GIEMSA BANDING PATTERNS IN NORMAL DONORS
AND IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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ABSTRACT

A method of obtaining banded chromosomes based on the DNA hybridization technique is described. Chromosome preparations treated with NaOH followed by incubation in phosphate buffer and staining in Giemsa showed distinct banding patterns which enabled their positive identification. Banding patterns from 50 normal donors were examined and a banding pattern for each chromosome established. The banding patterns were compared with those in the literature and the description published by the Paris Conference (1971): Standardization in Human Cytogenetics. There was good agreement between authors regarding the major bands. However, in this study 80 additional bands to those described by the Paris Conference were detected.

An important factor influencing band formation was the removal of proteins during fixation. The method of chromosome spreading was important with flame dried slides giving the best results. The age of the slides was also important, the best banding occurring on week old slides. Romanovsky stains were the only stains to give clear G banding.

Investigations on the nature of G bands indicated they were not composed of histone, but were composed of DNA. The denaturation and reannealing of DNA were not important in band formation and it is suggested that alterations in the configuration of DNA influences band formation.

The banding patterns of chromosomes from 20 patients with chronic lymphocytic leukaemia (C.L.L.) were found to be identical to those of the normal controls. There was a marked difference in size between members of some chromosome pairs in seven of the patients with C.L.L. The normal controls also showed this variation in size but the degree of difference appeared to be greater in the patients with C.L.L.
I. INTRODUCTION

1. PURPOSE OF THE INVESTIGATION

The last 13 years of intensive cytogenetic study has provided much information regarding chromosome abnormalities and leukaemia. Despite this wealth of information the precise role of chromosome abnormalities in leukaemia is still uncertain.

A major limitation of chromosome studies in leukaemia and malignant disorders has been that only gross abnormalities can be detected. These include additional or missing chromosomes and major structural rearrangements. However, point mutations, alterations in base sequence and the possible incorporation of viral DNA into the chromosome cannot be detected. Furthermore, little is known about the organisation of DNA, RNA, histones and acidic proteins within the chromosome, either in malignant or non-malignant conditions.

The description by Yunis et al. (1971), of a method of demonstrating areas of satellite DNA in human chromosomes prompted much of this study. Satellite DNA is a fraction of chromosomal DNA which, after isolation by any method gives a narrow unimodal band in CsCl because of common properties shared by its base sequences (Walker, 1971). Satellite DNA is usually composed of highly repeated sequences and Yunis's method seemed to offer promise as a means of localizing these areas in human chromosomes. The original purpose of this investigation was to study the distribution of satellite DNA in normal and leukaemic chromosomes.

It proved difficult to obtain results, similar to those published by Yunis et al., and the several attempts led to the development of a method, which instead of demonstrating large darkly staining areas of the chromosome, gave discrete banding patterns. The banding patterns were so specific that it was possible to
positively identify each chromosome, a hitherto impossible task.

A major portion of this investigation was therefore directed toward establishing a normal banding pattern, because at the time no normal banding pattern had been published. During the course of these studies various methods of obtaining banded chromosomes, some similar to the one described herein were published. These are referred to in the review of the literature. The majority, including the one described herein were found to be sometimes unreliable and studies were made into factors which might affect the banding mechanism.

In addition to providing a means of positively identifying each chromosome the method also offered clues as to the molecular organization of the chromosome. Investigations were carried out to determine the composition of the bands, i.e. whether they were composed of DNA, RNA, histone or acidic protein.

Once the reliability of the method had been improved and a normal banding pattern established, studies were made on the banding patterns from patients with chronic lymphocytic leukaemia. It was envisaged that chromosomes from these patients might show an altered banding pattern as evidence of their malignant condition. Particular attention was paid to the banding patterns of the G group chromosomes in view of the report of decreased length of the G group chromosomes in patients with chronic lymphocytic leukaemia (Fitzgerald, 1965).
II. REVIEW OF LITERATURE

1. CHROMOSOME IDENTIFICATION - DEVELOPMENT OF TECHNIQUES

Originally, identification of individual chromosomes of the human complement depended on the morphology of individual chromosomes using mainly overall length, centromeric position and to a lesser extent the position of the secondary constrictions. Using these criteria the human chromosome complement was divided into seven groups of which only two, the A group - chromosomes 1, 2 and 3, and the E group - chromosome 16, were further subdivided.

More recent techniques used for chromosome identification are reviewed below.

(a) Autoradiography

Autoradiography using $^{3}H$-Thymidine has been useful in identifying members of some chromosome groups, notably the B, D, E, and G groups, and in females one of the X chromosomes (Schmid, 1963; German et al., 1964; Yunis et al., 1964; Gianelli and Howlet, 1967; Morishima et al., 1962; Fitzgerald, 1971). Unfortunately the technique is laborious and difficult. The status of autoradiographic studies of human chromosomes has been excellently reviewed by Gianelli (1970).

(b) Fluorescence

The development by Caspersson et al. (1968), of U.V. fluorescence of chromosomes stained with quinacrine dyes, represented a major advance, which for the first time enabled the positive identification of both plant and animal chromosomes. Initial studies on hamster and bean chromosomes revealed, that when chromosomes stained with the fluorescent dye quinacrine mustard were viewed under U.V. light, each chromosome had a distinctive fluorescent pattern
(Caspersson et al., 1969). These authors suggested, that because quinacrine mustard contained a mustard group with an affinity for the N-7-atom of guanine (Brooks, 1964), the resultant fluorescent patterns represented differences in the base composition of the DNA in each chromosome. The fluorescent technique was applied to the study of human chromosomes, and with the aid of photoelectric scanning and densitometry each chromosome of the human complement was positively identified (Caspersson et al., 1970). In addition, the Karolinska group identified the Philadelphia chromosome as a No. 22, the extra chromosome in Down's syndrome as a No. 21 and the deleted B group chromosome in the "cri du chat" syndrome as a No. 5 (Caspersson et al., 1970a, 1970b, 1970c). The numbers assigned to each chromosome by the Paris Conference (1971), are based on the fluorescent patterns described by Caspersson et al. (1971).

Caspersson and his colleagues also investigated other DNA fluorescent agents for their banding ability and found similar, but less stable patterns with acriflavine and proflavine using a wide variety of plant and animal material (Caspersson et al., 1969a). Identical patterns to Q.M. were found with quinacrine dihydrochloride (O'Riordan et al., 1971), and these authors also identified the additional chromosome in Down's syndrome and the Philadelphia chromosome. The majority of fluorescent studies have used quinacrine dihydrochloride because of its greater availability. Quinacrine dihydrochloride lacks a mustard group indicating that the suggestion of Caspersson et al. of preferential binding of the mustard group to G-C areas was incorrect.

In a study of seven acridine derivatives Limon et al. (1970) found that only one, (3-Bromo-methoxy-9(4-dimethylaminobutylamino)-acridine-2HCl) stained identically to Q.M., while the remainder
showed overall fluorescence with no banding patterns.

Benzimidazol compound "33258 Hoechst" a fluorochrome totally unrelated to the quinacrine series was used to study the fluorescent patterns of mouse and hedgehog chromosomes (Hilwig and Gropp, 1972). The banding patterns were identical to those produced by the C banding technique of Arrighi and Hsu (1971), i.e. only the centromeric regions fluoresced brightly.

Ethidium bromide, a phenathridium dye gives fluorescent patterns in Trillium and Scilla that are the reverse of those of Q.M. (Caspersson et al., 1969a). Pearson et al. (1971), also reported a reciprocal pattern in the chromosomes of Bennett's wallaby when stained with ethidium bromide. The reciprocal patterns could not be convincingly demonstrated in the chromosomes from other mammals.

Acridine orange which has the remarkable property of fluorescing red in combination with single stranded DNA and green with double stranded DNA gave weak and variable bands with Trillium (Caspersson et al., 1969a). However, Bobrow et al. (1972) found a reverse banding pattern with acridine orange on slides incubated in phosphate buffer at 85°C for 30 minutes prior to staining. Reverse patterns with A.O. have also been reported by Costoldi et al. (1972), who heated slides to 96°C for ten minutes and cooled them to -20°C prior to staining.

(c) Centromeric Banding (C Banding)

C banding of human chromosomes originated with the report of Arrighi and Hsu (1971), of a method of localizing heterochromatin in cytological preparations. The method, essentially a modification of the DNA hybridization technique of Pardue and Gall (1970), consists of treating the slides with HCl, NaOH and RNase, incubating them in
2XSSC at 65°C for 16 hours and then staining in Giemsa. In a later publication it was recommended that the slides be heat-dried at 60°C for 15 minutes prior to treatment (Hsu, 1973). Human chromosomes treated this way show darkly-staining centromeric regions while the remainder of the chromosome is pale-staining. A notable feature of the method is that the secondary constrictions of chromosomes 1, 9 and 16 are intensely stained as is the distal portion of the Y chromosome.

The C banding technique has been applied to a wide variety of mammalian chromosomes and darkly-staining heterochromatin found in all cases (Hsu and Arrighi, 1971). Usually, the darkly staining heterochromatin occurred in the centromeric regions, but in the Chinese hamster it was situated in an interstitial position, while in the Seba fruit bat it was found situated terminally. Some species, e.g. the deer mouse and the Syrian hamster had completely heterochromatic chromosome arms.

Human chromosomes exhibit considerable polymorphism of centromeric heterochromatin (Craig-Holmes and Shaw, 1971). Notable variants were the secondary constrictions of chromosomes 1, 9 and 16 as well as the satellite and centromeric regions of the D, F, and G groups. In a further study (Craig-Holmes et al., 1973), found 31 C band variants among 20 unrelated individuals. In two of the families three of the six variants were transmitted.

The C banding technique has also been used in studies of mouse/human hybrid cells (Chen and Ruddle, 1971). While it was not possible to identify all chromosomes, the method was useful in distinguishing between mouse and human chromosomes.

Gagné et al. (1971) compared C banding patterns with fluorescent patterns by using a double staining procedure. Slides
were first treated by a modification of the technique of Arrighi and Hsu, stained in quinacrine and photographed. They were then washed in ethanol to remove the quinacrine and then stained in Giemsa. These authors found that the characteristic fluorescent patterns had been obliterated by the above treatment and the only chromosomes showing bright fluorescence were the A3, B4, Y and D group chromosomes.

A major drawback of Arrighi and Hsu's method and most modifications was the requirement for long incubation in buffer. This was overcome by the Barium saline Giemsa (BSG) technique described by Sumner (1972). In this technique chromosome preparations are treated with HCl, 5% barium hydroxide at 50°C for 5-15 minutes, followed by a one hour incubation in 2XSSC and staining in Giemsa.

A further rapid C banding technique has been described by Alfi and Menon (1973), who treated slides for five seconds in 0.0175N NaOH, rinsed thoroughly in distilled water and then heated in 12XSSC at 100°C for one minute. This extremely simple technique appears to give results that are comparable with the more tedious techniques.

A variation of the C banding technique has been reported by Bobrow et al. (1972a), who treated chromosome preparations with Giemsa, adjusted to pH 11 with dilute NaOH. The secondary constriction of chromosome 9 stains red with this technique, while the remaining heterochromatic areas are less intensely stained than with the C banding technique of Arrighi and Hsu.

In an analysis of factors affecting C band formation McKenzie and Lubs (1973), reported that good C banding could be obtained by treating heat dried slides with 0.2N HCl at 25°C, followed by incubation in 2XSSC for 18 to 24 hours. A brief exposure to NaOH
was recommended when slides had been air dried or flamed. An essentially similar technique has been reported by Lubs (1973).

A further modification of the C banding technique has recently been described by Forejt (1973). Slides 48-96 hours old are treated with 0.03-0.07M NaOH for 30-90 seconds followed by one hour in Sorensen buffer pH6.8 at 68°C.

(d) **G Bands**

There are numerous methods of demonstrating G bands which may be broadly classified as follows.

(i) **Incubation in Salt Solutions.**

The first reported technique for demonstrating the banded structure of human chromosomes was that of Sumner et al. (1971), who incubated chromosome preparations for one hour in 2XSSC (0.3M NaCl, 0.03M Na citrate) at 60°C followed by staining in Giemsa. Chromosomes treated in this way showed banding patterns that were in the main identical to fluorescent banding patterns. Striking differences were the secondary constrictions of chromosomes No. 1 and No. 16 which are faintly fluorescent but intensely stained with Giemsa and the distal half of the Y chromosome which stains intensely with quinacline, but not with Giemsa.

An essentially similar technique to the ASG was described by Ridler (1971), who identified the additional chromosome in Down's syndrome as No. 22.

Further modifications of the ASG technique have been described by Chaudhuri et al. (1971), (24hrs. in Sorensen's buffer at 62°C), Bhasin and Foerester (1972), (100mins. in 4XSSC at 65°C), and Grace and Bain (1972), (10mins. in concentrated buffer at 37°C). An interesting feature of the first method is that the incubation time could be reduced by raising the temperature to 66°C.
Dev et al. (1972), postulated that any treatment which removed divalent cations from the chromosome would give banding, and were able to obtain banded chromosomes by merely treating slides in Hanks BSS minus calcium and magnesium for 15-120 seconds at room temperature.

Identical patterns to those of the ASG technique were obtained by incubating slides for one hour in buffered sodium chloride pH7.5 at room temperature followed by five minutes in buffer pH6.8 and staining in Leishman (Bosman and Schaberg, 1973).

(ii) NaOH - Buffer.

A modification of the DNA hybridization technique (Pardue and Gall, 1970), was used by Drets and Shaw (1971), who were able to obtain distinct banding patterns. A major drawback of this method is the long incubation in buffer: 60-72 hours. In addition the method appears to reveal far fewer bands than any other method described.

Schnedl (1971), followed a 90 second treatment in NaOH with a 24 hour incubation in phosphate buffer and obtained superior results to those of Drets and Shaw (1971). Indeed, the number of bands described by Schnedl is far in excess of that of any other method. This is partly due to Schnedl concentrating on analyzing prometaphase chromosomes.

Treatment in NaOH followed by a two hour incubation in phosphate buffer also gives detailed banding patterns (Crossen, 1972a).

(iii) Heat - Giemsa

Yunis et al. (1971), heated chromosome preparations to 85-100°C in phosphate buffer for 10 minutes followed by rapid cooling to 0°C and then reincubation in buffer at 65°C for varying times. Chromosomes treated in this way did not show distinct banding patterns, but there were distinct dark staining areas of the
chromosomes. It was assumed that these darkly staining areas represented areas of the chromosomes that had reannealed after heat denaturation at 100°C.

Heating preparations to 69-70°C for three hours, cooling to 30°C and then staining for 20 minutes in Giemsa also gives banding patterns (Loomholt and Mohr, 1971). It was suggested that heating to 70°C may denature areas of the chromosome rich in A-T pairs as the transition from a double helical configuration to random coil is 69°C for A-T and 110°C for G-C. These authors also found that the technique could be applied to previously fluorochromed preparations, thus permitting a comparison between the two techniques.

Heating to 80°C in Sorensen’s buffer pH6.8 results in the majority of the prometaphases being banded while the contracted chromosomes are uniformly stained (Doyle and Bishun, 1972). These authors omitted colchicine from their cultures and found that banding patterns could also be obtained by dropping cells onto a hot slide, fiercely drying and staining in Giemsa.

(iv) Enzyme Pretreatment.

The use of proteolytic enzymes as a means of demonstrating the banded structure of human chromosomes was first described by Dutrillaux et al. (1971), who treated chromosome preparations with pronase 5μg/ml. for three to six minutes at 37°C and found that the chromosomes had a similar appearance to those stained with either quinacrine, or treated by denaturation. Two other French workers, Finaz and De Grouchy found that α-chymotrypsin also gave banded chromosomes (Finaz and De Grouchy, 1971). Other enzymes that give banding patterns are trypsin (Seabright, 1971; Wang and Federoff, 1972; Soheres, 1972), pankreatin (Müller and Rosenkranz, 1972) and papain (Howard et al., 1972). The greater
availability of trypsin, and the relative ease of the technique has resulted in many G banding studies being made using this technique. A disadvantage of the trypsin technique is that the chromosomes tend to swell causing a loss in morphology.

This distortion was overcome by combining the ASG and trypsin techniques to produce superbly banded chromosomes (Gallimore and Richardson, 1973). Slides were incubated in 2XSSC for three hours at 60°C followed by trypsin solution at 10°C for 90 seconds. A feature of this method is that slides were aged for seven days prior to treatment.

A modification of the trypsin technique was described by Sperling and Weisner (1972), who mixed the trypsin and stain together and by treating slides for 20 minutes in this solution, obtained banded chromosomes.

(v) Modified Giemsa.

Banding patterns can be obtained by merely raising the pH of the stain to pH9 (Patil et al., 1971). Despite the relative simplicity of the method few laboratories have been able to reproduce results similar to those described by Patil et al. (Miller et al., 1973). A feature of this method is, that unlike any other G band technique, the secondary constriction of chromosome 9 is positively stained.

Sanchez et al. (1973) also reported that pretreatment of the chromosomes was unnecessary and claimed good banding following staining in dilute Giemsa (1:100), for 20 minutes. Staining in heated Giemsa results in improved banding (Vass and Sellyez, 1972; Hoffman et al., 1972). The former authors used the ASG technique followed by staining in Giemsa at 40-45°C, while the latter group stained in Giemsa at 50-60°C following trypsin treatment.
(vi) Potassium Permanganate.

Incubation in 10mM potassium permanganate for 20-40 minutes at 0°C gives chromosomes with a differential staining pattern (Utakoji, 1972). The patterns are similar to those of other G banding techniques, but certain telomeric regions are swollen and stained faintly. These are on both arms of chromosomes 4, 7 and 9, on the long arms of chromosomes 2, 10, 13, 15 and 17, and the short arms of chromosomes 1, 5 and 16.

(vii) Protein Denaturation.

Treatment with various protein denaturing agents has also been found to give banded chromosomes. Slides incubated in 2M NaCl-5M urea for 20 minutes at 37°C, or dipped for two seconds in 0.07% mercaptoethanol, 2M urea, 0.05% sodium lauryl sulphate show good banding, although the latter treatment requires cautious handling (Kato and Yosida, 1972). Incubation in 8M urea in Sorensen's buffer for 10 minutes also results in banded chromosomes (Shiraishi and Yosida, 1972).

Yosida and Sagai (1972), found that a few seconds treatment in a mixture of 2XSSC in 0.1% sodium dodecyl sulphate at room temperature gave chromosomes with characteristic banding patterns.

A group of protein denaturing agents: 5M urea, sodium lauryl sulphate, sodium deoxycholate, nonidet P40 (an anionic detergent) and 7X (a laboratory glassware detergent) have been found to give banded chromosomes (Lee et al., 1973). The most effective were urea, sodium lauryl sulphate and 7X.

(viii) Prefixation techniques.

The addition of various compounds to lymphocyte cultures prior to harvest and fixation results in banded chromosomes. The majority of these compounds are known to bind to DNA.
Caratzali et al. (1972), added isoniazide, P.A.S. and pyrazinamide to cultures 12 and 24 hours prior to harvest and obtained chromosomes similar to those produced by the denaturation techniques.

Actinomycin D added to cultures at a final concentration of 5μg/ml for the final hour of culture gives identical patterns in human chromosomes to those produced by other G band techniques (Shafer, 1973). A further antibiotic, tetracycline when added to cultures for the final 24 hours also resulted in banded chromosomes (Meisner et al., 1973a).

In a study of a number of compounds which are known to bind to DNA Hsu et al. (1973), found that two, actinomycin D and azure B (a component of Giemsa), gave banding patterns. Cultures treated with ethidium bromide and Noglamycin resulted in chromosomes with what appeared to be a reverse banding pattern.

(ix) Hydrogen Peroxide.

Seabright (1973), reported that slides treated by the trypsin technique had to be left 5-7 days for optimum banding and recommended a technique whereby slides are placed in a horizontal position and flooded with H₂O₂ for 5-10 minutes. Strangely this method does not work by immersing slides in a coplin jar of H₂O₂.

(e) R Banding

A pattern that is the reverse of the G banding pattern has been reported by Dutrillaux and Lejeune (1971). The technique consists of incubating slides in phosphate buffer pH6.8 at 86-87°C for ten minutes and staining in Giemsa. The staining with this technique is usually very faint and preparations have to be viewed under phase-contrast to fully appreciate the banding patterns.
(Comings, 1972; Miller et al., 1973). In a later publication Durtrillaux (1973a) recommended keeping slides for eight days before treatment and substituting Earle's medium for phosphate buffer.

(f) N Banding

A technique which demonstrates nucleolus organisers has been recently described by Matsui and Sasaki (1973). The method which was designed to remove histones and nucleic acids involves incubation in 5% TCA at 85-90°C for 30 minutes, brief rinsing in tap water, reincubation in 0.1N HCl at 60°C for 30-45 minutes, and staining in Giemsa. In human metaphases the N bands are restricted to the acrocentric chromosomes, whereas in other animals their distribution varies and is thought to represent nucleolus organisers.

(g) T Banding

The terminal regions of many chromosomes are pale staining, but become positive staining with the T band technique. Two methods for demonstrating T bands have been described by Durtrillaux (1973). In the first, slides are given routine banding treatment followed by staining in acridine orange and observation with U.V. fluorescence. The second technique is also similar to the R banding technique except that the denaturation is carried out in acid buffer pH 5.1. The T banding technique appears to give results similar to those described by Bobrow et al. (1972), and Castoldi et al. (1972).

2. BANDING MECHANISM

Throughout the course of this investigation many papers related to the mechanisms of banding were published. As part of
this investigation was concerned with the banding mechanisms these are referred to in the discussion.

3. CHROMOSOME IDENTIFICATION IN MALIGNANT DISORDERS

One of the major drawbacks to chromosome studies of tumors is that it has not been possible to identify the missing or additional chromosomes in abnormal cell lines, nor could the origin of marker chromosomes be clearly defined. This has been overcome to some extent by the advent of fluorescent and Giemsa banding techniques. However, the number of fluorescent and G banding studies in malignant disorders, particularly the leukaemias is few, probably because the yield of metaphases is not great and chromosomes from these patients are often fuzzy and difficult to analyze.

(a) Haematological Disorders

(i) Chronic Granulocytic Leukaemia (C.G.L.)

