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Characterization of Protein Kinases Forming Acid-Labile Histone Phosphates in Walker-256 Carcinosarcoma Cell Nuclei†

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ABSTRACT: Two histone kinases, each of which catalyzes the transfer of the γ-phosphoryl group from ATP to a specific histone fraction forming acid-labile histone phosphates, have been partially purified from nuclei of Walker-256 carcinosarcoma cells. One of these enzymes preferentially phosphorylates histone IV (f2a1) at an optimum pH of 9.5 while the other preferentially phosphorylates histone I (f1) at an optimum pH of 6.5 [Smith, D. L., Bruegger, B. B., Halpern, R. M., and Smith, R. A. (1973), *Nature (London) 246,* 103]. Some of the properties of these two enzymes have been studied and compared. Both enzymes have an absolute requirement for Mg^{2+}, which could not be replaced by Mn^{2+} or Ca^{2+}, and both enzymes showed similar saturation levels for ATP and their respective histone substrates. Neither enzyme was stimulated by the presence of added cyclic AMP or cyclic GMP. The pH 9.5 kinase was strongly inhibited by relatively low concentrations of GTP or CTP and was also moderately inhibited by dATP and dGTP, although incubation of the enzyme with [γ-^{32}P]GTP showed GTP to be a poor phosphoryl donor. The pH 6.5 kinase was specific for ATP and unaffected by other nucleoside triphosphates. Histone IV from which the 18 carboxy-terminal amino acids had been removed by treatment with CNBr could also be phosphorylated by the pH 9.5 kinase.

In a previous study we presented evidence for the existence of at least two new histone kinases in cell nuclei of several normal rat tissues and Walker-256 carcinosarcoma (Smith *et al.*, 1973). All tissues studied had one or both of these histone kinases. These enzymes were unique in that the reactions they catalyzed led to the formation of acid-labile phosphoprotein linkages rather than the acid-stable phosphomonoesters that were formed by previously studied histone kinases (for example, Walsh *et al.*, 1968; Langan, 1968). Acid-labile bonds would have been destroyed in enzyme studies using the conventional trichloroacetic acid assay procedure for histone kinase or in whole animal experiments using acids to extract the histones. We reported that one of the new enzymes had a pH optimum of 9.5 and phosphorylated histone IV (f2a1)[1] most extensively, whereas the other enzyme had a pH optimum of 6.5 and phosphorylated histone I (f1) most extensively. Further investigation of the phosphorylated products has shown that the pH 9.5 kinase phosphorylates both of the histidine groups in histone IV to form 3-phosphohistidine and that the pH 6.5 kinase phosphorylates lysine groups in histone I (Smith *et al.*, 1973; B. B. Bruegger *et al.*, manuscript in preparation).

Both of these histone kinases were present at relatively high levels in the nuclei of Walker-256 carcinosarcomas, so the purified enzymes from this rapidly dividing tissue were deemed suitable for characterization. The comparison of these enzymes is presented in this study.

Materials and Methods

[γ-^{32}P]ATP and [γ-^{32}P]GTP. [γ-^{32}P]ATP was prepared (Glynn and Chappell, 1964) using carrier-free [^{32}P]orthophosphoric acid obtained from ICN Pharmaceuticals. The specific radioactivity of the [γ-^{32}P]ATP ranged from 3.1×10^9 to 9.5×10^9 cpm/μmol. [γ-^{32}P]GTP, with a specific radioactivity of 3.1×10^{10} cpm/μmol, was obtained from New England Nuclear. Concentration of these compounds was determined by absorbance at 259 nm, and purity was ascertained by descending paper chromatography in ethanol–1 M ammonium acetate (7:3).

Histones. Whole histone and histone I (f1) were obtained from Sigma Chemical Co., and the mixture of histones IV (f2a1) and IIb1 (f2a2) from calf thymus was obtained from Sigma Chemical Co. or isolated from whole histone (Oliver *et*

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[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; pH 6.5 kinase, histone kinase which forms acid-labile phosphates at pH 6.5; pH 9.5 kinase, histone kinase which forms acid-labile phosphates at pH 9.5. Histone nomenclature used is that of Rasmussen *et al.* (1962). An alternative nomenclature (Johns and Butler, 1962) is shown in parentheses when used.

al., 1972). Histone IV was obtained from the latter mixture by gel exclusion chromatography on a column of Bio-Gel P-100 (Oliver *et al.*, 1972). Cyanogen bromide modified histone IV was prepared following the procedure of DeLange *et al.* (1968a).

