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Electrofocusing Analysis of HeLa Cell Metaphase Chromosomes†

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ABSTRACT: The possibility of fractionating HeLa cell metaphase chromosomes by the electrofocusing technique has been investigated. By applying a modification of the standard procedure, which allows a considerable shortening of the electrofocusing time, these chromosomes could be resolved partially into groups in the pH range from 3.90 to 4.30. These groups, upon reelectrofocusing, banded at the same pH value as in the original run, with improved resolution and an apparent preservation of the morphological integrity of the chromosomes. Electrofocusing of the small HeLa cell chromosomes, previously separated by sedimentation velocity, produced an appreciable enrichment of rRNA genes in subfractions of these chromosomes.

Several reports have been published on the isolation and fractionation of metaphase chromosomes. The materials used in these studies were mammalian cell lines cultivated *in vitro* (Somers *et al.*, 1963; Lin and Chargaff, 1964; Salzman *et al.*, 1966; Huberman and Attardi, 1966, 1967; Mendelsohn *et al.*, 1968; Maio and Schildkraut, 1967; Schneider and Salzman, 1970), mouse ascites tumor cells (Cantor and Hearst, 1966), and mouse leukemia cells (Chorazy *et al.*, 1963). The fractionation procedures utilized were velocity sedimentation in sucrose gradients or in zonal rotors and filtration through porous stainless steel filters. These methods are based on differences in the size and morphology among metaphase chromosomes. In the present work, the applicability of the electrofocusing technique (Vesterberg and Svensson, 1966), which has been used successfully in the separation of macromolecules, to the fractionation of metaphase chromosomes has been investigated.

† Contribution No. 4299 from the Church Laboratory of Chemical Biology and Joint Contribution with the Division of Biology, California Institute of Technology, Pasadena, California 91109. *Received August 8, 1971.* Supported by U. S. Public Health Service Grants GM-14452 to M. A. R. and GM-11726 to G. A. Paper III in the series: Studies of Fractionated HeLa Cell Metaphase Chromosomes. Paper II is by Aloni *et al.* (1971).

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TABLE I: Conditions and Solutions Used for Electrofocusing.

	Standard[a]	Modified
Capacity (ml)	440 (LKB 8102)	110 (LKB 8101)
Polarity	Cathode at bottom Anode at top	Anode at bottom Cathode at top
Electrode solutions	Cathode solution: 1.6 ml of ethanolamine, 48 g of sucrose, and 56 ml of distilled H_2O Anode solution: 0.4 ml of concentrated H_2SO_4 and 40 ml of distilled H_2O	Cathode solution: 0.2 ml of ethanolamine and 10 ml of distilled H_2O Anode solution: 12 g of sucrose, 14 ml of distilled H_2O, and 0.2 ml of concentrated H_2SO_4
Density gradient solutions	Dense solution: 200 ml of 40% (w/w) sucrose solution and 8.0 ml of 40% ampholine (LKB), pH 3–6 Light solution: 204 ml of distilled H_2O, 3.0 ml of 40% ampholine (pH 3–6), and 1.0 ml of chromosome suspension ($\sim 10^9$ chromosomes in 2×10^{-3} M $CaCl_2$, 0.02 M Tris (pH 7), and 0.05% saponin) Above gives a total ampholine concentration in the column of 1%	Dense solution: 55 ml of 40% (w/w) sucrose solution, 0.4 ml of 40% ampholine (pH 3–5), and 1 ml of 5% Tween 80 Light solution: 55.25 ml of distilled H_2O, 0.15 ml of 40% ampholine (pH 3–5), and 1.0 ml of 5% Tween 80 Above gives a total ampholine concentration of 0.2%
Electrofocusing temperature (°C)	4	4
Power at start (W)	4 (starting voltage is 600 V)	1.5–2.0 (starting voltage is 500 V)
Filling rate (ml/min)	4	3
Emptying rate (ml/min)	2	1–2
Electrofocusing time	According to the experiment	According to the experiment

[a] When the 110-ml column (LKB 8101) was used in the standard procedure, the amounts of electrode and density gradient solutions were reduced to one-fourth, the ampholine concentration was 1 or 0.2%, as specified in the legends of the figures, and the power at the start and the filling and emptying rates were the same as in the modified procedure.

Materials and Methods

Chromosome Isolation. HeLa cells (S_3 clonal strain), grown in suspension as described by Amaldi and Attardi (1968), were blocked in metaphase by exposure for 15 hr to 0.005–0.01 μg/ml of vinblastine sulfate (Eli Lilly and Co.). Chromosomes were isolated from these cells at pH 3 according to Huberman and Attardi (1967), or at pH 7 according to Maio and Schildkraut (1967). Fractionation of chromosomes in glycerol–sucrose gradients was carried out as described by Huberman and Attardi (1967).

In most experiments, the chromosomes were labeled by exposing the cells (5×10^4/ml) for 48 hr to either [*methyl*-^{3}H]-thymidine (23 mCi/μmole; 1.25 μCi/ml, Amersham-Searle) or [2-^{14}C]thymidine (53 μCi/μmole; 0.003 μCi/ml, New England Nuclear); for labeling the chromosomal proteins, the cells were exposed for 72 hr to L-[^{14}C]leucine (316 μCi/μmole; 0.013 μCi/ml, Schwarz) in medium containing 2×10^{-4} M leucine.

The isolated chromosomes were kept at 4° in 2×10^{-3} M $CaCl_2$, 0.02 M Tris, pH 7 at 25°, and 0.05% saponin (FM), unless otherwise specified.

Electrofocusing was carried out on the whole population of chromosomes obtained by either method mentioned above, as well as on the individual groups of chromosomes as arbitrarily set by Huberman and Attardi (1967) and Aloni *et al.* (1971) (Figure 1).

STANDARD PROCEDURE. The electrofocusing columns used were LKB 8101 (110 ml) and LKB 8102 (440 ml) and the method employed was essentially that described in the LKB 8100 Ampholine Electrofocusing Instruction manual (LKB-Produkter AB). The columns were coated with Siliclad (Clay-Adams, Inc.) to minimize adhesion of chromosomes during draining. The solutions used and the conditions employed are summarized in Table I.

After choosing the electrode polarity, the central tube of the column was filled at 4° with the dense electrode solution by means of a syringe. The density gradient solution containing the sample was then introduced into the electrofocusing chamber from the LKB 8122 gradient mixer by a Sigma motor pump, followed by the light electrode solution. In some experiments, a solution containing 7×10^{-4} M $CaCl_2$ and 3×10^{-4} M $MgCl_2$ (CM) was substituted for distilled water in the density gradient solutions. In others, a protective light solution consisting of either distilled water or CM and ampholine (1%) was layered between the density gradient solution and the light electrode solution, while a dense protective solution consisting of 40% (w/w) sucrose solution (in H_2O or CM) and ampholine (1%) was layered between the dense electrode solution and the density gradient solution.

