Since Asp_{59} in the bovine enzyme corresponds to His_{59} in both dogfish and porcine trypsins, residue 59 cannot be a ligand in dogfish trypsin. It is possible, however, that the adjacent residue Asp_{60} serves the same role as Asp_{59} in the bovine enzyme, whereas in the porcine enzyme the corresponding site (His_{59}, Asn_{60}) obviously precludes calcium binding.

Acknowledgments

We thank Mrs. L. Black for her assistance in the preparation of dogfish trypsin, Dr. M. A. Hermodson, Mrs. B. Moody, Mrs. S. Balian, and Mrs. M. Jarvis for their assistance in the sequence analyses, and Mr. R. R. Granberg for his assistance in the amino acid analyses.

References

Ambler, R. P. (1965), *Biochem. J. 96,* 32P.
Bradshaw, R. A., Neurath, H., Tye, R. W., Walsh, K. A., and Winter, W. P. (1970), *Nature (London) 226,* 237.
de Haen, C., Neurath, H., and Teller, D. C. (1975), *Biochemistry* (in press).
Dickerson, R. E. (1971), *J. Mol. Evol. 1,* 26.
Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1,* 80.
Gross, E. (1967), *Methods Enzymol. 11,* 238.
Hermodson, M. A., Ericsson, L. H., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12,* 3146.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemsitry 11,* 4493.
Hermodson, M. A., Tye, R. W., Reeck, G. R., Neurath, H., and Walsh, K. A. (1971), *FEBS Lett. 14,* 222.
Kenner, R. A., Walsh, K. A., and Neurath, H. (1968), *Biochem. Biophys. Res. Commun. 33,* 353.
Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London) 214,* 652.
Mikes, O., Holeysovsky, V., Tomasek, V., and Sorm, F. (1966), *Biochem. Biophys. Res. Commun. 24,* 346.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Peterson, J. D., Nehrlich, S., Oyer, P. E., and Steiner, D. F. (1972), *J. Biol. Chem. 247,* 4866.
Robinson, N. C., Tye, R. W., Neurath, H., and Walsh, K. A. (1971), *Biochemistry 10,* 2743.
Schroeder, D. D., and Shaw, E. (1968), *J. Biol. Chem. 243,* 2943.
Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225,* 811.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 125.
Tye, R. W. (1971), Ph.D. Dissertation, University of Washington, Seattle.
Walsh, K. A. (1970), *Methods Enzymol. 19,* 41.
Walsh, K. A., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 884.
Walsh, K. A., and Wilcox, P. E. (1970), *Methods Enzymol. 19,* 31.
Wikler, M., Titani, K., and Putnam, F. W. (1970), *J. Biol. Chem. 245,* 2158.

Protein Content of Chromatin Fractions Separated by Sucrose Gradient Centrifugation†

D. Doenecke‡ and B. J. McCarthy*

ABSTRACT: When sheared chromatin is centrifuged in a steep sucrose gradient, two broad peaks are resolved. DNA extracted from both fractions has approximately the same molecular weight. The basis for this fractionation seems to be differential aggregation. The slowly sedimenting material shows a lower protein/DNA ratio than the rapidly sedimenting chromatin as judged by equilibrium density centrifugation in CsCl after formaldehyde fixation or under nonionic conditions. After selective removal of histone f1 and further shear, most of the slowly sedimenting chromatin material appears as free DNA in steep cesium chloride gradients. The data are consistent with several recent reports concerning the subunit structure of chromatin.

The earliest method to be developed for chromatin fractionation involved fragmentation and differential centrifugation (Frenster *et al.,* 1963). Although methods based upon other principles have since been developed, this same basic methodology has proved to be most popular and convenient for obtaining two chromatin fractions exhibiting different biochemical and physical properties. The centrifugation approach was later refined through the use of sucrose gradients (Chalkley and Jensen, 1968; Yunis and Yasmineh, 1971; Duerksen and McCarthy, 1971; McCarthy et al., 1973).

In the present communication we present further characterization of the two fractions obtained after shearing chromatin of *Drosophila* cells, Schneider's line 2 (Schneider, 1972). The results demonstrate a major difference in the protein/DNA ratio of the two peaks.

† From the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received October 31, 1974.* Supported by a grant from the National Science Foundation (GB 35704).

‡ Recipient of a research fellowship from the Deutsche Forschungsgemeinschaft. Present address: Physiologisch-chemisches Institut Lahnberge, D 355 Marburg Lahn, Federal Republic of Germany.

Materials and Methods

Tissue Culture Cells. Drosophila melanogaster tissue culture cells (Schneider, line 2) were used in all experiments described below. The cells were grown in Roller bottles at room temperature in Schneider's medium, supplemented with 0.5% Bactopeptone, 15% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml). Medium and serum were purchased from Pacific Biological Company (Berkeley), the antibiotics from Gibco (Grand Island, N.Y.).

Labeling of Cells. Radioactive compounds were from Schwarz/Mann. Cells were labeled by adding 100 μCi of [6-^{3}H]thymidine (8 Ci/mmol), 2 μCi of [2-^{14}C]thymidine (9.5 Ci/mol), or 100 μCi of [^{3}H]lysine (53 Ci/mmol) to 100 ml of Schneider's medium for 20 hr.

Preparation of Chromatin. Chromatin was prepared as described by Bonner et al. (1968). After centrifugation of the chromatin through 1.7 *M* sucrose in 0.01 *M* Tris (pH 8), the pellet was washed twice in 0.01 *M* triethanolamine (pH 7.8) by centrifugation at 8000*g* for 10 min and resuspending the chromatin in this buffer. Chromatin was sheared by passing through a French pressure cell at 3000 or 20,000 psi. The sheared chromatin was centrifuged at 16,000*g* for 20 min and the supernatant used for further studies. The sheared, soluble chromatin was obtained with a yield of 70–80% based upon the [^{3}H]thymidine radioactivity.