Most interest in chromosome identification in C.G.L. has centered on trying to identify the Philadelphia chromosome. This chromosome is present in the majority of cases of C.G.L. and appears as a G group chromosome with half its long arms missing. Autoradiographic analysis of the DNA replication patterns provided little conclusive evidence as to the identity of the deleted chromosome (Haines, 1965; Lima de Faria, 1967).

Fluorescent studies in C.G.L. revealed that the Ph1 belonged to the dull fluorescing pair of G chromosomes designated 22 (Caspersson et al., 1970a; O'Riordan et al., 1971). Further fluorescent studies in C.G.L. were carried out by Rowley (1973), who reported an additional dull fluorescing area on the long arms of one of the C9 pair of chromosomes and suggested that this
additional dull fluorescing material represented a translocation of the material deleted from the Phi to the long arms of chromosome No. 9. Further reports of the same structural change include that of Petit and Cauchie (1973), (one case identified by the ASG technique) and Dinauer and Pierre (1973), (bone marrow cells from ten cases identified by fluorescence). The T band technique was used by Van de Berghe (1973), in a study of seven patients, all of whom showed the translocation between chromosomes 22 and 9. In addition, Van de Berghe used trypsin G banding, and suggested that the translocation was interstitial, and not reciprocal, because the faint terminal band on the long arm of chromosome 9 was still present, leaving a gap between it and the more proximal dark band.

A case of C.G.L. in blast crisis with two Phi chromosomes and an additional C group chromosome identified by fluorescence as a No. 10 has been described by Beck and Chesney (1973). The authors felt that the quality of the preparation was not good enough to detect the additional dull fluorescing material on chromosome 9. However, Rowley (1973a), believed that the additional material was present at the end of the long arm of the right hand member of chromosome pair 9 in Fig. 2 of Beck and Chesney's case report.

The translocation between chromosome 22 and chromosome 9 is not entirely specific because Hayata et al., (1973) have reported a case where the translocation appeared to be between chromosomes 2 and 22.

A frequent finding in C.G.L. in blast crisis has been the loss of an E group chromosome and the gain of a metacentric chromosome of C group size which has been interpreted as an isochromosome for the long arm of chromosome 17 (De Grouchy et al., 1968; Engel et al., 1967). Using a modified Giemsa banding technique
Lobb et al., (1972), confirmed this interpretation in material from three patients with C.G.L. Rowley (1973), also identified the metacentric marker chromosome in four patients as an isochromosome for the long arm of chromosome 17.

(ii) Acute Leukaemia

An abnormal marker chromosome in bone marrow cells from one patient with acute myeloid leukaemia was identified by trypsin banding as an isochromosome for the long arm of chromosome 17 (Mitelman et al., 1973). Both fluorescence and Giemsa banding were used by Rowley (1973c), who identified a translocation between chromosome 8 and 21 in addition to a missing X chromosome in a patient with acute promyelocytic leukaemia. A case of acute myelocytic leukaemia also had the same translocation indicating that these abnormalities may be non-random (Rowley, 1973c). An additional C9 chromosome in about 35% of bone marrow cells from a patient with acute myelomonoblastic leukaemia was identified by a modified trypsin technique (Rutten et al., 1973). One member of a family carrying a balanced translocation between chromosomes 12 and 22 died of acute leukaemia (Hinkes et al., 1973). A further member of the family whose chromosomes were not examined also died of leukaemia while two other members of the family with the translocation were in good health. A mongol child with acute leukaemia was found to have an abnormal clone with 52 chromosomes (Berger et al., 1973). In addition to identifying the abnormal chromosomes as numbers 10, 13, 19, 21 and 22 a rearrangement of chromosome 1 was also detected. An abnormal No. 14 chromosome with two additional terminal bands was detected in 6 out of 35 bone marrow cells in one case of plasma cell leukaemia (Wurster Hill et al., 1973). Other markers were present, but the additional bands could not be identified. Monosomy 7 in bone marrow cells of a 4½ year old boy with erythroleukaemia has been described by Petit et al., (1973).
(iii) **Chronic Lymphocytic Leukaemia (C.L.L.)**

There are no reports of either Q or G banding in C.L.L. Autoradiographic studies have shown that the inherited marker chromosome Gp- described by Gunz *et al.* (1962), belongs to the G pair which is first to complete DNA synthesis; i.e. No. 22 (Fitzgerald, 1971).

(iv) **Polycythaemia**

An abnormal F group chromosome is present in a proportion of cases with polycythaemia (Millard *et al.*, 1968; Lawler *et al.*, 1970). The abnormal chromosome has been identified by the ASG technique as a No. 20 in four patients with polycythaemia (Reeves *et al.*, 1972). Rowley (1973b), used quinacrine fluorescence and Giemsa after NaOH in a study of one case with a mosaic chromosome pattern in the marrow. One cell line with 46 chromosomes had a translocation (46,XY,t(12;17)(q13;p11)) while the other cell line with 47 had an additional C9 chromosome and a translocation (47,X,mar(Y;1)(q12q21),+9). A further case had a mosaic pattern in bone marrow cells (46,XY,6q-/46,XY). Fluorescence revealed that the deleted chromosome was a No. 7 with a deletion distal to 7q22 (Rowley, 1973d).

(v) **Sideroblastic Anaemia**

An extra C chromosome, identified by fluorescence as a No. 8 was detected in bone marrow cells from one patient by Hellstrom *et al.* (1973).

(vi) **Multiple Myeloma**

An abnormal No. 14 chromosome with two additional bands was found in cells with 42 chromosomes from one case (Wurster Hill *et al.*, 1973). Other markers were present, but the origin of the additional bands could not be determined.
(vii) **Myelosclerosis**

An additional No. 9 chromosome in bone marrow cells was identified by the Gi11 technique in two patients (Davidson and Knight, 1973).

(viii) **Miscellaneous Haematological Disorders**

De la Chapelle et al. (1972), identified by both the ASG and fluorescent techniques an additional No. 8 chromosome in the bone marrow of two patients, one with intermittent severe pancytopenia, and the other with mild granulocytopenia and thrombocytopenia. De la Chapelle suggested that trisomy 8 may account for a significant proportion of all C trisomy bone marrows and may be causally related to ineffective erythropoiesis. A patient with essential thrombocytosis was found to have two additional C group chromosomes (Rowley, 1973b). One was identified as a No. 9 but the other could not be positively identified. Rowley (1973d), also found a mosaic karyotype (45,X/46,XY) in a patient who initially presented with pancytopenia. Later marrows showed megaloblastic erythroid features and a 45,XY-7 karyotype in 49 of the 50 cells examined. Two thirds of these cells contained a small chromosome fragment with uniform pale fluorescence and no distinct centromere after treatment in NaOH.

(b) **Tumors**

(i) **Burkitt's Lymphoma**

Initial fluorescent studies of 7 biopsies and 11 cultures from 12 patients with Burkitt's lymphoma revealed an essentially normal banding pattern (Manolov et al., 1971). In a further study of two cases, three marker chromosomes were identified as originating from A group chromosomes in one, while the other case had a Y chromosome with minimal fluorescence (Manolov et al., 1971a).
In a later publication an additional band on the long arm of chromosome 14 was found in all analyzable metaphases in both biopsies and cultures from 10 out of 12 patients (Manolov and Manolova, 1972). Other marker chromosomes were present, but not with the frequency of the abnormal 14.

(ii) Meningiomas

A common finding in human meningiomas is the loss of a G group chromosome (Zankl et al., 1971; Mark, 1970). Of the 18 tumors studied by fluorescence 17 had lost a No. 22 (Zankl and Zang, 1972; Mark et al., 1972).

(iii) Cervical Carcinoma

Normal chromosomes were found in two cases by Lin et al. (1973), while a third case had an extra No. 7 chromosome in 75% of the cells.

(iv) Ovarian Adenocarcinoma

Cells from ascitic effusions of two ovarian adenocarcinomas were studied by Tiepolo and Zuffardi (1973), using the fluorescence technique. Several chromosomes which appeared normal by conventional staining were identified as rearrangements, while several markers involving the long arm of chromosome 1 were also identified.

(v) Cell Lines

The fluorescent technique has been applied to the study of abnormal chromosomes in both human and animal cell lines and has resulted in much hitherto undetected information being gained (Miller et al., 1971; Lo Curto et al., 1972). The fluorescent technique has also been applied to the identification of chromosomes in mouse/human cell lines (Caspersson et al., 1970d), while the C banding technique has been used extensively by Chen and Ruddle (1971).
(vi) **Malignant Lymphoma**

Ten cases were studied by Reeves (1973), using the ASG technique. All cases contained abnormal cells with structurally abnormal chromosomes. Several of the changes were common to one or more of the tumors and the breakpoints were almost invariably in the light bands or centromeric regions. Fleischmann et al. (1971) used fluorescence in a study of three cases all of which had marker chromosomes. It was notable that all three contained a medium sized marker with a median centromere and even fluorescence, but the origin of the marker could not be determined. In a further case a marker of similar appearance was also found (Fleischmann et al., 1972). In addition, other markers were identified as parts of normal chromosomes, while others could not be positively identified.

(vii) **Malignant Melanoma**

Chen and Shaw (1973), analyzed the chromosome complement of a cell culture established from a human malignant melanoma. A mode of 45 was found with a distinctive ring chromosome derived from a No. 2 chromosome. In addition, at least five other structural rearrangements were detected.

4. **CHRONIC LYMPHOCYTIC LEUKAEMIA**

(a) **Statement on Chronic Lymphocytic Leukaemia (C.L.L.)**

Chronic lymphocytic leukaemia is a disorder of the lymphoproliferative system, usually occurring in adults, and in males more frequently than females in the ratio of 2:1. It is characterised by an increase in the number of lymphocytes circulating in the peripheral blood and their gradual infiltration into other organs. The leucocyte count ranges from 15,000 to
200,000/cu mm. with between 55% and 95% lymphocytes. The disease can vary from a benign, relatively asymptomatic one, characteristic of the older age group (60+), to an aggressive, usually fatal one more commonly occurring in the younger age group (30-50 years).

(b) **Chromosome Studies**

Chromosome studies of C.L.L. originated with the report by Ford et al. (1958) of one case of C.L.L. in which the bone marrow chromosomes were normal. All chromosome studies about this time were carried out on bone marrow material and include that of Baikie et al. (1959) one case, Ford et al. (1960) two cases, Bayreuther (1960) one case, Sandberg et al. (1961) three cases, Kinlough and Robson (1961) four cases. Normal chromosomes were found in all of these studies and mention is frequently made of the lack of divisions in the bone marrow. Bone marrow cells were also examined by Baserga and Castoldi (1965), who found 3 cells with a large submetacentric chromosome.

The first report of chromosome studies of peripheral blood lymphocytes is that of Gunz et al. (1962), who studied the chromosomes from lymphocyte cultures from 12 patients, 9 of whom had normal chromosomes, while two siblings had an inherited abnormality Gp- (Ch1), and the remaining patient had a similar abnormality. It was suggested that the Ch1 may predispose patients to the disease, but investigations of other cases of familial leukaemia have not revealed a similar chromosome (Fitzgerald et al., 1966). However, a third member of the family subsequently developed C.L.L., suggesting that in this family the Ch1 chromosome may be a predisposing factor (Fitzgerald and Hamer, 1969).
Further studies of peripheral blood lymphocytes, all with a normal chromosome complement include those of Nowell and Hungerford (1964) one case, Hayhoe and Hammouda (1965) nine cases, Ruffie et al. (1966) five cases, Oppenheimer et al. (1965 seventeen cases, Fitzgerald and Adams (1965) twenty nine cases, Woodliff and Cohen (1972) forty cases.

In an attempt to obtain more metaphases Goh (1967), cultured cells for six days. Two hundred and thirty metaphases from six patients were studied and pseudo-diploidy was found in 40% as compared with 3% in the normal controls. This high frequency was found in both treated and untreated patients. Six day cultures were also analyzed by Ducos and Colombies (1968), and Rozynkowa and Marczak (1968). The former authors found some abnormal metaphases from three patients, while the latter found normal chromosomes in five patients and an abnormal E 16 chromosome (E16q+), in the sixth. Lawler et al. (1968), did not find an increased incidence of aneuploidy in six day cultures, nor did they find a high frequency of pseudo-diploid cell in untreated patients.

Measurements of the length of the G group chromosomes in patients with C.L.L. have shown that the combined length of the G group chromosomes in male patients is significantly less than in normal controls (Fitzgerald, 1965). Fitzgerald suggested that this could be brought about by a greater degree of spiralization or condensation of the G group chromosomes, and that such heteropycnotic chromosomes may be the dense nuclear condensation that characterises the Grumeelee cell. Woodliff et al. (1972), also measured the length of the G group chromosomes in patients with C.L.L., but found no difference between the patients and the controls. However, the sample was smaller and the methodology different to that of Fitzgerald (1965).
A detailed analysis of 165 cells from seven patients revealed a marked increase in aneuploidy (20%) when compared with the normal controls (1.3%) (Rozynkowa and Maczak, 1970). This generalized increase in aneuploidy has also been noted by others (Berger and Parmentier, 1971; Woodliff and Cohen, 1972; Rastrick, 1972).

Chromosomes from lymph node preparations have also been examined but these also have shown normal chromosomes (Baker and Atkin, 1965; Baserga and Castoldi, 1965).

The above reports indicate that abnormal cell lines are extremely rare in C.L.L. However, a case with a positive cell line has been described by Fitzgerald and Adams (1965). This patient had a particularly aggressive form of the disease and had a cell line characterised by an abnormal G group chromosome, loss of an E group chromosome and gain of a C group chromosome. A patient with a raised γ-globulin production was found to have a A1/G translocation by Obara et al. (1971). It is noteworthy that the majority of cells carrying the translocation were found in three day cultures without PHA. This is the only report of C.L.L. lymphocytes dividing without PHA.

(c) Culturing C.L.L. Lymphocytes

One of the features of culturing lymphocytes from patients with C.L.L. is the reduced response of the lymphocytes to plant mitogens and products of microorganisms (Bernard et al., 1964; Sharman et al., 1966; Oppenheim et al., 1965; Shreck, 1967). Methods of increasing the yield of mitoses have included prolonging the culture to 5-6 days (Goh, 1967), adding the cell suspension to cold medium before incubating at 30°C for 48 hours (Moore et al., 1968) and freezing the patients' blood to -180°C before culturing (Ducos and Colombes, 1968). Despite all these technical
modifications the yield of mitoses is still less than normal.
III. EXPERIMENTAL STUDIES

1. MATERIALS AND METHODS

(a) Blood Culture Technique

The chromosome culture technique used throughout the course of this investigation was based on that of Coulson and Chalmers (1964). Approximately 15ml of peripheral blood was obtained from normal donors and defibrinated in a McCartney bottle containing glass beads. The defibrinated blood was removed from the fibrinogen clot, added to 3ml of warm sterile 3% gelatine in saline, and allowed to sediment at 37°C for approximately 30 minutes, by which time the majority of the red cells had settled. Five ml of the lymphocyte rich gelatine serum mixture was removed and added to culture bottles containing 3ml of TC 199 tissue culture medium (Difco) and 0.075ml of phytohaemagglutinin (Burroughs Wellcome). The cultures were incubated for three days at 37°C. Three hours prior to harvesting colchicine (Aqua Colchin, Parke Davis) was added to the cultures at a final concentration of 0.1µg/ml of culture. The culture was transferred from the culture bottle to a centrifuge tube by gentle pipetting and then centrifuged for five minutes at 500 r.p.m. Following centrifugation, the supernatant was replaced with 4ml of warm 0.075M KCl. The cells were mixed gently with the KCl, incubated for ten minutes at 37°C, and then fixed in three changes of freshly prepared chilled acetic methanol 1:3. Total time in fixative did not exceed 45 minutes. Chromosome spreads were made by the flame technique which allows 1-2 drops of the cell suspension to fall on a slide previously wetted with 20% ethanol and momentarily ignited. Chromosome spreads were also
made by an air drying technique whereby a slide is placed on a block of ice and one or two drops of cell suspension dropped onto the preformed film of condensation. Slides from both air dried and flamed preparations were dried in a current of warm air and stored in slide boxes until required.

(b) Isotope Labelling Technique

DNA was labelled by adding $^{3}$H-thymidine (specific activity 5000mCi/mM, Radiochemical Centre Amersham) to cultures at a final concentration of 2μCi/ml of culture for the final five hours of culture. Colchicine, 0.1μg/ml of culture was added for the final three hours of culture. The cells were washed twice in Hanks balanced salt solution to remove any surplus radioactivity and then treated as for routine blood cultures.

Chromosomal protein was labelled by adding $^{3}$H-lysine (specific activity 20mCi/mM Amersham) to cultures at a final concentration of 4μCi/ml of culture. The cultures were processed in the same manner as those to which thymidine had been added.

(c) Liquid Scintillation Analysis

Two ml samples of fixative from labelled cultures were mixed with 15ml of Dioxane based scintillation liquid (DBS) and counted in a Packard Tri Carb liquid scintillation counter. Buffer in which labelled preparations had been incubated was evaporated to 0.5ml, mixed with 17.5ml of DBS and counted as for the fixative. No correction was made for quenching.
2. DEVELOPMENT OF BANDING TECHNIQUE

The banding technique was developed from the DNA hybridization technique described by Pardue and Gall (1970), and by Jones (1970). These authors described a technique whereby radioactive complementary RNA (cRNA) is hybridized to DNA in cytological preparations. The chromosome preparations were denatured with NaOH and renatured in hot buffer containing radioactive cRNA. It was noted that the centromeric regions of the chromosomes in addition to being heavily labelled, were intensely stained with Giemsa stain.

At the beginning of this investigation slides were treated with 0.07M NaOH for varying times ranging from five seconds to five minutes (Table 1). It was apparent that the age of the preparation influenced the result, and in each experiment, both freshly made and week old slides were used. Treatment in NaOH caused total distortion of the chromosomes in fresh preparations (Figure 1), while week old preparations showed darkly staining areas around the centromere and faint bands distributed along the chromosome. However, the chromatids were fused and the morphology was indistinct (Figure 2). It was found that even older preparations were more resistant to the action of NaOH. Preparations a month or more old could often be treated for up to five minutes in NaOH without any effect on chromosome morphology or any indication of bands.

Incubation in 0.06M phosphate buffer (Na₂HPO₄/KH₂PO₄, pH 6.8) at 60°C was investigated and also gave varying results ranging from good banding on fresh slides to no banding on week old slides (Table 1).

Combinations of the two treatments, i.e. treatment in NaOH followed by incubation in phosphate buffer were tested and also
<table>
<thead>
<tr>
<th></th>
<th>0.07M NaOH</th>
<th>PO₄ Buffer</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07M NaOH</td>
<td>0.07M NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5secs</td>
<td>30secs</td>
<td>1min</td>
<td>2min</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>+</td>
<td></td>
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<td>5</td>
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<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>7</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

0 Distorted Chromosomes  + Slight Banding  ++ Poor quality Banding  +++ Good Banding
gave varying results (Table 1). However, one treatment gave more consistent results than the others, and this was adopted as the routine banding procedure. It is outlined below.

1. Immerse week old slides in 0.07M NaOH for two minutes.
2. Rinse in distilled water and transfer to phosphate buffer at 60°C for two hours.
3. Transfer the slides from the buffer into 10% Giemsa stain and stain for ten minutes.
4. Rinse in buffered distilled water and air dry.

**Figure 1.** Metaphase from a freshly made preparation treated for two minutes in 0.07M NaOH.

**Figure 2.** Metaphase from a week old preparation treated for two minutes in 0.07M NaOH.
# TABLE 2. Hypotonic Agents Tested

<table>
<thead>
<tr>
<th>No of Cultures Tested</th>
<th>Hypotonic Agent</th>
<th>Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>KCl</td>
<td>Good</td>
</tr>
<tr>
<td>7</td>
<td>Na-Citrate</td>
<td>Good</td>
</tr>
<tr>
<td>7</td>
<td>Distilled water</td>
<td>Poor</td>
</tr>
</tbody>
</table>

*Figure 3.* Metaphase from preparation in which distilled water was the hypotonic agent.
3. FACTORS OF CELL PREPARATION INFLUENCING BAND FORMATION

The experiments described in this section were undertaken to discover if the technical variables involved in the preparation of chromosome spreads influenced band formation.

(a) Hypotonic Treatment

Three commonly used hypotonic agents, 0.075M KCl, 0.93% sodium citrate and distilled water were tested for their effects on band formation. Cultures were harvested as previously described except that the cell suspensions were divided into three equal parts, one being treated with 0.075M KCl, the standard hypotonic agent used throughout this investigation, the second 0.93% sodium citrate and the third with distilled water. One slide from each treatment was then given routine banding treatment and the results are summarised in Table 2.

Chromosomes from cultures in which either KCl or sodium citrate had been used showed good banding, whereas chromosomes from cultures in which distilled water had been used were fuzzy and poorly banded (Figure 3).

(b) Fixation

The effect of fixation on banding was investigated by halving the cell suspension and fixing one half in acetic methanol 1:3, the standard fixative used throughout this investigation and the other half in one of the following fixatives: acetic methanol 1:1 (used by Saksela and Moorhead, 1962 to demonstrate the secondary constrictions and the heterochromatic X in female cells), acetic ethanol 1:3 (ethanol containing fixatives remove fewer histones than do methanol fixatives, Dick and Johns, 1968), acetic ethanol chloroform 1:6:3 (fixative used by Dutrillaux, 1971),
<table>
<thead>
<tr>
<th>No of Cultures Tested</th>
<th>Fixative</th>
<th>Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Acetic methanol 1:3</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>Acetic methanol 1:1</td>
<td>Fuzzy</td>
</tr>
<tr>
<td>8</td>
<td>Acetic methanol 1:3</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>Acetic ethanol 1:3</td>
<td>Good</td>
</tr>
<tr>
<td>8</td>
<td>Acetic methanol 1:3</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>Acetic ethanol chloroform 1:6:3</td>
<td>Good</td>
</tr>
<tr>
<td>8</td>
<td>Acetic methanol 1:3</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td>Acetic methanol Formalin 1:3:0.4</td>
<td>None</td>
</tr>
</tbody>
</table>

Figure 4. Metaphase from preparation fixed in acetic methanol 1:1.
and acetic ethanol formalin 1:3:0.4 (formalin containing fixatives preserve proteins Baker, 1969). In each experiment two slides, one from cells fixed in the standard fixative and one from cells fixed in one of the trial fixatives were given routine banding treatment and the banding compared.