MODIFIED PROCEDURE. The 110-ml column was filled as described above, using the conditions shown in Table I. (Notice that the chromosome sample is omitted in the filling step.) In the experiments in which a narrower pH range (3.80–4.30, see below) was used, enough of the ampholine was added to give a final concentration of 0.2%, and the volume of the

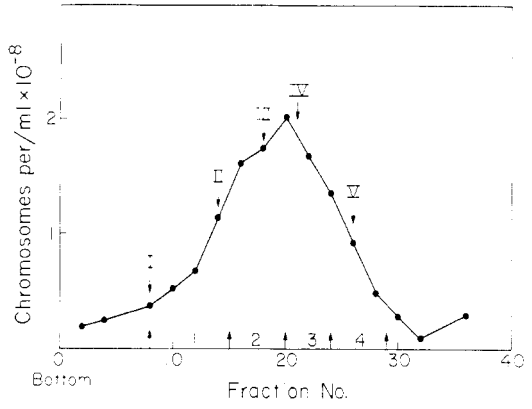

FIGURE 1: Distribution of chromosomes after centrifugation through a glycerol–sucrose density gradient. Chromosomes were isolated at pH 3 from about 10^9 HeLa cells, suspended in 10 ml of FM plus 3.3 ml of glycerol, layered onto a 140-ml linear gradient from 30% (w/w) glycerol in FM (at the top) to 30% (w/w) sucrose in FM (at the bottom), and centrifuged for 40 min at 450*g* (4°) (Huberman and Attardi, 1967). Fractions of 4 ml were collected through thin glass tubing inserted at the bottom of the gradient, and the chromosome concentration was determined by counting in a bacterial counting chamber. The arrows on the axis of abscissa indicate the cutoff points for pooling chromosome fractions into separate classes. The downward pointing arrows indicate the fractions utilized for amino acid composition analysis (see Table II).

density gradient solution was decreased accordingly. In the experiments using the modified procedure, the cathode was placed at the top, in order to introduce the chromosome sample (see below) above the pH range expected for the chromosome isoelectric points.

Electrofocusing was carried out at a starting voltage of 500 V, current ~2.0 mA. At the end of 12 hr, the voltage and the current were steady at 750 V and 0.8 mA, respectively. The power supply was disconnected and the valve to the inner chamber of the column closed. Two 5-ml fractions (corresponding to the top electrode solution), followed by 1.0-ml fractions (pH was measured in each fraction before withdrawal of the next fraction), were successively withdrawn from the top of the solution in the electrofocusing compartment until the fraction with a pH slightly less than 5 was reached. Then 1.5 ml more were removed, added to 1.5 ml of freshly homogenized (Dounce homogenizer) chromosome suspension in FM, and mixed gently with a wide-mouthed pipet. The resulting suspension was then pumped into the column at a rate of 3.0 ml/min. The withdrawn 1.0-ml fractions were diluted 1:1 with distilled water and pumped back into the column in the reverse order in which they had been removed, until the solution almost reached the upper electrode. The valve to the inner chamber was then opened, and 10 ml of fresh light cathode solution pumped in. Electrofocusing was conducted at constant voltage for the desired time.

DRAINING OF THE ELECTROFOCUSING COLUMN. The column was emptied as described in the LKB Instruction manual. Fractions of 2 and 0.5 ml were collected, respectively, from the 440- and 110-ml column by means of a Sigma motor pump at the flow rate indicated in Table I. These fractions were kept in an ice bath. Alternate fractions were analyzed for radioactivity and every fifth fraction for pH.

In the 13-min experiment, the column was drained with a Technicon proportioning pump at a flow rate of 2 ml/min, and 10-ml fractions were collected up to the region 1 cm below the chromosome band. Beyond this point, draining at a flow rate of 0.8 ml/min was accomplished by segmenting the effluent with air bubbles, thus avoiding mixing and improving the resolution.

PREPARATION OF AMPHOLINE SOLUTION, pH 3.8–4.3. The 0.5-pH unit range ampholine solution used in some experiments was prepared from the commercially available pH range 3–5, according to the method described in the LKB Instruction manual.

Ampholine solution (12.5 ml of 40%; pH 3–5) was added to the density gradient solution, resulting in a total ampholine concentration of 5%. After electrofocusing and draining, the fractions from the pH range 3.8–4.3 were pooled together, divided into five fractions (4.6 ml each) and stored frozen. Each of these was sufficient to give an approximately 0.2% ampholine for one electrofocusing run in the 110-ml column with the modified procedure. When this solution was used, the volume of the density gradient solution was decreased by 4.6 ml.

Analytical Methods. Radioactivity measurements were done by taking 25- or 100-μl aliquots of various fractions, and adding 1.0 ml of 5% trichloroacetic acid and 0.1 ml of bovine serum albumin (2 mg/ml). After chilling at 4° for 1 hr, the precipitates were collected on Millipore filters and washed thoroughly with cold 5% trichloroacetic acid, dried, and counted in toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene mixture in a Packard scintillation counter. The lysine-rich histones which had not been removed in the isolation of chromosomes at pH 3 (Huberman and Attardi, 1966) were presumably lost in the 5% trichloroacetic acid precipitation step; however, this loss does not affect the conclusions of the present work.

All pH measurements were made with a Radiometer Copenhagen Model 26 pH meter at 4°.

For determination of RNA and protein, the chromosome fractions obtained after electrofocusing were pooled and centrifuged at 15,000 rpm for 30 min in a SS-34 Sorvall rotor. The supernatants were dialyzed against distilled water (10× volume) to eliminate sucrose and ampholine, until complete removal of ampholine was shown by a negative Lowry test (Lowry *et al.*, 1951) in the dialysis medium. The dialysates were analyzed for RNA and protein by the orcinol test (Mejbaum, 1939) and the Lowry test, respectively.

For amino acid analyses, pellets obtained by centrifuging pooled fractions at 15,000 rpm for 30 min in the SS-34 rotor were washed three times with 10 ml of FM and then hydrolyzed in constant-boiling HCl. Amino acid analyses (Spackman *et al.*, 1958) of the hydrolysates were performed in a Beckman-Spinco Model 120B amino acid analyzer.

Optical densities were read in a Gilford Model 240 spectrophotometer.

For counting chromosomes, the fractions were centrifuged at 15,000 rpm for 30 min, and the pellets obtained were resuspended in 0.1 ml of FM. The chromosomes were counted in a bacterial counting chamber using a phase-contrast microscope. Slides were prepared according to Huberman and Attardi (1967).

Hybridization Studies. ISOLATION AND DENATURATION OF DNA. DNA was prepared from electrofocused chromosome fractions by a modification of the Marmur (1961) procedure, as detailed previously (Aloni *et al.*, 1971). Denaturation of DNA and removal of RNA was carried out by extensive dialysis against 0.3 N KOH for 18 hr at room temperature, followed by dialysis for 48 hr *vs.* 2 × SSC (SSC 0.15 M NaCl–0.015 M sodium citrate).

