DIFFERENTIAL STAINING OF HUMAN CHROMOSOMES

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CONTENTS

[1] For technical reasons this Paper has retained the page numbering of the original article.

Contents:

1.1. *Human cytogenetics before the development of differential chromosome staining techniques*

The diploid chromosome number in man is 46, viz. 22 pairs of autosomes and 1 pair of sex chromosomes. In the female the latter consists of two X chromosomes and in the male of one X and one Y chromosome. An abnormality in number or form of the chromosomes is usually associated with serious disturbances in the mental, physical, and / or reproductive development of the individual (for extensive reviews see, e.g., Levine, 1971; Hamerton, 1971).

In approximately 5 out of 1000 newborn infants a chromosomal aberration has been found (Jacobs et al., 1974; Nielsen and Sillesen, 1975). In spontaneous abortions a chromosomal abnormality occurs even in a much higher percentage than in newborn infants: 30-60% according to surveys carried out by Boué and Boué (1974) and by Creasy et al. (1976). An abnormal chromosome complement has also often been seen associated with various types of neoplasma (German, 1974; Mitelman and Levan, 1976). The study of the human chromosomes is therefore not only of purely scientific interest but also indispensible for the diagnosis of disorders associated with a chromosomal abnormality and for obtaining information on their pathogenesis, aetiology, mode of inheritance and recurrence risk.

Before the development of the differential staining methods the criteria for the identification of human metaphase chromosomes were very limited. As agreed upon in Denver (1960), London (1963) and Chicago (1966), the autosomal pairs were classified according to length, position of the centromere, and also the position of secondary constrictions when present (Fig.1), into 7 groups (A-G) and numbered 1-22. Figure 2 shows a human karyotype in which the chromosomes are arranged using this classification. As can be seen, many of the chromosomes within a particular group show similar features and only a few of the 22 autosomes can be easily distinguished. Changes in chromosome structure cannot be detected unless they alter the chromosomal shape. Although the autoradiographic analysis of the chromosomal DNA replication permitted identification of certain B-, D-, and E group chromosomes (Schmid, 1963; London Conference, 1963), the technique was too complicated and time-consuming for routine diagnostic use. After the initial period of describing chromosomal disorders based on gross karyotypic changes, the lack of better techniques for chromosomal identification proved to be a major restriction for further progress in human cytogenetics.

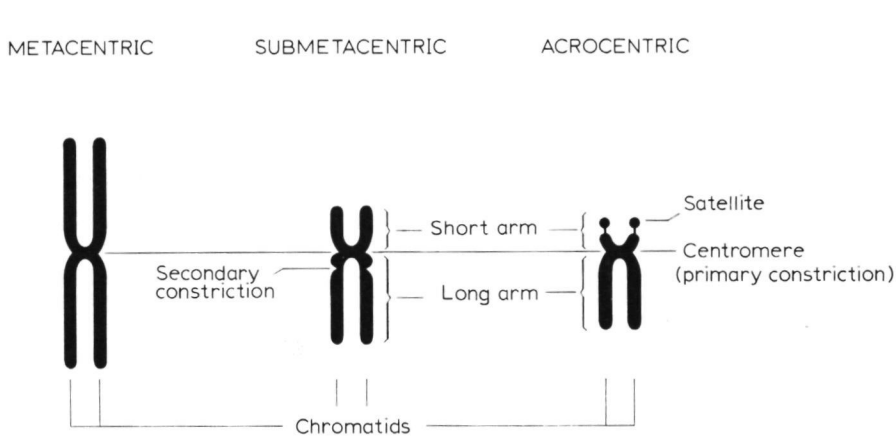

Fig. 1. Schematic representation of the morphological characteristics of human somatic metaphase chromosomes. According to the position of its centromere a chromosome is termed *metacentric* (centromere in or near the middle), *submetacentric* (centromere nearer one end of the chromosome; there is a distinct short arm and a long arm, often symbolized by the letters p and q, respectively), or *acrocentric* (centromere very near one end of the chromosome, which therefore has a very short short arm). The terminal regions of the chromatids are the"telomeres".

1.2. *The banding techniques*

A solution to the problem of identifying each individual chromosome was provided by Caspersson et al. (1968) when they developed their differential staining method. Studying metaphase preparations of several plant and animal species they observed that chromosomes treated with quinacrine mustard and examined with fluorescence microscopy showed bright and dark regions along their linear axis. The banding pattern obtained was characteristic for each homologue and permitted the identification of all chromosomes. This new technique appeared also suitable for the recognition of human chromosomes (Caspersson et al., 1970) and has contributed considerably to further progress in human genetics, especially in diagnostic cytogenetics. A more precise analysis of chromosomal aberrations in human malformation syndromes and in neoplastic disorders became possible. In addition, the technique could be applied for the identification of human chromosomes in meiosis (Caspersson et al., 1971a) and in somatic cell hybrids (Caspersson et al., 1971c). The fluorescence technique also permitted a more accurate karyotype analysis of

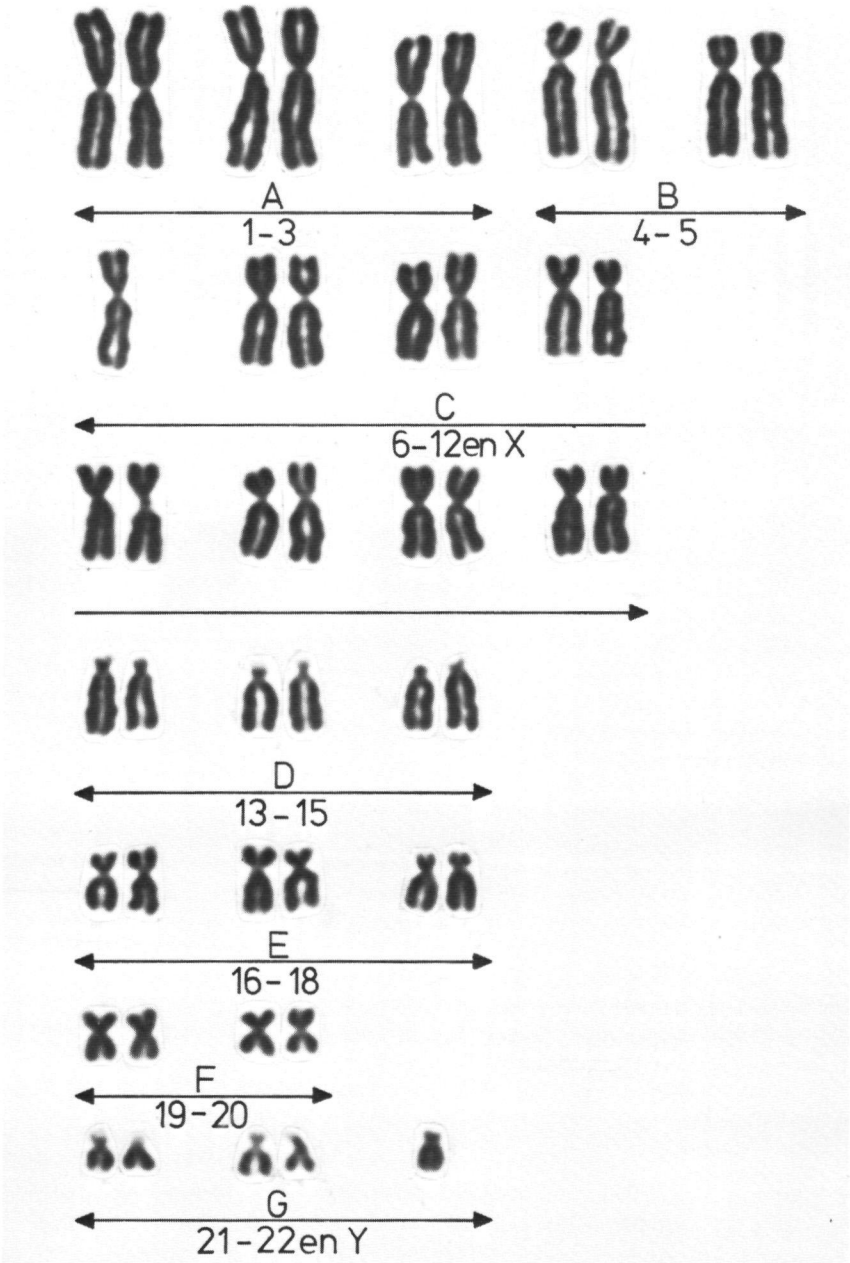

Fig. 2. Human male karyotype in which the chromosomes are arranged according to the Chicago classification (1966) (from Hustinx et al., 1975).

many species such as the scientifically important laboratory mouse Mus musculus (Francke and Nesbitt, 1971) and rat Rattus norvegicus (Miller et al., 1972) It also provided a new approach in the study of possible phylogenetic relations, e.g., between primates (Pearson et al., 1971) and between rodents (Miller, 1973).

The fluorescence technique has a number of disadvantages. The fluorescent light intensity fades rapidly during chromosome illumination with ultraviolet light, not only reducing the time available for microscopic observation but also increasing the exposure times required for making microphotographs. A further drawback of the technique is that the preparations cannot be made permanent. The subsequently developed techniques which use the non-fluorescent dye Giemsa did not have these disadvantages.

With the first Giemsa banding method mainly the centromeric areas of most chromosomes were more deeply stained (Pardue and Gall, 1970; Arrighi and Hsu, 1971). Soon after, modifications of this technique resulted in Giemsa stained bands similar in location and stain distribution to those produced by quinacrine. These Giemsa staining methods were rapidly introduced as either alternatives or supplements to the fluorescent technique.

To produce fluorescent bands the only treatment of the (fixed) chromosomes consists of their staining with a fluorochrome. In contrast to this, a pretreatment of the chromosomes is usually necessary to obtain banding patterns with Giemsa. It is amazing how many types of pretreatment are effective in doing this. Bands can be produced by heating the chromosomes at 87°C (Dutrillaux and Lejeune, 1971), by digesting them with enzymes (Dutrillaux et al., 1971), by treating them with alkali (Drets and Shaw, 1971; Schnedl, 1971) or warm salt solutions (Sumner et al., 1971a), or by incubating them with certain drugs before fixation (Shafer, 1973). Even a certain household detergent can be used for band production (Sperling, 1972)! The number of band-inducing pretreatments known is so high that it is surprising that these techniques were not sooner discovered.

The various differential staining techniques do not produce identical banding patterns. Chromosomal areas strongly staining with one technique might stain only weakly or not at all after the application of another technique. To discuss the results obtained so far and to establish "a standardized system of nomenclature to describe the chromosomes and chromosome regions revealed by the various new techniques" a group of cytogeneticists met in Paris on the occasion of the Fourth International Congress of Human Genetics

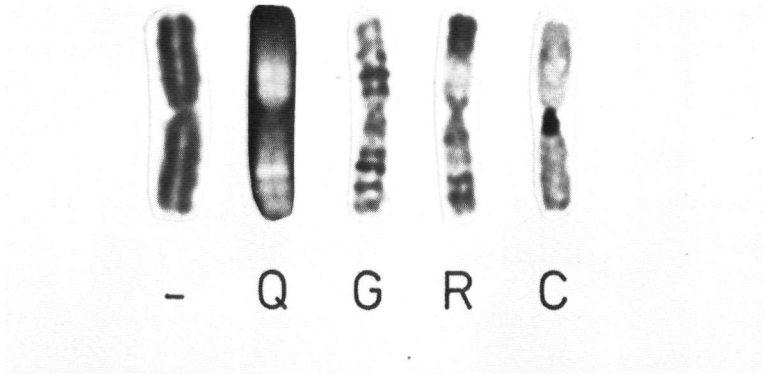

Fig. 3. The human chromosome No. 1 stained with various methods (- = "conventional" Giemsa-staining; Q = Q-banding; G = G-banding; R = R-banding; C = C-banding).

in 1971. The resulting "Paris nomenclature" (Paris Conference, 1971) has now been adopted by the majority of investigators and has greatly contributed to facilitate communication in the field of cytogenetics. In this report the banding patterns produced in human chromosomes were classified into four types (see also Fig. 3):

Q-BANDS

These bands are produced after staining with the fluorescent dyes quinacrine mustard or quinacrine dihydrochloride ("Atebrin"). The fluorescent karyotype originally described by Caspersson et al. (1971b) was selected as the basis for the designation of each chromosome.

C-BANDS

Some techniques in which Giemsa stain is used (Pardue and Gall, 1970; Arrighi and Hsu, 1971) produce dark bands at the centromeric regions and in a few other chromosomal areas. These are called C-bands. The differentiation provided by the C-banding techniques is insufficient to recognize each chromosome, in contrast to the one obtained with the Q-, G- and R-banding methods.

G-BANDS

These bands become visible after a variety of pretreatments followed by Giemsa staining. The resulting "G-banding pattern" agrees almost fully with the one obtained by quinacrine fluorochromes. In high quality preparations sometimes over 2000 bands per diploid chromosome complement may be distinguished (Yunis, 1976).

R-BANDS

"R-bands" are produced in chromosomes if the "controlled heat denaturation technique" of Dutrillaux and Lejeune (1971) is employed. This staining procedure, also using Giemsa dye, results in a pattern almost exactly the reverse of the G-banding pattern and for that reason is often called the "reverse- staining Giemsa method".

The banding techniques mentioned above are those most useful for diagnostic purposes, and each human cytogenetics laboratory should have the facilities to carry them out. In addition to the Q-, G-, C- and R-banding methods a number of other techniques has been developed since 1971 for the staining of certain specific chromosome areas, such as the telomeres (T-banding; Dutrillaux, 1973), the so-called "nucleolus organizer regions" in the short arms of the acrocentric chromosomes (N-banding; Matsui and Sasaki, 1973), and certain secondary constrictions (see *1.3.*). Some of the techniques involve the incorporation of base analogues (such as 5-bromodeoxyuridine, BrdU) into the DNA during culture and seem particularly useful for the microscopic study of DNA replication kinetics and the mechanisms of chromosomal breakage and repair (Latt, 1974; Dutrillaux et al., 1974).

For a review of most differential chromosome staining techniques at present available see, e.g., Dutrillaux (1975).

1.3. Interphase nuclei

In 1949 Barr and Bertram discovered a darkly staining body in the interphase nuclei of female cats not present in those of male cats. This "Barr-body" or "X-chromatin" has since been found in the large majority of female mammals, including man (Moore and Barr, 1953). It soon became clear that each Barr body represents an X chromosome in a condensed state. The study of human interphase nuclei for X-chromatin has become a valuable diagnostic tool in cases of disorders of sexual development (for reviews see, e.g., Moore, 1966 or

Hamerton, 1971).

The new differential staining techniques have widened the scope of the interphase nuclei studies. In 1970 Pearson et al. reported that with quinacrine staining the brilliant fluorescent segment characteristic for the long arm of the human Y chromosome (Zech et al., 1969) could easily be distinguished as a bright body in the interphase nucleus.This fluorescent chromatin was termed "Y-body" or "Y-chromatin". Normal males (XY) have one such bright fluorescent spot in their nuclei. It is absent in the nuclei of females (XX). In nuclei of males with two Y chromosomes (XYY) two spots are seen. The Y-chromatin can be demonstrated in practically any cell type (Pearson, 1972) and is often associated with the nucleolus (Bobrow et al., 1971a; Wyandt and Iorio, 1973). In a low percentage of the polymorphonuclear leukocytes it is present in "drumstick - like" nuclear appendages (Lamborot-Manzur et al., 1971).

The search for X- and Y-chromatin bodies in interphase nuclei provides a convenient and rapid method to determine, with a considerable degree of certainty, the sex chromosome complement of an individual. This is of practical importance as an abnormal number of sex chromosomes is the most frequently found chromosomal aberration in man (Jacobs et al.,1974, Nielsen and Sillesen, 1975). Both false-positive and false-negative results of sex chromatin examinations may occur, however, and to confirm the diagnosis a full metaphase analysis remains therefore necessary (Gardner, 1976). X- and Y-chromatin investigations are also carried out in prenatal diagnosis to determine the fetal sex, in tissue transplantations to distinguish donor from acceptor cells, and in more fundamental cell genetics (Pearson, 1972).

In addition to the X- and Y-chromatin staining methods a few other techniques have become available for the detection of chromatin bodies representing specific regions of certain autosomes in interphase nuclei. To be mentioned in this respect are the "G-11" staining method for the secondary constriction of chromosome No. 9 (Bobrow et al., 1972), the technique of Geraedts and Pearson (1973) for staining the secondary constriction of chromosome No. 1, and a modification of the "CT" technique for staining the short arms of certain acrocentric chromosomes (Scheres, 1976). In contrast to the X- and Y-chromatin staining methods, the latter techniques are only of minor importance for diagnostic cytogenetics. On the one hand they are less specific and therefore less reliable and on the other the chromosomal aberrations detectable with these specific staining methods and associated with clinical

abnormalities are rare.

The new interphase staining methods have been useful in the study of the internal organization of the interphase nucleus, and of the behaviour of certain chromosomal areas during the various stages of the mitotic cell cycle (Wyandt and Hecht, 1973) and during meiosis (Pearson and Bobrow, 1970; Stahl et al., 1973; Gagné et al., 1973). They have also revealed that a fairly high percentage of human spermatozoa carries a numerical chromosome aberration (Sumner et al., 1971b; Pawlowitzki and Pearson, 1972; Pearson et al., 1973b).

1.4. Chromosomal variants

The extensive use of the new differential staining techniques for the study of the chromosomes of normal and abnormal individuals has greatly increased our knowledge of the human chromosome complement. With the exception of Drosophila probably no other organism exists of which the karyotype is so well known as that of man. One of the most remarkable features of the human karyotype is the large number of variations seen in certain chromosomal areas. Considerable differences in size and staining behaviour of these areas may exist between individuals without affecting their phenotype. The chromosomes showing these variations are called variants. The new banding techniques have not only permitted a further analysis of the chromosomal variations already known but also revealed the existence of "entirely new" types. For instance, certain chromosomal areas as the centromeric regions of the acrocentric chromosomes may vary largely in Q-fluorescence intensity (Caspersson et al., 1971b). The well known variation in the size of the Y chromosome appears to be the result of differences in the length of its characteristic brightly fluorescent segment (Bobrow et al., 1971b). The size of the C-bands also appears to vary considerably in all chromosomes (Craig-Holmes and Shaw, 1971; McKenzie and Lubs, 1975). Some variants are more frequent in the population than other ones (Geraedts and Pearson, 1974). Their mode of transmission is a simple Mendelian one although possible exceptions have been reported (Geraedts and Pearson, 1974). Because such a large number of variants exists the human chromosome complement is so highly variable that among approximately 400 persons examined no two had identical karyotypes (Müller et al., 1975).

The origin and possible biological and evolutionary significance of this large karyotype variability in man is not yet understood. One assumes

that the variable areas are genetically inactive and that the variations therefore have no phenotypic effects. Some evidence exists, however, that at least the variable short arms of the acrocentric chromosomes code for ribosomal RNA and therefore may not simply be considered inactive (Henderson et al., 1972). Many variable regions contain certain types of highly repetitive DNA (Jones et al., 1972, Gosden et al., 1975). Within such regions unequal crossing-over (exchange of unequal chromosomal parts usually occurring following the pairing of the homologues in meiosis) might take place and provides a possible explanation for the origin of variants (Craig-Holmes and Shaw, 1971)

Chromosome variants may well be used as markers in, e.g., paternity testing (Jonasson et al., 1972a), for the assignment of gene loci to particular chromosomes with the aid of family studies (Hamerton and Cook, 1974), and to compare the cytogenetic characteristics of populations (Pearson et al., 1973a, Buckton et al., 1976). Chromosome variants have also been helpful in certain instances to determine in which parent of a child with, e.g , trisomy 21, trisomy 13, or triploidy, a meiotic disturbance had occurred (Mikkelsen et al., 1976, Jonasson et al., 1972b, Hara and Sasaki, 1975). In addition, the study of variants has shown that in cases of chronic myeloid leukemia associated with the so-called "Philadelphia chromosome" (Ph^1) the aberrant cells are very likely of monoclonal origin (Gahrton et al., 1974, Hossfeld, 1975).

1.5. The nature of the bands

Following the initial surprise caused by the discovery of the chromosomal banding patterns and the wide applicability of the new techniques to cytogenetics, the question arose as to which chromosomal properties formed the basis of the differential staining phenomena. Many investigators have considered the new methods as possible clues to the problems of the chemical and structural organization of chromosomes. Unfortunately, lack of precise knowledge on the interactions between Giemsa or quinacrine dyes and the chromosomal material is a major diffulty in the study of the banding mechanisms From the many small pieces of information which have nevertheless become available, it may be concluded that the matter is rather complicated. Several properties of the chromatin appear to influence its staining behaviour. For a comprehensive review of most of the theories on the mechanism of chromosomal band formation the reader is referred to, e.g., Comings

et al. (1973) and Comings (1974). A short summary of the principle factors possibly involved in band production is given below.

Initially it was believed that the properties of the chromosomal DNA play a major role in band formation. This idea was supported by the observed differences between band- and interband regions with respect to:
1. the base composition of their DNAs (Q- and G-positive bands are usually adenine- and thymidine-rich, Q- and G-negative regions are guanine- and cytosine-rich; Ellison and Barr, 1972; Dev et al., 1972; Weisblum and de Haseth, 1972), 2. the repetitivity of their DNAs (C-bands being highly repetitive, the remaining regions being largely intermediate or non-repetitive; Arrighi et al., 1971; Gosden et al., 1975), 3. the sensitivity of their DNAs to, e.g., alkali or heat (Bobrow and Madan, 1973; Chapelle et al., 1973; Dutrillaux and Covic , 1974), and 4. the timing of their DNA replication (Ganner and Evans, 1971; Calderon and Schnedl, 1973).

However, evidence is increasing that other factors than DNA may also be of importance in band formation. Mammalian metaphase chromosomes consist for more than one half of proteins, viz. the basic proteins or histones and the acidic or non-histone proteins (Maio and Schildkraut, 1967). That these chromosomal proteins are very probably involved in the band formation process is clearly illustrated by the observation that pronase, trypsin, other proteolytic enzymes, and protein denaturants are very potent band-inducing agents (Dutrillaux et al., 1971; Seabright, 1971; Wang and Fedoroff, 1971; Utakoji, 1972; Lee et al., 1972). An indication for a possible role of the histones has been found by, e.g., Brown et al. (1975), who observed that certain fractions of these proteins were able to prevent the G-banding of chromosomes. Whether or not histones figure in chromosome band formation remains a matter of controversy. According to some authors the histones are unimportant as they would be removed by the strongly acid fixation procedure (Sumner et al., 1973); other investigators have found, however, that at least certain histone fractions remain after fixation (Pothier et al., 1975). Comings and Avelino (1975) have thoroughly studied the binding of a Giemsa stain component to chromatin and have developed the theory that non-histone proteins are the determining factors in chromosome band formation. As a result of the banding pretreatments these proteins would cover the DNA in certain chromosomal areas making these inaccessible to the stain.

A number of authors believe the banding patterns to reflect a chromatin distribution pre-existing in fixed chromosomes which is only accentuated by

the pretreatments and the staining procedures used (McKay, 1973, Rodman and Taliani, 1973, Yunis, 1973). However, if this phenomenon is a major factor in G-band production, it could hardly be an explanation for the formation of the reverse- or R-banding pattern in which the chromatin concentrations are situated in negative G-band regions.

In summary, the cytochemical data at present available suggest that certain properties of the chromosomal DNA and proteins, and certain interactions between these components form the basis of the banding phenomena. The lack of a final answer to the question of the chemical nature of the chromosomal bands is of course in the first place caused by the complexity of the problem. It might, however, also partially be the result of the fact that, at least at present, most cytogeneticists are more interested in the practical use of the new banding methods than in understanding their physico-chemical nature.

2.1. Aim of this study

The research activities which led to the papers collected in this thesis started in the early phase of the"banding period" At the time only a few chromosome banding techniques had been described. The results obtained with these techniques were often unsatisfactory and varied greatly between laboratories. It was also at the time even more uncertain than at present which mechanism(s) were responsible for the differential staining phenomena.

The first aim of this study has therefore been the development of one or more techniques suitable for application in diagnostic cytogenetics. In Papers I, II, III, IV and V a number of new banding methods are described.

In the second place, an effort has been made to study the factors forming the basis of the differential staining results discovered. In particular the effects of certain other dyes than Giemsa on chromosomal band formation have been investigated. These studies, which at the same time have resulted in a number of the above-mentioned new staining effects, are described in Papers II, III, IV and V.

In one of the new staining methods, the CT technique, both alkali and several salts are used for pretreatment of the chromosomes. The role of the cations present during the formation of the CT- and related banding patterns has also been studied (Papers VI and VII).

Finally a number of clinical cytogenetic applications of the new banding techniques are presented in Papers VIII, IX and X.

2.2. Nomenclature of new banding techniques

In the 1975 Supplement of the Paris Conference (1971) it is recommended to use a three-letter code for describing (new) chromosome banding techniques In this code the first letter denotes the type of banding, the second letter the general technique, and the third letter the stain. For example, QFQ = Q-bands by Fluorescence using Quinacrine, and GTG = G - bands by Trypsin using Giemsa. In accordance with these proposals the new banding techniques described in this thesis should have the following three-letter codes:

GTF = G-banding by Trypsin using basis Fuchsin (Paper II)
CBS = CT-banding by Barium hydroxide using Stains-all (Paper IV)
CBF = CT-banding by Barium hydroxide using basic Fuchsin (Paper V)
RBS = R-banding by Barium hydroxide using Stains-all (Paper IV)
RBF = R-banding by Barium hydroxide using basic Fuchsin (Paper V).

Codification of the method for specifically staining the short arms of certain acrocentric variants (Paper IV) is difficult as it is not yet fully understood if these regions are variant C-bands or that they constitute a new type of band. In order to distinguish this staining method from the CBS- and RBS-techniques the following code is suggested.
ABS = A(crocentric)-banding by Barium hydroxide using Stains-all.

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PAPER I

Identification of Two Robertsonian Translocations with a Giemsa Banding Technique

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Summary. Using a trypsin banding technique a D/D and a G/G translocation were identified as D 13/14 and G 21/22.

Zusammenfassung. Mit Hilfe einer Trypsin Bandierungstechnik wurde eine D/D und eine G/G Translokation als D 13/14 und G-21/22 identifiziert.