The standard flame technique caused severe distortion of both chromosomes and cell nuclei from cultures fixed in either acetic ethanol 1:3 or acetic ethanol chloroform 1:6:3. To overcome this distortion, chromosome spreads from these cultures were made by the air drying technique described in the Methods.

Good banding was obtained with acetic methanol 1:3 and acetic ethanol 1:3 and there was little to choose between them (Table 3). Acetic methanol 1:1 gave chromosomes a fuzzy appearance (Figure 4). Acetic ethanol chloroform also gave banding but the morphology of the chromosomes was not as distinct as with the first two fixatives. Metaphases from preparations fixed in acetic methanol formalin 1:3:0.4 were poorly spread and did not band (Figure 5).

Figure 5. Metaphase from a preparation fixed in acetic methanol formalin. Note poor spreading and lack of banding.
**TABLE 4.**

*Time in Acetic Methanol 1:3 Fixative*

<table>
<thead>
<tr>
<th>Time</th>
<th>Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 minutes</td>
<td>Good</td>
</tr>
<tr>
<td>2 hours</td>
<td>Good</td>
</tr>
<tr>
<td>4 hours</td>
<td>Good</td>
</tr>
<tr>
<td>6 hours</td>
<td>Good</td>
</tr>
<tr>
<td>8 hours</td>
<td>Poor) Require</td>
</tr>
<tr>
<td>16 hours</td>
<td>Poor) more NaOH</td>
</tr>
<tr>
<td>24 hours</td>
<td>Poor)</td>
</tr>
<tr>
<td>48 hours</td>
<td>Poor)</td>
</tr>
</tbody>
</table>
To study the effect of time in fixative cells from five cultures were kept in the standard fixative for the following time intervals before chromosome spreading:

45 minutes, 2 hours, 4 hours, 6 hours, 8 hours, 16 hours, 24 hours, and 48 hours. A slide from each time interval was given routine banding treatment and the banding compared.

The longer cells were kept in fixative the more resistant they were to the action of NaOH. Cells left in fixative for up to six hours banded well with two minutes in NaOH whereas those kept in fixative for 24 hours or longer required three minutes in NaOH and in some instances did not band at all (Table 4).

(c) Interval Between Chromosome Spreading and Banding

Some slides were given routine banding treatment immediately they were dry, while other slides from the same culture were banded at daily intervals up to one week. Other slides from the same preparation were kept for periods ranging from one week to one year before banding.

Fresh preparations would often band after incubation in phosphate buffer alone as mentioned previously, while prolonged treatment (30 seconds to 5 minutes) in NaOH grossly distorted the chromosomes. However, brief treatment, 0-5 seconds in NaOH followed by incubation in phosphate buffer for two hours caused the chromatids to fuse and the bands appeared as thin darkly staining lines extending across the full width of the chromosome (Figure 6). Preparations 24 hours or older all required treatment with NaOH, the duration of treatment depending on the age of the slide. There was no absolute relationship, but as experience with the method was obtained, it became possible after microscopic examination of a trial slide to judge fairly accurately the length
Figure 6. Metaphase from a fresh preparation treated for 5 secs. in 0.07M NaOH followed by 2 hrs in P0₄ buffer. Note fused chromatids.

Figure 7. Week old metaphase from same preparation treated for 2 mins. in 0.07M NaOH followed by 2 hrs. in P0₄ buffer. Note distinct banding.
of NaOH treatment required. The best results were obtained by treating week old preparations for two minutes in 0.07M NaOH followed by two hours incubation in phosphate buffer at 60°C (Figure 7). Preparations three months or older gave unusual banding patterns and these are described in section V.

(d) **Chromosome Spreading Technique**

Chromosome spreads were made from routinely fixed cells by both the flame and air drying techniques as described in the methods. Air dried and flamed slides from five different preparations were given routine banding treatment immediately they were prepared, while further slides from the same cultures were kept for varying time intervals before banding as indicated in Table 5.

<table>
<thead>
<tr>
<th>Age of Slides</th>
<th>Air Dried</th>
<th>Flamed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly made</td>
<td>Total distortion</td>
<td>Total distortion</td>
</tr>
<tr>
<td>1 day</td>
<td>Total distortion</td>
<td>Poor banding</td>
</tr>
<tr>
<td>2 days</td>
<td>Total distortion</td>
<td>Poor banding</td>
</tr>
<tr>
<td>5 days</td>
<td>Some distortion</td>
<td>Reasonable banding</td>
</tr>
<tr>
<td>7 days</td>
<td>Reasonable banding</td>
<td>Good banding</td>
</tr>
</tbody>
</table>

Freshly made, one and two day old slides from air dried preparations showed totally distorted chromosomes (Figure 8). Chromosomes on freshly made flamed preparations were also totally distorted, but metaphases on one and two day old slides
showed definite but poor quality banding (Figure 8). Week old flamed slides showed consistently superior banding to air dried slides.

Figure 8. (a) Metaphase from air dried preparation 48 hours old given routine banding treatment. Note distorted chromosomes. (b) Flamed metaphase from same preparation treated identically to (a).

(e) Staining.

Giemsa stain (Gurr's R66), diluted in pH 6.8 buffered distilled water was used routinely. To obtain uniform staining, the time in stain and dilution were adjusted by trial for each batch of stain.

In view of evidence suggesting that electrolytes in the buffer used to dilute the stain play a part in the banding mechanism (Kato and Moriwaki, 1972), Giemsa was separately diluted in distilled water and deionised water. In each experiment three slides from the same culture were given routine banding treatment and then one stained in buffered Giemsa, one in distilled water Giemsa and, one deionised water Giemsa. The experiment was carried out five times with slides from different cultures.
Figure 9. Banded metaphase stained in Giemsa diluted in distilled water.

### TABLE 6

Time Interval Between Removal from Buffer and Staining

<table>
<thead>
<tr>
<th>Hours</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Good banding</td>
</tr>
<tr>
<td>2</td>
<td>Good banding</td>
</tr>
<tr>
<td>4</td>
<td>Good banding</td>
</tr>
<tr>
<td>8</td>
<td>Variable banding</td>
</tr>
<tr>
<td>16</td>
<td>Distorted chromosomes</td>
</tr>
<tr>
<td>24</td>
<td>Distorted chromosomes</td>
</tr>
<tr>
<td>48</td>
<td>Distorted chromosomes</td>
</tr>
</tbody>
</table>

Each entry is the mean result of the five experiments.
All diluents gave uniformly good banding (Figure 9) except that the morphology of the chromosomes was indistinct in one of the five trials stained in deionised water Giemsa.

The effect of stain temperature on band formation was examined by staining slides in Giemsa at different temperatures. Groups of four slides from the same culture were given routine banding treatment and stained in standard Giemsa; one slide each at temperatures of 0°, 20°, 37° and 60°C. Five trials were carried out with slides from different cultures.

There was equally good banding at all temperatures except at 60°C when no banding occurred.

During the standard banding procedure, slides were transferred directly from the phosphate buffer into the stain. However, delays in transfer caused poor banding and to investigate this effect further, seven slides from the same culture were removed from the buffer, stored at room temperature and stained at various time intervals as shown in Table 6. The experiment was repeated five times with further groups of seven slides from different cultures.

Delays in staining of up to three hours did not affect banding but longer delays had an adverse effect (Figure 10).

Figure 10. Banded metaphase left 24 hours before staining.
Figure 11. Metaphase and karyotype stained in Pinacyanol chloride.

Figure 12. Metaphase stained in cresyl violet.
Other chromosome stains were also investigated for their banding ability. Two slides from the same culture were removed from the buffer and one stained in standard Giemsa, and the other in one of the trial stains. Each stain was tested five times with slides from different cultures. Details of the staining procedures are given in the appendix.

The stains tested and the results are shown in Table 6 and Figures 11, 12 and 13. Only the Giemsa type stains gave satisfactory banding. Neither cresyl violet or orcein stained chromosomes were banded while pinacyanol staining resulted in variable banding.

### TABLE 6

<table>
<thead>
<tr>
<th>Stain</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giemsa</td>
<td>Good banding</td>
</tr>
<tr>
<td>May Grunwald</td>
<td>Faint banding</td>
</tr>
<tr>
<td>May Grunwald + Giemsa</td>
<td>Good banding</td>
</tr>
<tr>
<td>Cresyl violet</td>
<td>Distorted chromosomes Fig. 12</td>
</tr>
<tr>
<td>Orcein</td>
<td>No banding, unusual chromosomes Fig. 13</td>
</tr>
<tr>
<td>Pinacyanol chloride</td>
<td>Variable banding Fig. 11</td>
</tr>
</tbody>
</table>

Figure 13. Metaphase stained in orcein
<table>
<thead>
<tr>
<th>Buffer Tested</th>
<th>Banding Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06M Phosphate</td>
<td>Good</td>
</tr>
<tr>
<td>20 mM Phosphate</td>
<td>Good</td>
</tr>
<tr>
<td>2X SSC</td>
<td>Good</td>
</tr>
<tr>
<td>1M NaCl</td>
<td>Good</td>
</tr>
<tr>
<td>2M NaCl</td>
<td>Good</td>
</tr>
<tr>
<td>3.9% MgCl₂</td>
<td>Good</td>
</tr>
<tr>
<td>10% MgCl₂</td>
<td>Good</td>
</tr>
<tr>
<td>10% CaCl₂</td>
<td>Poor Fig. 14</td>
</tr>
<tr>
<td>45% Sucrose</td>
<td>Good</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Variable Fig. 15</td>
</tr>
<tr>
<td>Deionised water</td>
<td>Variable Fig. 16</td>
</tr>
</tbody>
</table>

**Figure 14.** Metaphase from preparation incubated for 2hrs in calcium chloride after treatment in 0.07M NaOH for 2mins.
4. INVESTIGATIONS ON THE NATURE OF G BANDS

The following experiments were carried out to obtain more precise information on the nature of G bands. In particular evidence was sought whether bands were composed of DNA, and whether denaturation and reannealing of DNA were important in band formation.

(a) Buffer Composition

Sumner et al. (1971) suggested that G banding reflected the rapid reannealing of highly repetitive DNA that had been denatured during fixation. The efficient reannealing of highly repetitive DNA is dependent on the ionic strength, viscosity, and temperature of the incubation buffer (Subirana and Doty, 1966; Britten and Kohne, 1968). Accordingly, buffers of varying strengths and viscosity were tested for their effects on band production (Table 8).

In each experiment, two slides from the same culture were first treated in 0.07M NaOH. One slide was then treated in the standard phosphate buffer and the other in one of the trial buffers. Treatment in both the standard and experimental buffers was for two hours at 60°C. Each buffer was tested five times.

The composition of the buffer appeared to have little effect as most buffers tested gave good banding (Table 8). However, chromosomes from preparations incubated in calcium chloride were indistinct (Figure 14), while chromosomes from preparations incubated in distilled or deionised water showed variable banding (Figures 15 and 16).

(b) Liquid Scintillation Analysis of Buffer

Many chromosomes in banded preparations appeared to have material removed from them. To investigate whether DNA and protein
Figure 15. Metaphase from preparation incubated in distilled water for 2hrs following treatment in 0.07M NaOH. Note similarity to C banding.

Figure 16. Metaphase from preparation incubated in deionised water for 2 hrs following treatment in 0.07M NaOH for 2mins.
were removed during incubation in buffer, chromosome preparations which had been labelled with either $^3\text{H}$-lysine or $^3\text{H}$-thymidine were given routine banding treatment and the buffer analysed as described in the methods.

Buffer in which thymidine labelled preparations had been incubated revealed considerable radioactivity (Table 9) whereas buffer in which lysine labelled preparations had been incubated showed minimal activity.

**TABLE 9. Analysis of Phosphate Buffer**

<table>
<thead>
<tr>
<th></th>
<th>$^3\text{H}$-Thymidine</th>
<th>$^3\text{H}$-lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Background</td>
<td>Sample</td>
</tr>
<tr>
<td>c.p.m.</td>
<td>c.p.m.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>898.20</td>
<td>26.43</td>
<td>40.71</td>
</tr>
<tr>
<td>5667.5</td>
<td>26.43</td>
<td>39.05</td>
</tr>
<tr>
<td>1230.23</td>
<td>33.36</td>
<td>50.66</td>
</tr>
<tr>
<td>863.26</td>
<td>33.36</td>
<td>44.76</td>
</tr>
<tr>
<td>1251.17</td>
<td>34.22</td>
<td>30.96</td>
</tr>
<tr>
<td>877.88</td>
<td>34.22</td>
<td></td>
</tr>
</tbody>
</table>

Each entry in Table 9 is the activity of the buffer in which 10 labelled preparations had been incubated.

(c) **Liquid Scintillation Analysis of Fixative.**

Fixation in acetic methanol 1:3 causes a loss of histone from calf thymus nuclei (Dick and Johns, 1968) and also removes DNA from cell preparations (Cleaver, 1967). To investigate whether similar losses occurred during fixation of chromosome preparations, four separate cultures were labelled with $^3\text{H}$-lysine and the fixative from these cultures analysed as described in the methods. Four further cultures were labelled with $^3\text{H}$-thymidine and the fixative from these cultures also analysed for their
<table>
<thead>
<tr>
<th>Sample c.p.m.</th>
<th>Background c.p.m.</th>
<th>Sample c.p.m.</th>
<th>Background c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4699.92</td>
<td>24.38</td>
<td>3083.62</td>
<td>24.38</td>
</tr>
<tr>
<td>163.69</td>
<td>24.38</td>
<td>2146.22</td>
<td>24.38</td>
</tr>
<tr>
<td>9240</td>
<td>74.96</td>
<td>1492</td>
<td>74.96</td>
</tr>
<tr>
<td>9920</td>
<td>74.96</td>
<td>3103</td>
<td>74.96</td>
</tr>
</tbody>
</table>

Each entry is the total radioactivity from one labelled culture.
radioactive content.

Fixative from both the $^3$H-thymidine labelled and $^3$H-lysine labelled cultures showed considerable radioactivity (Table 10).

(d) Treatment with Formalin

It has been suggested that banding procedures involve the denaturation and reannealing of DNA (Schnadl, 1971a). To test this possibility, cell preparations were treated with formalin which is known to prevent the reannealing of denatured DNA (Grossman et al., 1961). Slides were treated with formalin at three different stages: after removal from NaOH, during incubation in buffer and before staining (Table 11). Each treatment outlined in Table 11 was tested five times with slides from different cultures. In each experiment a control slide was given routine banding treatment to ensure banding was occurring.

No banding occurred on slides treated with formalin after removal from NaOH or during incubation in buffer. Slides treated with formalin before staining showed poor quality banding.

**TABLE 11. Effect of Formalin**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mins. 0.07M NaOH → 2mins. 4% formalin 2hrs. PO$_4$ Buffer 60°C → stained Giemsa</td>
<td>None</td>
</tr>
<tr>
<td>2mins. 0.07M NaOH → 2hrs. 4% formalin in PO$_4$ Buffer 60°C → stained Giemsa</td>
<td>None. Distorted chromosomes</td>
</tr>
<tr>
<td>Routine banding treatment → 2mins. 4% formalin → stained Giemsa</td>
<td>Poor</td>
</tr>
</tbody>
</table>
Formalin also acts as a hardening agent, i.e. it crosslinks protein groups to make the protein more resistant to physical and chemical treatments (Baker, 1969). To discover whether hardening of the DNA protein complex influenced band formation, freshly made preparations were treated in 4% formalin before standard banding treatment in NaOH and incubation in buffer. In each experiment an untreated slide from the same preparation was also given routine banding treatment. Ten preparations were studied.

Freshly made preparations pretreated with formalin showed good banding (Figure 17) but untreated preparations showed poor banding (Figure 18).

**Figure 17.** Metaphase from fresh preparation treated with formalin before banding. Note distinct chromosome morphology.

**Figure 18.** Metaphase from same preparation without formalin pretreatment. Note poor banding.
(e) **Histone Staining**

To determine whether the dark-staining bands were areas of the chromosomes rich in histones, preparations were stained by the fast green method of Alfert and Geschwind (1953), and by the alcian blue method of Labelle and Briere (1971). The fast green method, a traditional one, involves boiling in trichloroacetic acid, whereas the alcian blue method does not involve this rigorous pretreatment. Details of the staining procedures are given in the appendix. In each experiment two slides from the same culture were treated as outlined in Table 12, one being stained in fast green and the other in alcian blue. Each experiment was repeated five times.

**TABLE 12. Histone Staining**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mins. 0.07M NaOH → rinsed distilled water →</td>
<td>None. Pale stained fast green and alcian blue</td>
</tr>
<tr>
<td>stained fast green and alcian blue</td>
<td></td>
</tr>
<tr>
<td>Routine banding treatment → stained fast green and alcian blue</td>
<td>None. Pale stained fast green and alcian blue</td>
</tr>
<tr>
<td>Routine banding treatment → destained →</td>
<td>None. Pale stained fast green and alcian blue</td>
</tr>
</tbody>
</table>

No bands were seen on any of the preparations subjected to the above treatments, and the staining was always very pale.

(f) **DNA Staining**

To investigate whether the dark-staining bands were composed of DNA, slides were stained by the Feulgen reaction which, under properly controlled conditions, is specific for DNA (Dietch, 1966). Slides from different cultures were subjected to the treatments described in Table 13 on five separate occasions. In each experiment an unbanded unstained slide was given routine
Figure 19. Banded metaphase

Figure 20. Same metaphase, destained, Feulgen stained and then restained in Giemsa.
Feulgen staining to ensure that the staining reaction was satisfactory. Details of the staining and destaining procedures are given in the appendix.

**TABLE 13. Feulgen Staining**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>2mins. 0.07M NaOH → 2hrs. P0₄ buffer 60°C → Feulgen stained</td>
<td>None Pale staining</td>
</tr>
<tr>
<td>Routine NaOH/P0₄ Banding → Giemsa stained → destained → Feulgen stained</td>
<td>Faint bands in occasional metaphases</td>
</tr>
<tr>
<td>Routine banding treatment → destained → Feulgen stained → Giemsa stained</td>
<td>Poor Pale chromosomes</td>
</tr>
</tbody>
</table>

The first treatment was based on the assumption that the bands were present when the slides were removed from the buffer. Feulgen staining at this time hopefully would indicate if the bands were composed of DNA. However, there was no chromosome banding after this treatment and the staining was very pale.

In view of the pale staining of the above preparations, banded metaphases were destained and then restained by the Feulgen reaction. Metaphases subjected to this treatment were faintly stained and an occasional one showed faint chromosome banding. The most noticeable feature was that light G bands were faintly stained with Feulgen.

Metaphases from the second treatment were so faintly stained that it was impossible to take good quality photographs, even with phase contrast. To overcome this problem these metaphases were restained in Giemsa. Definite banding resulted from this treatment, but the morphology of the chromosomes was indistinct (Figures 19 and 20).
Figure 21. Banded metaphase.

Figure 22. Same metaphase as above destained and restained in cresyl violet. Note how some areas of the chromosomes are more heavily stained.
The lack of banding when slides were removed from buffer and Feulgen stained suggested that the acid hydrolysis destroyed the bands. To test this possibility two slides from the same culture were given routine banding treatment, and one slide stained in Giemsa, the other hydrolysed in 3.5N HCl at 37°C for 17 minutes before staining in Giemsa.

No banding was seen on the hydrolysed preparations from five samples tested, whereas all the control slides showed good banding.

(g) Permanency of Banding

To discover whether the destaining procedures affected the banding, banded preparations were photographed, destained and then restained in either cresyl violet or Giemsa. The metaphases were then re-photographed and the banding patterns compared. Details of the destaining procedures are given in the appendix. Eight preparations were investigated, four being stained with Giemsa and four with cresyl violet.

All restained preparations showed patterns identical to the original. After restaining in cresyl violet some areas of the chromosomes were more intensely stained than the original, but this was not a constant feature (Figures 21 and 22).

(h) Removal of DNA

Nucleic acids are effectively removed from cytological preparations by treatment in trichloroacetic acid at 100°C for 15 minutes (Dietch, 1966). Ten banded preparations were given this treatment. In all preparations the bands had disappeared and the chromosomes were faintly stained. Restaining in Giemsa did not improve the intensity of the staining or restore the bands.
Peripheral blood cultures from 25 female and 25 male donors were set up and harvested as described in section III. The age of the donors ranged from 16 to 60 years. Slides from each donor were given the routine banding treatment described in section III. Because one metaphase seldom showed the maximum number of bands for each chromosome, at least four well-banded metaphases from each donor were photographed, karyotyped and the banding patterns analyzed. A well-banded metaphase was one in which the chromatids were separate, and the bands appeared as discrete areas. Both early and late metaphases were examined so that the effects of contraction on banding could be ascertained. Where there was doubt about the maximum number of bands on a chromosome, the original material was examined microscopically. From a detailed analysis of 200 metaphases the banding patterns described below were compiled. The banding patterns have been described using the somewhat complicated nomenclature of the Paris Conference (1971). According to this nomenclature "each chromosome in the human somatic-cell complement is considered to consist of a continuous series of bands with no unbanded areas. The bands are allocated to various regions along the chromosome arms and delimited by specific chromosome landmarks. The bands and the regions they belong to are identified by numbers, with the centromere serving as the point of reference for the numbering scheme.

A chromosome landmark is defined as a consistent and distinct morphological feature that is an important diagnostic aid in identifying a chromosome. Landmarks include the ends of chromosome arms, the centromere and certain bands.
A region is defined as any area of a chromosome lying between two adjacent landmarks. The symbols p and q of the Chicago Conference (1966) are retained to designate, respectively, the long and short arm of each chromosome.

In designating a particular band, four items are required: the chromosome number, the arm symbol, the region number, and the band number within that region." A diagram of this system as applied to chromosome 17 is shown in Figure 23.

![Diagram of Paris Conference Nomenclature as applied to Chromosome 17.](image)

2. THE BANDING PATTERNS

(a) Chromosome 1

The largest and most intensely stained band on the short arm was band 1p31 which in uncontracted preparations appeared as two bands. Bands 1p21 and 1p33 also appeared as two bands in uncontracted preparations. In some instances the distal portion
of the short arm was pale-staining with little evidence of banding, while in others two distinct medium stained bands were visible. The long arm is characterised by a prominent dark-staining band in the centromeric region. The size of this band varied considerably between homologues in a number of cases. Of the 50 donors examined five showed distinct differences in the size of this region. Immediately below the centromeric band and often merging with it is a small dark band. Band 1q22 is a small band, and in most instances was fused to band 1q24. Band 1q31 is an intensely stained band, and is separated from two relatively similar bands by a distinctive non-staining band, 1q32.

