Separation and Quantitation of Intracellular Forms of Poliovirus RNA by Agarose Gel Electrophoresis†

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ABSTRACT: Intracellular poliovirus-specific RNA species can be measured directly by electrophoresis of total cytoplasmic nucleic acids through 1% agarose gels, resulting in the separation of single- and double-stranded forms of poliovirus RNA from each other and from HeLa cell 28S ribosomal RNA. Single-stranded RNA molecules differing by only 15% in length are resolved in this gel system. RNA species can be visualized as fluorescent bands appearing after staining of the gels with ethidium bromide and observation under ultraviolet illumination. The total amount of RNA can be determined by densitometric quantitation of the fluorescent response. In this way, the amount of poliovirus-specific RNA within the cytoplasm of HeLa cells infected for various times has been estimated. At 170-min postinfection, there are 0.67×10^5 molecules of single-stranded poliovirus RNA per cell and at 230 min, the amount has increased to 3.7×10^5 molecules/cell. Poliovirus double-stranded RNA reaches a maximum of 0.7×10^5 molecules/cell at 330 min after infection.

The size fractionation of high-molecular-weight nucleic acids by agarose gel electrophoresis (Sharp et al., 1973) provides greater resolution than techniques employing velocity sedimentation through density gradients. In addition, the physical characteristics of low percentage agarose gels make them more useful than the corresponding acrylamide gel systems. Gels of relatively low percentage agarose (0.6–1.4%) are easily formed, stained, or sliced for analysis. Such gels have been used for the separation of DNA fragments produced by restriction endonucleases (Sharp et al., 1973) and as a preparative technique for ribosomal RNA (Weil and Hampel, 1973) and ovalbumin mRNA (Rosen et al., 1975).

We have applied this gel system to an analysis of the intracellular forms of poliovirus RNA found within infected HeLa cells. Poliovirus RNA exists in the cell as single-stranded RNA (occuring in intact virions, associated with polyribosomes and as free cytoplasmic molecules), replicative intermediate RNA, and double-stranded RNA (Baltimore, 1969). While each of these species can be purified from infected cells, it has not been possible to directly quantitate the single- and double-stranded RNA molecules in the presence of cellular ribosomal species.

Estimates of the total amounts of poliovirus-specific RNA synthesized after infection have been made from the amount of labeled precursor found in viral species (Shatkin, 1962; Zimmerman et al., 1963). We report here the use of agarose gel electrophoresis for the separation of poliovirus single- and double-stranded RNA from other cellular RNA's and for the separation of single-stranded RNA of standard poliovirus from single-stranded RNA of defective-interfering (DI)[1] particles, a deletion mutant of poliovirus. We have detected and quantitated these RNA's by the ultraviolet light-stimulated fluorescence of ethidium bromide bound to both single- and double-strand molecules.

Methods

Growth of Cells and Virus. The growth of HeLa cells in suspension culture in Joklik's modified minimal essential medium supplemented with 7% horse serum has been described previously (Baltimore et al., 1966). The production and purification of poliovirus type 1 (Mahoney) and the labeling of viral RNA's with [^{3}H]uridine was performed as described elsewhere (Baltimore et al., 1966; Cole et al., 1971). The isolation and characterization of DI particles of poliovirus has been described in detail previously (Cole et al., 1971; Cole and Baltimore, 1973a).

Preparation of RNA. Poliovirus single-stranded RNA (from CsCl-purified virions) and single-stranded RNA from DI particles were prepared by sodium dodecyl sulfate–acetic acid lysis and sucrose gradient sedimentation (Granboulan and Girard, 1969; Spector and Baltimore, 1975). The purification of poliovirus double-stranded RNA from infected cells has been described before (Spector and Baltimore, 1975).

Total nucleic acids were prepared from cytoplasm of infected or uninfected HeLa cells. Cells were lysed with 1% Nonidet P40 in 0.01 M NaCl, 0.01 M Tris, pH 7.5. After removal of nuclei and debris, cytoplasmic extracts were treated with sodium dodecyl sulfate (final concentration = 1%) and extracted with phenol in the presence of chloroform–isoamyl alcohol (Spector and Baltimore, 1975).

