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Inhibition of Calf Thymus Deoxyribonucleic Acid Polymerase by Histones*

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ABSTRACT: The previously reported inhibition of deoxyribonucleic acid (DNA) synthesis by histones cannot be attributed solely to precipitation of primer DNA as a nucleohistone complex since present data demonstrate that an even greater inhibition occurs with the calf thymus DNA polymerase system under conditions (low Mg^{2+} concentration and low ionic strength) in which the complex of histones and heat-denatured DNA is soluble. Under both conditions the F1 fraction of histones is the most effective and the F3 fraction is the least effective inhibitor of DNA synthesis. Sonication of the insoluble nucleohistone complex (under conditions of higher Mg^{2+} concentration and ionic strength) disperses the gel but does not relieve the inhibition. At very low histone:DNA ratios there is a small "stimulation" of incorporation of [^{3}H]deoxyadenosine triphosphate into DNA in comparison with histone-free controls. There appears to be no specificity of the inhibition with respect to DNA bases since the incorporation of [^{3}H]deoxycytidine triphosphate by unfractionated histones (or fractions F1 and F3) is inhibited to the same extent as the incorporation of [^{3}H]thymidine triphosphate. At a Mg^{2+} concentration of 3.75 mM, the optimum concentration of potassium phosphate buffer is 30–40 mM for the DNA polymerase reaction with or without histones. At low concentrations (5 mM) of phosphate buffer (pH 7.4) the optimum concentration of Mg^{2+} is 3 mM for the DNA polymerase reaction in the absence of histones and 1–1.5 mM in the presence of histone fraction F1, with progressively increasing inhibition as the concentration is raised from 1.5 to 6 mM.

These data suggest the possibility of a cooperative role of histones and Mg^{2+} in one aspect of the control of DNA synthesis.

Studies in this and other laboratories (Bazill and Philpot, 1963; Gurley *et al.*, 1964; Billen and Hnilica, 1964; Schwimmer, 1965; Schwimmer and Bonner, 1965) have shown that histones inhibit various DNA polymerase systems *in vitro*, but the role of histones in the regulation of DNA replication *in vivo* is uncertain. Although the DNA polymerase of *Escherichia coli* provides a useful model system for investigation of effects of histones (Billen and Hnilica, 1964; Schwimmer, 1965; Schwimmer and Bonner, 1965), it is difficult to extrapolate these data and interpretations with confidence to the synthesis of DNA in animal systems in view of considerable differences, such as requirements for DNA primer, between the two enzyme systems. This problem is not entirely resolved by the use of DNA polymerases isolated from animal sources since several of these enzyme systems have been described with differing properties and primer requirements, and it is still uncertain whether these enzymes are primarily concerned with DNA replication or with DNA repair. A purified DNA polymerase from calf thymus (Bollum, 1960; Yoneda and Bollum, 1965) has an absolute requirement for single-stranded or de-

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TABLE I: Amino Acid Analyses of Various Fractions of Calf Thymus Histones.

Amino Acid	Moles/100 Moles of Total Amino Acids				
	Unfractionated	F1	F2a	F2b	F3
Lysine	15.1	27.5	10.0	13.8	9.4
Histidine	1.7	0.36	1.9	2.4	1.6
Arginine	8.3	1.9	11.6	7.8	12.8
Aspartic acid	5.1	3.1	5.0	5.6	4.4
Threonine	6.0	5.2	6.6	6.1	7.4
Serine	6.2	6.7	4.5	7.7	4.2
Glutamic acid	8.7	5.1	8.7	8.7	9.8
Proline	6.2	8.7	3.2	4.7	4.0
Glycine	8.7	6.8	10.4	7.1	8.8
Alanine	13.9	23.9	11.2	11.4	11.8
Cystine					Trace
Valine	4.7	4.2	6.7	6.7	6.0
Methionine	0.6	0.27	1.1	0.84	1.3
Isoleucine	3.3	0.97	5.4	4.2	5.6
Leucine	7.4	4.0	8.8	7.8	8.8
Tyrosine	2.4	0.55	2.8	3.4	2.7
Phenylalanine	1.8	0.84	2.2	2.0	2.4

natured DNA primers while a purified enzyme from regenerating rat liver (Mantsavinos and Munson, 1966) preferentially utilizes native, double-stranded DNA as primer. It is desirable to study the effects of histones on both of these DNA polymerase systems from animal sources, and the present paper is concerned with such a study of the DNA polymerase[1] of calf thymus for subsequent comparison with the enzyme system of regenerating rat liver.

Materials and Experimental Methods

Unfractionated histones were prepared from calf thymus by the method previously described by McAllister *et al.* (1963). Histone fractions F1, F2a, F2b, and F3 were isolated from whole calf thymus tissue by method I of Johns (1964) and were stored in the dry, solid state at −10°. They were analyzed for amino acid composition by the method of Spackman *et al.* (1958) with a Beckman (Spinco) amino acid analyzer, Model 120B. The analyses recorded in Table I are in fair agreement with the values of Johns (1964). The various fractions were found to be heterogeneous by electrophoresis on polyacrylamide gels by the method of McAllister *et al.* (1963) with the addition of 6 M urea. The histone solutions were analyzed for protein concentration by the method of Lowry *et al.* (1951).