Figure 24. Banding patterns of chromosome 1. (a) Typical patterns. (b) Most detailed pattern. Note variation in size of centromeric heterochromatin.
(b) **Chromosome 2**

The number of bands detected on chromosome 2 is largely related to the degree of contraction of the chromosome. The maximum number of bands detected on the short arm was six. The most commonly observed pattern was four bands of rather even staining intensity. However, in uncontracted preparations bands 2p24 and 2p16 appeared as split bands. The bands on the long arm are rather difficult to identify because they lie close together and stain with an even intensity. In good preparations 11 dark bands can be detected, the most distinctive being band 2q32 which can be further subdivided into two bands. Bands 2q12 and 2q14 were faint staining bands which were not always visible when a large pale-staining area adjoined the centromere.

Figure 25. Banding patterns of chromosome 2. (a) Most frequently observed patterns. (b) Most detailed pattern. Note pale-staining bands near centromeric region on the long arm.
Chromosome 3 has one of the most striking banding patterns, particularly in contracted preparations. In uncontracted preparations six bands can be detected on the short arm. Bands 3p22 and 3p24 in many instances fuse to form the distinctive dark band seen in contracted preparations. In many preparations the terminal band 3p26 was very small as if material had been stripped from it. Centromeric staining was most variable ranging from unstained to very intensely stained. In uncontracted preparations the broad dark band below the centromere appeared as three bands. The next two dark-staining bands also appeared as split bands with band 3q26 being slightly more distinctive.

Figure 26. Banding patterns of chromosome 3. (a) Most common pattern. (b) Most detailed pattern. Note the very small terminal band on the short arm.
(d) **Chromosome 4.**

The B group chromosomes are the most difficult of all to differentiate by their banding patterns. In contracted preparations the short arm of chromosome 4 has two dark-staining bands of even intensity, while in detailed preparations a further small light staining band, 4p13.1 was visible. Centromeric staining varied. Band 4q13 below the centromere tended to merge with the centromeric region, but in uncontracted preparations it appeared as two bands. There are four dark-staining bands of similar size and staining intensity in the middle of the long arm, and these are separated from the two distal bands by a distinctive light band 4q31. In uncontracted preparations the two distal bands appear as split bands.

**Figure 27.** Banding patterns of chromosome 4. (a) Most frequently observed pattern. (b) Maximum number of bands observed.
(e) **Chromosome 5**

There is one prominent dark-staining band 5p14 situated in the middle of the short arm. Above and below this band are smaller, less intensely stained bands. Band 5q12, immediately below the centromere appeared as two bands in uncontracted preparations. The next three bands on the long arm are of similar size and staining intensity. In detailed preparations the landmark band 5q31 was bisected by a small pale-staining band 5q31.2. Of the two remaining bands, the distal is the more prominent. This is in contrast to chromosome 4 where the two distal bands tend to be of similar size and intensity.

![Figure 28. Banding patterns of chromosome 5. (a) Most frequently observed pattern. (b) Maximum number of bands detected. Note the more prominent staining distal band on the long arm.](image)
There are two dark-staining bands situated distally on the short arm, and in many preparations they fuse to form one broad dark band. In extremely detailed preparations the landmark band 6p21 was bisected by band 6p21.1. The centromere was usually dark-staining with the bands immediately above and below tending to merge with it. The first two dark-staining bands on the long arm are close to the centromere and often fuse to form one band. In preparations of medium contraction four further bands can be detected. However, in uncontracted preparations these four bands appear as split bands.

Figure 29. Banding patterns of chromosome 6. (a) Most frequently observed pattern. (b) Maximum number of bands detected. Note how the distal bands on the short arm fuse to form one band as in the bottom right pair of chromosomes.
(g) Chromosome 7

The most distal band on the short arm 7p21 is very dark staining and in only one metaphase was there a suggestion that it may have been a split band. Band 7p12 is the smallest of the two remaining dark bands on the short arm and in many instances it fused with the centromere. Centromeric staining, although variable was usually dark-staining. The most commonly occurring pattern on the long arm was three dark-staining bands of which band 7q21 was the largest and most intensely stained. In uncontracted preparations these three bands all appear as split bands.

Figure 30. Banding patterns of chromosome 7. (a) Typical banding pattern. (b) Maximum number of bands. Note how the three darkly staining bands on the long arm appear as double bands in uncontracted preparations.
(h) **Chromosome 8**

Chromosome 8 has the least distinctive pattern of any of the C group chromosomes. In the majority of metaphases the short arm had two dark-staining bands but in uncontracted preparations band 8p22 appeared as two bands. The centromere is usually non-staining. The long arm, in most preparations had four bands with band 8q23 being the most distinctive. In uncontracted preparations the first three of these bands appeared as split bands, while band 8q24.2 was a single band.

**Figure 31.** Banding patterns of chromosome 8. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the distinctive bands 8q23 on the distal portion of the long arm.
(i) **Chromosome 9**

Chromosome 9 can often be identified in unbanded preparations by its characteristic secondary constriction. The most frequently occurring pattern on the short arm was two dark-staining bands of similar size and staining intensity (9p23 and 9p13). In uncontracted preparations both of these bands appeared as split bands. The centromere is dark-staining while the secondary constriction is non-staining. In contracted preparations two dark-staining bands of similar size, spaced evenly on the long arm were visible, but in uncontracted preparations these bands appear as split bands. In extremely uncontracted metaphases a small pale-staining terminal band was detected.

![Figure 32. Banding patterns of chromosome 9. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the distinctive non-staining secondary constriction on the long arm.](image)
Chromosome 10

In contracted preparations one dark-staining band (10p14), situated terminally was visible. However, in detailed preparations this band appeared as two bands, with band 10p14.1 being the larger and more intensely stained. Centromeric staining was variable, but usually Giemsa positive. In moderately contracted preparations the long arm had a most distinctive pattern of three dark-staining bands spaced equidistantly. The most proximal of these three bands was larger and more intensely stained than the others. In uncontracted preparations these three bands appeared as split bands.

Figure 33. Banding patterns of chromosome 10. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the distinctive pattern on the long arm.
(k) Chromosome 11

Chromosome 11 has a pattern that is superficially similar to chromosome 12. The two distal bands on the short arm tend to merge to form the broad dark band 11p14, while band 11p12 merges with the centromere. Centromeric staining was always Giemsa positive. Below the centromere, and often merging with it is a small dark band. Band 11p13 is a distinctive non-staining band which in extremely detailed preparations is bisected by band 11p13.2. Situated in the middle of the long arm are two dark-staining bands, which in contracted preparations merge, while in uncontracted preparations they each appear as split bands. The terminal band, which is of medium staining intensity is also a split band.

Figure 34. Banding patterns of chromosome 11. (a) Typical banding patterns. (b) Maximum number of bands observed. Note how the bands in the centre of the long arm tend to merge.
(1) **Chromosome 12**

Band 12p12, in the middle of the short arm is a broad dark band which in uncontracted preparations appears as three bands. The centromere is usually dark-staining, while immediately below there is a distinctive dark band which in most instances extends across the full width of both chromatids. In the middle of the long arm are three dark-staining bands which are separated from the paracentromeric band by a distinctive non-staining band, 12q13. In contracted preparations these three bands tend to fuse. The remainder of the long arm is less intensely stained but in good preparations two small bands, 12q24.2 and 12q24.4 were visible.

![Figure 35. Banding patterns of chromosome 12. (a) Typical banding patterns. (b) Maximum number of bands observed. Note how the three bands on the long arm fuse in contracted preparations.](image-url)
(m) **Chromosome 13**

The short arm of chromosome 13 stains with medium intensity and tends to merge with the centromere which is dark-staining. Band 13q13 appears as a split band in uncontracted preparations, and this region is much paler than the remainder of the chromosome. In uncontracted preparations band 13q21 also appears as a split band, but in preparations where it is fused, it is the most prominent band on the long arm. Band 13q31 is the more distinctive of the two remaining dark bands on the long arm.

**Figure 36.** Banding patterns of chromosome 13. (a) Typical banding pattern. (b) Maximum number of bands observed. Note how the bands near the centromere are much lighter staining than those at end of the chromosome.
Chromosome 14

The short arm of chromosome 14 stains with medium intensity as does the centromeric region. Immediately below the centromere is a small band of medium intensity which is followed by a dark-staining band. In contracted preparations these two bands merge. Band 14q23.3 is a very small band which usually fuses with band 14q23. Band 14q31 is very intensely stained and is the most distinctive band on the long arm. In uncontracted preparations band 14q31 is followed by a band of medium intensity and these two bands often fuse.

Figure 37. Banding patterns of chromosome 14. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the intensely stained band 14q31 on the distal portion of the long arm.
Chromosome 15

The short arm and centromere of chromosome 15 are dark-staining. Immediately below the centromere are two dark-staining bands, which in the majority of preparations fuse to form one broad dark band. In uncontracted preparations band 15q21 also appeared as a split band. Band 15q22.2 is a small faint band which was seen in only one preparation. The remaining two dark-staining bands are situated distally, lie close together and are less intensely stained than the proximal bands.

Figure 38. Banding patterns of chromosome 15. (a) Typical banding pattern. (b) Maximum number of bands observed. Note how the distal portion of the long arm is less intensely stained.
Chromosome 16 can usually be identified on morphological grounds alone. The short arm has two dark-staining bands which in contracted preparations fuse to form band 16p12. The centromere stains with medium intensity while immediately below is a very intensely stained band extending across the full width of both chromatids. This band often merged with the centromeric region and it stained with a far greater intensity than unbanded Giemsa stained preparations. Band 16q21 is the more intensely stained of the two remaining dark-staining bands although in some instances they stained equally.

Figure 39. Banding patterns of chromosome 16. (a) Typical banding patterns. (b) Maximum number of bands observed. Note the intensely stained band immediately below the centromere.
In contracted preparations the short arm appeared unbanded, while in detailed preparations a medium stained band situated terminally was visible. The centromere was always dark-staining. Band 17q12, immediately below the centromere usually extended across both chromatids and often merged with the centromeric band. The remaining two dark-staining bands on the long arm are separated from the centromeric band by a distinctive non-staining band 17q21. The most prominent of these two bands was 17q22, but occasionally they appeared to be of equal size.

Figure 40. Banding patterns of chromosome 17. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the distinctive non-staining band on the long arm.
Chromosome 18

In contracted preparations the short arm of chromosome 18 stains with an overall medium intensity. However, in extremely detailed preparations a small terminal band 18q11.2 was detected. The centromere is usually dark-staining and may extend part way up the short arm. Band 18q12 immediately below the centromere appears as a split band in uncontracted preparations. Band 18q22 also appears as a split band in uncontracted metaphases with the distal band being the larger.

Figure 41. Banding patterns of chromosome 18. (a) Typical banding pattern. (b) Maximum number of bands observed.
(s) Chromosome 19

The banding pattern of chromosome 19 varied considerably.
In many metaphases the centromeric region was intensely stained while the chromosome arms were pale and in some instances almost non-staining. In detailed preparations a small pale-staining band 19p13.2 situated terminally was detected. In the majority of metaphases the dark-staining band above the centromere fused with its counterpart on the long arm so that the pale-staining centromere was obliterated. The size of these bands varied considerably being quite small in some instances and extending half way up each arm in others. In extremely detailed preparations the long arm has two small bands (19q13.2 and 19q13.4) of medium intensity.

Figure 42. Banding patterns of chromosome 19. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the variation in size of the dark-staining centromeric bands.
(t) **Chromosome 20.**

In many metaphases chromosome 20 appeared unbanded even though the other chromosomes of the metaphase were well banded. The short arm has one dark-staining band 20p12 which, in extremely good preparations, can be subdivided into two. The centromere is usually dark-staining, but there are not the dark-staining bands associated with it as in chromosome 19. In detailed preparations band 20q12 appears as a split band with the distal band being the larger.

---

**Figure 43.** Banding patterns of chromosome 20. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the almost complete lack of banding in the bottom right pair of chromosomes. The rest of the chromosomes of the metaphase were well banded.
Chromosome 21

This is the smaller member of the G group and has an easily recognisable pattern. There are no bands on the short arm which is usually stained with a medium intensity as is the centromere. Satellites when present are also stained with a medium intensity. Immediately below the centromere is a broad dark band, 21q21 which extends across the full width of both chromatids. The distal portion of the long arm is usually pale-staining, but a small band 21q22.2 was observed in detailed preparations.

Figure 44. Banding patterns of chromosome 21. (a) Typical banding pattern. (b) Maximum number of bands observed. Note how the dark-staining band below the centromere extends across the full width of both chromatids.
Chromosome 22

Larger than chromosome 21, it also has a distinctive banding pattern. The short arm again cannot be subdivided and it also stains with a medium intensity as do the satellites when present. There is a dark-staining centromeric band which extends partly on to both the long and short arms. This centromeric band stained more intensely than the remaining bands. In the middle of the long arm is a dark-staining band 22q12. In contracted preparations this band appears in a distal position. However, in uncontracted metaphases a further band of medium intensity situated distally can be detected.

Figure 45. Banding patterns of chromosome 22. (a) Typical banding pattern. (b) Maximum number of bands observed. Note the intensely stained centromeric region and the dark-staining band in the middle of the long arm.
The short arm of the Y chromosome stains with a medium intensity while the centromere is pale or non-staining. In metaphases of medium contraction the long arm has two bands. The first, Yq11.2 is just below the centromere and in some preparations has stained more intensely than the remaining bands. The second band is situated distally and in uncontracted preparations has appeared as two bands (Yq12.1 and Yq12.3). Band Yq12.1 was the more prominent of these two bands.

Figure 46. Banding patterns of the Y chromosome. (a) Typical banding pattern. (b) Maximum number of bands observed.
(x) The X chromosome

In the majority of preparations the short arm of the X chromosome has two dark-staining bands. Band Xp21, situated in the middle of the short arm is the more distinct of these two bands. However, in some instances band Xp22.2 was stained as intensely as band Xp21. In detailed preparations a small band of medium intensity situated just above the centromere was visible. The small dark band immediately below the centromere also stains with medium intensity. Band Xq21 is the most prominent band on the long arm and in uncontracted preparations appears as two bands. There are three further dark bands on the long arm. There was no difference between the patterns of the two X chromosomes in female cells. There was also no difference between the X chromosome in male and female cells.

Figure 47. Banding patterns of the X chromosome. (a) Typical banding pattern. (b) Maximum number of bands observed. The 4 single X's in the bottom row are from male cells while the remainder are from female cells.
V. UNUSUAL BANDING PATTERNS

During the course of this investigation several preparations with unusual banding patterns were encountered. The experiments described in this section were carried out to determine under what conditions these unusual bands occurred, and whether they represented true variations in the banding patterns or were artifacts.

1. MATERIALS AND METHODS

Unstained slides which had been stored in slide boxes at room temperature for periods of time ranging from two weeks to one year were subjected to the routine banding treatment (2 mins 0.07M NaOH, 2 hrs. PO₄ buffer). Slides from 25 donors were examined.

To investigate whether these unusual bands resulted from treatment in NaOH, incubation in buffer or a combination of both, slides were treated for two minutes in 0.07M NaOH, rinsed in distilled water and stained, while further slides were incubated in phosphate buffer for two hours at 60°C without pretreatment in NaOH.

Fifty well-spread metaphases in which the chromosomes were readily identified were photographed and karyotypes made. The number of bands on each chromosome was recorded and the expected distribution of the bands calculated on the basis of the contribution of each chromosome group to the total length of the chromosome set. It was assumed that the probability of banding was proportional to the size of the chromosome group. The measurements of the Denver Conference were used for these
calculations. A similar analysis of the bands on the long and short arms of the B group chromosomes was also made.

2. RESULTS

Week old slides subjected to the routine banding treatment showed the typical banding patterns that have been described earlier. However, when three month old slides from the same preparation were given identical treatment no G bands were seen but instead a new type of band appeared as shown in Figure 48.

**Figure 48.** Metaphase showing unusually dark-staining bands located mainly near the centromere.

These bands were intensely stained, extended across the full width of the chromosome and in most cases were close to the centromere. Usually, there was only one band per chromosome, but if there was more than one band then both were stained equally, which is in contrast to G banding where the staining intensity of the bands varies greatly. The appearance of the banded metaphases varied greatly ranging from sharp chromosomes with distinct banding to others with distorted chromosomes and bizarre banding. Often the
chromosomes appeared as if material had been stripped from them and that the band was an accumulation of this material (Figure 49). In some instances G banding as well as unusual banding was observed. In these preparations areas adjacent to the bands also had looked as if material had been stripped from them (Figure 50).

Figure 49. Metaphase showing unusual bands. Note unusual light-staining areas adjacent to the bands.

Figure 50. Metaphase with both G and unusual banding present.
The distribution of the bands within the chromosomes differed significantly from a random expectation based on chromosome length (Table 14). Bands occurred more frequently than expected on the A and B group chromosomes and less frequently on the D, E, F and G groups. This difference was not merely a function of chromosome arm length, because the frequency of bands occurring on the short arms of the B group chromosomes was in excess of that expected on a random basis (Table 15).

An essential feature determining the formation of these unusual bands was the treatment with NaOH, because slides incubated in buffer without treatment in NaOH showed no banding, whereas those treated in NaOH alone did. However, the combined treatment of both NaOH and incubation in phosphate buffer gave more banded metaphases than did treatment with NaOH alone.

These unusual bands were found only on slides that were three months or more old. Slides less than three months old gave variable results, ranging from no banding of any sort to poor quality G banding. These unusual bands were found on all preparations that were three months old.
### TABLE 14

**Analysis of Bands within Chromosome Groups**

<table>
<thead>
<tr>
<th>Chromosome Group</th>
<th>Observed</th>
<th>Expected</th>
<th>$\chi^2$</th>
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<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>56.8</td>
<td>19.4</td>
</tr>
<tr>
<td>2</td>
<td>83</td>
<td>53.2</td>
<td>16.7</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>44.5</td>
<td>12.3</td>
</tr>
<tr>
<td>4-5</td>
<td>133</td>
<td>79.4</td>
<td>36.2</td>
</tr>
<tr>
<td>6-XX-12</td>
<td>251</td>
<td>265.5</td>
<td>.8</td>
</tr>
<tr>
<td>13-15</td>
<td>25</td>
<td>65.6</td>
<td>26.1</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>20.1</td>
<td>7.3</td>
</tr>
<tr>
<td>17-18</td>
<td>9</td>
<td>36.3</td>
<td>20.5</td>
</tr>
<tr>
<td>19-20</td>
<td>9</td>
<td>29.6</td>
<td>14.3</td>
</tr>
<tr>
<td>21-22</td>
<td>3</td>
<td>21.1</td>
<td>15.5</td>
</tr>
<tr>
<td>Y</td>
<td>0</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>679</td>
<td>679.0</td>
<td>175.0</td>
</tr>
</tbody>
</table>

$\chi^2 = 175.0$ with 10 d.f. $p < .001$

### TABLE 15

**Analysis of Bands on Long and Short Arms of B Group Chromosomes**

<table>
<thead>
<tr>
<th></th>
<th>Observed</th>
<th>Expected</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short Arm</td>
<td>79</td>
<td>36.3</td>
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<tr>
<td>Long Arm</td>
<td>55</td>
<td>97.7</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>134.0</td>
<td>68.9</td>
</tr>
</tbody>
</table>

$\chi^2 = 68.9$ with 1 d.f. $p < .001$
VI. GIEMSA BANDING PATTERNS IN CHRONIC LYMPHOCYTIC LEUKAEMIA

1. MATERIALS AND METHODS

Chromosome studies were made on patients with lymphoproliferative disorders who were attending the Haematology Clinic Christchurch Hospital. Peripheral blood cultures were attempted on 30 patients, 20 of whom yielded sufficient analysable metaphases, while the remaining 10 showed poor growth. The difficulty in culturing lymphocytes from patients with C.L.L. has already been noted. Of the 20 patients studied, 17 were diagnosed as having C.L.L., 2 had malignant lymphoma (patients N.B. and L.W.), and 1 lymphosarcoma (patient B.C.). Details of the patient’s age, sex, WBC, treatment and clinical status are shown in Table 16.

Chromosome cultures were set up in identical manner to the normal controls described in section III. A slight modification was made in cases with a high white count when only 2 ml of cell suspension was added to the culture medium plus 1 ml of AB serum. The majority of cultures were harvested after three days, but some were harvested at two days and some at five days. There was no appreciable difference in the yield of mitoses between the different culture times.