Introduction

Several staining techniques recently developed for karyotype analysis of human chromosomes result in banding patterns specific for each chromosome pair. These techniques include quinacrine fluorescence staining developed by Caspersson *et al.* (1970) and several variations of Giemsa staining in which it was first thought that denaturation followed by renaturation of chromosomal DNA were prerequisites for obtaining well defined banding in chromosomes (e.g. see Sumner *et al.*, 1971). However, Finaz and Crouchy (1971), Wang and Federoff (1971), and Seabright (1972) observed that similar banding patterns can be obtained by treating the chromosomes with proteolytic enzymes such as chymotrypsin and trypsin followed by staining with Giemsa or Leishman, which might suggest a relation between banding and the state of association between DNA and nucleoproteins. Even though the reason for banding is still unknown, it provides an excellent tool for identifying each chromosome in the karyotype. The purpose of the present communication is to report 2 cases of chromosomal aberrations as revealed by a modified banding technique (see Scheres 1972).

Method

Lymphocytes from peripheral blood were cultured as described by Moorhead *et al.* (1960) and harvested 4 hrs after adding colchicine. Subsequent to this they were treated with hypotonic KCl (0.075 M) for 8 min at 37°, fixed in 3:1 methanol-acetic acid mixture and spread by use of the flame drying method.

The slides were treated with 0.1% trypsin solution in isotonic phosphate buffered saline (pH 7.2) for 2—3 min at 10° followed by 2–3 min in Giemsa (10% Gurr's Improved R'66 in 1/15 M Sorensen buffer pH 6.8). Metaphases were photographed on 45C62 Scientia film (Agfa Gevaert).

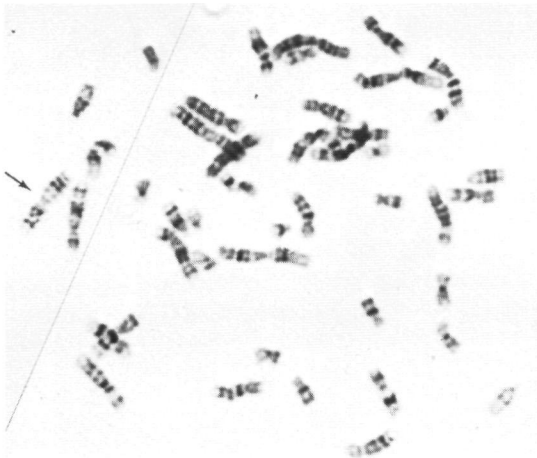


Fig. 1. Metaphase of case 1 after trypsin and Giemsa treatment; the arrow indicates the D-13/14 translocation chromosome

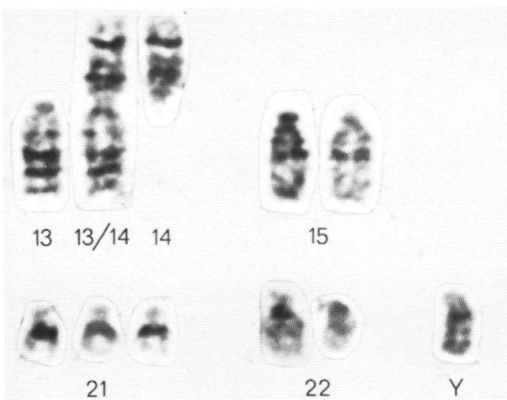
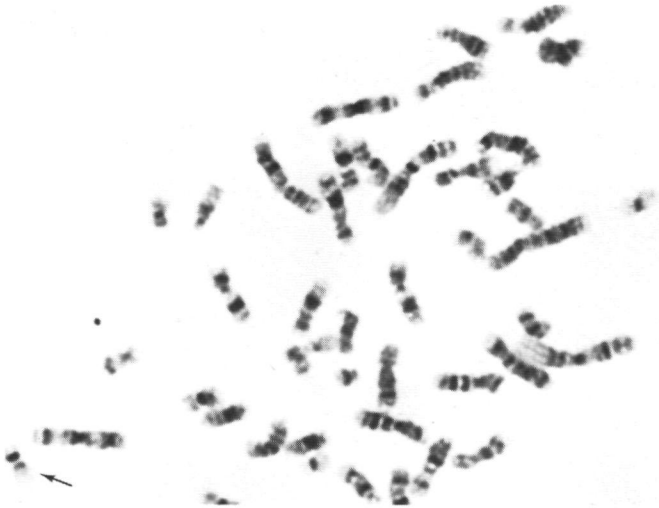


Fig. 2. Partial karyotype showing the groups D and G of case 1. The D-14 is mounted upside down to emphasize the similarity with the shorter arm of the translocation

Results and Discussion

Case 1. A conventional karyotype analysis of a 14 year old boy (LV) revealed the following abnormalities: 1 additional metacentric chromosome in group A, 2 missing D acrocentrics and 1 extra G. Since this boy was a patient with Down's syndrome the supernumerary G was tentatively identified as 21 and the extra A as a Robertsonian rearrangement between the two missing D acrocentrics. These tentative conclusions are confirmed by the Giemsa banding technique (Fig. 1), with the banding being similar to that reported by Finaz and Crouchy (1971). Following the notation suggested by these authors the 4 group D chromosomes



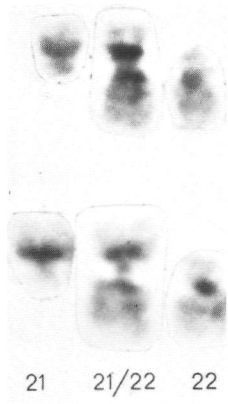


Fig. 3

Fig. 4

Fig. 3. Metaphase of case 2; the arrow indicates the G-21/22 translocation

Fig. 4. Group G chromosomes from 2 cells of case 2. Both 21's are placed upside down to facilitate comparison with the shorter arm of the translocation

were identified as 2 D-15's, 1 D-13 and 1 D-14. The banding patterns in the long and short arm of the additional A-like chromosome were similar to those seen in D-13 and D-14 respectively (Fig. 2), identifying it as a D-13/14 translocation (the most frequently found D/D translocation as described by Hecht and Kimberling in 1971). In addition, the banding pattern in the small supernumerary G made possible its identification as a G-21.

Case 2. This person (MP) was a 31 year old woman whose second and last pregnancy resulted in a male child with a G/G translocation type of Down's syndrome (the first resulted in abortion during the 5th month). A prior karyotype analysis without banding had suggested that the mother was a carrier of the translocation present in her son — 2 G's were missing and 1 extra submetacentric E-like element was found. In regard to the chance of further mongol children it was important to know whether the translocation was a G-21/22 or G-21/21, and banding strongly suggests the former — the short arm being similar to G-21, and the long arm to G-22 (Figs. 3 and 4).

These results clearly illustrate the utility of the new Giemsa technique in the characterization of chromosomal abnormalities. It provides a new approach to several problems in diagnostic cytogenetics, such as detection of minor chromosomal aberrations involved in clinical defects and screening of chromosomal variations as markers in linkage studies. Also a more basic need at present is to obtain an understanding of chemical composition, structure, and possible cyclic changes in chromosomes which make this banding possible.

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Banding of Human Chromosomes with Basic Fuchsin

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Summary In this paper a technique is described for the banding of human metaphase chromosomes with basic fuchsin The main characteristics of the G banding pattern obtained with this cationic triphenylmethane dye are

the secondary constriction regions of chromosomes Nos 1 and 16 are strongly stained, especially in the latter one,

the heterochromatic area of chromosome No 9, usually negative with most other G banding techniques, is clearly visible as an intensely stained band adjacent to the centromere,

the chromosomal outline is often very distinct, facilitating the study of the telomeres,

a number of chromosomal regions with bright Q fluorescence such as the polymorphic regions of the chromosomes Nos 3, 4, and Y also stain strongly with basic fuchsin

The basic fuchsin technique combines therefore properties of G , C , and Q-banding methods and seems very suitable for use in e g , family and linkage studies

Several triphenylmethanes closely related to basic fuchsin produce similar banding pat terns The band producing ability is, however, diminished in those dyes which contain methyl ated amino groups If the methyl groups are attached to the carbon atoms at the 3-positions in the phenyl rings, band formation seems unaffected

The way in which basic fuchsin and chromatin may interact as well as the possible mechanism(s) of band formation with this dye are discussed

Introduction

In 1968 Caspersson et al obtained a differential staining of metaphase chromo somes with quinacrine mustard This technique, soon known as Q banding, has become an important tool in cytogenetics, in particular for the analysis of trans locations and other structural rearrangements The observations of Caspersson et al were followed by a large number of investigations to clarify the physico-chemical basis of this banding phenomenon and by efforts to improve the tech nique During these studies several methods were developed in which nonfluo-rescent dyes were used to obtain various banding patterns (Paris Conference, 1971) Of the latter dyes only the Romanowsky stains such as Giemsa's, Leishman's, Wright's, and May Grunwald's gave a distinct G (Giemsa)-banding (Seabright, 1971, Lundsteen et al , 1973, Sumner and Evans, 1973, Crossen, 1973, Sanchez et al , 1973, Meisner et al , 1974) These dyes are all composed of one or more basic thiazine derivatives and the acid xanthene dye eosin A few basic thiazines on their own have also been reported to produce G-banding patterns (Lober et al , 1973, Comings, 1975)

In our laboratory a number of non-Romanowsky dyes were recently examined for their ability to produce chromosomal bands. With some triphenylmethane dyes such as basic fuchsin, excellent G-banding patterns were obtained. Basic fuchsin staining also made it possible to distinguish certain chromosomal variants which in general can only be recognized by the use of other banding methods. It is the purpose of this article to describe the characteristics of the banding pattern produced with basic fuchsin and to present the result of some studies on the mechanism of the band formation with this triphenylmethane dye.

Materials and Methods

Human venous lymphocytes were cultured according to a modified Arakaki and Sparkes (1963) microtechnique. Colchicine, in a final concentration of 1.0 μg/ml medium, was added 3 h before harvesting. The cells were then subjected to hypotonic treatment with 0.075 M KCl for 10 min at 37°C and fixed with several changes of 3:1 methyl alcohol-acetic acid. Chromosome preparations were made by dropping a small quantity of the final suspension onto clean, grease-free slides moistened with distilled water. After allowing 5—10 s for spreading, the excess water was removed and the preparations were dried by waving the slides vigorously through a warm air stream produced with the aid of a small flame. Care was taken to prevent overheating of the preparations or ignition of the chromosome suspensions.

Trypsin pretreatment of chromosomes was carried out at room temperature with a 0.1% solution of trypsin (Difco 1:250) in phosphate-buffered saline, pH 7.2. A small quantity of the solution was placed on one end of the slide and distributed at an even rate in about 10 s to the other end with the aid of a thin glass rod. The preparation was then rinsed with deionized water and immediately stained. In this way the effect of gradually increasing trypsin incubation periods could be studied on one slide.

For staining the chromosomes the following dyes were used:

from E. Merck: basic fuchsin (CI 42520), pararosaniline (CI 42500), methyl green (CI 42585), thionin (CI 52000), methylene blue (CI 52015), toluidine blue (CI 52040), and azure II;

from G. T. Gurr: basic fuchsin, rosaniline, new fuchsin, gentian violet, and Giemsa;

from J. T. Baker: basic fuchsin (CI 42510), and pararosaniline (parafuchsin, CI 42500);

from British Drug Houses: basic fuchsin (CI 42500), crystal violet (CI 42555), eosin (CI 54380), and Leishman's stain;

from Serva: basic fuchsin (CI 42500);

from Sigma: basic fuchsin;

from U. C. B. Brussels: basic fuchsin.

Thirty mg dye was dissolved in 30 ml of a 1:1:1 mixture of 0.1 N NaOH:formamide:water. With the aid of 1 N HCl the pH of the solution was adjusted to 10.5—11.0 or to a lower pH if necessary. The dye solutions were freshly prepared as after a few hours precipitation occurred, particularly in the basic fuchsin solution, usually resulting in loss of band-producing ability. The staining procedure consisted of placing the preparation in the dye solutions for 3 min, rinsing in deionized water. and air drying. The slides were examined without coverglass. Agfa-Gevaert Scientia 45C62, Scientia 50B65, or Copex Pan Rapid films were used for microphotography.

For interference contrast studies the Nomarski differential interference contrast system of Zeiss (Objective Inko f. Epiplan 40/0.85 Pol) was used.

Results

Basic Fuchsin. An acid or neutral solution of basic fuchsin (BF) stained metaphase chromosomes uniformly. Using an alkaline BF solution (pH 10.5 or above) produced a distinct G-banding pattern (Fig. 1). The formation of bands was improved by treatment of the chromosomes with trypsin solution before staining (Fig. 2). The characteristics of the G-banding pattern obtained were the following:

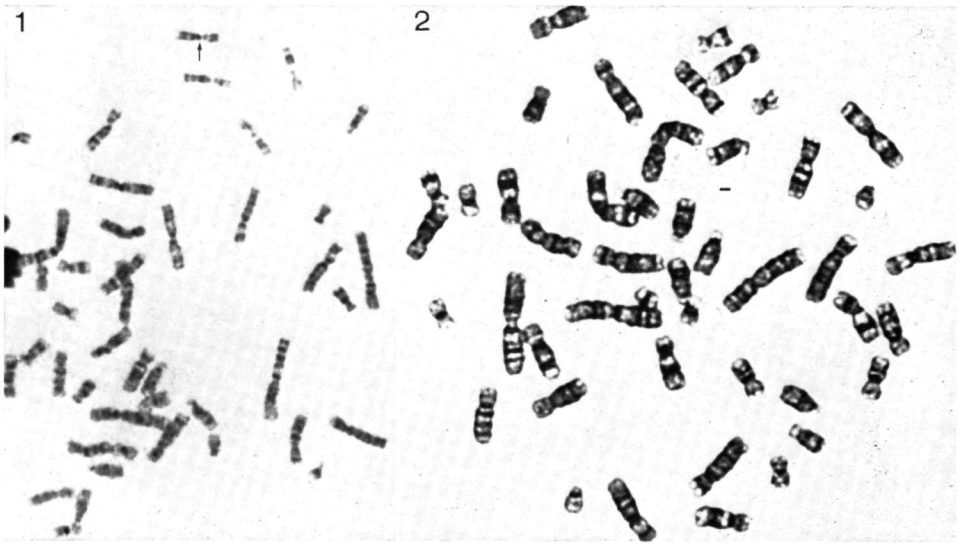


Fig. 1. Human metaphase chromosomes treated with an alkaline basic fuchsin solution: a G-banding pattern is visible. Notice distinct staining of heterochromatin in secondary constriction of chromosome No. 9 (*arrow*)

Fig. 2. Trypsin-pretreated, BF-stained metaphase showing a very distinct G-banding pattern

the heterochromatins in the secondary constrictions of chromosomes Nos. 1 and 16 were strongly stained, representing the most conspicuous chromosomal regions in the metaphase; in chromosome No. 16 they were usually more deeply stained than in chromosome No. 1;

the heterochromatin in the secondary constriction of chromosome No. 9, usually negative or only faintly stained in the G-banding patterns obtained with other techniques, was visible as a distinct band adjacent to the centromere (Fig. 3);

the chromosomes were usually slightly swollen and clearly delineated, facilitating the study of their poorly stained telomeres (Fig. 4);

in the metaphases of some individuals a strongly stained band became visible in the centromeric region of the long arm of one of the chromosomes No. 3 (Fig. 5); sometimes this occurred in both chromosomes No. 3; Q-band analysis revealed these to be the chromosomes No. 3 having brightly fluorescent material in their polymorphically variant regions.

The basic fuchsin-staining technique has therefore the advantage of not only showing a distinct G-banding pattern but also of permitting at the same time a study of some C- and Q-banding characteristics in human chromosomes. The method seems particularly suitable for the examination of chromosome No. 9, of its variants, and of rearrangements in which this chromosome may be involved. Figure 6 gives some examples of BF-stained chromosomes No. 9.

The BF-staining method also provides a means to detect chromosome No. 3 variants without the use of Q-fluorescence analysis. These variants may be easily recognized. In the chromosomes No. 3 without strongly fluorescent chromatin,

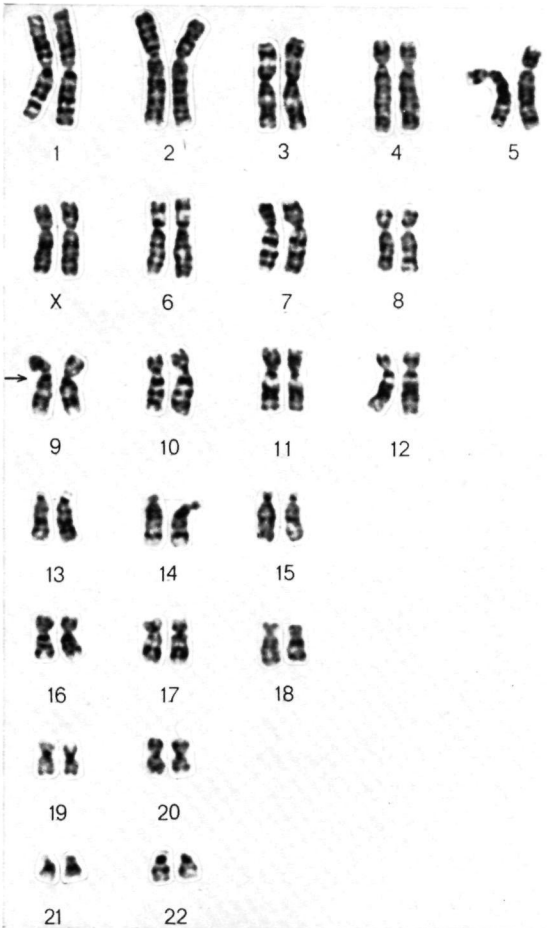


Fig. 3. Karyogram of a trypsin BF-treated metaphase; the *arrow* indicates the 9q heterochromatin

the long-arm band adjacent to the centromere usually stains less intensely than the neighboring band on the short arm. In this chromosomal variant therefore no difference is seen between the G-banding patterns obtained with the BF- or other staining techniques (Fig. 7a). In the chromosome No. 3 variant having an intensely fluorescent area, the BF technique produces a long-arm band which stains more strongly than the adjacent short-arm band (Fig. 7b and c). This long-arm band becomes more conspicuous if the chromosomes have been overtreated with trypsin (Fig. 7d). It is known that the intensity of the polymorphic fluorescent band in chromosome No. 3 may vary from barely discernible to bright (Pearson et al., 1973; Ferguson-Smith, 1974). The staining intensity of this area with the BF technique corresponds to its behavior after treatment with quinacrine mustard: brightly fluorescent polymorphic bands stain deeply with basic fuchsin; less intensely fluorescent ones more weakly.

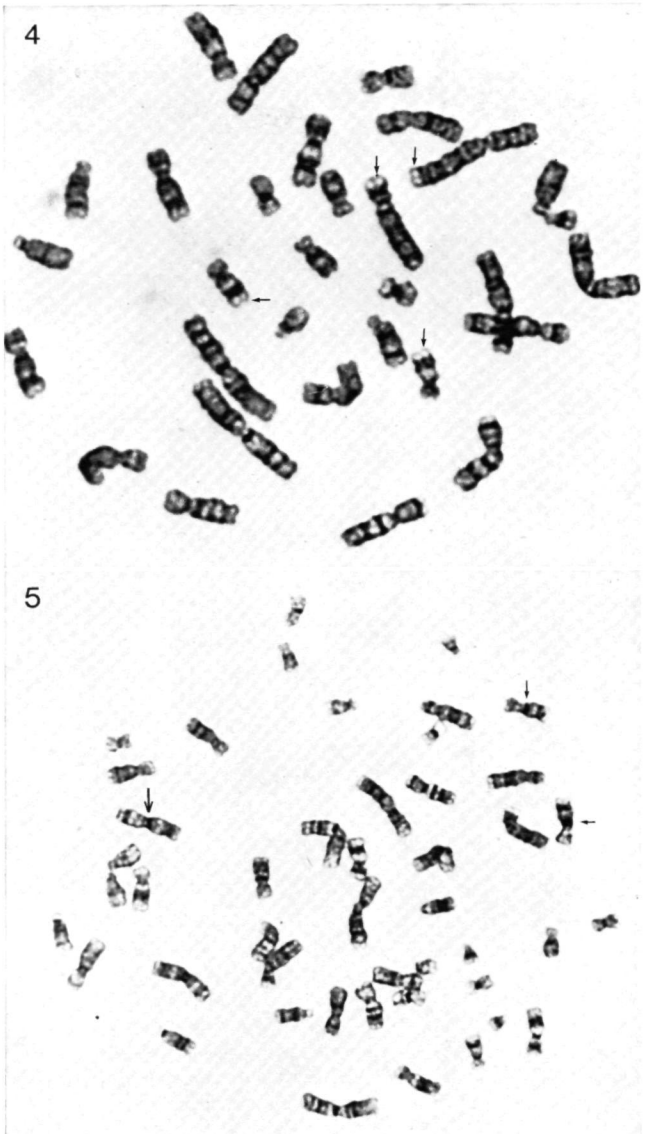


Fig. 4. Detail of a trypsin BF-treated metaphase; notice poorly stained but clearly outlined telomeric regions of a number of chromosomes (*arrows*)

Fig. 5. Trypsin BF-treated metaphase of individual having a chromosome No. 3 variant with a brightly Q-fluorescent area in polymorphic region; this region stained strongly (*large arrow*). 9q heterochromatin also visible (*small arrows*)

Other chromosomal areas known to have variable fluorescence were studied with the BF technique such as the distal part of the Y long arm, the long-arm region of chromosome No. 4 adjacent to the centromere, and the short arms and

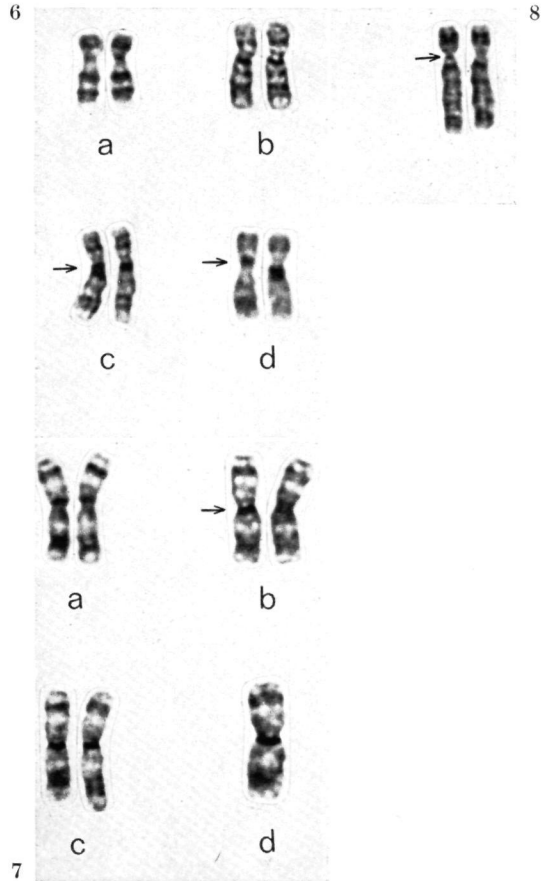
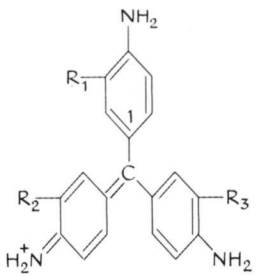
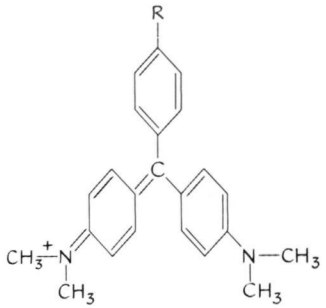


Fig. 6a—d. Chromosomes No. 9 stained with trypsin-Giemsa or with the trypsin-BF technique. (a) A pair of trypsin-Giemsa treated chromosomes No. 9: secondary constriction regions stain only faintly. (b) Chromosomes No. 9 treated with trypsin-BF technique: heterochromatin visible as a darkly stained band adjacent to centromere. (c) Trypsin BF-treated chromosomes No. 9 of individual having a variant chromosome No. 9 with an enlarged heterochromatic region; in this chromosome (*arrow*) two heterochromatic bands are visible. (d) Trypsin BF-stained chromosomes No. 9 of individual having a chromosome No. 9 (variant?) with pericentric inversion (*arrow*)

Fig. 7a—d. Chromosomes No. 3 stained with the trypsin-Giemsa or with the trypsin-BF technique. (a) Trypsin-Giemsa treated chromosomes No. 3: short-arm band adjacent to centromere usually stains more deeply than its neighboring band on long arm. (b) Chromosomes No. 3 of individual having one variant No. 3 with brightly Q-fluorescent chromatin in polymorphic region; after trypsin-BF staining this chromatin visible as an intensely stained band in centromeric region of long arm (*arrow*). (c) Trypsin BF-treated chromosomes No. 3 of individual having two brightly fluorescent No. 3 variants. (d) Chromosome No. 3 variant with brightly Q-fluorescent chromatin, stained with basic fuchsin after prolonged trypsin treatment

Fig. 8. Chromosomes No. 4 of individual with intensely Q-fluorescent chromatin in centromeric region on long arm of one chromosome No. 4; after trypsin-BF treatment this material appears as a small, but distinct band (*arrow*)





A

Pararosaniline : $R_1 = R_2 = R_3 = H$

Rosaniline : $R_1 = CH_3$; $R_2 = R_3 = H$

New fuchsin : $R_1 = R_2 = R_3 = CH_3$

B

Tetramethylpararosaniline : $R = NH_2$

Pentamethylpararosaniline : $R = NHCH_3$

Hexamethylpararosaniline (crystal violet) : $R = N(CH_3)_2$

Methyl green : $R = N(CH_3)_3^+$

Fig. 9A and B. Formulae of the triphenylmethane dyes used in these experiments. (A) Main components of basic fuchsin. (B) Other triphenylmethanes used

satellites of the acrocentric chromosomes. The intensely fluorescent Y chromatin, showing an inconstant staining behavior with other techniques (Paris Conference, 1971) stains consistently well with basic fuchsin. The brightly fluorescent chromosome No. 4 variant appears also distinguishable with the use of the BF technique (Fig. 8). Thus far we studied only a few individuals having this variant. With regard to the short arms and satellites of the acrocentric chromosomes a less consistent correspondence between the degree of fluorescence and the intensity of staining with basic fuchsin was noticed than e.g., in chromosome No. 3.

Basic Fuchsin Components and Related Triphenylmethanes. Basic fuchsin is a mixture of several cationic dyes of the triphenylmethane group. The main components are (Gurr, 1960): pararosaniline, rosaniline (basic magenta), and new fuchsin (new magenta). The last two are derived from pararosaniline by substituting methyl groups for one and three hydrogen atoms, respectively, at the 3-positions of the phenyl rings (Fig. 9a). To study the possible role of each of the BF components in the band formation, pararosaniline, rosaniline, and new fuchsin were investigated separately (Table 1). In chromosomes pretreated with trypsin, each compound produced a G-banding pattern identical in every respect to the one obtained with the complete basic fuchsin mixture. Apparently not the entire basic fuchsin complement is required for its band-producing properties.