Fractionation of Chromatin on Sucrose Gradients. Gradients were formed using equal volumes of 0.17 *M* sucrose and 1.7 *M* sucrose in 0.01 *M* triethanolamine (pH 7.8). Ultrapure sucrose (Schwarz/Mann) was used. Up to 1 ml of sheared chromatin, containing approximately 100 μg of DNA, was layered on top and the gradients were centrifuged in a Beckman SW40-Ti-rotor at 32,000 rpm, for 15 hr at 5°; 0.5-ml fractions were collected and 50-μl aliquots were removed for estimation of Cl_3CCOOH-precipitable radioactivity.

Determination of Molecular Weight. Chromatin was sheared at 3000 psi and fractionated on sucrose gradients or sheared at 20,000 psi in a French pressure cell. The various samples were then brought to 1 × SSC and 1% sodium dodecyl sulfate and extracted with water-saturated phenol at room temperature. After extraction with ether the aqueous supernatant was precipitated twice with two volumes of 95% ethanol. The samples were then centrifuged at 18,000*g* and the DNA was dissolved in Tris–borate buffer (0.09 *M* Tris, 0.09 *M* boric acid, and 2.5 m*M* NaEDTA (pH 8.3)) for electrophoresis; 25-μl samples containing approximately 10 μg of DNA were subjected to electrophoresis in 6% polyacrylamide slab gel (6% acrylamide, 0.4% 3-dimethylaminopropionitrile, and 0.2% bisacrylamide) for 2 hr at 40 mA. The gel was subsequently immersed in a solution of 4 μg/ml of ethidium bromide in water for 10 min and then photographed under irradiation with a long wavelength uv lamp. The gel pattern on the negative was then scanned at 650 nm in the gel scanner attachment of a Beckman Acta C III spectrophotometer. As a reference, SV 40 DNA was treated with *Haemophilus influenzae* restriction endonuclease III, which yields DNA pieces of defined molecular weight (Helling et al., 1974).

Fixation of Chromatin with Formaldehyde. Chromatin was fixed with formaldehyde by the method of Brutlag et al. (1969) at a formaldehyde concentration of 0.6% in most experiments. No differences in the equilibrium density patterns could be observed in the range of 0.4 and 1.0% formaldehyde in 0.01 *M* triethanolamine (pH 7.8). The chromatin solutions to be fixed, containing between 4 and 40 μg/ml in triethanolamine, were stirred vigorously into the appropriate formaldehyde solution. After standing in ice for 24 hr the samples were dialyzed for 24 hr against 0.01 *M* triethanolamine (pH 7.8) before being used in equilibrium density centrifugations.

Equilibrium Centrifugation in CsCl. In most experiments 10-ml gradients were used to cover a density range from 1.35 to 1.45 g/cm^3. The CsCl solutions (Harshaw Chemical, Optical Grade) were centrifuged in a fixed-angle Beckman 40 rotor at 33,000 rpm for 60 hr at 15°. Approximately 27 fractions were collected and the Cl_3CCOOH precipitable radioactivity was measured by diluting the fractions to 1 ml with buffer, adding 1 drop of bovine serum albumin solution (5 mg/ml), and making the fractions 10% in Cl_3CCOOH followed by filtration through Whatman GFC filters. After washing the filters with 5% Cl_3CCOOH, they were dried and counted.

Removal of Histone f1 with Purified Yeast RNA. Drosophila chromatin was prepared from cells doubly labeled with [^{3}H]lysine and [^{14}C]thymidine. After shearing and fractionation on a sucrose gradient, pooled fractions representing the two peaks were incubated overnight at 4° with purified yeast tRNA (10 μg of chromatin, 1 mg of tRNA in 1 ml of 1 m*M* $MgCl_2$, and 1 m*M* sodium phosphate buffer (pH 7.4)) (Ilyin et al., 1971). An aliquot of total chromatin was treated in the same way. Each sample was then placed on a column of Sepharose 4B (Pharmacia) equilibrated with 1 m*M* $MgCl_2$–1 m*M* sodium phosphate (pH 7.4) to remove the histone f1–tRNA complex.

Analysis of the histones in the tRNA fraction revealed undegraded f1 and traces of f2b and f2a2 as found earlier in similar experiments with mammalian chromatin (Ilyin et al., 1971). The remaining chromatin contained the other four histones but f1 was undetectable.

The excluded fraction was fixed with 1% HCHO at 0° for 24 hr and dialyzed against 1 m*M* sodium phosphate (pH 6.8). The dialyzed chromatin fractions were further sheared at 20,000 psi in the French pressure cell; 3 g of CsCl was added to 3.2 ml of chromatin solution and gradients were centrifuged at 42,000 rpm for 72 hr at 10° in the Beckman SW56 rotor.

Equilibrium Centrifugation in Conray. N-Methylglucamine-5-acetamido-2,4,6-triiodo-*N*-methylisophthalamate (Meglumine-iothalamate, Conray) was purchased from Mallinckrodt Pharmaceuticals, St. Louis, Mo. in 30-ml vials, containing 60% Conray solutions in 1 m*M* NaH_2PO_4–0.3 m*M* EDTA; 9-ml gradient solutions were formed by mixing chromatin samples, Conray stock solutions, and triethanolamine buffer to a final concentration of 33% Conray in 0.01 *M* triethanolamine. Gradients were centrifuged at 10° in a fixed-angle Beckman 40 rotor for 96 hr at 33,000 rpm. Between 25 and 30 fractions were collected by puncturing the bottom of the centrifugation tubes. The fractions were kept in ice, 1 drop of 5 mg/ml of bovine serum albumin was added, and the fractions were precipitated with ice-cold 95% ethanol as proposed by Ivarie and Pene (1970) for Renografin gradients. After filtration through Whatman GFC filters and several washes with 95% ethanol, 5 ml of 5% Cl_3CCOOH was filtered through the filters, followed by ethanol. After drying the filters were counted in toluene–Omnifluor. Density was calculated from refractive indices using a calibration curve based on weighing several dilutions of Conray stock solutions.