Slides from each patient were subjected to the same banding treatment as the normal controls. Ten well-spread metaphases from each patient were counted and at least five well-banded metaphases photographed and karyotyped. In assessing a well-banded metaphase, the same criteria used for the normal controls were adopted; i.e. separate chromatids with distinct banding. The banding patterns were then compared with the established normal patterns from section IV. Particular attention was paid to the G group
### TABLE 16

<table>
<thead>
<tr>
<th>Date Studied</th>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>WBC</th>
<th>% Lymphocyte</th>
<th>Date Diagnosed</th>
<th>Treatment</th>
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</thead>
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<td>27. 8.71</td>
<td>N.B.</td>
<td>M</td>
<td>56</td>
<td>10,000</td>
<td>63</td>
<td>7. 1.69</td>
<td>D.X.R. + Leukeran</td>
</tr>
<tr>
<td>6. 8.71</td>
<td>B.C.</td>
<td>M</td>
<td>40</td>
<td>14,000</td>
<td>57</td>
<td>12. 3.66</td>
<td>Leukeran until 30.1.70</td>
</tr>
<tr>
<td>22. 5.73</td>
<td>J.A.</td>
<td>F</td>
<td>75</td>
<td>16,000</td>
<td>60</td>
<td>24. 1.69</td>
<td>Leukeran</td>
</tr>
<tr>
<td>22. 5.73</td>
<td>F.P.</td>
<td>F</td>
<td>81</td>
<td>11,000</td>
<td>52</td>
<td>28. 1.63</td>
<td>Leukeran until 28.4.72</td>
</tr>
<tr>
<td>29. 5.73</td>
<td>J.M.</td>
<td>F</td>
<td>53</td>
<td>Not Available</td>
<td>14.12.70</td>
<td>Leukeran</td>
<td></td>
</tr>
<tr>
<td>30. 5.73</td>
<td>E.E.</td>
<td>M</td>
<td>65</td>
<td>Not Available</td>
<td>9.12.69</td>
<td>Untreated</td>
<td></td>
</tr>
<tr>
<td>10. 7.73</td>
<td>R.B.</td>
<td>M</td>
<td>64</td>
<td>12,000</td>
<td>60</td>
<td>27. 8.68</td>
<td>Leukeran</td>
</tr>
<tr>
<td>25. 7.73</td>
<td>J.C.</td>
<td>M</td>
<td>75</td>
<td>11,000</td>
<td>58</td>
<td>5. 2.73</td>
<td>Untreated</td>
</tr>
<tr>
<td>31. 7.73</td>
<td>A.S.</td>
<td>M</td>
<td>59</td>
<td>8,000</td>
<td>66</td>
<td>1. 9.70</td>
<td>Leukeran</td>
</tr>
<tr>
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<td>J.R.</td>
<td>F</td>
<td>51</td>
<td>25,000</td>
<td>60</td>
<td>30.11.71</td>
<td>Untreated</td>
</tr>
<tr>
<td>5. 8.73</td>
<td>L.W.</td>
<td>F</td>
<td>78</td>
<td>Not Available</td>
<td>11. 1.70</td>
<td>Leukeran</td>
<td></td>
</tr>
<tr>
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<td>N.T.</td>
<td>M</td>
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<td>32,000</td>
<td>76</td>
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<td>Leukeran</td>
</tr>
<tr>
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<td>G.W.</td>
<td>M</td>
<td>66</td>
<td>19,000</td>
<td>84</td>
<td>1. 7.73</td>
<td>Untreated</td>
</tr>
<tr>
<td>28. 8.73</td>
<td>S.P.</td>
<td>M</td>
<td>63</td>
<td>19,000</td>
<td>68</td>
<td>1. 5.72</td>
<td>Leukeran</td>
</tr>
<tr>
<td>18. 9.73</td>
<td>A.R.</td>
<td>F</td>
<td>67</td>
<td>21,000</td>
<td>59</td>
<td>18. 9.73</td>
<td>Untreated</td>
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<tr>
<td>31. 7.73</td>
<td>B.P.</td>
<td>M</td>
<td>63</td>
<td>19,000</td>
<td>68</td>
<td>3. 3.67</td>
<td>Leukeran until 2.6.72</td>
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<td>M</td>
<td>78</td>
<td>28,000</td>
<td>79</td>
<td>13. 2.73</td>
<td>Untreated</td>
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<tr>
<td>1.10.73</td>
<td>D.D.</td>
<td>F</td>
<td>68</td>
<td>12,000</td>
<td>63</td>
<td>17.11.72</td>
<td>Leukeran until 17.1.73</td>
</tr>
<tr>
<td>4.12.73</td>
<td>B.M.</td>
<td>M</td>
<td>31</td>
<td>4,000</td>
<td>88</td>
<td>13.11.73</td>
<td>Leukeran</td>
</tr>
<tr>
<td>22. 7.73</td>
<td>W.F.</td>
<td>M</td>
<td>65</td>
<td>14,000</td>
<td>52</td>
<td>26. 1.68</td>
<td>Untreated</td>
</tr>
</tbody>
</table>
chromosomes in view of the report of decreased length of the G group chromosomes in patients with C.L.L. (Fitzgerald, 1965), of an inherited abnormality of a G group chromosome in a family, of whom three members have C.L.L. (Fitzgerald and Hamer, 1969), and of a possible relationship of abnormalities of the small acrocentric chromosomes to tumour cells (Spiers and Baikie, 1972).
2. RESULTS

The results of the chromosome counts are shown in Table 17. There was a clear mode of 46 in all cases. Five cases (R.B., L.W., N.T., A.R., and J.C.) had one cell with an additional chromosome. In three of these cases (J.C., A.R., and N.T.), the additional chromosome had a banding pattern similar to a No. 21 (Plates 1, 2 and 3), while in the other two cases (R.B., and L.W.), the additional chromosomes had banding patterns similar to chromosomes 9 and 17 respectively (Plates 4 and 5). All patients had hypodiploid cells and normal cytogenetic analysis revealed no consistent chromosome loss.

A feature of cases N.B., J.A., B.D., A.S., N.T., E.E., and D.D., was the marked difference in size between members of a chromosome pair. (Plates 3, 6-11). These cases all had two or more cells showing marked homologue disparity (Table 18).

<table>
<thead>
<tr>
<th>Patient</th>
<th>No of Cells Showing Homologue Disparity</th>
<th>Chromosome Pairs Involved</th>
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</thead>
<tbody>
<tr>
<td>D.D.</td>
<td>4</td>
<td>12; 2 18; 6; X</td>
</tr>
<tr>
<td>N.T.</td>
<td>3</td>
<td>9 7; 7 17; 3</td>
</tr>
<tr>
<td>A.S.</td>
<td>4</td>
<td>17; 7 9 12; 9; 6</td>
</tr>
<tr>
<td>B.D.</td>
<td>4</td>
<td>9 17; 11 10 12; 1 5; 12</td>
</tr>
<tr>
<td>E.E.</td>
<td>2</td>
<td>3 4; 2</td>
</tr>
<tr>
<td>J.A.</td>
<td>3</td>
<td>6 3 11 9; 6 11; 1 2 11</td>
</tr>
<tr>
<td>N.B.</td>
<td>2</td>
<td>2 7 8; 2</td>
</tr>
<tr>
<td>Patient</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>---------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>N.B.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B.C.</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>J.A.</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>F.P.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>J.M.</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>E.E.</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>R.B.</td>
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<td>1</td>
</tr>
<tr>
<td>J.C.</td>
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<td>4</td>
</tr>
<tr>
<td>A.S.</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>J.R.</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>L.W.</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>N.T.</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>G.W.</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>S.P.</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>A.R.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B.D.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T.N.</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>D.D.</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>B.M.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>W.F.</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>
The overall incidence of homologue disparity in the 100 banded metaphases analyzed from patients with C.L.L. was 27%. A similar analysis of 100 metaphases from normal controls revealed an almost identical result — 30%. However, the degree of disparity appeared to be much greater in the patients with C.L.L.

Previous chromosome studies of patient N.B. had revealed a G group chromosome with enlarged short arms (Gp+). Banding indicated that this chromosome was a No. 22 and that the additional material was pale-staining. It was not possible to determine whether the additional material represented a translocation from another chromosome. In addition to the above abnormality this patient had three cells with structurally abnormal chromosomes. Banding allowed the positive identification of these abnormal chromosomes which were a dicentric chromosome involving chromosomes 14 and 9, a marker chromosome involving a translocation between chromosomes 1 and 2 and a pericentric inversion of a No. 5 chromosome (Plates 12, 13, 14).

Patient A.S. in addition to showing marked homologue variation also showed variation in the size of the centromeric heterochromatic area of chromosome 1. This patient also had one cell with a very small No. 3 chromosome and a fragment which resembled part of a No. 3 (Plate 9).

Patient B.C. had three metaphases with abnormal banding patterns. The chromosomes in these metaphases had unusual dark-staining bands which appeared similar to the unusual bands described in section V (Plate 15). However, the slides were banded within three days so it seems unlikely that they are abnormal bands which were due to aging. No abnormal banding patterns were seen in cells from any of the other patients.
Four patients, D.D., A.S., W.F., and L.W., all showed variation in size of the heterochromatic area of chromosome No. 1. The variation ranged from very small heterochromatic areas to others where there appeared to be duplication of the heterochromatic area (Plates 9, 11, 16 and 17).
VII. DISCUSSION

1. FACTORS OF CELL PREPARATION INFLUENCING G BAND FORMATION

All the steps involved in the preparation of chromosome spreads influenced band formation, but fixation had the most noticeable effect. Fixation is an integral part of chromosome preparation, but there is little information regarding its effects on chromosome structure. It was originally suggested that fixation in acetic methanol caused denaturation of the chromosomes (Sumner et al., 1971), but later these authors claimed that there was little denaturation during fixation (Sumner et al., 1973). This contrasts with the extremely detailed work of Rigler (1965), who reported that fixation in as little as 10% acetic acid caused more than two thirds of the helical structure of the chromosome to be destroyed.

Fixatives containing acetic acid remove histones from calf thymus nuclei (Dick and Johns, 1965), and from chromosomes (Sumner et al., 1973). Results presented in this investigation (page 50) show that lysine, a major component of F1 histone is removed during fixation in acetic methanol 1:3. ³H-thymidine was also removed but it is probable that this loss represented small molecular weight phosphorylated derivatives of DNA which are present within the cell rather than DNA (Cleaver, 1967). The removal of histones would appear to be important in band formation, because fixatives containing formalin which preserve histone did not give banding, (Evans, 1973; this investigation page 35).

Formalin is thought to combine with the amino groups of protein to form addition complexes which in turn are able to form methylene bridges with other free protein groups. The formation of
methylene bridges causes a hardening of the protein gel which may be responsible for the lack of banding when formalin-containing fixatives are used.

Acetic methanol fixation also causes the precipitation of nucleoproteins (Baker, 1969). Bound water normally present around the protein molecule is removed with the subsequent loss of the electric charge. This allows the protein molecules to move closer together and form new cross links. Long fixation times may cause further loss of water with the formation of further protein cross links and subsequent resistance to treatment with NaOH. An alternative explanation is that the loss of water molecules during long fixation may cause DNA to assume the C configuration, i.e. the form in which the helices are packed together as a result of partial drying. In this more tightly packed form DNA would be more resistant to the actions of NaOH. De la Chapelle et al., (1973) also found that the longer cells were kept in fixative the more resistant they were to the action of NaOH.

The method of chromosome spreading also has a considerable effect on the subsequent banding, and it has been claimed that flame dried preparations do not give banding (Miller et al., 1973). However, in this investigation all slides were made by the flame technique and banded successfully. Flaming of slides clearly does have an effect on the chromosomes allowing them to be treated more rigorously (page 39). Moreover, when flame dried preparations are stained with acridine orange some, or all of the chromosomes fluoresce red, indicating that the DNA is single stranded whereas airdried chromosomes show the green fluorescence of double stranded DNA, (De la Chapelle et al., 1971). These results suggest that flaming causes denaturation of the DNA, but it is extremely difficult to see how an already denatured preparation is more resistant to the action of NaOH. I think it more likely that during flaming the DNA
assumes a different configuration which prevents the intercalation of the acridine orange molecule and thus favours dye stacking and red fluorescence. Acridine orange staining is extremely difficult to interpret and results of this technique must be treated with extreme caution (Kasten, 1967; Sumner et al., 1973).

The time interval between slide preparation and banding treatment had a considerable influence, and it is clear that the aging of slides is an important factor in band formation. Improved results with slides aged 3-4 days have been reported with the ASG technique (Sumner, 1972), the Giemsa 11 technique (Bobrow et al., 1972), and the trypsin technique of Irwin et al., (1973). An interval of one week is recommended with the NaOH/PO₄ technique (Crossen, 1972), three other trypsin techniques (Ansede, 1972; Gallimore and Richardson, 1973; Seabright, 1973), and the R banding technique (Dutrillaux, 1973). However, there seems to be a limit to the beneficial effects of aging, and there is general agreement that slides three months or more old give variable or unsatisfactory banding (Evans et al., 1971; Schnedl, 1971). While the beneficial effects of aging in obtaining clearer banding patterns is recognised, little is known about the changes that take place in the chromosomal protein and DNA when slides are stored for a few days, and even less is known about the effects of prolonged storage which causes the unusual bands described in section V.

It has been suggested that as a result of fixation in acetic methanol, the chromosomes are in a "plastic" state and that with time the DNA/protein complex hardens (Crossen, 1972). Support for this suggestion comes from the experiments (page 51) where pretreatment of chromosome preparations with formalin — a known hardening agent (Baker, 1969), — gave clearer banding. The effects of formalin on chromosomal proteins have already been noted.
Changes in chromosomal fibres during air drying have been reported by Haapala (1967), who showed that air drying caused aggregation of the fibres in insect sperm and fungal chromosomes. If a similar aggregation takes place in mammalian chromosome fibres during air drying and storage, it would allow the formation of new cross links in a manner similar to long fixation times, and may account for the unusual effects of prolonged storage.

Slides stored for long periods of time fluoresce red when stained with acridine orange (Comings et al., 1973; Lubs et al., 1973), suggesting that slow denaturation is taking place. This could possibly explain the unusual results of aging. However, it is equally likely that there is a further loss of water during storage which would alter the configuration of the DNA, possibly to the C form. In this more tightly packed form the intercalation of the acridine orange would be prevented and the preparations would fluoresce red as observed.

The unusual bands described in section V, while in some respects similar to G bands did not have a specific distribution and must be regarded as artefacts resulting from prolonged storage. However, the occurrence of both the unusual bands and G bands in the same preparation is of importance because in these preparations the unusual bands could be interpreted as abnormal banding. For this reason prolonged storage which appears to be responsible for the unusual bands should be avoided.

The hypotonic solutions used in the preparation of chromosomes had little effect on banding. Potassium chloride which gave the best banding was used by Brooke et al. (1962) to uncoil and dissociate chromosomes, and Hungerford (1965), found that a higher concentration was non-disruptive and resulted in clear chromosome definition. Lam Po Tang (1968), also noted an improvement in the morphology of bone marrow chromosomes treated with KCl.
It appears that KCl improves chromosome morphology but is not involved in the banding mechanism. Poor banding resulted when Hanks BSS was used as a hypotonic agent but the reason for this is not clear (Lubs et al., 1973).
2. STUDIES ON THE NATURE OF G BANDS

Although there are conflicting reports regarding the composition of the dark-staining bands there is considerable evidence that they are areas of the chromosome rich in A-T base pairs. The majority of studies concerning the relationship of base pairs to banding have been carried out on Q banding. Since Q and G banding are identical with small exceptions, which have already been noted (page 9) these fluorescent studies are relevant here. The quinacrine bright areas of cytological preparations are rich in A-T base pairs which are quenched on addition of G-C containing polymers (Ellison and Barr, 1972; Weisblum and de Haseth, 1973). Furthermore, fluorescent antibody studies using antibodies specific for adenosine have shown a pattern identical to G banding (Dev et al., 1972a), whereas antibodies specific for cytosine gave a banding pattern identical to R banding (Schreck et al., 1973). These studies correlate well with the close similarity observed between Q banding and late replicating regions of the chromosome (Ganner and Evans, 1972; Calderon and Schmedl, 1973), and the A-T richness of late replicating DNA (Bostock and Prescott, 1971; Comings, 1972a). Paradoxically, the centromeric regions of mouse chromosomes, which are known to contain A-T rich satellite DNA fluoresce poorly, as do the centromeric regions of chromosomes 1, 9 and 16 which also contain highly repetitive A-T rich satellite DNA (Corneo et al., 1970; Jones and Corneo, 1971; Jones et al., 1973). To add to the confusion, the centromeric regions of chromosomes 1 and 16 are Giemsa positive by G banding techniques whereas the secondary constriction of chromosome 9 is Giemsa negative. These observations have led to the suggestion
that while base pair composition is important in band formation, the degree of repetition may also be important (Selander and De la Chapelle, 1973).

Feulgen staining has provided strong evidence that the bands are composed of DNA (Kato and Moriwaki, 1972; Rodman and Tahiliani, 1973; this investigation page 54). The complete absence of banding when slides are treated with TCA is a further indication that the bands are composed of DNA.

It has been suggested that histones take part in the banding mechanism (Meisner et al., 1973), and the association of arginine with G-C rich chromatin (Clark and Felsenfeld, 1972), and lysine with A-T rich chromatin (Combard and Vendrely, 1970), makes this suggestion most attractive. However, none of the histone staining methods in this investigation (page 52) showed banding, and as noted earlier, histones are removed during fixation and are therefore unlikely to take part in banding. Their removal during fixation would appear to be necessary to demonstrate banding.

As most banding methods were based on the DNA hybridization technique, it was assumed that Giemsa banding represented the denaturation and reannealing of DNA. However, results presented in this investigation show that the assumption is incorrect for the following reasons. Firstly, the finding of ³H-thymidine in the incubation buffer is clear evidence that DNA is not reannealed, but degraded during incubation in buffer. ³H-thymidine is a specific precursor of DNA and the activity found in the buffer can only have come from chromosomes and nuclei that had incorporated thymidine while the label was available. The possibility that the activity arose from the degradation of tritium during incubation can be ruled out, as the tritium label is stable even after several hours of boiling in acid or alkali — much severer conditions than
experienced during the incubation in buffer (Cleaver, 1967). Comings et al. (1973) found that DNA was not only removed during incubation in buffer but also during treatment with NaOH. These findings have been confirmed (Franke et al., 1973; Hsu and Arrighi, 1973) and are in contrast to the work of Sumner et al., (1973) who reported that incubation in buffer resulted in neither a loss nor a redistribution of the DNA. However, Sumner et al., (1973) used microdensitometry to monitor DNA loss whereas the other reports and this investigation all used radioactive labelling. The difference in methods may account for the discrepancy.

Secondly if the reannealing of DNA was involved in band formation the composition of the buffer should have influenced the banding as the efficient reannealing of DNA is dependent on the ionic strength, viscosity and temperature of the buffer (Subirana and Doty, 1966; Britten and Kohne, 1968). However, the various buffers tested in this investigation had no effect on banding indicating that differential renaturation is not important in band formation.

Thirdly, the reduced intensity of the Feulgen stain when banded preparations were destained, and then Feulgen stained is further evidence that the banding treatment removes DNA. The position regarding Feulgen staining of banded chromosomes is somewhat confused. When slides, which have been subjected to a banding procedure (either ASG or trypsin) are stained by the Feulgen reaction, no banding occurs (Comings et al., 1973; Summer et al., 1973; this investigation page 54). These observations have been interpreted by Comings and Sumner as indicating that DNA does not occur in variable amounts along the chromosome. However, chromosomes incubated in hot saline are collapsed undifferentiated structures and the bands do not form until the Giemsa stain is
applied (Ross and Gormley, 1973). Removing slides from hot saline or trypsin and placing them directly into hot acid is more likely to destroy the structure of the bands than to preserve them and the experiments described on page 56 show that such is the case.

Finally, the trypsin, sodium dodecyl sulphate, urea and potassium permanganate techniques can hardly be related to the denaturation and reannealing of DNA. These four compounds are protein denaturing agents and are more likely to cause a loosening of the DNA protein bonds. Furthermore, the prefixation techniques, in which compounds, known to bind to DNA are added to cultures (page 13) cannot be related to the denaturation and reannealing of DNA.

The composition of the stain is clearly important to the banding mechanism because only the Romanovsky group of stains and basic fuchsin give banding (Sumner and Evans, 1973; this investigation page 44). Romanovsky stains have a similar composition and are unique, being classified as neutral stains, capable of staining both basic and acidic groups (Conn, 1969). Giemsa is a mixture of four compounds (Azure A, Azure B, eosin and methylene blue) and when chromosomes are stained with it, a new compound is formed, which is magenta in colour and consists of two molecules of methylene blue and one of eosin (Sumner and Evans, 1973). These authors suggested that methylene blue may intercalate between bases and that two molecules of methylene blue may be bridged by one molecule of eosin. Sumner and Evans further hypothesized that banding may be a consequence of reduced dye-binding in those regions of the chromosome where the DNA molecules have become sufficiently dispersed to prevent bridging by the dye molecules.

Basic Fuchsin, the only other stain to give banding, is a single compound with a different structure to the compounds in Giemsa.
Although the precise mechanism of basic fuchsin banding is not known, it is probable that one or two of the phenyl rings of the molecule intercalate between base pairs of the DNA (Sumner and Evans, 1973).

Acetic orcein does not give G bands (Drets and Shaw, 1971; Vosa, 1973), but does give clear C bands in both plant and animal chromosomes (Vosa, 1973). C bands are more resistant to the actions of the banding techniques (Comings et al., 1973) and it is possible that the acetic acid in which the orcein stain is diluted destroys the G bands and the overall structure of the chromosome. The experimental orcein used in this investigation did not contain acetic acid and chromosomes stained by it were not banded but appeared similar to those described by Barnet et al., (1973), using haematoxylin. If the precise mechanism of orcein staining can be elucidated, it may provide additional information regarding the composition of centromeric heterochromatin. Unfortunately, the situation is complicated, because the exact composition of orcein is unknown (Conn, 1969) and in addition orcein consists of at least twelve different fractions (Musso and Beecken, 1957).

The rather inconsistent results with pinacyanol chloride provide little information regarding the banding mechanism. Pinacyanol chloride is a basic stain with a structure unrelated to the other stains which have given banding. There is no information regarding the specificity and mode of binding of pinacyanol.

When other stains were used, there was a complete absence of banding and the chromosomes were distorted. This suggests that as a result of the banding treatment the chromosomes are in a "plastic" state, and the Giemsa stain in some way stabilizes the chromosome. Support for this suggestion comes from electron microscope studies which indicate that chromosomes incubated in hot buffer are
collapsed undifferentiated structures, which on exposure to Giemsa become swollen with transverse ridges corresponding to the G bands (Ross and Gormely, 1973). Moreover, the lack of banding when slides are left 24 hours (page 42) is additional evidence for the stabilizing role of Giemsa.

It has been suggested that the electrolytes in the buffer used to dilute the stain play an important part in band formation (Shiraishi and Yosida, 1972), but in this investigation no difference was found with Giemsa diluted in either distilled or deionised water. This is in contrast to the findings of Kato and Moriwaki (1972), who reported that Giemsa diluted in distilled water did not stain the bands clearly. These authors used a different banding technique, and it seems more likely that the composition of the stain and not the diluent that is important in the banding mechanism.

Acridine orange staining has been used to study the banding mechanism but as noted earlier the results require cautious interpretation. Untreated chromosomes stained with acridine orange fluoresce green indicating that the DNA is double stranded, while those treated with either heat or NaOH fluoresce red (De la Chapelle et al., 1973; Bobrow and Madan, 1973). The first regions to fluoresce red when treated with NaOH are the para-centromeric regions of chromosomes 1, 9 and 16, and the unusual behaviour of these regions has already been noted. When C banded chromosomes are stained with acridine orange they fluoresce green with all the centromeric regions and the distal portion of the Y fluorescing brilliantly (De la Chapelle et al., 1973). These results are very difficult to reconcile with the reports that DNA is not only denatured but up to 80% of it removed during C band treatment (Comings et al., 1973; Franke et al., 1973). The failure by De la Chapelle and his co-workers to observe the strict conditions
for acridine orange staining laid down by Rigler (1965), may explain this discrepancy.

