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The Mechanism of C-Banding: Depurination and β-Elimination

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Abstract. C-banding of chromosomes involves the differential solubilization of fragmented DNA from euchromatin by three sequential treatments: 1. Acid, 2. Mild base, 3. Hot salt. The data indicate solubilization is effected by 1) depurination, 2) DNA denaturation, 3) chain breakage of the depurinated sites respectively in the three treatments. Conditions were found wherein each treatment in proper sequence was necessary for C-banding and the appropriate chemical reactions were measured in these treatment conditions. The acid treatment (0.2 N HCl) depurinates chromosomal DNA at the rate of 0.26×10^{-6} purines/dalton min to an alkaline molecular weight of 10^5 daltons but does not break the depurinated sites. Bleomycin can substitute for acid as a base removing agent. Sodium borohydride, by reducing the depurinated sugar's aldehyde thereby preventing chain breakage by the β-elimination reaction, reversibly inhibits DNA-extraction. Chain breakage at the DNA's apurinic sites occurs not in the 2 min mild alkali treatment where the half-life for breakage is 26 min but in the 18 h hot salt treatment where the half-life for chain breakage is 1–2 h. Most of the DNA extraction occurs in the hot salt as 10^5 dalton fragments as measured in formamide gradients. Bleomycin is introduced as a substitute for HCl; it removes nitrogenous bases from DNA in situ while better preserving the morphology of the final C-banded chromosomes.

Introduction

Banding of Giemsa stained chromosomes involves treatments which act upon a prepattern of longitudinal differentiation in the fixed chromosome to produce a banding pattern of differentially decreased Giemsa basophilia. Since Giemsa stains DNA but not protein (Comings and Avelino, 1975), one can decrease chromosome basophilia either by rendering the DNA inaccessible to dye, as in trypsin G-banding (Comings and Avelino, 1975) or by removing the DNA itself, as in the BrdU-Hoechst-Giemsa technique (Latt et al., 1977) and in stan-

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dard C-banding (Comings et al., 1973; Pathak and Arrighi, 1973). Overtrypsinization (Klinger, 1972) or warm formamide → hot salt (Hubble et al., 1976) treatments reveal the C-prepattern without appreciable DNA loss. This investigation concerns itself only with the standard C-banding procedures which reveal the C-prepattern by differential DNA extraction.

Before asking why DNA is differentially removed from euchromatin during C-banding leaving DNA mostly in heterochromatic regions, we must ask why DNA is removed at all. Since DNA in vivo is of chromosome size (Kavenoff et al., 1973), it must be fragmented extensively during the fixation and banding procedures to escape from the fixed chromosome. Photodegradation of dye sensitized BrdU substituted DNA followed by DNA fragment elution during the subsequent hot salt treatment appears to be the mechanism responsible for reduced basophilia of BrdU substituted chromatids (Latt et al., 1977). Sederoff et al. (1975) first proposed that DNA depolymerization during C-banding may be due to acid depurination with subsequent strand breakage in alkali and solubilization in hot salt. This report carefully defines the chemical reactions which depolymerize DNA during the C-banding procedure, and shows this depolymerization to be necessary for extraction of DNA from the fixed chromosome.

Standard C-banding involves four sequential chemical treatments before staining:

Fixation → Acid → Alkali → Hot Salt.

Conditions were found whereby each treatment in proper sequence is essential to the final C-banded product. Each treatment then causes one or more unique chemical or physical alterations of the chromosome necessary for the final selective solubilization of euchromatic DNA. Some chemical modifications of DNA which may occur during the various C-banding treatments are shown in Figure 1. With these reactions in mind, we have done the following: 1) By minimizing depurination during acid-alcohol fixation, we have made the acid treatment obligatory for DNA solubilization. 2) By substituting bleomycin for the acid treatment, we show depurination to be the essential chemical reaction of the acid treatment. 3) Since borohydride reduction of the aldehyde group produced by depurination reversibly inhibits DNA-solubilization, the necessary reaction of the alkali or hot salt treatments is to act on the aldehyde presented by the acid treatment thereby breaking the phosphodiester backbone of DNA by β-elimination. 4) By reducing the pH of the hot salt treatment, the alkali treatment becomes obligatory to DNA solubilization. 5) By measuring the kinetics of β-elimination, we show it not to occur appreciably during the alkali step but to occur during the hot salt treatment. Thus, DNA extraction involves a two step fragmentation process requiring first acid, then heat.

Materials and Methods

Cell Culture and Cytological Preparation. Several transformed mouse lines were prepared for cytological studies by fixing (24°) in methanol: acetic acid (3:1) and spreading on wet slides. For molecular

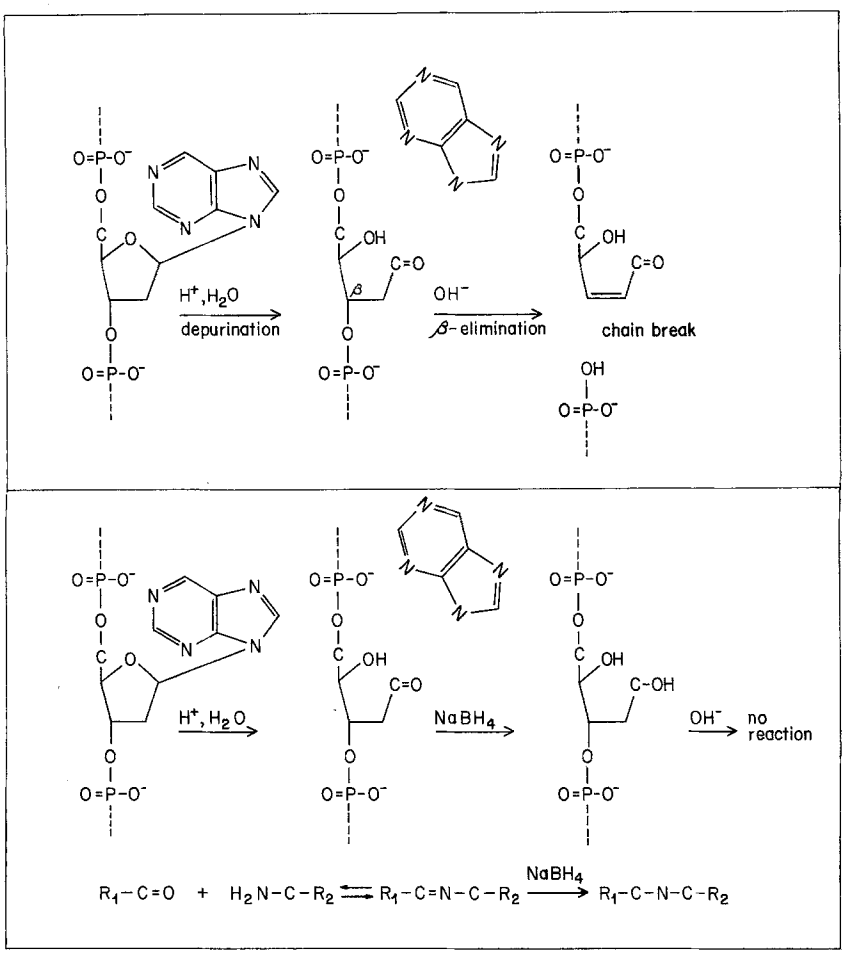

Fig. 1. Acid treatment of DNA protonates the N-glycosidic bonds of purines resulting in hydrolysis of purines from the pentose sugars without breaking the phosphodiester backbone directly (Tamm et al., 1952). The deoxyribose residue then exists in equilibrium between the closed furanose form and the open aldehyde form shown here (Overend, 1950). Bleomycin acts like acid, exposing aldehydes in the DNA (Müller and Zahn, 1976) and liberating nitrogenous bases while leaving the phosphodiester backbone intact (Ross and Moses, 1978). The deoxyribose residues which remain after acid or bleomycin treatment are labile sites along the phosphodiester backbone which break in acid, base or at elevated temperatures. β-elimination, activation of the pentose carbon β to the aldehyde, is the predominant chain breakage reaction of depurinated DNA in alkali (Bayley et al., 1961; Jones et al., 1968) and elevated temperatures (Lindahl and Andersson, 1972); it can be prevented by reducing the pentose aldehyde to an alcohol with sodium borohydride. The pentose aldehyde of depurinated DNA or the terminal α–β unsaturated sugar of cleaved DNA react with Schiff reagent, as in the Feulgen reaction (Swift, 1955), or may reversibly form Schiff bases with various protein groups such as the ε-amine of lysine. Subsequent borohydride reduction of the Schiff base could conceivably covalently link DNA to protein by a stable imide bond