Other Materials. Carboxymethylcellulose (CM-23) and DEAE-cellulose (DE-52) were obtained from Reeve-Angel, and Dowex-1 (AG1-X8), 100–200 mesh, was obtained from Bio-Rad. Cyanogen bromide was obtained from Eastman Kodak. GTP, CTP, dTTP, UTP, dGTP, and dCTP were purchased from Calbiochem; dATP and cyclic GMP were purchased from Sigma and cyclic AMP was purchased from Schwarz/Mann.

Analytical Methods. Homogeneity of histone fractions and CNBr modified histone IV was measured by polyacrylamide gel electrophoresis (Panyim and Chalkley, 1969). Protein concentration was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as standard, or when indicated by measuring the ultraviolet absorbance at 280 nm in a Beckman DU spectrophotometer. Histone concentration was assayed by the method of Lowry *et al.* (1951) using lysine-rich histone as standard or by measuring the ultraviolet absorbance at 230 nm. Radioactivity was counted in a Beckman LS-100 liquid scintillation counter in toluene-based scintillation fluid. Background, generally less than 80 cpm, was subtracted for each experiment.

Isolation and Fractionation of Nuclei. Walker-256 carcinosarcomas were removed from ether-anesthetized, exsanguinated Sprague-Dawley rats and used immediately or frozen in Dry Ice. All of the following procedures were carried out at 4°. Nuclei were prepared from Walker-256 carcinosarcoma cells essentially by the method of Chauveau *et al.* (1956). The tumor cells were prepared by mincing with scissors 300–400 g of fresh or frozen and thawed Walker-256 tumor in approximately 1200 ml of isotonic (0.25 M) sucrose containing 1 mM $MgCl_2$. The released cells were filtered through one layer of coarse cheesecloth, followed successively by filtration through one layer of fine cheesecloth and eight layers of fine cheesecloth, and the cells were washed several times by suspending in the isotonic sucrose solution and centrifuging at 120*g* for 10 min. After packing by centrifugation at 10,000*g* for 10 min, the cells were suspended in a small volume of 0.44 M sucrose containing 1 mM $MgCl_2$ with a tight fitting Teflon pestle and centrifuged at 120*g* for 10 min. The pellet was suspended in a small volume of 2.2 M sucrose containing 1 mM $MgCl_2$ and clean nuclei were prepared from the suspension by homogenizing with a motor driven Teflon pestle (0.005–0.007 in. clearance) for 5 min at high speed and centrifuging at 40,000*g* for 80 min. A small semisolid upper layer was carefully removed from the clear 2.2 M sucrose supernatant. The precipitate was reextracted several times by homogenizing in 1 M sucrose–1 mM $MgCl_2$ with a loose fitting Teflon pestle and centrifuging at 3000*g* for 10 min.

The resulting isolated nuclei were fractionated according to a scheme reported by Wang (1967). Three fractions were obtained: a Tris-Mg^{2+} soluble nucleoplasmic fraction containing nuclear soluble proteins and ribosomes; a chromatin acidic protein fraction obtained by dissociation from DNA in 1 M salt and recovery of the supernatant after reconstitution of histones with DNA by lowering the salt concentration; and the reconstituted DNA–histone fraction. The acidic protein fraction was the principal source of the histone kinases and was therefore used for further purification.

Partial Purification of the Histone Kinases. All purification operations were performed at 4°. The acidic protein fraction

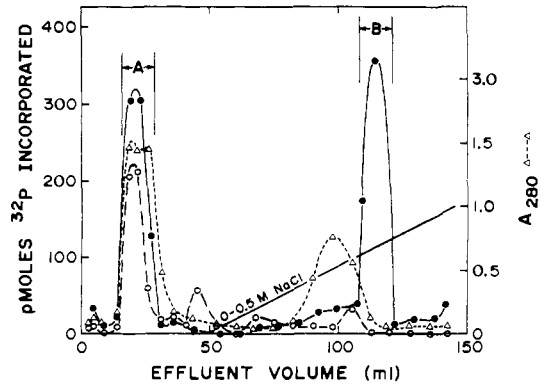

FIGURE 1: Chromatography of the pH 9.5 and pH 6.5 histone kinases on CM-cellulose, 1.0 × 30 cm. Elution was performed with 40 ml of starting buffer (0.01 M Tris-HCl buffer (pH 7.4) containing 30% glycerol and 1 mM 2-mercaptoethanol), followed by a linear gradient of 50 ml of starting buffer to 50 ml of 0.1 M Tris-HCl buffer (pH 7.4) containing 30% glycerol, 1 mM 2-mercaptoethanol, and 0.5 M sodium chloride. The fraction volume was 1.5 ml: (●) pH 9.5 kinase; (○) pH 6.5 kinase; (△) absorbance at 280 nm.

after precipitation with ammonium sulfate to 80% saturation was suspended in 0.01 M Tris-HCl buffer (pH 7.4) and dialyzed for 8 hr against 100 volumes (with two changes) of the same buffer. The solution was adjusted to pH 5 by the addition of 2 M acetic acid and centrifuged for 1 hr at 3000*g*. The pH of the supernatant was raised to 7.4 by the addition of 1 M Tris base. The sedimented material was homogenized with 10 volumes of 0.01 M Tris-HCl (pH 5). After centrifugation for 1 hr at 3000*g*, the sedimented material was discarded. The supernatant was adjusted to pH 7.4 and combined with the first supernatant. The combined supernatants were dialyzed for 8 hr against 100 volumes (with two changes) of 0.01 M Tris-HCl buffer (pH 7.4) containing 30% glycerol and 1 mM 2-mercaptoethanol and applied to a column of CM-cellulose, equilibrated with the same buffer. Elution was performed as described in the legend of Figure 1. Fractions were assayed for histone kinase activity at pH 6.5 and 9.5.

The fractions (labeled A) that were not retained on the CM-cellulose column but contained most of the protein were pooled as indicated in Figure 1 and applied to a column of DEAE-cellulose, equilibrated with 0.01 M Tris-HCl buffer (pH 7.4) containing 30% glycerol and 1 mM 2-mercaptoethanol. Elution was performed as described in the legend of Figure 2. Fractions were assayed for histone kinase activity at pH 6.5 and 9.5. Fractions of peak B in Figure 1 and peak A-2 in Figure 2 were pooled separately as indicated, dialyzed against 0.01 M Tris-HCl buffer (pH 7.4), containing 30% glycerol and 1

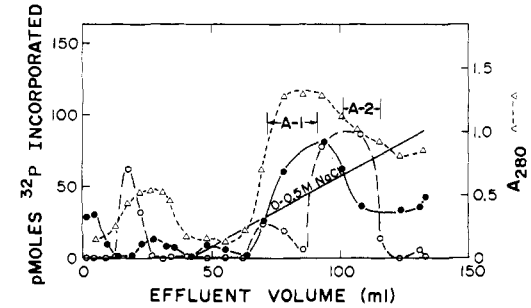

FIGURE 2: Chromatography of pooled histone kinases in peak A from Figure 1 on DEAE-cellulose, 1.0 × 20 cm. Elution was performed in an identical manner with that in Figure 1. The fraction volume was 1.5 ml: (●) pH 9.5 kinase; (○) pH 6.5 kinase; (△) absorbance at 280 nm.

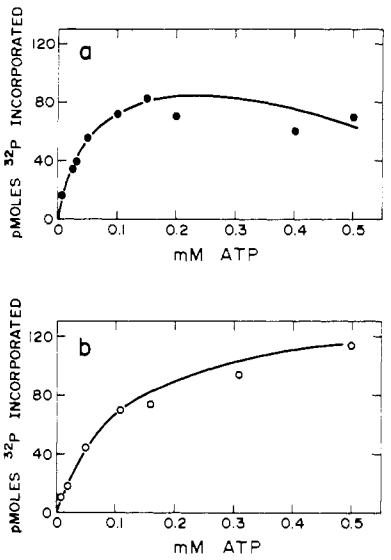

FIGURE 3: Activity of the enzymes as a function of ATP concentration. Incubations were carried out as described in the text, except for the variation in ATP concentration: (a) pH 9.5 kinase; (b) pH 6.5 kinase.

mM 2-mercaptoethanol, and concentrated by ultrafiltration, according to the procedure of Everall and Wright (1958). All fractions could be stored in 30% glycerol in the freezer without rapid loss of enzyme activity.

Assay for Acid-Labile Phosphorylating Activity. The pH 9.5 kinase activity was assayed by incubation in a final volume of 0.2 ml containing (unless otherwise indicated): 10 μmol of glycine–NaOH buffer (pH 9.5); 0.03 μmol of [γ-^{32}P]ATP, 1.5 $\times$ 10^6 cpm; 4 μmol of magnesium acetate; 0.03 μmol of EGTA; 20 μg of histone IV; and enzyme (peak B). The pH 6.5 kinase activity was assayed by incubation in a final volume of 0.2 ml containing (unless otherwise indicated): 10 μmol of maleate buffer (pH 6.5); 0.03 μmol of [γ-^{32}P]ATP, 1.5 $\times$ 10^6 cpm; 4 μmol of magnesium acetate; 0.03 μmol of EGTA; 20 μg of histone I; and enzyme (peak A-2). All reactions were started by addition of purified enzyme and carried out at 30°. The pH 9.5 kinase reactions were incubated for 5 min and the pH 6.5 kinase reactions for 10 min.

Measurement of the acid-labile phosphoryl groups incorporated into proteins was accomplished by a phenol extraction method, or alternatively, by an ion-exchange method, both of which are described by Boyer and Bieber (1967). The ion-exchange method, as used here, was modified to the extent that the initial Cl_3CCOOH step was eliminated and the 0.3 M NH_4OH was replaced by 10 mM NaOH. Since both methods gave nearly equivalent results, the ion exchange method was generally preferred because of its relative ease in assaying large numbers of fractions. When the phenol extraction method was used the reactions were stopped with 2 ml of 88% phenol (buffered at pH 8.0 with sodium phosphate) and added to 8 additional milliliters of the same solution. When the ion-exchange method was used, the reactions were stopped with 1 ml of a solution containing 7 M urea and 10 mM NaOH. A unit of enzyme activity was defined as that amount of enzyme which transferred 1 pmol of ^{32}P from [γ-^{32}P]ATP to recovered protein in 5 min (for pH 9.5 kinase) or 10 min (for pH 6.5 kinase).

Assay for Acid-Stable Phosphorylating Activity. Protein kinase of the usual type which incorporates phosphoryl groups into serine or threonine residues was assayed using the same reaction mixtures as used for the pH 6.5 and pH 9.5 kinases. The protein bound ^{32}P was determined by the method of DeLange *et al.* (1968b) as modified by Miyamoto *et al.* (1969).

Results

Enzyme Purification. The pH 6.5 and pH 9.5 histone kinases were both principally found in the acidic protein fraction. Very little activity could be detected in the deoxyribonucleoprotein fraction and none could be found in the nuclear-soluble protein fraction. Chromatography of the active pH 5 supernatant from the acidic protein fraction on CM-cellulose (Figure 1) resulted in two distinct profiles for the pH 6.5 kinase and pH 9.5 kinase. Approximately 55% of the pH 9.5 kinase was retained on the CM-cellulose column and was eluted by 0.35 M NaCl (peak B). The remaining 45% of the pH 9.5 kinase washed through the column along with all of the pH 6.5 kinase and most of the protein (peak A). Increasing the column dimensions did not reduce the proportion of pH 9.5 kinase that would not adsorb to the CM-cellulose, so it was assumed that these are two separate pH 9.5 activities. The purified pH 9.5 kinase (peak B) produced only acid-labile histone phosphates; thus, protein kinase of the usual type which incorporates phosphoryl groups into serine or threonine residues was not present in this fraction.

Chromatography on DEAE-cellulose (Figure 2) of peak A from the CM-cellulose column revealed that both the pH 6.5 kinase and the remaining pH 9.5 kinase adsorbed to the DEAE-cellulose. The pH 6.5 kinase activity was mostly eluted by 0.35 M NaCl (peak A-2), and the pH 9.5 kinase activity was eluted by 0.25 M NaCl (peak A-1), so the two activities could be partially separated from one another.