LABELING AND ISOLATION OF rRNA. HeLa cell ^{32}P-labeled

TABLE II: Amino Acid Analyses of Chromosome Fractions Separated by Sedimentation Velocity in a Glycerol–Sucrose Gradient.[a]

Amino Acid	Mole %				
	I	II	III	IV	V
Asp	7.86	7.79	7.34	6.24	6.00
Met-sulfone	1.04	0.58	3.96	3.29	2.41
Thr	5.68	5.35	5.96	6.17	5.88
Ser	6.25	6.06	6.39	6.45	6.27
Glu	9.09	9.84	6.11	6.35	6.86
Pro	4.97	4.92	4.43	4.60	4.72
Gly	9.99	9.57	9.00	9.97	9.54
Ala	9.10	8.86	7.93	9.07	8.78
Cys	1.35	1.07	1.04		
Val	6.08	6.22	6.45	6.55	
Met	1.24	1.58	1.16	0.92	trace
Ile	4.43	4.73	4.78	4.86	5.24
Leu	8.17	8.59	8.12	8.04	9.30
Tyr	2.56	2.53	2.69	2.85	2.24
Phe	2.91	2.96	3.72	3.67	3.37
His	2.28	2.26	5.36	3.62	5.41
Lys	9.55	9.65	8.78	9.82	10.53
Arg	7.35	7.34	6.69	7.58	7.00

[a] Fractions indicated by downward pointing arrows in the chromosome distribution in Figure 1 were utilized for amino acid composition analysis.

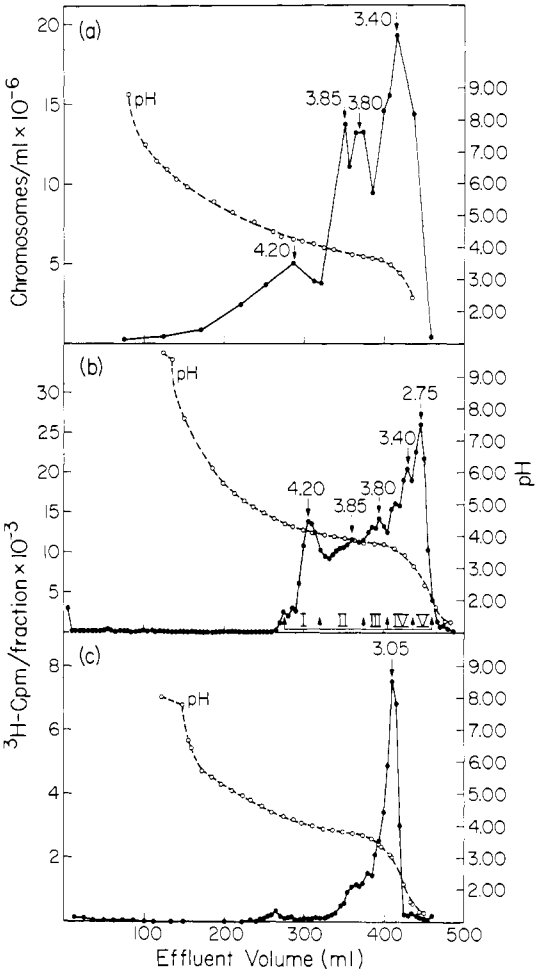

FIGURE 2: Electrofocusing of large chromosomes (class 1, Figure 1) by the standard procedure. (a) HeLa cell chromosomes were isolated at pH 3 and fractionated as shown in Figure 1. About 10^9 chromosomes of class 1 (see Figure 1) in 1 ml of FM were electrofocused for 48 hr, following the standard procedure, in the 440-ml column, 1% ampholine, pH range 3–6, starting voltage of 600 V. The voltage at the end of electrofocusing was 1000 V. Fractions of 2.5 ml were collected and analyzed for pH (○) and chromosome concentration (●). (b) [*methyl*-^{3}H]Thymidine-labeled large chromosomes were isolated and electrofocused as in parts a. Fractions of 2.5 ml were collected and analyzed for pH (○) and acid-precipitable radioactivity (●). Pooled fractions, as indicated by arrows, were used for amino acid analysis. (c) Reelectrofocusing of fraction I in part b, under the same conditions as in parts a and b. Fractions were analyzed for pH (○) and acid-precipitable radioactivity (●).

28S and 18S RNA components were isolated from purified ribosomal subunits as previously described (Amaldi and Attardi, 1968). To eliminate any possible DNA contaminant, the RNA samples were treated with electrophoretically, purified pancreatic DNase (Worthington, 20 μg/ml) in 0.05 M Tris buffer (pH 6.7 at 25°), 0.025 M KCl, and 0.0025 M $MgCl_2$ for 60 min at 22°, reextracted with dodecyl sulfate–phenol, precipitated with ethanol, dissolved in 2 × SSC, and run on 55 cm columns of Sephadex G-100 equilibrated with 2 × SSC at 22°. The specific activity of the final preparations varied between 5×10^5 and 7×10^5 cpm per μg.

RNA–DNA HYBRIDIZATION PROCEDURE. Hybridization of ^{32}P-labeled 18S and 28S RNA with DNA from fractionated chromosomes and isolation of RNA–DNA hybrids were carried out as described previously (Huberman and Attardi, 1967; Aloni *et al.*, 1971). All hybridization mixtures contained ^{32}P-labeled 28S RNA at an RNA:DNA ratio of 1:20 in combination with ^{32}P-labeled 18S RNA at an RNA:DNA ratio of 1:50. These ratios are sufficient to saturate the rRNA sites in HeLa cell DNA (Jeanteur and Attardi, 1969). In all cases, incubation was for 4 hr at 72°, and RNase digestion was carried out with 10 μg of enzyme/ml for 1 hr at 22°. As a control for nonspecific background, denatured DNA was incubated separately from labeled RNA and mixed with it before the RNase digestion step. All the hybridization values reported here have been corrected for background, determined as described above.

Results

Fractionation of Metaphase Chromosomes in a Glycerol–Sucrose Gradient. Figure 1 shows a typical distribution in a glycerol–sucrose gradient of HeLa cell metaphase chromosomes isolated and fractionated according to the Huberman–Attardi (1967) procedure. The chromosome distribution was arbitrarily cut into four sections, designated 1–4 in order of increasing sedimentation velocity, in a way roughly corresponding to that used by the above-quoted authors and by Aloni *et al.* (1971). Evidence for the efficiency of this type of fractionation, as judged from the distribution in the gradient of chromosomes of different morphological types and of the chromosomes carrying the rRNA genes, has been previously presented (Huberman and Attardi, 1967; Aloni *et al.*, 1971).