Calf thymus DNA was obtained from Gallard–Schlesinger Chemical Manufacturing Corp. and from Worthington Biochemical Corp. Viscosity of DNA solutions was determined at 36.7° in 0.15 M NaCl–0.015 M sodium citrate with a four-bulb viscometer (Cannon–Ubbelhode) and corrected to zero shear. The intrinsic viscosity $[\eta]$ was found to be 57.5 and 44 dl/g for the Gallard–Schlesinger and Worthington Biochemical DNA, respectively. The sedimentation coefficients were determined with a Spinco Model E ultracentrifuge, equipped for ultraviolet absorption measurements, with a 30-mm standard cell at a DNA concentration of 14 μg/ml in 0.15 M NaCl–0.015 M sodium citrate. Photographs were made at 4-min intervals and analyzed with a Densicord recording densitometer according to the procedure of Schumaker and Schachman (1957). The sedimentation coefficients were corrected to water at 20° and found to be 15.2 and 9.7 S for the Gallard–Schlesinger and Worthington Biochemical DNA, respectively.[2] Cesium chloride density gradient centrifugation of the DNA samples was performed in a Spinco Model L ultracentrifuge with a SW 39.1 rotor at 5° for 40 hr followed by collection of fractions from the bottom of the tubes (Buchler Instrument Co. tube sampler) for absorbancy determinations at 260 mμ. Both samples of DNA yielded

[1] Deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase (EC 2.7.7.7).

[2] By eq 2 of Eigner *et al.* (1962) the data for $s_{20,w}$ and $[\eta]$ yielded values for $s^0_{20,w}$ of 16.2 and 10.2 S for Gallard–Schlesinger and Worthington Biochemical DNA, respectively. From these values of $s^0_{20,w}$ eq 3 of Eigner and Doty (1965) gave molecular weights of 4.1 and 1×10^6 for Gallard–Schlesinger and Worthington Biochemical DNA, respectively, whereas the corresponding values calculated from $[\eta]$ were 11×10^6 for Gallard–Schlesinger DNA (eq 4′ of Eigner and Doty, 1965) and 3.4×10^6 for Worthington Biochemical DNA (eq 4 of Eigner and Doty, 1965). The discrepancies between estimates of the molecular weights of the DNA samples by the two methods may be attributed to polydispersity of the samples (Eigner and Doty, 1965). However, this polydispersity was not considered to be of serious consequence for the present study. A discrepancy in the opposite direction would have been more serious as indicative of partial denaturation of DNA.

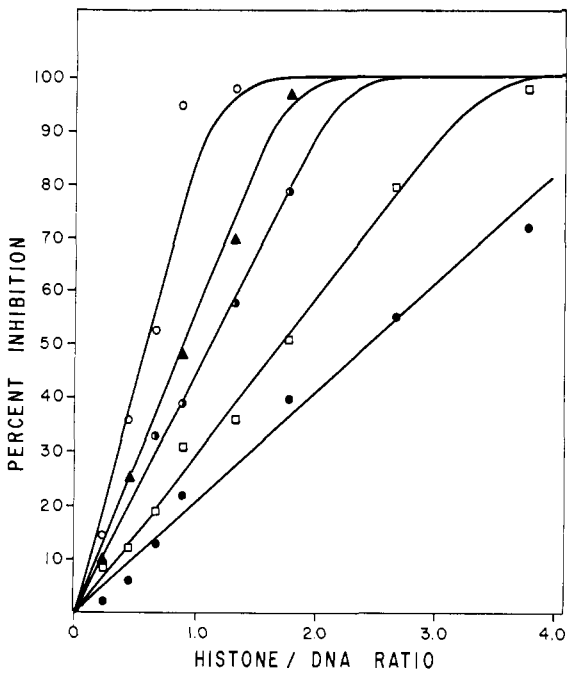

FIGURE 1: Effects of various histone fractions upon DNA synthesis under conditions (6.6 mM Mg^{2+} and 40 mM phosphate buffer) in which a portion of the nucleohistone precipitated as an insoluble complex. The final reaction mixture had the following composition: [^{3}H]dATP (1.4 × 10^6 cpm/μmole), dGTP, dCTP, and TTP, 100 μM each; 6.6 mM Mg^{2+}; 1 mM β-mercaptoethanol; 40 mM potassium phosphate, pH 7.4; heat-denatured DNA, 32.2 μg; calf thymus DNA polymerase (300 μg of protein); final volume 0.5 ml; 15 min at 37°. (○) Histone F1, (◑) histone F2a, (□) histone F2b, (●) histone F3, and (▲) unfractionated histones.

