesis. However, the health consequences for the individual cannot be estimated quantitatively or otherwise since little is known about the possible association of carcinogens or mutagenic agents with SCE (Erskine et al., 1984; Sorsa, 1984; Tice, 1984). Sorsa pointed out that another disadvantage of using SCE in occupational carcinogenic and mutagenic studies is that very few of such studies have been confirmed by independent findings. Perry

(1980) agreed that given the small number of chemicals that have been investigated rigorously using the SCE test, it is difficult to correlate this endpoint with animal carcinogenicity data or with other test systems commonly in use.

Danford (1983) noted that there is a high background level of SCE which may be in part due to the necessity of providing BrdU, known to induce SCEs, to the test organism in order to visualize exchanges. Some authors expressed concern as to whether or not BrdU incorporation affects the sensitivity of the SCE test to different chemical agents (Schvartzman and Tice, 1982; Morgan and Wolff, 1984) or whether the fact that this background frequency varies in different labs even in cells of the same line might not affect the test's accuracy (Perry, 1980). The possibility of synergistic interaction between BrdU substituted DNA of the test system and the test compound might also call into question the reliability of the assay (Franz, 1982; Schvartzman and Tice, 1982). Thus, according to Gebhart (1982), the SCE test is a measure of the co-activity of the test agent and BrdU which leads to the observation of co-mutagenicity rather than mutagenicity. In a study by Morgan and Wolff (1984), a comparison was made between cells in which chemical treatment was carried out before BrdU substitution was begun, and those in which cells were treated continuously with BrdU and the test agent. The results suggested that some SCE inducing agents affect substituted and unsubstituted DNA differently.

Those who feel that SCE may not yet be a reliable test for mutagenicity and carcinogenicity suggested that SCE may nevertheless provide a useful technique for exploring the mechanisms of chromosome breakage and repair (Solomon and Bobrow, 1975), serve as a complementary assay to other

assays for mutagenicity (Carrano and Moore, 1982; Hansteen, 1982; Anon, 1985a) or as an aid in obtaining additional insights into the molecular mechanisms of the cytogenetic activity of mutagens (Gebhart, 1982).

MATERIAL AND METHODS

Initial work concentrated on developing a technique for the differential staining of BrdU substituted chromosomes. Using the Feulgen staining procedure of Templaar et al. (1982) preliminary work was initiated with root tips of Hordeum vulgare L. Because differential staining was difficult to procure, variations of the FPG technique were tried. In barley a modification outlined by Grant and Zura (1982) was used. In Vicia faba, a modified version of Cortes and Andersson (1987) was used, as well as the original modified FPG technique of Kihlman and Kronborg (1975). The Feulgen staining procedure for SCE was also tried in Vicia.

Growing Roots from Seed

Barley:

The barley seed (cv. 'Laurier') was obtained from the E.A. Lods Agronomy Research Center, Macdonald College, McGill University.

Seeds were soaked in distilled water for 6 h followed by germination in 15 cm plastic petri dishes. The bottom of the petri dish was lined with about 0.5 cm of perlite and moistened with distilled water. This layer of perlite was covered with a layer of no. 4 filter paper which was also moistened. Seeds were placed on the filter paper. Enough space was left between the seeds so that they were not touching each other. Finally, the seeds were covered with a second piece of moist filter paper and the petri dish was covered and left in the dark at 18 C. Care was taken to leave a sufficient space between germinating seeds, as well as to assure that only a moderate amount of moisture was in contact with the seeds, as both

overcrowding and excessive moisture inhibit germination. Seeds were left for approximately 32-36 h at which point the radicle had grown to between 2 to 5 mm in length. The radicles were then ready for treatment with a base analogue.

Vicia faba:

Seeds of Vicia faba (cv. 'Aguadulce'; former name Long Pod Seville) were obtained from Charles Sharpe and Co. Ltd., Seed Producers, Sleaford, England.

Seeds were soaked in a 1% solution of sodium hypochlorite (commercial bleach) for 5 min followed by rinsing in running water for 10 min. This step was required since contamination rapidly develops in its absence. The seeds were then transferred to a large beaker in which they were left to soak overnight (14-20 h) in the dark at 18-20 C. The following day they were transferred to a shallow, glass baking dish 35 by 22 by 5 cm to which running water was supplied at a temperature of 18 C. This water was continually aerated by means of a small aquarium air pump. Germination was continued in the dark.

The outer seed coat was removed when the developing root reached approximately 1 cm. This step is necessary to guard against further contamination which may result from the presence of microorganisms sheltered beneath the seed coat. When the primary root tips reached 4.5 to 5.5 cm in length (approximately 7 days), they were decapitated so that the initiation of secondary roots would occur. Seedling shoots as well as root tips were removed. Since all primary roots do not reach the same length uniformly, shoots and root tips were removed on 3 to 4 consecutive days.

Seedlings were transferred in groups of 2 or 3 to individual 250 ml beakers. Seedlings were supported by a plastic screen at the mouth of the beaker so that only the radicles extended into the beakers. These were filled with distilled water and maintained in the dark with continuous aeration at 18 to 20 C for the number of days required in order that lateral roots appear and reach a length of approximately 0.5 to 2 cm (about 3 days). Seeds were then ready for treatment with a base analogue.

Base Analogue Treatment

Barley:

Root tips were treated for one cell cycle (12 h) with a 500 uM solution of Brd [5-bromodeoxyuridine ($5x10^{-4}M$), fluorodeoxyuridine ($5x10^{-8}M$), and uridine ($10^{-8}M$)], followed by a slightly prolonged second cell cycle, [12 h + (1-3h)], with a 25 uM solution of thymidine, [thymidine ($2.5x10^{-5}M$), uridine ($10^{-6}M$)] (Schubert et al., 1980).

Seedlings were transferred from the petri dishes in which they had been germinating into petri dishes prepared exactly as they had been at the germination stage except that the distilled water was replaced either by the BrdU or the thymidine solution.

When transferring seedlings from one petri dish to another each seedling was placed so that root tips were in contact with the bottom filter paper. Base analogue treatment was carried out in an 18 C incubator in the darkness. Due care was taken when handling solutions containing halogenated nucleosides as they are mutagenic (Lewin, 1985).

The prolongation of the second cell cycle allows for the fact that treatment of living plant cells with BrdU and other base analogues tends to

delay the cell cycle (Cortes and Gonzalez-Gil, 1982). Thus an increase in the second treatment period should yield the greatest possible number of substituted metaphases.

Vicia faba:

A number of workers appear to have different preferences both in terms of the concentration of base analogue they use, as well as in choosing whether to use either two cell cycles of BrdU, or one cell cycle of BrdU followed by a second cycle of thymidine.

For the Feulgen procedure both methods of treatment were used. In the first case, root tips were treated for two consecutive cell cycles with a 100 uM solution of BrdU [5-bromo-2'-deoxyuridine (10^{-4}M), 5-fluorodeoxyuridine (10^{-7}M), and uridine (5×10^{-6}M)] (Kihlman and Kronborg, 1975).

For the FPG technique in Vicia faba, two consecutive cycles of 100 uM BrdU were used (Cortes and Andersson, 1987). Treatments were carried out by replacing the distilled water in the 250 ml beakers with 100 uM BrdU or 100 uM thymidine for a period of two cell cycles as indicated previously. Base analogue treatment was carried out in an 18 C incubator in the dark with constant aeration.

Preliminary Feulgen Schedules for Differential Staining

Barley:

1) Excise root tips and wash. Treat with 0.002 M 8-Hydroxyquinoline for 2 h in a refrigerator (4 C).

2) Wash root tips and fix for:

1 h in glacial acetic acid in the refrigerator, followed by 14-20 h in 3:1 95% ethanol:glacial acetic acid in the refrigerator, then 15 min in 70% ethanol at 28 C (Tempelaar et al., 1982).

3) Wash root tips and treat with 5 M HCl at 37 C for 45-51 min.

4) Rinse at least 3 times in distilled water. Allow 2 min between rinses. Stain in basic fuchsin for approximately 15 min in the dark.

5) Macerate for 1.75 h in a solution of 5% pectinase and 0.5% cellulase adjusted to a pH of 4.7 with 0.2 M HCl (Tanaka and Ohta, 1982).

6) Squash root tips in 45% acetic acid and score using a phase contrast light microscope.

7) Store unsquashed root tips overnight in 70% ethanol in the refrigerator or make slides permanent by the Conger and Fairchild (1953) method.

Vicia faba:

1) Excise root tips and wash. Treat with 0.05% colchicine for 3 h at 18 C.

2) Wash root tips and fix for:

1 h in glacial acetic acid in the refrigerator, followed by 14-20 h in 3:1 95% ethanol:glacial acetic acid in the refrigerator, then 15 min in 70% ethanol at 28 C (Tempelaar et al., 1982).

3) Wash root tips and treat with 5 M HCl at 28° C for 80-85 min.

4) Rinse at least 3 times in distilled water. Allow 2 min between rinses. Stain in basic fuchsin for approximately 15 min in the dark.

5) Macerate for 1.25 h in a solution of 3% pectinase adjusted to a pH of 4.7 with 0.2 M HCl.

6) Squash root tips in acetic acid and score using a phase contrast light microscope.

7) Store unsquashed root tips overnight in 70% ethanol in the refrigerator or make slides permanent by the Conger and Fairchild method (1953).

Fluorescent-plus-Giemsa

The modifications of this technique in the literature are quite diverse. The simplest way to illustrate the differences between those which were attempted in barley and in Vicia in this study is by means of Table I, shown on the following page.

Examination of Some of the Factors Affecting Differential Staining in Vicia faba

In further experiments, some of the factors which might affect the success of differential staining in Vicia faba, with both the Feulgen procedure and the FPG technique, were examined.

Factors affecting the mitotic index:

In this section, unless otherwise mentioned, roots were grown as

TABLE I. FPG techniques according to original authors. Different FPG techniques identified by Roman numeral as reported by the author shown below.

I. Kihlman and Kronborg (1975).
II. Grant and Zura (1982).
III. Cortes and Andersson (1987).

TREATMENT I	II	III
BASE ANALOGUE		
100 uM BrdU 18 h 100 uM Thd 21 h	100 uM BrdU 17 h 100 uM BrdU 19 h	same as II
FIXATION		
0.05% colchicine 3 h 3:1 ETOH: acetic acid, cold, overnight (14–20 h)	same as I	0.05% colchicine 3 h 3:1 methanol:acetic acid prolonged fixation up to one week
RINSE		
0.01 M citric acid buffer	same as I	d H_2O
MACERATE		
0.5% pectinase in citric acid buffer pH 4.7, 75 min at 27 C	2 h at 22 C	2 h at 27 C
SQUASH		
in 45% acetic acid on slides subbed in a 10:1 mix of gelatin:chrome-alum.	XXX*	same as I
REMOVE COVER SLIP		
dry ice method	XXX*	same as I
HYDRATE		
absolute ETOH series: 95% etoh to d H_2O	XXX*	same as I
RNase		
prepared as follows: 1 mg RNase dissolved in 10 ml 0.5 x SSC. Place 200 ul onto squashed tissue. Incubate 1 h at 27.	XXX*	XXX*
RINSE		
in 0.5 x SSC	XXX*	XXX*

TREATMENT I	II	III
STAIN		
prepared as follows: 1 mg "33258 Hoechst" fluorochrome dissolved in 1 ml of ETOH. Then 1 ml of this solution is dissolved in 200 ml 0.5 x SSC. Stain for 20 min.	same as I	Stain for 25 min with simultaneous exposure to irradiation from a germicidal lamp.
Differentiation of sister chromatids may be improved by exposure to long wave UV for 30 min.	XXX*	XXX*
Store slides over d H_2O for 4 days in a moist chamber at 4 C.		XXX*
HYDROLYSIS		
Incubate at 0.5 x SSC for 60 min at 55 C.	same as I	Incubate in 5 M HCl for 20 min at 25 C.
RINSE		
in 0.017 M phosphate buffer, pH 4.6.	same as I	in d H_2O
STAIN		
in 3% Giemsa dissolved in same phosphate buffer 6-7 min.	same as I	XXX*
MOUNT		
Air dry and mount. END	same as I END	XXX*
		MACERATE Incubate in 0.1 M HCl for 2 min at 60 C.
		SQUASH in 45% acetic acid; leave preparations for some minutes in 0.017 M phosphate buffer, pH 6.8.
		STAIN in 2% Giemsa dissolved in the same buffer for 8 min.
		MOUNT Air dry slides and mount.

*This step is either not performed or is performed at another time.

described in the Material and Methods section titled "Growing roots from seed". Root tips were fixed and stained according to the following schedule:

1) Excised root tips were treated for 2 h with 0.05% colchicine at room temperature and fixed overnight (14-20 h) in Carnoy's fixative: 3:1 95% ethanol:45% glacial acetic acid.
2) Root tips were hydrolyzed for 6 min. in M HCl in a water bath at 60 C and rinsed 3 times in distilled H_2O.
3) They were then stained in Feulgen reagent for 45 min. and rinsed 3 times in distilled H_2O.
4) Stained root tips were macerated in a solution of 5% pectinase, 0.5% cellulase, dissolved in a 0.01 M citric acid ($C_6H_8O_7 \cdot H_2O$) sodium citrate ($Na_3(C_7H_5O_7 \cdot 2H_2O)$) buffer, adjusted to a pH of 4.7 (Kihlman and Kronborg, 1975), and incubated at 37 C for 1 h.

Macerated root tips were squashed in 45% acetic acid and mitotic indexes were scored using a phase contrast light microscope. An analysis of variance and Duncan's multiple range test were carried out to test for differences between treatments.