weight determinations, NIH Swiss embryo V16 mouse cells were grown in McCoy's medium, 20% Fetal Calf serum, 5 μg/ml thymidine, 0.1 μg/ml FdU, 1.0 μCi/ml ^{3}H-thymidine to $^2/_3$ confluence in plastic flasks. Loose cells were shaken off and the media replaced with conditioned medium containing 0.06 μg/ml Colcemid. After 4 h, metaphase cells were shaken off, swollen for 15 min in 0.074 M KCl and fixed in methanol:acetic acid (3:1). Preparations containing less than 93% metaphase cells were discarded.

Anhydrous fixation: resuspend chilled pellet of swollen cells in 3 ml of 3:1 fixative, 0° C, immediately pellet cells, 3 min, resuspend immediately and repeat 3 times. Spread fixed cell suspension on 24° C dry microscope slides. Standard fixation: resuspend swollen cells (24° C) in 0.5 ml 0.074 M KCl, slowly add 4 ml fixative, let stand 20 min, 24° C, pellet cells, resuspend in fixative, repeat 3×. Spread fixed cells onto cold wet slides. Each slide contained $5–10 \times 10^4$ CPM in a circle of 24 mm diameter.

ϕX 174 phage DNA, 6×10^5 cpm/ml, 1,250μg/ml in 50 mM Tris 20 mM pH 7.5 (a gift from Robb Moses and Steven Ross), originally RFI (supercoiled form) was degraded to 3–5% supercoiled from as measured by filter retention (Kuhnlein et al., 1976). Bleomycin (Bristol Labs, Syracuse, N.Y.) was made to 1 mg/ml in 10 mM Tris pH 7.5 and stored at 4° C.

ϕX Depurination in HCl. 5 μl ϕX DNA, 20 μl 3 mg/ml salmon sperm DNA in 0.10 mM NaCl 1 mm EDTA + 0.1 ml 0.25 N HCl. Incubate 24° C. Depurination in anhydrous fixative required a precipitation step. 5 μl ϕX DNA, 50 μl salmon sperm DNA and 5 μl 5 M NaCl were mixed (0° C), precipitated with 300 μl fixative (MeOH/HAc: 3/1), and resuspended in fixative (24° C). Depurination was terminated by centrifugation and titration of the pellet.

$NaBH_4$ Reduction. Sodium borohydride reduction was according to Hadi et al. (1973). Slides were placed in 40 ml of 0.5 M KPi, 1 mM EDTA pH 6.5. Sodium borohydride (Mg^{2+} free) 0.5 g was dissolved in 5 ml H_2O. One third of this was added to the buffer at three 10 min intervals (24° C) followed by another 30 min reaction period; pH was maintained below 8.5 by adding HCl. ϕX DNA was depurinated in 0.2 N HCl 24° C, neutralized with KPi and used directly or dialyzed against 0.01 M NaCl, 1 mM EDTA. Subsequent alkali treatment in 8.5 mN NaOH, 0.3 M NaCl was stopped by back titration with HCl. Carrier DNA (20 μg) was added, the solution was brought to 4 ml of 0.5 M KPi, 1 mM EDTA pH 6.5 and reduced with $NaBH_4$ as previously described. Two volumes of −20° C ethanol stopped the reaction and a two phase system formed after storage at −20° C. 80–90% of the ^{3}H-DNA was recovered from the lower (about 0.5 ml) phase by spooling, rinsed in ethanol, and dissolved in alkaline loading buffer. This unique two phase system, which simplifies reduction of small amounts of DNA, did not form unless the $NaBH_4$ was allowed to react for a few minutes.

Alkaline Isokinetic Gradient Analysis. Chromosomal DNA was digested off the slide, 37° C, 1 h, with 0.1 ml of 1% Sarkosyl, 0.01 M Tris, 0.02 M EDTA, 1 mg/ml Pronase, 50 μg/ml salmon DNA, pH 8.0, placed between microslide and coverslip. The slide was rinsed with 0.1 ml of 1.5 N NaOH, 30 mM EDTA, and with 0.1 ml H_2O. The combined rinses, 0.3 ml, were layered over isokinetic 5–20% sucrose gradients (4.8 ml of 0.3 N NaOH, 0.7 M NaCl, 10 mM EDTA) and spun 20° C, 49,000 rpm, 4–6 h in albumin coated cellulose nitrate tubes in a SW50.1 rotor. ϕX DNA was loaded in the same final mixture. Gradients were longitudinally assayed for TCA precipitable radioactivity by the paper strip method (Carrier and Setlow, 1971). Molecular weights were calculated using the peak of ^{3}H-ϕX DNA as a 1.7×10^6 dalton marker in the sixth tube of each centrifuge run. Number average molecular weights were calculated by computer assuming mobility was proportional to molecular weight exp (0.38) and migration started 0.2 ml from the top of the loading solution. An unbiased second party determined boundaries for the activity distribution in each gradient.

Depurination rates were calculated as described in Figure 2. Here, a hypothetical molecule of molecular weight M = 100 is nicked at a linear rate so that after 8 h, there are 8 nicks and 9 fragments of number average molecular weight $M_n = 11.1$. Knowing the initial and final Mn, one can calculate the number of nicks $= (M_o/M) - 1 = k't$ where t is time and k' is the nick rate for a molecule of size M_o. Randomly nicked populations of macromolecules have a Montroll-Simha (1940) distribution where M_n, M_w (weight average) and the molecular weight at the peak of the distribution are all proportional; $M_n < M_p < M_w$ and $M_w = 2M_n$ (Tanford, 1961). Since our untreated ϕX DNA

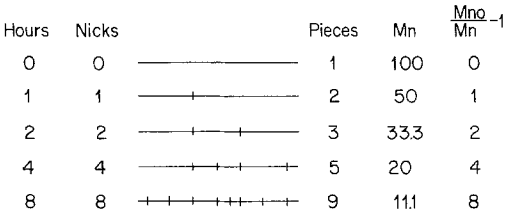

Fig. 2. Nick rate calculation. – A hypothetical polymere is nicked at the rate of 1 nick/h. Number average (M_n) rather than weight average (M_w) molecular weights are used in calculations because only M_n is independent of the position of the nicks (Tanford, 1961). The number of nicks introduced into a molecule or population of molecules is determined from the initial (t=0) and final M_n by the equation nicks$=(M_{no}/M_n)-1$. Note that if eight lesions instead of nicks were introduced followed by a treatment which slowly nicks at lesions and a Mn determination of the partially nicked DNA, then one could use this same equation to determine the rate at which lesions are converted into nicks

population was not randomly nicked, M_n instead of peak molecular weight had to be used in the weight ratio equation for nicking rate.

Formamide (Fisher) gradients were 4.8 ml, 5–20% sucrose, 1 mM EDTA and adjusted to pH 7 (indicator paper) with HCl and spun in the SW50.1 rotor at 28° C for 15 h. In these gradients, the sedimentation rate of DNA 25% of the distance from the top of the gradient was twice that of DNA half way through the gradient. An approximate correction for changing sedimentation rate was incorporated into the computer program to roughly determine M_n.