HeLa 28S rRNA was prepared by sedimenting total cytoplasmic nucleic acids through a linear 15–30% (w/v) sucrose gradient in 0.5% sodium dodecyl sulfate, 0.15 M NaCl, 0.1 M Tris, pH 7.4, at 64 000*g* for 14 h at 21 °C.

All RNA species were adjusted to 0.4 M sodium acetate and precipitated with 2.5 volumes of 95% ethanol at −20 °C. RNA preparations were stored as ethanol suspensions and portions for gel analysis were obtained by centrifugation in a Brinkman microcentrifuge for 10 min at 4 °C.

Quantitation of single-stranded RNA in solution was performed by measurement of the 260-nm absorbance and conversion to concentration units by employing the factor 1 A_{260}

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[1] Abbreviations used are: DI, defective interfering; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

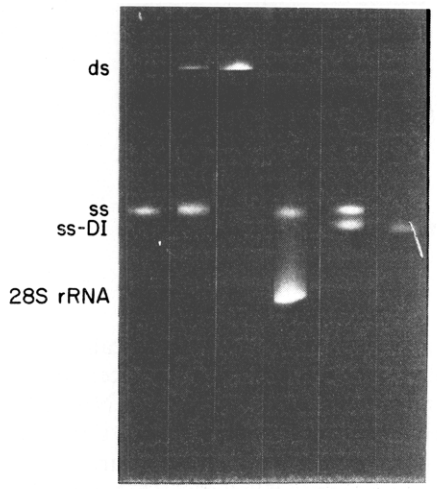

FIGURE 1: Electrophoresis of selected RNA species through 1% agarose gels. Samples of each RNA were prepared and electrophoresed as described under Materials and Methods. The gels were photographed under ultraviolet light illumination after staining with ethidium bromide. Lane A, poliovirus ss (single-stranded) RNA; lane B, poliovirus ds (double-stranded) and ss RNA; lane C, poliovirus ds RNA; lane D, poliovirus ss RNA and HeLa 28S rRNA; lane E, poliovirus ss RNA from standard virions and DI (1) particles; lane F, DI (1) particle ss RNA.

unit = 40 μg of RNA/mL. Poliovirus double-stranded RNA was quantitated by labeling viral RNA with [^{3}H]uridine and determining the specific activity of the single-stranded RNA prepared from the same cells. This technique was necessary, since we observed a variable amount of nonviral nucleic acid in some preparations of double-stranded RNA.

Agarose Gel Electrophoresis. We have used a procedure which is a modification of that described by Sharp et al. (1973). Agarose was melted in 40 mM Tris, 10 mm acetate, 1 mM EDTA, pH 7.3 (E buffer), containing 10% (v/v) glycerol. Agarose gels (1%, w/v) were cast in Plexiglas tubes (0.6-cm i.d. × 15-cm long) and were prerun in E buffer at 10 V/cm.

Ethanol precipitates of RNA species were washed with 95% ethanol and dried in vacuo and dissolved in 10 μL of 0.5% sodium dodecyl sulfate in 0.15 M NaCl, 0.1 M Tris, pH 7.5. Five microliters of 60% (w/v) sucrose containing 0.2% (w/v) bromophenol blue was added and the samples were layered onto the gels under the running buffer. Electrophoresis was carried out at 10 V/cm but not over 8 mA/gel. Under these conditions, the dye will move about 10 cm in 4 h. Slower running times (lower voltages) resulted in sharper double-stranded RNA bands but caused broadening of the single-stranded RNA bands.

Ethidium Bromide Staining and Quantitation. After electrophoresis, the gels were placed in ethidium bromide, 0.5 μg/mL in E buffer for 30 min at room temperature. The gels were rinsed with E buffer and observed under ultraviolet light.