1 **Removal of seedling shoots and cotyledons:**

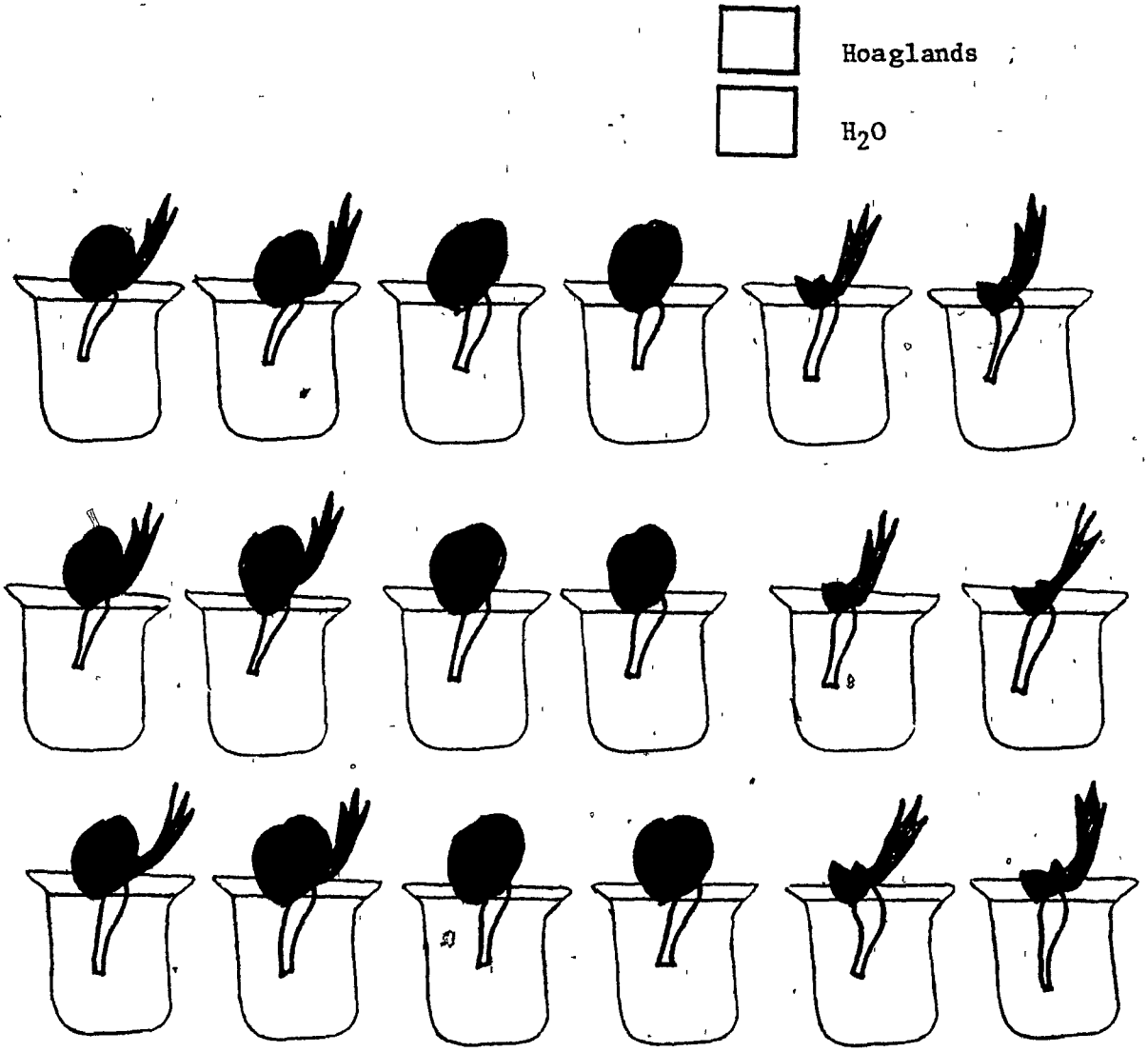
Some authors include the removal of growing shoots from young Vicia faba seedlings in order to encourage the growth of secondary roots (Kihlman and Andersson, 1984), while at least one author has suggested that high concentrations of BrdU are needed to offset the store of thymidine

precursors that are available to the plant by way of the cotyledon (Tempelaar, 1987; personal communication). To test how secondary root tip initiation and cell division might be influenced by the removal of either seedling shoots or seedling cotyledons, the following experiments were carried out.

Each experiment was made up of six treatments, each having three replicates, as shown in Figure 1. Two treatments in each experiment were control treatments and only primary root tips were excised. In another two treatments the seedling shoots and the primary root tips were excised. In the final two treatments the seedling cotyledons and the primary root tips were excised. One half of each double set of treatments was supplied with tap water, and the other half was supplied with Hoagland nutrient solution (Appendix II). The only difference between the first and the second experiment was that in the second experiment 1) a Hoagland nutrient solution of 1 part Hoaglands 8 parts water (Scheid, 1976) was used and 2) the primary root tips were cut on the first day and the seedling shoots and cotyledons were cut when the first signs of secondary root initiation were observed.

Secondary root tips were collected and fixed when they had reached a length of approximately 0.5 to 2.0 cm. After staining and maceration mitotic index counts were made as follows: 100 cells were counted at random, ten times, in each of three root tips chosen randomly from each replicate and these were recorded as percentages per root tip. An analysis of variance and Duncan's multiple range test were carried out to test for differences between treatments.

Figure 1 Removal of seedling shoots and cotyledons



CONTROL:
Experiment #1
Removal of primary root tips on day 1

Experiment #2
Same as above

REMOVAL OF SHOOTS:

Removal of primary root tips and seed shoots on day 1

Removal of primary root tips on day 1 and removal of seed shoots upon the appearance of secondary roots

REMOVAL OF COTYLEDONS:

Removal of primary root tips and seed cotyledons on day 1

Removal of primary root tips on day 1 and removal of seed cotyledons upon the appearance of secondary roots

ii Unifilar vs. bifilar substitution of the base analogue BrdU at various concentrations:

This experiment was made up of 7 treatments, each having 3 replicates. In the contol treatment, tap water alone was supplied to the growing seedlings. The remainder of the treatments were supplied for either 21 or 41 h with the base analogue BrdU, in order to obtain either unifilar or bifilar substitution, respectively. BrdU was supplied to the seedlings at 50, 100 and 500 uM upon the initiation of secondary roots. The base analogue solutions were supplemented with 0.5 uM fluorodeoxyuridine (FdU) and 5.0 uM uridine (Kihlman and Kronborg, 1975).

Secondary root tips were collected and fixed when they had reached a length of approximately 0.5 to 2.0 cm. After staining and maceration, mitotic index counts were made as described in the previous section and an analysis of variance and Duncan's multiple range test were carried out to test for differences between treatments.

iii Increasing concentration of the base analogue BrdU and the thymidylate synthetase inhibitor fluorodeoxyuridine (FdU):

In this experiment 10 treatments of 3 replicates each were set up much the same way as in the previous section. All treatments were supplied for 21 h (one cell cycle) with various concentrations of the base analogue BrdU. In the control treatment tap water alone was supplied to the growing seedlings. The remaining 9 treatments were divided into 3 groups of 3 and upon the initiation of secondary roots, were treated as follows:

Treatments 2, 3 and 4 were treated with 50 uM BrdU and 5 uM uridine, and supplemented with 0, 0.1 and 5.0 uM FdU, respectively.

Treatments 5, 6 and 7 were treated with 100 uM BrdU and 5 uM uridine, and supplemented with 0, 0.1 and 5.0 uM FdU, respectively.

Treatments 8, 9 and 10 were treated with 500 uM BrdU and 5 uM uridine, and supplemented with 0, 0.1 and 5.0 uM FdU, respectively.

Secondary root tips were collected and fixed when they had reached a length of approximately 0.5 to 2.0 cm. After staining and maceration, mitotic index counts were made as described in the previous sections. Statistical analysis was carried out as described above.

Factors affecting the Feulgen procedure for the differential staining of sister chromatids:

1 Maceration:

Table II illustrates the diversity of maceration protocols found among the staining techniques reported by different authors. In order to choose the optimal procedure for the maceration of Vicia faba root tips, secondary root tips were generated, fixed and stained, as in the previous sections, without base analogue substitution. Seven root tips, selected randomly from a group of 18 plants were placed in each of 18 vials and treated at 37 C as follows:

1, 2 and 3% pectinase for 1 and 2 h each

1, 2 and 3% pectinase, supplemented with 0.5% cellulase for 1 and 2 h each.

10:1 45% acetic acid: M HCl for 1, 2 and 3 h

0.1 M HCl for 1, 2 and 3 h

Table II. Maceration techniques according to original authors. All authors used *Vicia faba*.

Author(s)	0.01 M citric acid–sodium citrate buffer pH 4.7	Concentration of enzymes	Temp C	Time (min)
Kihlman & Kronborg (1975)	yes	0.5% pectinase	27	75
Grant & Zura (1982)	no	0.01 N HCl	60	2
Tempelaar et al. (1982)	yes	2% pectinase	28	15
Vosa (1981)	no maceration treatment specified.			
Schubert et al. (1980)	yes	1% pectinase 2% cellulase	37 37	120 90
Cortes & Andersson (1987)	yes	0.5% pectinase	27	120

The enzymes pectinase and cellulase were dissolved in a 0.01 M citric acid—sodium citrate buffer, pH 4.7. The other solutions were made up in tap water.

After the maceration was complete, root tips were stored in 70% ethanol at 4 C. Three out of 7 root tips from each vial were squashed in 45% acetic acid and chromosome squashes were scored on overall appearance, quality of staining, chromosome morphology and the appearance of the cytoplasm.

ii **Duration of 5 M HCl acid hydrolysis:**

In this section the Feulgen procedure for the differential staining of sister chromatids is described as follows:

1) Root tips were grown as described in the Material and Methods section entitled "Growing roots from seed".
2) Excised root tips were treated for 2 h with 0.05% colchicine at room temperature and fixed overnight (14-20 h) in Carnoy's fixative 3:1 95% ethanol: glacial acetic acid.
3) Root tips were washed in distilled water and hydrolyzed in 5 M HCl at 28 C for the following time periods:

Trial #1: 68-72-76-80-84-88 and 94 minutes

Trial #2: 50-58-62-64-68-72-76 and 80 minutes

and then rinsed at least 3 times in distilled water.

4) They were then stained in Feulgen reagent for approximately 15 minutes in the dark and macerated for 1 h and 45 min in 3% pectinase, 0.5% cellulase dissolved in a 0.01 M citric acid-sodium citrate buffer pH 4.7, at 37° C.

5) Root tips were squashed in 45% acetic acid and scored using a phase contrast light microscope.

Slides were scanned for the presence and frequency of chromosomes containing either sister chromatid differentiation or sister chromatid exchanges.

iii SO_2 water wash of Feulgen stained root tips:

The Feulgen procedure for differential staining of sister chromatids was carried out as described above and root tip maceration was followed by a 10 min wash in SO_2 water (10 ml of 10% $K_2S_2O_5$ + 10 ml M HCl in 200 ml of tap water) as described in the protocol of Tempelaar et al. (1982). Comparisons were made between root tip squashes in which an SO_2 wash had been applied and those in which it had not.

iv Duration of root tip fixation in Carnoy's:

The feulgen staining schedule for differential staining of sister chromatids described in the previous section was followed and root tips chosen randomly from 20 plants were fixed for 1, 4, 7, 10, 13 and 22 h. Acid hydrolysis was carried out for 80, 83 and 86 minutes. For each time of fixation there were six vials, two for each time of hydrolysis. This

allowed one set of root tips to be examined immediately to determine the short term effects of varying fixation time, while the second set of vials was examined one month later, to determine whether any differences could be seen after a one-month storage period. All root tips were stored in 70% ethanol at 4 C.

Root tips were squashed in 45% acetic acid. Three root tips were examined from each vial. Mitotic indices were counted and observations were made with respect to the presence, quantity, and resolution of sister chromatid differentiation and exchange, as well as the quality of staining, chromosome morphology and the appearance of the cytoplasm.

Factors affecting the FPG staining procedure:

Based on the results from the above experiments, and according to the various authors of the FPG protocol studied here, Vicia faba seedlings were grown as described previously and root tips were treated with BrdU, fixed, and squashed according to the following schedule:

1) A 100 uM solution of BrdU, supplemented with 0.5 uM FdU and 5 uM uridine was provided to the growing root tips for 19 h and followed immediately by treatment for 21 h with a 100 uM solution of thymidine supplemented with 0.5 uM FdU and 5 uM uridine.
2) Root tips were then removed and treated for 3 h with a 0.05% solution of colchicine and fixed in 3:1 95% ETOH:glacial acetic acid for 7 h at 4 C.

3) Root tips were then macerated in a solution of 3% pectinase, 0.5% cellulase, dissolved in a 0.01 M citric acid buffer, at 37 C for 2 h.

4) Maceration was followed by squashing the cells in 45% acetic acid on 95% ETOH cleaned slides which have been coated with Albumen fixative from Fisher.

5) Cover slips are then removed by the dry ice method and hydrated via 100-95-85-70-50-30% ETOH-H_2O series.

The remaining sections describe the examination by various authors of factors which might affect the FPG techniques for sister chromatid differentiation.

1 Incorporation of an RNAse treatment into the FPG staining protocol:

Among the three FPG staining protocols for SCE in Vicia faba, discussed in this paper, only the authors who first reported SCE in Vicia faba (Kihlman and Kronborg, 1975) carried out an RNase digestion before staining (see Table I).

In the present study, the RNAse digestion was also added to the protocol of Cortes and Andersson (1987) as follows: After chromosome squashes are hydrated in an ethanol series from 95% ethanol to H_2O, 200 ul of RNAse solution are placed onto the squashed tissue and incubated for 60 minutes at 27 C. The RNAse solution is made as described in Table I.

Comparisons were made between cells in which RNAse digestion had been carried out and those in which it had not.

ii **Incorporation of trypsin digestion into the FPG staining protocol:**

Incorporation of trypsin digestion was made as follows: after the RNAse digestion described above was completed, the tissue was again rinsed in 0.5 x SSC. Then, 200 uM of 0.1% or 0.25% trypsin, dissolved in a phosphate buffer pH 7.02, was placed onto the squashed tissue and incubated at room temperature for 1, 3, 5 and 10 min. Preparations were rinsed again in 0.5 x SSC and stained in "33258 Hoechst" according to the techniques of Kihlman and Kronborg (1975) or Cortes and Andersson (1987) as described in Table I, with the additional modifications described in the beginning of this section. Comparisons were made between the chromosome staining of those cells which had undergone a trypsin digestion and those which had not.

RESULTS AND DISCUSSION

Feulgen Staining in Barley

Figure 2A illustrates a metaphase spread of barley chromosomes with differential staining of sister chromatids according to the Feulgen technique. As is often the case, not all of the chromosomes in this, or in any of the photographs presented in Figure 2, would have been suitable for scoring, even though they show clear differential staining and obvious sister chromatid exchange. This is because of overlap, and twists in individual chromosomes which are a common occurrence and one of the reasons why the tissue must be sufficiently macerated before it is squashed.

Although in this work sister chromatid differentiation was sometimes obtained in barley using the Feulgen procedure for differential staining of sister chromatids (Vosa, 1981; Tempelaar et al., 1982), repetition of these results was difficult to obtain. When the procedure was repeated, sister chromatid differentiation was often not obtained and at no time when chemical pesticide treatment was applied was sister chromatid differentiation obtained.