Results

Biochemistry

To assure that all potentially alkali labile bonds are cleaved during alkali sedimentation, six slides were 0.2 N HCl treated for 30 min, pronase digested and the extracts were pooled and stored at 0° C. Aliquots were subjected to the following treatments before layering over alkaline gradients: 0.5 N NaOH, 0.01 M EDTA at 24° C for 18, 1.5 and 0.25 h; 1.0 M Glycine, 0.01 M EDTA pH 12.8, 24° C for 18 and 1.5 h. Radioactivity in all gradients sedimented identically with M_n equal to 0.064. Lindahl and Andersson (1972) stated that alkali lability of the first apurinic site of the denatured form of covalently circular PM2 DNA is incomplete after a few hours in 0.5 N NaOH; they used glycine to catalyze alkaline lability. Our results show alkaline lability of relaxed DNA to be complete during the short time required to load and start the centrifuge; our alkaline gradients therefore reflect total alkali lability and not a partially labilized intermediate.

DNA is solubilized from chromosomes only during the last two treatments, alkali and hot salt; the % DNA extracted (Table 1) is in accord with other observations (Comings et al., 1973; Francke et al., 1973). With extensive base treatment, 75% of the DNA was removed resulting in very faintly staining chromosomes. Short alkali treatments selectively solubilized DNA of low alkaline MW (Table 1) and in general, solubilized DNA sedimented a little slower than residual DNA.

Table 1. DNA extracted during banding. – Fixed metaphase cells were dried onto the inner surface of small test tubes, HCl treated (0.2 N, 15 min, 25° C) and processed for C-banding. The reagent supernatants were filtered through a 0.2 micron nucleopore filter, pronase digested and layered onto gradients. Water rinses were only assayed for TCA precipitable radioactivity. After the 65° C pH 7.3 treatment, the glass was shattered and one piece was stained with Giemsa and scored for C-banding on a scale of 0–5. The chromosomes were pronase digested for centrifugation and alkaline molecular weights (daltons) determined

	$M_n \times 10^6$	%CPM	C-banding
NaOH (30 sec) supernate	0.069	8.8	
65° C supernate	0.167	43	
65° C remains	0.179	48	Good; +4.0
NaOH (5 min) supernate	0.138	21	
65° C supernate	–	53	
65° C remains	0.180	26.5	faint staining; +2.3

Table 2. Depurination rates. – Chromosomes or ϕX DNA were partially depurinated in 0.2 N HCl at 25° C, for the indicated times and alkaline number average (M_n) or weight average (M_w) molecular weights determined. Separate rotor runs are separated by a space. Weight average molecular weights are about twice their respective number average molecular weights except for undegraded ϕX DNA, which has not a randomly cleaved distribution, and the highly degraded DNA, which did not migrate far into the gradient. The depurination rate constant is in units of purines removed per dalton of DNA per minute by 0.2 N HCl, 24° C. Rate constants from chromosomes fixed by the anhydrous method are indicated by asterisk

Chromosomal DNA				ϕX DNA			
min HCl	$M_n \times 10^6$	$M_w \times 10^6$	$k \times 10^{-6}$	min HCl	$M_n \times 10^6$	$M_w \times 10^6$	$k \times 10^{-6}$
M_o	0.223	0.430		M_o	1.02	1.43	
3	0.180	0.361	0.357	3	0.327	0.643	0.692
15	0.166	0.374	0.103	15	0.141	0.274	0.407
60	0.069	0.135	0.167	80	0.024	0.057	0.509
360	0.021	0.077	0.120				
				M_o	0.536	1.196	
M_o	0.24	0.42		30	0.054	0.113	0.557
15	0.10	0.19	0.389				
15	0.12	0.22	0.278	M_o	1.41	1.86	
				15	0.122	0.257	0.499
M_o	0.135						
30	0.074		0.203	M_o	1.20	1.65	
				5	0.348	0.797	0.408
						average	k=0.512
M_o	0.301	0.675					
15	0.0945	0.211	0.438[a]				
		average	k=0.262				

[a] From anhydrous fixed chromosomes

Depurination rates in any one experiment were calculated from the equation in Figure 2, $(M_o/M_t)-1=k't$ where k′ is the rate of purine loss for a molecule of size M_o. The intrinsic depurination rate k of DNA is $k=k'/M_o=(M_o/M_t)-1/M_ot$. The average depurination rate for chromosomal DNA, 0.262×10^{-6} purines/dalton, min (Table 2) indicates that during the standard 15 min HCl treatment, an average of one purine is lost for every 793 bases (0.26×10^6 daltons). The depurination rate for free DNA is about twice that of DNA in fixed chromosomes (Table 2). In one experiment with slides stored 46 days 24°, the initial M_o was 0.135×10^6 daltons and the average k from 7 different slides and two separate experiments was only 0.107×10^{-6}. These slides, however, did not C-band by the standard procedure and required more extensive NaOH treatment to reveal marginally acceptable C-bands. This observation shows the importance of parallel cytological controls, offers an explanation for old slides not banding, suggests that some of the variation in k determinations of fixed chromosomes is due to slide aging, and shows the depurination rate difference between free and chromosomal DNA to depend on the history of the fixed chromosome.

Depurination is a hydrolytic cleavage (Fig. 1) which cannot occur in anhydrous mixtures of methanol-acetic acid unless the fixative is diluted with water (Table 3). This observation is the rationale for the anhydrous fixation method in which cold fixative is quickly added to cells; they are subsequently prepared to experience a minimum time in semiaqueous conditions. DNA from such chromosomes had alkaline molecular weights which averaged 1.7× larger than DNA from standardly fixed more depurinated preparations. Anhydrous fixation results in chromosomes which require subsequent depurination to C-band but, because it produces poorly spread metaphase preparations, it is not recommended for routine cytology.

Sodium borohydride repairs alkali sensitive sites in depurinated DNA. By reducing the pentose-aldehyde, it prevents β-elimination (Hadi et al., 1973; Fig. 1). Excepting the first set of data in Table 4, repair is maximal after 15 min of reduction and the 1 h treatment routinely used was considered sufficient to repair all ultimately repairable lesions.

To test if β-elimination occurs in C-banding's NaOH step, ϕX-DNA was partially depurinated and treated for various times with 0.0085 N NaOH (Table 5), a concentration which is just sufficient for C-banding. Sites not cleaved

Table 3. Depurination in fixative. ϕX DNA or chromosomes were exposed to fixative (3 parts methanol; 1 part acetic acid) 24° C, aqueous fixative 24° C, or Bleomycin (1.0 mg/ml) 4° C and alkaline molecular weights determined. *k* is bases removed/dalton min

	$M_n\times10^6$	$k\times10^{-6}$
Untreated ϕX DNA	0.910	
ϕX in fixative, 5 h	0.845	
ϕX in 50% fix: 50% H_2O, 5 h	0.0846	0.0357
ϕX in Bleomycin, 2 days	0.117	0.0037
untreated chromosomes	0.17	
chromosomes in fixative, 5 h	0.17	

Table 4. Rate of repair of alkali sensitive sites. ϕX DNA was partially depurinated, neutralized and either dialyzed then reduced or reduced directly with sodium borohydride. Reduced DNA was recovered by spooling before alkaline molecular weight determination

	$M_n \times 10^6$
ϕX DNA	0.5365
↓	
Depurinated 30 min	0.0538
↓	
5 min $NaBH_4$	0.2935
20 min $NaBH_4$	0.2690
60 min $NaBH_4$	0.6528
ϕX depurinated 15 min and dialyzed	
↓	
15 min $NaBH_4$	0.713
60 min $NaBH_4$	0.702
ϕX depurinated 15 min and dialyzed	
↓	
15 min $NaBH_4$	0.631
60 min $NaBH_4$	0.636