Related to basic fuchsin are those triphenylmethanes derived from pararosaniline by replacing one or more N-hydrogen atoms by methyl groups (Fig. 9b). Of this group we studied a few viz. gentian violet which is a mixture of tetra-, penta-, and hexamethylpararosaniline, crystal violet (hexamethylpararosaniline), and methyl green ("heptamethylpararosaniline"). Only gentian violet and crystal violet produced G-banding patterns (Table 1). The banding was, however, inferior to the one obtained with basic fuchsin. We also noticed that the heterochromatin

Table 1. Ability of alkaline dye solutions[a] to produce G bands in human chromosomes; the effect of pretreatment with trypsin

Dye	Band production without trypsin pretreatment	Band production after trypsin pretreatment	Remarks
Triphenylmethanes:			
Basic fuchsin	+	+++	G + 9
Pararosaniline	+	+++	G + 9
Rosaniline	+	+++	G + 9
New fuchsin	+	+++	G + 9
Gentian violet	+	++	G + 9
Crystal violet	0	++	G
Methyl green	0	0	
Romanowsky dyes:			
Giemsa	0	0[b]	
Leishman	0	0[b]	
Thiazine dyes:			
Methylene blue	+	+	G
Azure II	+	+	G
Thionin	0	0	
Toluidine blue	0	0	
Methylene blue-Eosin	0	0	
Azure II-Eosin	0	0	

0 = no banding; + = faint banding; ++ = good banding; +++ = very good banding. G = G-banding pattern. G + 9 = G-banding pattern in which the 9q heterochromatin stains strongly.

[a] The dyes are dissolved in 1:2 formamide-water mixture (pH 10.5—11.0).

[b] In neutral, aqueous solutions these dyes gave very good banding.

of chromosome No. 9 was not well stained with crystal violet which is the most methylated band-producing triphenylmethane in this series (Fig. 10).

Differences in Dye Samples. The proportions in which each of the main components are present in the various brands of basic fuchsin may vary greatly (Mowry and Kasten, 1975). For that reason we tested a number of basic fuchsin samples from various manufacturers (see Materials and Methods). Although the differences in the patterns obtained with the brands used were small, the UCB and BDH dyes appeared to produce slightly better results.

The Effect of pH. The pH of the BF solution also influences the banding pattern produced in trypsin-pretreated chromosomes. A pH of 10.5—11.0 gives the best results. Below a pH of 10 the banding pattern becomes less distinct because the G-negative regions gradually take up more stain. At a pH below 7.0 hardly any banding remains.

Staining of the heterochromatin of the chromosome No. 9 with BF appears possible at each pH examined although at lower pHs this chromosome is usually difficult to recognize.

Romanowsky and Thiazine Dyes. A number of Romanowsky and thiazine dyes were tested for their ability to produce bands under the same experimental

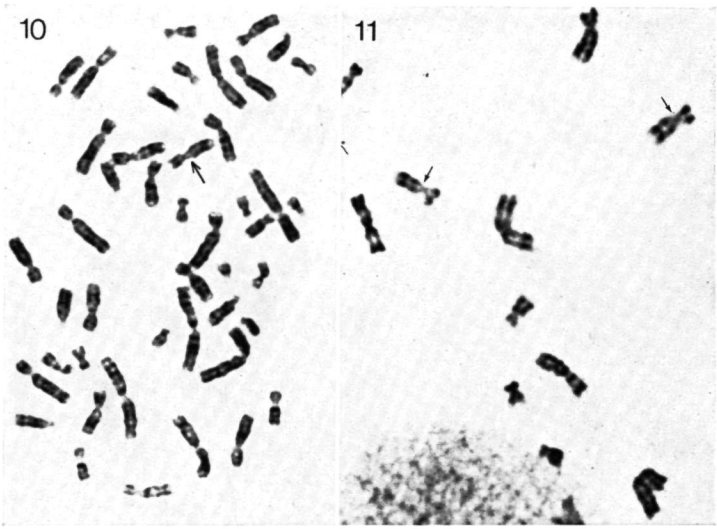


Fig. 10. Trypsin-pretreated chromosomes stained with crystal violet: secondary constriction region of chromosome No. 9 only weakly tinted (*arrow*)

Fig. 11. Trypsinized metaphase chromosomes treated with azure II: the 9q heterochromatin only weakly stained (*arrows*)

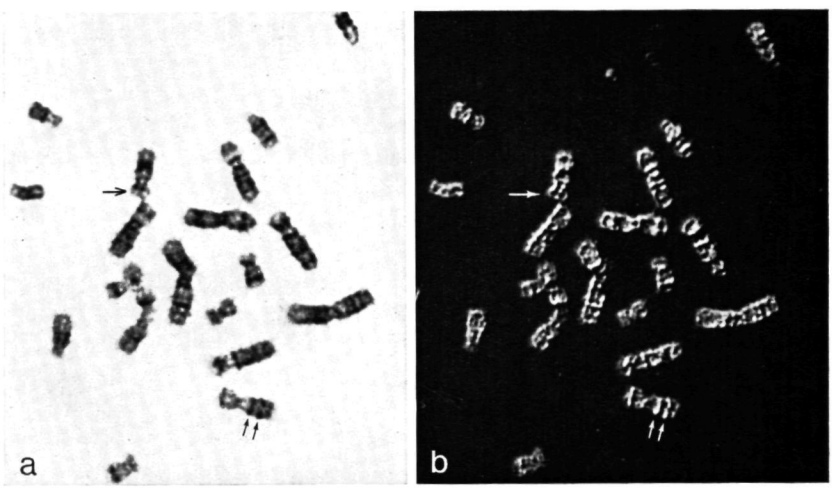


Fig. 12a and b. Comparison of bright field- and Nomarski interference contrast appearance of trypsin BF-treated chromosomes. (a) Banded chromosomes as seen with bright field optics. (b) Nomarski interference contrast appearance of same chromosomes; the G-positive areas appear as ridges (*small arrows*), the G-negative regions sometimes as cavities (*large arrow*)

conditions as used for BF staining (Table 1). Only methylene blue and azure II gave banding patterns. They differed from the one obtained with basic fuchsin: the chromosomes stained with methylene blue or azure II appeared less swollen,

were not clearly outlined, and the heterochromatins in the chromosomes No. 9 (Fig. 11) and in the variant No. 3 chromosomes with brightly fluorescent areas remained unstained.

The presence of eosin in the methylene blue or azure II solutions had an adverse effect on the banding pattern produced (Table 1).

The Effect of Salt. Increasing the Na^+ concentration in the basic fuchsin solution by the addition of sodium chloride had no negative effect on the chromosome staining intensities obtained. With a concentration of 0.25 M Na^+ the intensity was even higher than after the use of the standard BF solution with 0.03 M Na^+.

Morphologic Aspects. To determine whether trypsin-BF treatment results in morphologic changes comparable to those caused by the trypsin-Giemsa techniques (e.g., Ross and Gormly, 1973; Cervenka et al., 1973) the chromosomes were studied by Nomarski interference contrast microscopy. In trypsin-BF treated chromosomes the positive band areas appeared as raised structures in the chromatids and the negative regions often as cavities (Fig. 12a and b). The elevated regions and the cavities were absent in chromosomes left unstained after treatment with trypsin. Basic fuchsin and probably the other band-producing triphenylmethanes as well cause therefore very important structural changes in trypsin-treated chromosomes during the elicitation of the G-bands.

Discussion

Our results demonstrate that a number of triphenylmethane dyes, particularly basic fuchsin, are suitable for G-band formation in human chromosomes. Sumner and Evans (1973) have also examined the band-producing ability of basic fuchsin. These authors considered the BF-induced G-banding pattern inferior to that obtained with Giemsa. In our studies in which we used dyes dissolved in alkaline formamide-water mixtures, basic fuchsin and its component triphenylmethanes gave banding patterns clearly superior to those obtained with Giemsa or with the thiazines. With the basic fuchsin method it is also possible to stain the heterochromatin of the chromosome No. 9 and to recognize the brightly fluorescent variants of the chromosomes Nos. 3, 4, and Y. The BF-banding pattern is thus even more informative than the G-banding obtained with other techniques. The basic fuchsin-staining method may therefore be very useful in e.g., family and linkage studies in which marker chromosomes recognition plays an important role. A further advantage of the BF technique is the usually very clear chromosomal outline especially facilitating the study of the poorly stained telomeric areas. For these reasons we intend to adopt the BF method temporarily in clinical cytogenetics along with the routinely used trypsin-Giemsa technique (Scheres, 1972). After carrying out a sufficient number of chromosome analyses we shall then be able to decide which of the two methods gives the most consistent results and is the least susceptible to occasional minor changes in the preparative technique.

In the G-banding patterns obtained with most techniques the heterochromatin in the chromosome No. 9 secondary constriction remains negative (Paris Conference, 1971). Except for our basic fuchsin method only a few other G-banding techniques stain this region as far as we know. According to Chen (1974), the 9q

heterochromatin is sometimes well stained if the ASG method of Sumner et al. (1971) is used for banding. The so-called Giemsa-9 technique of Patil et al. (1971) and the modified trypsin-Giemsa method of Merrick et al. (1973) are also capable of staining this heterochromatic area. In the latter one the chromosomes are slightly overtrypsinized before staining, in the former technique no pretreatment is used before the chromosomes are stained in an alkaline Giemsa solution. The use of an alkaline Giemsa solution (pH 11.6) also forms the basis of the so-called Giemsa-11 technique (Bobrow et al., 1972; Gagné and Laberge, 1972) which specifically stains the heterochromatin in the secondary constriction of chromosome No. 9. From the above data it may be concluded that a trypsin pretreatment or a high pH of the dye solution make this region more accessible to the stain. In the basic fuchsin technique these factors are combined, which possibly explains why the 9q heterochromatin consistently stains well with this method. Overtrypsinization of the chromosomes enhances the stainability of this region with BF. The same is observed in the heterochromatic regions of chromosomes Nos. 1 and 16. Merrick et al. (1973) also found better stained heterochromatins in these chromosomes when longer trypsinization periods were used. In the BF technique the chromatin in the brightly Q-fluorescent regions of variant chromosomes No. 3 stains also more deeply after overtrypsinization.

An interesting observation is also that the chromosome No. 9 heterochromatin does not stain in the G-banding pattern obtained with crystal violet, the most methylated band-producing triphenylmethane dye used in our experiments. Possibly the interaction between the dye and the heterochromatin of the chromosome No. 9 is inhibited by the methylated amino groups. These groups also seem to impair the band-producing capability of the triphenylmethanes: increasing the number of N-methyl groups in these dyes results in a less distinct G-banding pattern. However, as there was no difference seen between the banding patterns obtained with the nonmethylated pararosaniline or the methylated compounds rosaniline and new fuchsin—containing one and three methyl groups, respectively, at the 3-positions in the phenylrings—one might conclude that the presence of methyl groups at these positions plays no major role in the band formation with the triphenylmethanes. According to Comings (1975) methyl groups also seem to be of importance for chromosome banding with the thiazine dyes. However, for these stains the situation seems to be different: the band-producing ability of the thiazines appears to depend on the presence of at least one methyl group. For example, the nonmethylated dye thionin consistently failed to give good banding (Table 1: Comings, 1975).

The mechanism(s) of the interaction between band-producing dyes and chromatin remain(s) uncertain. According to Sumner and Evans (1973) the formation of a complex consisting of one eosin molecule and two methylene blue molecules attached ionically to the DNA is necessary for band production with Giemsa. On the other hand, Löber et al. (1973) have found that methylene blue alone can give satisfactory banding. Good G-banding was also obtained by Comings (1975) using methylene blue or any of the azures alone. From their results it might therefore be concluded that a complex formation between eosin, thiazines, and DNA as suggested by Sumner and Evans (1973) is not required for obtaining bands. In an effort to gain an insight into the mechanism of G-banding, Comings (1975)

and Comings and Avelino (1975) have further studied spectrophotometrically the binding of methylene blue to DNA and to fixed and unfixed chromatin. They concluded that methylene blue is bound to chromatin by side stacking interaction with the phosphate groups in the DNA. According to these authors the banding pretreatments (e.g., heat-, salt-, or trypsin incubation) might produce such a change in the nucleoprotein complex that the DNA in the interbands becomes more effectively covered by the proteins. This would hinder the interaction of the cationic dye molecules with the anionic DNA phosphates, leaving the interbands unstained. The theory that the chromosomal proteins are involved in the differential staining is further supported by the observations of Brown et al. (1975) that certain histone fractions were able to prevent the G-banding of chromosomes with Giemsa.

As we studied only a few aspects of BF staining, it is difficult to establish whether the binding of the basic fuchsin to the chromatin resembles the methylene blue-chromatin interaction. Ionic binding of basic fuchsin to substrates with an opposite charge (e.g., DNA phosphates) does not seem to be of much importance as chromosome staining not only remains uninhibited by the presence of relatively high Na^+ concentrations but is even somewhat enhanced. In addition, the ionization degree of the amino groups in basic fuchsin might become reduced by the high pH used in our experiments. If ionic binding plays only a minor role in the BF-staining technique or perhaps none at all, the question arises as to which other binding mechanism or mechanisms are involved in the formation of banding patterns with this dye. Scott (1967) studied the interaction between chromatin and methyl green, a triphenylmethane closely related to basic fuchsin. He considered it likely that one or two phenyl rings of the dye intercalate between the base pairs of DNA. The remaining nonintercalated ring or rings would protrude from the pile of base pairs and fit into the major groove of the DNA. Such intercalation might also occur in the basic fuchsin banding. The high pH of the BF-staining solution may loosen the nucleoprotein complex to some extent, possibly facilitating the intercalation of the dye molecules. In the interbands known to contain chromatin less susceptible to alkali (Bobrow and Madan, 1973; de la Chapelle et al., 1973; Scheres, 1976) the dye molecules might less easily intercalate. This would then result in a weaker staining of these regions.

A binding of basic fuchsin to chromatin by intercalation might also explain why the BF-staining intensity of certain polymorphic chromosomal areas corresponds to their Q-fluorescence intensity, as quinacrine also binds predominantly by intercalation (Comings et al., 1975). In contrast to these polymorphic regions the heterochromatins of the chromosome Nos. 1, 9, and 16 do not show a correlation between their Q fluorescence and BF-staining intensity. They stain deeply with BF but fluoresce only weakly. This discrepancy may be caused by the predominantly AT-rich satellite DNAs which are present in these regions (Jones et al., 1973; Gosden et al., 1975), and which are probably the reason for the marked alkali sensitivity observed in these heterochromatic areas (de la Chapelle et al., 1973; Bobrow and Madan, 1973). Treatment with the high pH solution of basic fuchsin would increase even more the above-mentioned loosening of the nucleoprotein complex in these chromosomal regions, resulting in their deep staining. The weak Q fluorescence of these areas is perhaps the result of the presence of

interspersed quenching GC base pairs (Pachmann and Rigler, 1972; Weisblum, 1973; Sclander and de la Chapelle, 1973; Latt et al., 1974), of special protein-DNA interactions (Comings et al., 1975), or of an alteration in DNA configuration (Gottesfeld et al., 1974) which might interfere with the quinacrine intercalation (Schreck et al., 1974).

The BF-banding pattern characteristics are very similar to those obtained with 2,7, di-t-butyl proflavine (DBP, Distèche and Bontemps, 1974). Chromosomes stained with this fluorochrome show a "Q"-banding pattern in which the heterochromatins of the chromosome Nos. 1, 9, and 16 are brightly fluorescent. Müller et al. (1973) studied the properties of this quinacrine-related fluorochrome and found that it binds specifically to AT base pairs. The similarity between the DBP- and BF-banding patterns may be an indication that BF stains mainly AT-rich chromatins. For a better interpretation of the interaction between BF and chromatin additional, more fundamental experiments will have to be carried out.

Although the binding mechanisms of basic fuchsin and Giemsa to chromatin may differ, both dyes cause similar structural changes in trypsin-pretreated chromosomes. With the aid of Nomarski interference contrast microscopy, the positive bands become visible as ridges on the chromatids after staining with basic fuchsin (own results) or Giemsa (Schnedl, 1973; Cervenka et al., 1973; Schuh et al., 1975). This phenomenon is in accordance with the electron-microscopic observations that the G-positive areas appear as raised structures (Gormley and Ross, 1972; Ross and Gormley, 1973; van der Linden and Pearson, 1973), and that they contain more and very densely packed chromatin fibrils (Ruzicka and Schwarzacher, 1974; Burkholder, 1975; Bahr et al., 1973).

Although the mechanism of the BF-banding pattern formation remains uncertain the basic fuchsin staining method may contribute to cytogenetics because it permits the simultaneous study of G-, and of some C- and Q-banding characteristics of human chromosomes.

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PAPER III

Production of C and T Bands in Human Chromosomes after Heat Treatment at High pH and Staining with "Stains-All"

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Summary. Heat treatment of human chromosomes in $Ba(OH)_2$ solution (pH 13), followed by incubation in 2 SSC and subsequent staining with "Stains-all" produces a banding pattern which is comparable to that of the R- and T-systems. However, some differences have been noted.

Zusammenfassung. Hitzebehandlung von menschlichen Chromosomen in $Ba(OH)_2$-Lösung (pH 13), gefolgt von Inkubation in 2 SSC sowie Färbung mit "Stains-all", ruft ein Bandmuster hervor, das mit dem des T- und R-Systems vergleichbar ist. Es werden jedoch einige Unterschiede wahrgenommen.

Introduction

Recently, the use of a low pH has been suggested as a prerequisite for obtaining R-bands in heat-treated human chromosomes (Sehested, 1974). Similarly the T-band (terminal band) system, which apparently represents a constituant of the R-system (Dutrillaux, 1973), has been reported to occur after heat treatment at a pH below neutral. These results have prompted us to present a technique by which reverse bands can be produced following heat treatment of chromosomes in a strongly alkaline $Ba(OH)_2$ solution.

Material and Method

Chromosome preparations are obtained from peripheral blood cultures as described earlier (Scheres, 1972). Slides are incubated for 40 min in a saturated aqueous solution of barium hydroxide pH 13.2 at 60°C, rinsed in distilled water and subsequently placed in 2 SSC (0.3 M sodium chloride and 0.03 M tri-sodium citrate, pH 7.2) for 1 hr at the same temperature. After being washed in distilled water, preparations are stained for 2 min in 10% Giemsa (Gurr's R 66) in Sørensen buffer (1/15 M KH_2PO_4 and 1/15 M Na_2HPO_4, pH 6.8), or in a 0.005% solution of the cationic dye "Stains-all", = 'SA' (4,5,4',5'-Dibenzo-3,3'-diethyl-9-methyl-thiacarbocyanine bromide, SERVA) in 1+1 formamide-water mixture (pH 8.5).

Results

When treated chromosome preparations are stained with Giemsa, staining of centromeric regions (C-banding) can be observed in almost all chromosomes but some chromosomes show faint bands. The localization of these bands corresponds to that of the R-bands. However, when using the SA instead of the Giemsa

staining procedure the faint bands become much more distinct. In addition to C banded cells a number of metaphases are found with a T-banded appearance (Fig. 1). Most cells show characteristics of both types of banding (Figs. 2 and 3).

Fresh preparations mostly result in incomplete C banding and in many cells only the centromeric heterochromatine of chromosome 1 is strongly stained (Fig. 4). Cells with pronounced T banding are usually obtained if preparations older than 2 weeks are used.

Results are similar when incubation in 2 SSC is omitted from the procedure, but in that case the chromosomes exhibit a distorted appearance. Incubation in 2 SSC without previous bariumhydroxide treatment results in G banding after staining with Giemsa or SA.

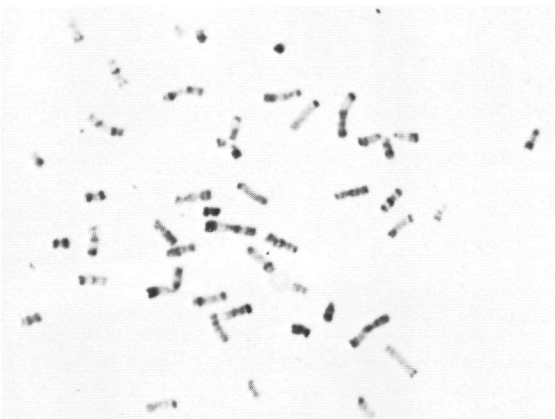

Fig. 1. Metaphase after heat treatment and staining with "SA": Many chromosomes show heavy staining of telomeric regions

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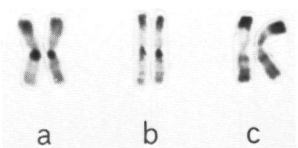

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Fig. 2. Metaphase with reverse bands and distinct staining of centromeric regions (arrows) especially in chromosomes 1, 9, and 16. (Female carrier of a 4/15 translocation, see Hustinx, 1974)

Fig. 3a—c. Pairs of chromosomes 1 after heat treatment and staining with SA: a From typically C banded cell; b from cell with both C and T characteristics and c from cell with pronounced T banding

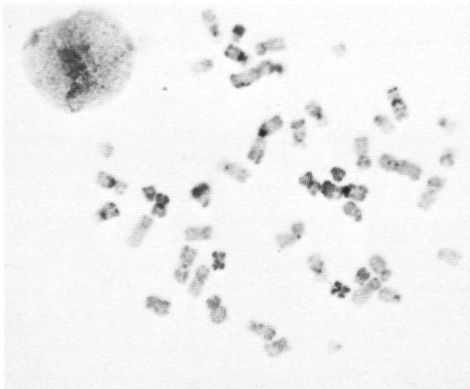

Fig. 4. SA-stained metaphase from a fresh preparation: Note the prominent staining of the paracentromeric heterochromatine in chromosomes 1

Discussion

Dutrillaux (1973) and Sehested (1974), both using incubation in sodium phosphate and staining with Giemsa, have found that T and R banding occur at a pH below neutral. However, our results clearly show that reverse banding of human chromosomes can also be obtained by pretreatment with heat in strongly alkaline bariumhydroxide solution (pH 13). This leads to the conclusion that the type of banding which will appear after a given heat pretreatment depends upon both the kind of salt and the pH used. This corresponds well to the observations recently reported by Eiberg (1973), who obtained reverse banding in human chromosomes by heat treatment at pH's ranging from 5.2 to 8.6 in solutions of various organic and inorganic salts.

Close analysis of the banding pattern obtained with this technique reveals the following feature: In addition to terminal and reverse bands some chromosomes (especially chromosomes 1, 9, and 16) show staining of the paracentromeric heterochromatine. Distinct centromeric staining in these chromosomes is absent in the R and T patterns as originally described by Dutrillaux and Lejeune (1971) and Dutrillaux (1973). Thus this banding pattern apparently constitutes a somewhat different system which makes visible both C banding and T banding characteristics, and therefore in our laboratory has tentatively been called the "CT technique".

The mechanism which causes this banding remains obscure. It is quite possible that the formamide which has been shown to cause denaturation of DNA in mammalian chromosomes (de la Chapelle, 1973) plays an important role in the production of these bands.

Further experiments to elucidate the factors which might influence this banding phenomenon—such as pH, temperature and duration of incubation, ions present, and concentration of formamide—are now being undertaken.

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CT Banding of Human Chromosomes

Description of the Banding Technique and Some of Its Modifications

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Summary. A technique is described for staining centromeric areas and reverse, mainly telomeric bands in human chromosomes. With this "CT" technique karyotyping of C-banded metaphases is possible without previous or subsequent use of other banding methods. The method consists of an alkaline pretreatment at 60°C with $Ba(OH)_2$, followed by salt incubation in 2 × SSC at 60°C and staining with the cationic dye "Stains-all". In a series of experiments the influence of the variables in the procedure was studied, with the following main results:

1) $Ba(OH)_2$ treatment alone and subsequent staining produces a distinct reverse banding pattern in which the secondary constriction of chromosome 9 is positive.

2) The 2 × SSC incubation in the CT procedure causes the $Ba(OH)_2$ induced reverse bands to become weaker; the centromeric regions, however, become very prominent.

3) If the temperature of the 2 × SSC treatment is raised to 85°C, the CT technique results in a specific staining of the short arm regions of some probably variant acrocentric chromosomes. The interphase nuclei of individuals possessing such acrocentrics usually show very distinct chromocentres after this treatment; in the polymorphs these chromocentres are often situated along the nuclear membrane.

The mechanisms which may form the basis of the staining results obtained, and the possible significance in human cytogenetics of the techniques described, are discussed briefly.

Introduction

Since the development of the fluorescent banding technique (Caspersson *et al.*, 1969) many other methods for differential staining of human chromosomes have been described. The resulting banding patterns were divided into 4 main groups by a committee of the International Conference on Standardization in Human Cytogenetics (Paris Conference, 1971):

the Q bands produced by quinacrine dyes, the G bands obtained with Giemsa staining after various pretreatments, the "reverse" or R bands caused by the application of Dutrillaux and Lejeune's procedure (1971) giving a pattern almost exactly the reciprocal of the Q and G patterns, and the C bands revealed by Pardue and Gall's (1970) *in situ* hybridization procedure or its modifications (Arrighi and Hsu, 1971).

In human chromosomes C banding techniques stain the paracentric heterochromatins and the distal half to two-thirds of the Y long arm. The remaining chromosomal regions are only slightly tinted. Consequently karyotyping in C banded metaphases requires previous or subsequent Q, G or R banding. Recently

we developed a method by which additional areas, mainly telomeric bands, are stained and which enables us to identify C banded chromosomes. We termed it the CT technique (Scheres, 1974). It is a modification of the C banding method of Sumner (1972) and consists of heat pretreatment of the chromosomes with $Ba(OH)_2$ solution followed by incubation in $2 \times$ SSC and staining with the cationic dye "Stains-all" (4,5,4′,5′-dibenzo-3,3′-diethyl-9-methyl-thiacarbocyanine-bromide). Dahlberg *et al.* (1969) have used this dye as a sensitive stain for electrophoretically separated nucleic acids. They noted that RNA was stained purple, DNA blue and proteins red and named the dye therefore "Stains-all". It appeared to be an excellent stain for CT banding (Scheres, 1974). In this paper we present a complete description of the CT technique and report the results of studies carried out to determine the influence of a number of variables in the procedure. During the investigations a new, simple technique was also found for obtaining distinct R bands as well as a method for specifically staining the short arms of a number of acrocentric chromosomes in metaphase and interphase nuclei of some individuals.

Materials and Methods

Human venous lymphocytes were cultured according to a modified Moorhead *et al.* method (1960). Colchicine, in a final concentration of 0.1 μg/ml medium, was added 3 hrs before harvesting. The cells were then subjected to hypotonic treatment with 0.075 M KCl for 10 min at 37°C and fixed with several changes of 3:1 methyl alcohol-acetic acid. Chromosome preparations were made by dropping small quantities of the final suspension onto clean, grease-free slides moistened with distilled water. After 5—10 sec spreading, the excess water was removed and the preparations were dried by waving the slides vigorously through a warm air stream produced with the aid of a small flame. Care was taken to prevent overheating of the preparations or ignition of the chromosome suspensions.