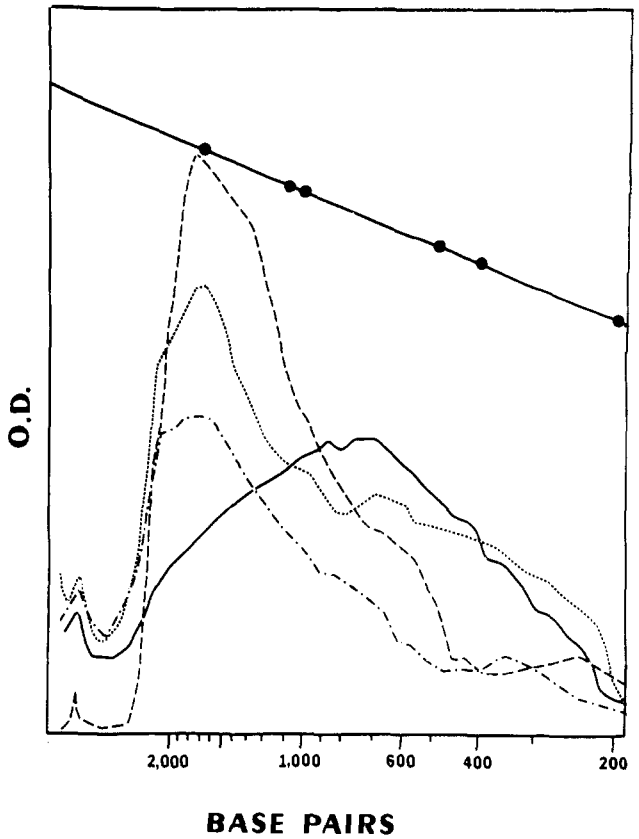

FIGURE 1: Determination of molecular weight of DNA extracted from chromatin, sheared at 3000 psi (- · · ·), fractionated into slowly (- - -), and rapidly (· · ·) sedimenting portions on a sucrose gradient and DNA from chromatin, which was sheared at 20,000 psi (—). Top line (●—●): reference DNA molecules, obtained by digesting SV40 DNA with *H. influenzae* restriction endonuclease. OD scan of a photograph of 6% polyacrylamide slab gel (see Materials and Methods).

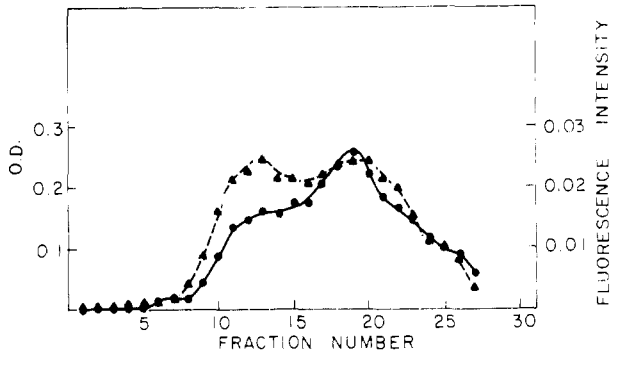

FIGURE 2: Sucrose gradient fractionation of labeled chromatin from *Drosophila melanogaster* tissue culture cells and binding of ethidium bromide. Chromatin was prepared as described under Materials and Methods, sheared at 3000 psi and fractionated on a gradient from 0.17 to 1.7 *M* sucrose in 0.01 *M* triethanolamine (pH 7.8) at 32,000 rpm in a SW40 Beckman swinging bucket rotor for 15 hr; 0.5-ml fractions were collected and the optical density was measured at 260 nm (●—●); 0.25-ml aliquots of the fractions were mixed with 10 μl of an ethidium bromide solution, 1 mg/ml in 0.01 *M* Tris buffer (pH 8.0). Excitation 546 nm, emission 590 nm. Fluorescence intensity (▲- - -▲).

Results

General Properties of Fractionated Chromatin. When chromatin from Schneider's cells is sheared at 3000 psi in the French pressure cell and centrifuged on a 0.17–1.7 *M* sucrose gradient, two broad peaks are obtained (McCarthy et al., 1973). As shown in Figure 1, the analysis of the molecular weight of both peaks shows a broad distribution between 1000 and 2000 base pairs. When chromatin is sheared at 20,000 psi, the molecular weight of DNA in the chromatin pieces is reduced to between 500 and 1100 base pairs.