Table 5. Rate of alkali lability. – ϕX DNA was partially depurinated, 0.2 N HCl, 15 min, 24° C, neutralized and dialyzed before alkali treatment, $NaBH_4$ reduction and alkaline molecular weight determination. The 0.00085 N alkali contained 0.3 M NaCl. The 0.5 N NaOH treatment, which had been shown to labilize all potentially labile sites and the NaCl treatment, which we assumed broke no bonds, were considered equivalent to an infinitely long time and zero time respectively in alkali. Labile sites remaining after time t in 0.0085 N alkali $= (M_{nt}/M_{n\infty}) - 1$ and are plotted in Figure 3

Time in NaOH		$M_n \times 10^6$	Labile sites remaining
0	0.4 M NaCl, 1 h → $NaBH_4$	0.911	2.70
4	4 min, 0.0085 N NaOH → $NaBH_4$	0.882	2.59
30	30 min, 0.0085 N NaOH → $NaBH_4$	0.581	1.362
60	60 min, 0.0085 N NaOH → $NaBH_4$	0.361	0.467
∞	60 min, 0.5 N NaOH → $NaBH_4$	0.246	0

by NaOH were repaired by reduction with $NaBH_4$ before Mn determination. In dilute NaOH, the alkali labile sites are slowly cleaved (Table 5) at a certain probability per unit time. The halflife for cleavage of one of these sites in dilute NaOH (Fig. 3) is 26 min. At this rate, negligibly few (2.6%–10%) sites would be cleaved during the 1–4 min NaOH treatment minimally required for C-banding.

Amino acids (Lindahl and Andersson, 1972) catalyze β-elimination and the protein in fixed chromosomes may possibly do likewise. However, a 4 min treatment of chromosomes with 0.01 N NaOH, which sufficed to C-band the chromosomes and should have removed about 18% of the DNA (Table 1), did not significantly cleave alkali labile lesions (Table 6 compare row #3 with row #4). Thus, the alkali treatment minimally required for C-banding cleaves free depurinated DNA and depurinated chromosomal DNA to a very minor

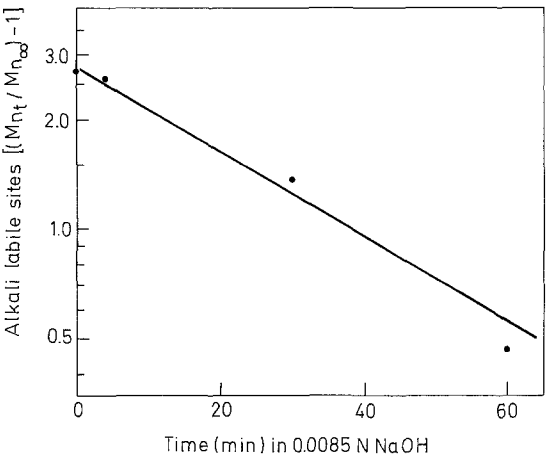

Fig. 3. The rate of alkali lability plotted from data in Table 5 as labile sites remaining versus time in 0.0085 N NaOH. The rate constant for alkaline lability (reciprocal of the time required to break 63% of the labile bonds) is 4.3×10^{-4} sec^{-1} which corresponds to a half-life of 26 min for any one labile bond

Table 6. $NaBH_4$ reduction of chromosomal DNA. – Chromosomes fixed by the anhydrous or standard methods were treated with 0.2 N HCl, 15 min, 24° C; 0.01 N NaOH, 0.3 M NaCl, 24° C, or $NaBH_4$ 1 h and alkaline molecular weights determined. Three experiments on different slide batches fixed differently are shown

#	Treatment	$M_n \times 10^6$
1	Untreated anhydrous fix	0.30
2	HCl	0.094
3	HCl → NaOH (4 min) → $NaBH_4$	0.29
4	HCl → $NaBH_4$	0.30
5	$NaBH_4$	0.52
6	Untreated standard fix	0.24
7	HCl	0.10
8	HCl → $NaBH_4$	0.24
9	HCl	0.12
10	HCl → $NaBH_4$	0.29
11	$NaBH_4$	0.37
12	Untreated anhydrous fix	0.35
13	HCl → $NaBH_4$	0.44
14	HCl → $NaBH_4$	0.50

extent. This may not be true for banding procedures which utilize stronger (0.07 N) alkali.

The remaining data in Table 6 simply show by alkaline molecular weight increases that $NaBH_4$ treatment in situ is capable of repairing fixative or HCl induced alkali labile lesions in chromosomal DNA. Since $NaBH_4$ does extract some chromosomal DNA which may preferentially be of smaller size, the M_n of DNA remaining in situ after $NaBH_4$ treatment may slightly overestimate the total M_n. In any case, depurinated sites are rendered alkali stable by in

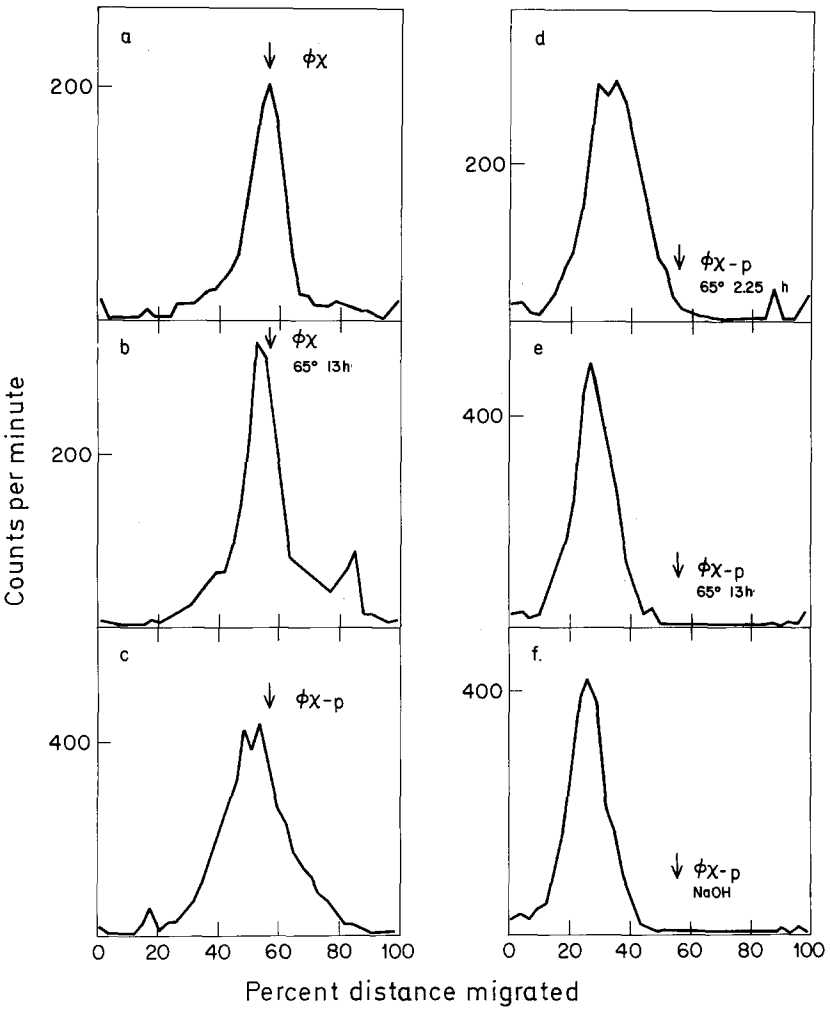

Fig. 4a–f. Formamide gradients. – ^{3}H-ϕX DNA (95% supercoiled), 1.5 mg/ml salmon DNA was freeze-thawed 10X reducing the supercoiled form to 50%: total volume was 30 µl stock ϕX. **a** 44 µl ϕX stock in 0.25 M NaPi, 3 mM EDTA, pH 7.3. **b** as above but incubated 65° C, 13 h. The remaining ϕX stock was partially depurinated 15 min, 24°, 0.2 N HCl, and the precipitate redissolved. **c** depurinated ϕX (ϕX-p) 0.25 M NaPi, 3 mM EDTA pH 7.3. **d, e** As above but incubated 2.25 and 13 h 65° C. **f** 40 µl depurinated ϕX in 0.5 N NaOH, 1 h, 24° C was neutralized with HCl. All reaction mixtures were 0.1 ml and made to 0.3 ml with formamide, heated 65° C for 5 min before formamide gradient sedimentation

situ reduction with $NaBH_4$. While repair of acid induced alkali labile lesions is complete in vitro (Table 4), it is not complete in situ (Table 6 compare #4–#5, #10–#11). This could mean that acid induces some breaks in chromosomal DNA or that all depurinated sites in situ are not accessible to reduction.