The following solutions were used in the experiments:

a saturated, aqueous solution of $Ba(OH)_2$ (pH $\pm$ 13) for alkaline treatment of the chromosomes at different temperatures;

$2 \times$ SSC (0.3 M sodium chloride and 0.03 M trisodium citrate, pH 7.2) for salt incubation at different temperatures.

Staining of chromosomes was performed for 10 min with a 10% solution of Giemsa (Gurr's Improved R'66) in Sørensen buffer (1/15 M KH_2PO_4 and 1/15 M Na_2HPO_4, pH 6.8), or with a 0.005% solution of "Stains-all" (4,5,4′,5′-dibenzo-3,3′-diethyl-9-methyl-thiacarbocyanine-bromide, Serva) in a 1:1 formamide-water mixture (pH 8.2).

Results

In the initial experiments $Ba(OH)_2$-pretreated (40 min at 60°C) and subsequently $2 \times$ SSC-incubated (40 min at 60°C) preparations were stained with Giemsa, resulting in C banding of the metaphase chromosomes. Substituting "Stains-all" for Giemsa in the procedure, however, gave distinct non-centromeric bands in addition to the well stained paracentric heterochromatins (Fig. 1). Closer investigation revealed these bands to belong to the reverse banding system. The telomeric regions of most chromosomes were particularly well stained. The banding pattern obtained was sufficiently defined to permit the identification of all chromosomes. Fig. 2 is an example of a typical CT karyogram. Of the C bands the centromeric heterochromatins of chromosomes 1, 3, 5, 6, 7, 9, 16 and

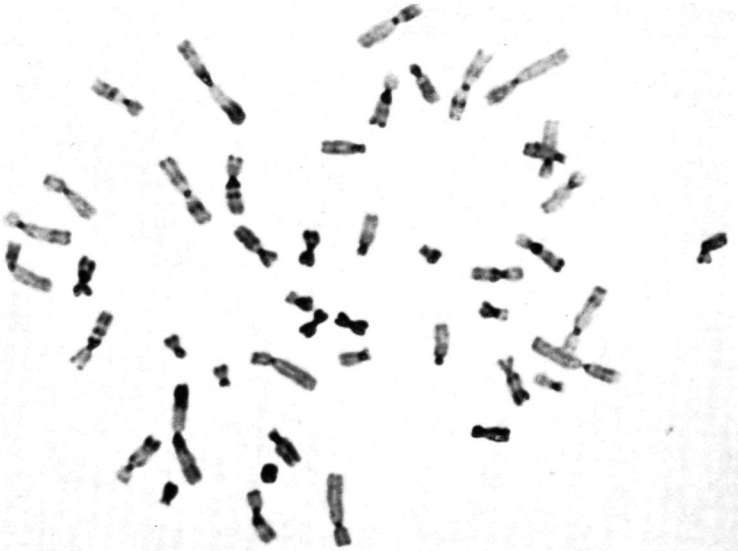

Fig. 1. CT-banded human metaphase (case MJ 150974 MR) treated with $Ba(OH)_2$ at 60°C, $2 \times$ SSC at 60°C, and "Stains-all"; note the prominent staining of both centromeric and telomeric regions

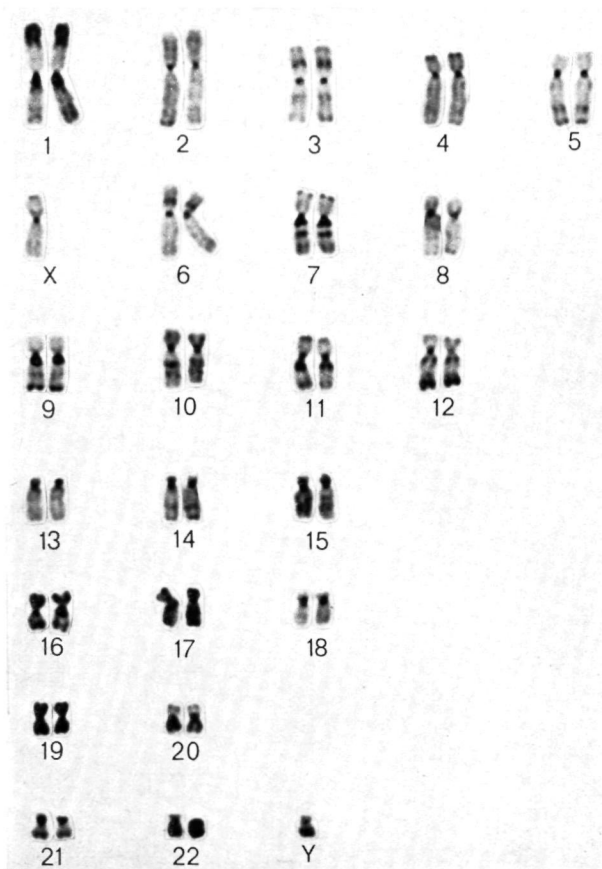

Fig. 2. Karyogram of the CT-banded metaphase of Fig. 1 (case MJ 150974 MR)

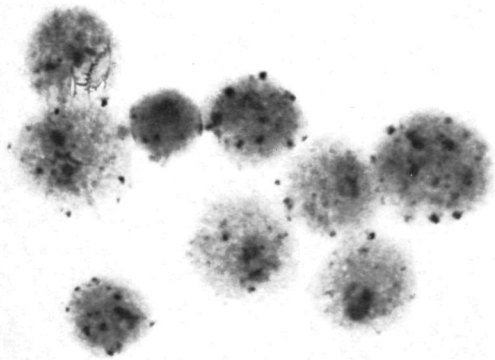

Fig. 3. Interphase nuclei of lymphocytes stained with the CT banding method; the chromocentres are clearly visible (case WB 630703)

18, as well as the distal part of chromosome Y, are the most clearly stained regions. The most conspicuous non-centromeric bands are the telomeres of the chromosome arms 1p, 4p, 5q, 7p, 8q, 9q, 11p, 12q, 14q and 21q in addition to a number of interstitial bands of the chromosome arms 1p, 3p, 5q, 6p, 7q, 11q, 12q and 20q. Chromosomes 16 and 17 and, above all, chromosomes 19 and 22 are almost entirely and deeply stained. With the exception of the C bands these characteristics are therefore very similar to those of the T banding pattern (Dutrillaux, 1973). In the interphase nuclei of the preparations examined, a variable number of chromocentres of different sizes becomes visible with the CT banding method (Fig. 3).

With the CT staining method both C and T bands can therefore be examined simultaneously. In some metaphases of a preparation the C band characteristics predominate, in others the T bands are more pronounced. Because of these inconsistent results, and also to determine the effect of a number of variables, a series of experiments was carried out. The test results of the influence of $Ba(OH)_2$ and $2 \times$ SSC treatment alone or in combination are listed in Table 1. From our series of experiments it can be concluded that, for the staining of centromeric as well as telomeric and interstitial bands (CT banding), both pretreatment with $Ba(OH)_2$ and $2 \times$ SSC are necessary. It has become obvious that $Ba(OH)_2$ treatment alone produces telomeric and other reverse bands but no centromeric bands (Fig. 4). For the production of the latter, subsequent incubation in $2 \times$ SSC is required. The reverse banding pattern obtained after $Ba(OH)_2$ treatment differs only in one respect from the R banding pattern described by Dutrillaux and Lejeune (1971) *viz.* that the heterochromatin in the secondary constriction of chromosome 9, negative in the original R banding, becomes stained (Fig. 5). From the results listed in Table 1 it can also be concluded that "Stains-all" produces very distinct R and CT bands while Giemsa is more suitable for G and C banding.

In the following experiments the optimal conditions for obtaining CT and reverse banding patterns were determined.

Table 1. Effects of $Ba(OH)_2$- and 2 × SSC treatments on human chromosomes

Pretreatment			Staining		Banding type
$Ba(OH)_2$ 60°C pH 13	2 × SSC 60°C pH 7.2	2 × SSC 85°C pH 7.2	"Stains-all"	Giemsa	
Treatment sequence					
+			+		R + 9[a]
+				+	Weak R + 9
+	+		+		CT
+	+			+	C
	+		+		Weak G
	+			+	G
		+	+		R
+		+	+		Some acrocentrics

[a] R + 9 = Reverse banding pattern in which the paracentric heterochromatin of chromosome No. 9 is positive.

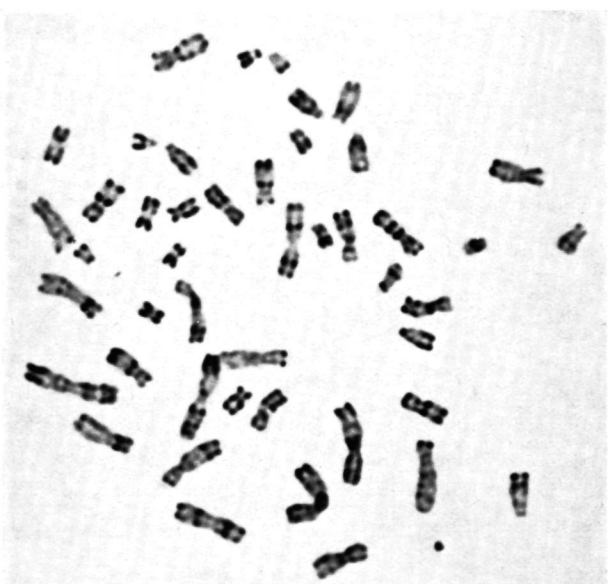

Fig. 4. Human metaphase (case LT 581111) treated with $Ba(OH)_2$ at 60°C and stained with "Stains-all", resulting in a distinct reverse banding pattern

a) $Ba(OH)_2$ Incubation. Using a saturated $Ba(OH)_2$ solution (pH ± 13) at 60°C the optimal incubation period was 10 min. Shorter times did not produce enough differential staining; longer periods were harmful to the chromosomes. Incubation at a temperature of 85°C for 30—60 sec caused so much damage that

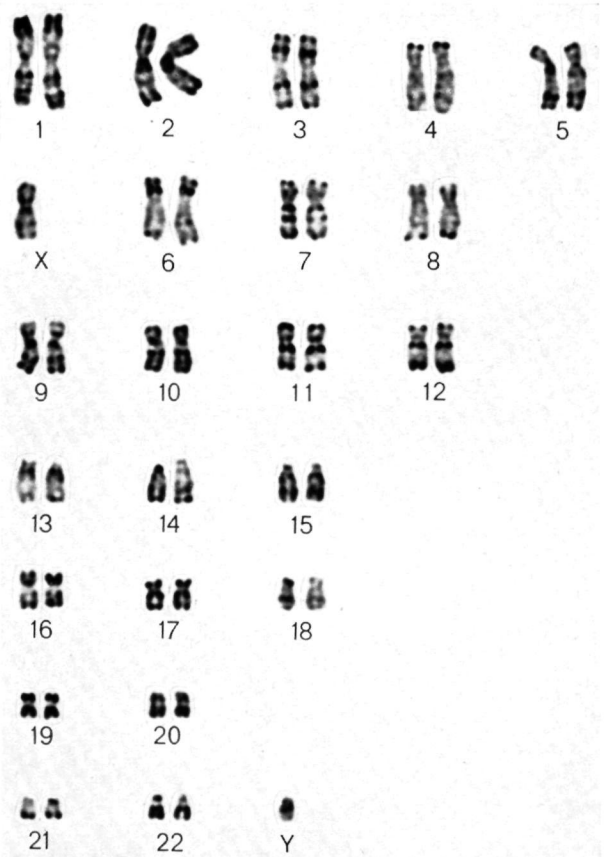

Fig. 5. Karyogram of the reverse banded metaphase shown in Fig. 4 (case LT 581111). Note the positive staining of the heterochromatin in the secondary constriction of chromosomes No. 9

only telomeric band remnants could be seen. At temperatures below 40°C, or at pH below 12, unsuitably long incubation periods were required to obtain banding.

b) 2 × SSC Incubation. The most important results of our experiments to test the influence of the 2 × SSC incubation were:

without previous bariumhydroxide treatment, incubation of the preparations in 2 × SSC for 1 hr at 60°C and pH between 4 and 9 produced G banding; at a temperature of 85°C a R banding pattern was obtained;

after previous $Ba(OH)_2$ treatment, incubation in 2 × SSC at 60°C and pH between 4 and 9 yielded the CT banding pattern. The incubation period in 2 × SSC at 60°C optimal for CT banding was about 30 min. Shorter periods gave less pronounced C bands while after longer incubation the reverse and telomeric bands induced by the $Ba(OH)_2$ treatment became fainter and only the centromeric heterochromatins remained clearly visible. The influence of the temperature of

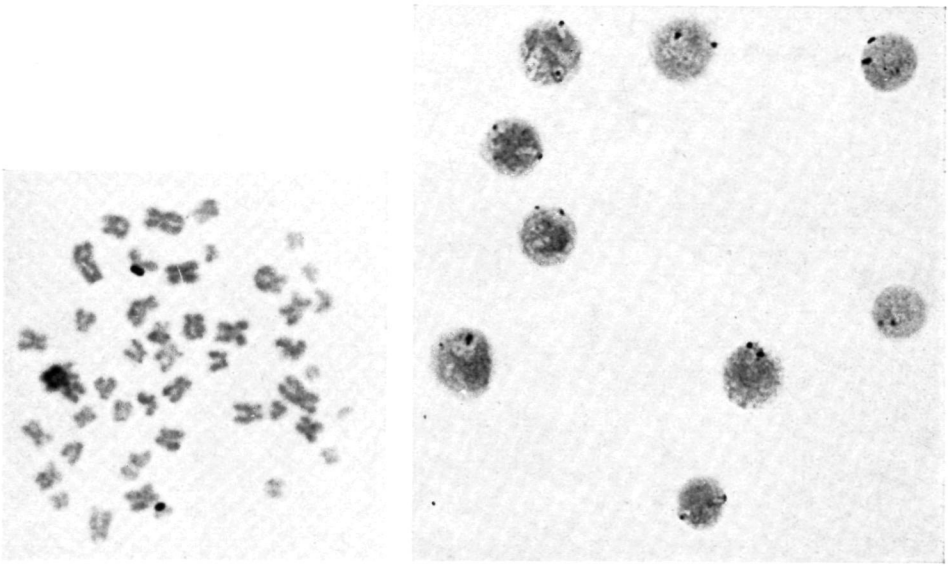

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Fig. 6. Specific staining (barium hydroxide at 60°C, 2 × SSC at 85°C, "Stains-all") of the short arm regions of 2 acrocentric chromosomes; the individual (case ZT 750808) has a chromosome 13 and a chromosome 21 of the probably variant types described in the text

Fig. 7. Specific staining of 2 chromocentres in lymphocytic interphase nuclei of the individual mentioned in Fig. 6 (case ZT 750808)

the 2 × SSC incubation on the chromosomes can be summarized as follows: at temperatures below 60°C the staining of C bands diminished progressively and under 40°C only the $Ba(OH)_2$ induced reverse banding pattern remained. Raising the incubation temperature to 70°C intensified the C band formation process but the reverse bands became less distinct. At temperatures above 85°C the ability of the entire chromosome to become stained decreased rapidly and a banding pattern was no longer visible.

In the metaphases of a number of individuals the treatment with $Ba(OH)_2$ at 60°C and 2 × SSC at 85°C resulted in a specific, very pronounced staining of the short arm areas of some acrocentric chromosomes (Fig. 6). Examination with other banding techniques established the chromatin of these short arm areas to be Q and G negative but R, T and C positive. In similarly treated interphase nuclei of these individuals, strongly stained chromocentres were seen in a number equal to that of the variable areas observed in the metaphases (Fig. 7). We therefore assume these chromocentres to represent these areas in the interphase of the cell cycle. It was noticed that in the polymorphs these heterochromatic bodies were usually situated along the nuclear membrane and that in some cells they seemed to form a nodule (Fig. 8).

c) Preparation of Chromosomes. The fixation time, the methods of preparing the slides and their age appeared to be of importance to the quality of the banding

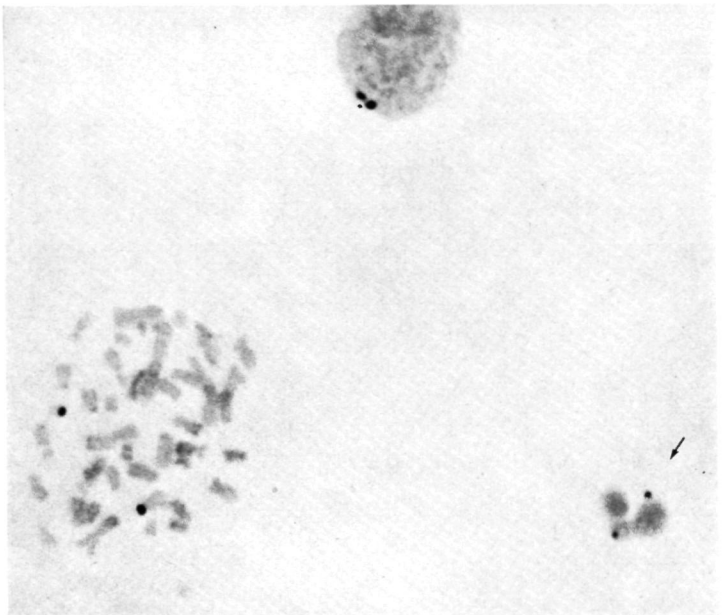

Fig. 8. Specific staining of short arm regions and chromocentres in metaphase and interphase nuclei of the individual mentioned in Figs. 6 and 7 (case ZT 750808). Note that one of the chromocentres in the polymorph is represented as a nodule (arrow) on its nuclear membrane

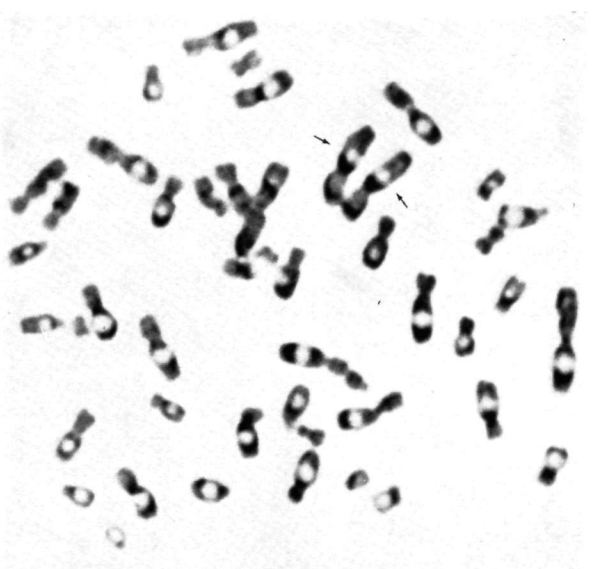

Fig. 9. Freshly prepared chromosome spread (case LT 581111) treated with $Ba(OH)_2$ at 60°C and stained with "Stains-all". Most chromosomes show "vacuoles" which in homologous chromosomes often occur at similar sites (see *e.g.* chromosomes No. 2 indicated by arrows)

pattern obtained. Slides from suspensions in fixative for more than 1 month, ignition dried chromosome spreads, or preparations older than 2 weeks, proved unsuitable as they usually gave only weak C banding after CT treatment and the chromosomes were only faintly stained. Freshly prepared chromosomes proved too sensitive to alkaline heat treatment and to distend strongly. The swelling was perhaps also the cause of the "vacuoles" particularly seen in R negative regions. In homologous chromosomes they often occurred at homologous sites (Fig. 9).

The best results were obtained using 2- to 3-day-old slides prepared from fresh cell suspensions as described in "Materials and Methods". For that reason these have been used in our series of experiments.

Routine Methods

Based on the experiments described the following new staining techniques are now routinely used in our laboratory:

a) Reverse Banding

1. Place slides in saturated $Ba(OH)_2$ solution at 60°C for 10 min.
2. Rinse in running distilled water.
3. Remaining $BaCO_3$ crystals may be removed by gently rinsing in water of pH 4—5.
4. Stain with "Stains-all" for 10 min.
5. Rinse and dry.

b) CT Banding

1 and 2: as for a).
3. Incubate in 2 × SSC, pH 7.2 at 60°C for 30 min.
4. Rinse in running distilled water.
5. Stain with "Stains-all" for 10 min.
6. Rinse and dry.

c) Specific Staining of the Short Arms of Some Acrocentrics

1 and 2: as for CT banding.
3. Incubate in 2 × SSC, pH 7.2 at 85°C for 2 min.
4, 5 and 6: as for CT banding.

Multiple Techniques

The "reverse" and CT banding methods described can also be carried out after Q band analysis of the chromosomes. However, UV light has an adverse effect: the cells previously studied with fluorescence microscopy usually have a diminished clarity of banding pattern as compared with adjacent, unexposed metaphases. CT banding can also be applied to metaphases already studied after staining with a trypsin-Giemsa method (Fig. 10a and b). Chromosomes treated with trypsin, however, are very sensitive to the action of $Ba(OH)_2$ and 2 × SSC and require short incubation periods.

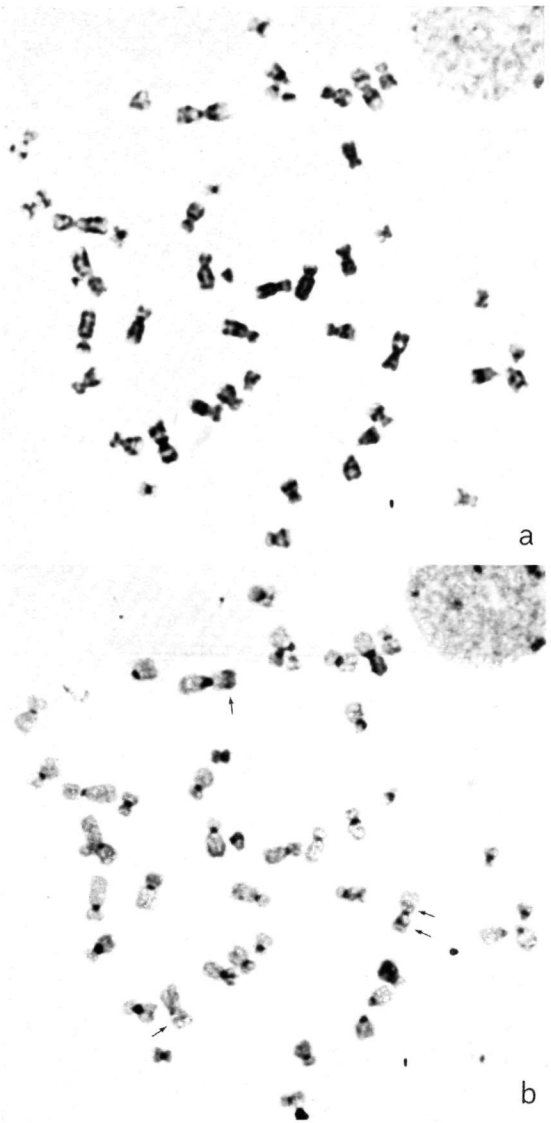

Fig. 10a and b. Dual G and CT banding of human chromosomes (case LV 660411). (a) Metaphase stained with a trypsin-Giemsa banding method (Scheres, 1972). (b) The same cell after subsequent CT treatment. Notice the strong staining of most centromeric regions and the presence of faint reverse bands in some G-negative areas (arrows)

Discussion

It is an important feature of the CT banding pattern described that, in addition to centromeric heterochromatins, most telomeric and some interstitial bands belonging to the "reverse" system are stained, permitting the identification of

all chromosomes. If other C banding methods are employed karyotyping becomes possible only after using a second banding technique. The simple new procedure has now been applied routinely for some time in our laboratory to study C bands and their variations in normal and abnormal human chromosomes (Rutten *et al.*, 1974: Hustinx *et al.*, 1975).

The process causing the CT banding pattern involves: 1) the production by an alkaline treatment ("denaturation") of a reverse banding pattern in which, after staining, the centromeric regions are negative, 2) the subsequent salt incubation ("renaturation") which makes the centromeric regions heavily stainable: the $Ba(OH)_2$-induced reverse bands become weaker and only their most resistent areas, the telomeres, remain visible.

Compared to the large number of G banding techniques published R banding methods have remained surprisingly few (see for review: Dutrillaux, 1975: Verma and Lubs, 1975). The reverse banding pattern obtained after alkaline heat treatment and the use of "Stains-all", is very distinct and intensely stained. For human chromosomes we prefer the use of the reverse banding technique developed in our laboratory to the R banding method with Giemsa stain (Dutrillaux and Lejeune, 1971) because in our experience it proved difficult to obtain adequate staining intensities with the latter technique.

The physicochemical basis for R band formation remains uncertain. In most procedures the chromosomes are strongly denaturated to obtain well differentiated R bands. With regard to this the observations of Bobrow and Madan (1973) and of De la Chapelle *et al.* (1973) are of interest. They noticed that the R and T bands contain chromatins which—probably as a result of a specific base composition of the DNA or of the presence of special chromosomal proteins—are less susceptible to thermal and alkaline denaturation than the chromatins in the interband regions. This agrees with our experience that the telomeres are the areas most resistant to $Ba(OH)_2$ treatment. Another recently suggested factor, which may play a role in differential chromosome staining, is that the banding pretreatments might produce such a change in the nucleoprotein complex that the proteins cover the DNA more effectively in the interbands. This would prevent the dye molecules from reaching the DNA phosphates, leaving the interbands unstained (Comings and Avelino, 1975).

An important difference between our reverse banding technique and those described by others is the use of barium hydroxide for alkaline denaturation instead of the more usual sodium hydroxide. Substituting NaOH (pH 13) for $Ba(OH)_2$ in our reverse or CT banding procedures causes the chromosomal material to disappear within seconds (Scheres, unpublished results). The use of $Ba(OH)_2$ might also be the cause of the positive staining of the heterochromatin in the secondary constriction of chromosome 9. In contrast, this region does not stain in the R banding patterns obtained with other methods (Dutrillaux, 1975). As Gosden *et al.* (1975), and Jones *et al.* (1973) proved with the aid of *in situ* hybridization techniques, the heterochromatin in this region contains the highest concentration of satellite DNAs in the human chromosome complement. The presence of these highly repetitive satellite DNAs, or of special chromosomal proteins associated with them, might influence the stability and staining behaviour of

this region. It has also been observed that one of the satellite DNAs present in the chromosome 9q heterochromatin is rich in GC base pairs and has an increased resistance to thermal denaturation (Saunders *et al.*, 1972).