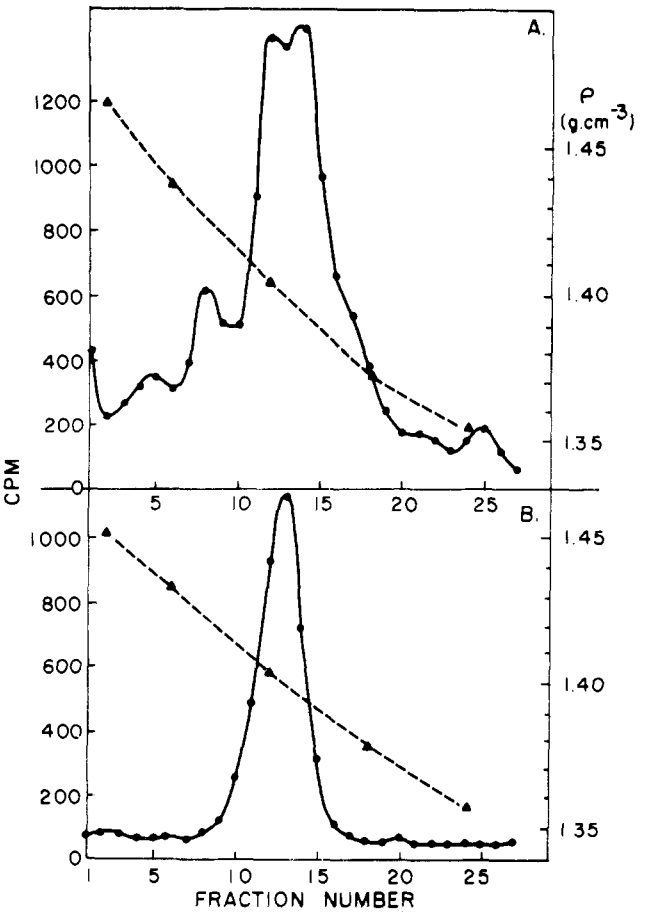

FIGURE 3: Cesium chloride gradient of sheared and unsheared chromatin. Buoyant density pattern of chromatin from *Drosophila melanogaster* tissue culture cells. Chromatin was extracted from cells, which had been labeled for 20 hr with [6-^{3}H]thymidine (1 μCi/ml of Schneider's medium). Fixation with 0.7% formaldehyde at 0° for 24 hr, followed by dialysis against 0.01 *M* triethanolamine (pH 7.8). Lower frame, unsheared chromatin; upper frame, chromatin sheared at 3000 psi. (●—●) Cl_3CCOOH-precipitable radioactivity; (▲- - -▲) density.

Since the sucrose gradient is steep and not isokinetic, the two peaks actually represent material with a large difference in sedimentation behavior. An example of this separation is given in Figure 2. These two peaks differ in several properties including in vitro template capacity and thermal denaturation profiles (McCarthy et al., 1973). As shown in Figure 2, they also differ in their capacity to bind ethidium bromide. This result suggests that the DNA of the slowly sedimenting peak is more available to ligands. This result is consistent with the differences in thermal denaturation profiles which also suggest that the DNA of slowly sedimenting chromatin is less stabilized against melting by associated proteins.

Buoyant Density of Fractionated Chromatin in CsCl. After fixation with HCHO, chromatin may be centrifuged in a CsCl gradient to a position reflecting its protein/DNA ratio (Brutlag et al., 1969; Ilyin et al., 1970). This procedure therefore offers a sensitive approach to comparing the protein/DNA ratio of fractionated chromatin. Total chromatin bands at a density of approximately 1.40 g cm^{-3} (Figure 3). When sheared at 3000 psi a broader distribution

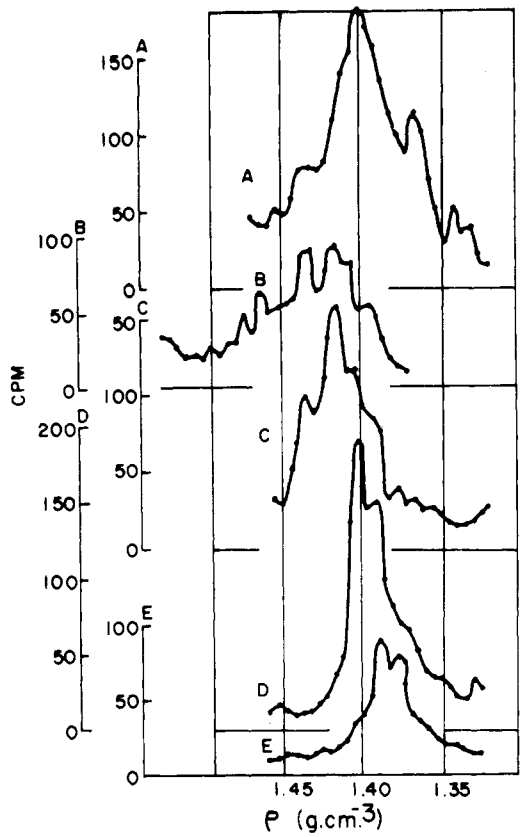

FIGURE 4: Buoyant density patterns in cesium chloride of total sheared chromatin and pooled fractions from four different segments of a sucrose gradient as shown in Figure 2. Each sample was treated with 0.5% formaldehyde and dialyzed against 0.01 *M* triethanolamine before equilibrium density centrifugation. (A) Sheared unfractionated chromatin; (B) slowly sedimenting chromatin fraction; (C) intermediate region between slowly and rapidly sedimenting chromatin fractions; (D) peak fractions of rapidly sedimenting chromatin; (E) tail region of rapidly sedimenting chromatin fraction (as fractions 21-25 in Figure 2). (●—●) Cl_3CCOOH-precipitable radioactivity.

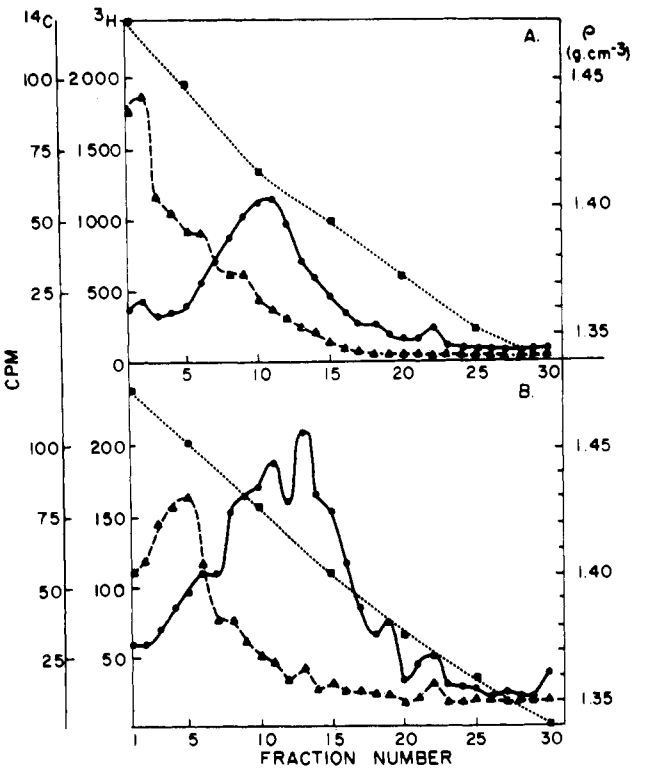

FIGURE 5: Cesium chloride gradient of mixed chromatin fractions. Buoyant density pattern of formaldehyde fixed chromatin fractions from two differentially labeled chromatin preparations, separated on sucrose gradients (as shown in Figure 2) into a slowly and a rapidly sedimenting portion. (A) Pooled fractions from slowly sedimenting chromatin, labeled with [^{3}H]thymidine, were mixed with rapidly sedimenting, [^{14}C]thymidine labeled, chromatin fractions and then fixed with formaldehyde. (▲- - -▲) ^{3}H cpm; (●—●) ^{14}C-cpm; (■ · · ·■) density). (B) Same fractions as in A, but fixed with formaldehyde prior to being pooled before equilibrium centrifugation.

results with significant shoulders at densities up to 1.45.

In order to establish any possible relationship between the distribution of chromatin in CsCl and the fractionation by sedimentation, several fractions from the sucrose gradient were fixed and centrifuged to equilibrium (Figure 4). When the most slowly sedimenting fraction was examined in this way, a broad distribution was obtained with densities ranging from 1.40 to 1.50. In contrast, the most rapidly sedimenting fractions banded at lower densities, approximately 1.38.

As a further demonstration of the relationship between the distribution of chromatin in sucrose and CsCl gradients, fractions from two separate sucrose gradients were mixed and centrifuged together. Two sucrose gradients were run in parallel, one containing chromatin from cells labeled with [^{14}C]thymidine, the other containing [^{3}H]thymidine-labeled chromatin. Fractions representing the two peaks were mixed and then fixed with formaldehyde or separately fixed and then mixed (Figure 5). In both cases a clear separation was obtained in the CsCl gradients with the respective fractions appearing at the same positions as in Figure 4.

Equilibrium Centrifugation of Native Chromatin. Although the results from the CsCl gradient centrifugation indicate a clear difference in protein/DNA ratio, the necessity for fixation complicates any detailed interpretation. Rickwood et al. (1973) have introduced a method for banding chromatin to equilibrium in which the necessity for fixation is obviated. We have followed the same approach using a similar but not identical substance, *N*-methylglucamine-5-acetamido-2,4,6-triiodo-*N*-methylisophthalamate (Conray) as the density medium. In equilibrium gradients of this material, DNA bands at approximately 1.17, RNA at 1.21, protein at 1.28, and chromatin at 1.23 g cm^{-3} (Figure 6).

When sheared chromatin, fractionated on a sucrose gradient, was centrifuged to equilibrium in the same way two rather broad peaks were obtained, differing in mean density from 1.24 to 1.22 (Figure 7). Again this result is consistent with a lower protein/DNA ratio in the slowly sedimenting chromatin. Since the difference in density was small and the peaks overlapped an attempt was made to perform a double isotope mixing experiment of the type illustrated in Figure 5. In fact, aliquots of the same sucrose fractions used in this experiment differentially labeled with ^{3}H and ^{14}C were mixed and banded in Conray. However, as shown in Figure 8, the distribution of ^{3}H and ^{14}C overlapped and was almost coincident. The most likely explanation appeared to be that the two chromatin fractions, although differing in protein/DNA ratio, interact, aggregate, and band at the same position.

To test this notion similar sucrose gradient fractions were fixed with formaldehyde either prior to or subsequent to being mixed together. In both cases a clear difference in the distribution of ^{3}H and ^{14}C was obtained demonstrating that slowly sedimenting chromatin has a lower density and thus

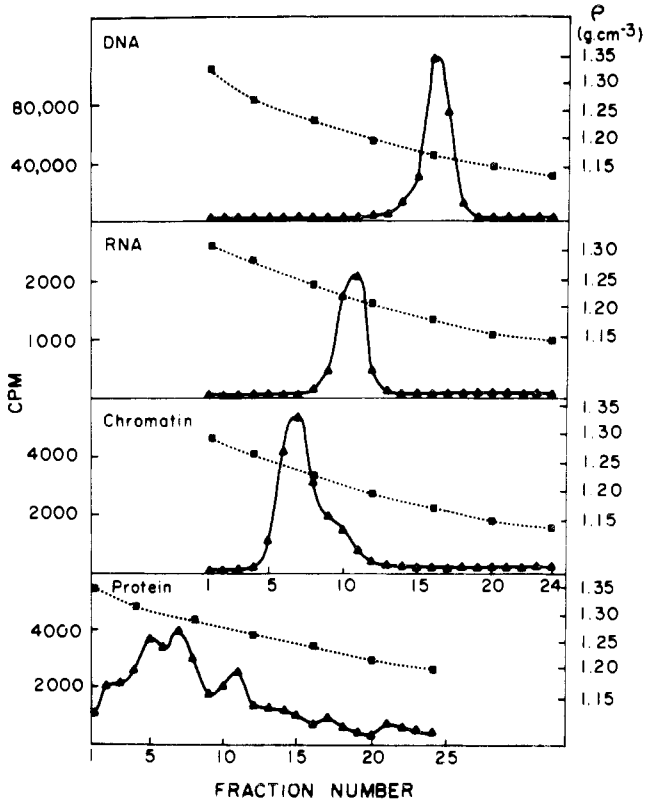

FIGURE 6: Buoyant density of different macromolecules after 96 hr of centrifugation at 33,000 rpm in Conray in a fixed angle Beckman rotor 40 at 10°. DNA, ^{3}H-labeled DNA, purified from *Drosophila melanogaster* tissue culture cell chromatin; RNA, ^{3}H-labeled 18S ribosomal RNA isolated from polysomes, prepared out of [^{3}H]uridine-labeled *Drosophila* cells (2 μCi of [^{3}H]uridine/ml of Schneider's medium); chromatin, prepared as described under Materials and Methods from *Drosophila* tissue culture cells; protein, catalase (National Biochem. Corp., Cleveland, Ohio) was labeled by reductive alkylation following the procedure of Rice and Means (1971).

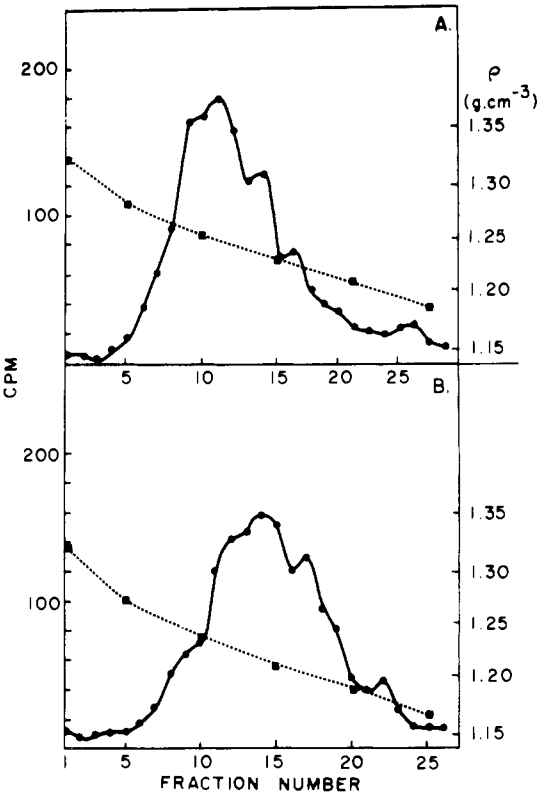

FIGURE 7: Conray density gradient of different chromatin fractions, previously separated on a sucrose gradient (0.17–1.7 *M* sucrose) after 96 hr at 10° in a fixed angle Beckman 40 rotor at 33,000 rpm. Upper frame, chromatin fractions sedimenting rapidly in sucrose gradients (●—●); lower frame, slowly sedimenting sucrose fractions (●—●); density (■ · · · ■).

a lower protein to DNA ratio than does the rapidly sedimenting fraction (Figure 9).

Removal of Histone f1 from Fractionated Chromatin. Since histones are the major protein constituents of chromatin, it seemed most probable that the differences in protein content are a reflection of the distribution of histones along the DNA. Ilyin et al. (1971) demonstrated that it is possible to remove histone f1 selectively from chromatin by incubation with yeast tRNA and 1 m*M* Mg^{2+}. After this procedure, in which no other histones are removed, they were able to demonstrate the existence of short regions of DNA essentially devoid of proteins (Varshavsky and Georgiev, 1973). The same approach was used to test for the possibility that such regions are unequally distributed between the two sucrose gradient fractions. Fractions representing the two peaks were prepared, treated to remove histone f1, fixed with HCHO, and sheared at 20,000 psi to produce fragments containing DNA of approximately 800 base pairs. Each preparation was then centrifuged to equilibrium in CsCl in a SW56 rotor with total sheared chromatin treated in the same way as reference (Figure 10). In the total chromatin about 20% of the ^{14}C label, representing the DNA, banded at a density corresponding to free DNA. More than 95% of the [^{14}C]DNA in the slowly sedimenting fraction appeared at the density of DNA. In contrast only a few percent of free DNA was derived from rapidly sedimenting chromatin. Thus slowly sedimenting chromatin contains most of the regions which give rise to free DNA upon removal of histone f1.

Discussion

Fragmentation by shear gives rise to two fractions of chromatin which differ in several properties. One is rapidly sedimenting and the other sediments at a rate consistent with its molecular size (Chalkley and Jensen, 1968). The basis of this difference appears to be aggregation. This fractionation has been exploited by many workers who have examined various physical and biochemical properties of the two fractions. The slowly sedimenting fraction has many of the properties expected for active or transcribable chromatin. The average thermal denaturation temperature is lower (McCarthy et al., 1973) and the ability to support RNA synthesis in vitro with added *Escherichia coli* or homologous polymerase is greater (Murphy et al., 1973; McCarthy et al., 1973). In contrast the rapidly sedimenting chromatin shows an enrichment of satellite DNA known to be localized in heterochromatin (Yunis and Yasmineh, 1971; Duerksen and McCarthy, 1971).