Apurinic sites undergo β-elimination at elevated temperatures and this was

tested for ϕX DNA using the hot salt conditions of C-banding and subsequent sedimination through neutral formamide under loading and running conditions where DNA is single stranded and apurinic sites are not labile. The rationale here is depurination does not cause single strand breaks (Fig. 1) and formamide, as opposed to alkali, denatures DNA without inducing single strand breaks at apurinic sites. DNA mobility in the formamide gradient thus reveals the linear density of single strand breaks rather than the density of apurinic sites plus breaks. Figure 4a shows the mobility of single stranded linear ϕX DNA; the denatured form of double stranded covalently circular ϕX DNA (50% of the sample) pelleted. Heat treatment (Fig. 4b) did not appreciably alter mobility but it did introduce some nicks because little radioactivity pelleted. HCl depurination (Fig. 4c) also had little effect on mobility as expected if it introduced no nicks. However, when the depurinated ϕX DNA was heated (Fig. 4d and e) or treated with strong alkali (Fig. 4f), mobility decreased significantly; this is consistent with cleavage at apurinic sites. These gradients were not isokinetic; sedimentation slowed appreciably as radial distance increased (unpublished data). When this non-linear sedimentation rate was incorporated into the assumptions (Fig. 2) used to calculate alkali-lability kinetics, the half-life of an apurinic site at 65° C pH 7.3 was 0.7–2 h and the corresponding rate constant, $1\text{–}2.6\times10^{-4}$ sec^{-1}, is close to that reported (2.4×10^{-5} sec^{-1}) at 70° C pH 7.3 where lability was shown to be by β-elimination (Lindahl and Andersson, 1972). Comparing gradients c, d and f, in Figure 4 one sees that most of the heat or alkali labile bonds are broken in 2.25 h at 65° C. Similar kinetic measurements on chromosomal DNA were not attempted because the progressive solubilization of DNA during heat treatment necessitates more control gradients than a rotor accommodates. One experiment is in accord with in situ heat lability of apurinic chromosomal DNA. Two slides were carried through the standard C-banding procedure including 18 h, 65° C, pH 7.3. One slide was $NaBH_4$ treated. DNA from both slides sedimented identically ($M_n=0.13\times10^6$ daltons) in alkaline sucrose indicating the absence of $NaBH_4$ repairable sites in the chromosomal DNA which remained in situ.

Thus, we may reasonably conclude that apurinic sites of chromosomal DNA are broken in the early stages of C-banding's hot salt treatment and not appreciably broken during the NaOH treatment. Suspecting DNA to be extracted from chromosomes during C-banding because it is fragmented during the process, we have shown that during C-banding the phosphodiester backbone of DNA is cleaved by a two step process involving depurination during both fixation and acid treatment and by labilization (β-elimination) of the depurinated sites during the hot salt treatment. That this cleavage is essential to chromosomal DNA solubilization and not just an unimportant side reaction remains to be shown. However, we also learned that depurination does not occur in anhydrous fixative, that a bleomycin treatment for two days produces the same density of alkali labile lesions as the 15 min acid treatment (Table 3) and that the lesions can be repaired by $NaBH_4$ reduction. We shall now apply this chemical knowledge to modify the C-banding procedure itself and see if the proposed chemical mechanism is consistent with DNA extraction.

Cytology

The extensive cytological data presented in Tables 7, 8 and 9 extends the work of McKenzie and Lubs (1973) and will only be summarized with occasional numerical references to treatment conditions by row number. C-banding here consists of three sequential steps, acid-base-hot salt. Conditions were found whereby each step is, of itself, necessary for a C-banded product. Then, a particular step was modified or substituted hopefully to ascertain the chemical or physical change(s) which make that particular step necessary for DNA extraction.

Slide aging is important to almost all banding techniques. Slides stored without dessication at 24° C for 5 days or more showed the effect of aging; NaOH treatment had to be extended to produce good C-bands. Increasing the HCl treatment aided banding of old slides to a limited extend. Extremely aged slides lost basophilia from heterochromatin and euchromatin at equal rates during the banding process whatever extremes of conditions were used. Freshly prepared chromosomes swelled excessively during the NaOH treatment. Storing slides at $-20°$ C or $-70°$ C in a dry N_2 atmosphere inhibited the aging effect in that 3 day or 3 week old slides banded similarly. Such slides were used in subsequent experiments.

Chromosomes treated with trypsin lose Giemsa basophilia of their R-bands (i.e., they G-band) without DNA loss (Comings et al., 1973). Such chromosomes do, however, stain uniformly with ethidium bromide (data not shown) indicating chromosomal DNA is much more accessible to ethidium than to Giemsa. Since both the Giemsa and ethidium staining patterns after the same C-banding treatments were similar (Table 8), we shall interpret decreased Giemsa stainability as a DNA loss.

Separation of the alkali effect from the hot salt effect: If the alkali treatment is extensive, fair C-bands can be produced without hot salt (data not shown). When the alkali treatment is mild, hot salt is always required for DNA extraction as determined by decreased staining of euchromatin (Table 7). An NaOH treatment is less necessary for C-banding if the hot salt is alkaline but reducing the pH of the hot salt to 7.3 makes a prior NaOH treatment obligatory for C-band production (McKenzie and Lubs, 1973; Table 8 #12 and #13). Most workers use 2× or 6× SSC in their C-banding procedure and adjust the pH to 7 where citrate ($pK_2 = 5.5$) does not buffer well and wherein at 65° C, Na_2O, CaO and SiO_2 leach from the glass slides raising the pH to 10 in a few hours (Eiberg, 1973). The final pH actually depends on how many slides are cooking. Such leached cations have not been directly tested for their effects on C-banding (Scheres, 1977). When ten slides were incubated 65° in 40 ml of 0.25 M NaPi pH 8.3, 65° C, 3 days, the pH remained constant but the glass slides were basophilic to Giemsa and a crude elemental analysis was positive for calcium (results not shown). Slides incubated similarly at pH 7.3 65° C did not stain with Giemsa and no calcium was detected in the solution. Thus, the NaPi, EDTA buffer used at pH 7.3 maintains pH and ionic composition of the incubation buffer, prevents the glass slide from staining like a chromosome and makes a previous alkali treatment necessary for C-banding.