Table I shows typical purification data for the enzymes stud-

TABLE I: Partial Purification of the pH 9.5 and pH 6.5 Histone Kinases.[a]

Fraction	Total Protein (mg)		Total Act. (pmol of ^{32}P Incorpd)		Sp Act. (pmol of ^{32}P Incorpd/mg of Protein)	
	pH 9.5 Kinase	pH 6.5 Kinase	pH 9.5 Kinase	pH 6.5 Kinase	pH 9.5 Kinase	pH 6.5 Kinase
Acidic protein	108	108	44,820	31,860	415	295
pH 5.0 supernatant	40	40	41,000	26,400	1,025	660
CM-Cellulose (peak B)	1.6		32,000		20,000	
CM-Cellulose (peak A)		16		20,640		1290
DEAE-Cellulose (peak A-2)		6		11,200		1867

[a] Walker-256 carcinosarcoma, 310 g, was used as enzyme source. Purification procedures are described in the text.

TABLE II: Comparison of [γ-^{32}P]ATP and [γ-^{32}P]GTP as Phosphoryl Donors Using pH 9.5 Kinase.[a]

Labeled Phosphoryl Donor	pmol of ^{32}P Incorpd at Concns of Labeled Phosphoryl Donor (mM)		
	0.05	0.15	0.30
[γ-^{32}P]ATP	135	234	228
[γ-^{32}P]GTP	16	45	54

[a] Incubations were carried out as described in the text, except for the replacement (where appropriate) of 0.03 μmol of [γ-^{32}P]GTP (1.5×10^6 cpm) for 0.03 μmol of [γ-^{32}P]ATP (1.5×10^6 cpm).

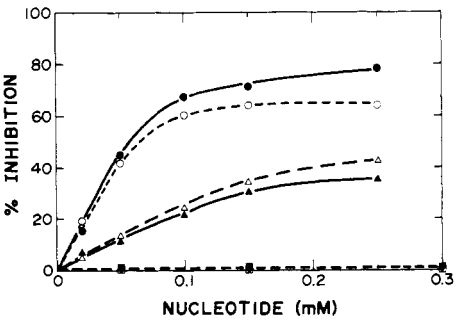

FIGURE 4: Effect of various nucleoside triphosphates on the activity of the pH 9.5 kinase. Incubations were carried out as described in the text, except for the addition of the nucleoside triphosphates other than ATP in the concentrations indicated: (○) GTP; (●) CTP; (▲) dATP; (△) dGTP; (▼) UTP; (▽) dTTP; (□) dCTP.

ied here: the pH 6.5 kinase (peak A-2) and the pH 9.5 kinase (peak B).

Conditions Aiding Stability of the Enzymes. Although sulfhydryl compounds were not required in the incubation mixtures of either of the enzymes studied, the presence of 1 mM 2-mercaptoethanol was required in the enzyme solutions to prevent loss of activity. Enzyme solutions were also routinely stored at −20° in 20–30% glycerol. Under these conditions, the pH 9.5 kinase lost approximately 50% of its activity within 15 days, but the pH 6.5 kinase was stable for several months.

Effect of Varying Incubation Time and Amount of Enzyme. The activity of the pH 9.5 kinase was linear with reaction time for 5 min, and the activity of the pH 6.5 kinase was linear with reaction time for at least 10 min. Both histone kinase activities were proportional to enzyme concentration up to 230 μg/ml of enzyme protein.

Effect of Cyclic AMP and Cyclic GMP. Cyclic AMP or cyclic GMP, when added to the reaction mixture at various levels between 10^{-2} and 10^{-11} M together with 0.4 μmol of theophylline, had no effect on the activity of either the pH 9.5 kinase or the pH 6.5 kinase.

Effect of Metal Ions. The dependence of the activity of the pH 6.5 kinase and the pH 9.5 kinase on Mg^{2+} concentration was examined. For the pH 9.5 kinase, the K_m for Mg^{2+} was 5.5 mM. For the pH 6.5 kinase, the K_m for Mg^{2+} was 3.1 mM. Very slight inhibition was observed at 100 mM Mg^{2+} for both enzymes. The Mg^{2+} dependence of both enzymes was absolute and Mg^{2+} could not be replaced by Mn^{2+} or Ca^{2+} in any of the concentrations tested (from 1 to 50 mM).

ATP Saturation. The pH 6.5 kinase and the pH 9.5 kinase were tested for saturating concentrations of the phosphoryl donor, ATP (Figure 3). The saturating concentration of ATP for both enzymes was approximately 0.15 mM. Slight inhibition of the pH 9.5 kinase occurred at 0.5 mM ATP, but no inhibition by high ATP concentration was observed for the pH 6.5 kinase up to 0.5 mM ATP. For the pH 9.5 kinase and the pH 6.5 kinase, double reciprocal plots showed that the K_m values of the enzymes for ATP were approximately 5.5×10^{-5} and 1.7×10^{-4} M, respectively.

Effect of Other Nucleoside Triphosphates. Various nucleoside triphosphates were studied for their effect on the activity of the histone kinases. The effect on the pH 9.5 kinase is shown in Figure 4. The formation of ^{32}P-labeled acid-labile histone phosphates by this enzyme was greatly reduced by the presence of GTP or CTP; an inhibition of 60–70% was observed in the presence of 0.15 mM of these nucleotides. dGTP and dATP moderately inhibited the enzyme, indicating an inhibition of 30–35% at a concentration of 0.15 mM. UTP, dTTP, and dCTP had no effect on the enzyme activity. None of the above nucleotides had any effect on the activity of the pH 6.5 kinase.

To determine whether the inhibitory effect of certain nucleoside triphosphates, such as GTP, on the pH 9.5 kinase was attributable to competition with the ATP as phosphoryl donor [γ-^{32}P]ATP was replaced by [γ-^{32}P]GTP in the standard assay system (Table II). [γ-^{32}P]GTP was capable of transferring its phosphoryl group to form acid-labile histone phosphates in the presence of the pH 9.5 kinase, but only to the extent of 20–25% of phosphoryl group incorporated from [γ-^{32}P]ATP. GTP remained a potent inhibitor of the pH 9.5 kinase activity at concentrations up to 0.15 mM even under conditions in which the overall nucleoside triphosphate (ATP and GTP) concentration remained constant at 0.15 mM (Figure 5). Thus the observed inhibition was not merely an effect of high total nucleoside triphosphate concentration, but possibly the inhibition by GTP (and the similar inhibition by CTP, dATP, and dGTP) may be the result of regulatory phenomena. As shown in Figure 5, 50% inhibition was observed at a ratio of GTP:ATP of 0.25.

Effect of Varying Histone and Acidic Protein Concentration. The pH 6.5 kinase and pH 9.5 kinase activities were studied for saturating concentrations of their preferred phosphoryl acceptors, histone I and histone IV, respectively (Figure 6). Because acidic extraction is one of the conditions for histone isolation, the histones can be considered to be completely stripped of acid-labile phosphoryl groups prior to their addition to the

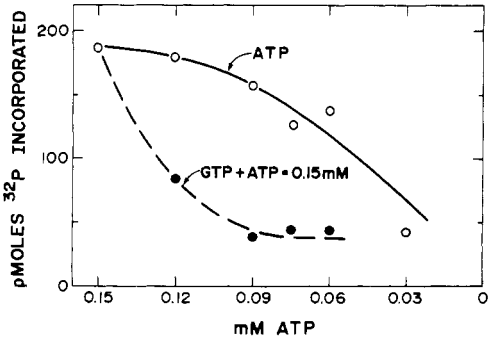

FIGURE 5: Inhibition of the pH 9.5 kinase by GTP under conditions where the total concentration of nucleoside triphosphates (ATP and GTP) is constant. Incubations were carried out as described in the text, except for the appropriate reductions in ATP concentration and additions of GTP: (○) pH 9.5 kinase activity with ATP as the only nucleoside triphosphate present: (●) pH 9.5 kinase activity with GTP present such that ATP + GTP = 0.15 mM.

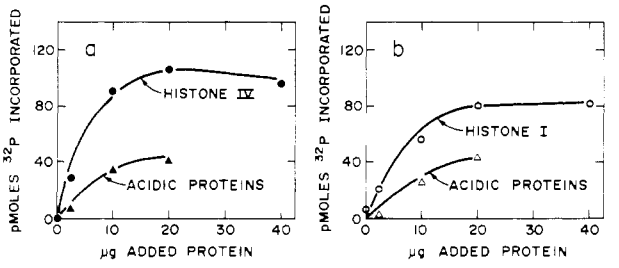

FIGURE 6: Activity of the enzymes as a function of histone and chromatin acidic protein concentration. Acidic protein fraction was heated at 80° for 15 min to remove histone kinase activity which is normally present in this fraction. Incubations were carried out as described in the text, except for the amount and/or types of protein added: (a) pH 9.5 kinase; (●) histone IV (f2a1); (▲) acidic protein fraction; (b) pH 6.5 kinase; (○) histone I (f1); (△) acidic protein fraction.

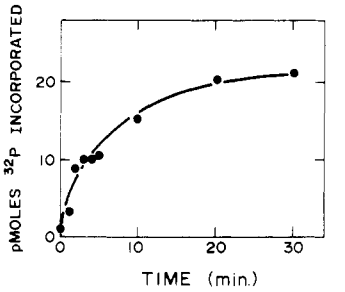

FIGURE 7: Incorporation of phosphoryl groups into CNBr-modified histone IV as a function of incubation time. Incubations were carried out as described in the text, except for the replacement of 20 μg of histone IV by 17 μg of CNBr-modified histone IV. CNBr-modified histone IV was prepared as described in the text.

reaction system. For both enzymes, the saturating concentration of histone was approximately 0.10 mg/ml. The K_m values for histone of the pH 9.5 kinase and the pH 6.5 kinase, respectively, were approximately 0.033 mg/ml (2.9 μM histone IV) and 0.048 mg/ml (2.4 μM histone I).[2]

In addition to the proteins examined for their ability to serve as substrates as discussed in a previous publication from our laboratory (Smith *et al.*, 1973), heat inactivated acidic protein fraction was tested as a substrate for the pH 9.5 kinase and the pH 6.5 kinase to see whether any of the nonhistone chromatin proteins (acidic proteins) could be phosphorylated by these enzymes (Figure 6). The acidic proteins were capable of acting as phosphoryl acceptors for both enzyme systems but were considerably less effective than the respective histones. Other proteins that have been tested with either enzyme were unphosphorylated or only slightly phosphorylated. These include casein, protamine, and bovine serum albumin.

Cyanogen Bromide Modified Histone IV. Histone IV from which the 18 carboxy-terminal amino acids have been removed with CNBr was tested as a phosphoryl acceptor for the pH 9.5 kinase. Figure 7 shows the time course of ^{32}P incorporation into this molecule. The modified histone IV was capable of being phosphorylated by the enzyme. However, although the CNBr modified histone IV does not aggregate in solution and thus alleviates one of the problems sometimes encountered with histone IV in solution (Ziccardi, 1973), the incorporation of phosphoryl groups into this molecule does not seem to be enhanced over intact histone IV and in fact appears to occur more slowly.

Discussion

At least two different enzymes that form acid-labile histone phosphates have been demonstrated in Walker-256 carcinosarcoma (Smith *et al.*, 1973). The present studies demonstrate that the pH 6.5 kinase, which preferentially phosphorylates histone I (f1), and the pH 9.5 kinase, which preferentially phosphorylates histone IV (f2a1), are separate enzymes although both enzymes have a number of characteristics in common. The histone kinases showed separate elution profiles on CM-cellulose chromatography. In this paper the properties of the histone kinases separated on this column were studied, after further purification of the pH 6.5 kinase on DEAE-cellulose, which separated this latter enzyme from another, undefined, kinase active at pH 9.5.

With respect to most parameters other than pH optimum and histone specificity, the pH 6.5 kinase and pH 9.5 kinase were quite similar. For example, neither enzyme was stimulated by cyclic AMP, unlike most of the histone kinases that form acid-stable phosphomonoesters (Walsh *et al.*, 1968; Langan, 1968; Miyamoto *et al.*, 1969). Cyclic GMP likewise had no effect on the enzymes studied here. Both enzymes were similar in their dependence on Mg^{2+}, and saturating concentrations of the phosphoryl donor (ATP) were similar for both enzymes, as were the saturating concentrations of the phosphoryl acceptor histone I or IV. Both enzymes were also able to phosphorylate acidic proteins although much less effectively than the appropriate histones. The acidic protein fractions employed, as judged by acrylamide gel chromatography (Panyim and Chalkley, 1969), contained a small amount (about 10%) of a protein with mobility similar to histone IV. Thus the interpretation of this result is not currently clear. The pH 9.5 kinase phosphorylated CNBr-treated histone IV much more slowly than untreated histone IV, indicating that the intact histone may be required for complete phosphorylation.