Table II shows the amino acid composition analysis of individual chromosome fractions taken from the distribution presented in Figure 1. It appears that the amino acid composition of chromosomes of different sedimentation velocity is similar. The somewhat greater deviations in amino acid

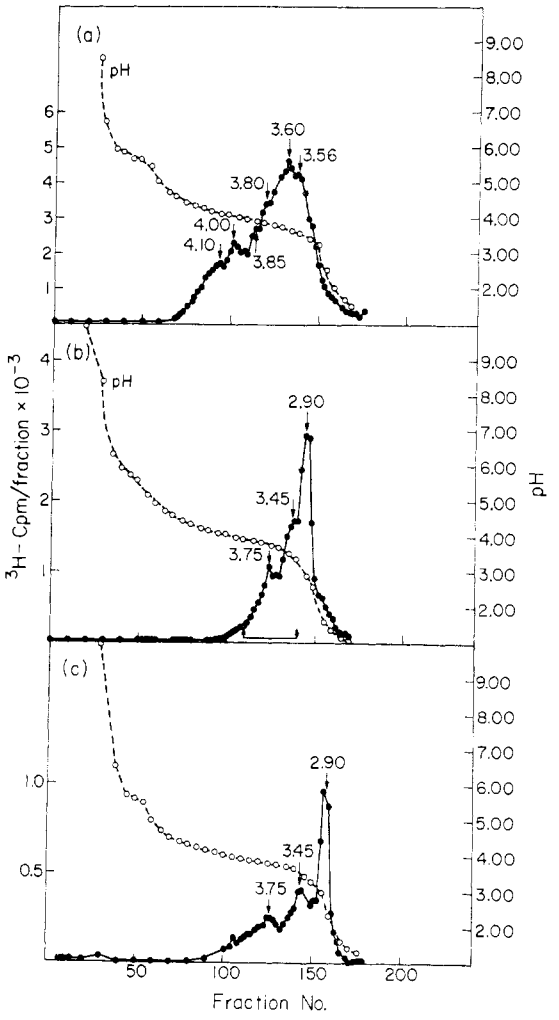

FIGURE 3: Effect of method of isolation of chromosomes on their electrofocusing behavior. About 10^9 [*methyl*-^{3}H]thymidine-labeled chromosomes (whole population) were electrofocused as in Figure 2, except that the ampholine concentration was 0.2%, Ca^{2+} and Mg^{2+} were added to the density gradient solutions to 7×10^{-4} and 3×10^{-4} M, respectively (see below), and the electrofocusing time 24 hr. Collected fractions (2.5 ml each) were analyzed for pH (○) and acid-precipitable radioactivity (●). (a) Chromosomes isolated at pH 7 (Maio and Schildkraut, 1967); (b) chromosomes isolated at pH 3 (Huberman and Attardi, 1967); (c) pooled fractions 110–140 in part b were reelectrofocused under the same conditions.

TABLE III: Amino Acid Analyses of Fractions Obtained by Electrofocusing Large Chromosomes Previously Separated by Sedimentation Velocity in a Glycerol–Sucrose Gradient.[a]

	Mole %						
	Before Electro-	After Electrofocusing					
Amino Acid	focusing	I	II	III	IV	V	I (Rerun)
Asp	8.36		10.0	12.2	9.6	16.1	39.06
Met-sulfone	2.62						
Thr	5.47		5.4	5.6	4.4	5.0	2.49
Ser	6.38		7.2	9.9	6.1	6.3	3.35
Glu	8.30		12.0	15.0	27.6	15.7	30.94
Pro	4.60		5.0	3.4	4.0	5.1	2.81
Gly	9.72		10.4	12.0	8.9	9.0	4.42
Ala	8.32		8.2	7.2	6.6	7.8	4.09
Cys	Trace			4.6			
Val	5.64		5.8	5.9	4.5	5.1	2.68
Met	1.53		1.7	1.3	1.3	1.3	0.58
Ile	4.49		4.5	3.4	3.5	3.4	1.57
Leu	8.26		7.7	6.4	5.9	6.8	2.99
Tyr	2.13		1.9		2.1	1.2	0.72
Phe	3.92		4.4		4.1	2.8	
His	2.87					2.0	
Lys	10.11		8.8	7.2	6.3	6.6	2.04
Arg	7.25		7.0	6.2	5.1	5.5	2.26

[a] See Figure 2b,c for pooled chromosome fractions used for amino acid analysis.

composition in the smallest chromosomes (fraction V) may reflect contamination of these chromosomes by cytoplasmic proteins (Mendelsohn *et al.*, 1968).

Electrofocusing of Metaphase Chromosomes by the Standard Procedure. Figure 2a shows the results obtained by electrofocusing for 48 hr the chromosome fraction designated as sedimentation class 1 or large chromosomes (see Figure 1). A plot of chromosome counts *vs.* fraction number gave a continuous distribution with resolved peaks at pH 4.20, 3.85, 3.80, and 3.40. When ^{3}H-labeled large chromosomes were electrofocused under the same conditions, radioactive peaks were found at the same positions, plus an additional one at pH 2.75, as shown in Figure 2b. Amino acid composition analysis of fractions pooled as indicated in Figure 2b showed in all fractions an increase in the proportion of acidic amino acids and a decrease in that of basic amino acids with respect to the overall amino acid composition of the large chromosomes before electrofocusing (Table III); furthermore, there was a pattern of increasing acidic and decreasing basic amino acids from fractions occurring at less acid to more acid pH (II–V, Table III).

The above results seemed to indicate the applicability of the electrofocusing technique for the fractionation of chromosomes. However, reelectrofocusing for 48 hr of fraction I in Figure 2b (peak pH 4.2) resulted in a shift of the peak to a more acid area, pH 3.05 (Figure 2c). Amino acid analysis of the material from this peak showed an increase in acidic and a decrease in basic amino acids with respect to the values expected for this fraction (Table III). When chromosome fractions electrofocused for 24 hr (Figure 3b, pH 3.75, fractions 110–140) were reelectrofocused for another 24 hr, there was, on the contrary, no obvious shift, but rather an enrichment of the peak at pH 3.75 and an improved resolution of this component from other peaks at pH values 3.95 and 3.45 (Figure 3c). Also in the latter rerun, however, an acidic peak was present (at pH 2.90), and in amount greater than expected from contamination of the rerun fractions by the pH 2.90 component present in the first run. These results indicated the need for further studies on the effects of ampholine concentration, method of isolation of the chromosomes, use of freshly prepared or aged chromosomes (stored frozen or at 0°), chromosome concentration, and time of electrofocusing.

In order to reduce possible complex formation between ampholine and chromosomes, in subsequent experiments the ampholine concentration was decreased from 1 to 0.2%: this did not affect the shape of the pH gradient nor the electrofocusing profile of the chromosomes.