The dark staining G bands of chromosomes processed according to the ASG, R and trypsin techniques, fluoresce red when stained with acridine orange, while the pale-staining bands fluoresce green (Bobrow and Madan, 1973). These results indicate that the dark-staining bands are single stranded DNA while the light-staining bands are double stranded DNA. It should be noted that both Comings et al., (1973) and Sumner et al., (1973), found overall green fluorescence with trypsin digested chromosomes. These conflicting results emphasize the great variability of acridine orange staining in the hands of different workers.

Many of the banding techniques involve protein denaturing agents (page 13), and as acidic proteins are the only proteins remaining in association with DNA after fixation they are thought to be implicated in band formation (Comings et al., 1973; Sumner and Evans, 1973). Daniel and Lam Po Tang (1973) proposed that Giemsa stained the non-disrupted protein remaining after the chromosomes have been banded but the bulk of evidence indicates that Giemsa stains DNA and not protein.

Because protein denaturing agents give banding it has been assumed that protein denaturation takes place. However, the validity of this assumption must be questioned, because Sehested (1973) has obtained banding with inactivated trypsin. This indicates that some other property, such as the reduction of surface tension as suggested by Sehested is responsible for banding, rather than proteolytic digestion. Furthermore, the wide variety of protein denaturants, each with a different mode of action indicates that differences in protein are not important.
From results presented in this investigation and those in the literature I believe that the configuration of the DNA within the chromosome is the main factor involved in band formation. The configuration of DNA is influenced by humidity (Wilkins, 1963), association with basic proteins (Bram, 1971), and base sequence (Bram, 1971a) and these three factors all play a part in band formation.

I propose that banding can be explained on the following basis:

1. During fixation in acetic methanol histones, particularly lysine rich histones are removed from the chromosome. The experiments on page 48 confirm this suggestion. Lysine is associated with A-T rich DNA (Combard and Vendrely, 1970) which has a different configuration from other DNA (Bram, 1971). The removal of lysine would expose the underlying A-T rich DNA to the subsequent banding treatment.

2. During banding treatment by whatever means the configuration of the DNA is altered allowing the A-T rich areas to stain intensely while the G-C areas are pale-staining. The various treatments would alter the configuration of DNA by, either modifying the relationships of DNA with the acidic proteins, or by removing some base pairs. There is no direct evidence that the relationships of the acidic proteins and DNA are altered but the experiments on page 46 indicate that thymidine is removed during incubation in buffer.

Quinacrine banding can also be explained on the same hypothesis. As noted earlier there is considerable evidence which indicates that quinacrine binds to A-T rich DNA. These areas would be more accessible to the quinacrine stain because lysine which is associated with A-T rich DNA has been removed during fixation. The lack of
staining of areas known to contain highly repetitive DNA A-T sequences can be explained by the highly repetitious nature of these areas which prevents intercalation of the quinacrine.

The prefixation techniques can also be explained in that actinomycin D preferentially binds to G-C rich sequences (Sobell et al., 1971), thereby preventing the uptake of the Giemsa stain by those areas and permitting conventional Giemsa staining by the A-T rich areas.
3. NORMAL BANDING PATTERNS

The aim of this part of the investigation was to establish a normal banding pattern and to determine how much variation occurred. It was immediately apparent that the degree of contraction of the chromosomes greatly influenced the banding patterns. Many of the prominent dark bands in chromosomes of medium contraction were found to be two bands in uncontracted chromosomes, and once allowance for this was made there was a remarkable degree of banding consistency.

Often one member of a chromosome pair was more contracted than the other and consequently showed less bands. Similarly, there was variation in the banding between chromatids of the same chromosome. Often a band would appear very small on one chromatid and large and dark-staining on the other. Furthermore, a single band on one chromatid might appear as two smaller bands on the other. However, these variations were never consistent, but emphasize the need to analyse more than one karyotype from each person.

During the course of this study several descriptions of the banding patterns of human chromosomes were published and these are compared in Table 19. The patterns described by Drets and Shaw (1971), have been omitted from the table because it was extremely difficult to reconcile their patterns with this study and others in the literature. Drets and Shaw detected fewer bands and also numbered the chromosomes differently. A number of discrepancies were also found between the patterns of this study and those described by Shiraishi and Yosida (1972). These are due entirely to the numbering adopted by Shiraishi and Yosida, and when
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interchanges between chromosomes 9 and 12, 14 and 15, 19 and 20, and 20 and 21 are made there is good agreement. A single discrepancy was found in the patterns described by Schnedl (1971a), but by interchanging chromosomes 17 and 18 good agreement was also found. The patterns described by the Paris Conference (1971): Standardization in Human Cytogenetics are included but it should be noted that these patterns are derived from Q, G, and R banding methods, whereas the patterns described in this study and in the table are all based on G banding methods.

In general there is good agreement between authors with regard to the major bands. However, the number of bands reported varied considerably ranging from 119 (Evans et al., 1971) to 210 (Schnedl, 1971a). It is notable that this study and the reports of Schnedl, Bigger et al., and Shiraishi and Yosida all describe more bands than the Paris Conference. This is due in part to many of the wide dark bands described by the Paris Conference being split bands and a summary of these is given in Table 20.

**TABLE 20**

<table>
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<tr>
<th>Paris Conference Bands which Appear as Split Bands</th>
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<td>1p31</td>
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<td>8q12, 8q21, 8q23</td>
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<td>9p12, 9q21</td>
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<td>10q21, 10q23, 10q25</td>
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In addition there are other striking differences which are detailed below.

Chromosome 1. The distal portion of the short arm of chromosome 1 is described by the Paris Conference as being pale-staining. In this study it was found to be Giemsa positive with at least two bands. Schnedl also reported Giemsa positive bands in this region.

Chromosome 4. Band 4p16 is shown as being pale-staining and of equal size as 4p15. This was not found to be the case in this study, nor did Schnedl find this.

Chromosome 6. Band 6p21.2 was not recorded by the Paris Conference, but has been described by Schnedl.

Chromosome 8. The broad dark band 8q21 was always seen as two bands, while band 8q23 was the most prominent band on the long arm. Band 8q24.2 is an additional band which has also been described by Schnedl and Bigger et al.

Chromosome 11. The small faint band 11p13.2 is a new band which has not been recorded by any other investigator.

Chromosome 12. There are at least two additional dark bands on the long arm of chromosome 12. Schnedl detected three while in this investigation two were detected.

Chromosome 14. The distal portion of the long arm is described as being pale-stained but a band of medium intensity can be seen. This band has also been detected by Schnedl.

Chromosome 15. Bands 15q22 and 15q25 lie much closer than depicted by the Paris Conference. Band 15q23.1 is an additional band which has also been reported by Schnedl.

Chromosome 18. The short arm is depicted as unbanded whereas in this study it was found to have a small terminal band which has also been described by Schnedl and Bigger et al.
Chromosome 19. Both arms are described by the Paris Conference as being unbanded. However, the short arm has one band situated terminally while the long arm has two bands. These additional bands have been recorded by all authors bar Seabright and Evans et al.

Chromosome 21. The small dark band situated terminally is not recorded by the Paris Conference but, has been described by Schnedl and Bigger et al.

Chromosome 22. The long arm has an additional small band situated distally. This band has also been recorded by Schnedl and Bigger et al.

The X Chromosome. There are two additional dark-staining bands on the short arm. These are situated on either side of band Xp21.

These results indicate that caution should be used in accepting as a normal standard, the bands described by the Paris Conference. Nevertheless they serve as a useful guide and no doubt, at a later conference the results of more recent studies will be incorporated.
4. BANDING PATTERNS IN CHRONIC LYMPHOCYTIC LEUKAEMIA

The majority of cells from patients both treated and untreated had a normal karyotype and this is in agreement with other reports (Oppenheim et al., 1965; Fitzgerald and Adams, 1965; Lawler et al., 1968). There was an increase in the number of aneuploid cells and this tendency has been noted by others (Rozynkowa and Marczak, 1968; Woodliff and Cohen, 1972; Rastrick, 1972). None of the cells with 47 chromosomes can be considered cell lines, because they were isolated occurrences. The finding of three cells with what appeared to be an additional No. 21 chromosome is of interest but the numbers are too small to be significant.

The occurrence of homologue disparity was no greater than that of the normal controls. However, the degree of disparity appeared to be much greater in six of the C.L.L. patients. Four of these patients were being treated with Leukeran at the time of chromosome study while the fifth had received treatment earlier. However, it is unlikely that the treatment is responsible for homologue disparity, because nitrogen mustards usually cause chromosome breaks (Evans and Scott, 1969). Furthermore patient E.E. had not received any treatment but showed homologue disparity. Fitzgerald (1965), suggested that abnormal spiralization may be responsible for the decreased length of the G group chromosomes in patients with C.L.L. It is possible that abnormal spiralization is a feature of C.L.L. cells affecting all chromosomes, and not only the G group. Further studies of larger numbers of patients may resolve this point.
The significance of the abnormal chromosome 22p+ in patient N.B. is difficult to assess. Abnormal chromosomes such as 22p+ occur in 3% of the population (Court Brown, 1967), and it is unlikely that the abnormal chromosome is related to the patient's condition, although the possibility that it may be, cannot be ruled out entirely. This patient had received both chemotherapy and radiotherapy (Table 16), and it is probable that the three structurally abnormal chromosomes resulted from this treatment. The abnormalities were typical of those caused by radiation and chemotherapy and although it was possible to identify the abnormal chromosomes the sample was too small to ascertain if any particular chromosome was preferentially involved. Radiation induced chromosome breaks occur more frequently in the light interband regions (Seabright, 1973) and this appeared to be the case in the three abnormal cells.

The significance of the three cells with abnormal bands in patient B.C. is also difficult to assess. While the abnormal bands were similar to the unusual bands described in section V it is unlikely that they are the same because the slides were banded within three days. The patient had been treated with Leukeran and the treatment may have been responsible for the unusual banding. However, Sumner et al. (1973) found no abnormal banding patterns in leucocyte cultures treated with nitrogen mustard.

The variation in size of the heterochromatic area of chromosome 1 would seem to be of little significance because the frequency was the same as that found in a study of C band heterochromatin in normal controls (Craig-Holmes and Shaw, 1973). The failure to find either chromosome or banding pattern anomalies in patients with C.L.L. suggests that they are not present in this disease. However, before this can be stated categorically one vital question must be answered; namely whether the metaphases
examined are derived from normal or leukaemic lymphocytes.

Untreated blood cultures from patients with C.L.L. contain few, if any, mitoses. This is in contrast to cultures from patients with acute leukaemia and chronic granulocytic leukaemia, where metaphases are present after 12-24 hours in culture without mitogenic additives, and are probably derived from leukaemic cells. The lack of divisions in cultures from patients with C.L.L. necessitates the addition of a mitogenic stimulant such as PHA. The response of C.L.L. lymphocytes to PHA is variable, usually being delayed and less than normal controls (Bernard et al., 1964; Fitzgerald and Adams, 1965; Oppenheim et al., 1965; Schreck, 1967; Smith et al., 1972).

Immunofluorescent studies (Johnson and Klein, 1970; Papamichail et al., 1972) and a double antibody autoradiographic technique (Wilson and Nossal, 1971) have shown that human lymphocytes can be divided into two classes. These are immunoglobulin bearing lymphocytes (bone marrow derived or B cells), and non-immunoglobulin bearing lymphocytes (thymus derived or T cells). The proportion of T and B cells has been estimated at 60% and 34% respectively (Wilson and Nossal, 1971; Papamichail et al., 1971). However, this classification may not be as precise as originally thought because recent work indicates that there is a third class of lymphocytes with both T and B cell markers (Dickler et al., 1974), and in addition Marchalonis et al. (1973) reported that there are equal amounts of Ig on both T and B cells. Despite these conflicting results there appears to be broad agreement that there are two classes of lymphocytes (Nature 247: 175, 1974).

Applying these techniques to C.L.L. lymphocytes it has been shown that the majority of lymphocytes from patients with C.L.L. are of the immunoglobulin bearing or B cell type (Wilson and Nossal, 1971;
Aisenberg and Bloch, 1972; McLaughlin et al., 1972). In addition separation studies have shown that C.L.L. lymphocytes can be separated into two populations one rich in T cells and responding to plant mitogens and poor in T cells and not responding to plant mitogens (Wybran et al., 1973). However, more recent work indicates that not all cases of C.L.L. are exclusively B cell leukaemias, and there may in fact be T cell leukaemias as well (Piessens et al., 1973).

Experimental studies with mouse lymphocytes indicates that PHA selectively stimulates T cells to divide. Whether these findings hold true for man is not clear and there is yet no unequivocal evidence that PHA is a T cell stimulant in man.

If PHA proves to be a specific T cell stimulant and C.L.L. a B cell leukaemia then the majority of chromosome studies in C.L.L. including this one have been carried out on essentially normal lymphocytes, and the finding of abnormal chromosomes is not surprising. If, on the other hand, PHA is not a specific T cell stimulant, but is capable of stimulating both T and B cells then it is possible that chromosome studies have been made on both T and B cells. Whether these studies have been made on leukaemic T and B cells is very difficult, if not impossible, to assess. The problem will remain unsolved until methods of isolating leukaemic cells, and stimulating them to divide, can be found.

The conclusion that can be drawn from these results is that the C.L.L. lymphocytes which respond to PHA, whether they be T or B cells do not have abnormal banding patterns. There is a suggestion that chromosomes from some patients may show a greater degree of homologue disparity than normal controls. Whether this is a feature of C.L.L. chromosomes can only be ascertained from more extensive studies of normal and leukaemic chromosomes.
VIII. SUMMARY

1. The literature on methods of chromosome identification and their application to the study of malignant disorders, particularly the leukaemias is reviewed and discussed. The literature regarding chromosome studies in chronic lymphocytic leukaemia is also reviewed.

2. A method of obtaining banded chromosomes is described. The method involves treating chromosome preparations with 0.07M NaOH for 2 minutes, incubating them in 0.06M phosphate buffer at 60°C for 2 hours and then staining in Giemsa.

3. The technical variables of cell preparation were investigated for their effect on band formation. Liquid scintillation analysis of the fixative revealed that the removal of proteins during fixation was necessary for band formation. In addition cells fixed in formalin-containing fixatives, which preserve protein did not show banding. The chromosome spreading technique was also important with flame dried slides giving the best results. The time interval between chromosome spreading and banding treatment was also important, the best banding occurring on week old slides. Romanovsky stains were the only stains to give clear G banding.

4. Investigations on the nature of G bands indicated that the composition of the buffer had little effect on the quality of the banding. Liquid scintillation of the buffer indicated that thymidine was removed during incubation in buffer. These results indicate that the denaturation and reannealing of DNA are not important in band formation. Histones do not appear to be involved in band formation because neither of the two histone staining methods gave banding. The bands appear to be composed of DNA, because
Feulgen staining of previously banded preparations resulted in the occasional metaphase showing faint banding, while slides treated with TCA which removes DNA showed no banding. The results of the study are discussed in relation to others in the literature, and it is concluded that the configuration of DNA is important in band formation.

5. The banding patterns of chromosomes from 50 normal donors were examined and a banding pattern for each chromosome established. The patterns of this study were compared with those in the literature and with the description published by the Paris Conference (1971). In general there was good agreement between authors regarding the major bands. However, in this study 80 additional bands to those described by the Paris Conference were recorded. The detection of many of the additional bands, was due in part to many of the wide dark bands described by the Paris Conference being two bands. There were also other differences and it is suggested that caution be exercised in accepting the patterns of the Paris Conference as a normal standard.

6. Preparations which had been stored for periods of time in excess of three months were found to show unusual banding patterns. These bands were intensely stained, located mainly near the centromere and statistical analysis revealed that their distribution on the chromosomes was not random. However, the unusual bands appear to be artefacts brought about by the aging process and it recommended that care should be taken not to confuse them with G bands.

7. The banding patterns of chromosomes from 20 patients with chronic lymphocytic leukaemia were analysed and compared with the established normal patterns. There was no difference in the banding patterns between the patients and the normal controls. However, there was a marked difference in size between members of some chromosome pairs in seven of the patients with chronic
lymphocytic leukaemia. The normal controls also showed this variation in size but the degree of difference appeared to be greater in the patients with chronic lymphocytic leukaemia.
REFERENCES


PLATE 1. Karyotype from patient J.C. Note the additional No. 21 chromosome.

PLATE 2. Karyotype from patient A.R. Note the additional No. 21 chromosome.
PLATE 3. Karyotype from patient N.T. Note the additional No. 21 chromosome.

PLATE 4. Karyotype from Patient R.B. Note the additional chromosome with a banding pattern similar to a No. 9.
PLATE 5. Karyotype from patient L.W. Note the additional chromosome with a banding pattern similar to a No. 17.

PLATE 6. Karyotype from patient N.B. Note the difference in size between members of chromosome pairs 2, 7 and 8, and the enlarged short arms of one member of chromosome pair 22.
PLATE 7. Karyotype from patient J.A. Note the difference in size between members of chromosome pairs 3, 6 and 11.

PLATE 8. Karyotype from patient B.D. Note the difference in size between members of chromosome pairs 1, 10 and 12.
PLATE 9. Karyotype from patient A.S. Note the difference in size between members of chromosome pairs 7 and 9. Also note the small No. 3 chromosomes and the additional fragment.

PLATE 10. Karyotype from patient E.E. Note the difference in size between the two No. 2 chromosomes.
PLATE 11. Karyotype from patient D.D. Note the difference in size of the heterochromatic areas of chromosome 1 and the homologue disparity of chromosomes 2 and 18.

PLATE 12. Karyotype from patient N.B. Note the abnormal marker chromosome involving chromosome 1 and 2. There is a No. 17 missing and an additional chromosome of C group size.
PLATE 13. Karyotype from patient N.B. showing a dicentric chromosome formed from chromosomes 14 and 9. There is also a small dot present.

PLATE 14. Karyotype from patient N.B. showing a pericentric inversion of a No. 5 chromosome.
PLATE 15. Metaphase from patient B.C. showing chromosomes with unusual bands.

PLATE 16. Metaphase from patient L.W. Note variation in size of the heterochromatic area of chromosome 1.
PLATE 17. Metaphase from patient W.F. showing variation in the size of the heterochromatic area of chromosome 1.
XI. APPENDIX

STAINING METHODS

   1. Incubate slides for 3 hours at 37°C in the following solution. 1000 U.S.P. units of Heparin, 0.9gm NaCl, 25ml of 0.2M EDTA. (sodium ethylenediamine tetraacetate), 0.1M phosphate buffer to make 100ml.
   2. Rinse slides in running tap water for 5 minutes.
   3. Stain for 30 minutes in a 1% aqueous solution of Alcian Blue 8GX (G.T. Gurr London).
   4. Rinse thoroughly in distilled water and dry.

   A. Preparation of the Schiff reagent.
      1. Dissolve 1gm of basic fuchsin (E. Merck Darmstadt) in 200ml of boiling distilled water. Shake thoroughly, cool to 50°C and then filter.
      2. Add 20ml of 1N HCl to the filtrate and cool to 25°C.
      3. Add 1gm of sodium metabisulphite, shake and store in a tightly stoppered flask in the dark for 12-24 hours.
      4. Add 1gm of activated charcoal, shake for 1 minute, filter and store in a tightly stoppered amber bottle at 4°C.
   B. Preparation of Bisulphite rinses.
      1. 10mls 1N HCl.
      2. 10mls of 10% sodium metabisulphite.
      3. 150mls of distilled water.
      The rinse must be made immediately before use.
C. Staining Procedure.

1. Place slides in distilled water for 1 minute followed by 1 minute in 1N HCl.
2. Hydrolyse in 3.5N HCl at 37°C for 17 minutes.
3. Transfer slides to 1N HCl for 1 minute followed by distilled water for 1 minute.
4. Stain in Schiff solution to 30 minutes.
5. Rinse slides three times in bisulphite rinse followed by distilled water.


1. Remove nucleic acids by treating slides in 5% TCA (Trichloracetic acid) for 20 minutes at 90°C.
2. Rinse slides in cold 5% TCA followed by several rinses in distilled water.
3. Stain at room temperature for 30 minutes in a 0.1% aqueous solution of Fast Green FCF (Allied Chemical) made up in 0.005 phosphate buffer, pH 8.0-8.1. (5ml of a 1% aqueous solution diluted with 45ml of phosphate buffer and the pH adjusted with 0.07M NaOH.
4. Rinse in distilled water and dry.


A. Preparation of Stain.

1. Dissolve 4gm carmine in 15ml of distilled water and 1ml of concentrated HCl.
2. Mix well and boil for ten minutes while stirring frequently.
3. Cool, add 95ml of 85% ethanol and filter.

B. Staining.

Slides were transferred directly from the phosphate buffer into the stain and stained for ten minutes after which they were rinsed in buffered distilled water and air dried.
5. Aceto Carmine. From Darlington and LaCour (1950)

A. Preparation of Stain.
   1. Mix 45ml of acetic acid and 45ml of distilled water and bring to boil.
   2. Add 0.5gms of carmine, shake well, cool and filter.

B. Staining
   Slides were transferred directly from the phosphate buffer into the stain for 15 minutes after which they were rinsed in buffered distilled water and air dried.

6. Orcein

   Standard orcein was found to be totally disruptive to the chromosomes and a modified orcein was used.

A. Preparation of stain.
   1. Dissolve 0.5gms of orcein in 10ml of ethanol.
   2. When thoroughly dissolved add 45ml of phosphate buffer and shake well.

B. Staining.
   Slides were transferred directly from the buffer into the stain for 15 minutes after which they were rinsed in buffered distilled water and air dried.

7. Cresyl Fast Violet

A. Preparation of stain.
   1. Dissolve 2gms of stain in distilled water, shake well and filter.
   2. Leave overnight at room temperature and then dilute 3 parts of stain with 2 parts of distilled water. Filter before use.

B. Staining.
   Slides were transferred directly from the buffer into the stain for 15 minutes after which they were rinsed in distilled water and air dried.
8. **Pinacyanol Chloride**
   
   **A. Preparation of stain.**
   
   1. Dissolve 50mg of stain in 5ml of ethanol and shake well.
   2. Add 4.5ml of 0.06M phosphate buffer and again shake well.
   