The major difference observed between the two enzymes was the inhibitory effect of GTP, CTP, dATP, and dGTP on the pH 9.5 kinase. The pH 6.5 kinase was unaffected by all nucleoside triphosphates tested other than ATP. Although the significance of this effect is not yet clear, it might point out a regulatory mechanism for the pH 9.5 kinase, such as competition of certain nucleoside triphosphates with ATP for catalytic sites, which might not operate in the pH 6.5 kinase. Alternatively, the inhibition may result from the conformational changes produced by interaction of histone IV in solution with GTP, CTP, and ATP (Wickett and Isenberg, 1972).

Other work in our laboratory has shown that the pH 9.5 kinase phosphorylates both of the histidine residues in histone IV, whereas the pH 6.5 kinase phosphorylates lysine residues in histone I (Smith *et al.*, 1973; B. B. Bruegger *et al.*, manuscript in preparation).

[2] The following molecular weights are assumed: 11,282 for histone IV and 20,250 as an average for the histone I molecules (DeLange and Smith, 1971).

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Occurrence and Distribution of Acid-Labile Histone Phosphates in Regenerating Rat Liver†

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ABSTRACT: A distinct class of histone kinases producing acid-labile, alkali-stable phosphates was found in regenerating rat liver and other tissues [Smith, D. L., Bruegger, B. B., Halpern, R. M., and Smith, R. A. (1973), *Nature (London) 246,* 103]. The present study shows that the activity of one of these kinases in regenerating rat liver reached its highest level during the period of DNA synthesis after partial hepatectomy. After *in vivo* administration of ^{32}P-labeled sodium phosphate the distribution of ^{32}P included both histone acid-stable and acid-labile phosphates which were separated by chromatography on Bio-Rex 70. The majority of acid-labile phosphorylation occurred on histones I and IV isolated from regenerating rat liver while the histones isolated from normal liver were less phosphorylated. Acidic proteins were also highly phosphorylated yielding both acid-labile and acid-stable forms. Proteolytic digestion of phosphorylated histone fractions isolated from regenerating liver by chromatography showed that acid-labile phosphate was on lysine in histone I while that in histone IV was on histidine. Acid-labile phosphorylation of acidic proteins occurred on both histidine and lysine residues. Electrophoresis at pH 10 on cellulose acetate revealed similar mobilities for the acid-labile histone phosphates whether they were isolated from *in vivo* or *in vitro.*

The formation of acid-stable histone phosphates has been demonstrated in a number of previous studies (Ord and Stocken, 1969; Kleinsmith *et al.,* 1966; Gutierrez and Hnilica, 1967; Balhorn *et al.,* 1971, etc.), and several histone kinases which produce *O*-phosphoserine or *O*-phosphothreonine have been described (Langan, 1969; Yamamura *et al.,* 1970). While it has been suggested that histones may be responsible for the control of gene expression (Stedman and Stedman, 1951) and it is possible that phosphorylation of the histone components of chromatin may result in a change of the template activity of DNA, the role of phosphorylation of the relatively limited number of types of histone molecules has not been entirely settled.

The preceding paper described two kinases which catalyze phosphorylation of histones I and IV in Walker-256 carcinosarcoma (Smith *et al.,* 1974). These enzymes which form acid-labile histone phosphates have also been found in other rat tissues, including regenerating rat liver. In the present study we have examined these enzyme activities in relation to the period of DNA synthesis in regenerating rat liver, and we determined the patterns of *in vivo* incorporation of radioactive phosphorus into various histone fractions and acidic proteins of regenerating rat liver.

Materials and Methods

Chemicals. [γ-^{32}P]ATP was prepared as described in the preceding paper (Smith *et al.,* 1974). [^{3}H]Thymidine was purchased from Schwarz/Mann. Bio-Rex 70 (200–400 mesh) was obtained from Bio-Rad Laboratories. Histone IV was a gift from Dr. Robert DeLange, and other histones were obtained from the Sigma Chemical Co.

Preparation of Phosphoamino Acids. N^{ϵ}-Phosphoryllysine was prepared by the method of Zetterqvist and Engström (1967). Preparations of phosphoarginine and phosphohistidine were carried out by the methods of Marcus and Morrison (1964) and DeLuca *et al.* (1963), respectively.

Partial Hepatectomies. Female Sprague-Dawley rats (250–350 g) were subjected to partial hepatectomy under ether

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