Figure 3a shows the radioactivity profile obtained when a

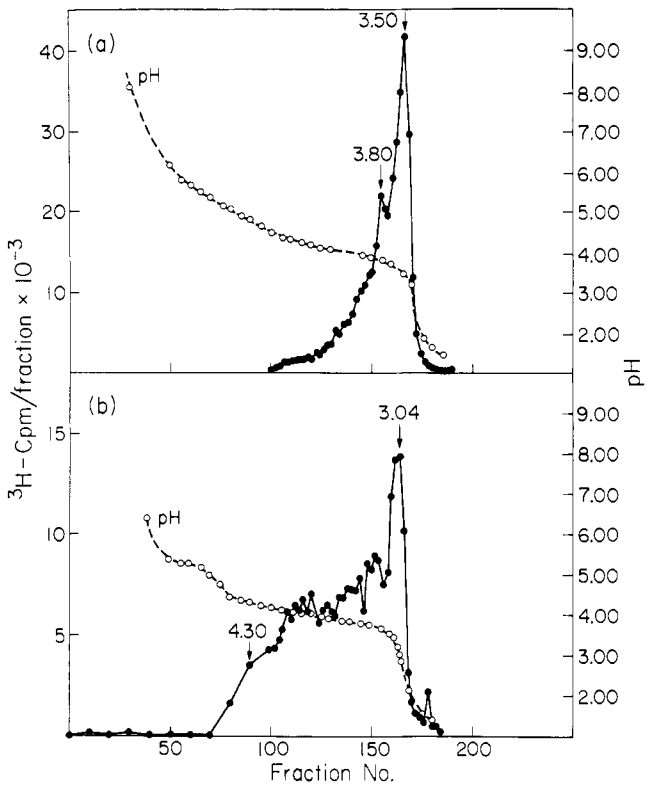

FIGURE 4: Effect of Ca^{2+} and Mg^{2+} on electrofocusing behavior of aged chromosomes (kept frozen at −20°). About 10^9 [*methyl*-^{3}H]-thymidine-labeled chromosomes (sedimentation class 1 (see Figure 1) of chromosomes isolated at pH 3; kept frozen for about a month) in 1 ml of FM were electrofocused as in Figure 2, except that the ampholine concentration was 0.2%; pH range 3–5. Fractions of 2.5 ml were analyzed for pH (○) and acid-precipitable radioactivity (●). (a) Ca^{2+} and Mg^{2+} omitted in the density gradient solutions; (b) Ca^{2+} and Mg^{2+} were added to the density gradient solutions to a final concentration of 7×10^{-4} and 3×10^{-4} M, respectively.

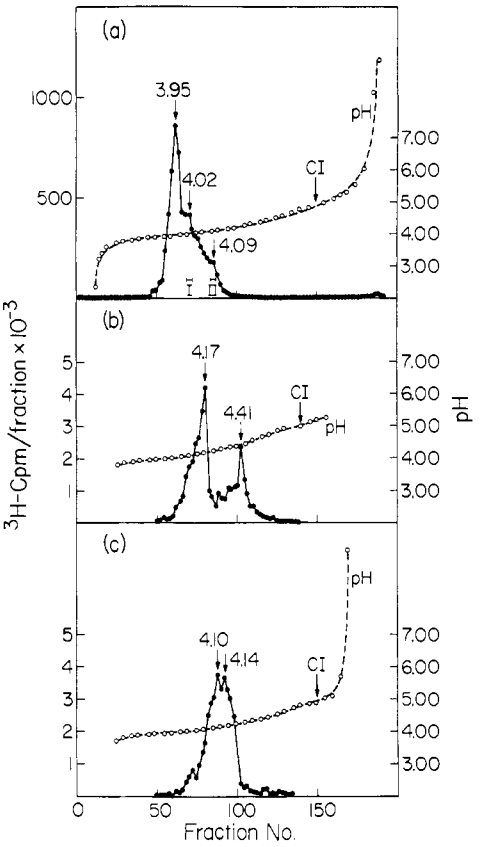

FIGURE 5: Electrofocusing of the total chromosome fraction using the modified procedure. (a) Following the modified procedure described in Material and Methods, and using 0.2% ampholine, pH range 3–5, about 10^9 [*methyl*-^{3}H]thymidine-labeled chromosomes (total population, isolated at pH 3) in 1.5 ml of FM were mixed with 1.5 ml of the gradient solution with the appropriate pH (4.88) and introduced (arrow marked CI in pH curve) into the LKB 110-ml column. Electrofocusing was carried out at a starting voltage of 500 V and terminated after 3 hr at a volatge = 700 V. Fractions of 0.5 ml were collected and analyzed for pH (○) and acid-precipitable radioactivity (●). Using the same conditions, pooled fractions 69–70 (I) and 83–85 (II) were reelectrofocused separately (b and c, respectively): in the former, CI = 5.04, and in the latter, CI = 4.86.

total chromosome population prepared at neutral pH according to Maio and Schildkraut (1967) was electrofocused for 24 hr. The distribution of the chromosomes was shifted to a less acid pH range when compared to that obtained by electrofocusing the total chromosome population isolated at pH 3 following the Huberman–Attardi (1967) procedure (Figure 3b).

Chromosomes which had been stored frozen at −20° for almost a month, upon being electrofocused, gave a profile of radioactivity which was different from that of freshly prepared chromosomes (Figure 4a). The effect of aging was reversed by adding $CaCl_2$ and $MgCl_2$ to the density gradient solutions to a final concentration of 7×10^{-4} and 3×10^{-4} M, respectively (Figure 4b).

Varying the chromosome concentration did not seem to affect the electrofocusing profile of radioactivity. Under our experimental conditions, by using a total amount of about 10^9 chromosomes (freshly prepared), reproducible results were obtained.

Spreads on slides of chromosomes from electrofocused fractions were indistinguishable in the phase-contrast microscope from those of the chromosomes before electrofocusing, without any evidence of size fractionation. As an exception, chromosomes collected in strongly acid regions (pH < 3) after prolonged electrofocusing appeared to be fragmented and with a looser texture.

Electrofocusing of Metaphase Chromosomes by the Modified Procedure. Although the above results indicated the occurrence of some kind of fractionation, stripping of proteins from the chromosomes seemed also to have taken place during the prolonged exposure to the electric field. Lowry tests performed on the supernatants obtained by centrifuging the electrofocused chromosome fractions, after dialysis to remove the ampholine and sucrose, gave a positive test for protein. So did orcinol tests for RNA. Analyses of the proteins and RNA were qualitative and were not pursued further, since the primary consideration was to determine the optimum conditions for obtaining morphologically intact chromosomes.