Fluorescent-plus-Giemsa in Barley

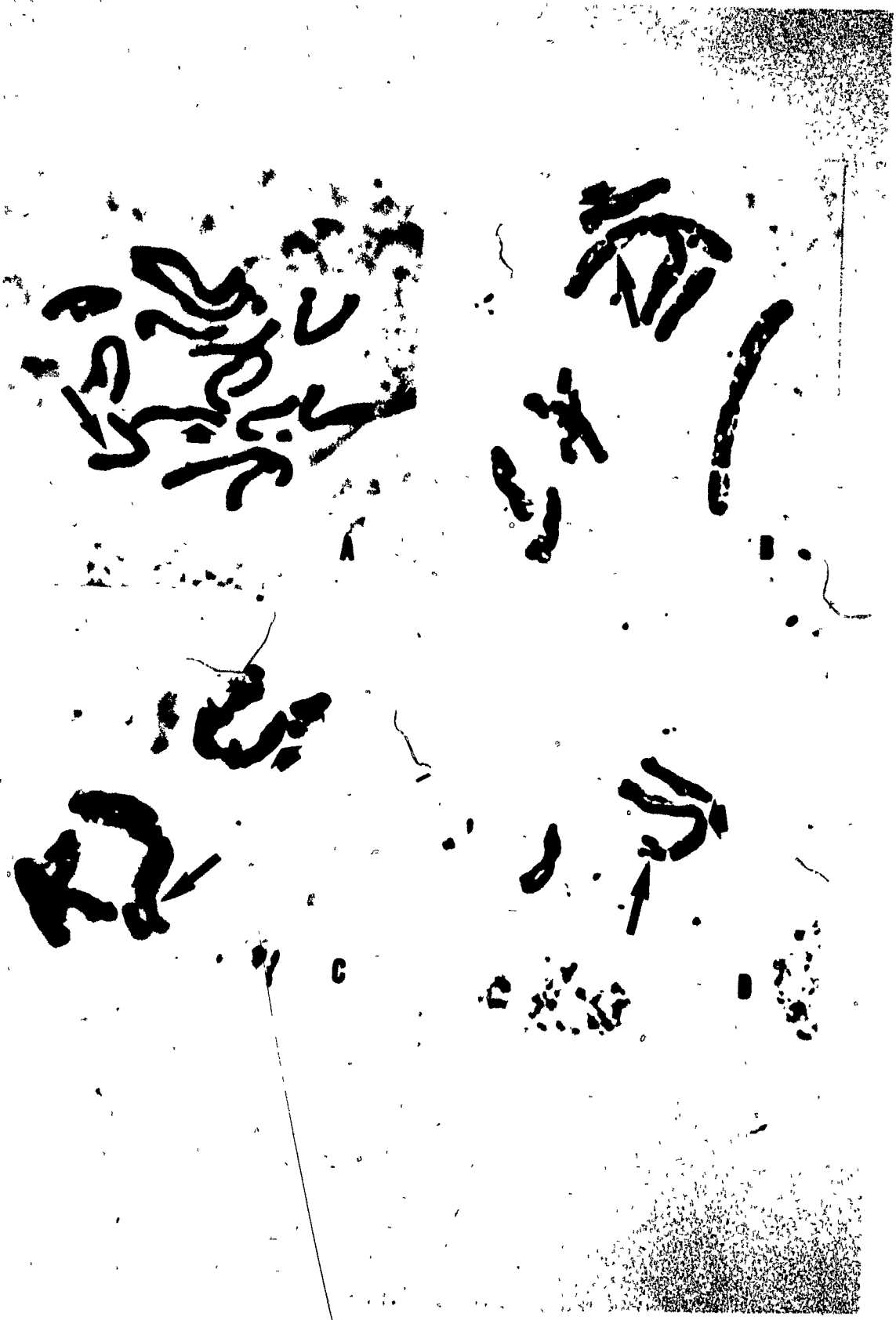
To obtain sister chromatid differentiation in barley by means of FPG staining, the technique of Grant and Zura (1982) was attempted. In their protocol whole root tips were treated exactly as squashed tissue is treated in the modified technique of Khilman and Kronborg (1975) and chromosome squashes were made after staining was carried out. The chromosomes obtained in this work, were stained lightly by Giemsa but were enlarged, and no

FIGURE 2

Differential sister chromatid staining in barley and Vicia faba according to the Feulgen procedure.

A Sister chromatid exchange, indicated by the long arrow, in barley chromosomes bifilarly substituted with BrdU at a concentration of 500 uM. A twist in the chromosome is indicated by the short arrow. x ca. 1510.

B,C,D Sister chromatid exchange in Vicia faba chromosomes unifilarly substituted with BrdU at a concentration of 100 uM. Sister chromatid exchange and chromosome twists are indicated by long and short arrows, respectively. x ca. 1243, 1510 and 888.



differential staining or fluorescence was detected.

Feulgen Staining in Vicia faba

Sister chromatid differentiation was attempted in Vicia faba using the Feulgen technique, primarily because this material had already been used by a number of workers to analyze the effects of chemical mutagens on the frequency of SCE. Although in the initial trials Vicia chromosomes stained quite darkly, and some differentiation was obtained in a few cases, contrast was not distinct enough to count interchanges accurately. Often, both chromatids stained so darkly that chromatid differentiation was barely distinguishable. Increased duration of hydrolysis did not result in further differentiation. Instead it seemed to yield an overall reduction in chromosome staining and in chromosome morphology.

Fluorescent-plus-Giemsa Staining in Vicia faba

In the initial trials, differential staining of sister chromatids was attempted in Vicia faba by means of the FPG technique of Kihlman and Kronborg (1975) as well as by the modified version of Cortes and Andersson (1987). The two techniques are similar, as can be verified in Table 1, differing essentially in base analogue substitution and by a hot acid pretreatment for Giemsa staining which was added by the latter authors. Neither protocol yielded sister chromatid differentiation in the present study.

Examination of Some Factors Affecting Differential Staining in Vicia faba

The results presented in the following three sections make up the

remainder of the data to be presented in this thesis. These results represent a study which was undertaken to examine a number of factors which were thought likely to play a role in obtaining successful differentiation of sister chromatids in Vicia faba by means of the Feulgen procedure for differential staining or by the FPG technique. This study was initiated because of the number of unsuccessful attempts which had been made to study the effect of pesticide application on the frequency of SCE in Vicia faba as a measure of genetic toxicity.

Along with my own results, I also discuss some of the differences among the techniques which I followed, as well as others found in the literature. These include, the temperature and duration of acid hydrolysis in the Feulgen procedure, the use of an RNAse treatment and a trypsin digestion in the FPG technique, and in general, the growth and treatment of Vicia faba seedlings, the concentration, duration and substitution of the base analogue BrdU, cell wall maceration and fixation time.

Factors affecting the mitotic index:

The initial consideration in the present study was to determine what questions needed to be asked about the different components of the differential staining procedures in order to identify the most favorable sequence of steps which would lead to the clear differential staining of the sister chromatids.

i Removal of seedling shoots and cotyledons:

It has been widely reported that plant cells do not readily incorporate BrdU into their DNA (Vosa, 1976; Evans and Filion, 1980;

Gonzalez-Gil and Navarrete, 1982; Uggla and Natarajan, 1982). Tempelaar (personal communication) suggested that BrdU uptake may be limited because of the ample supply of nutrients from the endosperm, implying that thymidine or thymidine precursors might be readily synthesized by means of nutrients available to the growing seedling by way of the cotyledons, thus allowing the young plant to bypass the uptake of BrdU and continue to incorporate the normal base thymidine.

In the first stage of this work I examined the effect of removing possible endogenous sources of nutrients to the young seedlings; that is, seedling shoots and seedling cotyledons. I reasoned that if such removal resulted in only a slight or no decrease in the mitotic index that the use of such plants would have a definite advantage over the use of whole plants, since they would be expected to incorporate greater amounts of BrdU, by virtue of the need created by the removal of the plant shoots or cotyledons, of an exogenous source of nutrients.

In the first experiment which was carried out, either the seedling shoots, or the seedling cotyledons, were removed at the same time as the primary root tips were removed. Almost none of these plants gave rise to secondary roots indicating that the seedlings could not easily recover after the simultaneous removal of major meristematic centers and/or cotyledons. The control plants which were grown in tap water had mitotic indices ranging from 7 to 8.3% (Appendix IX) in individual root tips, while those grown in Hoagland's developed such severe contamination that no primary roots developed. Of a total of 18 plants in this experiment, only two, excluding the controls, developed any secondary root tips. When the experiment was repeated with the excision of seedling shoots or cotyledons being performed

only after the excision of the primary root tip and the subsequent development of secondary roots, enough numerical data were obtained to do a statistical analysis. Mitotic index counts were obtained from all plants except one, which developed no secondary roots. The analysis of variance (Appendix III) showed no signigicant differences between the treatments. However, since the coefficient of variability (CV) was well above 20% (Appendix VIII), no conclusion could be drawn about the non-significance of the results. The high CV was due to a high variance within treatments which led to a high error term. An example of the high variance is seen among the plants grown in a diluted Hoagland's solution from which the cotyledons were removed. Mitotic indices for individual root tips fell in a range between 0 and 14.4% (Appendix IX). This was a surprising result since such a high rate of cell division was not seen even in individual root tips among the control plants. But while individual root tips showed a broad range of values for the mitotic indices for plants in which the cotyledons or shoots had been removed, the treatment means were always lower than the controls.

Because the variance of the mitotic index within the controls was not great, falling in a range between 3.4% to 6.7% (Appendix IX), it is suggested that the high variance within non-control treatments was due to the differential responses of individual plants as a result of the removal of shoots or cotyledons. While one plant may be able to overcome the removal of these plant parts which is expressed in a normal or even sporadically high mitotic index, another plant will recover less easily and some plants may even die, as seen here, with the reduction of mitotic indices to zero or the failure of the plant to give rise to secondary roots. In this experiment, the treatment means show a reduction in the mitotic

index when compared to the controls. While these results were not significant, neither were they conclusive. As a result, it was decided to standardize the protocol for the remainder of the study using whole plants. The reasons for this are as follows. Evans and Filion (1980) reported a lower proportion of metaphase cells per slide with increasing concentrations of BrdU and suggested a toxic effect resulting from the base analogue. If treated plants were indeed expected to experience further reduction of cell division in the root meristem following BrdU treatment, it was reasoned that those plants with the highest mitotic index would have an advantage over those in which cell division, and therefore DNA replication, had already been artificially reduced by the removal of shoots and cotyledons. In fact, Tempelaar et al. (1982) noted that when plants with a high mitotic index were used, the yield of plants showing sister chromatid differentiation could be doubled, suggesting a relationship between more actively dividing meristems and increased BrdU uptake. They also suggested that the use of whole plants was superior to those from which the shoots or cotyledons had been removed.

While, in a number of studies, seedling shoots were removed at the same time as the primary root tips were excised (Kihlman, 1975b; Kihlman, 1971; Kihlman and Andersson, 1984), the reason for their removal is not discussed in any of the papers. I can only speculate that this was done to further encourage the initiation of secondary root tips. Whether the shoot plays a role in the transport of nutrients which might effect thymidine uptake is not clear. In any case, in this study, its removal does seem to supress cell division in the secondary roots which develop after its removal, when compared to control plants.

Finally, since little or no difference was seen in the means between treatments in which seedlings were grown in tap water and those grown in a dilute Hoagland's solution (1 part Hoagland's: 8 parts water), seeds were germinated and seedlings were grown in tap water for the remainder of the study.

ii Unifilar vs. bifilar substitution of the base analogue BrdU:

A number if workers have used one cell cycle of 100 uM BrdU followed by a second cell cycle of 100 uM thymidine, stating that unifilar substitution resulted in a more marked differential contrast (Kihlman and Kronborg, 1975; Kihlman et al., 1978; Kihlman and Andersson, 1984; Andersson, 1985). However, Schvartzman and Cortes (1977) suggested that equally good results can be obtained with either type of substitution.

Schubert et al. (1980) also carried out differential staining following a FPG staining schedule. They used barley root tips as the test material and treated the roots with a 500 uM solution of BrdU followed by a second cycle in 25 uM thymidine, likewise giving rise to material which was unifilarly substituted. Schubert et al. did not comment on their preference.

Cortes and Andersson (1987) on the other hand, treated with two consecutive rounds of 100 uM BrdU, stating simply that this substitution gives optimal results for their procedure.

Both Vosa (1981) and Tempelaar et al. (1982), who used the Feulgen reaction to obtain differential staining, employed two successive rounds of 100 uM BrdU. Neither worker discussed the possibility of using successive rounds of BrdU and thymidine. Tempelaar indicated that his results show

that chromosomes treated with high concentrations of BrdU (e.g., up to 1000 uM BrdU) show greater contrast between darkly and lightly staining chromosome pairs, commenting however, that higher base analogue concentrations resulted in an increase in the number of spontaneous exchanges. In a personal communication (1987), Tempelaar indicated that poor results are often a result of lack of incorporation of base analogue, and stated that he obtained good results with 1000 uM BrdU and no FdU. Such high concentrations, however, are not used by researchers who use SCE as a bioassay for chromosomal damage caused by putative mutagens. Concentrations as low as 50 uM were used by Vosa (1981), presumably since with increasing concentration of base analogue the number of spontaneous exchanges caused by the actual incorporation of the base analogue also increases, a disadvantage since this means an increase in the frequency of background exchanges. Andersson (1985) stated that a concentration of 100 uM of BrdU was the lowest concentration that consistently gave rise to good differentiation in Vicia faba. He suggested that higher concentrations should be avoided since they result in an increased baseline level of SCE.

a) Barley:

When staining of barley chromosomes was carried out in this study according to the Feulgen technique of Tempelaar and coworkers, the most favorable results were obtained using 500 uM BrdU for two consecutive cell cycles. A 100 uM treatment did not result in clear differentiation, nor did one cell cycle of BrdU treatment followed by a second cell cycle in thymidine. With respect to base analogue concentration, these results are similar to those obtained by Schubert (1980), the only author who worked

with barley, and by Vosa (1981) and Tempelaar et al. (1982) with respect to the use of two consecutive cell cycles of BrdU. For the FPG technique in barley, bifilar or unifilar substitution was used depending on whose procedure was being attempted. Cortes and Andersson (1987) worked with bifilarly substituted chromosomes, and Kihlman and Kronborg (1975) worked with unifilarly substituted chromosomes. As reported earlier no sister chromatid differentiation was obtained in barley using the FPG technique.

b) **Vicia faba**:

The objective of this experiment, in which BrdU was supplied to growing seedlings for a single cell cycle, or for two consecutive cell cycles at various concentrations, was to determine the concentration and the type of substitution, unifilar or bifilar, which would least adversely affect the rate of cell division (the mitotic index) in growing seedlings. The analysis of variance (Appendix IV) showed no significant differences between treatments in either of the two identical runs of this experiment. The CV's for the analyses were both well over 20% (Appendix VIII). The implications of a high CV are discussed in the previous section. In this case, the high variance was noted within all the treatments, including the controls. While some of this variance can be attributed to the differential tolerance of individual plants to treatment with BrdU, this explanation does not hold for the control plants.

In general, plants seemed to have a low survival rate (four plants died), or a reduced rate of cell division, when treated for two consecutive cell cycles at any of the three concentrations of BrdU. Treatment means for cells which had been exposed to two cell cycles of the base analogue were at

least halved when compared to unifilarly substituted cells at the same concentration (Appendix X). These results are in agreement with Dolezel and workers (1986), who noted the negative impact of BrdU treatment for two cell cycles on the mitotic activity of root meristem cells. No trend toward a decrease in the mitotic index was noted in plants when BrdU substition was increased.

iii <u>The thymidylate synthetase inhibitor fluorodeoxyuridine (FdU):</u>

The objective of this experiment was to study the effect of FdU in conjunction with the base analogue BrdU on the mitotic index of <u>Vicia faba</u> seedlings.

The rational for supplementing FdU to BrdU treatment solutions has been attributed, by most authors, to its suppression of cellular synthesis of thymidylic acid through the inhibition of thymidylate synthetase (Kihlman and Kronborg, 1975; Schvartzman and Cortes, 1977; Cortes et al., 1980, Kihlman and Andersson, 1984; Escalza et al., 1985; Andersson, 1985; Cortes and Andersson, 1987). Tempelaar et al. (1982) have suggested that the recommended concentration of 0.1 uM FdU improved chromatid differentiation, but that at high concentrations of BrdU, such as 1000 uM, FdU is no longer required to obtain good differential staining. However, Kihlman and Andersson (1984) reported that even at high BrdU concentrations, if FdU is not added, poor differential staining is obtained. Other authors have suggested that the use of FdU is uneccessary or that the increase in the number of root tips actually incorporating BrdU was negligable, even when it was supplemented (Vosa, 1976; Kihlman and Andersson, 1987).

While most authors have used the concentration of FdU originally

proposed by Kihlman and Kronborg (1975), some use concentrations as low as 0.001 uM (Schvartzman et al., 1979a; Schvartzman and Hernandez, 1980; Gutierrez, et al., 1981; Gutierrez and Lopez-Saez, 1982).