In our previous comparisons of the chemical composition of the two sucrose gradient fractions, no large difference in protein/DNA ratio was evident (McCarthy et al., 1973). However, it is now clear from results of more sensitive approaches that this ratio is lower for the slowly sedimenting chromatin. In Conray gradients, densities obtained for the rapidly and slowly sedimenting chromatin suggested protein/DNA ratios of 0.9 and 1.9. These figures represent histone and non-histone proteins. In CsCl the protein/DNA ratios appear at 0.87 and 1.39 as calculated from the rela-

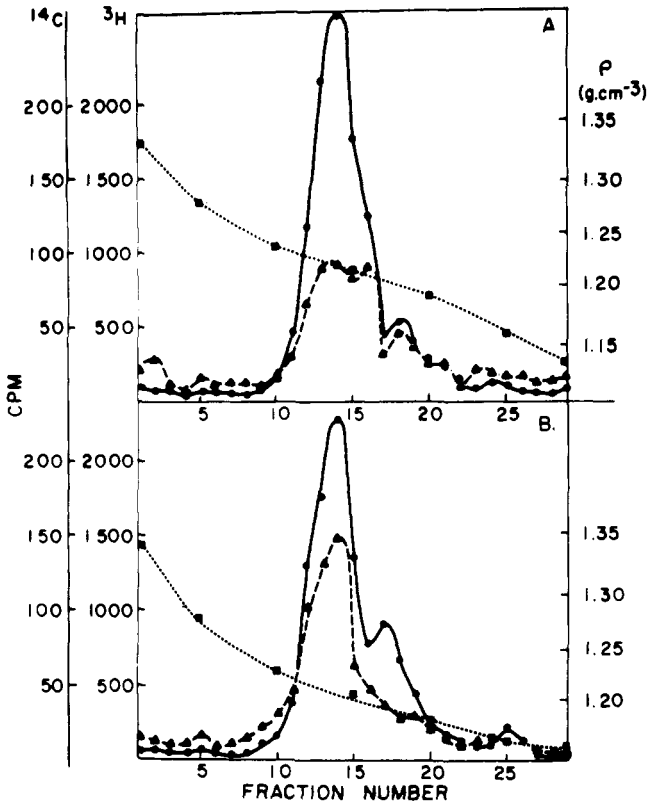

FIGURE 8: Conray density gradient of chromatin fractions from differentially labeled preparations, which had been fractionated on sucrose gradients. Upper frame, ^{3}H-labeled chromatin (●—●) from rapidly sedimenting sucrose gradient fractions mixed with ^{14}C-labeled fractions (▲- - -▲) from the slowly sedimenting portion of the same sucrose gradient; lower frame, ^{3}H-labeled chromatin (●—●) from slowly sedimenting portion, mixed with ^{14}C-labeled chromatin from the rapidly sedimenting fraction (▲- - -▲). Density (■ · · · ■).

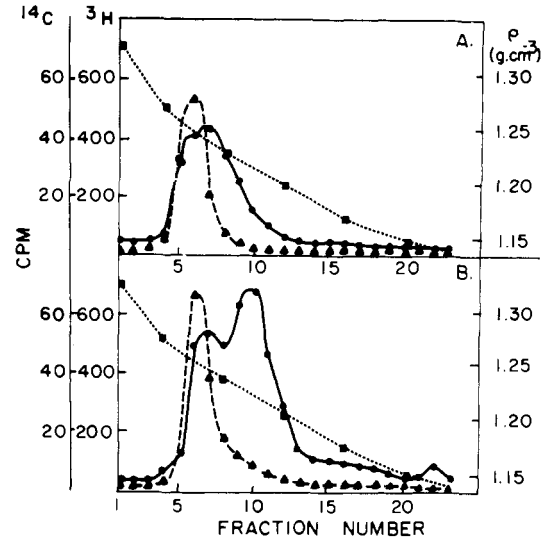

FIGURE 9: Conray density gradient of formaldehyde fixed, differentially labeled chromatin from the peak fractions of two sucrose gradients. Upper frame, the peak fractions of the rapidly sedimenting chromatin (^{3}H-labeled, ▲- - -▲) was mixed with the peak fraction of the slowly sedimenting chromatin from a different sucrose gradient (^{14}C-labeled, ●—●) and then fixed with 0.6% formaldehyde; lower frame, the same fractions (^{3}H-labeled, rapidly sedimenting chromatin (▲- - -▲); and ^{14}C-labeled, slowly sedimenting chromatin (●—●)) were fixed with 0.6% formaldehyde separately and after complete fixation combined into one Conray gradient; centrifugation for 96 hr at 10°, 33,000 rpm in a fixed angle Beckman 40 rotor. Density (■ · · · ■).

tionship proposed by Brutlag et al. (1969). These two independent estimates are in fact in reasonable agreement since, although the histones are fixed with high efficiency to the DNA, most non-histone proteins fail to be fixed (Doenecke and McCarthy, 1975). Therefore the difference must reflect mainly the distribution of histones since they are the major class of chromosomal protein. Most of the difference is attributable to the four histones other than f1 since, when this histone is specifically removed from the slowly sedimenting chromatin peak, most of it bands at the density of free DNA after fixation and centrifugation in CsCl. Therefore we conclude that the clusters of slightly lysine-rich and arginine-rich histones f2b, f2a2, f3, and f2a1 (Kornberg, 1974) are irregularly distributed along the chromatin and that, after shearing, fragments rich in these histones preferentially aggregate and sediment rapidly in a sucrose gradient. In contrast, chromatin fragments in which these histones are underrepresented sediment slowly at rates consistent with their molecular weight.

We have previously reported only minor differences in the ratio of histone f1 to the other four histones between the two peaks (McCarthy et al., 1973). We can only attribute this discrepancy to increased resolution in the sucrose gradient fractionation or to poor quantitation of histones in the gels. More recent experiments using isotope dilution to quantitate the relative amounts of the five histones in each peak confirm the present conclusion that slowly sedimenting chromatin is deficient in all of the histones except f1 (Levy, Nasser, and McCarthy, unpublished results).