   **B. Staining.**
   
   Slides were transferred directly from the buffer into the stain for one minute, differentiated for one minute in buffered distilled water and air dried.

9. **Destaining**

   **A. Method 1.**
   
   Slides were treated in 45% acetic acid for 20 seconds, rinsed in distilled water and air dried.

   **B. Method 2.**

   Slides were treated for 30 minutes in methanol, rinsed in distilled water and air dried.
PHOTOGRAPHIC TECHNIQUE

Apparatus: Olympus FA microscope with Olympus PM6 35mm photographic attachment. Tungsten lamp.

Technique: A suitable metaphase was focussed with the X100 objective, and the iris diaphragm closed completely. The field diaphragm was three quarters closed, the light level turned up to 6 volts and the light compensation lever moved to high. The above settings resulted in the chromosomes having a reddish colour against a yellowish background and consistently gave high quality negatives. No filters were used and exposure was for 3 seconds.

Film: Ilford Microneg Pan Type B. ASA 12-20.

Developer: Agfa/Gevaert G170p Diluted 1:3 for 5 minutes at 20°C. Intermittent agitation.

Printing: Prints were made on grade 2 (normal) Ilfaprint paper and processed in a Ilfaprint Processor.

The above technique was used throughout the course of this investigation and gave prints with good contrast and resolution.
Factors Influencing Giemsa Band Formation of Human Chromosomes*

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Received January 3, 1973

Summary. Factors influencing a Giemsa banding method in which slides are treated with NaOH and then incubated in phosphate buffer were investigated. The study indicated that the removal of chromosomal proteins during fixation in acetic methanol is important for band formation. When fixatives containing formalin were used no banding occurred. Histones do not appear to be involved in band formation as neither of the two histone staining methods tested gave banding patterns. The age of the slide preparations was important, the best banding occurring on slides a week old. Romanovsky stains were the only stains to give banding, other stains resulted in distorted chromosomes. The composition of the incubation buffer had little effect on the quality of the banding. However, liquid scintillation analysis of the phosphate buffer in which 3H-thymidine labelled preparations had been treated, revealed that thymidine is removed during incubation in buffer, and suggests that the degradation of thymidine is an important factor in band formation.

Introduction

There are now many methods which reveal a banded structure of human chromosomes and allow the positive identification of each chromosome. The methods originated with the work of Caspersson and his colleagues (Caspersson et al., 1970) who described the fluorescent "Q" bands resulting from staining with quinacrine mustard. Giemsa-staining centromeric "C" bands were demonstrated by Arrighi and Hsu (1971) using a modification of the DNA hybridization technique. "G" bands result from Giemsa staining after appropriate pretreatment and methods for obtaining "G" bands include incubation in saline (Summer et al., 1971; Bowbrow, 1971), incubation in phosphate buffer after pretreatment with NaOH (Schnedl, 1971; Crossen, 1972), treatment with trypsin (Seabright, 1971; Wang and Federoff, 1971), urea (Shiraishi, 1972), sodium dodecyl sulphate (SSD) (Yosida and Sagai, 1972), and potassium permanganate (Utakoji, 1972). A different type of banding with a reverse pattern, "R" bands, has been described by Dutrillaux (1971).

Little is as yet known about the mechanisms involved in the formation of the darkly staining bands. Denaturation and renaturation of chromosomal DNA (Summer et al., 1971; Schnedl, 1971 a), and solubilization or extraction of chromosomal proteins have both been suggested (Kato and Moriwaki, 1972). It has been shown that the darkly staining centromeric regions of mouse chromosomes contain

*This work was supported by the W. H. Travis Trust and the Canterbury and Westland Division of the Cancer Society of New Zealand.
satellite DNA (Pardue and Gall, 1970), and also the darkly staining centromeric regions of human chromosomes 1, 9, and 16 (Jones and Corneo, 1972; Hsu et al., 1972). It is not clear however, whether other darkly staining bands are composed of DNA, and even less is known about the nature of the pale staining areas.

Many of the different methods for obtaining banded chromosomes have given inconsistent results in this and other laboratories. The present report describes factors which influence band production, and presents evidence that the reannealing of DNA is not involved in band production.

Materials and Methods

Cytogenetic Preparations

Peripheral blood cultures from normal donors were set up and harvested after three days. Aqua Colchin (Parke Davis) was added 2 hours prior to harvest at a final concentration of 1 μg/ml of culture. To obtain uncontracted chromosomes final doses of 0.05 μg/ml were added for up to 4 hours prior to harvest. The cells were given hypotonic treatment for 10 minutes in 0.075 M KCl and fixed in three changes of freshly prepared chilled acetic-methanol 1:3. Total time in fixative did not exceed 45 minutes. Cells were spread immediately by the flame technique which allows 1–2 drops of the cell suspension to fall on a slide previously wetted with 20% ethanol and momentarily ignited.

Banding Technique

The banding technique used as previously described (Crossen, 1972), consisted of treating the slides with 0.07 M NaOH, incubating them for 2 hours in phosphate buffer at 60°C and then staining in Giemsa (Gurr’s R66). The time spent in NaOH was adjusted after a trial slide had been examined and ranged from 5 seconds to 4 minutes. Similarly the intensity of the stain was found to vary with each batch of stain and the time of staining and dilution was adjusted after trials had been carried out.

Experimental Procedures

Fixation. The effect of fixatives on banding was investigated by halving the cell culture suspension and fixing one half in acetic-methanol 1:3 and the other half in one of the following fixatives-acetic-methanol 1:1, acetic-ethanol 1:3, acetic-ethanol-chloroform 1:6:3, and acetic-methanol-formalin 1:3:0.4. Each fixative was tested on cultures from at least eight different people. The flame technique caused severe distortion of chromosomes and cell nuclei from cultures fixed in either acetic-ethanol 1:3 or acetic-ethanol-chloroform. To overcome this distortion slides from these cultures were made by the air-drying technique whereby 1–2 drops of the cell suspension were dropped on a slide previously wetted with 20% ethanol and momentarily ignited.

Labelling Studies. DNA was labelled by adding 3H-Thymidine (3H-Tdr) (specific activity 5000 mc/mM; Radiochemical Centre Amersham) at a final concentration of 2 μc/ml of culture for the final 5 hours of culture. Colchicine 1 μg/ml was added for the final 3 hours of culture. The cells were washed twice in Hanks BSS and then treated as for routine blood cultures. 2 ml samples of fixative were mixed with 15 ml of DBS and counted in a Packard Tri Carb liquid scintillation counter. The phosphate buffer used for banding the 3H-Tdr-labelled preparations was evaporated to 0.5 ml, mixed with 17.5 ml of DBS and counted as for the fixative. No correction was made for quenching. Chromosomal protein was labelled by adding 3H-Lysine (specific activity 20 Ci/mmol Amersham) to cultures at a final concentration of 4 μc/ml. The experimental procedure was as for the cultures to which thymidine had been added.

1 Dioxane Based Scintillator.
Buffer Composition. The following buffer solutions were tested: 0.6M P0₄, 20 mM P0₄, 2 × SSC (0.3 M NaCl-0.03 M sodium citrate), 2 M NaCl, 3.9% MgCl₂, 10% MgCl₂, 10% CaCl₂, 45% sucrose, distilled water and deionised water. Slides were treated in the above solvents for 2 hours at 80°C after first being treated with NaOH.

Effect of Formalin. The effect of formalin pretreatment was studied because of its known actions in preventing the renaturation of DNA and as a hardening agent (Grossman et al., 1961; Baker, 1969). Investigations were made by 1. Treating slides for 10 minutes in 4% formalin before banding. 2. Incubating in phosphate buffer containing 4% formalin. 3. Treating slides for 2 minutes in 4% formalin prior to staining.

Staining Methods

a) Chromosomes. Giemsa stain diluted in buffered distilled water was routinely used. However the use of deionised water to dilute the stain and the effects of temperatures of 0°, 2°, 37°, and 60°C were also studied. Other stains that were investigated for their banding ability were Aceto Carmine, Snow’s Alcoholic Carmine, Cresyl Violet and May Grünwald.

b) Histones. Histones were stained by the Fast Green method of Alfert and Geschwind (1933) and the Alcian Blue method of Labelle and Brière (1971). Cells were stained at the following times. 1. On removal from NaOH. 2. On removal from buffer. 3. After the routine banding treatment slides were destained in methanol for 30 minutes and then stained.

c) DNA. Slides were treated by the Feulgen reaction at the same three stages as the histone investigations. Destaining was for 20 seconds in 45% acetic acid and the Feulgen method of Fand (1970).

In all experiments care was taken to ensure that all slides used in experimental series were of the same age, as this was found to influence results considerably.

Observations

Effect of Time

The time interval between slide making and staining pretreatment greatly influenced band production (Crossen, 1972a). Freshly made slides could be banded by incubation in phosphate buffer without pretreatment in NaOH. Indeed, if treated for times in excess of 1 minute, grossly distorted chromosomes resulted. However, brief treatments in NaOH, ranging from 5–15 seconds, often resulted in preparations in which the chromatids could not be distinguished from each other and the bands appeared as thin dark staining lines extending the full width of the chromosome (Fig. 1a). Slides 24 hours or older all required treatment with NaOH, the time being dependant on the age of the slide. The most detailed banding patterns, in which the chromatids were separate and the bands appeared as distinct sharply defined areas, were obtained from slides one week old treated for 2 minutes in NaOH (Fig. 1b). Slides three months or older gave unsatisfactory banding.

Effect of Fixation

Different fixatives had a noticeable effect on the quality of banding. Clearest banding was obtained after the use of acetic-methanol 1:3 or acetic-ethanol 1:3. Acetic-methanol 1:1 resulted in preparations with indistinct chromatids and chromosomes with a “fuzzy” appearance. Acetic-ethanol-chloroform 1:6:3 gave reasonable banding. Fixatives containing formalin yielded few well spread metaphases and these had no banding. Finally, the longer cells were kept in fixative the more treatment they required with NaOH.
Fig. 1a and b. Giemsa banded metaphases. a) Fresh preparation treated for 5 seconds in 0.07 M NaOH, 2 hours PO₄ Buffer 60°C. b) Week old preparation treated for 2 minutes NaOH, 2 hours PO₄ at 60°C

**Labelling Studies**

Liquid scintillation counting of the fixative revealed that considerable amounts of both ³H-Lysine and ³H-Thymidine were removed during fixation (Table 1).

<table>
<thead>
<tr>
<th>Sample c.p.m.</th>
<th>Background c.p.m.</th>
<th>³H-Thymidine c.p.m.</th>
<th>Background c.p.m.</th>
<th>³H-Lysine c.p.m.</th>
<th>Background c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4699.92</td>
<td>24.38</td>
<td>3083.62</td>
<td>24.38</td>
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<td></td>
</tr>
<tr>
<td>163.69</td>
<td>24.38</td>
<td>2146.22</td>
<td>24.38</td>
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<tr>
<td>9240</td>
<td>74.96</td>
<td>1492.92</td>
<td>74.96</td>
<td></td>
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<tr>
<td>9920</td>
<td>74.96</td>
<td>3103.76</td>
<td>74.96</td>
<td></td>
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</tr>
</tbody>
</table>

The counts per minute were considerably above those of the background standards. Similar analysis of the phosphate buffer showed that large amounts of thymidine were removed during the 2 hour incubation period, but not lysine (Table 2).

**Buffer Composition**

The composition of the buffer had little effect as banding was obtained with all buffers tested. Phosphate and 2× SSC gave the most consistent and detailed
Fig. 2a and b. Metaphases incubated in deionised water for 2 hours at 60°C

Table 2. Analysis of phosphate buffer

<table>
<thead>
<tr>
<th>Sample c.p.m.</th>
<th>Background c.p.m.</th>
<th>Sample c.p.m.</th>
<th>Background c.p.m.</th>
</tr>
</thead>
<tbody>
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<td>898.20</td>
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</tr>
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<tr>
<td>877.88</td>
<td>34.22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

results. Calcium chloride gave chromosomes with an indistinct appearance. Inconsistent results were obtained with deionised and distilled water. In some instances good banding was obtained (Fig. 2a) while in others swollen chromosomes with darkly staining centromeric regions and pale non-staining areas resulted (Fig. 2b).

Effect of Formalin

In fresh preparations where treatment with NaOH caused the chromatids to fuse pretreatment with formalin resulted in the chromatids becoming distinct (Fig. 3) with clear banding (Fig. 3a). No banding occurred when slides were incubated in phosphate buffer containing formalin (Fig. 3b). Banded chromosomes were found on slides treated with formalin prior to staining, but the chromatids were fused and the morphology indistinct.
Staining Methods

a) Chromosomes. There was no difference in the quality of the banding when the Giemsa stain was diluted in buffered distilled water, plain distilled, or deionised water (Fig. 4). Only two stains gave banding. These were Giemsa and May Grünwald. May Grünwald by itself gave weak banding but when followed by Giemsa was adequate. Other stains gave distorted chromosomes (Fig. 5). The temperature of the stain had little adverse effect except at 60°C when no banding
occurred. On the other hand the time interval between removal from buffer and staining was important. Slides could be removed from buffer and stained up to 8 hours later and banding occur. However after 24 hours no banding occurred, and the chromosomes had a distorted appearance (Fig. 6). Slides which had been banded, then destained in acetic acid and restained in Giemsa, gave identical patterns to the original although the staining was pale and blue, compared to the original red. Similarly, banded slides which were destained and then restained in Cresyl Violet gave banding patterns identical to the original, except that
pale staining areas were now stained and some of the bands were more intensely stained (Fig. 7a and b).

b) Histones. Neither of the histone staining methods gave banding patterns at any of the stages at which they were treated. Fast Green staining of acetic-methanol fixed slides was always faint whereas acetic-methanol-formalin fixed preparations gave intense staining.

c) DNA. Slides treated by the Feulgen reaction whether at the time of removal from buffer or NaOH gave pale staining without banding. When banded slides were destained and then restained by the Feulgen reaction very faint banding occurred in the occasional metaphase. The most noticeable feature was that non-staining areas were now stained. Furthermore the Feulgen reaction was always less intense than that in slides which had not been subjected to any banding treatment.

Discussion

The exact nature of Giemsa banding is not clear. Because the original methods were based on DNA hybridization techniques it was thought that Giemsa banding represented the denaturation and reannealing of repetitive DNA. However, this idea is clearly untenable for the following reasons. Firstly, the finding of $^3$H-Thymidine in the incubation buffer is clear evidence that DNA is not reannealed, but degraded during the incubation in buffer. $^3$H-Thymidine is a specific precursor of DNA and the activity found in the buffer can only have originated from chromosomes and nuclei that had incorporated thymidine during the period the label was available. The possibility that the activity arose from the degradation of tritium during incubation in the buffer can be ruled out as the tritium label is stable, even after several hours of boiling in acid or alkali—much severer conditions then experienced during the incubation in buffer (Cleaver, 1967). It was not possible to ascertain if the thymidine that was removed represented non-repetitive DNA as suggested by Sumner et al. (1971). However, the finding of thymidine in the incubation buffer is highly significant and clearly demonstrates that DNA is involved in band formation. Secondly, if the reannealing of DNA was involved in band production the composition of the buffer should have had a marked effect, as efficient reannealing of DNA is dependant on the ionic strength, temperature, and viscosity of the buffer (Britten and Kohne, 1968; Subirana and Doty, 1966). Furthermore the reduced intensity of the Feulgen stain following destaining and restaining is further evidence that the banding treatment removes DNA. Finally the trypsin, SSD, urea, and potassium permanganate methods can hardly be related to the denaturation and reannealing of DNA.

Fixation in acetic-methanol clearly plays an important part in band formation. The complete lack of banding when fixatives containing formalin are used suggests that the removal of proteins, especially histones, is important in band formation. Formalin is thought to combine with amino groups of protein, particularly lysine, to form addition complexes which are able to form methylene bridges with other free protein groups, with the subsequent hardening of the protein gel (Baker,
The hardening, and preservation of the protein gel, may be responsible for the lack of banding when formalin containing fixative are used.

The action of acetic-methanol fixation on chromosome structure is a subject that has received little attention. Acetic-methanol is known to cause precipitation of nucleoproteins (Baker, 1969), and during fixation the bound water normally present around the protein molecule is removed with the subsequent loss of the electric charge. This allows the protein molecules to move closer together and form new cross links. The effect of long fixation times may be to cause further loss of water with the formation of further protein cross links and subsequent resistance to treatment with NaOH.

Fixatives containing acetic acid remove histones from calf thymus nuclei (Dick and Johns, 1963), and results presented in this paper show that lysine is lost when human lymphocytes are treated with acetic-methanol. ³H-Thymidine was also removed but it is probable that this loss represented small molecular weight phosphorylated derivatives of DNA that are present within the cell rather than DNA (Cleaver, 1967). The removal of histones and the formation of new protein cross links appears to play some part in the banding mechanism but raises the question, that while fixation in acetic-methanol gives good chromosome morphology, does it preserve the original chemical composition of the chromosome?

In an earlier publication (Crossen, 1972a) it was suggested that as a result of fixation in acetic-methanol the chromosomes were in a denatured state and that with time, the DNA protein complex hardened. This concept has been further strengthened by the experiments in which slides were treated with formalin prior to banding. The action of formalin on chromosomal proteins has already been noted and it would appear that the formation of new protein cross links, whether by time or formalin, is responsible for the clearer banding. The distinct effect that time has on the subsequent treatment and banding patterns probably explains the variable results of many of the reported banding methods. An interval of 3-4 days is recommended by Bowbrow et al. (1972) when using the Giemsa 11 technique, and improved results with the trypsin technique have been obtained by leaving slides a week before treatment (Ansede, 1972). It seems, that some, as yet unidentified process, takes place on the slide which results in clearer banding. This process may be the further loss of water molecules with the subsequent formation of new protein cross links.

It has been suggested that the electrolytes in the buffer used to dilute the Giemsa stain may play an important part in band formation (Shiraishi and Yosida, 1972). No difference was found using either distilled or deionised water to dilute the Giemsa stain when using the banding method described in this paper. This is in contrast to Kato and Moriwaki (1972), who found that Giemsa diluted in deionised water did not stain the bands clearly. However these authors used different techniques to obtain the banding patterns.

The composition of the stain is clearly crucial to banding. The majority of authors have used Giemsa although the use of Leishman has been reported (Seabright, 1971). The finding that May Grünwald also gives banding is not surprising as it is a further member of the Romanovsky group of stains. These stains have a similar composition and are unique, being classified as neutral
stains, capable of staining both basic and acidic groups (Conn, 1969). However, information regarding the mechanism of routine Romanovsky staining is meagre.

The complete lack of banding and the distorted chromosomes that occur from the use of other stains suggests, that as a result of the banding treatment, the chromosomes are in a plastic state and that the Giemsa stain in some way stabilizes the chromosome. This stabilization is permanent because it is possible to destain banded preparations and restain with either Giemsa or Cresyl Violet and banding reappear. Moreover, the lack of banding when chromosomes are left for 24 hours before staining is additional evidence for the stabilizing role of Giemsa.

The question arises as to the composition of the darkly staining bands? The darkly staining centromeric regions of chromosomes 1 and 16 demonstrated by "G" band techniques, and the dark staining area of chromosome 9 demonstrated by the Giemsa 11 technique (Bowbrow et al., 1972) contain satellite DNA (Jones and Corneo, 1972; Hsu et al., 1972). The remaining dark staining bands would also appear to be composed of DNA as a close relationship between late labelling areas, "Q" bands and "G" bands has been reported (Ganner and Evans, 1972). Furthermore the quinacrine bright material of cytological preparations has been shown to contain areas rich in A-T base pairs (Weisblum and de Haseth, 1972).

Little is at present known about the composition of the pale nonstaining areas. However the Feulgen positivity of these areas indicates that they also are composed of DNA. These regions while composed of DNA must have a different composition from the darkly staining areas. It is possible that the non-staining areas are regions which have lost thymidine. This would explain the finding of thymidine in the incubation buffer and is in keeping with the observation that the quinacrine bright material is rich in A-T base pairs (Weisblum and de Haseth, 1972).

While the removal of histones appears to be important for band formation they do not appear to take part in the staining reaction. Both histone staining methods used in this investigation did not reveal a banded pattern and it seems that considerable histone has been removed from the chromosome.

From results presented in this paper it appears that Giemsa banding results from 1. The removal of proteins, particularly histones, by fixation in acetone-methanol. 2. Further denaturation and loosening of the DNA protein bonds by NaOH. 3. The removal of DNA, possibly non-repetitive DNA rich in A-T base pairs during the incubation in buffer. 4. The binding of the Giemsa stain to areas of the chromosome that are rich in A-T base pairs.

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References

Giemsa Band Formation of Human Chromosomes


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Unusual Chromosome Bands Revealed by Aging*

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Summary. An unusual form of chromosome banding is described. The unusual bands were found only on 3 month old slides subjected to a routine banding procedure. The bands were intensely stained, located mainly near the centromere and statistical analysis revealed that their distribution on the chromosomes was not random. While these unusual bands have similarities to G bands, they do not have a specific distribution and care should be taken not to confuse them with G bands.


Giemsa banding is now firmly established as a simple and reliable method for the positive identification of human chromosomes. The bands may be demonstrated by a variety of techniques (Sumner et al., 1971; Seabright, 1971; Schnedl, 1971a; Crossen, 1972a; Shiraishi and Yosida, 1972; Utakoji, 1972; Gallimore and Richardson, 1973), and while there is variation in the number of bands reported by authors there is general consensus with regard to the major bands. Furthermore, the banding patterns appear to be remarkably constant because to date no variant banding patterns have been reported. In addition to G bands, a reverse banding pattern (R bands) and a terminal banding pattern (T bands) have also been described (Dutrillaux and Lejeune, 1971; Dutrillaux, 1973).

This report describes a further type of banding that was encountered during a study of factors influencing G band formation. As this unusual banding occurs only on slides that have been aged for periods of 3 months or more they have provisionally been called A bands.

Materials and Methods

Chromosome preparations were made from short term cultures of defibrinated gelatine sedimented blood. Hypotonic treatment was for 10 min in 0.075 M KCl and fixation consisted

* This work was supported by the W. H. Travis Trust, and the Canterbury and Westland Division of the Cancer Society of New Zealand.
of three changes of freshly prepared chilled acetic methanol 1:3. Chromosome spreads were made by the flame technique.