A number of authors have studied the effects of increasing the concentration of FdU supplemented to the BrdU treatment solutions. Schvartzman and Cortes (1977) showed that 0.1 uM FdU is the minimum concentration which severely depressed the mitotic index when administered alone to root tips of Allium cepa, and that 100 uM BrdU was needed to restore normal mitotic activity. Schvartzman and coworkers (1979a) reported that variations in concentration of up to 0.05 uM FdU do not significantly modify either the duration of the cell cycle or the yield of SCEs. In Tradescantia, Andersson (1985) showed that a marked supression of the mitotic index is not obtained until concentrations of 5 uM FdU are used, and that 0.5 uM BrdU restored mitotic activity to the control level, while 100 uM was needed to obtain good differential staining. Escalza et al. (1985) demonstrated that when FdU was given for two consecutive cell cycles in conjunction with BrdU the frequency of SCEs was enhanced in a dose dependent manner with saturation at 0.5 uM FdU. They also reported that concentrations of above 1 uM FdU the prolific activity of the meristem was insufficient for SCEs to be scored. These authors employed a FdU concentration of 0.5 uM. In Allium sativum (garlic), Dolezel and workers (1986) found that 0.5 uM FdU was needed to inhibit mitotic activity. A pronounced reversion of mitotic activity was related to the application of rather high concentrations of BrdU. For this reason the FdU concentration was lowered to 0.1 uM and reversion to almost normal levels of cell division was obtained at 200 uM BrdU. In Allium cepa, Pardo and coworkers (1987)

reported that FdU in the range of 0.001 to 0.1 uM produced a dose and time dependent decrease of the number of cells in mitosis. They reversed this effect by means of 100 uM uridine.

Whether FdU actually enhanced BrdU uptake in plants was probably first shown by Escalza et al. (1985). They substituted root tip cells with [^{3}H]BrdU in the absence or presence of FdU and showed that the incorporation of this radioactively labelled base analogue was dramatically enhanced when cells were simultaneously treated with FdU.

In the present study, an analysis of variance (Appendix V) showed significant differences between treatments in which 0, 0.1 and 5 uM FdU were supplemented to treatment solutions of 50, 100 and 500 uM BrdU. A Duncan's multiple range test showed that the mitotic indices obtained in treatments where 5 uM FdU had been supplemented were significantly lower than the control, less than 1%. The treatment of 500 uM BrdU unsupplemented with FdU was also significantly lower than the control (Appendix XI). No other differences were found between the treatments and indeed the means for the remaining treatments fell within a range of 2.06 and 3.56% (Appendix XI). Again the CV for this experiment was well above 20% (Appendix VIII), due to a large variance within treatments. But in this case significant differences were detected in spite of the high CV This means that differences between treatments were greater than the differences within treatments. A finding of significant differences between treatments is highly significant.

It was decided that bifilar substition with 100 uM BrdU, supplemented with 0.1 uM FdU would be used throughout the remainder of this study. The reasons are as follows. While little difference is seen in the mitotic

indices of plants treated with or without FdU supplementation at low concentrations of BrdU, evidence seems to support the suggestion that its use does indeed enhance BrdU incorporation.

500 uM BrdU was the highest concentration used to study the effects of increasing BrdU concentration on mitotic index. While it did not appear to reduce cell division, it has been reported by some to increase differential contrast. Such a high concentration of BrdU was not chosen for use in further experiments since, in general, high concentrations of this analogue are not recommended for use in SCE bioassays for mutagenicity testing. This is because of the increase in baseline SCE frequencies they induce and reports that BrdU itself is a mutagen.

Because of the halving of the mitotic index which appeared to occur when bifilar substitution was carried out at any given concentration of BrdU, unifilar substitution was chosen for the remainder of the study.

Factors affecting the Feulgen procedure for differential staining of sister chromatids:

i. Maceration

The rigid plant cell wall has been cited by many authors (Kihlman, 1975b; Kihlman and Kronberg, 1975; Gonzalez-Gil and Navarrete, 1982; Kihlman and Andersson, 1984) as one of the major disadvantages of using plant cells as biological material for the study of SCE when compared to the use of animal cells. Cortes (1980) suggested that the small width and the distortion of the chromosome structure produced by the squash technique leaves the problem of cell wall maceration unresolved.

As pointed out by Kihlman (1975b), in order to obtain the separation of root tip cells which is necessary for good chromosome squashes to be made, the pectic substances in the middle lamella of the cell wall must be dissolved. The majority of authors carry out cell wall maceration by means of an enzyme prepartion, usually pectinase and less frequently with pectinase and cellulase. Kihlman points out that these procedures may change the morphology of the chromosomes and the composition of their chemical constituents. He suggests that such changes render certain types of cytological analyses, such as the analysis of the effect of chemicals on the frequency of SCEs, extremely difficult.

Kihlman and Kronborg (1975) macerated fixed root tips by means of 0.5% pectinase dissolved in a 0.1 M citric acid-sodium citrate buffer adjusted to a pH of 4.7. at 27 C for 75 minutes. These authors pointed out that while root tips stained by the Feulgen technique, are sufficiently macerated when root tips are exposed to 1 M HCl at 60 C for 7 minutes prior to staining, it would be impossible to incorporate such an acid hydrolysis into the FPG technique because of the drastic chemical changes that such a treatment would have on chromosomal DNA. These authors noted that while satisfactory maceration was obtained at low concentrations of HCl and at lower temperatures (0.1 M HCL at 35 C for 10 minutes), that the differential contrast obtained in such cases was poor. However, according to the same authors, chromosome morphology following pectinase maceration was somewhat less well preserved than after acid hydrolysis. Apparently, for these authors, the trade-off was between the preservation of chromatid differentiation and the loss of chromosome morphology. It is interesting to note that in later papers, several authors (Gonzalez-Gil and Navarrete,

1982; Cortes and Andersson, 1987) added a rather harsh acid hydrolysis step (5 M HCl at 25 C for 20 minutes) to the FPG technique, not for the purpose of maceration, but in order to improve the quality of sister chromatid differentiation.

Vosa (1976) macerated root tips in 45% acetic acid for 3 to 5 minutes, but reported that while enough intact metaphases were usually obtained, the root tips were nevertheless tough and difficult to squash. Schvartzman and Cortes (1977) compared maceration of plant cell walls obtained by using a hot mixture of 10:1 45% acetic acid: 1 M HCl, to that obtained by the standard pectinase maceration. He concluded that while both methods yielded satisfactory results in terms of obtaining good chromatid differentiation, chromosome spreading was better in squashes obtained by the pectinase treatment.

While many authors have followed the original maceration technique of Kihlman and Kronborg (1975), some have prolonged the treatment to 2 hours (Kihlman and Sturelid, 1978; Kihlman et al., 1978; Kihlman and Andersson, 1984; Cortes and Andersson, 1987), whereas others have increased the temperature to 37 C while shortening the time of maceration to 1 hour (Schvartzman and Cortes, 1977; Cortes, 1980; Cortes et al., 1980; Cortes and Gonzalez-Gil, 1982). Others have increased the concentration of pectinase (Tempelaar et al., 1982, Dolezel et al., 1986). Tempelaar and co-workers (1982) preferred to shorten the time of maceration to 15 minutes, claiming that prolonged maceration contributes to loss of staining intensity. Grant and colleagues (1981) macerated root tips with a 0.1 M solution of HCl at 60 C for 2 minutes. Still others squash root tips in 45% acetic acid with no prior maceration (Evans and Filion, 1980; Vosa, 1981). Finally, some

authors macerate using the enzyme pectinase in combination with cellulase (Schubert et al., 1979; Andersson, 1985; Dolezel et al., 1986).

Kihlman and Andersson (1984) have pointed out that impurities in enzymes used for root tip maceration may affect the protein and nucleic acid components of chromosomes in such a way that the differential staining for scoring of SCE is impaired.

In this work I found that the use of cellulase greatly improved the plasticity of the cells, and therefore, led not only to improved cell squashes but also to an enhancement of chromosome spreading. Thus, there were many fewer overlapping and clumped chromosomes in metaphase and more chromosomes spread singly on the slide. I found that the best maceration which could be obtained while still preserving good chromosome staining and morphology, as well as a clear cytoplasm, was at a pectinase concentration higher than any recommended, and at the highest temperature of any recommended by the workers mentioned above. Results obtained with a 3% solution of pectinase dissolved in a citric acid-sodium citrate buffer adjusted to a pH of 4.7 and treated at a temperature of 37 C for 2 hours were good, and chromosome spreading was enhanced by the addition of 0.5% cellulase. At a concentration of pectinase lower than 3% and a treatment duration of less than 1.75 hours, or at a temperature lower that 37 C, a good monolayer of cells was not obtained.

If one compares the results from the Feulgen procedure and the FPG technique, it is clear that the hydrolysis treatment in the Feulgen staining technique does lend to improved maceration of cells which subsequently undergo enzyme digestion. However, I found that this acid maceration alone was not sufficient to give rise to good chromosome squashes without enzyme

digestion. The fact that acid hydrolysis aids in cell wall maceration, is evident when one examines and compares the photographs in Figures 2 through 7, in which the Feulgen procedure was carried out and in which an acid hydrolysis was carried out prior to enzyme maceration, with those in Figures 8 and 9, in which root tips were stained according to the FPG technique, and cell squashing took place directly after maceration and no other treatment was given to the cells prior to squashing. In the former, the chromosomes are well spread and many are seen as single chromosomes, whereas in the latter, chromosomes are more often than not clumped together at metaphase, or lying in an overlapping configuration with other chromosomes.

The improvement rendered by cellulase treatment, while not discussed by any of the authors who employed it, is probably due to the actual softening of the cellulose of the cell wall. While pectinase softens and dissolves the pectin which binds cells together, the cellulase actually begins to dissolve the cell wall so that it provides less resistance to the pressure of squashing the cell and chromosomes tend to react to this force by being dispersed within the cell.

While Kihlman and Andersson (1984) reported that impurities in enzymes used for maceration may affect the morphology and the staining of differentiated chromosomes, Andersson (1985) reported that solutions prepared from some batches of cellulase destroyed the chromosome structure. He reported, as did Kihlman and Andersson (1984) that the quality of the preparations is improved by leaving the roots in distilled water for half an hour before the squash preparations are made.

The problem of cell wall maceration has hardly been resolved since it is still being discussed in the literature. While some authors note that

maceration with enzymes can impair the quality of staining, the present study shows that satisfactory maceration is not obtained when pectinase concentrations of less than 3% are employed. Maceration is especially important in techniques for sister chromatid differentiation, particularly in cases where this endpoint is to be used for assaying putative mutagens, since in order to score the exchanges, clumping and overlapping of chromosomes must be minimized while the integrity of staining must be maximized.

ii Duration of 5 M HCl Acid Hydrolysis:

Differential staining was carried out in Vicia faba according to the Feulgen procedure. Root tips were treated from 50 to 90 minutes with 5 M HCl at 28 C. In general, poor results were obtained at the low and high end of the range at which root tips were exposed. For example, at 50 minutes of hydrolysis no sister chromatid differentiation was seen and both chromatids stained darkly with Feulgen. Between 58 to 68 minutes, sister chromatid differentiation became apparent but differential contrast was poor. Many slides showed no differential staining, sister chromatids remained indistinguishable in terms of staining intensity. Between 80 and 84 minutes of hydrolysis, excellent differentiation was obtained in some metaphases. At 88 minutes of hydrolysis and longer, some differentiation was still apparent but chromosomes began to take on a faded or fuzzy appearance and differential contrast was no longer clear at extended periods of hydrolysis. These findings are in good agreement with results obtained in Vicia faba according to the Feulgen procedure by other authors who treated differentially substituted chromosomes over an extended period of

time (Vosa, 1981; Tempelaar et al., 1982). The latter author suggests that decreased staining intensity which appears in conjunction with extended periods of hydrolysis is due to the depolymerization of the DNA. Since BrdU substituted DNA resists acid hydrolysis compared to unsubstituted DNA there is therefore a specific duration of acid hydrolysis treatment which gives rise to good differential contrast. In the present study this range was found to be between 80 and 86 minutes. This is slightly longer than recommended by Tempelaar and coworkers, who hydrolyzed at the same temperature.

It may be seen from Table III that among different authors, the acid hydrolysis step in the Feulgen staining procedure varies somewhat. All workers used 5 M hydrochloric acid. The variations have consisted of different treatment temperatures and, thus, different durations of acid hydrolysis required to obtain differential staining. The general pattern is that increasing temperature requires a decrease in the period of time required to obtain differential staining.

iii SO_2 wash:

In Tempelaar's work (1982) the maceration of root tips is followed by a 10 min wash in SO_2 water (10 ml of 10% $K_2S_2O_5$ + 10 ml 1 N HClin 200 ml of tap water). The purpose of this step according to the author is to remove excess Feulgen reagent. In the present study no difference was seen between preparations in which an SO_2 wash was given and in which no SO_2 wash was applied.

Table III. Temperature and duration of HCl acid hydrolysis in the Feulgen procedure. All authors used a concentration of 5M HCl.

Author	Organism	Temp C	Time needed to obtain differential staining
Vosa (1981)	*Vicia faba*	18	90-120 min
Tempelaar et al. (1982)	*Vicia faba*	28	40-70 min
Castillo-Ruiz (personal communication)	*Vicia faba*	28	80 min
Present study:	barley	37	45-51 min
	Vicia faba	28	80-95 min

iv Duration of root tip fixation in Carnoy's:

The duration of root tip fixation was examined to see if fixation could be related to the quality of staining obtained in the differential staining techniques.

Fixation was obtained by most authors by means of 3:1 95% ETOH-glacial acetic acid, while in a few cases 3:1 methanol-acetic acid was employed (Kihlman and Kronborg, 1975; Kihlman and Andersson, 1984; Cortes and Andersson, 1987). In general, treatment was carried out overnight (14-20 h) at 4 C, even though some authors fixed at slightly different temperatures or for slightly different time periods. Several authors have fixed in ETOH-acetic acid 3:1 at -20 C overnight (Kihlman, 1975b; Tempelaar et al., 1982; Kihlman and Andersson, 1984). These authors specified that root tips could be stored for several days, weeks or months at this temperature. In the present work, however, fixation at -20 C resulted in chromosomes which were very difficult to distinguish as a result of poor staining, and cell contents which were murky and unclear, presumably because of poor fixation as a result of freezing.

Some discrepancy exists among author's results in regard to which duration of fixation gives the best results. While some authors fix overnight (Kihlman and Kronborg, 1975; Vosa, 1981), Schubert et al. (1980) fixed for 1 hour. Grant and Zura (1981) fixed for 2 hours, stating that fixation for longer periods does not improve the preparation and renders the material more difficult to squash. Cortes and Andersson (1987), on the other hand state that fixing time is a crucial factor. They found that sister chromatid differentiation is improved by prolonged fixation time, at least overnight but no more than one week.

In Figures 3 and 4, photographs of chromosomes fixed for increasing durations with 3:1 ethanol-acetic acid and stained by the Feulgen procedure are presented. Cells in Figure 3 were observed immediately after staining while those in Figure 4 were observed after one month of storage in 70% ethanol at 4 C. The duration of acid hydrolysis is also given. No sister chromatid differentiation was seen after 1 hour of fixation. It was found that good differential staining can be obtained at a wide range of fixation times. Qualitative data obtained in this study were analyzed by means of the X^2 test for homogeneity. Observations about the quality of staining, chromosome morphology, and the appearance of the cytoplasm were pooled into three classes: poor (+, +1 and +2), good (++, ++1 and ++2) and excellent (+++). The X^2 test for homogeneity requires that either each observation have at least an expected value of 1, or that 20% of the observations have an expected value of 5. Since neither the treatment in which observations were made before, nor the one in which observations were made after root tips were stored for one month, fulfilled one of the above requirements, the two treatments had to be pooled.