In 1970 Pardue and Gall studied the *in situ* hybridization of complementary RNA to fixed murine metaphase chromosomes. They used 2 × SSC as an environment favourable to reassociation of the nucleic acids. During these studies they observed the first C banding pattern. 2 × SSC has since been widely used in other banding techniques. Depending on the conditions used in our experiments a heat treatment with 2 × SSC results in (Table 1): 1) a G banding pattern, 2) a R banding pattern, 3) a CT banding pattern and 4) specific staining of the short arm heterochromatin in certain acrocentric chromosomes.

Results 1 and 2 might have been expected as the pretreatments used are very similar to the ASG technique of Sumner *et al.* (1971) for G banding and the controlled thermal denaturation method of Dutrillaux and Lejeune (1971) for R banding. A 2 × SSC incubation after alkaline pretreatments (results 3 and 4) has a rather strong effect on the chromosomes. We did not carry out studies to determine whether any loss of chromosomal material occurred during the various treatments. An analysis with the aid of phase and interference contrast microscopy (Scheres, unpublished results), as well as a visual comparison between staining intensities, showed that $Ba(OH)_2$ incubation causes slight, but subsequent 2 × SSC treatment considerable, loss of chromosomal material. This is in agreement with the Comings *et al.* (1973) results obtained in biochemical and densitometric studies that, using a C banding method comparable to our CT technique, about 60% of the total DNA and a certain amount of the proteins were extracted from the chromosomes. The DNA loss was much larger in the euchromatic than in the heterochromatic areas. Similar results were obtained by Pathak and Arrighi (1973) and very recently by Schmiady *et al.* (1975) in autoradiographic experiments on C-banded chromosomes. Schmiady *et al.* (1975) noticed that the BSG (barium hydroxide/saline/Giemsa) method (Sumner, 1972) for staining of constitutive heterochromatin—a procedure very similar to the CT technique— also removes a larger quantity of DNA from the chromosome arms than from the centromeric heterochromatins. The theory that this selective loss of DNA plays an important role in the production of C banding patterns is further supported by the electron microscopic studies of Ross and Gormley (1973) and Burkholder (1975).

In addition to the discovery of two new techniques useful in human cytogenetics (*viz.* the CT and the new reverse banding techniques), the finding of a method for staining the short arms of some acrocentric chromosomes specifically is also an interesting result of our experiments. The underlying mechanism of this selective staining process is not yet known. It has been shown, however, that the short arms of the human acrocentric chromosomes contain the genes coding for ribosomal RNA (Henderson *et al.*, 1972; Evans *et al.*, 1974). The DNA of these cistrons is very rich in GC base pairs (Sinclair and Brown, 1971; Amaldi and Attardi, 1968) and therefore perhaps more resistant to the denaturating effect of the barium hydroxide treatment at 60°C and subsequent 2 × SSC incubation at 85°C. Possibly the short arm regions, specifically stained with our method, contain an extra large amount of this GC-rich DNA. It is also possible that a special type of heterochromatin occurring in the short arms of these

probably variant acrocentrics is responsible for the selective staining. On the other hand, specific chromosomal proteins might influence the denaturation resistance and staining behaviour of these regions. In this respect it is of interest to mention the findings of Matsui and Sasaki (1973), who developed a technique for specifically staining the short arms of all 10 human acrocentric chromosomes (N banding). From their results they concluded that certain structural non-histone proteins linked to the nucleolus organizer regions (NOR) in the short arms of these chromosomes were responsible for the selective staining (Matsui, 1974; Funaki *et al.*, 1975).

The acrocentrics specifically stained by our method resemble the variant 15p+ described by Yoder *et al.* (1974). Our discovery was made during the examination of an individual possessing 2 of these acrocentrics *viz.* a chromosome No. 13 and a chromosome No. 21. Two strongly stained chromocentres were visible in many interphase nuclei. In the nuclei of the polymorphs, which were slightly swollen as a result of hypotonic treatment, these chromocentres were usually attached to the membrane, sometimes seemingly on its outside. A peripheral position of other heterochromatic bodies in polymorphs has been demonstrated by Atkin and Baker (1974) using the BSG technique. The chromocentres seem to have this feature in common with the X and Y chromatins which are known to occur in these cells in the form of "drumsticks" (Davidson and Smith, 1954; Lamborot-Manzur *et al.*, 1971). It is likely that the minuscule nuclear appendages and "small clubs" sometimes seen in the polymorphs of both males and females (Davidson and Smith, 1961) contain these and possibly other heterochromatins.

The detection of specific areas in the interphase has previously been possible only for X chromatin (Moore and Barr, 1954), fluorescent Y chromatin (Pearson *et al.*, 1970) and heterochromatin in the secondary constrictions of chromosomes No. 1 (CsCl denaturation technique of Geraedts and Pearson, 1973) and 9 (Giemsa-11 technique of Bobrow *et al.* (1972) and of Gagné and Laberge (1972)). Our new method for staining the short arms of certain acrocentrics makes an additional technique available for demonstrating very specific human chromosome regions in interphase nuclei. We intend to investigate the applicability of this method to interphase nuclei of other tissues. This might contribute to the knowledge of the structure, position and behaviour of the short arms of the various human acrocentrics in somatic and meiotic cells.

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R- AND CT-BANDING OF HUMAN CHROMOSOMES WITH BASIC FUCHSIN

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Summary. A method is described for the staining of distinct R- and CT-banding patterns in human chromosomes with the aid of the triphenylmethane dye basic fuchsin. The results obtained illustrate the applicability of this dye in diagnostic as well as in more fundamental cytogenetic studies

Introduction

A considerable number of laboratory procedures exists for the production of the various banding patterns in human chromosomes. They can be divided into those using fluorochromes and those in which non-fluorescent dyes are applied. With a few exceptions (e.g., Stahl and Vagner-Capodano, 1974; Scheres, 1974; Scheres and Merkx, 1976) in the latter methods so-called Romanowsky dyes such as Giemsa's, Leishman's, Wright's, and May-Grunwald's are used for the production of G-, R- and other bands. A number of non-Romanowsky dyes were recently examined in our laboratory for their ability to produce chromosomal bands With some triphenylmethanes, in particular with basic fuchsin (BF), excellent G-banding patterns were obtained. The BF banding pattern appeared even more informative as a number of chromosomal variants could be distinguished which normally can not be recognized in the G-banding pattern produced with Giemsa (Scheres and Merkx, 1976). Because of the good results obtained with basic fuchsin in G-band formation we decided to investigate its possible value in the production of other banding patterns. The present paper describes the results obtained if BF is used for the staining of R- and CT-bands in human chromosomes.

Materials and Methods

Chromosome spreads were prepared from cultured human peripheral lympho-

cytes using a previously described method (Scheres, 1976). To produce an R-banding pattern the preparations were pretreated with a saturated solution of $Ba(OH)_2$ (pH $\pm$ 13.0; 10 min; 60°C) and rinsed with deionized water. Staining was carried out for 3 min using a 0.1% solution of basic fuchsin (UCB Bruxelles) in a 1:1:1 mixture of 0.1N NaOH:formamide:water, adjusted to a pH of 10.5 with the aid of 1N HCl. Previously this BF solution had shown to be very suitable for the formation of G-bands (Scheres and Merkx, 1976). For the production of CT-bands the chromosomes were pretreated with $Ba(OH)_2$ as described above, subsequently incubated for 30 min at 60°C in 2xSSC (= 0.3M sodium chloride and 0.03M tri-sodium citrate, pH 7.2) and than stained with the basic fuchsin solution.

Results and Discussion

Figure 1 shows a metaphase pretreated with $Ba(OH)_2$ and stained with BF. Very distinct "reverse" bands are visible, permitting homologue identification and recognition of chromosomal aberrations. Figure 2 gives an example of a structural rearrangement in a metaphase stained with this $Ba(OH)_2$-basic fuchsin technique.

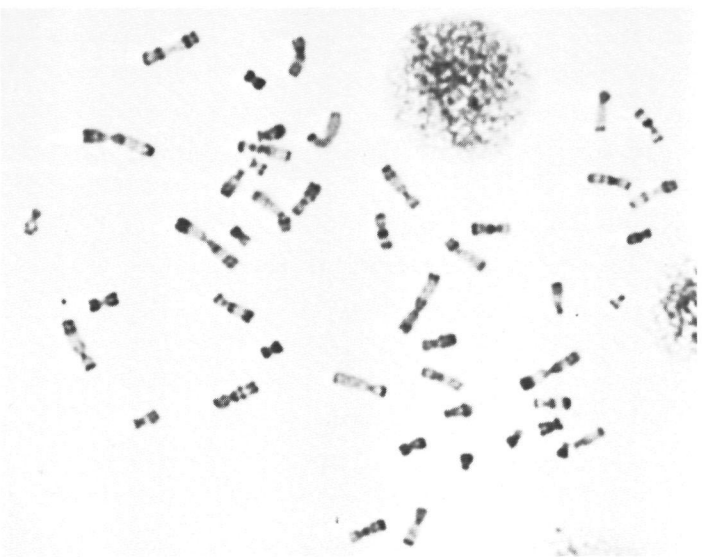

Fig. 1. Human metaphase stained with basic fuchsin after pretreatment with $Ba(OH)_2$: distinct R-bands can be seen.

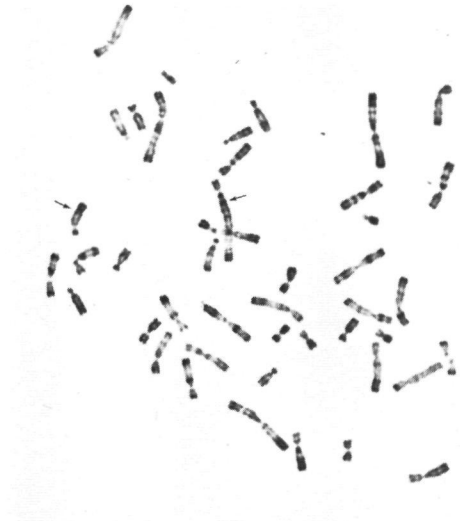

Fig. 2. Metaphase of an individual having a translocation between a chromosome No.2 and a No.10 (arrows); $Ba(OH)_2$-BF technique.

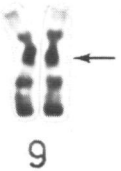

Fig. 3. Pair of chromosomes No.9 from a metaphase treated with $Ba(OH)_2$ and stained with BF: the heterochromatin in the secondary constriction (arrow) stains positive.

The characteristics of the R-banding pattern produced with basic fuchsin are very similar to those obtained with the cationic dye "Stains-all" after previous treatment of the chromosomes with $Ba(OH)_2$ (Scheres, 1976). The heterochromatin in the secondary constriction of chromosome No. 9 is well stained with basic fuchsin (Fig.3) as was also observed after the use of "Stains-all" (Scheres, 1976). In contrast to this, the 9q heterochromatin remains negative in the R-banding pattern obtained with the "controlled heat denaturation technique" of Dutrillaux and Lejeune (1971).

Basic fuchsin can also be used successfully for the production of a so-called CT-banding pattern in which both centromeric as well as reverse and telomeric bands are simultaneously visible. Figure 4 gives an example of a metaphase pretreated as in the CT-banding technique and stained with basic fuchsin.

As far as we know basic fuchsin is the 4th non-fluorescent dye which can be used for R-banding. The other dyes are Giemsa (Dutrillaux and Lejeune, 1971), pseudo-isocyanine which is also a fluorochrome (Vagner-Capodano et al., 1976), and "Stains-all" (Scheres, 1976). For the production of R-bands with

Fig.4. Metaphase pretreated as in the CT-banding technique and stained with basic fuchsin.

the BF technique described in the present paper, the chromosomes are treated with strong alkali-- i.c. $Ba(OH)_2$-- before staining with basic fuchsin. It is interesting to note that after $Ba(OH)_2$- treatment a similar R-banding pattern can be obtained if instead of BF the chemically unrelated dyes Giemsa or "Stains-all" are used (Kanda, 1976; Scheres, 1976). This might indicate that the dyes used in this R-banding method play only an unspecific role and that the banding pattern which appears after staining, merely reflects the chromatin distribution induced by the $Ba(OH)_2$-pretreatment. This theory is supported by the recent observation that a distinct R-banding pattern can already be seen in $Ba(OH)_2$- treated but unstained chromosomes when studied by Nomarski interference contrast optics (Scheres, manuscript in preparation).

In a previous paper we reported that basic fuchsin is an excellent stain for G-bands and that it can be used for the recognition of certain C- and Q-band polymorphisms (Scheres and Merkx, 1976). As demonstrated in the present article basic fuchsin is also very suitable for producing R- and CT-banding patterns and may therefore be used in clinical cytogenetics. The stain may also be of importance in more fundamental studies of the mechanism

of G- and R-band formation. One of the reasons for its possible role in this type of research are its fluorescent properties which may be advantageous in, e.g., quantitative microscopic studies of the chromosomal banding patterns.

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PAPER VI

CT Banding of Human Chromosomes

The Role of Cations in the Alkaline Pretreatment

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Summary This paper describes a study of the role of certain cations in the alkaline pretreatment step used in the CT technique for chromosome band formation Treatment of human chromosomes with ammonium bases or with the hydroxides of the monovalent alkali metals Na, K, or Li resulted in their rapid disintegration, unless very short treatment periods or diluted solutions were used In the latter cases a subsequent staining produced a weak G banding pattern The chromosomes appeared to be much less sensitive to treatment with the hydroxides of the divalent alkaline earth metals Ba, Sr, Ca, and Mg Staining after exposure to these hydroxides yielded R banding patterns The reduced alkali sensitivity of the chromosomes and the reverse banding pattern formation observed are probably the result of a chromatin stabilization by the divalent cations of the alkaline earth metals It is proposed that not only in the R band formation with the hydroxides of the alkaline earth metals but also in that obtained by other techniques, chromosome stabilization plays an important role

Introduction

C banding techniques usually stain only the constitutive heterochromatins of human chromosomes and do not allow the recognition of all chromosomes Recently a new C banding method was described (Scheres, 1974) which provided a means to identify the human chromosomes by staining the centromeric heterochromatins as well as the bands belonging to the R ("reverse") system In particular, most telomeric regions were stained deeply For these reasons the banding method was named the CT technique The procedure consists of a pretreatment of the chromosomes with a strong alkali, viz a saturated $Ba(OH)_2$ solution, followed by their incubation in $2 \times SSC$ and staining with "Stains all" The reason for using this dye was that it stains the telomeric bands more strongly than does Giemsa's solution (Scheres, 1974) A study of the various steps employed in the CT technique showed that the $Ba(OH)_2$ treatment induced a very distinct R-banding pattern (Scheres 1976) This was an unexpected result as an alkaline treatment with NaOH or KOH followed by Giemsa staining usually produces the reciprocal pattern a G banding (Schnedl, 1971 Drets and Shaw, 1971 Berger, 1972, 1975) It seems therefore that the divalent cations present in the alkaline pretreatment contribute to the band formation with the CT technique Thus it was considered that a further study of the role of cations in the CT banding process might lead to a better understanding of the mechanism which underlies this differential staining phenomenon

This article reports the most significant results from a series of experiments in which the role of cations in the first step of the CT-staining procedure (the alkaline treatment of chromosome) was studied

Materials and Methods

Chromosome spreads were prepared from cultured peripheral lymphocytes using a previously described method (Scheres, 1976) After the various pretreatments employed in the experiments, the chromosomes were stained for 3 min in a 0 005% solution of Stains all (4,5,4',5', dibenzo 3,3' diethyl 9 methyl thiacarbocyanine bromide, Serva) in a 1 1 formamide-watermixture

Results

To determine to what extent the cations present may influence the result of alkaline treatment of chromosomes, preparations were incubated at 60°C in solutions of various bases The solutions used contained either one of the hydroxides of the monovalent alkali metals sodium, potassium, and lithium, of the divalent alkaline earth metals barium, strontium, calcium, and magnesium, or of ammonium, tetramethylammonium, or tetraethylammonium Table 1 lists the results of this series of experiments Both the ammonium bases and the hydroxides of the monovalent alkali metals appeared to have a strong disintegrating effect on the chromosomes In most experiments no stainable material was left after a treatment period of 10 s at pH 13 If even shorter times or diluted solutions were used, a faint

Table 1
The effect of various hydroxides on human chromosomes (incubation temperature 60°C)

Reagent	pH	Incubation time	Result after staining
Monovalent cations			
NaOH	13 0	10 s	complete disintegration
KOH	13 0	10 s	complete disintegration
LiOH	11 7	10 s	complete disintegration
NH_4OH	12 6	10 s	complete disintegration
$N(CH_3)_4OH$	12 8	10 s	complete disintegration
$N(C_2H_5)_4OH$	12 9	10 s	complete disintegration
NaOH	11 9	1 s	weak G banding
KOH	11 2	2 s	weak G banding
LiOH	11 7	1 s	weak G banding
NH_4OH	11 9	2 s	weak G banding
$N(CH_3)_4OH$	12 8	1 s	weak G banding
$N(C_2H_5)_4OH$	12 9	1 s	weak G banding
Divalent cations			
$Ba(OH)_2$	13 1	10 min	R banding
$Ba(OH)_2$	13 1	60 min	R banding
$Sr(OH)_2$	12 7	10 min	R banding
$Ca(OH)_2$	12 5	20 min	R banding
$Mg(OH)_2$	9 9	60 min	weak R banding

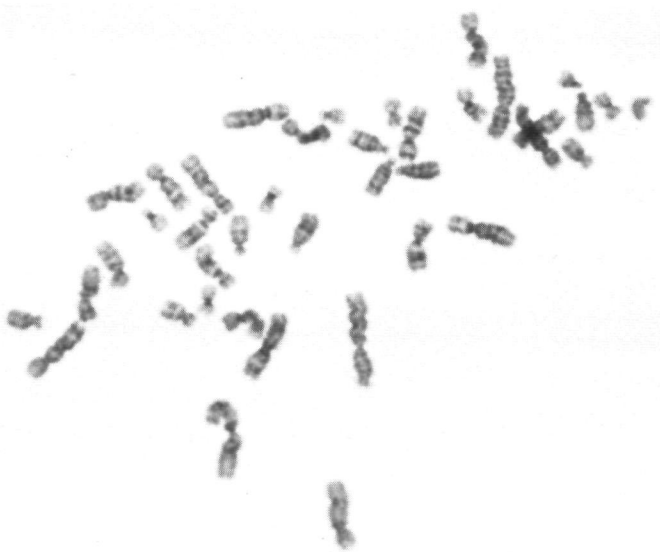

Fig. 1. Human metaphase chromosomes treated with NH_4OH (60°C, 2 s, pH 11.9) before staining; notice presence of G bands

G-banding pattern became visible after staining (Fig. 1). The result of a treatment with one of the hydroxides of the divalent alkaline earth metals was entirely different. These caused much less damage to the chromosomal structure than the bases of the previous group. Even after a 1-h incubation period strongly stainable chromosomal areas remained. In addition, all of them produced similar R-banding patterns (Fig. 2). The characteristics of the pattern obtained with one of these hydroxides, viz. $Ba(OH)_2$, have been previously described in detail (Scheres, 1976). It was also noticed that the R-band producing ability of these bases corresponded with their base strength: $Ba(OH)_2$ was the most effective, followed by $Sr(OH)_2$, $Ca(OH)_2$, and $Mg(OH)_2$.

An effort was made to determine whether the induction of R-bands by hydroxides of divalent alkaline earth metals was based on the presence of divalent cations or on the absence of monovalent cations. A number of experiments was performed with various solutions containing not only a monovalent alkali metal hydroxide or an ammonium base but also a divalent alkaline earth metal cation and vice versa (Table 2). These experiments showed that the monovalent cations apparently did not alter the result of chromosome treatment with alkaline earth metal hydroxides. In contrast, divalent cations appeared to cause a remarkable change in the effect of the ammonium bases and the monovalent alkali metal hydroxides. The strong destructive effect of the latter disappeared after the addition of divalent cations and a distinct R-banding with the characteristics of the $Ba(OH)_2$-induced pattern was obtained instead of the weak G-banding normally produced by the ammonium bases or the alkali metal hydroxides (Fig. 3).

Fig. 2. Human metaphase chromosomes treated with $Ba(OH)_2$ (60°C, 10 min, pH 13.1) before staining; a pronounced R-banding pattern has been produced

Table 2. The effect of mono- and divalent cations on the reaction of human chromosomes to treatment with various hydroxides at 60°C

Reagent		Test conditions		Results after staining
hydroxide[a]	cation added[b]	pH	incubation time	
NaOH	Na^+	12.2	1 s	G banding
NaOH	Ba^{++}	11.6	10 min	R banding
NaOH	Ca^{++}	11.7	10 min	R banding
KOH	Ba^{++}	11.6	5 min	R banding
$N(CH_3)_4OH$	Na^+	12.5	1 s	G banding
$N(CH_3)_4OH$	Ba^{++}	12.5	10 min	R banding
$N(C_2H_5)_4OH$	Na^+	12.5	1 s	G banding
$N(C_2H_5)_4OH$	Ba^{++}	12.7	10 min	R banding
$Ba(OH)_2$	Na^+	12.8	10 min	R banding
$Ba(OH)_2$	Ba^{++}	12.7	10 min	R banding
$Sr(OH)_2$	Na^+	12.4	5 min	R banding

[a] The high pH solutions mentioned in Table 1 were used.

[b] Cations were added as 0.1M solutions of the chlorides; in some solutions precipitation occurred, resulting in a gradual decrease of pH.

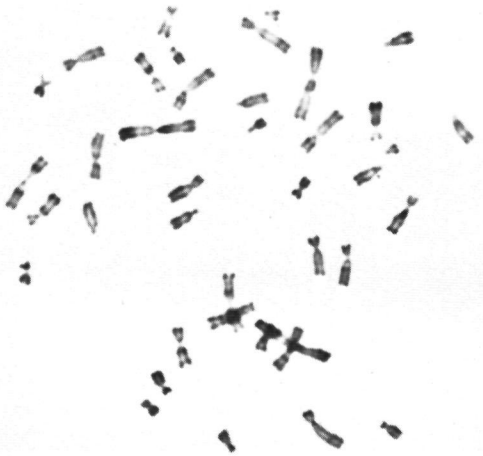

Fig. 3. Metaphase treated with solution of $N(C_2H_5)_4OH$ to which Ba^{++} ions had been added; no G, but distinct R bands can be seen

Discussion

The results obtained illustrate clearly that the cations studied in these experiments strongly influence the effect of alkaline heat treatment of chromosomes. If only monovalent cations are present the chromatin is extremely alkali-sensitive and a rapid disintegration occurs unless the exposure times are very short or diluted solutions are used. In the latter cases a weak G-banding pattern is produced. However, if divalent cations are present during alkaline heat treatment (as is the case in the CT technique) the opposite pattern (R-banding) is obtained and the chromosomes become markedly resistant to alkali. Although incubation at 60°C with, e.g., NaOH of pH 13 resulted in a rapid and complete loss of chromatin, the chromosomes were able to withstand a similar treatment with $Ba(OH)_2$ over 1 h. Divalent cations have thus a strong stabilizing influence on the chromosomes. Previously, a similar effect of divalent cations on isolated, unfixed chromosomes has been described by, amongst others, Chorąży et al. (1963) and Maio and Schildkraut (1967). Recently McKay (1973) reported that divalent cations could also stabilize fixed chromosomes and for that reason might play a role in chromosomal behavior to banding pretreatments. A possible role of divalent cations such as Ca^{++} or Mg^{++} in G-banding has also been suggested by Dev et al. (1972) and by Comings et al. (1973).

An intriguing question arising from the studies reported in this paper is why chromosome treatment with hydroxides of alkaline earth metals produces an R-banding pattern and one with ammonium bases or with hydroxides of monovalent alkali metals results in G-banding or no banding at all. The most important difference between these treatments seems to be the presence or absence of divalent cations as a stabilizing factor. Since in our experiments R-banding of chromosomes was obtained with all treatments in which both denaturation by strong alkali and stabilization by divalent cations occurred simultaneously, it is

tempting to conclude that a combination of denaturation and stabilization is required for R-band formation It is interesting to note that in most other R banding procedures described—at least those in which Giemsa's stain is employed —also some combination of a strongly denaturing and a stabilizing factor was used In the method of Dutrillaux and Lejeune (1971) for example, and in some of its modifications (Schested, 1974, Pangalos and Frezal, 1974) the high incubation temperature (85—90°C) causes denaturation while the low pH, necessary for a good R- or T band differentiation (Dutrillaux, 1973, Dutrillaux and Covic, 1974, Sehested, 1974), has a strongly stabilizing effect on the chromosomes (Huberman and Attardi, 1966, Chorąży et al, 1963, Scheres, manuscript in preparation) In the so called NaOH F method for the production of R-bands (Wyandt et al, 1974), the NaOH treatment is the denaturing factor, while the formalin to which the chromosomes are simultaneously exposed, has been shown to have a clear chromatin stabilizing influence (Traganos et al, 1975, Scheres, manuscript in preparation) We are therefore inclined to assume that a strong *denaturation* of *stabilized* chromosomes indeed forms the basis for the production of R- or T bands with Giemsa's stain or other nonfluorescent dyes such as Stains all

In which way a combination of denaturation and stabilization results in such banding patterns remains uncertain The following information might contribute to an explanation of the process It is known that the chromatin of the R bands is slightly more alkali resistant than that of the G-bands (Bobrow and Madan, 1973, de la Chapelle et al, 1973) If the chromosomes are treated for example with NaOH in the absence of extra stabilizing factors such as divalent cations, the small difference in alkali resistance might be insufficient to protect the R-bands more than the G bands against disintegration As a result of an almost uniform loss of material from all areas, the chromatin might than remain in the G banding pattern distribution which is believed to exist already in untreated chromosomes (Bahr et al, 1973, Yunis and Sanchez, 1973, McKay, 1973, Golomb and Bahr, 1974) If, however, during the alkaline treatment stabilizing factors such as divalent cations are present, the slight difference in alkali resistance between the R- and the G bands might become accentuated It is possible that, as a result of a special base composition or other properties of the nucleoprotein complex in the R bands (such as the DNA conformation or its superhelicity) these regions become more effectively stabilized than the G bands This will result in a smaller loss of material from the R-bands than from the G bands and, after staining, in the appearance of an R banding pattern

The purpose of the experiments carried out has been to obtain a better understanding of the CT banding mechanism by studying the role of cations in this differential staining process Cations could be demonstrated to exert a pronounced influence on the reaction human chromosomes to the alkaline pretreatment, which forms the first step in the CT staining procedure In further investigations we shall study whether cations play an equally important role in the salt incubations which the CT technique and many other banding methods use as "renaturation" steps after previous "denaturation" of the chromosomes with alkali

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CT BANDING OF HUMAN CHROMOSOMES
The effect of cations on C-band formation

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Summary. A number of salt solutions was tested for their ability to replace 2xSSC in the production of C-bands with the so-called CT technique. Those containing monovalent cations, except for the ones with Ag^+-ions, were capable of inducing C-bands in $Ba(OH)_2$-pretreated human chromosomes. Caesium cations were the most effective in this respect. In contrast to the effect of the monovalent cations studied, di- or polyvalent cations (and Ag cations) were incapable of producing C-bands, and appeared to stabilize the $Ba(OH)_2$-induced reverse banding pattern. This stabilizing effect could already be obtained with relatively low cation concentrations and persisted even when C-band producing cations were present at the same time.