**Experimental Procedures**

Unstained slides which had been stored in slide boxes at room temperature for periods of time ranging from 2 weeks to 1 year were subjected to the banding technique previously described (Crossen, 1972a). Slides from 25 donors were examined.

To investigate whether A bands resulted from treatment in NaOH, incubation in buffer, or a combination of both, slides were treated for 2 min in NaOH, rinsed in distilled water and stained, while a further set of slides were incubated in phosphate buffer for 2 hrs at 60°C without pretreatment in NaOH.

Fifty well spread metaphases in which the chromosomes were readily identified were photographed and karyotypes made. The number of bands on each chromosome was recorded and the expected distribution of the bands calculated on the basis of the contribution of each chromosome group to the total length of the chromosome set. It was assumed that the probability of banding was proportional to the size of the chromosome group. The measurements of the Denver conference were used for these calculations. A similar analysis of the bands on the long and short arms of the B group chromosomes was also made.

**Results**

Week old slides subjected to routine banding treatment showed the typical G banding patterns that have been previously described (Crossen, 1972a). However, when 3 month old slides from the same preparation were given identical treatment no G bands were seen, but instead a new type of band appeared (Fig. 1). These bands were intensely stained, extended across the full width of the chromosome and in most cases were positioned close to the centromere. Usually there was only 1 band per chromosome, but if there was more than 1 band then both stained with an even intensity, which is in contrast to G banding where the intensity of the bands varies considerably. The appearance of the banded meta-

![Fig. 1. Metaphase showing darkly staining A bands located mainly near the centromere](image-url)
phases varied greatly ranging from preparations where the chromosomes were sharp and the banding distinct, to others where there was extreme distortion with bizarre banding. Often the chromosomes had the appearance that material had been stripped from them and that the band was an accumulation of this material (Fig. 2). In some instances G banding as well as A banding was observed (Fig. 3). In these preparations areas adjacent to the A bands also had the appearance that material had been stripped from them.
Table 1

<table>
<thead>
<tr>
<th>Chromosome group</th>
<th>Observed</th>
<th>Expected</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>56.8</td>
<td>19.4</td>
</tr>
<tr>
<td>2</td>
<td>83</td>
<td>53.2</td>
<td>16.7</td>
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<tr>
<td>3</td>
<td>68</td>
<td>44.5</td>
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<td>4–5</td>
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<td>6–XX–12</td>
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<td>13–15</td>
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<td>175.0</td>
</tr>
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$\chi^2 = 175.0$ with 10 df; $P < 0.001$.

Table 2. Analysis of bands on long and short arms of B group chromosomes

<table>
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<th>Observed</th>
<th>Expected</th>
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<tbody>
<tr>
<td>Short arm</td>
<td>79</td>
<td>36.3</td>
<td>50.2</td>
</tr>
<tr>
<td>Long arm</td>
<td>55</td>
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<tr>
<td><strong>Total</strong></td>
<td>134</td>
<td>134.0</td>
<td>68.9</td>
</tr>
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</table>

$\chi^2 = 68.9$ with 1 df; $P < 0.001$.

The distribution of the bands within the chromosomes differed significantly from that expected on a random basis (Table 1). Bands occurred more frequently than expected on the A and B group chromosomes and less frequently on the D, E, F and G groups. This difference was not merely a function of chromosome arm length, because the frequency of bands occurring on the short arms of the B group chromosomes was far in excess of that expected on a length basis (Table 2).

An essential feature determining band formation was the treatment in NaOH because slides incubated in buffer without treatment in NaOH showed no banding, whereas those treated in NaOH alone did.

A bands occurred only on slides that were 3 months or more old. Slides less than 3 months old gave variable results ranging from no banding of any sort, to poor quality G banding. A bands were found on all 25 preparations that were 3 months old.

Discussion

An essential feature for the formation of A bands is the aging process, and it is becoming increasingly clear that the aging of slides is an important factor in G band formation. Improved results with slides 3–4 days old have been reported...
with the ASG technique (Sumner, 1972), the Giemsa 11 technique (Bobrow et al., 1972), and the trypsin technique of Irwin et al. (1973), while an interval of 1 week is recommended with the NaOH/PO₄ technique (Crossen, 1972b), and two other trypsin techniques (Ansede, 1972; Gallimore and Richardson, 1973). However there seems to be a limit to the beneficial effects of aging, because there is general agreement that slides 3 months old give variable or unsatisfactory banding (Evans et al., 1971; Schnedl, 1971b; Crossen, 1973). While authors recognize the beneficial effects of aging in obtaining clearer banding patterns little is known about the changes that take place in the chromosomal protein and DNA while the slides are stored for a few days, and even less is known about the effects of prolonged storage which results in A banding.

A bands, while in some respects similar to G bands do not have a specific distribution and therefore must be regarded as artefacts resulting from prolonged storage. However, the occurrence of both A and G bands in the same preparations is of extreme importance because in these preparations, A bands could be interpreted as abnormal banding patterns. For this reason, prolonged storage which appears to be responsible for A band formation should be avoided. By treating slides at the optimum time confusion will be avoided and accurate G banding result.

Acknowledgement. I am grateful to Dr P. H. Fitzgerald for his advice in the preparation of this manuscript.

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Giemsa banding patterns of human chromosomes*

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A method of obtaining differentially stained chromosomes is described. The method based on the treatment of the chromosomes with NaOH and subsequent incubation in phosphate buffer and Giemsa staining gave distinct banding patterns in both blood and bone marrow chromosomes. The banding patterns in some instances agreed closely with those of other methods but in others they were dissimilar. A probable explanation for the differences in banding patterns is the various treatments involved in producing banded chromosomes. While repetitive DNA may play a part in band formation, other factors may also be important.

Identification of individual chromosomes of the human complement has depended largely on the morphology of individual chromosomes, mainly overall length, centromeric position and, to a small extent, the position of secondary constrictions. Unfortunately, many chromosomes show only minor morphological differences which cannot be used for identification. Autoradiography using ³H-thymidine has been useful in identifying some of these chromosomes, notably members of the B and D groups, but the technique is laborious and difficult. The development of U. V. fluorescence of chromosomes stained with acridine dyes (Caspersson 1970) is a big advance, which, for the first time, has enabled the positive identification of each human chromosome. The more recently developed Giemsa banding techniques (Dutrillaux & Lejeune 1971, Sumner et al. 1971, Ridler 1971, Patil et al. 1971, Drets & Shaw 1971, Yunis et al. 1971, Arrighi & Hsu 1971, Schendel 1971) show even greater promise as simple and inexpensive methods of identifying human chromosomes. The purpose of this paper is to describe the technique in this laboratory and to present a detailed description of the banding pattern of each chromosome.

Material and Methods
Peripheral blood cultures from normal donors were set up and harvested after three days. Aqua Colchin (Parke Davis) was added 2 hrs prior to harvesting at a final concentration of 1ug/ml of culture. The cells were incubated for 10 mins in .075M KCl, fixed in acetic methanol 1:3 and made into slides by the “flaming” technique. Bone marrow chromosomes were

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prepared using the method of Lam Po Tang (1968).

The banding pattern was produced by a modification of the DNA hybridization technique of Pardue & Gall (1970). The slides were treated with 0.07M NaOH for 2 mins, rinsed in distilled water, and then incubated at 60°C for 2 hrs in 0.06M phosphate buffer pH 6.8. The slides were then transferred directly into 10% Giemsa (Gurr R.66) for 8 mins, rinsed in buffered distilled water pH 6.8 and air dried. Suitable metaphases in which the banding pattern was clear were photographed, and karyotyped. Where there was doubt about the precise number of bands, reference was made to the original material. This was necessary where the bands were faint or close together.

Results

The above method gave reproducible and distinct banding patterns in up to 80% of the metaphases from both blood and bone marrow preparations. Best results were obtained from cells in early metaphase, although chromosomes with a greater degree of contraction also gave suitable results. The intensity of the bands was found to vary considerably, ranging from intensely stained bands as in chromosomes 3, 16, and the centromeric region of 19, to small pale bands such as are found on the distal portion of many of the chromosomes. Distinctive non-staining areas were also found to be an aid in identification, especially in chromosomes 3, 6, and 19. The banding patterns presented in Fig. 8 were determined from an analysis of 50 metaphases. As the banding patterns were similar to those obtained by fluorescence, I have adopted the numbering system of Casperson (1971). This is in agreement with the decision of the Orly-Paris Conference (1971) on human chromosome nomenclature. The centromere has been used as a starting point in reference to numbered bands on each chromosome arm.

The staining patterns

Chromosome 1 (Fig. 1)

This chromosome which is nearly meta-centric has distinctive banding patterns on each arm. There are six distinct bands on the short arm, the largest and most intensely stained being the second. The terminal three bands are very faintly stained and can only be identified in uncontracted preparations. The long arm is characterised by a prominent dark staining band in the centromeric region followed closely by a small dark stained band. In some preparations this latter band could not easily be distinguished from the centromeric band. The remaining four bands occur as two groups of two. The second of these bands (band 4) is the most prominent.
Chromosome 2 (Fig. 1)
The bands of this chromosome are difficult to distinguish since in most preparations they stain with an even intensity. In uncontracted and lightly stained metaphases, four evenly spaced bands can be distinguished on the short arm. Of these, the second in the middle of the short arm is the most intensely stained. The bands on the long arm are difficult to identify because they also lie close together and stain with an even intensity. In good preparations six bands spaced evenly along the length of the long arm can be identified. In some preparations the bands nearer the centromere appear to be more lightly stained, but this is not a constant feature.

Chromosome 3 (Fig. 1)
This chromosome has a staining pattern unlike that of any other chromosome. There is a distinctive wide, dark stained band on either side of the centromere. The short arm has two minor bands associated with the first band and these are separated by a large unstained area from two distal bands, the first of which is the more prominent. On the long arm a second band lies very close to the wide centromeric band. Two darkly stained bands in the distal half of the arm and a smaller pale terminal band complete the pattern.

Chromosome 4 (Fig. 2)
There are three bands on the short arm of chromosome 4. The first is just above the centromere, whereas the second and third are distal. The long arm has six bands. The first is the most prominent and is followed by three evenly spaced bands which are separated from the distal pair by a distinctive unstained area.

Chromosome 5 (Fig. 2)
Chromosome 5 can be readily distinguished from chromosome 4 because it has only two bands on the short arm, one proximal and the other distal. The long arm has banding similar to that of chromosome 4 but lacks the distinctive band below the centromere.

Chromosome 6 (Fig. 3)
This is the largest and most distinctive member of the C group. There are two darkly stained bands on the short arm. The first involves the centromeric region and the second is terminal. These two bands are
separated by a distinctive unstained area. The long arm also has a dark stained band extending from the centromere. It is followed by a prominent band, two less intensely stained bands, and a terminal pale band.

**Chromosome 7 (Fig. 3)**
The only distinctive band on the short arm is in a distal portion. The remainder of the short arm stains with an intensity that is normally seen in chromosomes stained routinely with Giemsa. The centromeric region in some preparations has stained lightly. There are two distinctive bands and a third lighter band on the long arm.

**Chromosome 8 (Fig. 3)**
The short arm has two bands: one situated just above the centromere and the other distally. The long arm has a lightly stained proximal band which may not be detected in overcontracted preparations. Second and third bands are in the middle and near the end of the long arm respectively. The bands on chromosome 8 are the least distinctive of the C group because there are no distinctive non-stained areas.

**Chromosome 9 (Fig. 3)**
The short arm has a band close to the centromere and a dark terminal area which in elongated preparations shows as two
bands. The long arm has an extensive pale area corresponding to the secondary constriction characteristically found on chromosome 9. Two dark stained bands follow and the distal portion of the arm is pale.

Chromosome 10 (Fig. 3)
Only one band, which has a distal position, can be distinguished on the short arm of chromosome 10. In many preparations the whole of the short arm stained evenly and made identification of this band very difficult. There are three bands on the long arm: the first is the most heavily stained, and is found just below the centromere, the second is in the middle of the long arm, and the third is at the end.

Chromosome 11 (Fig. 3)
A single broad dark stained band is present at the end of the short arm of chromosome 11. The long arm shows a prominent band immediately below the centromere. This is separated by a distinctive unstained area from a darkly stained median area which in good preparations can be identified as three distinct bands.

Chromosome 12 (Fig. 3)
The pattern is superficially similar to chromosome 11 but can be distinguished from that chromosome by the short arm which has a thin pale distal band and an associated larger, darker stained band. Furthermore, the centromere and only a small area of the long arm adjacent to the centromere are darkly stained. There is a median, broad, dark double band and a small pale band terminally.

The X Chromosome (Fig. 3)
There is a prominent band in the middle of the short arm and a fainter distal band. In many preparations these bands merge and appear as a single dark band. The centromere is usually unstained, although in some preparations staining has been observed. The long arm has three equally spaced bands, the first being the most prominent.

Chromosome 13 (Fig. 4)
None of the acrocentric chromosomes have distinctive bands on the short arms, which is not surprising in view of their short size. The centromeric region of chromosome 13 stains intensely, as do the first three bands. The terminal band is less intensely stained. The bands of chromosome 13 often tend to merge, and can be difficult to recognize.

Chromosome 14 (Fig. 4)
The two dark stained bands just below the centromere make chromosome 14 easily recognisable. They are followed by a small pale band in the middle and an intensely stained band near the end. In uncontracted preparations a further pale stained small band situated terminally can be seen.
Chromosome 15 (Fig. 4)
This chromosome also has two dark stained bands just below the centromere, but they are much wider than those of chromosome 14. The third band is very faint and may not be detected. The terminal band is broader and more intensely stained than that of chromosome 14.

Chromosome 16 (Fig. 5)
A dark stained band occurs in the middle of the short arm. In uncontracted preparations two further bands can be recognised: one situated immediately above the centromere and the other in a terminal position, but these are rarely visible. A very intensely stained band occurs on the long arm close to the centromere, and this is followed by bands of lesser intensity in the middle of the arm and at the end.

Chromosome 17 (Fig. 5)
A faint band can sometimes be distinguished at the end of the short arm. The centromere and a small area of the long arm adjacent to it are dark staining. Two distal bands can be detected, the first being the more prominent.

Chromosome 18 (Fig. 5)
As with chromosome 17 there is a faint band on the short arm. The centromere is usually non-staining. In contrast to chromosome 17 there is a prominent band below the centromere and a single darkly stained band terminally.

Chromosome 19 (Fig. 6)
This is one of the most easily recognised chromosomes because only the centromere and a small area of the short arm stain...
intensely, whereas the remainder of the chromosome is very pale.

**Chromosome 20 (Fig. 6)**

The banding pattern of chromosome 20 has been found to vary considerably. In many cases no distinct bands could be detected even though other chromosomes were banded clearly. The band which was detected with the most frequency was situated distally on the short arm. The centromere is usually darkly stained. There are two faint bands on the long arm; one situated in the middle and the other at the end.

**Chromosome 21 (Fig. 7)**

There is no difficulty in distinguishing chromosomes 21 and 22. The long arm of chromosome 21 has a dark band just below the centromere. In uncontracted preparations this appears as two bands, the proximal band being very narrow.

**Chromosome 22 (Fig. 7)**

This chromosome can be readily identified by the darkly stained centromeric region. Also in uncontracted preparations a terminal pale band can be detected on the long arm.

**The Y Chromosome (Fig. 7)**

The Y chromosome can often be identified by its morphological appearance but it also has a distinctive banding pattern. There are three bands on the long arm; the first is pale and near the centromere, whereas the other two, on the distal half of the arm, are intensely stained. The distal two bands appear to correspond to the brightly fluorescent area of the Y described by Zech (1969).

**Discussion**

Development of Giemsa banding techniques originated with the work of Pardue & Gall (1970), who found that when mouse chromosomes were treated with NaOH, and incubated in 2xSSC in the presence of 3H-labelled satellite DNA, the centromeric regions were not only labelled, but also intensely stained with Giemsa. Pardue & Gall (1970) considered that the intense stain reflected the rapid annealing of highly repetitive DNA present in these regions. Arrighi & Hsu (1971) used a similar method but omitted the 3H-labelled DNA. They found that the centromeric regions of human chromosomes also stained deeply with Giemsa and suggested that these heterochromatic areas contained repetitive DNA which renatured readily under the conditions of treatment. Yunis et al. (1971), using heat denaturation in phosphate buffer, found that the centromeric regions took up Giemsa stain, and notably, that with longer renaturation times other specific regions of the chromosomes also stained. The darkly stained areas corresponded to those known to be late replicating. This work was extended by Sumner et al. (1971) and Bobrow (1971 see ref. 3) both of whom found that alkali denaturation was un-
necessary, and that distinct banding patterns resulted from the incubation of chromosome preparations in 2xSSC for 1 hr. Further methods, all of which give distinct banding patterns, have been described (Dutrillaux & Lejeune 1971, Patil et al. 1971, Drets & Shaw 1971, Schendl 1971).

While all the reported methods give banding patterns, there is great variability in both the clarity and the number of bands, and this is probably a result of the different procedures used. One important factor is the denaturation pretreatment. The procedure of Arrighi & Hsu (1971), using HCl and RNase, demonstrated only the heterochromatic regions of the centromere. This rather vigorous pretreatment would remove histones and other non-acidic proteins which might play an important part in band production. An entirely different picture
emerges when heat denaturation in phosphate buffer is used, as described by Dutrillaux & Lejeune (1971). These authors present a banding pattern that is in many respects the direct converse of that obtained by other methods. A striking example is chromosome 3, which is shown by Dutrillaux & Lejeune (1971) to have a lightly stained pericentric region, whereas other authors report it as darkly stained (Sumner et al. 1971, Ridler 1971, Patil et al. 1971, Drets & Shaw 1971, Schendl 1971). Yunis et al. 1971 who use heat denaturation in phosphate buffer, also present a karyotypic pattern which is different from that of other methods. We, and others (Drets & Shaw 1971, Schendl 1971) have found that treatment with NaOH is necessary for the production of clear bands. However, distinct banding has been obtained without the use of alkali pretreatment (Sumner et al. 1971, Ridler 1971, Patil et al. 1971).

Only two renaturation buffers have been reported; SSC, in concentrations ranging from × 2 (Sumner et al. 1971, Ridler 1971) to × 12 (Drets & Shaw 1971), and .06M phosphate buffer (Dutrillaux & Lejeune 1971, Yunis et al. 1971, Schendl 1971). Both buffers appear to give adequate banding patterns. The time in buffer has ranged from 1 hr (Sumner et al. 1971) to 72 hrs (Drets & Shaw 1971). The long incubation time would appear to reduce the banding because Drets & Shaw (1971) report fewer bands than other authors. This is particularly noticeable in chromosomes 5 and 15. The common choice of temperature for reassociation is 60°C, but higher temperatures have been reported (Dutrillaux & Lejeune 1971, Yunis et al. 1971). These higher temperatures probably account in part for the different banding patterns obtained by these authors.

The unique properties of Giemsa stain are undoubtedly important for band formation because all authors report its use. Indeed, Patil et al. (1971) have obtained banded chromosomes by merely raising the pH of the Giemsa stain to 9. They found the banding pattern to be similar to that produced by U.V. fluorescence, with the notable exception of the Y chromosome which was pale staining, whereas the distal half of this chromosome fluoresces brightly. Drets & Shaw (1971) have found that acetic orcein did not produce banding, and we have had a negative result with cresyl violet which we routinely use as a chromosome stain. Sumner et al. (1971) report that methylene blue gives only weak banding, so it would appear that some, as yet unidentified, property of the combination of eosin and methylene blue is a factor in the production of specific banding.

The hypotonic solution used in the preparation of the chromosomes may be a factor in band formation. Our experiments have given good banding with KCl or Na-citrate but we obtained little or no banding with distilled water. KCl was used by Brooke et al. (1962) to uncoil and disassociate chromosomes, and Hungerford (1965) found that a higher concentration was less disruptive and gave clear chromosome definition. Lam Po Tang (1968) also noted improved morphology of bone marrow chromosomes treated with KCl. It is possible that KCl and Na-citrate have an effect on chromosome structure which is important in band formation.

Sumner et al. (1971) have suggested that fixation in acetic methanol denatures the DNA and that imperfect annealing occurs when the slides are air dried. When the chromosomes are incubated in 2xSSC the non-repetitive DNA is degraded and does not stain. Rowley & Bodmer (1971), on the other hand, suggest that single stranded DNA may be more readily denatured during the prolonged incubation in buffer and that the Giemsa may be binding to double stranded DNA.
However, one must doubt the validity of assuming that the darkly stained bands do represent repetitive DNA, if only for the following reasons. Firstly, the banding pattern reported by Dutrillaux & Lejeune (1971) appears to be the complement of that produced by other techniques (Sumner et al. 1971, Ridler 1971, Patil et al. 1971, Drets & Shaw 1971, Schendel 1971), and if all dark stained areas are repetitive DNA, it follows that many chromosomes are composed largely of repetitive DNA. This is a most improbable situation because Corneo et al. (1970) have shown that human DNA has about 2.5% of satellite DNA containing highly repetitious DNA, and a further 15% of moderately repetitious main band DNA. Furthermore, repetitive DNA is thought to be genetically inactive, and if the darkly stained areas represent repetitive DNA, then many chromosomes are composed almost entirely of repetitious sequences which serve no useful coding purpose. Secondly, if these darkly stained areas are heterochromatic, then one would expect the late labelling heterochromatic X chromosome of female cells to be intensely stained—a fact not yet reported.

The centromeric regions of mouse chromosomes have been shown to contain highly repetitive DNA (Pardue & Gall 1970), and it is likely that the darkly stained centromeric regions of human chromosomes demonstrated by Arrighi & Hsu (1971) likewise contain highly repetitive DNA. At present, no radioactive annealing studies with human satellite DNA have been carried out to ascertain if either the centromeric regions, or the darkly stained Giemsa bands, contain highly repetitive DNA. Factors other than repetitive DNA may well play a part in the formation of banding patterns.

Whatever the nature of the Giemsa stained bands, this technique will play an important part in chromosome identification because of its technical simplicity, and the specific banding pattern it produces. The detailed patterns should help disclose the exact nature of chromosome rearrangements, and the precise identification of the abnormal chromosomes found in human leukaemia and other tumours. Variant banding patterns will also be detected in the human population. Polymorphism of human constitutive heterochromatin has been described (Craig-Holmes & Shaw 1971), and we have found a man with atypical banding of the Y chromosome. Refinements of technique will undoubtedly lead to a standard pattern and to the detection of further abnormalities.

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References


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