Table 7. C-Banding: Standard fixation ± 65° C pH 8.3. – Appearance of standardly fixed slides scored on a scale of 0–4. *C* is C-band quality; Eu is darkness of euchromatin; swelling was measured relative to unswollen, untreated chromosomes. Treatments were 0.2 N HCl, 24° C, 15 min; 0.01 N NaOH, 0.3 M NaCl, 24° C, 2 min; $NaBH_4$, 1 h. After treatment, slides were broken in half. The bottom half was Giemsa stained directly (lower set of numbers in parentheses) and the upper half was incubated 18 h, 65°, 0.25 M NaPi, 3 mM EDTA, pH 8.3 before staining. Each datum is from 4 slides treated on 3 different days. The degree of C-banding is roughly the ratio (basophilia of heterochromatin/basophilia of euchromatin). Darkness of euchromatin indicates how much DNA was extracted before staining. Since the slides were scored both with and without a hot salt treatment, one can ascertain DNA extraction before and after hot salt extraction

#	Treatment		C	Eu	Swelling
1	HCl → NaOH	+65° C	4	0	3
		−65° C	(2)	(2)	(2)
2	HCl → $NaBH_4$ → NaOH		1	2	2
			(0)	(3)	(2)
3	HCl → $NaBH_4$ → HCl → NaOH		4	1	3
			(2)	(2)	(3)
4	HCl → NaOH → $NaBH_4$		3	1	3
			(1)	(1)	(3)
5	$NaBH_4$ → HCl → NaOH		4	1	2
			(2)	(3)	(2)
6	$NaBH_4$ → NaOH		1	2	2
			(0)	(3)	(2)
7	NaOH → HCl		3	1	3
			(0)	(3)	(3)
8	NaOH		3	1	4[a]
			(1)	(2)	(2)
9	–		1	3[b]	1
			(0)	(6[c])	(0)

[a] Very swollen almost invisible. [b] This is a standard G-banded preparation.
[c] Untreated chromosomes stained very darkly

Bleomycin in the presence of a sulphydryl reducing agent (Ross and Moses, 1978; Povirk et al., 1977) or DNAse I are agents which nick DNA. These nicking treatments alone suffice when excessive to entirely solubilize DNA from the chromosome. When used to supplant the HCl treatment in C-banding they induced faster DNA solubilization and marginally acceptable C-bands (#35, #38). DNA in the euchromatin of fixed mouse chromosomes is more accessible to DNAse I than is DNA in heterochromatin (Burkeholder and Weaver, 1977). Thus, we conclude that DNA breaking treatments, while enhancing DNA solubilization, can only partially supplant depurination in the C-banding procedure even when the breaks preferentially occur in euchromatin.

Necessitating the acid treatment: Mouse chromosomes were chosen for these experiments because they C-band easily. With normal fixation protocols, they C-band without an acid treatment (#8, #32). When fixed by the anhydrous method, however, an acid treatment is definitely required (#15, #31). HCl treatments longer than 15 min followed by the remaining C-banding treatments produced faster DNA solubilization but poorer C-bands. Bleomycin could completely replace the acid requirement (#21, #22; Fig. 6), when its reaction

Table 8. C-Banding: Anhydrous fixation and 65° pH 7.3. – Appearance of anhydrous fixed slides was scored on a scale of 0–5. *G* is quality of G-banding; *C-Eth* is quality of C-banding after destaining, RNAse treatment and ethidium bromide staining. Bleomycin treatments were for 3 days unless noted. All slides, unless noted, were treated 65° C, 0.25 M NaPi, 3 mM EDTA, pH 7.3, 18 h before Giemsa staining. Fifteen cells were scored from each coded slide and scores were stored and averaged in a hand calculator. Each datum is an average from two pair of slides treated 1 and 3 days after chromosome preparation

#	Treatment					C	Het	Eu	G	C-Eth
10	HCl	NaOH			65°, 2 h	3	4	2	2	3
11	HCl	NaOH				5	5	1	0	5
12	HCl					1	3	3.5	3	2
13	HCl				pH 8.3	3	4	3	1	
14	–					0	5	4	3	0
15		NaOH				0	3.5	3.5	1	1
16	HCl	$NaBH_4$	NaOH		65°, 2 h	0.5	4	3.5	4	2
17	HCl	$NaBH_4$	NaOH			2	4	3.5	3	2
18	HCl	$NaBH_4$	HCl	NaOH		5	5	1	0	5
19	HCl	$NaBH_4$	HCl			3.5	5	2.5	0.5	3
20	HCl	NaOH	$NaBH_4$			4	3	1	0.5	3.5
21	Bleo	NaOH	(30 sec)			5	4	1	0	4
22	Bleo	NaOH				5	5	1.5	0	3.5
23	Tris[a]	NaOH				0	3	3	2	0
24	Bleo	$NaBH_4$	NaOH			4	5	2.5	0	3.5
25	Bleo	$NaBH_4$	HCl	NaOH		5	5	1	0	5
26	Bleo					2	5	4.5	0	0.5
27	Bleo	$NaBH_4$				2	4	3	0	0
28	Bleo	(1 day)	NaOH	(30 sec)		3.5	4	2	0	2
29	Bleo	(1 day)	$NaBH_4$	NaOH (30 sec)		1.5	3	2.5	0	0

[a] 3 days, 4° C, 10 mM Tris pH 7.5

time was extended to 3 days; this reduces the DNA's alkaline molecular weight (Table 3) to the same degree as would a 15 min acid treatment (Table 2). These data implicate depurination as the reaction of import during the acid step because a modified fixation procedure designed specifically to minimize hydrolytic depurination made subsequent depurination or depyrimidation essential to DNA solubilization and because the two conditions, mild acid and bleomycin at pH 7.4, appear to have only one common chemical effect on fixed chromatin, DNA base removal.

Sodium borohydride treatment of acid treated chromosomes (#2, #16, #17), standardly fixed chromosomes (#6), and bleomycin treated chromosomes (#29), inhibited C-banding by preserving the euchromatin's basophilia. Inhibition was totally reversed by a subsequent acid treatment (#3, #18; Fig. 5). These data constitute the final proof that phosphodiester cleavage is responsible for DNA solubilization during C-banding. Solubilization requires lesions, lesion repair (Table 6) inhibits solubilization, and lesions are finally converted to breaks only in the hot salt step (Fig. 4) where most solubilization occurs (Table 1).

Cytochemical experiments unfortunately must often lack a control. For example, HCl treatments not only expose DNA pentose-aldehydes but also change the chromosomal protein. To show depurination and not the other

Table 9. DNase C-banding. – Slides, with one exception, were prepared by the anhydrous method and treated; 0.2 N HCl, 15 min, 24° C; 0.01 N NaOH, 0.3 M NaOH, 2 min, 24° C; DNase buffer 0.1 M NaCl, 0.01 M Tris, 5 mM $MgCl_2$, pH 7.3, 24° C before incubation at 65° C in 0.25 M NaPi, 3 mM EDTA, pH 7.3, 18 h. Bleomycin treatment was 1 mg/ml, 10 mM Tris, pH 7.5, 4° C. Bleo-SH is the bleomycin conditions stated above + 2 mM beta-mercaptoethanol

#	Treatment		C-bands	Euchromatin
30	HCl	NaOH	5	1
31	Buffer	NaOH	0.5	4
32[a]	Buffer	NaOH	2.5	3
33	0.025 µg/ml DNase	NaOH	0	5
34	0.25 µg/ml DNase	NaOH	1	4
35	2.5 µg/ml DNase	NaOH	3	2
36	25 µg/ml DNase	NaOH	nothing stained	
37	Bleo (1 day)	NaOH	4.5	1
38	Bleo-SH (3 min)	NaOH	2.5	3
39	Bleo-SH (15 min)	NaOH	nothing stained	

[a] Standard fixation

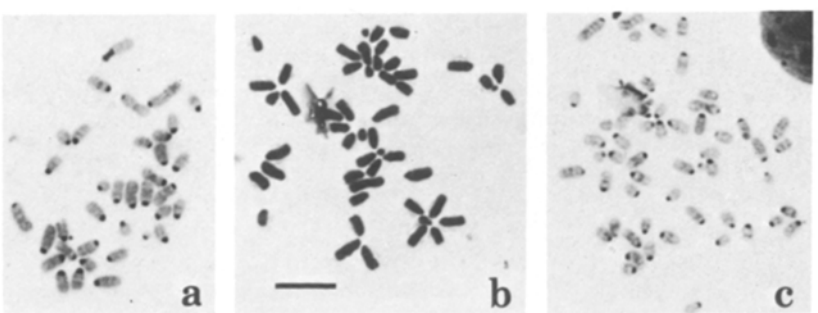

Fig. 5a–c. Representative examples of chromosomes from Table 7. **a** Standard C-banding: HCl → NaOH → 65°. **b** Sodium borohydride inhibition: HCl → $NaBH_4$ → NaOH → 65° C. **c** Reversal of inhibition: HCl → $NaBH_4$ → HCl → NaOH → 65° C. Bar is 10 µm

side-effects of acid to be the reaction of import, another non-acidic treatment was used to expose pentose aldehydes and a further treatment was devised to reduce the aldehydes. Often, one cannot provide controls for the side effects of a treatment such as $NaBH_4$ where the pH rises to 8.5, the Na concentration rises to about 1.0 molar, many non-DNA components are probably reduced, and ^{3}H-chromosomal DNA is solubilized (data not shown). The molecular weight data (Table 5; Fig. 4), which show the DNA's aldehydes are not acted upon until the final 65° C treatment, would predict that reducing the aldehydes

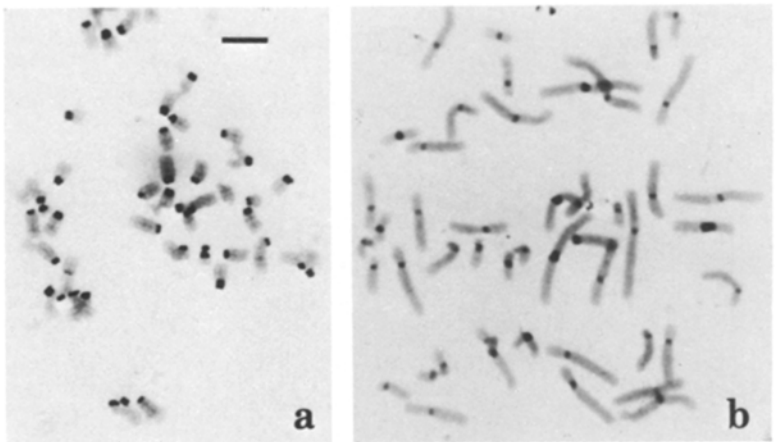

Fig. 6a and b. Bleomycin (3 day treatment) induced C-bands in chromosomes fixed by the anhydrous method. **a** Mouse fibroblast. **b** Human leukocyte from a 9 qh^+ proband. When bleomycin supplants the acid step in C-banding, chromosomes are less swollen and sister chromatids are often distinguishable. Bar is 10 μm

either before or after the NaOH treatment should prevent DNA solubilization. While $NaBH_4$ reduction before NaOH does inhibit DNA solubilization in hot salt (#2, #16, #17), reduction after NaOH results in solubilized DNA (#20). This later result is contrary to expectation until one realizes the DNA is being extracted by the $NaBH_4$ treatment and not by the hot salt (Table 7 #4, compare $\pm 65°$ C in the euchromatin column). NaOH treatment, by a means other than phosphodiester backbone cleavage (Table 6 row 3), somehow alters the chromosome so that solubilization is the primary effect of the hot salt treatment and a side-effect of the $NaBH_4$ treatment.

Discussion

Nuclear DNA is of chromosome size (Kavenoff et al., 1973) with single stranded nicks every $6–17 \times 10^7$ daltons (Hozier and Taylor, 1975) and alkali labile sites about every 10^8 daltons (Filippidis and Meneghini, 1977). DNA from unfixed chromosomes has an alkali molecular weight of 10^8 daltons which is reduced to 10^7 daltons after hyptonic treatment of the cells (Wray and Stubblefield, 1972) and further reduced to $M_n = 0.22 \times 10^6$ daltons if the chromosomes are fixed and dried (Table 2). The 15 min acid depurination treatment of C-banding only reduced this alkaline M_n by half (Table 2), a reduction which at first seems minor compared with the drastic reductions which occurred prior to it, but when one takes into consideration depurination introduced both during fixation and during the acid treatment (Table 6), one sees that depurination during these two steps decreases the alkaline M_n from 5×10^5 to 1×10^5 daltons. Since apurinic sites are cleaved early in the hot salt treatment (Fig. 4), DNA is mostly extracted during the hot salt step (Table 1) at a single strand molecular weight of 10^5 daltons.

What determines the rate of DNA removal from fixed chromosomes, DNAs molecular weight or protein? On a weight basis, more protein than DNA is extracted during C-banding (Comings et al., 1973; Francke et al., 1973) suggesting, as an extreme argument, that DNA removal may be a direct consequence of protein removal. Extreme trypsinization alone produces chromosomes which vary from C-banded by ethidium to completely dissolved (unpublished results) showing that DNA removal can indeed be a passive consequence of protein removal. Alternatively, DNase can remove almost all DNA while leaving the protein framework morphologically intact. Therefore, both DNA size and protein are important factors. When trypsinized or very freshly prepared chromosomes are taken through the C-banding procedure, the resulting chromosomes appear as ethidium ghosts: if very old preparations or formaldehyde post fixed chromosomes are used, the standard C-banding procedure removes almost no ethidium stainability (unpublished results). Thus, the state of chromosomal protein does alter the rate of DNA removal during C-banding. However, for standard chromosome preparations which are neither extremely aged nor fresh and otherwise untreated, the data show that one must decrease the single strand molecular weight of chromsomal DNA during the standard C-banding procedure in order to increase the rate of DNA extraction in hot salt to a rate necessary for a bandable product.

Chromosomal DNA extraction during Feulgen acid hydrolysis, where depurination, DNA denaturation, depolymerization by cleavage of depurinated sites, and DNA extraction all occur in the same acid solution, demonstrates much of the chemistry of C-banding. After acid treatment, the aldehydes of either the saturated or α–β unsaturated, depurinated pentose residues (Fig. 1) remaining in the chromatin matrix are stained red by the colorless Schiff reagent. The hydrolysis curve, color after staining versus time in acid, ascends to a peak and then descends. The ascent is due to aldehydes liberated by acid depurination while the descent is due to solubilization of DNA fragments produced by strand breakage during prolonged hydrolysis. Color intensity is always proportional to residual aldehydes (Duijndam and van Duijn, 1975; Anderson and Kjellstrand, 1975) indicating equal or total accessibility of all aldehydes to Schiff reagent. However, the various reactions which determine residual aldehyde concentration have different rates in different chromosome regions. Procedures which accentuate these differences produce Feulgen banded chromosomes wherein stain distribution is not proportional to the original DNA distribution (Greilhuber, 1975; Ennis, 1975). Individual reaction rates have been studied. The rate constant for DNA depurination in vitro pH 2.4, is 120× faster than the rate constant of depolymerization (Pollmann and Schramm, 1961). For methanol-alcohol fixed lymphocytes, the in situ depurination rate is only 30× faster than the DNA solubilization rate, both processes being first order linear (Duijndam and van Duijn, 1975). The depurination rate of DNA in vitro at pH 1.5 is reduced to half by addition of polylysine or polyarginine (Miano and Ferrini, 1969). DNA in heterochromatin depurinates slower in strong acid (Mittermayer et al., 1971) by a factor of 2.3 (Duijndam and van Duijn, 1975) than does DNA in euchromatin. Chromosomal DNA depurination rates are unchanged by formalin fixation (Andersson and Kjellstrand, 1975) but are

reduced by alcohol fixation (Mittermayer et al., 1971). These data are consistent with in situ depurination rates of alcohol-acid fixed chromosomal DNA being slower than rates of DNA in vitro (Table 2) and show that proteins can change the rate of chromosomal DNA extraction by slowing the fragmentation process.