Statisitical analysis showed that there is a significant difference in the quality of chromosome staining and in the appearance of the cytoplasm due to the duration of fixation. In Figure 5, the number of observations are plotted against the quality of staining recorded for each observation at each time of fixation. The trend is toward good to excellent staining in a majority of the root tips at and after 7 hours of fixation, with the 7 hour treatment yielding the greatest number of good to excellent observations when compared to the other treatments. A similar result is seen for the effect of fixation on the appearance of the cytoplasm (Figure 6). In the

FIGURE 3

Vicia faba chromosomes fixed between 1 hour and 22 hours, stained according to the Feulgen procedure, and examined immediately.

A 4 hours of fixation, 80 minutes of 5 M HCl hydrolysis, x ca. 775.

B 7 hours of fixation, 86 minutes of 5 M HCl hydrolysis, x ca. 775.

C 10 hours of fixation, 86 minutes of 5 M HCl hydrolysis, x ca. 775.

D 13 hours of fixation, 83 minutes of 5 M HCl hydrolysis, x ca. 715.

E. 22 hours of fixation, 86 minutes of 5 M HCl hydrolysis, x ca. 681.


A
B
C
D
E

FIGURE 4

Vicia faba chromosomes fixed between 1 and 22 hours, stained according to the Feulgen procedure, and examined after storage for one month at 4 C.

A 4 hours fixation, 80 minutes of 5 M HCl hydrolysis, x ca. 1050.

B 10 hours fixation, 86 minutes of 5 M HCl hydrolysis, x ca. 1165.

C 13 hours fixation, 83 minutes of 5 M HCl hydrolysis, x ca. 845.

D 22 hours fixation, 80 minutes of 5 M HCl hydrolysis, x ca. 755.

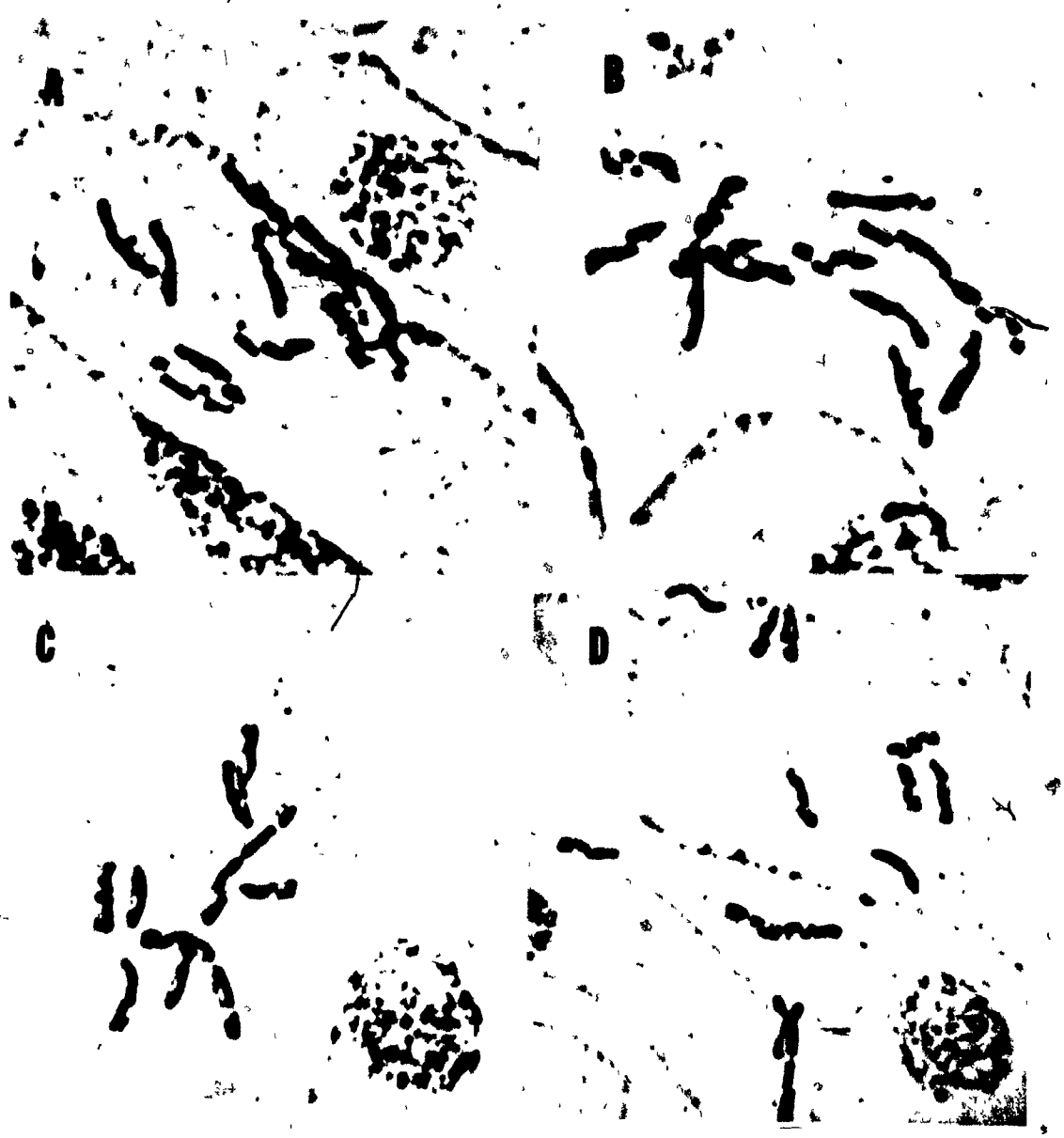

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FIGURE 5

The effect of fixation time on the quality of staining. Poor (+, +1 and +2), good (++, ++1 and ++2) and excellent (+++).

A 1 hour of fixation.

B 4 hours of fixation.

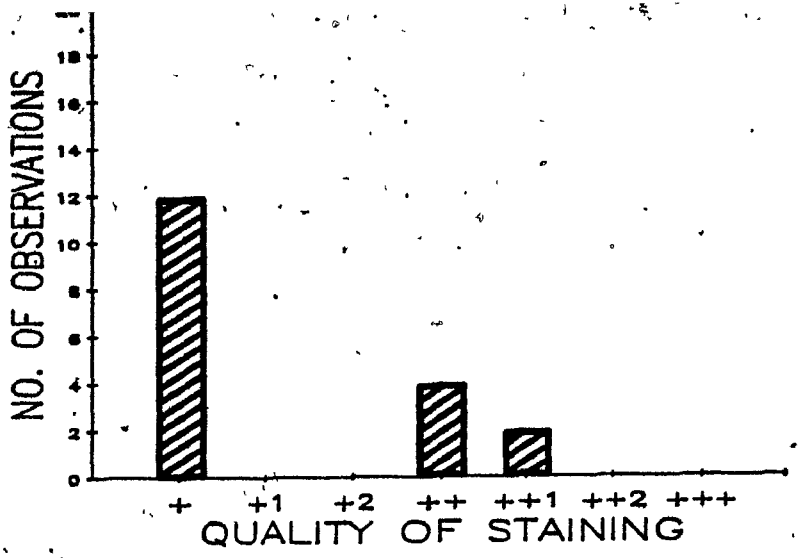
C 7 hours of fixation.

D 10 hours of fixation.

E 13 hours of fixation.

F 22 hours of fixation.

A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QUALITY OF STAINING

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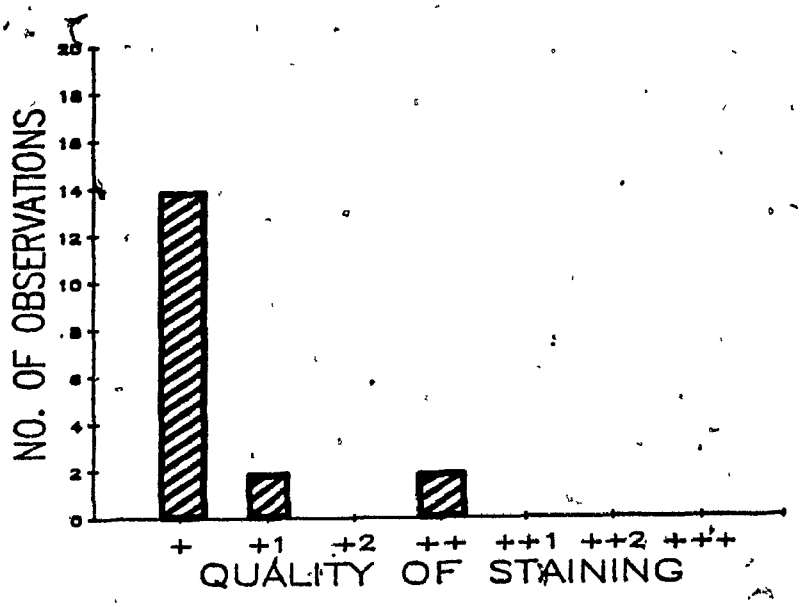
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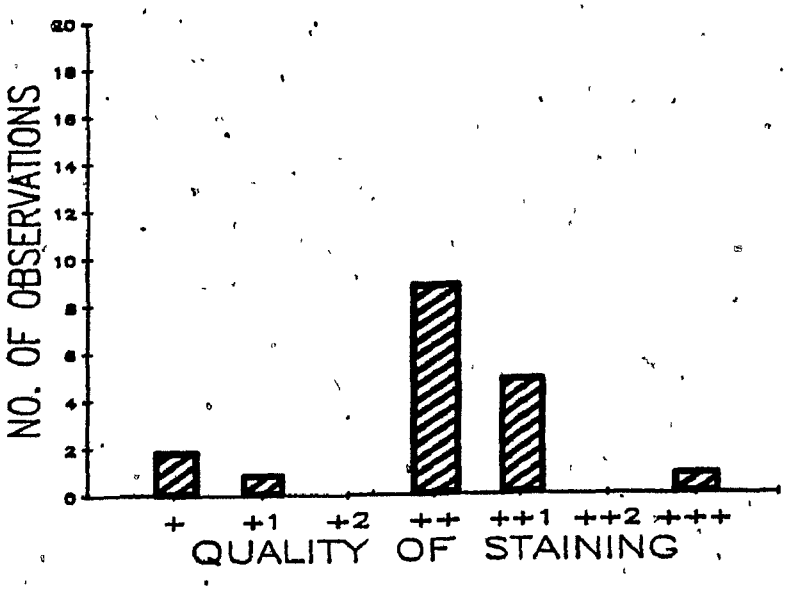
QUALITY OF STAINING

C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QUALITY OF STAINING

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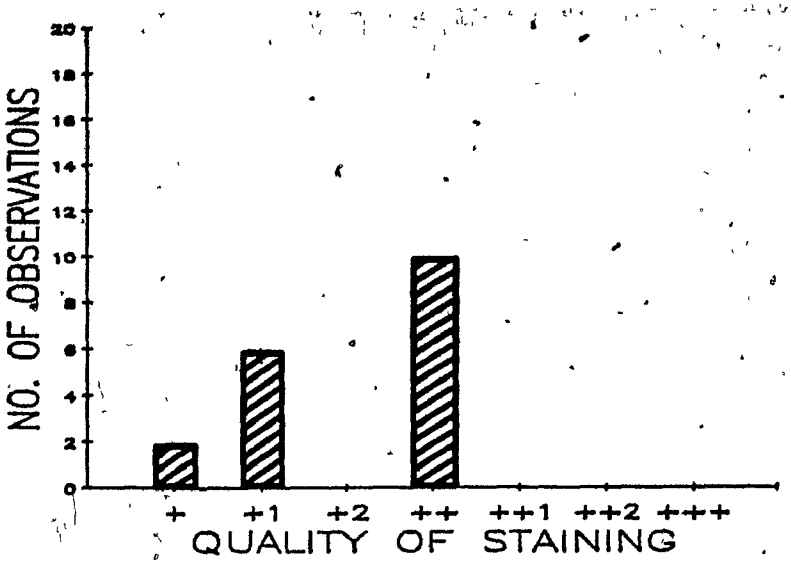
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QUALITY OF STAINING

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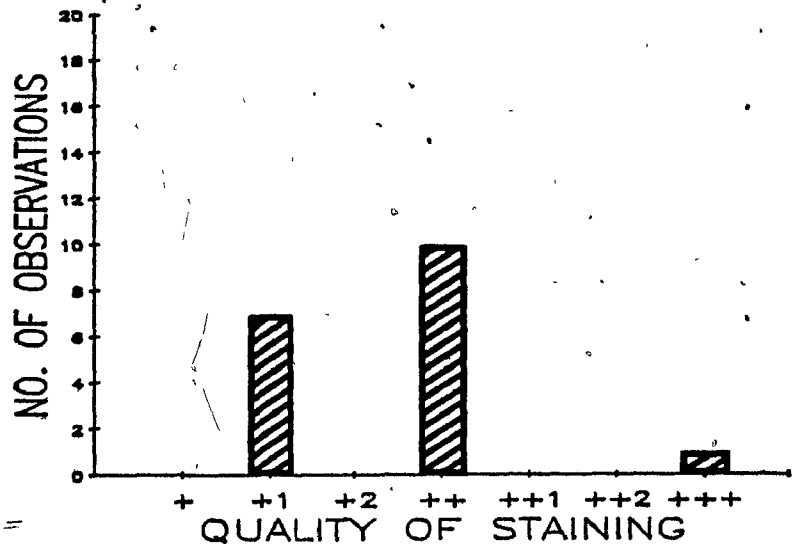
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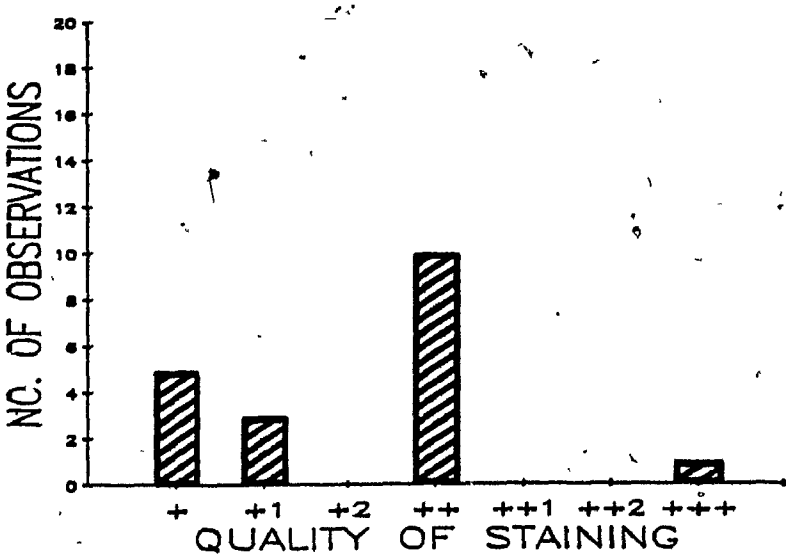
QUALITY OF STAINING

F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QUALITY OF STAINING

latter case, the cytoplasm is clear at fixation times in a range between 7 and 13 hours. Bubbly cytoplasm appears to be charactaristic of fixation times of 4 hours and less, while the cytoplasm becomes increasingly more grey as fixation time is prolonged.

Not infrequently, metaphase chromosomes of the same root tip did not all yield differentially stained chromosomes. In Figure 7, examples of such a discrepancy in staining among chromosomes of the same root tip are shown. Duration of fixation and of hydrolysis are indicated. That only a fraction of metaphases of a given root tip may give rise to differential staining has been reported by other workers (Evans and Filion, 1980), and Kihlman (1975b) suggested that the root tip meristem of plants contain cells with differential sensitivities to chemical treatments due either to their position in the root tip or to the different duration of mitotic cell cycles present in the same root tip. Whether the occurrence of metaphase chromosomes in the same root tip with apparently differential responses to staining may be due to differential incorporation of BrdU by individual cells or to some other factor is not clear.