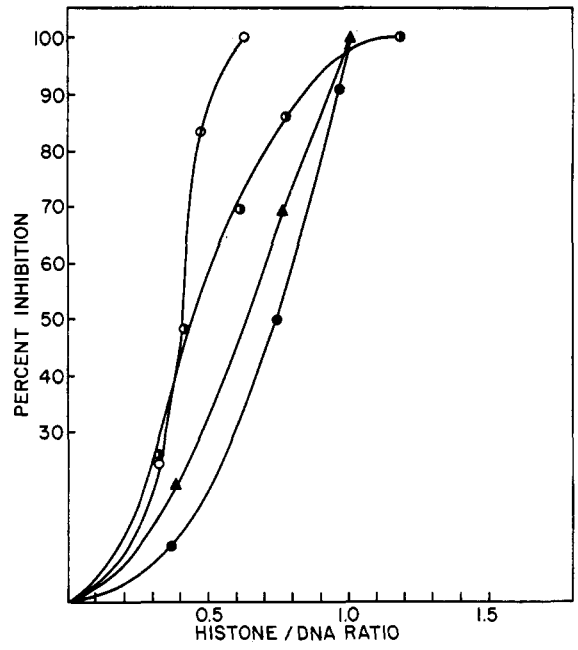

FIGURE 2: Effects of various histone fractions upon DNA synthesis under conditions (3.75 mM Mg^{2+} and 5 mM phosphate buffer) in which the reconstituted nucleohistone remained in solution. Each reaction mixture contained: [^{3}H]dATP (4.5 × 10^5 cpm/μmole), dGTP, dCTP, and TTP, 100 μM each; 3.75 μM Mg^{2+}; 1 mM β-mercaptoethanol; 5 mM potassium phosphate, pH 7.3; heat-denatured DNA, 26.8 μg; calf thymus DNA polymerase (300 μg of protein); final volume 0.4 ml; 20 min at 37°. (○) Histone F1, (◑) histone F2a, (●) histone F3, and (▲) unfractionated histones.

symmetrical bands with no evidence of denaturation of the DNA. Heat denaturation of the Gallard–Schlesinger DNA yielded a hyperchromicity of 39–40% at 260 mμ which approaches the maximal optical density change (Mahler *et al.*, 1964). The hyperchromicity observed for alkaline denaturation was about 29% which approaches the maximum for this procedure (Hotchkiss, 1957). The two samples of DNA gave essentially the same results when used in the experiments described in this paper.

Heat-denatured DNA for use as primer for the DNA polymerase reactions was prepared by heating the solutions of native DNA (2 mg/ml in phosphate buffer) at 90° for 10 min followed by rapid cooling in an ice bath. The sedimentation coefficient ($s_{20,w}$) of heat-denatured DNA (Worthington Biochemical) was found to be 14.7 S. DNA concentrations were determined by the diphenylamine method (Burton, 1956) and by direct determination of spectrophotometric absorbancy at 260 mμ for native DNA and at 268 mμ for DNA heated at 90° for 20 min in 0.5 N perchloric acid.

Deoxyribonucleoside triphosphates were obtained from Calbiochem, Inc. [^{3}H]dATP, [^{3}H]dCTP, and [^{3}H]-TTP (each 1–2 Ci/mmole) were obtained from Schwarz BioResearch, Inc., and were diluted with unlabeled deoxyribonucleoside triphosphates to the specific activities stated in the legends to the figures.

Calf thymus DNA polymerase was prepared by the method of Bollum (1960), and fraction D was used in all experiments after assay for DNase and for terminal nucleotidyl transferase activity. For the DNase assay 0.1 ml of enzyme solution (728 μg of protein) was incubated for 30 min at 37° with ^{14}C-labeled DNA (isolated by the procedure of Marmur (1963) from *E. coli* grown in medium containing [2-^{14}C]thymidine), 18,138 cpm/assay, in 0.024 M potassium phosphate buffer (pH 7.3) containing 6.6 mM $MgCl_2$ in 1-ml total volume. No acid-soluble radioactivity was detectable, which indicates the absence of DNase activity under the conditions of this assay.

The DNA polymerase preparations also were tested for the presence of terminal nucleotidyl transferase activity (Yoneda and Bollum, 1965; Gottesman and Canellakis, 1966) by incubating 0.1 ml of enzyme solution (300 μg of protein) for 30 min at 37° with [^{3}H]dATP under the conditions of Figure 1 except for omission of dGTP, dCTP, and TTP. The amount of radioactivity incorporated into acid-insoluble product under these conditions was less than 5% of that incorporated in the presence of the four deoxyribonucleoside triphosphates.