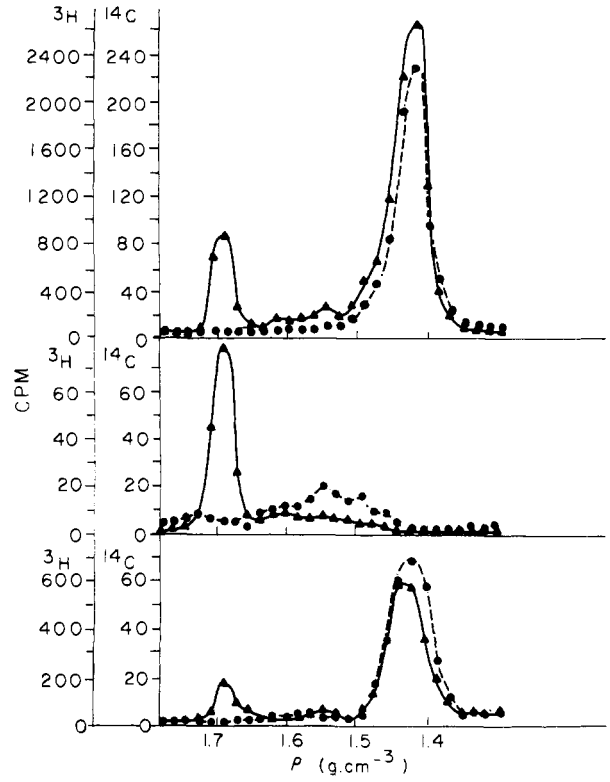

FIGURE 10: CsCl equilibrium centrifugation of chromatin treated with yeast-tRNA to remove histone f1. Total sheared chromatin or chromatin fractionated on sucrose gradients was treated to remove histone f1, dialyzed, fixed with HCHO, further sheared at 20,000 psi, and centrifuged in the SW56 rotor at 42,000 rpm for 72 hr after addition of CsCl. Upper frame, total chromatin; middle frame, slowly sedimenting sucrose fraction; lower frame, rapidly sedimenting sucrose fraction. (●- - -●) [^{3}H]Lysine. (▲—▲) [^{14}C]thymidine.

It is not possible to make a detailed interpretation of this difference in aggregation. Nevertheless a simple model may be offered. Suppose that the histone clusters can interact with one another and that they are monovalent. Then if a chromatin fragment contains only one histone cluster it can interact with only one other fragment to produce a dimer. Conversely any chromatin fragment containing two or more histone clusters may participate in multiple interactions leading to aggregation. If histone clusters cover approximately 200 base pairs and are spaced 200 base pairs apart, then randomly cleaved fragments of chromatin containing between 1000 and 2000 base pairs will contain only a few histone clusters. Further, if histone clusters are irregularly spaced or if shearing is nonrandom with respect to the location of the histones, then similar size fragments may contain quite different numbers of clusters and differ widely in aggregation behavior.

This overall view of the structure of chromatin is in agreement with the conclusions of Varshavsky et al. (1973). They conclude that stretches of DNA as long as 4000 base pairs may exist in chromatin free of all histones except f1. Such a view is also consistent with the electron microscopic study of Olins and Olins (1974) who observed globular nucleohistone subunits separated by linear threads. Likewise the globular subunits observed by Sahasrabuddhe and van Holde (1974) may also represent a basic histone clustered chromatin subunit (Kornberg, 1974). However, in all these cases the conclusion is subject to possible preparational artifacts in which histones are displaced along the DNA strand during chromatin preparation, shearing, or removal of histone f1.

References

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H. H., Olivera, B., and Widholm, J. (1968), *Methods Enzymol. 12B,* 3.

Brutlag, D., Schlehuber, C., and Bonner, J. (1969), *Biochemistry 8,* 3214.

Chalkley, R. G., and Jensen, R. H. (1968), *Biochemistry 7,* 4380.

Doenecke, D., and McCarthy, B. J. (1975), following paper.

Duerksen, J. D., and McCarthy, B. J. (1971), *Biochemistry 10,* 1471.

Frenster, J. H., Allfrey, V. G., and Mirsky, A. E. (1963), *Proc. Natl. Acad. Sci. USA 50,* 1026.

Helling, R. B., Goodman, H. M., and Boyer, H. W. (1974), *Virology 14,* 1235.

Ilyin, Yu. V., Varshavsky, A. Ya., and Georgiev, G. P. (1970), *Mol. Biol. 4,* 821.

Ilyin, Yu. V., Varshavsky, A. J., Mickelsaar, U. N., and Georgiev, G. P. (1971), *Eur. J. Biochem. 22,* 235.

Ivarie, R. D., and Pene, J. J. (1970), *J. Bacteriol. 104,* 839.

Kornberg, R. D. (1974), *Science 184,* 868.

McCarthy, B. J., Nishiura, J. T., Doenecke, D., Nasser, D. S., and Johnson, C. B. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 763.

Murphy, E. C., Jr., Hall, S. H., Shepherd, J. H., and Weiser, R. S. (1973), *Biochemistry 12,* 3843.

Olins, A. L., and Olins, D. E. (1974), *Science 183,* 330.

Rice, R. H., and Means, G. E. (1971), *J. Biol. Chem. 246,* 831.

Rickwood, D., Hell, A., and Birnie, G. D. (1973), *FEBS Lett. 33,* 221.

Sahasrabuddhe, C. G., and van Holde, K. E. (1974), *J. Biol. Chem. 249,* 152.

Schneider, I. (1972), *J. Embryol. Exp. Morphol. 27,* 353.

Varshavsky, A. J., and Georgiev, G. P. (1973), *Mol. Biol. Rep. 1,* 87.

Varshavsky, A. J., Ilyin, Yu. V., and Georgiev, G. P. (1973), *Mol. Biol. Rep. 1,* 201.

Yunis, J., and Yasmineh, W. G. (1971), *Science 174,* 1200.