Fluorescent bands in the gels were photographed using a Kodak 23A red filter and Kodak TXP523 film. Negative images of the gels were scanned in a Canalco Model GII microdensitometer equipped with an automatic baseline trace system for determining relative areas under peaks.

Results

Resolution of RNA Species. The resolution of RNA species by electrophoresis through gels of 1% agarose was studied using various pure poliovirus RNA species and 28S HeLa ribosomal RNA. After staining the gels with ethidium bromide, the RNAs were visible as fluorescent bands under ultraviolet light illumination (Figure 1). Poliovirus single-stranded RNA migrated as a narrow band (Figure 1, lane A) and was well separated from poliovirus double-stranded RNA (Figure 1, lane B and C). The resolving power of the gel system was demonstrated best by the separation of a mixture of single-stranded RNA's obtained from standard virions (lane A) and from poliovirus DI (1) particles (Figure 1, lane F). DI (1) is a spontaneously arising deletion mutant of poliovirus whose RNA is 15.6% smaller than that of standard poliovirus (Cole and Baltimore, 1973a,b; Hewlett and Baltimore, unpublished results). These two RNA species were well resolved by agarose gel electrophoresis (Figure 1, lane E).

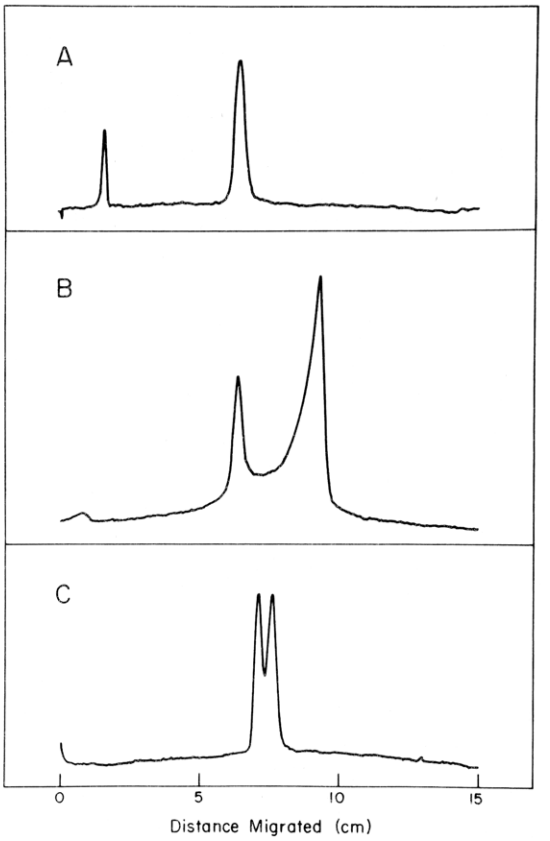

FIGURE 2: Microdensitometer tracings of RNA species electrophoresed through 1% agarose gels. Negative images of selected gels described in Figure 1 were scanned as described under Materials and Methods. Panel A, gel in Figure 1, lane B; panel B, gel in Figure 1, lane D; panel C, gel in Figure 1, lane E.

Poliovirus single-stranded RNA could also be separated from HeLa cell 28S ribosomal RNA (Figure 1, lane D). Electrophoresis of total cytoplasmic nucleic acids in 1% agarose was therefore used for the direct quantitation of poliovirus RNA species.

Microdensitometer tracings (Figure 2) of negative images of gels shown in Figure 1 (lanes B, D and E) also demonstrate the resolving power of this system. Relative areas under the peaks in tracings of these types were used to quantitate amounts of RNA in the gels.

Quantitation of Ethidium Bromide Staining. Known amounts of single- or double-stranded poliovirus RNA and of HeLa cell 28S ribosomal RNA were electrophoresed through 1% agarose gels and the fluorescence of the ethidium bromide stained bands was analyzed as described under Materials and Methods. A linear relationship between the amount of RNA applied to the gel and the intensity of the fluorescence was observed (Figure 3). Linearity extended from 0.1 to 3.0 μg of

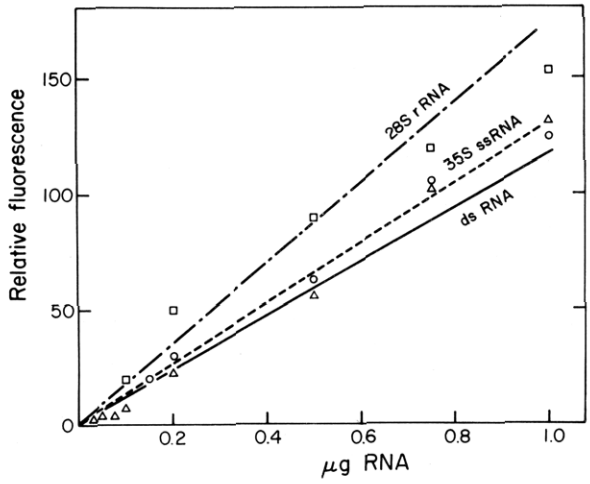

FIGURE 3: Standard curves for quantitations of RNA species by ethidium bromide fluorescence. Known amounts of each RNA were electrophoresed as described under Materials and Methods. Quantitation by microdensitometric scanning of photographic negatives was used to give a relative number for fluorescent intensity. (△) Poliovirus ds (double-stranded) RNA; (○) poliovirus 35S ss (single-stranded) RNA; (□) HeLa 28S rRNA.

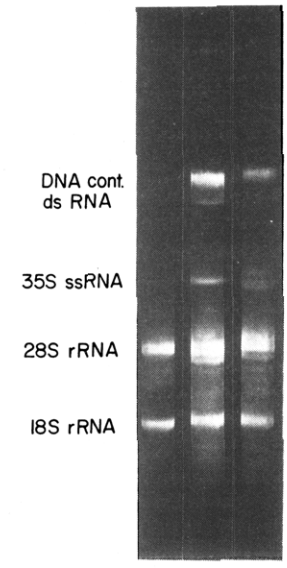

FIGURE 4: Electrophoresis of HeLa cell cytoplasmic nucleic acids in 1% agarose gels. Samples of cytoplasmic nucleic acids were analyzed as described under Materials and Methods. Lane A, uninfected cells; lane B, poliovirus-infected cells; lane C, cells infected with a mixture of standard poliovirus and DI (1) particles (ss = single-stranded, ds = double-stranded).

RNA. The slopes of the curves for single- and double-stranded RNA were found to be similar. A curve of single-stranded poliovirus RNA electrophoresed in the presence of HeLa 28S ribosomal RNA gave the same slope as single-stranded viral RNA alone. Although the response for double-stranded RNA is not greater than single-stranded RNA, lower amounts of double-stranded RNA can be detected due to the fact that double-stranded RNA forms a narrower band upon electrophoresis which is more easily quantitated by microdensitometry. Thus, the lower limit of detectability for poliovirus RNA species was 0.1 μg for a single-stranded and 0.03 μg for double-stranded.

Quantitation of Intracellular Poliovirus RNA. Cytoplasmic nucleic acids from HeLa cells were electrophoresed through 1% agarose gels. Figure 4 shows the patterns obtained for uninfected cells (lane A), cells infected with standard poliovirus (lane B) and cells infected with a mixture of standard poliovirus and DI (1) particles (lane C). The uppermost band is deoxyribonuclease sensitive and contains molecules of heterogeneous size (data not shown). This probably represents contaminating DNA from the nucleus. This region may also contain poliovirus replicative intermediate RNA (Flanegan and Baltimore, unpublished data). The second band from the top in lane B is poliovirus double-stranded RNA, and the corresponding doublet in lane C is due to the mixture of standard and DI (1) molecules. The third band in lane B contains poliovirus single-stranded RNA, and in lane C a doublet which resolves the standard and DI (1) RNAs. The next two major bands are HeLa 28S and 18S ribosomal RNA, respectively. The minor bands visible between the major ribosomal species are not reproducible and may be artifacts. HeLa 4S RNA migrates as a broad band with the dye front and has been electrophoresed out of the gels in Figure 4.