The experiments clearly show that the cations present during salt incubations, generally used in the CT- and several other banding methods, can exert a pronounced influence on the behaviour of the chromosomes.

Introduction

In human chromosomes the CT technique (Scheres, 1974) not only stains C-, but also "reverse"-, mainly telomeric bands (T-bands; Dutrillaux, 1973). A more detailed study of this differential staining phenomenon showed the reverse bands to be induced during the first step of the CT-procedure, the alkaline pretreatment of the chromosomes with $Ba(OH)_2$ (Scheres, 1976a) The C-bands were produced during the second step of the procedure, viz. the incubation of the $Ba(OH)_2$-treated chromosomes in 2xSSC solution. In further experiments (Scheres, 1976b) it was found that certain cations were able to exert a pronounced influence on the reaction of the chromosomes to the CT-banding treatment. In contrast to monovalent cations, divalent cations such

as Ba^{++}, Ca^{++}, Mg^{++} and Sr^{++} appeared to have a strong chromosome stabilizing effect and to play an important role in the formation of the R-banding pattern which results from the alkaline pretreatment of the CT technique (Scheres, 1976b). In the present paper we describe a number of experiments showing that the cations used also are an important factor in the second step of the CT technique, the induction of C-bands with 2xSSC.

Materials and Methods

Chromosome spreads were prepared from cultured peripheral lymphocytes using a previously described method (Scheres and Merkx, 1976). To obtain a CT-banding pattern in the chromosomes the preparations were treated for 10 min at 60°C with a saturated aqueous solution of $Ba(OH)_2$ (pH $\pm$ 13.0), subsequently incubated for 30 min at 60°C in 2xSSC (= 0.3 M sodium chloride and 0 03 M tri-sodium citrate, pH 7.2) and stained with "Stains-all" (Scheres, 1974).

Results

In the CT technique the C-bands are induced by incubation of $Ba(OH)_2$-pretreated chromosome in 2xSSC. One may ask whether the composition of the 2xSSC solution is essential to obtain these bands. In an effort to answer this question a series of experiments was carried out in which 2xSSC was replaced by 0.1 M solutions of other salts or by deionized water. The results obtained are summarized in Tables 1 and 2. From these the following main conclusions may be drawn.

- A number of salt solutions may replace 2xSSC to induce C-bands in $Ba(OH)_2$-pretreated chromosomes. CsCl solution proved even more effective than 2xSSC (Fig. 1) It is evident that all C-band inducing salts are compounds of monovalent metals or of ammonium.
- None of the salts of di- or polyvalent metals studied is suitable for the induction of C-bands. The $Ba(OH)_2$-produced R-banding pattern does not change after incubation of the chromosomes in these salts and seems to have become stabilized by them (Fig. 1). It is noteworthy that the salts of the monovalent metal silver (examined were $AgNO_3$ and Ag-acetate) have the same effect as those of the di- or polyvalent metals
- If divalent cations are added to a solution of a monovalent metal salt the C-band inducing capability of the latter is lost and the $Ba(OH)_2$-dependent R-band pattern remains.

Table 1. **Effect of monovalent cations on $Ba(OH)_2$-pretreated and on unpretreated human chromosomes (Incubation conditions 60°C, 30 min).**

Reagent	*pH*	*Effect obtained on $Ba(OH)_2$-pretreated chromosomes*	*Effect obtained on unpretreated chromosomes*
2xSSC	7.2	CT	G
H_2O	6.2	C (c.d.)	G (n.d.)
NaCl	8.2	CT	G
- ClO_3	6.7	CT	G
- SO_4	8.6	CT	G
- S_2O_3	7.0	CT	G
- S_2O_5	3.4	-	n.d.
- F	8.3	CT	n.d.
- CO_3	10.9	c.d.	c.d.
- HCO_3	8.7	CT	G
- HPO_4	9.1	CT	G
- H_2PO_4	4.6	CT	G
- $OOCCH_3$	8.0	CT	G
- $O_7C_6H_5$	9.3	CT	G
KCl	8.7	CT	G
-S_2O_5	4.0	CT	G
-H_2PO_4	4.4	CT	G
-$Fe(CN)_6$	7.4	CT	G
LiCl	6.7	CT	G
- SO_4	6.7	CT	G
- CO_3	10.8	c.d.	-
RbCl	8.1	CT	G
CsCl	7.0	CT (C)	G
NH_4Cl	5.5	CT	G
- SO_4	5.2	CT	G
$N(CH_3)_4Cl$	6.2	CT	G
$TlNO_3$	5.1	CT	G
- SO_4	7.3	CT	-
$AgNO_3$	8.2	R	GI
- $OOCCH_3$	7.1	R	GI

CT = CT-banding pattern; C = C-banding pattern; G = G-banding pattern; R = R-banding pattern; GI = Intermediate b.p. (see text) ; n.d. = no differentiation; c.d.= complete disintegration of chromosomes; - = not tested

Fig. 1a. Human metaphase stained after $Ba(OH)_2$- treatment at 60°C: a distinct "reverse" banding pattern is visible.

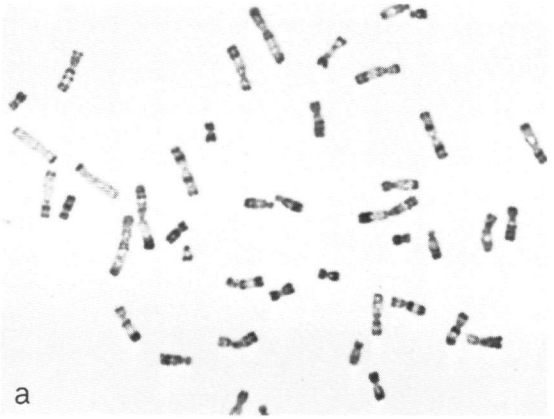

Fig. 1b. Metaphase stained after $Ba(OH)_2$-pretreatment and subsequent incubation in a 0.1 M solution of CsCl: very prominent C-bands have been induced.

Fig. 1c. Metaphase chromosomes stained after $Ba(OH)_2$-pretreatment and subsequent incubation in 0.1M $La(NO_3)_3$: the $Ba(OH)_2$-induced R-banding pattern remains and no C-bands are seen.

Table 2. Effect of di- and polyvalent cations on $Ba(OH)_2$-pretreated and on unpretreated human chromosomes (Incubation conditions 60°C, 30 min).

Reagent	*pH*	*Effect obtained on $Ba(OH)_2$-pretreated chromosomes*	*Effect obtained on unpretreated chromosomes*
$BaCl_2$	5.9	R	GI
$-(NO_3)_2$	5.8	R	n.d.
$MgCl_2$	6.2	R	GI
$CaCl_2$	6.5	R	GI
$SrCl_2$	7.2	R	GI
$BeCl_2$	2.8	R	GI
$CuCl_2$	3.5	R	n.d.
Hg_2Cl_2	3.6	R?	n.u.d.
$ZnCl_2$	5.8	R	GI
$MnCl_2$	6.5	R	GI
$FeCl_2$	2.8	n.u.d.	n.d.
$FeCl_3$	1.6	n.u.d.	n.d.
$AlCl_3$	2.2	R	-
$CrCl_3$	1.9	R	-
$NiCl_2$	5.1	R	n.d.
$CoCl_2$	5.2	R	n.d.
$CdCl_2$	5.4	R	GI
$La(NO_3)_3$	4.9	R	GI
$Pb(OOCCH_3)_2$	6.1	R	GI
$UO_2(OOCCH_3)_2$	4.2	R	GI
$Mg(OOCCH_3)_2$	7.7	R	GI
$CaCl_2$+CsCl	5.8	R	G(I)

R= R-banding pattern; G= G-banding pattern; GI= Intermediate banding pattern (see text); n.d.= no differentiation; n.u.d.= no uptake of dye; - = not tested

- The type of anion in the salt solution does not appear to be of much importance for C-band formation.
- In the absence of salt (viz. in deionized water) a strong swelling and disintegration of the $Ba(OH)_2$-pretreated chromosomes occur. Sometimes a very faint C-band pattern formation is also observed.

The observed R-band stabilizing effect of the divalent cations could already be obtained at relatively low cation concentrations. The lowest stabilizing

Ba-,Mg- and Ca concentrations seemed to depend on the condition of the preparations and varied between 0.5 and 5 mM.

In other experiments some stabilization of R-bands and an inhibition of C-band formation could also be obtained with monovalent cations if present in sufficiently high concentrations, e.g., in a saturated solution of NaCl or of $(NH_4)_2SO_4$. The most effective C-band inducing agent in our experiments, CsCl, was, however, unable to stabilize the R-banding pattern even in a saturated solution.

Both the stabilization of R-bands with divalent cations and that with high concentrations of monovalent cations appeared to be reversible. If the chromosomes after a "stabilizing" salt incubation were treated with 2xSSC solution, a subsequent staining could produce a C-band pattern.

Stabilizat on of R-bands and inhibition of C-band production during salt incubation of $Ba(OH)_2$-pretreated chromosomes could also be achieved by the addition of formalin to a concentration of about 10%, of ethanol to a concentration of approximately 75%, or by reducing the pH of the solution to below 4.0

In the experiments described above the chromosome preparations were always treated with $Ba(OH)_2$ before the salt incubations. If the $Ba(OH)_2$-treatment was omitted the results of incubations with solutions containing mono- or divalent cations appeared to differ only slightly. Most monovalent cations produced weak and sometimes hardly visible G-band patterns at 60°C At this temperature divalent cations usually induce a faint "intermediate" banding pattern in which some G- and a few R-bands seem to be present at the same time (Tables 1 and 2).

Discussion

The mechanism of C-band formation in alkali-pretreated chromosomes is not fully understood At first it was thought that the C-band formation resulted from a rapid reassociation of the alkali-denatured highly repetitive satellite DNAs which occur in the C-bands (Arrighi and Hsu, 1971, Jones et al., 1973, Gosden et al., 1975) Whether the DNA is double-stranded or not does not seem to play an essential role in C-band production, however, as has been made plausible by amongst others Stockert and Lisanti (1972), Comings et al (1973), Lubs et al.(1973) and in this laboratory (Scheres, manuscript in preparation) It is now generally accepted that the C-bands are the chromosomal

areas most resistant to the extraction of chromatin by alkaline or salt treatments. After these treatments sufficient stainable material would remain only in the C-bands (Comings et al., 1973; Pathak and Arrighi, 1973, Schmiady et al., 1975). Chromosomal proteins in these areas seem to be of importance in the C-band formation (Comings et al., 1973). It has also been observed that considerable structural alterations ("collaps", Ross and Gormley, 1973) have occurred in C-banded chromosomes.

In the experiments described in this paper a number of important observations were made which possibly contribute to a clarification of the C-banding mechanism. It has been found that incubation in a solution with monovalent cations is necessary to obtain good C-band differentiation in $Ba(OH)_2$-pretreated chromosomes. Caesium cations, having the largest ion size of the alkali metals tested, appeared to be the most effective C-band inducing agent of this group. It is noteworthy to mention here that Meisner et al.(1973) as well as Geraedts and Pearson (1973) also found caesium salts to be very suitable to make centromeric constitutive heterochromatins stainable. However, as the relatively large monovalent tetramethylammonium cations were clearly less effective than Cs-ions, the C-band producing capability of a monovalent cation does not seem to be directly correlated with its size.

A second important observation made in our investigations is that di- or polyvalent metal cations can prevent the production of C-bands and the structural changes connected with it. This inhibition occurs even if in addition to the divalent cations C-band inducing monovalent cations are present at the same time. The $Ba(OH)_2$-induced R-band pattern appears to become stabilized by the di- or polyvalent metal cations. This finding clearly corresponds with the results of a previous study showing that the cations of the divalent alkaline-earth metals protect the chromosomes against the denaturing action of alkali and for that reason play an important role in the production of a "reverse" banding pattern with a modified CT technique (Scheres, 1976a; Scheres, 1976b). The results of the experiments described in the present paper clearly demonstrate that the chromosome stabilizing divalent cations can also influence the reaction of the chromosomes to the salt treatments which, both in the CT technique and in several other banding methods, are used for the production of various banding patterns.

The results obtained support the hypothesis of amongst others McKay (1973) and of Dev et al. (1972) that divalent cations could play a role in banding of chromosomes by stabilizing their structure. On the other hand,

Kato and Moriwaki (1972) and also Eiberg (1973), who studied extensively the influence of various salts on chromosome banding, did not mention such chromosome stabilizing effect of divalent cations. These investigators, however, used chromosome preparations which were not pretreated with alkali. The difference in effect of the monovalent cations on the one hand and the di- and polyvalent cations on the other is under these conditions much less spectacular than if alkali-pretreated chromosomes are used (Tables 1 and 2).

A chromosome stabilizing influence of divalent cations has been described before by Maio and Schildkraut (1967) and by Chorazy et al. (1963) who studied unfixed, isolated metaphase chromosomes. It is worthwhile to mention that the lowest stabilizing cation concentrations in our experiments with fixed chromosomes are of the same order of magnitude as those reported to stabilize unfixed chromosomes (Maio and Schildkraut, 1967).

Whether the stabilization of chromosomes by di- and polyvalent cations is based on interactions of the ions with the chromosomal DNA, with the chromosomal proteins, or with both, remains as yet unexplained. A large number of investigations has been carried out with regard to the possible interactions between metals and the DNA or the bases, nucleosides and nucleotides of which it is composed (for extensive reviews see Izatt et al., 1971 or Eichhorn, 1973). As the most important possible explanations for the stabilization of the DNA double helix by di- or polyvalent cations can be mentioned: neutralization of negatively charged phosphates repelling each other, and / or the formation of cross-links between phosphates and/ or bases in opposite strands. If the latter process would indeed play a role in the chromosome stabilization seen in our experiments it might account for the cations of the monovalent metal Ag to have a similar effect. It is well known that Ag cations are capable of forming very stable cross-links between opposite DNA base moieties (Izatt et al., 1971). Other types of cross-links by divalent cations resulting in DNA stabilization have been suggested by Mejbaum-Katzenellenbogen and Maskos (1971). According to the theory of these authors they may be formed by, e.g., Mg^{++}-ions between neighbouring phosphates in the same or in opposite helical strands. The result of this process would be the formation of a compact, stabile DNA aggregate.

Chromatin stabilization by divalent cations might also be the result of their action on the DNA/protein complex. Cation-mediated binding of protein to the DNA might be related to this stabilization process (Maskos, 1971; Mil-

ler,1973). Such theory is supported by Johnson et al.'s observation (1972) that divalent cations transformed the DNA of isolated nucleohistone from the B- into the C configuration resulting in a tightening of the nucleoprotein complex and it becoming less vulnerable to environmental factors.

It should be taken into account that the above hypotheses concerning the possible interactions between metal ions and the DNA or the DNA/protein complex are based on data obtained from studies on isolated, often highly purified components. One has therefore to be careful in simply extrapolating these theories to the much more complex "in situ" conditions of the metaphase preparations. For that reason, additional and more extensive experiments will be needed to explain more fully the observed effects of cations on the formation of C-bands in human chromosomes.

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PAPER VIII

Trisomy for the short arm of chromosome No. 10

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The clinical and cytogenetic data are presented of a child with multiple congenital malformations, including cystic kidneys. A trisomy for at least the larger part of the short arm of chromosome No. 10 in association with a translocation between chromosomes Nos. 10 and 14 was found in peripheral blood lymphocytes and skin fibroblasts. Both the mother and a younger sib were balanced carriers of the translocation.

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As far as we know, patients with a complete trisomy of chromosome No. 10 have not been described. A patient with a karyotype 46,XY/47,XY,+10 has been reported (Higurashi et al. 1969, Nakagome et al. 1972).

The patient described in this paper had a trisomy for at least the larger part of the short arm of chromosome No. 10 in association with a translocation between chromosomes No. 10 and 14.

Case Report

The proband (D.G. 710807) was the first child of a 17 year old mother and a 23 year old father who are not related. A progressive intrauterine growth retardation was observed from the 23rd week of pregnancy on. Placental functions seemed unimpaired, since the estriol and pregnanediol excretion in 24 h urine was normal. An immature child, weight 2120 g, and length 43 cm, was born after a gestation of 41 weeks. The placenta, the amniotic membrane and the umbilical cord showed no abnormalities. A few minutes after birth, the Apgar score was 2 and the plasma pH 7.02. Administration of $NaHCO_3$ resulted within 2 h in a blood pH of 7.28. A mild cyanosis persisted. A few hours after birth, petechiae developed on the child's back and legs; the platelet count was then 50000/mm^3.

When physically examined, a number of abnormalities was seen. The patient had bilateral epicanthal folds, a broad nasal bridge and hypertelorism. Her ears were low-set, slanted and dysplastic. She had a retrognathia and a medial palatoschizis (Figs. 1, 2). The cranial sutures were wide; the anterior fontanel was 3×3 cm, the posterior fontanel was 4×4 cm. The cardiac sounds were loud but without murmur; the heart rate was 160 beat/min. A marked hypotonia was present. The external genitalia were normal female. A unilateral hip dislocation existed, as well as club feet which could not be corrected manually. Both hands were flexed at the wrist and the thumbs were adducted. In the palms of the hands, abnormal flexion creases were seen (Fig. 3). The child had

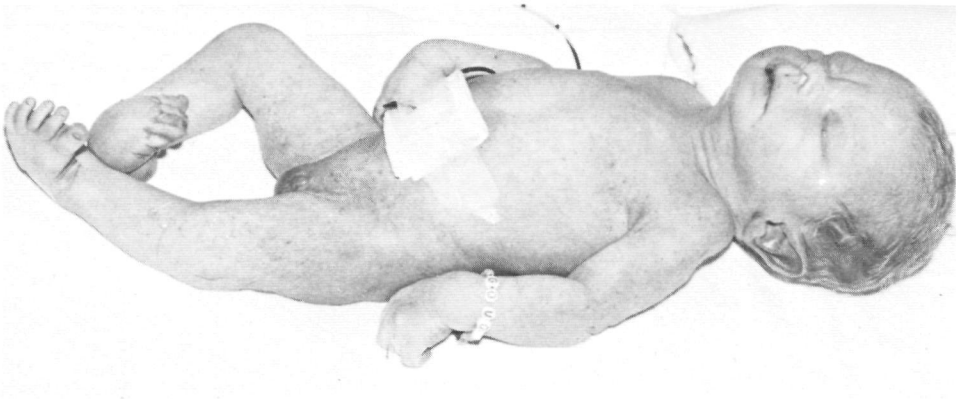

Fig. 1. Postmortem photograph of the proband

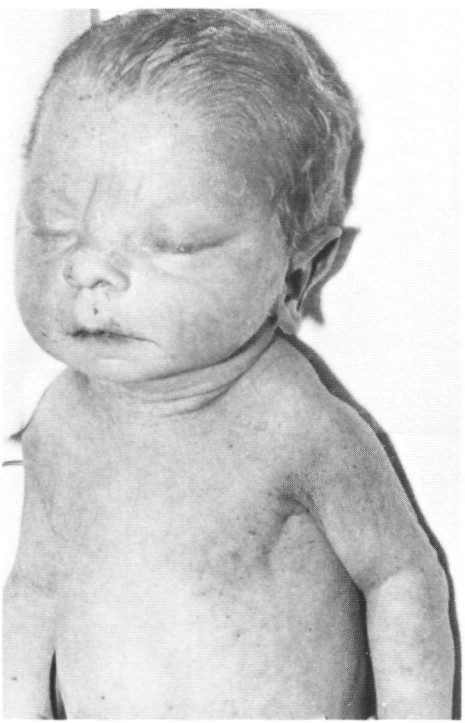

Fig. 2. Postmortem photograph of the proband, close-up

dimples on the shoulders, elbows and knees. The electrocardiogram was very pathologic, indicating a severe cardiac dysfunction.

The platelet count decreased gradually to 12000/mm^3, whereas the hemoglobin value was 11.0g%, and the number of white cells 18300/mm^3 of which 25% were lymphocytes.

The child died 48 h after birth as a result of a respiratory distress syndrome. A total of only 0.5 ml. urine was produced.

At autopsy, additional malformations were noticed. The right kidney consisted of fetal renal tissue with many cysts. The left kidney was a cystic mass with sporadic glomeruli and tubules not connected with the ureter. The left ureter ended blindly at a distance of 4 cm from the urinary bladder. In addition, a coarctatio aortae and hyaline membranes were found.

Cytogenetic Investigations

Chromosome studies of the proband were carried out on peripheral lymphocyte cultures using a modification of the method

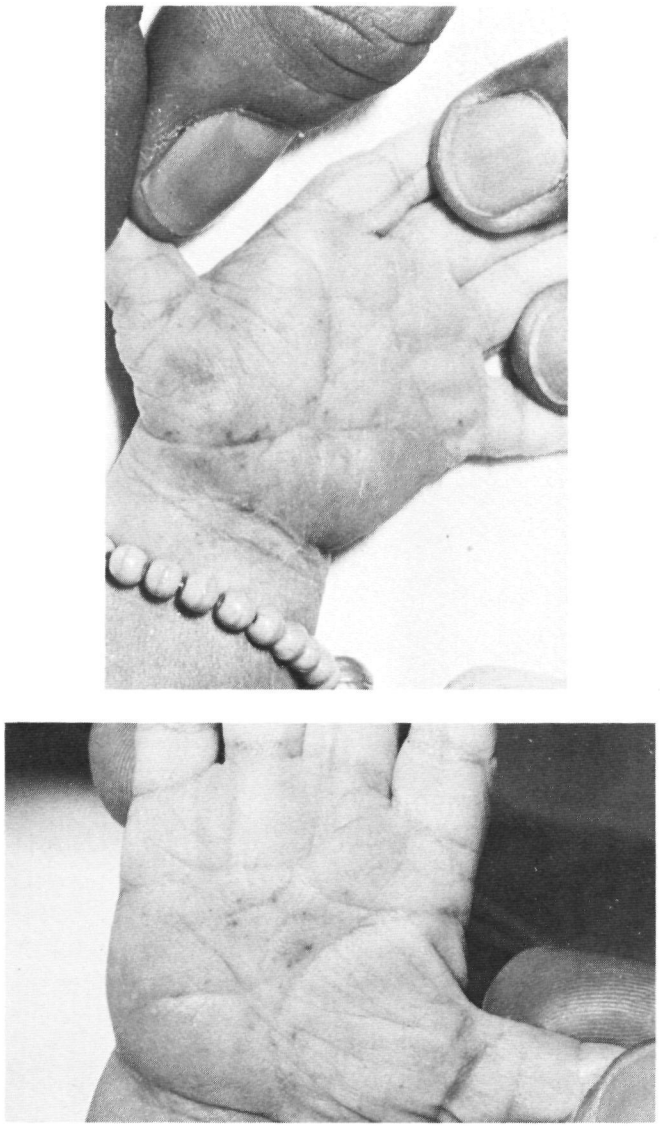

Fig. 3. The hands of the proband

described by Moorhead et al. (1960), and on fibroblasts cultivated from skin obtained shortly after death. Peripheral lymphocyte cultures of the proband's relatives were examined. Chromosome preparations were stained with Unna-blue (Turpin & Lejeune

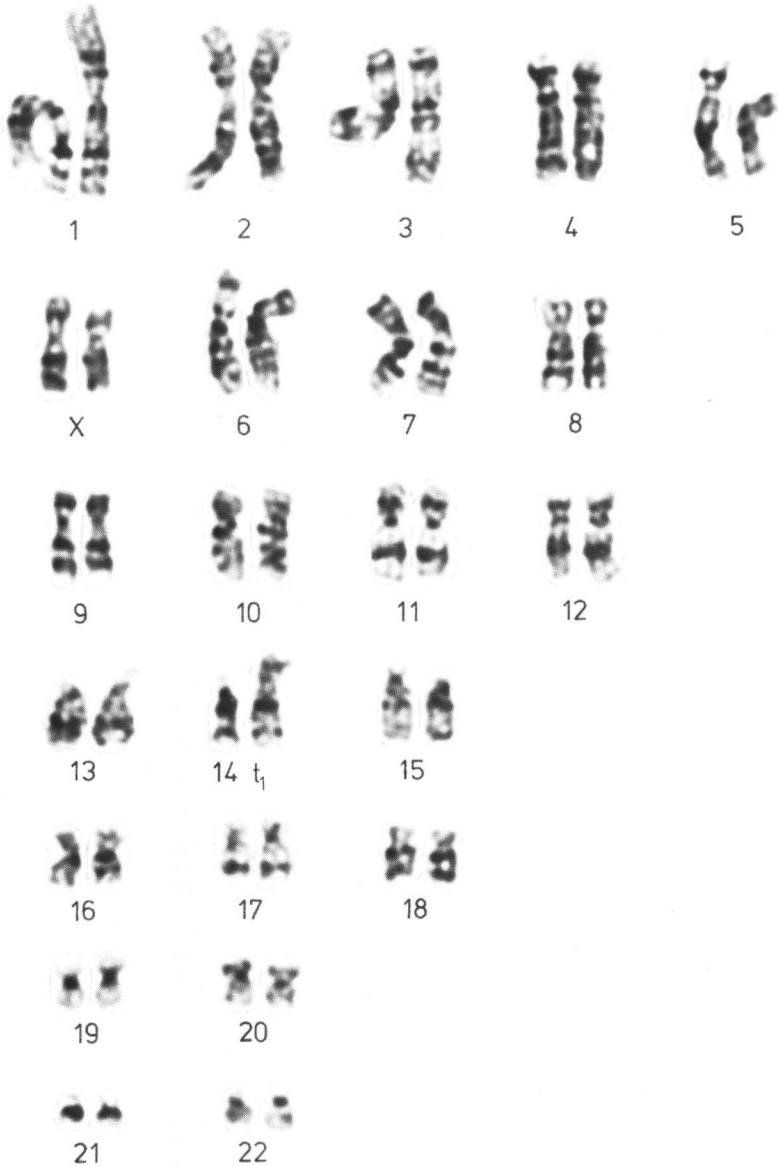

Fig. 4. Karyotype of the proband: t_1 is the large translocation element

1965) or with a modification (Scheres 1972) of Seabright's (1972) trypsin-Giesma banding method.