In view of the above mentioned stripping of proteins from the chromosomes, it became necessary to modify the standard procedure in such a way as to reduce the electrofocusing time of the chromosomes. This was done by introducing the chromosomes as a layer at an appropriate pH in an already established pH gradient. The narrow pH range used, 3.8–4.3, was chosen as a result of preliminary experiments using this modification and a pH range 3–5 (as an example, see Figure 5a), which showed that the peaks of radioactivity were between 3.9 and 4.3. The pH appropriate for introduction of the chromosomes (CI in Figures 5 and 7) was chosen as that

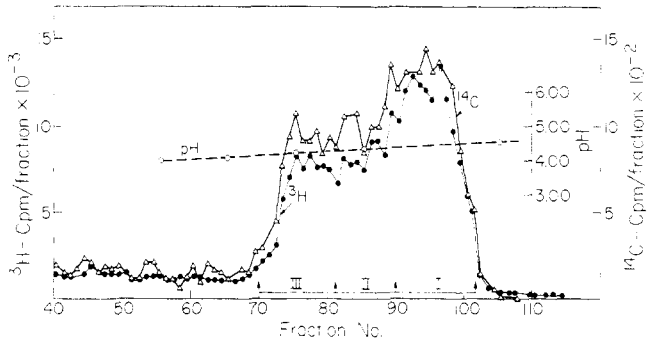

FIGURE 6: Electrofocusing of [*methyl*-^{3}H]thymidine- and [^{14}C]leucine-labeled chromosomes (total population, isolated at pH 3) according to the modified procedure. The pH range was 3.80–4.30 (0.2% ampholine), voltage constant at 700 V, CI = 4.99, electrofocusing time = 13 min. Fractions of 0.5 ml were collected and analyzed for pH (○), ^{14}C cpm (△), and ^{3}H cpm (●). The fractions were pooled arbitrarily into three groups (designated I, II, and III in order of decreasing basicity), from which DNA was extracted and hybridized with RNA, as described in Materials and Methods.

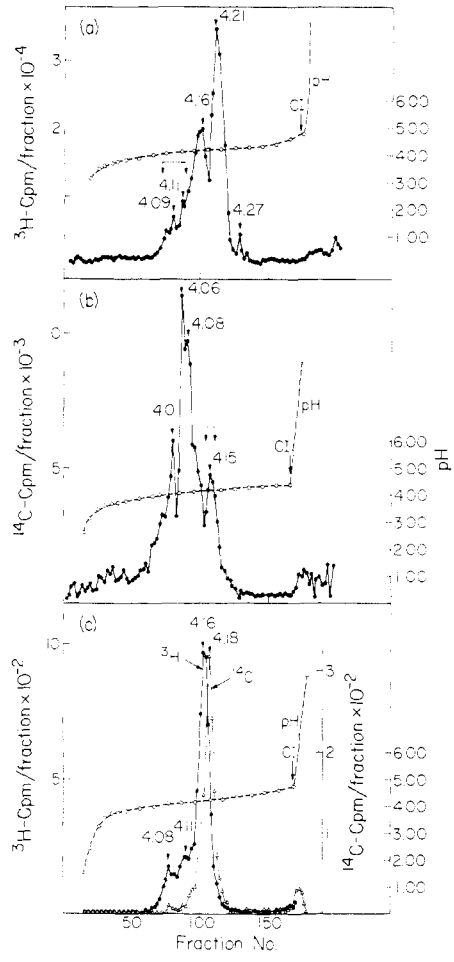

FIGURE 7: Reelectrofocusing, according to the modified procedure, of a mixture of electrofocused chromosome fractions from differently labeled populations. (a, b) Using the modified procedure and 0.2% ampholine in the pH range 3.8–4.3, about 10^{9} chromosomes (total population from [*methyl*-^{3}H]thymidine- (a) or [2-^{14}C]thymidine- (b) labeled cells, isolated at pH 3) were electrofocused at a constant voltage (700 V) for 4.5 hr. Fractions of 0.5 ml were collected and analyzed for pH (○) and radioactivity (●). In part a, the electrofocused chromosomes were introduced at CI = 4.72, in part b at CI = 4.57. In part c fractions 71–91 from part a and fractions 105–115 from part b were mixed and electrofocused under the same conditions as in parts a and b; CI = 4.75. (○) pH, (●) ^{3}H cpm, (△) ^{14}C cpm.

which lay on the pH curve just before the curve rose abruptly.

In this modification of the electrofocusing procedure, there was a tendency of chromosomes to aggregate, which was accentuated when Ca^{2+} and Mg^{2+} were added to the density gradient solutions to a final concentration of 7×10^{-4} and 3×10^{-4} M, respectively; addition of Tween 80 to the same solutions to a final concentration of 0.1%, in the absence of divalent cations, substantially eliminated this aggregation.

Using the modified procedure, it was observed that, immediately after electrofocusing was started, the chromosomes began to move and, within 15 min, two to four bands were discernible by their turbidity. When electrofocusing was allowed to continue, the bands seemed to diffuse. Figure 6 shows a 13-min run, Figure 5a a 3-hr run, and Figure 7a,b, 4.5-hr runs. As appears in Figure 6, after a 13-min run, the chromosomes are distributed in the pH range from 4.15 to 4.56. The bands visible with the naked eye are barely recognizable in the chromosome profile, possibly due to mixing during draining. In this experiment, the chromosomes had been isolated from cells labeled with both [^{3}H]thymidine and [^{14}C]leucine. It can be seen in Figure 6 that the ^{3}H and ^{14}C profiles correspond very closely; this suggests a relatively constant ratio of DNA to protein throughout the chromosome distribution. Analysis of the supernatant obtained after chromosome centrifugation did not reveal any [^{14}C]leucine-labeled protein.

After a 3-hr run (Figure 5a), there was a shift toward lower pH's of the chromosome distribution. Reelectrofocusing areas I and II in Figure 5a gave an improved resolution, as shown in Figure 5b,c. In Figure 5c, the chromosomes from area II were introduced into the established pH gradient at almost the same pH (CI = 4.86) as in Figure 5a (CI = 4.88). These chromosomes, upon reelectrofocusing, were found in their original position. In Figure 5b, the chromosomes from area I in Figure 5a were found at a pH higher than the original (4.02), with a major peak at pH 4.17 and a smaller peak at pH 4.41. This may be due to the pH at which they were introduced (CI = 5.04).

Further evidence for fractionation was obtained from the results of a double-labeling experiment. Using the modified procedure, ^{14}C-labeled and ^{3}H-labeled chromosomes were electrofocused separately at a constant voltage (Figure 7a,b). Reelectrofocusing a mixture of chromosomes obtained from the pH extremes of the two runs (fractions 70–90 from the run of Figure 7a and 105–115 from that of Figure 7b) gave results shown in Figure 7c. As expected, the ^{3}H-labeled chromosomes were collected first, followed by the ^{14}C-labeled chromosomes. Superposition of Figure 7c on 7a or 7b shows the ^{3}H-labeled area and the ^{14}C-labeled area in approximately their original positions. There was also an improved resolution in these areas.

RNA–DNA Hybridization Experiments. Other evidence for the occurrence of fractionation was obtained from RNA–DNA hybridization experiments. It was shown by Huberman and Attardi (1967) that DNA complementary to high molecular weight rRNA is confined to the smaller HeLa cell chromosomes (possibly, exclusively to groups D and G). These chromosomes are found in sedimentation class 4 (see Figure 1). Thus, electrofocusing the small chromosomes could, if further fractionation were achieved, give an enrichment of the rRNA sites in some subfraction, as determined by RNA–DNA hybridization experiments.