HeLa cells were infected with poliovirus at a multiplicity of infection of 20 plaque-forming units per cell. RNA synthesis was followed by incorporation of [^{3}H]uridine into Cl_3-AcOH-insoluble material (Figure 5). At various times during the linear phase of RNA synthesis and at a time late in infection, portions of the culture were taken for preparation of total cytoplasmic nucleic acids. Samples were electrophoresed

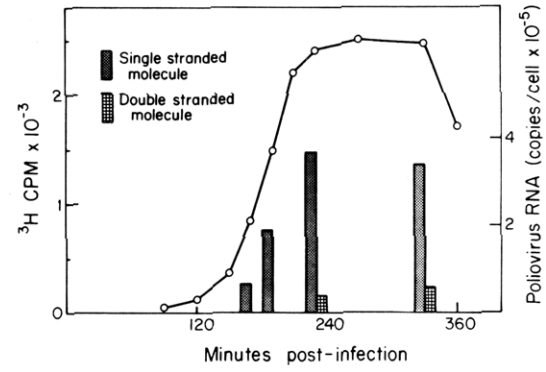

FIGURE 5: Time course of synthesis of poliovirus RNA in HeLa cells. HeLa cells were infected with poliovirus at 37 °C as described under Materials and Methods and treated with actinomycin D (5 μg/mL) and samples were taken at various times for analysis of poliovirus-specific RNA. (○) Incorporation of [^{3}H]uridine into Cl_3AcOH-insoluble material. The bars represent calculated molecules/cell at each indicated time, as described in Table I.

through 1% agarose gels and the quantity of poliovirus double- and single-stranded RNA and the amount of HeLa 28S ribosomal RNA was determined using the appropriate standard curves. Table I lists the amounts of each RNA found in each analysis and the ratio of single- or double-stranded RNA to 28S ribosomal RNA. These later data were used to calculate the number of molecules of poliovirus RNA per cell at each time point (Table I; Figure 5). This calculation assumes a molecular weight for 28S ribosomal RNA OF [/76 × []^6 (Wellauer and Dawid, 1973), 5 × 10^6 28S molecules/cell (Weinberg and Penman, 1968), and 2.5 × 10^6 for the single-strand molecular weight of poliovirus (Granboulan and Girard, 1969). The amount of poliovirus single-stranded RNA rises from 0.6 × 10^5 molecules/cell at 170 min to a peak of 3.73 × 10^5 molecules/cell at 230 min after infection. Poliovirus double-stranded RNA was not detectable prior to 230 min after infection. At this time we found 0.4 × 10^5 molecules/cell and at 330 min the value had risen to 0.7 × 10^5 molecules/cell.

TABLE I: Quantitation of Poliovirus RNA in HeLa Cell Cytoplasm at Various Times after Infection.[a]

Postinfection (min)	RNA (μg) 28S rRNA	RNA (μg) Poliovirus ss RNA	RNA (μg) Poliovirus ds RNA	μg of ss RNA/μg of 28S RNA	μg of ds RNA/μg of 28S RNA	Poliovirus RNA ((molecules/cell) × 10^{-5}) ss	Poliovirus RNA ((molecules/cell) × 10^{-5}) ds
170	1.32	0.025	ND	0.019		0.67	
190	0.93	0.050	ND	0.054		1.90	
230	1.32	0.140	0.030	0.106	0.023	3.73	0.40
330	1.14	0.110	0.045	0.097	0.040	3.41	0.70

[a] Abbreviations used: ss, single-stranded; ds, double-stranded; ND, not detected. The number of molecules and RNA per cell was calculated as follows: Poliovirus RNA molecules/cell = (μg of poliovirus RNA/μg of 28S rRNA)(mol wt of 28S rRNA/mol wt of poliovirus RNA)(28S rRNA molecules/cell). Assuming that the molecular weight of poliovirus ss RNA = 2.5×10^6 (Granboulan and Girard, 1969); the molecular weight of poliovirus ds RNA = 5.0×10^6 ($2 \times$ ss mol wt); the molecular weight of HeLa 28S rRNA = 1.76×10^6 (Wellauer and Dawid, 1973); the molecular weight of HeLa 28S rRNA = 5×10^6 molecules/cell (Weinberg and Penman 1960).

The lower limit of detectability in our hands was set by the necessity to keep the amount of 28S ribosomal RNA in the gel within the linear range of measurement. Therefore, we could have detected less than 10^4 molecules/cell at the two earliest time points.

Discussion

Electrophoresis of high-molecular-weight RNA molecules through 1% agarose gels allows the separation of poliovirus-specific RNAs from cellular RNAs. Relatively small differences in size can be detected with this system: poliovirus single-stranded RNAs purified from standard virions and from DI (1) particles (RNAs that differ by approximately 15%) can be resolved by the system. The different RNA species can be quantitated in the gels.

The separation shown (Figure 1, lane E, and Figure 2, panel C) is sufficient to allow measurement of the relative amounts of standard and DI RNA in some cases. However, electrophoresis of [^{3}H]- or [^{14}C]uridine-labeled RNA's and measurement of radioactive material in gel slices gave more consistent results (Hewlett and Baltimore, unpublished data). Agarose gel electrophoresis has also been used for the separation of RNA species from murine sarcoma viruses (Olshevsky and Baltimore, unpublished results).

The staining of single-stranded RNA with ethidium bromide can be used as a quantitative measure of the amount of RNA present in a gel. Ethidium bromide has been previously used in a fluorometric assay for the synthesis of reovirus single-stranded RNA (Kapuler, 1971), but the nature of the binding of ethidium bromide to single-stranded RNA is not clear.

The quantitation of intracellular poliovirus single- and double-stranded RNA by this method agrees well with previous estimates. Total viral single-stranded RNA synthesized after infection had been previously estimated to be about 2×10^5 molecules/cell (Baltimore, 1969). We found a maximum at 230-min postinfection of 3.73×10^5 molecules/cell. The decrease in single-stranded RNA by 330 min is probably due to degradation of RNA not incorporated into mature virions. Total double-stranded RNA late in infection was reported to be about 20% of the total RNA synthesized (Baltimore and Girard, 1966). We find 7×10^4 molecules/cell or about 20% of the total single-stranded RNA present at 330 min after infection.

The steady-state level of poly(A)-containing mRNA in the HeLa cell cytoplasm has been estimated between 5 (Singer and Penman, 1973) and 2% (Lodish, 1971) of the total ribosomal RNA. If we assume that the average cell mRNA molecule is 1200 nucleotides long, we can calculate the molar ratio of poliovirus mRNA to cell mRNA at 170 min postinfection as 0.04–0.1.

Within the first 3 h after infection, poliovirus inhibits host cell translation such that less than 5% of the total cell mRNA can be found in polyribosomes, even though these molecules remain intact (Willems and Penman, 1966; Koschel, 1974; Fernandez-Muñoz and Darnell, 1976). Our results indicate that this inhibition is not due to the synthesis of a large pool of poliovirus mRNA during the early stages of infection, in agreement with the observed kinetics of the decline of host protein synthesis. Therefore, the mechanism of protein synthesis inhibition may involve either a very large difference in initiation rates between poliovirus and cellular mRNA's or the action of a translational control factor produced by the virus.

These data also confirm another previous indirect estimate. Cole and Baltimore (1973b) suggested that if cells are infected with an equal mixture of standard virions and DI particles, the intracellular pool of RNA would have approximately equal amounts of the two RNAs. As seen in Figure 4, lane C, when the amounts of DI and standard RNA are directly visualized, the equal production of the two RNA's is evident. Equal amounts of the two double-stranded RNA's can also be seen.

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Serological Analysis of the H3–H4 Histone Complex†

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ABSTRACT: The H3–H4 histone complex has been prepared by mild methods of salt extraction from calf thymus chromatin. Its composition was characterized electrophoretically and its size corresponded to that of a tetramer. It was studied serologically with antibodies to both acid-extracted histones and to the complex itself. In quantitative complement fixation reactions, antibodies to the H3–H4 histone complex reacted more strongly with the complex than with either acid-extracted H3 or H4 alone, or a 1:1 mixture of the two histones. Antibodies to isolated H3 or H4 reacted at least as effectively with the complex as with the individual histones; however, in both cases, 1:1 mixtures of the H3 and H4 were less reactive than either the homologous histone alone or the salt-extracted preparation. The results seen with all three antibody systems indicated that reactions of the salt-extracted complex were not simply the sum of reactions of the H3 and H4 components. This suggested that unique structural features are present in the tetramer. Antibodies to the H3–H4 complex or to acid-extracted H3 or H4 did not give complement fixation reactions with chromatin, but differed in reactivity with chromatin as measured by an absorption assay. Whereas antibodies induced by the H3–H4 complex were readily absorbed by insoluble chromatin, those induced by acid-extracted H3 or H4 were not. Both the electrophoretic and serological properties of the H3–H4 preparation remained stable during storage in 50 mM sodium acetate, pH 5, at −20 °C for 1 year and after repeated freezing and thawing.

In recent years, a great deal of attention has been focused on the role of histone–histone interactions in maintaining the structure of chromatin. Chromatin fragments prepared by nuclease digestion (Hewish and Burgoyne, 1973; Noll, 1974; Sahasrabuddhe and Van Holde, 1974) have been seen by electron microscopy to consist of a series of uniform beadlike structures (Olins and Olins, 1974). Kornberg (1974) proposed that these beads are the primary structural subunits of chromatin and are composed of an octamer of two each of histones H2A, H2B, H3, and H4, around which the DNA of chromatin is wrapped. This model further proposed that the structure consists of a unique $(H3)_2(H4)_2$ tetramer and a linear H2A–H2B oligomer (Kornberg, 1974).

The association of H3 and H4 in a tetramer or dimer–tetramer equilibrium has been characterized further (Roark et al., 1974; D'Anna and Isenberg, 1974; Sperling and Bustin, 1975; Thomas and Kornberg, 1975; Lewis, 1976a) and found to be the most strongly associating of several possible histone–histone interactions that can occur in solution (D'Anna and Isenberg, 1974; Sperling and Bustin, 1975). It provides the minimal requirement for association with DNA in a way that reconstitutes some aspects of native chromatin structure (Camerini-Otero et al., 1976). Further, the association prevents self-aggregation of the individual histone components (Rubin and Moudrianakis, 1975; Sperling and Bustin, 1975). This suggests there may be a unique structure for the H3–H4 complex and raises the question of whether the complex, as isolated from chromatin under mild conditions, has the same structure as the complex that is reconstituted from individual acid-extracted components. In this article, we report serological studies of this question. Immunizing rabbits with H3–H4 histone–RNA complexes (Stollar and Ward, 1970), we obtained antibodies to the H3–H4 complex, as did Mihalakis et al. (1976). With these antisera and antisera to the isolated H3 and H4, we have found that the H3–H4 complex isolated by the mild procedures of van der Westhuyzen and von Holt (1971) differs from a mixture of the two acid-extracted histones.

Materials and Methods

Histone Isolation. Chromatin was prepared from fresh calf thymus (50 g) by the method of Busch (1968), and histones were then extracted by a modification of the procedure of van der Westhuyzen and von Holt (1971). All procedures were carried out at 4 °C. Briefly, histones were extracted from chromatin with 2.0 M NaCl–50 mM sodium acetate–50 mM sodium bisulfite. DNA was precipitated by the addition of protamine sulfate (Sigma, grade I, recrystallized two times from H_2O). The resulting supernatant, containing histones and excess protamine, was applied to a Sephadex G-50 column (8.5 × 100 cm) and eluted with 50 mM sodium acetate–50 mM

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