According to the classical analysis of the metaphases of the proband's lymphocytes, one of the D group chromosomes seemed to

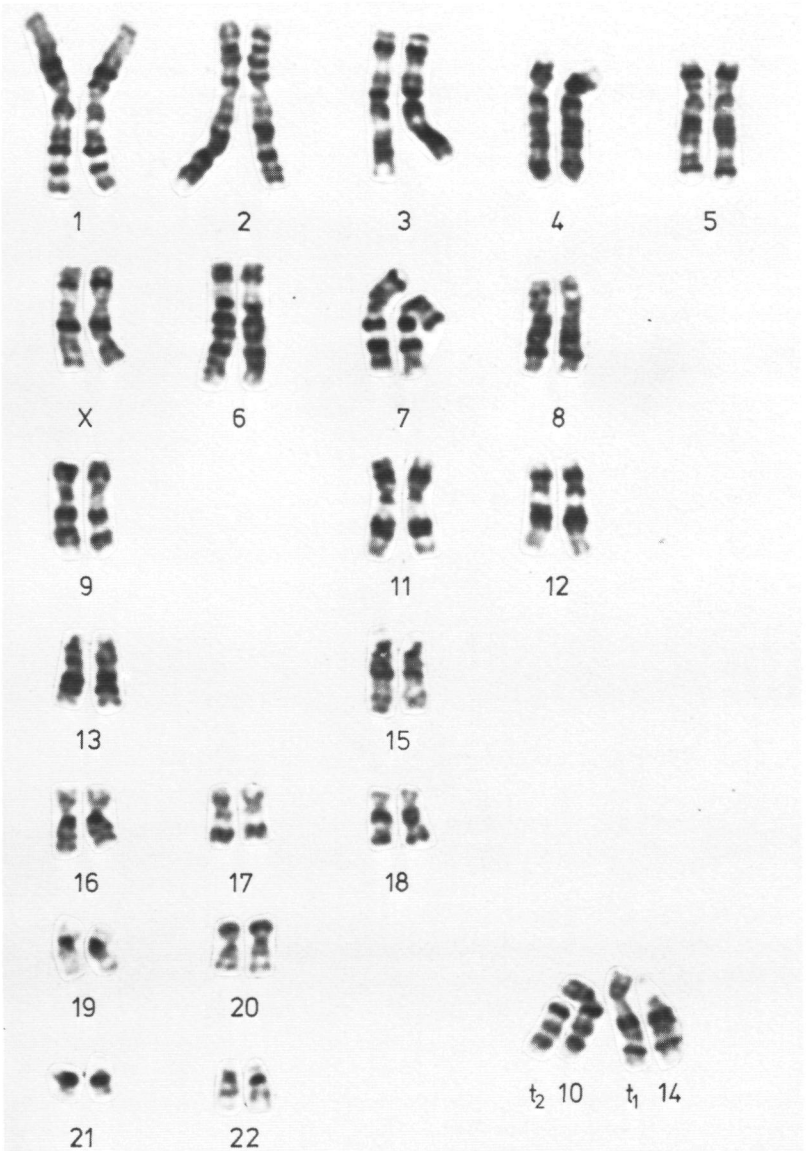

Fig. 5. Karyotype of the proband's mother: t_1 is the large and t_2 the small translocation element

have been replaced by a C group chromosome. A study of the fibroblast metaphases, using the trypsin-Giesma staining method, showed that the missing D chromosome was a chromosome No. 14 and that the extra C chromosome consisted of the long arm of

Fig. 6. A metaphase of the proband's mother. Note the association between t_2 and a chromosome No. 21 (arrow)

chromosome No. 14 and probably of the short arm of chromosome No. 10 (Fig. 4).

When examined in the classical way, no chromosomal aberrations were found in the lymphocyte metaphases of the proband's parents, but the trypsin-Giemsa staining method showed that the lymphocytes of her mother had two abnormal chromosomes. These abnormal chromosomes were also present in the proband's phenotypically normal younger brother. These persons proved to be carriers of a reciprocal translocation between a chromosome No. 10 and a chromosome No. 14 (Fig. 5). The large translocation element had a long arm of a chromosome No. 14 and a short arm of a chromosome No. 10. The small translocation element had a long arm of a chromosome No. 10 whereas satellites were seen on the short arm; in a number of cells, this abnormal chromosome was found in association with a D or G group chromosome (Fig. 6).

The points of breakage in the chromosomes Nos 10 and 14 are in or near the centromeres of these chromosomes, but their exact location could not be established

The parents and the only sib (a sister) of the proband's mother had normal karyotypes

Discussion

The proband is trisomic for at least the larger part of the short arm of chromosome No 10 and hemizygotic for at least a segment of the short arm of chromosome No 14 The uncertainty with regard to the accurate exchange points does not allow a more precise description of the proband's chromosomal aberration

The most notable symptoms in our patient are serious intrauterine growth retardation, typical facial abnormalities consisting of hypertelorism, a broad nasal bridge, bilateral epicanthal folds, low-set dysplastic ears, retrognathia, palatoschizis, joint abnormalities and cystic kidneys In addition, a thrombocytopenia was present

The only symptoms common to our patient and to the 46,XY/47,XY,+10 patient of Higurashi et al (1969) and Nakagome et al (1972) are hypertelorism, low-set dysplastic ears, retrognathia and joint abnormalities A few patients with a mosaic trisomy C (Bijlsma et al 1972) show more similarity with our patient The most striking resemblance in symptomatology exists between our patient and that described by Juberg et al (1970) both had intrauterine growth retardation, bilateral epicanthal folds, hypertelorism, a depressed nasal bridge, low-set ears, palatoschizis, micrognathia, articular abnormalities (e g hip dislocation), bilateral polycystic kidneys, and death shortly after birth Reinwein et al (1966) reported on a dysplastic child that died 10 min after birth In addition to multiple malformations of the face and limbs, cystic kidneys were found in this patient Analysis showed the patient to have a mosaic chromosomal pattern with a cell line having an extra C group chromosome and another with an extra chromosomal fragment

Pfeiffer & Meyer (1967) found cystic kidneys in a child that died 2 h after birth A chromosomal analysis was not carried out, but the patient's mother had a translocation between a C group chromosome (No 10) and a D group chromosome Possibly this patient was trisomic for part of the short arm of chromosome No 10 The interpretation of the translocation in which the mother's C chromosome was involved was made before the introduction of the banding techniques

As far as we know, our patient is the first proven case of a trisomy 10p In view of the symptomatology of this, and of some selected cases reported to have extra C chromosome material (Juberg et al 1970, Pfeiffer & Meyer 1967, Reinwein et al 1966), early death from respiratory distress and the presence of bilateral cystic kidneys may form part of a syndrome characteristic for a trisomy 10p However, the symptomatology of such a syndrome can only become known when more proven cases have been described

Since the proband's maternal grandparents have normal chromosome patterns, her mother is the first person in the pedigree to have this reciprocal translocation The balanced carriers of a translocation, as reported in our proband's family, cannot be discovered with the classical staining technique The importance of the use of chromosome banding methods in the genetic counseling of such cases is evident

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PAPER IX

A Presumptive Tetrasomy for the Short Arm of Chromosome 9

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Summary For the identification of an extra chromosome in a female child a number of recently developed staining techniques were used

The extra element found in 86% of the patient's peripheral lymphocytes resembles an isochromosome for the short arm of chromosome No 9, in which case a tetrasomy for this arm would exist The clinical findings in our patient were compared with those in patients reported to have extra chromosome 9 material

Introduction

Ghymers *et al* (1973) described a male child with multiple congenital malformations having a partial tetrasomy of chromosome No 9 in his lymphocytes, the karyotype of the fibroblasts from a skin biopsy was normal

We report on a child with multiple congenital malformations in which an extra chromosome of the size of chromosome No 16 occurred in 86% of her lymphocytes The application of a number of recently developed staining techniques enables us to interpret—with a fair degree of certainty— the extra chromosome to consist of double the material of the short arm of chromosome No 9, resulting in a tetrasomy for this arm

Case Report

The proband (H A 660203), a girl, is the second child of a mother who was 31 at the time of delivery and a father who was then 32 The mother's first pregnancy had ended in a spontaneous abortion Between the abortion and the proband's birth a phenotypically normal boy was born to the unrelated parents He was delivered by Caesarean section because of a narrow pelvis The third pregnancy was complicated around the 12th week by severe loss of blood per vaginam requiring a 3 week hospitalization period After discharge she had taken Duphaston® for 3 months The pregnancy was terminated by vacuum extraction The child's weight at birth was 3400 g and no asphyxia or jaundice was reported during the newborn period

The first months of life caused few problems The parents noticed, however, a slower development of the proband compared with their first child A substantial increase of her head circumference was observed for the first time at the age of 8 months Although no marked increase of the head circumference was seen in the next 2 years, a connection between the retarded psychomotoric development and a possibly existing hydrocephalus was suspected At the age of 2 5 years when the fontanel was still 3 × 4 cm, lumbopneumoencephalography confirmed the diagnosis hydrocephalus A Spitz-Holter drain was then inserted As she was

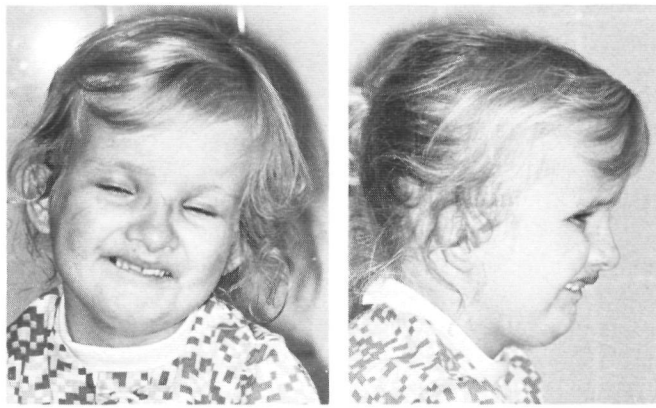

Fig. 1. The proband at the age of 7.5 years

still unable to walk at the age of 3 because of a flaccid paresis of the leg musculature, a training program was begun with the help of braces.

At the age of 4.5 years she started wearing spectacles for her marked myopia (—15 and —18). Speech development was very poor and at the age of 5 years she was only able to say a few words. Her hearing seemed unimpaired. A psychological examination at the age of 5 years showed an intellectual level of a 3-year-old. Her mental capacities were those of an imbecile (Stutsman score: 41; Terman-Merill score: 48). Her social adjustment, however, developed rather well.

She was able to sit with help at the age of 1 year, sit-up unaided when she was 2 years old, stand upright at the age of 3 years; she was able to say a few words at 4 years old and became continent in the daytime at the age of 6.

Myopia (ranging from —4 to —11) occurs frequently among the relatives of the proband's mother. A first cousin of the mother suffers from osteomalacia and another one is a Down's syndrome patient. A first cousin of the proband's father died 2 days after birth from complications of a spina bifida. One of the father's brothers has both a boy with a cheilognathopalatoschizis and one with Down's syndrome.

At the age of 6 the proband was again fully investigated: she measured 106 cm ($< P3$), weighed 18 kg, and had a head circumference of 55 cm ($> P98$). Her forehead was prominent and the fontanels were closed. She showed hypertelorism with bilateral epicanthal folds, a convergent squint and a highly arched palate. Both external ear canals were narrow, the auricles slightly abnormally shaped and protruding (Fig. 1).

The mandible was small, but X-ray examination showed all permanent teeth to be present. A grade II cardiac murmur with a marked degree of splitting of the second sound was heard. A thorax X-ray examination and an electrocardiogram showed no abnormalities. The liver extended 1 cm below the right mid-costal margin and the spleen was only barely palpable. The external genitalia showed no anomalies. The fifth fingers were short and curved which at X-ray examination appeared to be the result of a brachymesophalangy. Roentgenological examination showed a pseudo-arthrosis of the right clavicle without callus formation, no deformities of the spine or abnormalities in the intravenous pyelogram. Osseous maturation was between 6 and 7 years. She had pedes planovalgae.

A neurological examination showed the cranial nerves intact, a normal muscle tone, a slightly lowered muscular strength but no signs of atrophy. The plantar reflexes were of normal intensity and symmetrical. The electroencephalogram was underdeveloped for the patient's age and on the left side a little slower than on the right one.

The laboratory examinations of blood and urine showed no abnormalities, in particular with regard to blood clotting, glucose tolerance test, lipid composition, amino acid excretion, the Alcian blue test, and metachromatic material Syphilis, toxoplasmosis, and leptospiroses could be excluded

Dermatoglyphic Studies

The dermatoglyphic patterns of hands and feet of the patient are outlined in Fig 2 Table 1 contains the ridge counts of the fingers and toes The a—b ridge counts of the hand palms are 40 on the right and 39 on the left side The soles of the feet have simian creases

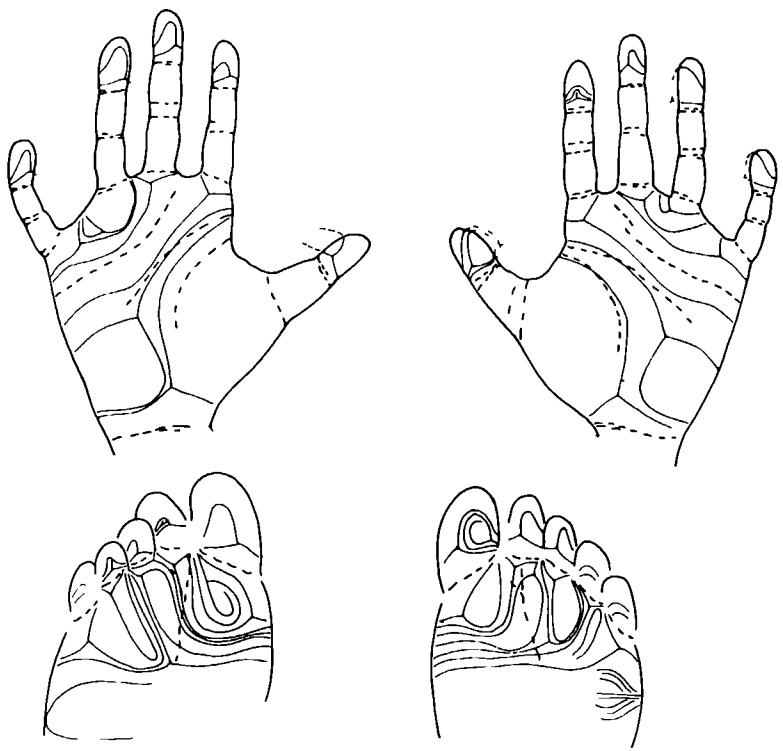

Fig 2 The dermatoglyphic patterns of hands and feet of the patient

Table 1 The ridge counts on the patient s fingers and toes

	Left					Right					Total ridge count
	I	II	III	IV	V	I	II	III	IV	V	
Fingers	15/0	5/0	9/0	13 0	10/0	20/17	0	5/0	10/0	6/0	93
Toes	15/0	1/0	6/0	0	0	11/6	7/0	7/0	0	0	47

Table 2 The phenotypes of the blood groups and serum factors of the patient and her parents

Patient	A_1	MNS—	P_1(+)	Lu(a—)	CcDee	K	Fy(a+)
Father	A_1	MNS	P_1 +	Lu(a)	ccDee	K—	Fy(a+)
Mother	A_1	MNS—	P_1( )	Lu(a—)	CcDee	K	Fy(a+)

Patient	Gc1—1	Hp2 1	Gm(z—a—x—f—n—g—b+)	Inv(1—)	Am(1+,2)
Father	Gc1—1	Hp2 1	Gm(a x f n g b+)	Inv(1 a—)	
Mother	Gc1 1	Hp2—1	Gm(a—x—f+n—g—b+)	Inv(1—a—)	

Blood Groups and Serum Factors

The phenotypes of the blood groups and serum factors of the patient and her parents show no deviation from the expected inheritance pattern (Table 2)

Cytogenetic Studies

Interphase nuclei of buccal smear cells were examined for the presence of X chromatin (orcein staining) and of Y chromatin (QM fluorescence method of Caspersson *et al*, 1970)

Chromosome examinations of the patient and her parents were carried out on lymphocyte cultures using a modification of the method described by Moorhead *et al* (1960) The chromosome metaphases of these cultures were stained according to the classical method (Turpin and Lejeune, 1965) as well as with a modification (Scheres, 1972) of Seabright's method (1972) The patient's metaphases were also examined with the use of the fluorescence method (Caspersson *et al*, 1970), the Giemsa 11 staining method (Bobrow *et al*, 1971), the CT staining method according to Scheres (1974) and with an experimental method staining the centromeric regions of a number of chromosomes, mainly those of Nos 16, 19, and 20 (Scheres, unpublished results)

Results

In 55 of 100 examined interphase nuclei of buccal smears X chromatin was present Quinacrin mustard fluorescence of buccal smears interphase nuclei showed no Y chromatin

In 92 of 107 (86%) lymphocyte metaphases examined, an extra chromosome was found Studied after classical staining, the extra chromosome resembled chromosome No 16

After analyzing metaphases using the trypsin Giemsa method, the extra chromosome was believed to consist of double the material of the short arm of chromosome No 9 (Figs 3 and 4)

More credence was attached to this supposition after the extra chromosome was examined with the QM fluorescence method and the CT staining technique (Figs 5 and 6) the latter shows both C and T band characteristics The extra chromosome did not have the paracentromeric heterochromatin typical for the long arm of chromosome No 9 (Giemsa 11 staining method) or that of chromosome No 16 (Fig 7)

The patient's parents did not have chromosomal aberrations

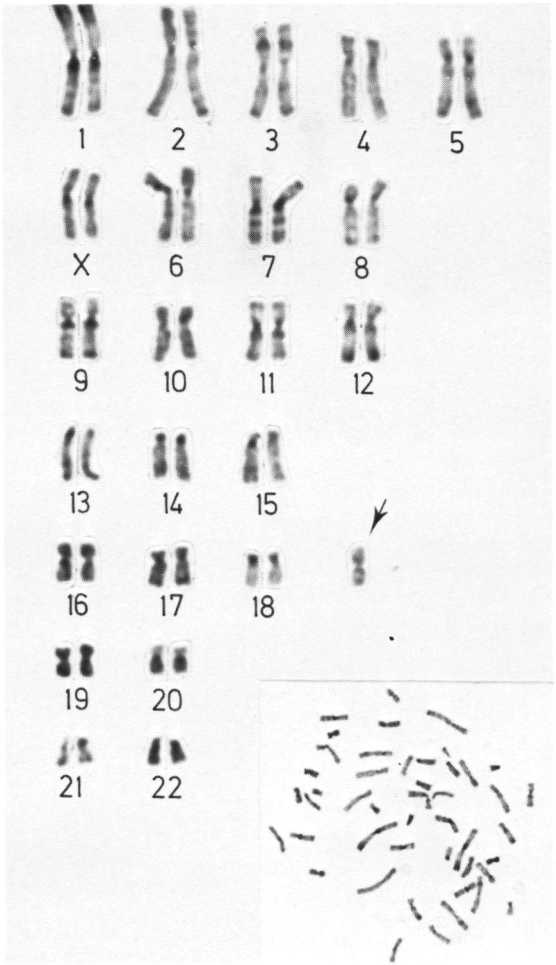

Fig. 3. Metaphase and karyogram of a cell with 47 chromosomes stained according to the trypsine-Giemsa technique

Fig. 4. Examples of the extra chromosome from different metaphases. The upper row shows the extra chromosome compared to a normal No. 9 chromosome from the same cell (trypsine-Giemsa technique)

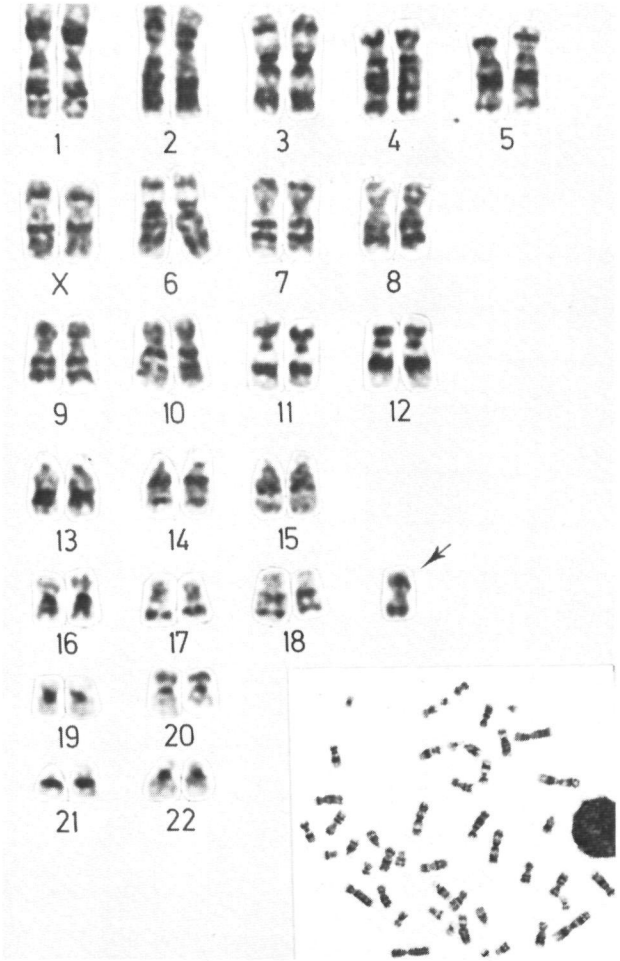

Fig. 5. Karyogram of a cell with 47 chromosomes (CT banding technique)

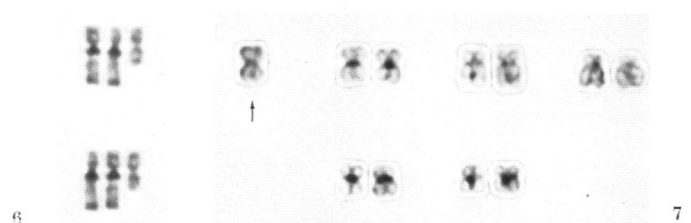

Fig. 6. Comparison of the extra chromosome with the short arms of both chromosomes No. 9 (detail of Fig. 5)

Fig. 7. Extra chromosome (arrowed) and chromosomes Nos. 16—20 (E and F groups) from a cell treated with a technique preferentially staining the centromeric regions of chromosomes Nos. 16, 19, and 20. Note absence of centromeric staining in the extra chromosome

Discussion

An extra chromosome was present in 86% of the lymphocyte metaphases of the patient We consider the probability to be very high that this extra chromosome consists of double the material of the short arm of chromosome No 9, perhaps an isochromosome 9p, however, with the methods available no complete certainty as to the correctness of our interpretation can be obtained

Ghymers *et al* (1973) described a patient with an extra chromosome in his lymphocyte culture The extra chromosome not only had twice the short arm of a chromosome No 9 but also double the paracentromerically situated heterochromatin of the long arm of that chromosome The extra chromosome was not present in cultures of his fibroblasts A hydrocephalus internus is the only abnormality common to Ghymer's patient and ours The dissimilarity in symptomatology between the 2 patients might be explained by a difference in distribution of the pathological cell lines in the patient's body and/or in the composition of the extra chromosome Feingold and Atkins (1973) described a trisomy 9 patient who resembled ours in having severe mental retardation, hydrocephalus and retrognathia Haslam *et al* (1973) reported a patient with a mosaic 46,XY/47,XY,+9, comparing this patient with our case, we found that the following clinical symptoms were similar severe mental retardation, short statute, receding chin, high arched palate, and hydrocephalus The occurrence of a hydrocephalus in the patients described by Ghymers *et al* (1973), Feingold and Atkins (1973), Haslam *et al* (1973) and in this paper is noteworthy

Rethoré *et al* (1973) characterize the clinical entity found in patients with a trisomy 9p as follows severe mental retardation (IQ + 50), moderate microcephaly, enophthalmus, antimongoloid slant of the palpebral fissures, mild hypertelorism, protruding ears, aplasia of the central area of the antihelix, globulous nose, downward slanting of the corners of the mouth, hypoplasia of a number of phalanges, abnormal transverse palmar flexion creases, absence of a digital triradius or fusion of the triradii b and c and unequal distances between the flexion creases of the fingers Of the symptoms listed by Rethoré *et al* (1973), the patient described in this paper had severe mental retardation, mild hypertelorism, protruding ears, hypoplasia of the triradius c in the left palm Our patient only slightly resembles those described in Rethoré's *et al* (1973) article on trisomy 9p patients

With regard to the origin of a chromosome pattern as found in the described patient, several hypotheses can be made, the most likely ones appear to us to be

1) A disturbance of meiosis I on the paternal or maternal side in which two of the four chromatids of a bivalent 9 are rearranged in such a way that a chromosome is formed that morphologically can be interpreted as an isochromosome This chromosome, together with a normal chromosome No 9, would then go to 1 gamete as a result of a faulty distribution The mosaic pattern may be the result of a loss of the abnormal chromosome during subsequent mitoses

2) A selective endoreduplication involving the entire short arm of chromosome No 9 and its centromere during meiosis I or during one of the postzygotic mitoses

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Trisomy-9 in the Bone Marrow of a Patient with Acute Myelomonoblastic Leukaemia

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SUMMARY The case history of an adult patient with acute myelomonoblastic leukaemia is presented. The leukaemia developed 4 yr after the first irradiation for a Wilms' tumour. Approximately 35% of the bone marrow cells had 47 chromosomes and were trisomic for chromosome 9.

Deviant karyotypes of bone marrow cells occur in a significantly higher percentage of patients with acute myeloblastic leukaemia (AML) than in healthy individuals or in patients without diseases of the haemopoietic system (Sandberg *et al*, 1961; O'Riordan *et al*, 1970). Although hypoploidy is found in most patients with numerical aberrations of the chromosome pattern, patients with hyperploid cell lines have been described as well (e.g. Sandberg *et al*, 1964, 1968; Sandberg & Hossfeld, 1970). In a few patients with myeloproliferative diseases, hyperploid bone marrow cells have been fully analysed (de la Chapelle *et al*, 1972; Hellström *et al*, 1971). Trisomy of chromosome 8 has been found in these cases. The occurrence of a high percentage of trisomy-9 bone marrow cells was observed in the patient with acute myelomonoblastic leukaemia described in this paper.

CASE HISTORY

The patient, a man born on 26 June 1939, was submitted to surgery on 14 February 1969 for a tumour of the right kidney which proved to be a Wilms' tumour. Postoperative irradiation was given over a period of 6 weeks, using orthovoltage apparatus (half-value thickness 3 mm of Cu) and applying a mid-line depth dose of 2000 rads at the level of the renal fossa.