Factors Affecting the FPG staining procedure:

While some progress was made in modifying the Feulgen procedure to obtain improved differential staining in Vicia faba and barley chromosomes, positive results were not obtained using the FPG techniques. In the following section the effect of RNAse and trypsin treatments on the FPG staining protocol were examined.

FIGURE 6

The effect of fixation time on the appearance of the cytoplasm. Poor (+, +1 and +2), good (++, ++1 and ++2) and excellent (+++).

A 1 hour of fixation.

B 4 hours of fixation.

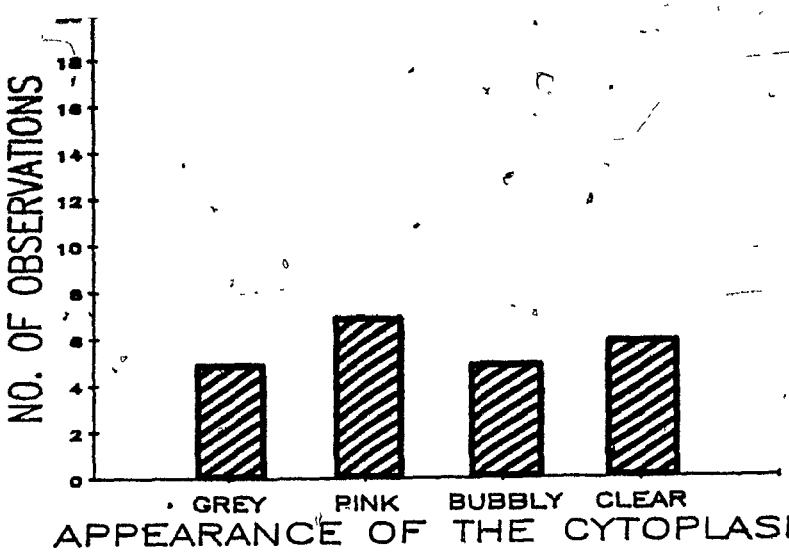
C 7 hours of fixation.

D 10 hours of fixation.

E 13 hours of fixation.

F 22 hours of fixation.

A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APPEARANCE OF THE CYTOPLASM

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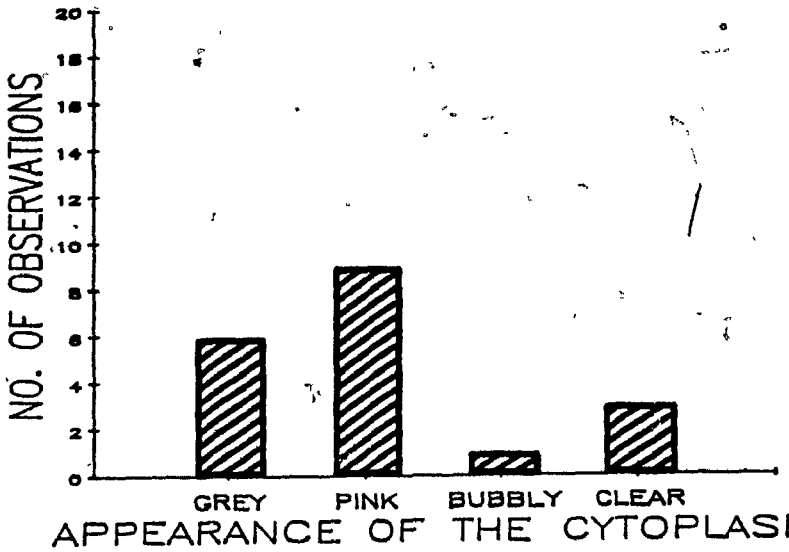
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APPEARANCE OF THE CYTOPLASM

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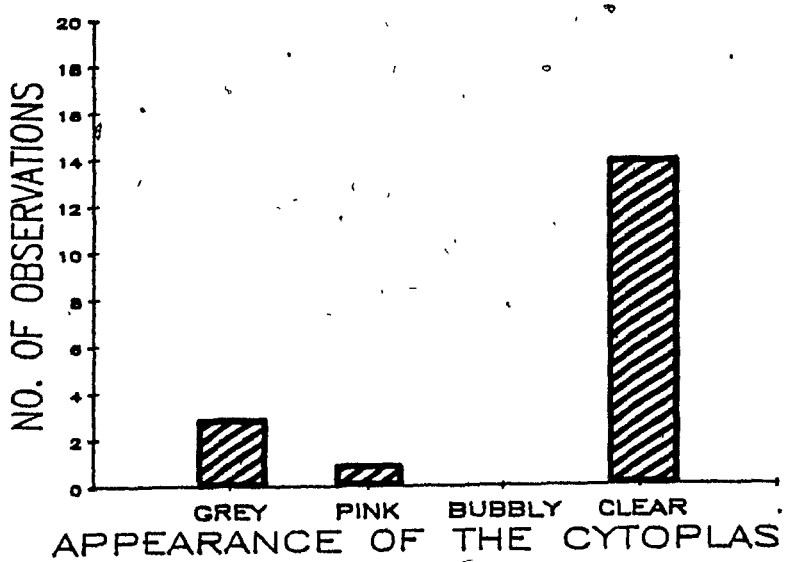
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APPEARANCE OF THE CYTOPLASM

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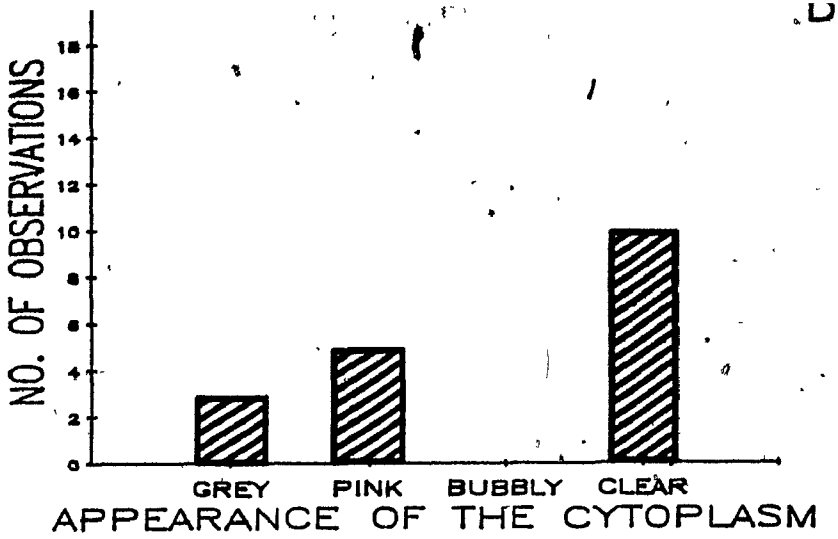
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APPEARANCE OF THE CYTOPLASM

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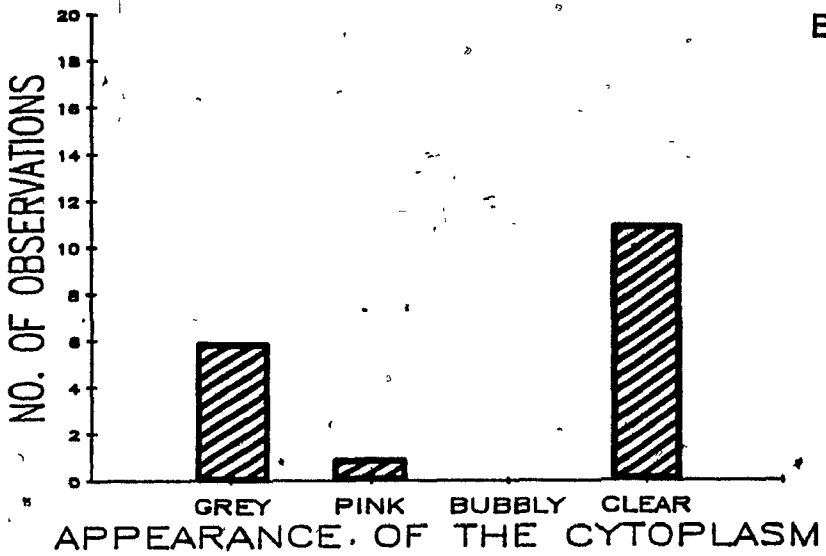
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APPEARANCE OF THE CYTOPLASM

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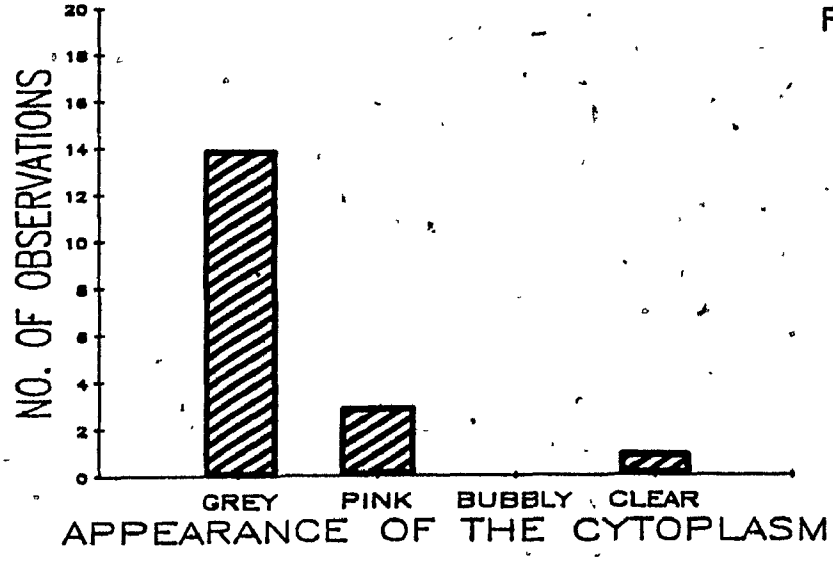
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APPEARANCE OF THE CYTOPLASM

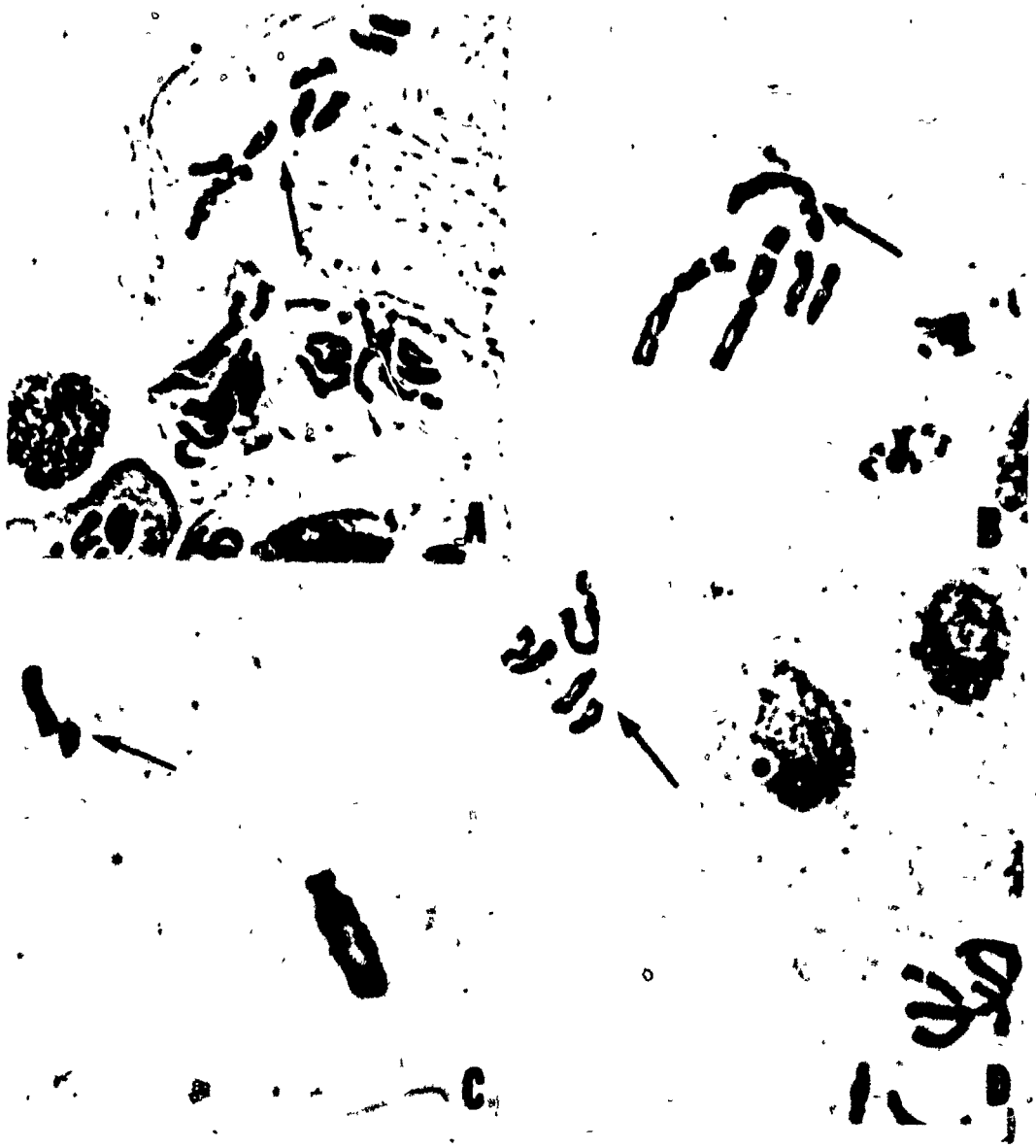
FIGURE 7

Examples of differential staining in Vicia faba in which metaphase chromosomes from the same root tip do not all show differential staining.

A,B 13 hours of fixation, 83 minutes of 5 M HCl hydrolysis. Arrow points to the chromosome(s) in which sister chromatid differentiation is present, x ca. 569 and 827 respectively.

C 4 hours of fixation, 83 minutes of 5 M HCl hydrolysis. Arrow points to the chromosome in which sister chromatid differentiation is present, x ca. 3897.

D 10 hours of fixation, 86 minutes of 5 M HCl hydrolysis. Arrow points to the metaphase in which SCE is present, x ca. 637.