TABLE IV: Hybridization with ^{32}P-Labeled rRNA of DNA from Fractions Obtained by Electrofocusing Small Chromosomes.[a]

Fraction	pH Range	Rel Hybridization	Distribution of rRNA Genes[b] (%)
I	4.35–3.88	1.86	37.8
II	3.80–3.68	1.41	30.1
III	3.65–3.45	1.28	16.1
IV	3.40–3.02	1.00	16.0

[a] Electrofocusing was carried out for 24 hr at a starting voltage of 600 V, using the standard procedure (see Figure 8 for pooled fractions). [b] Obtained by multiplying the relative hybridization value for each chromosome cut by the fraction of total [^{3}H]thymidine label in that cut, and expressing the product as per cent of the total.

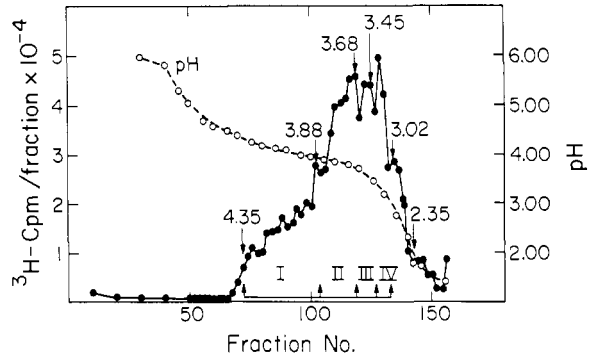

FIGURE 8: Electrofocusing of small chromosomes using the standard procedure. About 10^9 [*methyl*-^{3}H]thymidine-labeled small chromosomes (sedimentation class 4 (see Figure 1) of chromosomes isolated at pH 3; stored frozen) in 1.0 ml of FM were electrofocused as in Figure 4b, but for 24 hr. Fractions of 2.5 ml were collected, and analyzed for pH (○) and acid-precipitable radioactivity (●). The fractions were pooled arbitrarily into four groups, from which DNA was extracted and hybridized with rRNA, as described in Materials and Methods.

Figure 8 shows an electrofocusing run of small chromosomes. A continuous distribution with several partially resolved peaks was obtained. This was divided into four fractions, and the DNA extracted from each of these was hybridized with ^{32}P-labeled rRNA prepared from HeLa cells. The results shown in Table IV clearly indicate an enrichment of the rRNA genes in the less acid among the small chromosomes. The fairly broad pH distribution of the chromosomes carrying the rRNA genes may reflect the different properties of the individual D and G chromosomes, which are those containing a nucleolar organizer (see Huberman and Attardi, 1967).

Other hybridization results are shown in Tables V and VI, which concern experiments carried out with chromosome fractions obtained by electrofocusing whole chromosome populations. The relative hybridization values show a very slight enrichment of rRNA genes in the least acid fraction after the 13-min run and an appreciable increase in that after the 4.5-hr run. From the comparison of Table VI to Table IV it appears that with the increase in electrofocusing time from 4.5 to 24 hr there has been both a shift toward lower pH's of the whole distribution of chromosomes carrying the rRNA genes and a change in the shape of the distribution. If the latter reflects a fractionation of different types of nucleolar organizer-containing chromosomes, it would appear that the removal of protein occurring during prolonged electrofocusing plays a useful role in this respect.

Discussion

The applicability of electrofocusing to the fractionation of chromosomes is suggested by the following observations made by using the standard procedure. (1) Starting with different chromosome preparations (isolated in the same manner) and using different methods of analysis, similar patterns showing a continuous distribution of chromosomes with peaks at the same pH values were found. (2) Amino acid analysis of the electrofocused fractions, compared to that of chromosomes before electrofocusing, showed a change in amino acid composition, as well as differences from one fraction to the other. In view of the similarity in amino acid pattern between chromosome fractions of different sedimentation velocity, and of the progressive effects of electrofocusing on amino acid composition, it seems likely that the amino acid differences between electrofocused chromosome fractions are

TABLE V: Hybridization with ^{32}P-Labeled rRNA of DNA from Fractions Obtained by Electrofocusing a Whole Chromosome Population for 13 min.[a]

Fraction	pH Range	Rel Hybridization	Distribution of rRNA Genes (%)
I	4.31–4.56	1.11	44.8
II	4.26–4.30	1.05	28.5
III	4.15–4.25	1.00	26.7

[a] Electrofocusing was carried out for 13 min at a constant voltage of 700 V, using the modified procedure (see Figure 6 for pooled fractions).

TABLE VI: Hybridization with ^{32}P-Labeled rRNA of DNA from Fractions Obtained by Electrofocusing a Total Chromosome Population for 4.5 hr.[a]

Fraction	pH Range	Rel Hybridization	Distribution of rRNA Genes (%)
I	4.20–4.24	1.57	27.2
II	4.18–4.20	1.14	29.2
III	4.14–4.18	1.04	24.5
IV	4.06–4.14	1.00	19.1

[a] Electrofocusing was carried out for 4.5 hr at a constant voltage of 700 V, using the modified procedure. The electrofocusing profile was similar to that of Figure 7b with a slight overall displacement toward higher pH's: the cuts chosen would correspond in Figure 7b to fractions 55–82 (IV), 83–96 (III), 97–103 (II), and 104–122 (I).

the result of differential removal, during the run, of basic proteins from the various chromosomes, rather than expression of intrinsic differences in their protein complement; however, no direct evidence on this point has been obtained. (3) The distribution of chromosomes isolated at pH 7 was shifted to pH values less acid than that obtained by electrofocusing chromosomes isolated at pH 3. This result is consistent with the observation made by several investigators (Huberman and Attardi, 1966; Maio and Schildkraut, 1967; Salzman *et al.*, 1966; Hearst and Botchan, 1970) that chromosomes isolated at neutral pH have more basic proteins (acid soluble) than those isolated at pH 3. (4) Divalent cations (Ca^{2+} and Mg^{2+}), which have been shown to stabilize the metaphase chromosome structure (Chorazy *et al.*, 1963; Somers *et al.*, 1963), when used in the electrofocusing of 1 month-old chromosomes (stored at $-20°$), reversed the effect of aging on the electrofocusing behavior of chromosomes, giving a profile similar to that obtained by electrofocusing fresh chromosomes. This suggests that working with chromosomes that are morphologically intact gives reproducible results. (5) The experiments of hybridization with ^{32}P-labeled rRNA of DNA extracted from the chromosome fractions obtained by electrofocusing the small chromosomes showed an enrichment of the rRNA sites in subfractions of these chromosomes, thus providing further evidence for fractionation.

The observation that a similar distribution in a pH gradient was obtained by electrofocusing, under comparable conditions, large and small metaphase chromosomes (see, for example, Figures 4b and 8), indicates that the charge differences detected here were not correlated with size differences. This has been confirmed by phase-content examination of electrofocused fractions of total chromosome populations. However, the RNA–DNA hybridization results obtained with the small chromosomes strongly suggest that, within the same size class, charge differences occur between different types of chromosomes. If the recently developed refined methods of chromosome staining, which allow identification of individual pairs of human chromosomes (Drets and Shaw, 1971; Patil *et al.*, 1971), can be applied to electrofocused material, it may be possible to provide a morphological evidence of this fractionation.