DNA Polymerase Assay. Two different reaction conditions were used. One, which yielded precipitates of nucleohistone, was prepared according to Bollum (1960) and is described in the legend to Figure 1. The other, based on conditions used by Bonner and Huang (1966) in a study of RNA polymerase, is described in the legend to Figure 2 and is characterized by a lower concentration of Mg^{2+} and of phosphate buffer such that the nucleohistone complexes were completely soluble. The re-

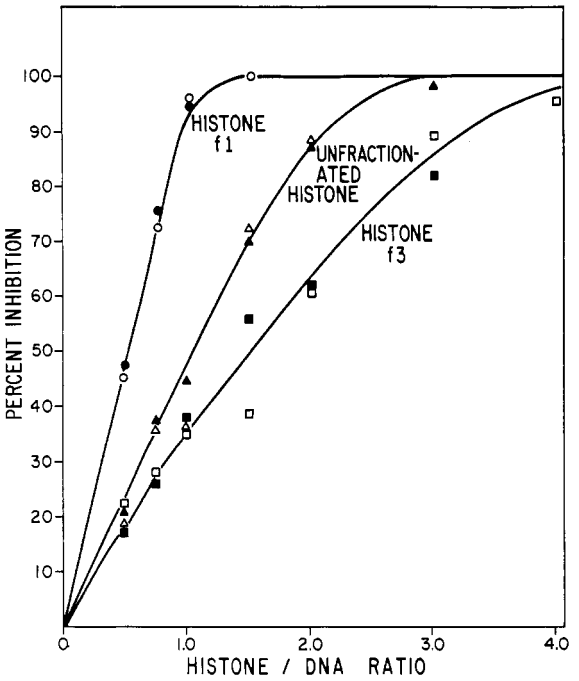

FIGURE 3: Inhibition by various histones of incorporation of [^{3}H]dCTP (open symbols) and [^{3}H]TTP (solid symbols) into DNA by calf thymus DNA polymerase primed with heat-denatured (90°, 10 min) DNA. The reaction conditions were similar to those of Figure 1.

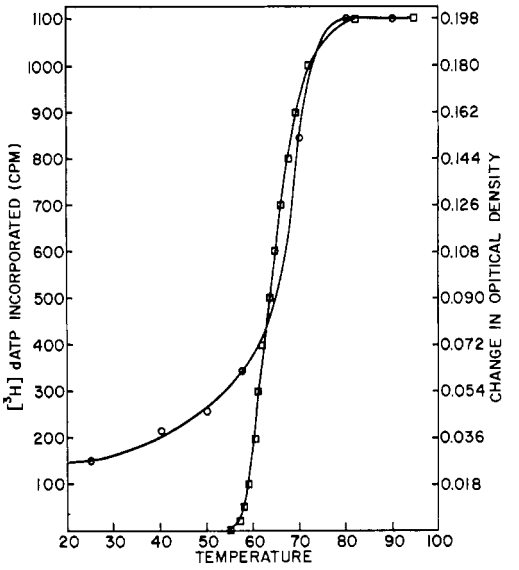

FIGURE 4: Melting profile of calf thymus DNA (obtained from Worthington Biochemical Corp.) determined spectrophotometrically (□—□) in 5 mM potassium phosphate buffer (pH 7.3), T_m = 64.2°, and determined enzymatically (○—○) by use of heated DNA (10 min at the indicated temperature, followed by rapid cooling) as primer for the DNA polymerase reaction with [^{3}H]dATP by the method described in the legend to Figure 1. (T_m = 67° by the enzymatic method.)

action mixtures were prepared and incubated in small (10 × 75 mm) test tubes.

For the experiments with an insoluble nucleohistone complex the addition of the components was as follows. The DNA and histone solutions were introduced on opposite sides of the tube, then the buffer solution containing the deoxyribonucleoside triphosphates and Mg^{2+} was added, and the contents of the tube were mixed before addition of 0.1 ml of the enzyme (300 μg of protein). Incubation was for 15 min at 38°.

The reaction solution for the soluble nucleohistone complex was prepared as follows. The DNA solution was added, then the buffered solution containing the four deoxynucleoside triphosphates. The histones were added and the solution was mixed before adding Mg^{2+}. Enzyme (300 μg) was added, and the solution was again mixed before incubation at 37° for 20 min.

The incorporation of [^{3}H]dATP into DNA was measured by pipetting 0.1 ml of the reaction solution onto Whatman No. 1 paper strips (1 × 3 in.) and precipitating the DNA into the strips by dropping the latter into 5% trichloroacetic acid. When necessary, 1 N NaOH was added to the reaction solution before application to strips to solubilize any precipitate which had formed. The dilution factor was taken into account when reporting the counts per minute incorporated.

The strips were washed twice with 5% trichloroacetic acid and twice with 95% alcohol, and after drying at room temperature they were immersed as cylinders in scintillation vials containing 16 ml of a solution of 5 g of 2,5-diphenyloxazole and 0.8 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per liter of toluene for counting in a scintillation spectrometer (Packard Instrument Co., Model 3003). The counting efficiency was approximately 5%.

It should be pointed out that the inhibitory effects of histones were determined under conditions in which the concentration of DNA primer in the system was less than that required to give maximum velocity of incorporation of radioactive deoxyribonucleotides into DNA. In all tables and figures the histone/DNA ratios are by weight. In Figures 1–3 the inhibitions by histones are expressed as "per cent inhibition" = $100(c - a)/c$, where a and c are the counts per minute of labeled deoxyribonucleotide incorporated into acid-insoluble material (DNA) in the presence and absence of histones, respectively.

The melting profile of the primer DNA (Gallard–Schlesinger) (Figure 4) was obtained by means of a Gilford multiple-sample recording spectrophotometer, Model 2000, with a thermostated cuvet compartment which was programmed for the temperature interval of 25–100° to increase at the rate of 0.8–1.0°/min.

In certain experiments (Table II) reconstituted nucleohistones and DNA were sonicated with a Branson Sonifier, Model S-100 with the microtip. An output-power setting of 1.0 was used, and the instrument was tuned for maximum output. The nucleohistone and DNA solutions were contained in ice-cooled test tubes during sonication.

In preparation for the experiments involving variation in Mg^{2+} concentration (Figure 5), the DNA and histone solutions in 5 mM phosphate buffer (pH 7.4) were dialyzed at 4° for 20 hr against 5 mM phosphate–

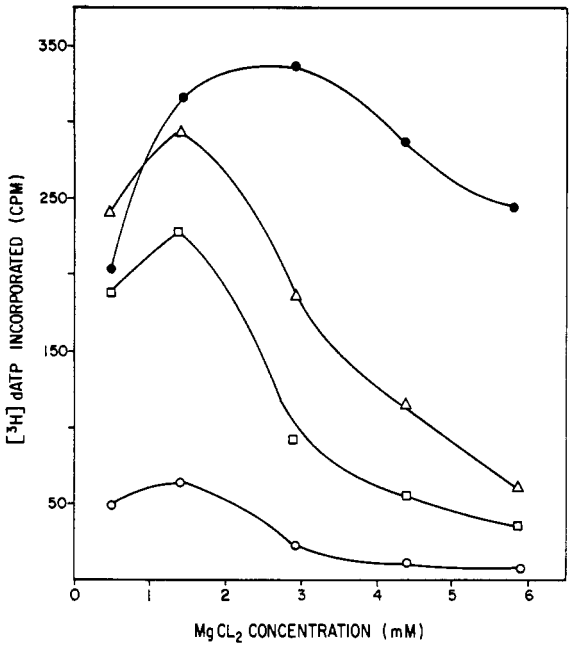

FIGURE 5: Effects of variation in Mg^{2+} concentration upon incorporation of [^{3}H]dATP into DNA in the presence and absence of histone fraction F1. The reaction conditions were identical with those of Figure 8 except that the potassium phosphate (pH 7.4) concentration was kept constant at 5 mM and the $MgCl_2$ concentration was varied. The symbols have the significance of those in Figure 8.

0.1 mM EDTA to remove traces of Mg^{2+} and then for 20 hr against 5 mM phosphate buffer to remove EDTA prior to use in these experiments.

TABLE II: Effect of Sonication upon the Priming Activity of Calf Thymus DNA and of Reconstituted Nucleohistones in the Calf Thymus DNA Polymerase System.[a]

Type of DNA	Duration of Sonication (sec)	cpm Incorp into DNA
Native DNA	0	31
	20	191
	40	232
Native DNA	20	749
Sonicated, then heated at 90° for 10 min	40	677
Native DNA + histones (histone/DNA ratio = 1.26)	0	20
	20	48
Heat-denatured DNA (90° for 10 min)	0	446
	20	468
Heat-denatured DNA (as above) + histones (histone/DNA ratio 1.25)	0	53
	20	54

[a] Unfractionated calf thymus histones were used in these experiments in which the sonication and the polymerase assays were as described in Methods and in the legend of Figure 1, respectively, with [^{3}H]dATP as labeled precursor.

Results and Discussion

The data of Figure 4 show fair agreement between the T_m of calf thymus DNA determined by a spectrophotometric and an enzymatic method, and these data confirm the conclusion of Bollum (1960) that the DNA polymerase of calf thymus has a requirement for single-stranded DNA as primer.

By comparison of the data of Figure 2 with those of Figure 1 we conclude that precipitation of primer DNA (heat denatured) by histones is not a necessary requirement for the inhibition of DNA synthesis by histones; in fact, the inhibition occurs at lower histone/DNA ratios under the conditions of low Mg^{2+} and phosphate buffer concentration (Figure 2) which yield soluble nucleohistone complexes. Under both conditions histone fraction F1 is the strongest and fraction F3 the weakest inhibitor of DNA synthesis if one defines the weakest inhibitor as that which requires the highest histone/DNA ratio to attain 50% inhibition. There is a smaller difference between the histone/DNA ratios for 50% inhibition by the various histone fractions under the conditions which yield soluble nucleohistone complexes (Figure 2) than under the conditions which yield precipitates of nucleohistones (Figure 1). This can be explained upon the basis that precipitation of DNA primer by histones under the conditions of Figure 1 augments the inhibition which results from "binding" of DNA by histones in the absence of precipitation, and the various histone fractions differ considerably in the histone/DNA ratio at which precipitation of nucleohistone occurs under the conditions of Figure 1. Thus, the histone/DNA ratios at which precipitation of nucleohistone commences under the conditions of Figure 1 are approximately as follows: F1, 0.1; F2a, 0.25; F2b, 0.25; and F3, 0.55. The ratios for precipitation of 50% of the primer DNA are: F1, 0.8; F2a, 1.6; F2b, 1.4; and F3, 3.2.

Sonnenberg and Zubay (1965) concluded that the inhibition of DNA-dependent RNA polymerase by histones can be attributed to the formation of an impenetrable, gelatinous nucleohistone complex which prevents access of enzyme to the DNA primer. They reported that sonication of the nucleohistone resulted in breakdown of the gel structure and a marked increase in priming activity of the complex. It seemed desirable to determine whether the priming ability of insoluble nucleohistone complexes (formed under the conditions of Figure 1) for the DNA polymerase reaction could be increased by sonication. These and related experiments are recorded in Table II. The data in this table indicate that the priming ability of native DNA for the calf thymus DNA polymerase is considerably increased by sonication for 20 or 40 sec, but there is a further increase in priming activity if the sonicated DNA is heated at 90°

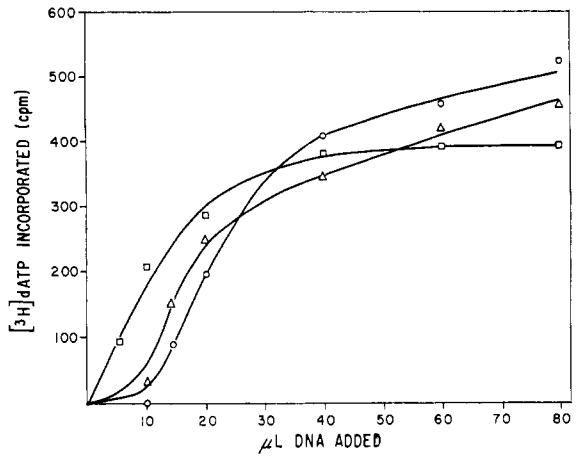

FIGURE 6: Effect of histone fraction F1 on incorporation of [^{3}H]dATP into DNA in 6.6 mM Mg^{2+} and 40 mM potassium phosphate (pH 7.4) under reaction conditions similar to those in legend to Figure 1. In a particular series the quantity of histones was kept constant ((□) histone-free controls, (△) 12.7 μg of histone F1, and (○) 21.2 μg of histone F1), and the amount of heat-denatured DNA was varied. The added DNA solution had a concentration of 1.37 μg/μl. Each point is an average of three determinations.

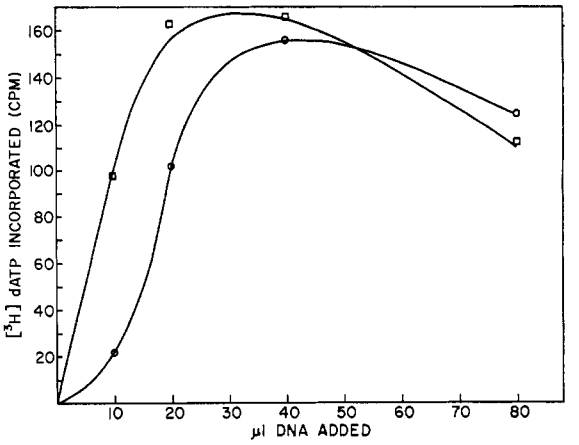

FIGURE 7: Effect of histone fraction F1 on incorporation of [^{3}H]dATP into DNA in 5 mM phosphate and 3.75 mM Mg^{2+} under conditions similar to those in the legend to Figure 2. In a particular series the quantity of histone was kept constant ((□) histone-free control and (○) 6.21 μg of histone F1), and the amount of heat-denatured DNA was varied. The added DNA solution had a concentration of 1.34 μg of heat-denatured DNA/μl. Each point is an average of three determinations.

for 10 min. These results are consistent with the conclusion of Doty *et al.* (1958) that sonication of double-stranded DNA results primarily in double-chain scission, but the moderate increase in priming activity by sonication alone does suggest that some conversion to the single-stranded form may occur during sonication.

Native DNA in a complex with unfractionated histones has low activity as primer, and there is only a slight increase in activity when the complex is sonicated for 20 sec (Table II) despite the fact that the gelatinous complex becomes finely dispersed during sonication. These results suggest that the histones may stabilize the double-stranded DNA against the partial conversion to the single-stranded form which seems to occur when native DNA alone is sonicated, or that the histones form primer-inactive complexes with the single-stranded DNA.

Heat-denatured DNA shows only a small increase in priming activity when sonicated for 20 sec (Table II). Heat-denatured DNA complexed with histones exhibits the expected strong inhibition in comparison with the DNA alone, but there is no increase in priming activity when the complex is sonicated for 20 sec, although the gelatinous complex does become finely dispersed during sonication. Thus, in the case of the DNA polymerase system the repression of priming activity of denatured DNA by histones cannot be attributed sole y to the formation of an impenetrable gel as proposed by Sonnenberg and Zubay (1965) for an RNA polymerase system.

In Figures 6 and 7 are recorded the results of experiments in which the concentration of histone fraction F1 was kept constant while the concentration of DNA was varied. At low DNA concentrations the expected inhibition is noted, but at high DNA concentrations a small augmentation of incorporation of [^{3}H]dATP into DNA is observed under both conditions of assay. Similar results were obtained with the other histone fractions. It is of interest that the plot of incorporation *vs.* DNA concentration is sigmoidal in the presence of histones in contrast to the hyperbolic curve which is found in the absence of histones. These curves resemble the graphs of velocity *vs.* substrate concentration for allosteric enzymes. However, a detailed kinetic analysis will be necessary to determine the significance of these curves.

An attempt was made to investigate the possibility that histones might have some specificity of combination with the heat-denatured DNA primer. If such specificity occurred, the different histone fractions might affect the composition of the product DNA in a manner which might be detected by a difference between the degree of inhibition of incorporation of [^{3}H]dCTP and [^{3}H]TTP by the various histones. For example, if one of the histone fractions used in this experiment had greater affinity for regions of the DNA with high (A + T)/(G + C) ratio, then the inhibition of incorporation of [^{3}H]TTP would be expected to be greater than the inhibition of incorporation of [^{3}H]dCTP. However, the experiments with unfractionated histones and with fractions F1 and F3 (Figure 3) failed to yield any evidence of such specificity.

The data of Figure 8 show that the optimum concentration of potassium phosphate for the DNA polymerase reaction is 30–40 mM when the Mg^{2+} concentration is 3.75 mM, and this optimum concentration is not altered in the presence of various proportions of histone fraction F1. The inhibition of the DNA polymerase reaction by histones in comparison with corresponding histone-free controls is minimal at the optimum phosphate concentration and increases on both sides of the optimum.

At a phosphate concentration of 5 mM an optimum Mg^{2+} concentration of approximately 3 mM is observed for the DNA polymerase reaction in the absence of his-

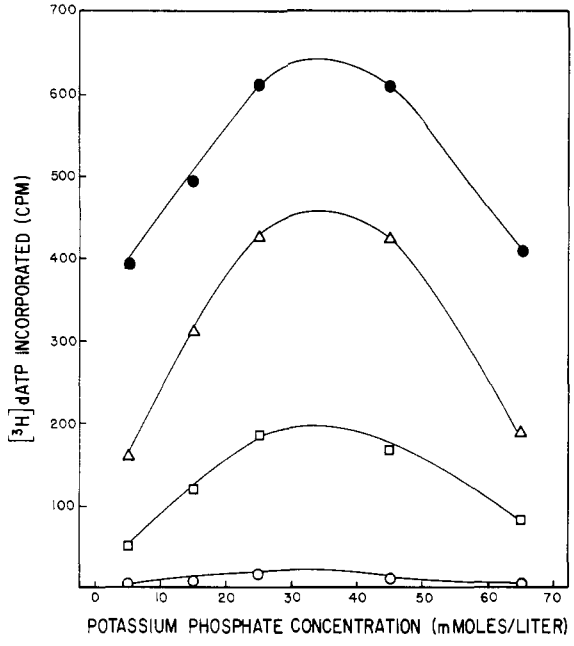

FIGURE 8: Effects of variation in phosphate buffer concentration upon incorporation of [^{3}H]dATP into DNA in the presence and absence of histone fraction F1 under conditions in which the nucleohistone complex was soluble. The reaction mixture contained: the four deoxyribonucleoside triphosphates (including [^{3}H]dATP, 2.3 $\times$ 10^5 cpm/μmole), 100 μM each; 3.75 mM $MgCl_2$; 1 mM β-mercaptoethanol; potassium phosphate (pH 7.4), in the final concentrations indicated on the abscissa; 16.3 μg of heat-denatured (90°, 10 min) DNA; total volume 0.4 ml. (●) No histones present; (△) histone/DNA ratio 0.15; (□) histone/DNA ratio 0.25; (○) histone/DNA ratio 0.4.

tones, but the optimum concentration of Mg^{2+} is shifted to lower concentrations (1–1.5 mM) in the presence of histone fraction F1, and the inhibition increases markedly as the concentration of Mg^{2+} is raised from 1.5 to 6 mM (Figure 5).