C
D

i RNAse treatment:

High RNA content has been cited as one of the disadvantages in using plant cells to achieve differential staining. Giemsa stains both RNA and DNA. Thus an RNAse treatment is included in the FPG technique in order to clear the cells of RNA which might otherwise mask the chromosomes. Gonzalez-Gil and Navarrete (1982) suggested that nuclear and cytoplasmic RNA can interfere with differential staining of sister chromatids. Kihlman and Kronborg (1975) found that the use of RNAse improves the contrast between sister chromatids but is not absolutely necessary. Andersson (1985) and Cortes and Andersson (1987) suggested that an RNAse treatment eliminates background staining caused by cytoplasmic RNA. But according to Cortes and Andersson, this step is no longer necessary since the hot acid treatment included in their staining procedure hydrolyzed nuclear and cytoplasmic RNA. In general, this study bears out the assertion of Cortes and Andersson. Figure 8 allows the comparison of chromosomes stained according to the FRG technique of Cortes and Andersson, with and without an RNAse treatment. No difference was seen in chromosome preparations obtained by either method. In Figure 8D, cells with heavy RNA staining of the cytplasm by Giemsa are shown. Such staining of RNA, however, was seen infrequently.

ii Trypsin digestion

Trypsin digestion has been used to obtain Giemsa banding in both plant and animal cells (Chuprevich et al., 1973; Wiscovich et al., 1974; Wang, 1972; Scheid and Traupe, 1973). Scheid (1976) and Scheid and Traupe (1977) used trypsin digestion in combination with Hoechst 33258 staining to obtain differential staining of *Vicia faba* chromosomes bifilarly substituted with

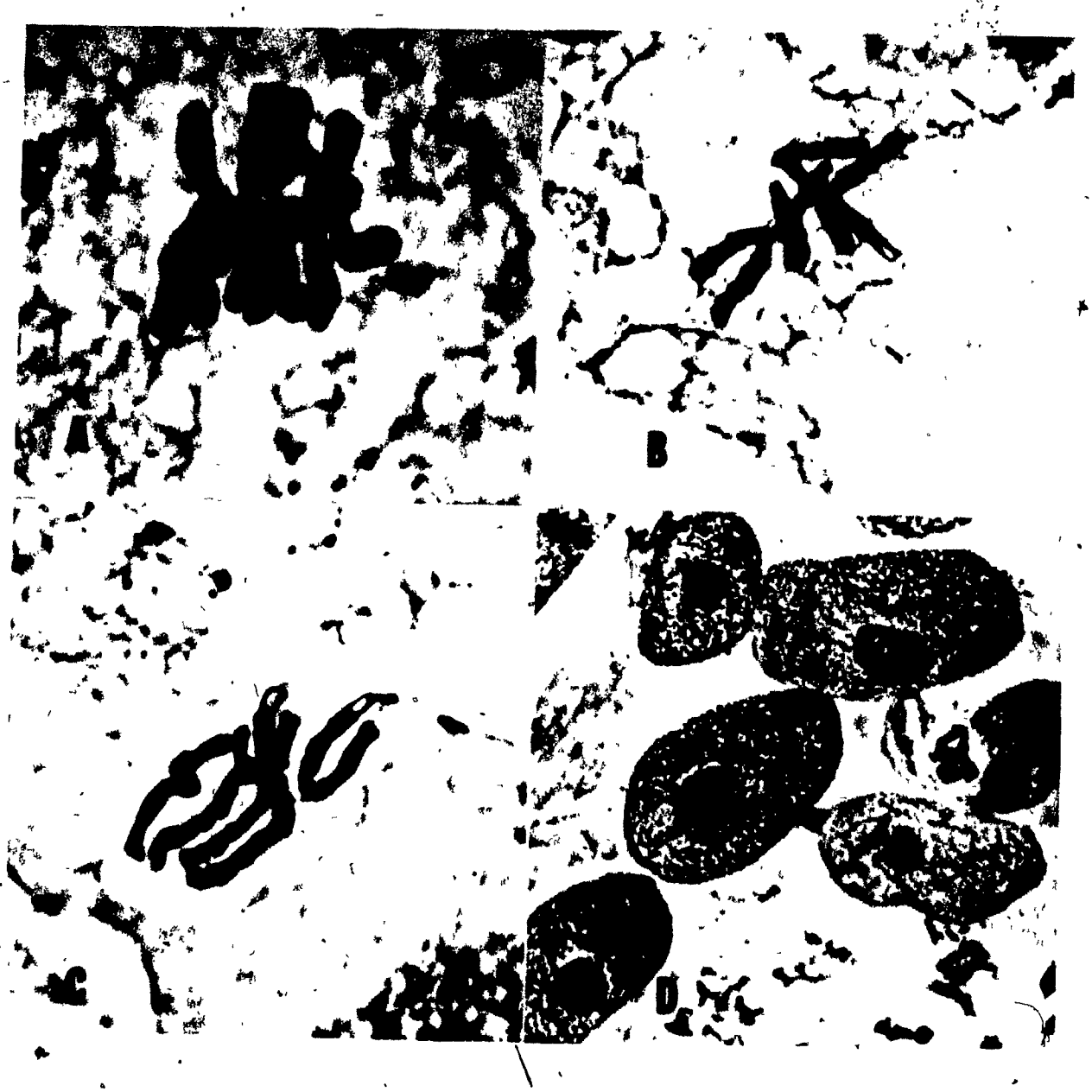
FIGURE 8

Vicia faba chromosomes stained according to the FPG technique of Cortes and Andersson (1987) with and without RNAse treatment.

A Original protocol without RNAse treatment. No chromatid differentiation present, x ca. 1037.

B,C FPG staining with RNAse treatment. No chromatid differentiation present, x ca. 798 and 1146 respectively.

D Cells stained by means of the original FPG protocol with no RNAse treatment showing unhydrolyzed RNA in the cell cytoplasm, x ca. 491.


B
D

BrdU. It has been suggested that the direct action of trypsin is to hydrolyze the protein component of nucleoprotein (Chuprevich et al., 1973), which has already been denatured by cell fixation (Wang, 1972). Scheid (1976) and Scheid and Traupe (1977) noted rapid preferential dissolution of one sister chromatid after trypsin digestion of bifilarly substituted, Hoechst 33258 stained chromosomes of Vicia faba. No differential dissolution was seen without trypsin digestion. They argued that single strand breaks preferentially induced by UV treatment in the doubly substituted chromatid led to its rapid dissolution upon trypsin digestion.

Several workers have suggested differential protein binding properties for BrdU substituted DNA (Lin and Riggs, 1972; Gordon et al., 1973; Lapeyre and Bekhor, 1974; Gordon et al., 1976). Differential properties of BrdU substituted DNA, both with respect to its susceptibilty to single strand breaks induced by UV light, as well as to altered protein binding properties, coupled with the findings of Scheid (1976) and Scheid and Traupe (1977), that trypsin treatment led to the differential dissolution of one sister chromatid, suggested that a trypsin digestion incorporated into the FPG technique, in which staining with 33258 Hoechst and Giemsa are combined, might lead to differentiation of sister chromatids, where before none had been observed. Trypsin concentration, temperature and duration of the treatment were chosen from among authors who had used trypsin for the purpose of obtaining Giemsa banding. Most authors used comparable concentration and temperature ranges.

In the present study, while increasing time and concentration of trypsin treatment led to obvious deterioration of chromosomes stained by the FPG techniques (Figure 9), no sister chromatid differentiation was seen as a

result of such treatment.

The Feulgen Procedure for Differential Staining

The number of publications on SCE studies using higher plants account for about 3% of the approximately 2672 articles published in this field since 1974 (Schvartzman, 1987). Of the approximately 76 papers which fall into this category, two have been published in which the Feulgen technique is used (Vosa, 1981; Tempelaar et al., 1982). In both papers, staining is carried out in Vicia faba.

Tempelaar et al. (1982) suggested that hydrolysis by 5 M HCl is the crucial step for obtaining diffential contrast by means of the Feulgen procedure. These workers showed that among the three varieties of Vicia faba with which they worked, "Threefold white", "Early longpod" and "Akerbona primus", the duration of 5 M HCl hydrolysis at 28 C required to give rise to differentiation, varied as much as 15 minutes among the three varieties. The maximum duration required for any variety was 70 minutes. In the present study, the Vicia cultivar "Aguadulce" showed good differential staining when chromosomes were hydrolyzed at 28 C from 80 to 86 minutes.

In barley, modifications which led to improved differential staining included, an increase in BrdU concentration to 500 uM, the bifilar substitution of chromosomes and an increase in the temperature of acid hydrolysis to 37 C.

While the Feulgen technique for differential staining of plant chromosomes has received little attention from workers in the field, it is shorter and less complicated than the FPG technique. In the present study,

FIGURE 9

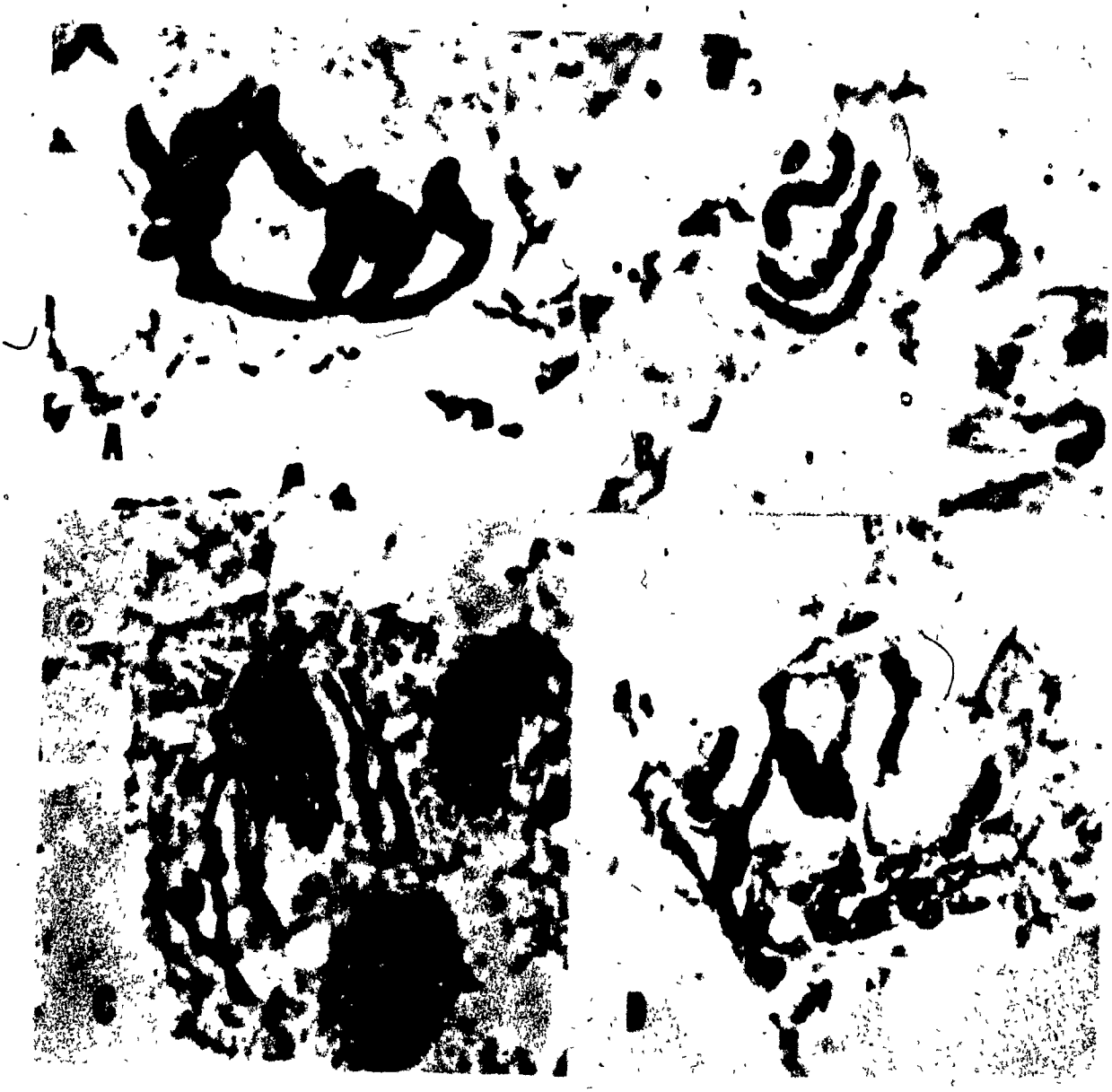
Vicia faba chromosomes stained according to the FPG technique with trypsin digestion.

A Original FPG technique of Kihlman and Kronborg (1975) with no trypsin digestion. No chromatid differentiation present, x ca. 1109.

B FPG technique of Kihlman and Kronborg (1975) with trypsin treatment of 0.1 % for 1 minute. No chromatid differentiation present, x ca. 1228.

C FPG technique of Cortes and Andersson (1987) with trypsin treatment of 0.25% for 1 minute. No chromatid differentiation present, x ca. 818.

D FPG technique of Cortes and Andersson (1987) with trypsin treatment of 0.25% for 5 minutes. No chromatid differentiation present, x ca. 818.



the Feulgen procedure yielded the most promising results for the development of a staining technique for use as a bioassay in testing for putatative mutagens or other DNA damaging substances.

The Fluorescent-plus-Giemsa Technique

While the modifications of the FPG technique are too numerous to discuss here, an attempt will be made to touch upon those which are relevant to this study.

Kihlman and Kronborg (1975) suggested that some magnification of a structural difference between chromatids containing differentially substituted DNA must take place between the time when the root tips are exposed to 33258 Hoechst and when they are exposed to Giemsa stain, in order for good differentiation to be obtained. They go on to say that such a process appears to be a function of time and temperature and may also be dependent on the light conditions during this period. They feel that the main effect of heating during this period is to speed up this process.

The warm buffer treatment between staining with 33258 Hoechst and Giemsa is replaced by a warm HCl acid treatment by Cortes and Andersson (1987). The aim of the acid hydrolysis, according to them, was not only to hydrolyze nuclear and cytoplasmic RNA, but also to solubilize and extract photodegraded DNA. This hydrolysis was first incorporated into the FPG technique in Allium cepa by Gonzalez-Gil and Navarrete (1982) who showed that it drastically improved differential staining when applied directly before Giemsa staining. They reported that in the absence of hydrolysis only 15% of metaphases showed differential staining while acid hydrolysis increased the percentage to 80%. With prolonged acid hydrolysis these

authors reported a progressive loss of staining capacity of the chromosomes. They suggested that HCl treatment washed out RNA and most of the chromosomal proteins which may interfere with differential staining, and that as a result, the Giemsa dye behaves as if it were specific for DNA. With respect to Giemsa staining, some authors have stated that duration of staining is crucial (Kihlman and Kronborg, 1975; Andersson, 1985). The latter, claimed, that Giemsa staining should be kept as short as possible since when staining exceeds 4 to 4.5 minutes, overstaining results making scoring difficult or impossible. Among other authors, variations in concentration for Giemsa staining range between 0.5% and 3%, with staining duration ranging between 4 and 9 minutes (Kihlman and Kronborg, 1975; Evans and Filion, 1980; Andersson, 1985; Luo et al., 1988).

Goto et al. (1978) carried out a study in rat femur bone marrow cells, in which various factors involved in the FPG technique were analyzed by means of microspectrophotometry. Those factors which Goto found influenced differential staining were, concentration of Hoechst 33258, pH of mounting medium, temperature during UV-exposure and the wavelength of UV light. Goto suggested that the existence of the various modifications indicates that technical problems of this method have not yet been solved.

He found that heating of cells especially during UV irradiation enhanced sister chromatid differentiation e.g., UV-exposure in buffer at 50 C gave improved sister chromatid differentiation. Among artificial light sources he found a 15 watt blacklight to be the best since the maximum absorption of DNA-bound Hoechst 33258 occurs at 360 nm, whereas the peak wavelength of blacklight is 355 nm as compared to germicidal light (254 nm) and fluorescent light (455/555 nm). He concluded that there are probably

two main categories of the Giemsa method for sister chromatid differentiation, one using UV sensitivity of heavily BrdU substituted DNA and the other, using thermal stability e.g., resistance to hot acid extraction. The findings of Goto with respect to the wavelength of UV light and heating of material during exposure to UV irradiation are not in agreement with the findings of Cortes and Andersson (1987). The latter authors concluded that the emission spectrum of the light source seems to play an important role in obtaining good differential staining, but noted that when a germicidal lamp radiating mainly at 254 nm was substituted for one radiating at 280-380 nm, the result was unsatisfactory, even after prolonged exposure. They concluded that the short wave UV dependence is probably explained by the fact the DNA bound Hoechst has an absorption maximum near 345 nm. Cortes (1980) stained meristem cells in Hoechst 33258 with simultaneous UV exposure and other authors have followed suit (Cortes and Andersson, 1987). Luo et al. (1988) suggested that the use of high pressure mercury lamps (450 W Foshan bulbs) improved the standard FPG method for SCE. However, variations in UV irradiation of Hoechst stained cells where not attempted in the present study.