Lung metastases were diagnosed in September 1971. The patient first received a 5-day course of 500 μg actinomycin-D intravenously daily. Afterwards he was treated by irradiation of the lungs, using telecobalt apparatus, 2250 rads were applied for 24 days, and by further administration of actinomycin-D (5 × 500 μg). In November 1971 he received a supplementary 1000 rads in 5 days to the left lung together with 5 × 500 μg actinomycin-D. A metastasis in the second cervical vertebra was detected in December 1971. Local irradiation was given, applying 3000 rads in 2 weeks. At the same time the patient received 5 × 500 μg actinomycin-D. In February 1972 a metastasis in the right phrenicocostal sinus and one above the right hemidiaphragm required supplementary application of 1500 rads and intravenous administration of 6 × 500 μg actinomycin-D. The patient then remained free from symptoms until

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September 1972, when pain in the right leg developed. This proved to be due to metastatic growth in the pubic bone. The pain disappeared following the local application of 3000 rads.

Late in November 1972 the patient developed spontaneous cutaneous haemorrhages of the arms and legs and fever. He was admitted to hospital on 15 December His temperature was 39 2°C No pathological lymph nodes were palpable. There was gingivitis and a white film was observed in the right tonsillar fossa Crepitating rales were audible over both lungs The liver was palpable 4 cm below the costal margin The spleen was not palpable

Laboratory findings. ESR 110 mm in 1 hr; Hb 6 9 g/100 ml; PCV 20%. WBC 41 000/μl with 45% myeloblasts. Platelet count 8000/μl. Alkaline phosphatase 4.3 units (normal value up to 3.5 units). Serum asparate aminotransferase (SGOT) 42 u/l (normal value up to 25 u/l); serum alanine aminotransferase (SGPT) 42 u/l normal value up to 25 u/l); serum lactate dehydrogenase 464 u/l (normal value up to 175 u/l), serum creatinine 60 mg/l (norml value up to 12 mg/l) *Candida albicans* was cultured from a throat swab. Blood, sputum and urine cultures remained negative. The bone marrow proved to contain more than 90% myelomonoblasts (Fig 1).

The condition was diagnosed as acute myelomonoblastic leukaemia. The patient was given blood transfusions and antibiotics from 15 December 1972 Cytostatic medication was started on 21 December: 2 × 200 mg cytosine arabinoside being given daily by intravenous injection. The temperature remained very high The patient developed marked dyspnoea with signs of pericarditis and cardiac decompensation and died on 24 December 1972.

The post-mortem revealed fibrous pericarditis and pleurisy. The pericardial cavity contained 450 ml haemorrhagic fluid. The liver was enlarged (2340 g) and there were several subcapsular metastases with a diameter of 3–4 cm which at microscopic examination proved to originate from the Wilms' tumour. In addition there were two small metastases in the lung, which was oedematous. The abdominal cavity contained 1.5 litres of haemorrhagic fluid. Small haemorrhages were observed in the epicardium, subpleural space, liver, spleen, peritoneum, duodenum, jejunum, ileum, colon, gingiva and the enlarged left kidney.

CYTOGENETIC STUDY

Direct chromosome preparations were made from bone marrow obtained 21 December 1972 using a modification of the technique of Tjio & Whang (1962). In Unna blue-stained specimens, 13 of the 53 euploid cells found were microscopically analysed. One of these cells was karyotyped. The chromosome pattern in these cells was interpreted as normal for a male. In 29 additional cells found in the same specimens, the number of chromosomes proved to be 47 Five of these cells were microscopically analysed, and an extra C-group chromosome was found in each. No other numerical or structural abnormalities of the chromosomes were observed Five cells with 47 chromosomes were fully typed after staining by a modification (developed in our laboratory) of the trypsin-Giemsa 'banding' technique (Scheres, 1972). Each of these cells contained three no. 9 chromosomes. Expansion of the study by the staining which is characteristic for the secondary constriction area of chromosome 9 (Bobrow *et al*, 1972) confirmed this finding (Fig 2).

DISCUSSION

The patient described showed three unusual features. The first lies in the relative rarity of the

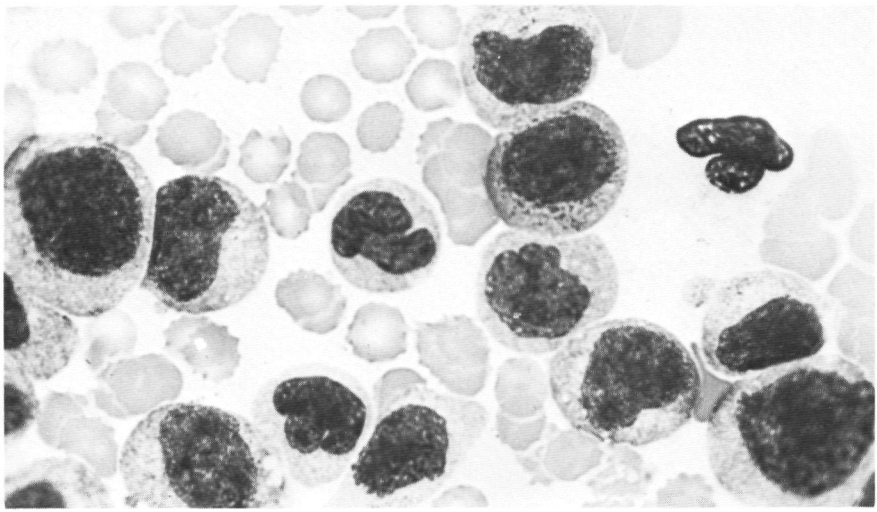

FIG 1. Bone marrow (May-Grünwald, × 800).

FIG 2. Bone marrow cell metaphase stained by the method of Bobrow *et al* (1972). Although the general appearance of the chromosomes is fuzzy, three C-group chromosomes clearly show the dark spots considered typical for the secondary constriction areas of chromosome 9.

(*Facing p* 392)

tumour type for his age (29 yr). A collected series of 1106 cases of Wilms' tumour reported by Klapproth (1959) included 49 cases in adults. The second unusual feature is the development of a second malignancy in the same individual. It may well be that the acute myelomonoblastic leukaemia resulted from the renal fossa irradiation carried out 4 yr earlier. The subsequent irradiations and the actinomycin-D therapy may also have exerted an influence. As Court Brown & Doll (1965) have demonstrated, the risk of dying from leukaemia is maximal 3–5 yr after irradiation. The type of leukaemia commonly seen in such cases is the acute myeloid form. This, too, is an argument in favour of irradiation as an aetiological factor. The third unusual feature is the presence of trisomy of chromosome 9 in *c* 35% of the bone marrow cells. So far as we could ascertain, this chromosomal aberration has not previously been established with certainty in a patient with AML.

Deviant karyotypes are known to occur frequently in the bone marrow in patients with AML, and the anomalies are very variable (Woodliff, 1971). In a large number of patients with AML a statistical analysis has been made (Sandberg *et al*, 1968) in an effort to establish whether one or more groups of chromosomes were involved in the disease process more frequently than might be expected according to the laws of probability. This analysis was particularly directed to the possibility of a disproportionately frequent occurrence of C-group and G-group aberrations. However, Sandberg and his co-workers concluded that none of the chromosomal groups showed numerical aberrations more frequently than might be expected from the number of chromosomes in each group. It is possible, however, that individual chromosomes are more frequently absent or, on the other hand, occur in numbers larger than normal. Sandberg *et al* (1964) suggested trisomy of chromosome 6 or of chromosome 9 in a few instances, but no complete certainty could be obtained by conventional methods.

Identification of each of the human chromosomes is now possible, since the introduction of banding techniques. By the combined use of the trypsin–Giemsa technique and secondary constriction 9 staining, trisomy of chromosome 9 was clearly shown in the patient with AML described in this paper. The possibility of a certain role of chromosome 9 in diseases of the haematopoietic system has recently also been pointed out by Rowley (1973). In nine consecutive patients with chronic myelogenous leukaemia and with one or more Ph' chromosomes an amount of additional material approximately equal to the amount missing from the Ph' chromosome appeared added to the long arm of chromosome 9. This suggests a translocation between the long arm of 22 and the long arm of 9, producing a 9 q+ chromosome.

A possible correlation between chromosomal aberrations on the one hand and myeloproliferative changes on the other can be established only after a study of a large number of bone marrow specimens from patients, and identification of each and every supernumerary or absent chromosome.

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At the end of this thesis it appears desirable to make a few remarks on the most important results described in its various Papers.

The first aim of the studies carried out has been the development of differential chromosome staining methods for use in diagnostic or in more fundamental cytogenetics. The six new staining techniques described in Papers I through V resulted from these efforts. One of the methods, viz. the trypsin-Giemsa technique for the staining of G-bands (Paper I), is at present routinely used in our diagnostic cytogenetic laboratory (see for example Papers I, VIII, IX and X). The CBS-, CBF-, RBS- and RBF-techniques (Papers IV and V) are being used, although less frequently, to study structural chromosome aberrations in order to determine as exactly as possible the points of breakage and the position of the centromeres. These methods may be of special importance when the terminal chromosome regions are affected. The latter areas often stain only faintly with G-banding methods, but in contrast can be made clearly visible and recognizable using the new R- and CT-banding techniques described above.

The method for specifically staining the short arms of certain acrocentric variants (the ABS-technique, Paper IV) appears, at least for the time being, of less diagnostic significance as the variants distinguishable with this technique occur only infrequently. However, in more fundamental cytogenetics this method might be very useful to study the variant areas both in metaphase and interphase nuclei. At present the location of the "ABS-chromocentres" in the interphase nuclei of phytohaemagglutinin-stimulated lymphocytes is being investigated in our laboratory. These chromatin bodies seem often associated with the nucleolus, as has also been reported for the Y-chromatin (Bobrow et al., 1971), and for the chromocentres which can be made visible with the aid of the so-called N-banding technique (Funaki et al., 1975).

The ABS method might also be useful in the study of non-disjunction during male meiosis. Thus far the detection of specific chromocentres in human spermatozoa has only been possible for the Y-chromatin and for the heterochromatins of chromosomes Nos. 1 and 9. Using the Nos. 1-, 9-, and Y-staining methods, non-disjunction rates of one to two percent for each of these chromosomes have been found (Pearson et al., 1973). If for the remaining chromosomes the non-disjunction rate would be similar, approximately half

of the human spermatozoa would have a numerical chromosome aberration. ABS-staining of the spermatozoa of individuals with ABS-positive variants should enable us to determine the non-disjunction rate of five additional autosomes, the chromosomes Nos. 13, 14, 15, 21 and 22. Such a study might provide a means to obtain evidence in support of or in disagreement with the hypothesis that a very high percentage of the human spermatozoa does not have the correct chromosome number.

The ABS-technique might also be used as a supplementary method in the comparison of the karyotype of man and those of the primate species closest related to him (see, e.g , Grouchy et al , 1973, Pearson, 1973) Such investigations might reveal whether or not the type of chromatin characterized by the ABS-method occurs only in man

In this postscript the trypsin--basic-fuchsin technique (GTF-method) deserves some further comments As discussed in Paper II, the basic fuchsin-stained G-banding pattern has certain characteristics which make the GTF-method in some way preferable to the GTG-banding techniques. However, the possibility to use the GTF-method in automated chromosome analysis was not mentioned in that paper. As in many other branches of cytology efforts are being made in diagnostic cytogenetics to use automation in the time-consuming microscopic examination of chromosome preparations. At present, efficient automation is restricted in its application by, amongst others, the inadequacy and the insufficient standardization of the preparative and the staining phases Particularly the often very fuzzy appearance of GTG-stained chromosomes may be a hindrance. The sharp outline of chromosomes stained with the GTF-method may make this technique a valuable tool for a further standardization in automated chromosome analysis

The second purpose of our investigations has been the study of the physico-chemical nature of the chromosome bands. Although each new staining result obtained did mean another phenomenon to be explained, our experiments have nevertheless provided a number of valuable data which might contribute to a clarification of the mechanism(s) of band formation. For example, it has been clearly demonstrated that the ability to produce G-bands is not restricted to Giemsa or related Romanowsky stains but that also those of an entirely different class, viz triphenylmethane dyes, can very well be used. Using the latter staining compounds to obtain bands, similar structural changes occurred in the chromosomes as with Giemsa. This means that a specific interaction be-

tween chromatin and the various components of the Giemsa mixture (Crossen, 1973, Sumner and Evans, 1973; Meisner et al , 1974) does not fully explain the process of G-band formation

The lack of information on the interaction between the complex Giemsa dye and the chromatin has always been a problem in the study of the banding mechanism. The GTF-method might simplify this problem as it has now become possible to produce distinct G-bands with basic fuchsin, a less complex stain than Giemsa, or even with a single component of basic fuchsin, e.g , pararosaniline (Paper II). The binding of basic fuchsin or of its constituents to chromatin, as occurring in the Feulgen reaction, is one of the most intensively studied histochemical processes. The mode of interaction between basic fuchsin and the chromatin in chromosome banding might well differ from that in the Feulgen reaction. Even so, the use of this dye may provide a means to better interpret the differential chromosome staining phenomena by taking advantage of the knowledge obtained from previous Feulgen studies.

Another interesting observation made (Papers VI and VII) is that cations may play an important role in chromosomal band formation These ions were already known to affect chromosome morphology (Maio and Schildkraut, 1967, McKay, 1973) and in some cases were able to suppress the production of bands (Miller, 1973). Our discovery reported in Paper VI, that alkaline denaturation of chromosomes in the presence of monovalent cations induces a G-banding pattern and that divalent cations have the opposite effect (R-banding pattern), shows very clearly that cations may indeed be of considerable importance in differential chromosome staining As suggested in Papers VI and VII this is probably connected with a chromatin stabilization by the divalent cations. It also indicates that, in addition to the already known differences between band- and interband regions, e.g , in the base composition and repetitivity of their DNAs, etc. (see General Introduction 1.5.), these chromosomal segments vary in their capacities to bind cations. It will be our aim to determine in further studies the reason(s) for the observed differences in cations binding properties It is also intended to investigate how divalent cations stabilize chromatin and protect it from denaturation by alkali (Paper VI)

Since the introduction of biological staining the microscopist has learned to rely on staining results which he was unable to explain A good example is the May-Grunwald--Giemsa technique which has considerable value for dif-

ferentiating blood cells and therefore is a major tool in medicine. Another example is Gram's staining method, one of the most frequently used techniques in bacteriology. The mechanisms of both staining methods are still unknown. A similar situation has now developed in cytogenetics. Although the mechanism responsible for differential chromosome staining is as yet not understood, the new methods have already revolutionized cytogenetics, especially human cytogenetics, and have become indispensable in the continuing search for a better understanding of the fine structure, the molecular organization, the behaviour and the function of the mammalian chromosome.

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In this thesis a number of new techniques has been described for the differential staining of human chromosomes:

1. A trypsin-Giemsa method to stain G-bands (Paper I).
2. A second technique to produce G-bands. In this method, which also uses a trypsin pretreatment, the chromosomes are not stained with Giemsa, but with the triphenylmethane dye basic fuchsin (BF). The BF-banding gives more information than G-banding with Giemsa, as, in addition to G-bands, also certain Q- and C-band variants can be distinguished (Paper II).
3. The CT technique, in which both C-bands and some "reverse",mainly telomeric (T-)bands are stained with the cationic dye "Stains-all". In contrast to other C-banding methods, the CT technique permits the identification of all chromosomes (Papers III and IV).
4. A new R-banding technique, which is a modification of the CT technique. As the secondary constriction of chromosome No. 9 stains positive, the pattern obtained differs from the "standard" R-banding pattern (Papers IV and V).
5. A method for specifically staining the short arms of certain acrocentric variants. It also permits the detection of these variant regions in interphase nuclei (Paper IV).

In addition, the staining experiments described in this thesis have provided some interesting data concerning the possible mechanism(s) underlying the differential staining phenomena:

1. In contrast to data from literature, a few other dyes than Giemsa or related Romanowsky stains may well be used for nonfluorescent banding of human chromosomes. For instance, the cationic dyes "Stains-all" and basic fuchsin are very suitable for R- and CT-banding. In addition, basic fuchsin and each of its component triphenylmethanes produce excellent G-banding patterns (Papers II, IV and V).
2. G-band formation with basic fuchsin produces similar structural changes in the chromosomes as are known to occur after G-banding with Giemsa: after staining the positive band areas appear as raised structures (Paper II).
3. The CT technique reveals both C- and T-band characteristics in human chromosomes. This simultaneous presence of two types of bands can be explained as follows:

- the alkaline pretreatment used in the CT technique induces an R-band pattern in which the centromeres remain negative after staining,
- the subsequent salt incubation makes the centromeric regions heavily stainable, the reverse bands, however, become weaker and of these mainly the telomeric bands remain (Paper IV)

4 Cations play an important role in the band formation with alkali If only monovalent cations are present during alkaline pretreatment a rapid chromosomal disintegration occurs, if the pretreatment is short enough, a G-banding pattern is obtained However, if divalent cations are present, some chromosomal areas become markedly resistant to alkali, and the opposite pattern, viz R-banding, is obtained. These results are probably caused by a chromatin stabilization by divalent cations (Paper VI)

5 Cations are also important factors in the production of C-bands With the exception of Ag-ions, all other monovalent cations tested were able to induce C-bands in $Ba(OH)_2$-pretreated chromosomes, caesium cations were the most effective In contrast, di- and polyvalent cations, and Ag-ions were not suitable for C-band formation Ca^{++}- ions were even able to prevent the C-band inducing effect of caesium cations These results are probably also an expression of a chromatin stabilization by di- and polyvalent cations (Paper VII)

In the last part of this thesis three cases are described in which the new differential chromosome staining techniques have been used for cytogenetic diagnosis Paper VIII reports on a chromosomally abnormal child whose parents seemed to have normal karyotypes as judged from conventionally stained metaphases. However, G-band analysis showed the mother to carry a balanced translocation between a chromosome No 10 and a chromosome No. 14

Paper IX describes a child with a presumptive tetrasomy for a part of an autosome (No 9), an extremely rare type of chromosomal aberration

Finally, paper X reports on the use of some differential chromosome staining techniques for the diagnosis of acquired trisomy-9 associated with a certain type of acute leukaemia

Dit proefschrift beschrijft in de eerste plaats een aantal nieuwe technieken voor de differentiele kleuring van menselijke chromosomen:

1. Een trypsine-Giemsa methode voor de kleuring van G-banden (Artikel I).
2. Een tweede techniek voor het zichtbaar maken van G-banden. Bij deze methode worden de chromosomen na voorbehandeling met trypsine echter niet gekleurd met Giemsa, maar met de triphenylmethaankleurstof basische fuchsine. Het met basische fuchsine verkregen bandenpatroon geeft meer informatie dan G-bandering met Giemsa, omdat naast G-banden ook bepaalde Q- en C-band varianten te onderscheiden zijn (Artikel II)
3. De CT-techniek, met behulp waarvan tegelijkertijd C-banden en bepaalde "reverse", voornamelijk telomere banden (T-banden) gekleurd worden met de basische kleurstof "Stains-all". Met de CT-techniek kunnen alle chromosomen worden geidentificeerd, wat bij gebruik van andere C-banderingsmethoden niet mogelijk is.
4 Een nieuwe R-banderingstechniek, die een modificatie is van de CT-techniek. Het hiermee verkregen bandenpatroon verschilt van het "standaard" R-bandenpatroon, omdat de secundaire constrictie van chromosoom no. 9 positief kleurt (Artikelen IV en V).
5. Een methode voor de specifieke kleuring van de korte armen van bepaalde acrocentrische varianten. Met behulp hiervan kunnen de variante gebieden ook in interphase-kernen worden aangetoond (Artikel IV).

Daarnaast hebben de kleuringsexperimenten enkele gegevens opgeleverd omtrent het mechanisme dat aan de chromosomenbandering mogelijk ten grondslag ligt:

1 In tegenstelling tot gegevens uit de literatuur kunnen ook enkele andere niet-fluorescerende kleurstoffen dan Giemsa of verwante Romanowsky-mengsels worden gebruikt voor de bandering van menselijke chromosomen.Zo zijn bijv. de kleurstoffen "Stains-all" en basische fuchsine geschikt voor R- en CT-bandering.Met basische fuchsine en elk van de triphenylmethanen waaruit het is samengesteld kunnen uitstekende G-bandenpatronen worden verkregen (Artikelen II, IV en V).
2. Bij G-bandering met behulp van basische fuchsine treden dezelfde structurele veranderingen in de chromosomen op als na G-bandering met Giemsa· de positieve bandgebieden worden als richels zichtbaar (Artikel II).
3. De CT techniek maakt zowel C- als T-banden in menselijke chromosomen zicht-

baar. De gelijktijdige aanwezigheid van deze twee typen banden kan als volgt worden verklaard:

- de alkalische voorbehandeling van de chromosomen induceert een R-bandenpatroon waarin de centromeren na kleuring negatief blijven,
- door de daaropvolgende zoutincubatie worden de centromere gebieden sterk kleurbaar; het R-bandenpatroon wordt echter zwakker, zodat daarvan alleen de telomere banden overblijven (Artikel IV)

4. Kationen spelen een belangrijke rol bij de bandvorming met behulp van logen. Indien tijdens alkalische behandeling slechts éénwaardige kationen aanwezig zijn treedt een snelle desintegratie van de chromosomen op; is de voorbehandeling kort genoeg, dan wordt een G-bandenpatroon geïnduceerd. Indien echter tweewaardige kationen aanwezig zijn, worden sommige chromosoomgedeelten opvallend alkali-resistent en wordt het tegengestelde, nml een R-bandenpatroon verkregen. Deze resultaten zijn vermoedelijk het gevolg van een chromatine-stabilisatie door tweewaardige kationen (Artikel VI).
5 Kationen zijn ook belangrijke factoren bij de vorming van C-banden gebleken. Met uitzondering van Ag-ionen waren alle onderzochte eenwaardige kationen in staat C-banden te induceren in met $Ba(OH)_2$- voorbehandelde chromosomen; caesium kationen waren in dit opzicht het meest effectief. Twee- en meerwaardige kationen, en Ag-ionen bleken echter niet geschikt voor C-bandering, Ca^{++}-ionen waren zelfs in staat het C-band inducerend vermogen van caesium-ionen te niet te doen. Ook deze resultaten zijn waarschijnlijk een gevolg van een chromatine-stabilisatie door twee- en meerwaardige kationen (Artikel VII)

In het laatste gedeelte van dit proefschrift worden 3 toepassingen van de nieuwe differentiele chromosoomkleuringen in de cytogenetische diagnostiek beschreven. Artikel VIII betreft een chromosomenaberratie bij een kind wiens ouders, volgens analyse met behulp van conventionele chromosoomkleuring, normale karyotypen leken te bezitten G-band analyse bracht echter aan het licht dat bij de moeder een gebalanceerde translocatie tussen een chromosoom no. 10 en een chromosoom no. 14 aanwezig was

Artikel IX beschrijft een kind met een mogelijke tetrasomie voor een deel van een autosoom (i.c. chromosoom no. 9) hetgeen een buitengewoon zeldzame chromosomale afwijking is.

In artikel X tenslotte wordt een voorbeeld gegeven van het gebruik van

differentiele chromosoomkleuringen in de diagnostiek van haematologische stoornissen, in dit geval een acute leukaemie gepaard gaande met een verworven trisomie van chromosoom no. 9.

De schrijver van dit proefschrift werd geboren te Heerlen op 4 mei 1946 als zoon van Josephus Hubertus Scheres en Maria Hubertina Ramakers. Hij doorliep de Lagere School te Pey-Echt (Patricius-school, O.L.V.v.Schilberg-school), bezocht het Bisschoppelijk College te Sittard, en behaalde in 1965 het diploma Gymnasium-B. In september van dat jaar werd hij ingeschreven als student biologie aan de Katholieke Universiteit te Nijmegen, waar hij in juni 1968 het kandidaatsexamen en in november 1970 cum laude het doctoraalexamen aflegde (hoofdvak Genetica, bijvakken Zoölogie en Botanie). Hierna was hij gedurende ongeveer een jaar te Rijswijk wetenschappelijk medewerker van het Instituut voor Experimentele Gerontologie (Gezondheidsorganisatie TNO). In juli 1971 huwde hij te Echt Catharina Johanna Rosalie Heijmans. Sedert augustus van dat jaar is hij verbonden aan de Faculteit der Geneeskunde en Tandheelkunde van de Katholieke Universiteit te Nijmegen; hij maakt als wetenschappelijk medewerker deel uit van de cytogenetische groep van het Anthropogenetisch Instituut.

1

G-bandering van menselijke chromosomen kan ook met andere dan de Romanowsky kleurstoffen worden verkregen.

Dit proefschrift.

2

Bij onderzoek naar het mechanisme van chromosoombandering is het gebruik van enkelvoudige kleurstoffen te verkiezen boven dat van mengsels.

Dit proefschrift.

3

Door behandeling met logen kunnen in menselijke chromosomen bandenpatronen worden geïnduceerd. Daarbij speelt de aard van de aanwezige kationen een belangrijke rol.

Dit proefschrift.

4

Er zijn goede redenen de drie-lettercode voor banderingstechnieken te herzien.

Dit proefschrift.

5

De naam chiasma dient niet te worden gebruikt voor de quadriradiale configuraties zoals die bijvoorbeeld in mitotische metafasen van patiënten met het syndroom van Bloom kunnen worden aangetroffen.

Kunh, E.M.: Chromosoma (Berl.) 57, 1-11 (1976).

6

Chromosomenanalyse is onmisbaar bij onderzoek naar de aetiologie en pathogenese van maligne aandoeningen.

7

Geneesmiddelen dienen ook te worden getest op hun vermogen in menselijke cellen uitwisselingen tussen zusterchromatiden te veroorzaken.

8

Bij het gereedmaken van gebandeerde menselijke karyogrammen voor publicatie dient door deskundigen gewaakt te worden voor fouten tegen de Parijse nomenclatuur.

Het inbrengen van getransformeerde cellen in een embryo vormt een mogelijkheid hun vermogen tot differentiatie te onderzoeken.
Mintz, B., Illmensee, K.: Proc. nat. Acad. Sci. USA 72, 3585-3589 (1975).

10

Analoog aan de regelingen betreffende laboratoriumonderzoek met radioactieve stoffen dient ook het werken met virus aan een vergunningen- en contrôlesysteem te worden onderworpen.

11

Syndroombenamingen die patiënten of hun familieleden onaangenaam kunnen treffen, moeten worden vermeden.

12

Het anoniem publiceren van wetenschappelijk werk maakt promoveren op artikelen onmogelijk zonder afbreuk te doen aan de anonimiteit.
Garfield, E.: Current Contents 19(26), 5-7 (1976).

13

Meer aandacht voor de etymologie op middelbare scholen zal de interesse van de leerlingen in de taal doen toenemen.

14

De laatste stelling bij een proefschrift leidt vaak de aandacht onevenredig veel van de dissertatie af.

Nijmegen, 5 november 1976

Stellingen behorende bij het proefschrift: "Differential staining of human chromosomes" van J.M.J.C. Scheres.