Two models exist to explain the extraction of fragmented chromosomal DNA during acid hydrolysis of chromosomes. The diffusion model (Scott, 1974) proposes that DNA fragments are retained within the protein matrix during hydrolysis if their diffusion rate constants are high: addition of polyethylene glycol to the acid hydrolysis reaction, which should slow diffusion of polymers but not small molecules, greatly retarded DNA solubilization without affecting the depurination rate. A linker model (Kjellstrand and Lamm, 1976) proposed that DNA fragments can quickly diffuse from the protein matrix but are retained until all bonds connecting them to the fixed protein matrix are broken. Since the rate of DNA solubilization during acid hydrolysis of fixed chromatin greatly increased when salt to 1 M was added (Kjellstrand, 1977), a change which does not influence diffusion rates appreciably, some DNA-protein bonds may exist which are salt labile or at least something more than DNA depolymerization controls the rate of DNA loss from hydrolyzing chromatin. Both these models may be partly true. Most importantly, they help explain how alcohol (Mittermayer et al., 1971) or, more effectively, formalin (Andersson and Kjellstrand, 1975) fixation, by altering the protein matrix, reduces the rate of DNA extraction during Feulgen hydrolysis and why DNA in decondensed (eu)chromatin is removed more quickly than DNA in condensed (hetero)chromatin (Mittermayer et al., 1971; Duijndam and van Duijn, 1975).

Burkeholder and Weaver (1977) showed that DNase I reduces DNA to an acid soluble form faster in unfixed euchromatin than in heterochromatin and that this differential rate is due to a tightly binding protein and not to differential condensation. When fixed mouse chromosomes were briefly treated with DNase (0.5 µg/ml, 37° C, 1 min), acid hydrolyzed and stained with Schiff reagent, C-bands were obtained. Thus, limited nicking suffices to appreciably accelerate the rate of chromosomal DNA extraction during acid hydrolysis.

A review of DNA extraction from chromatin during the DNA denaturing conditions of strong acid reveals that DNA must first be depolymerized and that the rate of depurination and extraction can be very sensitive to protein-DNA interactions, fixation, degree of condensation, salt conditions and DNA nicking by previous treatments. DNA extraction during C-banding occurs not in acid but mostly during the DNA reannealing conditions of the hot salt treatment (Comings et al., 1973; Table 1).

The % helicity of chromosomal DNA is a critical parameter if DNA is extracted by depolymerization. The data in Table 2 shows the single stranded molecular weight of chromosomal DNA after standard acid depurination and β-elimination to be 1.2×10^5 daltons = 360 nucleotides. The stable minimum length for a region of double stranded DNA to remain double stranded under our hot salt conditions is about 20 nucleotides (Thomas, 1966). Single strand nicks would have to occur approximately every 40 neucleotides to allow double stranded DNA to depolymerize in hot (65° C) salt. Thus, for DNA randomly nicked every 360 nucleotides to be solubilized because of this limited degree

of nicking, the DNA must be single stranded during solubilization. Either the acid treatment or the NaOH treatment could do this.

Chromosomal DNA, once alkali denatured, remains mostly single stranded (Kurnit, 1974). Early investigators believed C-bands to be caused by a selective reannealing of highly repetitive DNA in heterochromatin (Arrighi and Hsu, 1971; Yunis and Roldan, 1971) during the final hot salt treatment. Acridine orange staining showed denatured chromosomes to fluoresce red, and to fluoresce green after short times in annealing conditions (Stockert and Lizanti, 1972; Chapel et al., 1973; Comings et al., 1973); DNA extracted from the "reannealed" green fluorescing chromosomes by prolonged pronase digestion banded in cesium gradients as double stranded (Comings et al., 1973). Pretreatment of denatured chromosomes under hot salt annealing conditions before in situ hybridization showed a loss of hybridizable DNA which increased with pretreatment time (Alonso, 1973). Some of these results remain unexplained but the initial conclusion, DNA reanneals in situ, was wrong. Kurnit (1974) used short pronase digestion times to recover DNA from chromosome preparations and assayed helix content by CsCl bouyant density of S1 endonuclease digestion. He found at least 80% helix content in fixed chromosomes and less than 30% helix content in subsequently denatured (0.07 N NaOH, 24°, 3 min) chromosomes. Annealing conditions ($2 \times$ SSC, 65° C, 18 h) did not noticeably alter the % helicity of either satellite or mainband DNA remaining on the alkali denatured slide. When Szabo et al. (1977) analyzed in situ hybridization kinetics in ($2 \times$ SSC, 50% formamide, 40°), conditions which probably remove little DNA from fixed chromosomes (Hubbel et al., 1976), RNA-chromosomal DNA hybridization kinetics were first order; DNA reannealing did not occur at least after the first few minutes of hybridization conditions. Acid denatured (0.2 N HCl, 25°, 20 min) and alkali denatured (0.01 N NaOH, 25°, 2 min) chromosomes had 10% and 30% respectively of their initial 5S-DNA sequences accessible for hybridization. Alonso (personal communication) has measured the hypochromic (A_{260}) shift of individual chromosomes after the various C-banding treatments. He found alkali but not acid produced a sizeable hypochromic effect, alkali and acid permanently denaturing 82% and 18% respectively of the chromosomal DNA. Again, reannealing did not occur during a subsequent hot salt incubation. Thus, during C-banding, alkali treatment but not acid treatment produces the degree of irreversible DNA denaturation necessary for depolymerization of 360 nucleotide long single stranded fragments.

Cytological data are consistent with this conclusion. Treatments such as HCl → 65° (Tables 7 and 8), which exclude an NaOH treatment, produced the darkest euchromatin, as consistent with little DNA denaturation, little DNA depolymerization and extraction. Bleomycin treatment does not denature DNA and Bleomycin → 65° C (#26) chromosomes showed dark euchromatin while HCl → 65° C (#12) chromosomes showed lighter euchromatin, as consistent with HCl but not Bleomycin inducing some irreversible denaturation and subsequent DNA extraction. Also, if one assumed that the high salt $NaBH_4$ treatment extracts denatured DNA, this would explain why $NaBH_4$ treatment after NaOH treatment but not before NaOH treatment extracts DNA (Tables 1 and 8). If DNA is to denature irreversibly, the strand pairs must unwind. This may

distort the chromosome and indeed, almost all of the swelling noted in Table 7 occurs during the NaOH step. Acid does not distort chromosomes (unpublished results). The normal C-banding sequence, using HCl or bleomycin, nicks DNA after denaturation; the bleomycin-SH (Ross and Moses, 1977) or DNase I C-banding sequence (Table 9) nicks DNA before denaturation. Denaturing DNA confined in a protein matrix should unwind in a different fashion if it is extensively nicked. This may explain why nicking after denaturation produces better C-banding than nicking before denaturation. These combined observations suggest the function of the NaOH treatment is to produce a degree of DNA denaturation unattained by acid treatment so that the following hot salt treatment can depolymerize and solubilize the DNA fragments.

With this chemistry in mind, one may now ask what property is responsible for the selective solubilization of DNA from euchromatin. One possibility, differential depurination rates, was suggested in the Feulgen hydrolysis discussion. Here euchromatic DNA would solubilize faster than heterochromatic DNA because it was more extensively depurinated and hence fragmented more severely. Alternatively, all DNA may be fragmented equally with protein interactions or partial reannealing determining the differential ratio of solubilization. The biochemistry of fixed chromatin is likely too complex to yield simple chemical answers to the banding patterns it presents. However, a slight understanding of this chemistry should aid the presently rather stochastic search for better banding techniques.

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