Additional factors which might have been examined in this study, to identify those steps which would require modification in order to obtain chromatid differentiation by means of the FPG technique, are duration and concentration of Giemsa staining, and variations in concentration and duration of fluorochrome staining. Wavelength and duration of UV exitation of fluorochrome stained chromosomes may also play a role.

CONCLUSIONS

The initial work described in this study was carried out first in Hordeum vulgare (barley) and then in Vicia faba in order to establish a staining procedure for sister chromatid exchange. Sister chromatid exchange was then to be used as a bioassay with which to test the effects of several pesticides on the frequency of SCE after roots where exposed to various concentrations of pesticides. As outlined above, an adequate staining procedure which could be replicated with fidelity, was not found.

Therefore a study of a number of the steps involved in both the Feulgen procedure and the FPG technique for differential staining in plants, was attempted, in the hope of shedding light on the problems which were initially encountered.

In general, the results suggest that when embarking on a study of sister chromatid exchange in plants, factors which affect the outcome of differential staining should be examined before proceeding, even if sister chromatid differentiation has already been reported for the species. This suggestion is made not only because of the difficulties which were encountered here but also because of the discrepancies concerning the SCE staining protocols which exist in the literature, even among authors working with the same species and the same staining techniques.

Differential staining is influenced by a number of factors. Those which affect seedling growth and BrdU uptake of growing seedlings are important if sister chromatid differentiation is to be obtained. A variety of steps carried out after the root tips are harvested, including fixation and the staining which follows fixation have been shown to affect both the quality of staining and SCE.

Other factors are important to consider if this technique is to be used in mutagenicity testing. Among these are the concentration of the base analogue, and of FdU, as well as the ability to obtain good metaphase spreading when the cells are squashed.

As might be predicted, treatments such as the removal of plant shoots and cotyledons, and the application of the base analogue BrdU and FdU led to reductions in mitotic index of most plants. The use of FdU was not eliminated however, since it has been shown to enhance the uptake of radioactively labelled BrdU (Escalza et al., 1985). However, the lowest possible concentration of BrdU was employed, and whole plants were treated.

This study shows that the variance within treatments when plant seedlings were treated, was consistently high, implying that a range of tolerance to a given treatment exists among individual plants, even of the same cultivar. This variance caused the results of statistical analyses to be inconclusive with respect to the differences between treatments. Others have reported that plants of the same or different cultivars respond differently to the Feulgen procedure and the FPG technique respectively (Tempelaar et al., 1982; Dolezel et al., 1986).

Fixation was found to have a significant influence on the quality of staining in this study, and a fixation time of 7 hours is suggested to maximize the quality of staining at least for the Feulgen procedure. Fixation should be carried out at 4 C and storage of stained root tips for up to one month in 70% ethanol does not seem to adversely affect staining.

For qualitative data such as cytological observations of staining quality, it was found that a large amount of data are required in order to carry out a statistical analysis.

After careful study, good differential staining was obtained by means of the Feulgen procedure in Vicia faba. Differential staining was also obtained in barley by means of this staining technique. While many papers report good differential staining in Vicia faba according to the FPG technique, and a variety of modifications have been published, differential staining by means of the FPG technique was not obtained in the present study.

In conclusion, perhaps the comments of a few of the authors, with respect to their own staining techniques, would be of interest. Tempelaar et al. (1982) for example stated, "the merit of the process is its rapidity and lack of complicated steps so that the results are not to a large extent dependent on the skill and experience of the investigator". He goes on to say, "when carefully performed the Feulgen procedure yields very reproducible results with chromatin from a variety of sources." Cortes and Andersson (1987) commented that in general while most of the FPG procedures allow good differential staining they are sometimes not as reliable as techniques performed on animal materials. However, while this statement was not discussed, their own technique was described as "quick and simple and has the advantage of high reproducibility." Finally Cortes and Andersson (1987), claimed that their technique is superior to the Feulgen procedure outlined by Tempelaar. Unfortunately, the present study did not bear out the evaluations of any of the authors with respect to the simplicity of their respective staining techniques.

Perhaps, the difficulties encountered in at least some of these studies could have been more readily acknowledged. Furthermore, in many instances the authors could have explicitly stated which steps or treatments

were likely to adversely affect the results, thereby avoiding to give the mistaken impression that the procedures were unproblematic.

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APPENDIX I

Procedure for preparing leuco-basic fuchsin

1. Dissolve 1 g basic fuchsin by pouring it over 200 ml boiling distilled water.

2. Shake well and cool to 50 C.

3. Filter: Add 30 ml N HCl to the filtrate.

4. In a fume hood add 3 g $K_2S_2O_5$.

5. Allow solution to bleach for 24 h in a tight-stoppered bottle, in the dark.

6. After 24 h in the dark add 0.5 g decolorizing carbon. Norit, a proprietary vegetable carbon, is recommended.

7. Shake well for about one minute and filter rapidly through coarse filter paper.

8. Store in a tightly-stoppered bottle in the dark, at 4 C.

APPENDIX II

Hoagland Nutrient Solution

Taken from: Plant Tissue Culture Methods, L.R. Wetter and F. Constabel (eds.) National Research Council of Canada, Saskatoon, Saskatchewan, 1982. p. 134.

Macronutrients	mM	g/l
$Ca(NO_3)_2.4H_2O$	4.0	.94
$MgSO_4.7H_2O$	2.0	.52
KNO_3	6.0	.66
$NH_4H_2PO_4$	1.0	.12
Sequestrene 330 Fe	---	.07

Micronutrients (stock solution)	
H_3BO_3	28
$MnSO_4.H_2O$	34
$CuSO_4.5H_2O$	1.0
$ZnSO_4.7H_2O$	2.2
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.0
H_2SO_4 (conc.)	5.0 ml

0.1 ml of the micronutrients is mixed with 1 litre of the macronutrients and the pH is adjusted to 6.7.

APPENDIX III

Effect of the removal of *Vicia faba* seedling cotyledons and shoots on the mitotic index.

Source of Variation	DF	MS	F val	Prob F
TRT	4	0.00966337	0.94	<0.4816

APPENDIX IV

Effect of unifilar vs. bifilar substitution of the base analogue BrdU at different concentrations on the mitotic index of *Vicia faba* seedlings.

First run:

Source of Variation	DF	MS	F val	Prob F
TRT	6	0.013475325	2.17	< 0.1191

Second run:

Source of Variation	DF	MS	F val	Prob F
TRT	6	0.0030121433	0.32	< 0.9135

APPENDIX V

Effect of increasing concentrations of FdU supplemented to treatment solutions of BrdU on the mitotic index of *Vicia faba* seedlings.

Source of Variation	DF	MS	F val	Prob F
TRT	9	0.01781882	2.65*	< 0.0462

* Significant at the 0.05 level

APPENDIX VI

Effect of the duration of fixation on the quality of staining.

Source of Variation	DF	X^2 value	Prob X^2
Duration of fixation	5	22.22**	$p < 0.005$

** Significant at the 0.01 level

APPENDIX VII

Effect of the duration of fixation on the appearance of the cytoplasm.

Source of Variation	DF	X^2 value	Prob X^2
Duration of fixation	5	28.69**	$p < 0.005$

** Significant at the 0.01 level

APPENDIX VIII

List of the Coefficients of Variability (CVs)

Experiment:	CV
Effect of the removal of *Vicia faba* seedling cotyledons and shoots on the mitotic index.	53.13%
Effect of unifilar vs. bifilar substitution of the base analogue BrdU at different concentrations on the mitotic index of *Vicia fava* seedlings.	
First run:	59.75%
Second run:	84.49%
Effect of increasing concentrations of FdU supplemented to treatment solutions of BrdU on the mitotic index of *Vicia faba* seedlings.	70.59%

APPENDIX IX

Effect of the removal of *Vicia faba* seedling cotyledons and shoots on the mitotic index. A. Hoagland's nutrient solution control, A1. Water control, B. Cotyledons cut and seedlings grown in Hoaglands, B1. Cotyledons cut and seedlings grown in water control, C. Shoots cut and seedlings grown in Hoaglands, C1. Shoots cut and seedlings grown in water. Data in percent; SE = standard error.

Treatment	Experiments						
	1	Mean	2	Mean	3	Mean	Average and SE
A	4.4		6.1		5.8		
	3.4	4.3	8.8	6.3	4.0	4.8	5.13 ± 0.54
	5.0		3.9		4.7		
A1	6.1		6.1		3.5		
	5.0	5.2	6.7	6.3	1.8	3.1	4.87 ± 0.53
	4.5		6.2		4.1		
B	2.7		0.8		1.7		
	14.4	8.9	1.5	1.1	0	0.97	3.66 ± 1.64
	9.6		1.1		1.2		
B1	0		0				
	0		0.1		No secondary roots developed		
	0.2		0				

... Appendix Table IX Continued

Appendix Table IX continued

Treatment		Experiments				
C	0	4.8		4.7		
	0	2.4	3.5	4.0	3.87	2.46 ± 0.65
	0.2	3.2		2.9		
C1	1.6	2.2		3.9		
	1.9 2.03	3.0	2.7	2.8	2.7	2.48 ± 0.26
	2.6	3.0		1.4		

APPENDIX X

Effect of unifilar vs. bifilar substitution of the base analogue BrdU at different concentrations on the mitotic index of *Vicia faba* seedlings. First run. Data in percent; SE = standard error.

Treatment	Experiments						
	1	Mean	2	Mean	3	Mean	Average and SE
A. control							
	5.4		2.7		1.4		
	4.6	4.5	0.3	1.07	4.3	3.5	3.02 ± 0.66
	3.5		0.2		4.8		
B. BB-BT substitution, 50 uM BrdU							
	0.5		0		0.7		
	1.8	0.97	0	0	0.2	0.3	0.42 ± 0.19
	0.6		0		0		
C. BT-TT substitution, 50 uM BrdU							
	1.9		1.4		0.1		
	2.6	2.73	0	0.8	0.5	0.2	1.24 ± 1.29
	3.7		1.0		0		
D. BB-BT substitution, 100 uM BrdU							
	2.6		1.2		0.9		
	1.7	2.10	0.1	0.73	0.5	0.70	1.18 ± 0.26
	2.0		0.9		0.7		

...Appendix X continued

Appendix X continued

Treatment	Experiments						
	1	Mean	2	Mean	3	Mean	Average and SE
E. BT-TT substitution, 100 uM BrdU							
	2.6		1.4		2.5		
	3.5	2.77	1.6	1.77	5.2	3.77	2.77 ± 0.39
	2.2		2.3		3.6		
F. BB-BT substitution, 500 uM BrdU							
	1.1		1.5		0		
	2.2	1.67	3.3	2.83	0.1	0.03	1.51 ± 0.46
	1.7		3.7		0		
G. BT-TT substitution, 500 uM BrdU							
	3.3		2.3		3.6		
	5.9	3.87	2.8	2.50	3.4	2.80	3.06 ± 0.42
	2.2		2.3		3.6		

APPENDIX XI

Effect of unifilar vs. bifilar substitution of the base analogue BrdU at different concentrations on the mitotic index of *Vicia faba* seedlings. Second run. Data in percent; SE = standard error.

Treatment	Experiments						
	1	Mean	2	Mean	3	Mean	Average and SE
A. control							
	3.2		0.3		2.2		
	4.7	3.37	0.1	0.13	2.6	2.4	1.97 ± 0.52
	2.2		0		2.4		
B. BB-BT substitution, 50 uM BrdU							
	0.2		0.8		0.5		
	0.6	0.47	1.0	1.70	2.2	0.90	1.02 ± 0.34
	0.6		3.2		0		
C. BT-TT substitution, 50 uM BrdU							
	1.7		0.3		5.3		
	1.3	1.47	1.2	1.07	5.1	5.0	2.51 ± 0.64
	1.4		1.7		4.6		
D. BB-BT substitution, 100 uM BrdU							
	1.3		0.8		0.1		
	1.3	1.33	1.4	1.07	0	0.03	0.81 ± 0.21
	1.4		1.0		0		

...Appendix XI continued

Appendix XI continued

Treatment	Experiments					
	1	Mean	2	Mean	3	Mean	Average and SE
E. BT-TT substitution, 100 uM BrdU							
	1.0		3.0		1.1		
	0.4	1.70	2.7	2.37	0	0.43	1.50 ± 0.44
	3.7		1.4		0.2		
F. BB-BT substitution, 500 uM BrdU							
	0		0.8		1.7		
	0	0.03	0.6	0.83	1.4	1.70	0.85 ± 0.25
	0.1		1.1		2.0		
G. BT-TT substitution, 500 uM BrdU							
	1.6		1.7		0.2		
	1.6	1.97	2.8	2.40	0.2	0.23	1.53 ± 0.36
	2.7		2.7		0.3		

APPENDIX XII

Effect of increasing concentrations of FdU supplemented to treatment solutions of BrdU on the mitotic index of *Vicia faba* seedlings. Data in percent; SE = standard error.

Treatment	Experiments						
	1	Mean	2	Mean	3	Mean	Average and SE
1. control							
	2.1		4.3		3.1		
	2.8	2.63	6.8	5.77	2.5	2.40	3.60 ± 0.60
	3.0		6.2		1.6		
2. 50 uM BrdU							
	3.9		1.8		1.1		
	2.1	3.40	1.8	1.40	4.0	2.13	2.31 ± 0.45
	4.2		0.6		1.3		
3. 50 uM BrdU, 0.1 uM FdU, 5 uM Urd							
	3.3		1.4		3.8		
	1.9	2.73	2.4	1.90	2.0	2.07	2.23 ± 0.34
	3.0		1.9		0.4		
4. 50 uM BrdU, 5 uM FdU, 5 uM Urd							
	0.3		0		1.1		
	0.1	0.13	0		0.1	1.03	0.39 ± 0.22
	0		0		1.9		

...Appendix XII continued

Appendix XII continued

Treatment	Experiments					
	1	Mean	2	Mean	3	Mean	Average and SE
5. 100 uM BrdU							
	0.7		3.3		2.5		
	0.2	1.03	5.0	3.17	3.6	3.20	2.47 ± 0.52
	2.2		1.2		3.5		
6. 100 uM BrdU, 0.1 uM FdU, 5 uM Urd							
	2.9		0.4		1.0		
	3.6	3.20	0.4	0.67	1.8	2.30	2.06 ± 0.48
	3.1		1.3		4.3		
7. 100 uM BrdU, 5 uM FdU, 5 uM Urd							
	0.3		0.1		0		
	0.3	0.20	0.1	0.07	0		0.09 ± 0.04
	0		0		0		
8. 500 uM BrdU							
	0.4		0.2		0.3		
	0.9	0.70	0.7	0.60	0	0.33	0.54 ± 0.11
	0.8		0.9		0.7		

...Appendix XII continued

Appendix XII continued

Treatment	Experiments					
9. 500 uM BrdU, 0.1 uM FdU, 5 uM Urd						
	1.1		0		0.1	
	1.9	3.13	0.3	0.10	0	0.33	1.09 ± 0.69
	6.4		0		0	
10. 500 uM BrdU, 5 uM FdU, 5 uM Urd						
	0		0		0.9	
	0		0	0.03	0.6	0.70	0.24 ± 0.12
	0		0.1		0.6	