Discussion

The results presented here clearly demonstrate that the inhibition of the calf thymus DNA polymerase reaction by histones cannot be attributed to precipitation of primer DNA as an insoluble nucleohistone complex. Akinrimisi *et al.* (1965) have shown by the method of equilibrium dialysis that histones can bind to denatured as well as native DNA to yield soluble complexes under certain conditions. With the soluble system used in the present experiments (Figure 2) the histone/DNA ratios for 50% inhibition of DNA synthesis were lower than those reported by Billen and Hnilica (1964) and by Hnilica and Billen (1964) for the *E. coli* DNA polymerase system with native DNA as primer despite the fact that precipitation of nucleohistone occurred in the latter system. The inhibitions reported in Figure 2 also are greater than those found by Schwimmer (1965) for reconstituted nucleohistone (with native DNA) as primer for the *E. coli* DNA polymerase and by Gurley *et al.* (1964) for the rat liver DNA polymerase with native DNA–histone complexes. Evidently the low ionic strength of the reaction conditions of Figure 2 increases the binding of histones to heat-denatured DNA which more than compensates for the fact that histones bind less tightly to heat-denatured DNA than to native DNA (Akinrimisi *et al.*, 1965). The relative order of inhibitory effects of the various histone fractions is approximately the same for the DNA polymerase systems of calf thymus and of *E. coli* (compare present data with those of Hnilica and Billen, 1964; Schwimmer, 1965). The lysine-rich histone fraction F1 is the strongest inhibitor despite the fact that the arginine-rich histones bind to DNA more tightly (Akinrimisi *et al.*, 1965).

The apparent lack of DNA-base specificity of the inhibition of DNA synthesis by various histone fractions (Figure 3) is in contrast to the results of experiments (Skalka *et al.*, 1966) with a DNA-dependent RNA polymerase which showed that the product formed in the presence of histones had a base ratio and nearest neighbor frequency which differed from that produced in the absence of histones. However, the lack of specificity of inhibition of DNA synthesis by histones is a reasonable finding in view of the cellular and genetic requirement for complete and nonselective replication of DNA prior to cell division.

The data of Figures 5 and 8 show that changes in ionic composition (particularly Mg^{2+} concentration) of the reaction mixture can markedly affect the extent of inhibition of the calf thymus DNA polymerase system by histones. It seems probable that the downward shift of the optimum Mg^{2+} concentration for the DNA polymerase reaction in the presence of histone fraction F1 (Figure 5) can be attributed to release of Mg^{2+} ions from binding to template DNA through competition by histones for the binding sites on DNA, thus raising the concentration of free Mg^{2+} ions for binding at the active site of the enzyme. For the same reason the inhibitory concentrations of Mg^{2+} are lower in the presence of histones. These relationships suggest the possibility that intranuclear changes in Mg^{2+} concentration could play a cooperative role with histones in contributing to the mechanisms for the control of DNA synthesis.

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The Fractionation of Quaternary Ammonium Complexes of Nucleic Acids. Evidence for Heterogeneity of Ribosomal Ribonucleic Acid*

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ABSTRACT: A method for the fractionation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) as their cetyltrimethylammonium complexes is described. The complexes when precipitated on an inert support are eluted at characteristic sodium chloride concentrations. Low molecular weight RNA, ribosomal ribonucleic acid (rRNA), and DNA are separated from each other by means of a salt gradient. rRNA is separated into two fractions, the relative amounts of which are altered by heating or by repeated freezing and thawing. This separation appears to be a function of the conformation of the RNA rather than of its size. Each of the rRNA fractions is separated into 16S and 23S RNA after centrifugation through a linear sucrose gradient. Differences in the nucleotide frequencies of the rRNA present in each of the fractions are evident after digestion of the fractions with pancreatic ribonuclease or ribonuclease T_1. These results show that the two fractions contain RNA molecules which are chemically distinct and are taken to indicate that heterogeneity exists within the 16S and 23S species of rRNA.

Long-chain aliphatic ammonium compounds combine with polyanions to form water-insoluble complexes which dissolve in salt solution when the salt concentration is above a certain critical level. The critical salt concentration is a function of the nature of the salt, the structure and size of the polyanion, and the structure of the aliphatic ammonium cation. It is constant for a given polyanion when a standard salt and an aliphatic ammonium cation are used. This property of the quaternary ammonium complexes of the polyanions has been extensively used in the isolation and fractionation of acidic polysaccharides (Scott, 1960). Nucleic acids also form complexes with aliphatic quaternary ammonium compounds. The complexes are insoluble in water, but soluble in organic solvents and in strong salt solutions. These properties have been utilized for the isolation and purification of both DNA and RNA, and tRNA has been fractionated as the alkylammonium salt (Kirby, 1964a; Kelmers, 1966; Brown, 1963; Jones, 1963; Burkard *et al.*, 1965; Abel-Sadron *et al.*, 1961).

A technique which has been used in the fractionation of mixtures of acidic polysaccharides relies on the precipitation of the quaternary ammonium complexes of the polyanions on an inert support and the extraction of the precipitated complexes by salt solutions of increasing concentration (Scott, 1960; Antonopoulos *et al.*, 1961, 1965). This method of fractionation depends on differences in the critical salt concentration of

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