The results obtained in the present work indicate that, during electrofocusing, some stripping of protein from the chromosomes occurred. Thus, a Lowry test for protein was positive, after dialysis to remove the ampholine, in the supernatants obtained by centrifuging the electrofocused chromosome fractions. This stripping resulted in a change of charge and therefore a change in isoelectric point. This is indicated by the shift to a lower pH of chromosome fractions separated in a 48-hr run upon reelectrofocusing for another 48 hr, and by the tremendous increase in acidic and decrease in basic amino acids of the reelectrofocused fractions. The stripping of protein, and the resulting change in isoelectric point, could be greatly reduced by decreasing to 24 hr the electrofocusing time in the first and second run.

Protein denudation of the chromosomes may be due to prolonged exposure to an electric field, as carried out in the standard procedure. In this procedure, the chromosomes were introduced with the density gradient solution, so that they were distributed throughout the electrofocusing compartment. Whether the proteins removed by prolonged electrofocusing are constituents of the metaphase chromosomes as they exist *in vivo*, or whether they are adventitiously adsorbed during chromosome isolation is not known.

It was possible to shorten the electrofocusing time by using a modified procedure, in which the chromosomes were introduced as a layer at an appropriate pH in a preformed pH gradient. This reduced the denudation effect to a minimum. As a result, when chromosomes labeled with [^{14}C]leucine were electrofocused for 13 min, no radioactive protein was found in the supernatant. The appearance of discrete visible bands at this time presumably reflects the beginning of fractionation, but the physical basis of their formation is not known. In the 3-hr run some protein was already found in the supernatant. However, this loss of protein did not appear to affect the behavior of electrofocused chromosomes in rerun experiments. In fact, chromosome fractions separated by the modified electrofocusing procedure in a 3- or 4.5-hr run, upon electrofocusing under identical conditions, banded at the same pH values as in the first run. These results, and the results mentioned above concerning rerun experiments after 24-hr electrofocusing by the standard procedure, indicate that the loss of protein from chromosomes, under our experimental conditions, is, at least at start, a relatively slow process, and does not affect appreciably the electrophoretic behavior of chromosomes within the first 9 hr, and possibly 24 hr, of exposure to the electrical field. As a consequence, reproducible results are obtained in run and rerun experiments carried out under strictly identical conditions, if the overall time of electrofocusing is kept within the limits indicated above.

From the results presented in this paper and the above discussion it appears that the isoelectric point of metaphase chromosomes is not a fixed quantity, but that rather its value is strongly dependent upon the experimental conditions used and, in particular, is subject to a continuous, though slow, change when the chromosomes are exposed to an electrical field.

Conclusion

The results of this work indicate that metaphase chromosomes, in as much as they possess a charge, can be fractionated electrophoretically in a pH gradient. Using the modified electrofocusing procedure described here, which allows a drastic shortening of the electrofocusing time, chromosomes form a continuous distribution in the pH range from 3.90 to 4.30 with partially resolved groups. Upon reelectrofocusing under identical conditions, these groups band at the same pH value as in the original run, with improved resolution of the components and apparent preservation of the morphological integrity of the chromosomes.

The appreciable enrichment of rRNA genes observed in chromosome fractions obtained by electrofocusing the small chromosomes, previously separated by sedimentation velocity, illustrates the potential value of combining the two methods of fractionation. It is possible that with cell lines having fewer chromosomes than HeLa cells, like those from Drosophila, Chinese hamster, or Kangaroo rat, the electrofocusing method, either alone or in combination with the sedimentation velocity method, may yield single chromosome species.

Acknowledgment

We gratefully acknowledge John Racs for the amino acid analyses and Mrs. LaVerne Wenzel and Benneta Keeley for their technical assistance.

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Elapid Neurotoxins. Purification, Characterization, and Immunochemical Studies of α-Bungarotoxin†

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ABSTRACT: The purification to homogeneity of α-bungarotoxin (α-Bgt) from the venom of *Bungarus multicintus* by starch electrophoresis followed by gradient elution on carboxymethylcellulose is described. Characterization of the purified toxin was carried out with respect to its amino acid composition, isoelectric point (p*I*), minimal molecular weight, N-terminal amino acid, and its electrophysiological characteristics. The amino acid composition of α-Bgt was found to correspond to its general basic nature (p*I* = 9.19 for iodinated [^{125}I]α-Bgt). Minimal molecular weight determination from the amino acid composition data gave a value of 7904, in agreement with a value of 8000 found by sodium dodecyl sulfate disc gel electrophoresis. Quantitative determination of the N terminus as isoleucine allowed estimation of the purity of this preparation of α-Bgt to be at least 99%. Electrophysiological characterization of α-Bgt and its purified iodinated analog showed both preparations to be effective in blocking the acetylcholine response of frog rectus abdominus muscle. Rabbit antibodies against purified α-Bgt were purified by removal of macroglobulin on Sephadex G-200. Subsequently the remaining immunoglobin G was coupled to cyanogen bromide activated Sepharose 2B. The binding capacity and conditions for eluting bound toxin from the toxin specific immunosorbent were investigated.

The recent interest (Changeux *et al.*, 1970; Miledi *et al.*, 1971; Cooper *et al.*, 1971)[1] in low molecular weight protein neurotoxins for the study of neurochemical processes has necessitated their fractionation, purification, and characterization. Of the elapid neurotoxins, only the purification and immunochemical characteristics of cobrotoxin from *Naja naja atra* have been fully described (Yang, 1964; Chang and Yang, 1969). Among the elapid neurotoxins investigated by Lee (1970) are those from *Bungarus multicintus*, for which he reports the amino acid composition of two (α and β) of this venom's many components. However, the purification of α-bungarotoxin (α-Bgt),[2] which blocks neuromuscular transmission by acting at the postsynaptic membrane of the neuromuscular junction (Lee, 1970), has yet to be described in full (Changeux *et al.*, 1970; Lee, 1970), although its amino acid sequence has been reported (Mebs *et al.*, 1971). We report here a purification procedure and some chemical properties of α-Bgt as well as the preparation of toxin-specific antibodies.

† Contribution No. 4293 from the Church Laboratory of Chemical Biology, California Institute of Technology, Pasadena, California 91109. *Received August 9, 1971.* Supported by U. S. Public Health Service Grant GM-14452.

‡ Recipient of a National Institutes of Health Fellowship GM-46182-01 from the National Institute of General Medical Sciences.
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[1] See companion papers in this issue.

[2] Abbreviations used are: Bgv, venom from *Bungarus multicintus;* α-Bgt, α-bungarotoxin; IgG, immunoglobin G; IgM, macroglobin; AcCh, acetylcholine; AcChE, acetylcholine esterase; AcChR, acetylcholine receptor; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate.