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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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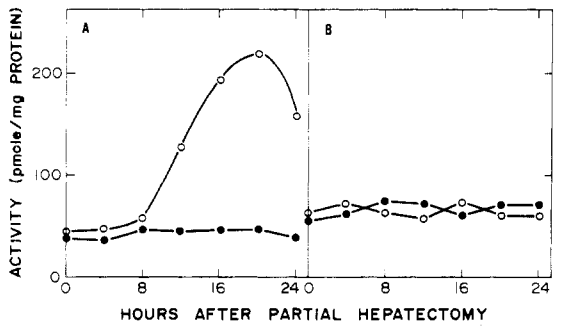

FIGURE 1: The levels of histone kinase activity found in rat regenerating and normal livers. Reaction mixtures for the pH 9.5 kinase contained in 0.2 ml, 10 μmol of glycine-NaOH buffer (pH 9.0), 0.03 μmol of [γ-^{32}P]ATP (1.5×10^6 cpm), 4 μmol of magnesium acetate, 0.03 μmol of EGTA, and 20 μg of whole histone. The pH 6.5 enzyme was assayed by incubating in the same mixture except that glycine-NaOH buffer was replaced by maleate buffer (pH 6.5). Nuclei were used as the enzyme source. All reactions were carried out at 30° for 5 min: (○) regenerating rat liver nuclei; (●) sham-operated rat liver nuclei; (A) pH 9.5 enzyme activity; (B) pH 6.5 enzyme activity.

anesthesia by the method of Higgins and Anderson (1931) with removal of about two-thirds of the liver. The livers were allowed to regenerate for appropriate periods. Control rats were sham-operated and sacrificed at intervals corresponding to those of the partially hepatectomized animals.

Nuclei Preparation for Enzyme Assay. Liver nuclei from normal (sham-operated) and partially hepatectomized rats were prepared by the method of Chauveau *et al.* (1956).

Enzyme Assay. Reaction mixtures are described in the legend of Figure 1. All enzyme assays were performed by the method described in the preceding paper (Smith *et al.*, 1974). Unless otherwise specified, protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Isotope Administration and Isolation of Chromatin. Female Sprague-Dawley rats (250–350 g), either sham-operated or partially hepatectomized 15 hr previously, were injected intraperitoneally with [^{32}P]orthophosphate in 0.9% NaCl solution at a dose of 2 mCi/100 g body weight. After 1.5 hr, the animals were sacrificed by decapitation, and the livers were removed and placed in ice-cold 0.25 M sucrose containing 0.025 M Tris-HCl (pH 7.5) and 0.005 M $CaCl_2$. After the adhering connective tissues were removed, the nuclei were isolated by pelleting through a 2.2 M sucrose solution by the method of Chauveau *et al.* (1956). Purified chromatin was prepared by extraction of the nuclei with 0.15 M NaCl and it was subsequently washed three times with a solution of 0.05 M Tris (pH 7.5), followed by 0.15 M NaCl.

Isolation of Chromosomal Proteins. Total chromosomal proteins were isolated by dissociation of the washed chromatin in 2.5 M guanidinium chloride containing 0.1 M sodium phosphate buffer (pH 7.0). Removal of DNA was accomplished by ultracentrifugation at 90,000*g* for 10 hr.

Acid and Base Treatment of Chromosomal Proteins. In separate aliquots acid-stable phosphoryl linkages were for the most part eliminated by treatment at 60° for 10 min with 0.1 M NaOH while acid-labile phosphoryl linkages were eliminated by treatment at 60° for 10 min with 0.1 N HCl. This treatment was followed by dialysis at 4° overnight against two changes of 2 l. each of 0.01 M phosphate buffer (pH 7.0) to remove the free ^{32}P-labeled inorganic phosphate.

Bio-Rex 70 Column Chromatography. Separation of chromosomal proteins was carried out on a Bio-Rex 70 (0.6 × 60 cm) column. The elution was performed with a total of 50 ml of a linear gradient of 8–13% guanidinium chloride in 0.1 M phosphate buffer (pH 6.8) prior to elution with 40% guanidinium chloride in the same buffer. Protein elution was monitored by the Cl_3CCOOH turbidity method (Bonner *et al.*, 1968).

Proteolytic Digestion. The separate peaks from the Bio-Rex column were pooled and dialyzed against two changes of 1-l. each of 0.01 M Tris buffer (pH 8.0) and concentrated by lyophilization. Each fraction was then dissolved in 1 ml of 0.1 M Tris buffer (pH 8.0) and incubated with trypsin (0.5 mg) at 37° for 2 hr. After heat denaturation of the trypsin at 60° for 15 min and subsequent cooling to room temperature, the enzymatic digestion was continued by adding 25 units of Pronase. The mixture was now incubated at 37° for 24 hr, after which proteolytic digestion was complete. The mixture was analyzed by ascending chromatography on Whatman No. 3 paper with the following solvent system: isopropyl alcohol–ethanol–water–triethylamine (30:30:39:1).

Thymidine Incorporation. The rats were injected intraperitoneally at various times after partial hepatectomy with [^{3}H]thymidine (20 μCi/100 g body weight). Animals were sacrificed 1 hr after the injection, and the rapidly excised livers were processed as described by Munro and Fleck (1967). The incorporation of [^{3}H]thymidine was determined in a scintillation counter, and DNA concentration was measured by the diphenylamine method of Burton (1956).

Cellulose Acetate Electrophoresis. Since phosphoramidates are extremely acid labile, it was necessary to use an alkaline electrophoretic system for separation of acid-labile histone phosphates. For this purpose, we employed a system similar to that described by Machicao and Sonnenbichler (1971), in which histones are separated by rapid electrophoresis at pH 10 on cellulose acetate strips. At this pH phosphoramidates readily undergo transphosphorylation to uncharged amines; therefore, we used a pH 10.0 buffer containing 0.01 M sodium borate, 0.01 M EDTA, and 6 M urea, instead of the original buffer described by Machicao and Sonnenbichler (1971). For standards in these experiments, we prepared the appropriate histone phosphate in a standard enzyme reaction mixture using γ-^{32}P-labeled ATP and the appropriate histone and histone kinase as previously described (Smith *et al.*, 1974). The acid-labile ^{32}P-labeled histone phosphates from regenerating liver were isolated by chromatography on a cation exchange resin, as described above, and, in preparation for electrophoresis, were dialyzed after elution from the Bio-Rex 70 column against Tris buffer (50 mM, pH 7.5), lyophilized, and then dissolved in 1 ml of the pH 10 electrophoretic buffer and applied to the cellulose acetate strip. Electrophoresis was carried out for 20 min at 140 V.

Results

Histone Kinase Activities in Rat Liver. Regenerating rat liver nuclei contain two histone kinases producing acid-labile histone phosphates, one active at pH 6.5 and another active at pH 9.5. The enzymes from regenerating liver are similar in every respect, so far examined, to those enzymes isolated from Walker-256 carcinosarcoma (Smith *et al.*, 1974). Sham-operated rat liver nuclei contain about the same level of the pH 6.5 kinase, but only negligible levels of the pH 9.5 kinase can be detected (Smith *et al.*, 1973). Figure 1 shows that during liver regeneration the pH 6.5 kinase activity remained approximately constant, whereas the pH 9.5 kinase activity increased at 12 hr, reached a peak at 18 hr, and began to decline at 24 hr after partial hepatectomy. Sham-operated rat liver showed very low

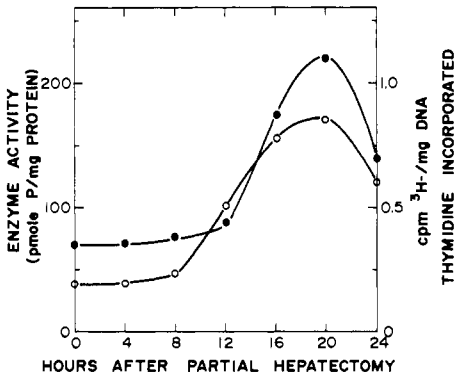

FIGURE 2: Correlation of histone kinase activity with DNA synthesis after partial hepatectomy. The pH 9.5 kinase activity was assayed as described in Figure 1. The administration of [^{3}H]thymidine and determination of thymidine incorporation were performed as described under Materials and Methods: (O) enzyme activity; (●) [^{3}H]thymidine incorporation.

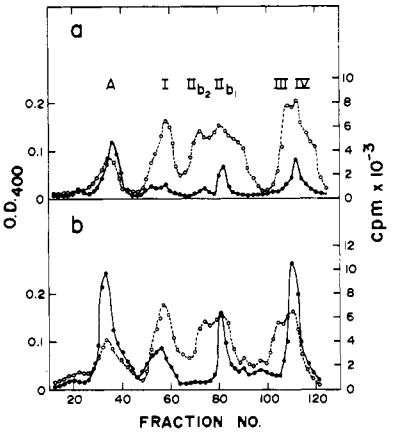

FIGURE 3: Chromatography of acid-stable histone phosphates and acidic proteins on Bio-Rex 70 resin. Incorporation of radioactive inorganic phosphate, isolation of histones and acidic proteins, removal of acid-labile phosphates, and chromatographic separation were performed as described under Materials and Methods. Calf thymus histones (3 mg) were added as carriers during column chromatography. A refers to acidic proteins; histones are referred to by fraction designation, using the nomenclature of Rasmussen *et al.* (1962): (a) sham-operated rat liver; (b) regenerating rat liver (18 hr after partial hepatectomy); (O) OD_{400} (Cl_3CCOOH turbidity); (●) radioactivity (^{32}P incorporated *in vivo*).

pH 9.5 enzyme activity throughout the period tested.

Temporal Relationship of Histone Kinases in Regenerating Rat Liver with DNA Synthesis. To study the temporal relationship of the histone kinase activity during regeneration, DNA synthesis was determined by assaying the incorporation of [^{3}H]thymidine into DNA at various times after partial hepatectomy. Figure 2 shows a marked correlation of the pH 9.5 histone kinase activity with DNA synthesis after regeneration begins. In the several experiments performed it always appeared that the increase in histone kinase activity just preceded the increase in DNA synthesis.

Phosphorylation of Histones in Vivo. We were interested in the extent of formation of acid-labile histone phosphates in normal and regenerating livers during the DNA synthesis period 18 hr after partial hepatectomy. Figure 3 shows that histones I, IIb1, and IV were phosphorylated forming acid-stable histone phosphates to a greater extent in regenerating rat liver than in sham-operated rat liver. These results are quite similar to those obtained by Sung *et al.* (1971), and served as a qualitative control on our slightly different isolation procedure. Figure 4 shows the results obtained for the acid-labile histone phosphates. Clearly, histones I and IV were more extensively converted to acid-labile histone phosphates in the regenerating liver than in the normal liver in sham-operated animals. Other histones formed acid-labile phosphates to only a limited extent at 18 hr after partial hepatectomy. The nonhistone chromosomal proteins (acidic proteins) were extensively phosphorylated forming both acid-labile and acid-stable phosphates in either regenerating or sham-operated normal livers.

Identification of Acid-Labile Phosphoamino Acid. The acid-labile phosphorylated fractions from the Bio-Rex 70 columns were pooled and analyzed for identification of phosphoamino acids. Figure 5 shows that while the acidic proteins were phosphorylated on both lysine and histidine residues, only phospholysine could be found in digests of histone I and only phosphohistidine could be found in digests of histone IV.

Electrophoretic Comparison of Acid-Labile Histone Phosphates. Having isolated the acid-labile histone phosphates from regenerating rat liver by a standard technique, we were interested in further evidence that the phosphorylated species were the modified histones and not just contaminants leaving phosphoryl groups. Since the usual electrophoretic techniques for histones involve pH values at which P–N bonds are rapidly hydrolyzed, we chose electrophoresis at pH 10 on cellulose acetate as described above. As standards in these experiments, we used purified histones I and IV which were phosphorylated with the usual *in vitro* conditions using the pH 6.5 and 9.5 histone kinases partially purified from Walker-256 carcinosarcoma (Smith *et al.*, 1974). After the enzyme reactions were completed, the reaction mixture was applied to a small anion exchange resin (Dowex-1) and the ^{32}P-labeled histone phosphates were collected in the eluate. Each eluate was lyophilized and then redissolved in the electrophoresis buffer and applied to the cellulose acetate strip (5.5 cm × 7 cm). Electrophoresis was carried out for 20 min at 140 V after which each strip was cut into 0.5-cm segments and the radioactivity in each determined. In our original experiment we used histone IV labeled with [^{3}H]arginine as substrate together with the pH 9.5 kinase and [γ-^{32}P]ATP. The nonphosphorylated histone IV moved toward the negative pole while the phosphorylated histone containing both ^{32}P and ^{3}H moved toward the positive pole. Figure 6a

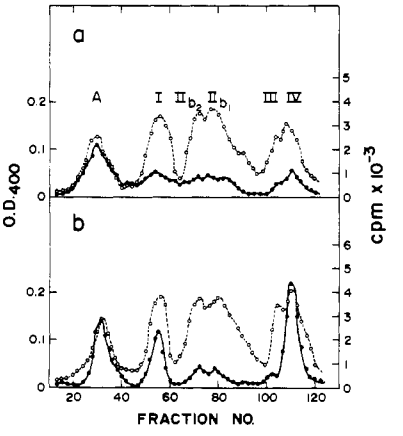

FIGURE 4: Chromatography of acid-labile histone phosphates and acidic proteins on Bio-Rex 70 resin. Incorporation of radioactive inorganic phosphate, isolation of histones and acidic proteins, removal of acid-stable protein phosphates, and chromatographic separation were performed as described under Materials and Methods. Calf thymus histones (3 mg) were added as carriers during column chromatography: (a) sham-operated rat liver; (b) regenerating rat liver (18 hr after partial hepatectomy); (O) OD_{400} (Cl_3CCOOH turbidity); (●) radioactivity (^{32}P incorporated *in vivo*).

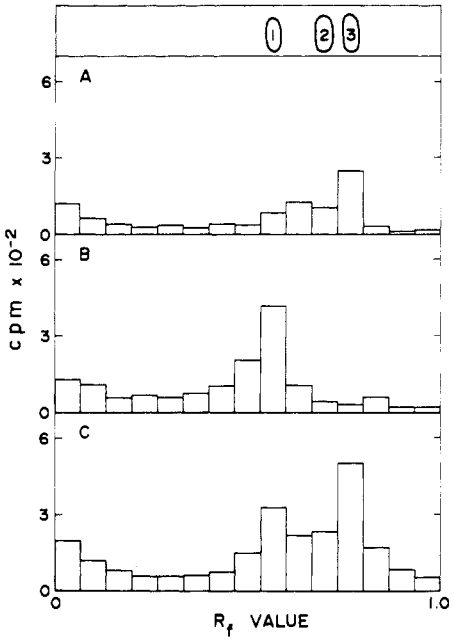

FIGURE 5: Paper chromatography of acid-labile phosphoamino acids on Whatman No. 3 paper. Base treatment and Bio-Rex 70 chromatography of extracted proteins and proteolytic digestion of pooled fractions of acidic proteins, histone I, and histone IV were carried out as described under Materials and Methods. Ascending paper chromatography was performed in the following system: isopropyl alcohol–ethanol–water–triethylamine (30:30:39:1). The paper strips were cut into 15 fractions of 1 cm each and these fractions were counted: (A) histone I; (B) histone IV; (C) acidic protein. Standard phosphoamino acids: (1) phosphohistidine; (2) phosphoargine; (3) phospholysine.

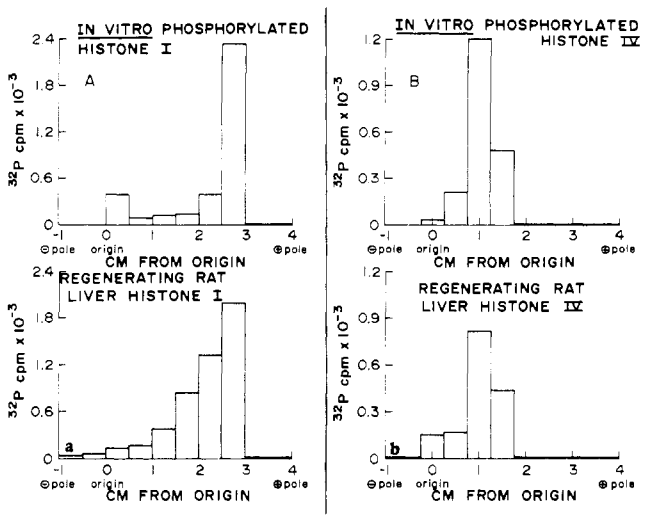

FIGURE 6: Electrophoretic analysis of acid-labile phosphorylated histones I and IV on cellulose acetate strips. The histone phosphates from regenerating liver were isolated by chromatography on a Bio-Rex 70 column as described in the text and as shown in Figure 4, except that no carrier histones were added. The phosphorylated histones from peaks I and IV were dialyzed and freeze dried as indicated in the text. The *in vitro* phosphorylated histones were prepared from standard reaction mixtures employing either the pH 6.5 or 9.5 histone kinases described earlier (Smith *et al.*, 1974). The reaction mixtures were run over small Dowex-1 columns as previously described, then dialyzed and freeze dried for electrophoresis as above.

shows the electrophoretic mobility of histone I phosphorylated *in vitro* as described and as isolated from regenerating liver by cation exchange chromatography. The majority of the ^{32}P had the same mobility when obtained from either system; however, in the sample obtained in the enzyme reaction mixture we always observed a smaller peak of radioactivity which remained close to the origin. The results in Figure 6b show that histone IV isolated from regenerating liver and that isolated from a pH 9.5 histone kinase reaction mixture have almost identical electrophoretic mobilities.

Discussion

The findings of this study show that in addition to the previously demonstrated acid-stable histone phosphates (for example, Kleinsmith *et al.*, 1966) acid-labile histone phosphates are formed *in vivo*. Our evidence, so far, indicates that it is histones I and IV which are principally modified in this manner, although to a much lesser extent histones IIb_1, IIb_2, and III may also contain acid-labile phosphates.

The isolation of N^{ϵ}-phosphoryllysine and 3-phosphohistidine from histones I and IV which in turn were isolated from specific kinase reactions and from regenerating liver, together with the almost identical electrophoretic mobility of each of the pairs of modified histones, clearly support our conclusion that acid-labile phosphorylations occur on specific histones *in vivo*.

Presently we know very little about the turnover of the acid-labile histone phosphates and since so much time is consumed in the isolation of nuclei and from them the modified chromosomal proteins, our results may only show a portion of the acid-labile histone phosphates formed *in vivo*. To date kinases capable of forming acid-labile phosphates have been detected in nuclear extracts only for histone I and histone IV (Smith *et al.*, 1974). However, earlier experiments (Smith, 1974), in which the ratio of total acid-labile protein phosphate to acid-stable protein phosphate formed in isolated Walker-256 carcinosarcoma nuclei varied from about 1.0 to 3.0, suggest that there may well be a rapid turnover of nuclear phosphorylated proteins. Thus our results shown in Figure 4 may only represent the most kinetically stable of the acid-labile histone phosphates formed.

Appropriate proteolytic digestion of the acid-labile histone phosphates isolated from regenerating rat liver by chromatography on Bio-Rex 70 resin revealed the same phosphorylated amino acids as were detected in the kinase studies (Smith *et al.*, 1973; B. B. Bruegger *et al.*, manuscript in preparation). Thus, although it is clear that N^{ϵ}-phosphoryllysine occurs in histone I *in vivo* neither the number of such phosphorylations nor their location in the protein sequence is known. Similarly, with the acid-labile histone IV phosphate our studies have revealed 3-phosphohistidine as the only phosphorylated amino acid isolated. Whether the considerably more labile 1-phosphohistidine does not occur or is converted to 3-phosphohistidine during isolation is not presently known. Histone IV has been shown to contain only two histidine residues, at positions 18 and 75 (DeLange *et al.*, 1969), and although the stoichiometry and position of phosphorylation in the enzymically produced (pH 9.5 kinase) histone IV phosphate have been investigated (B. B. Bruegger *et al.*, manuscript in preparation) we were unable to determine these parameters in the histone phosphates isolated in this work.

The biological role of the acid-labile histone phosphates is not known. They may have functions similar to those postulated for other types of histone phosphates such as roles in gene activation (Kleinsmith *et al.*, 1966), DNA replication (Balhorn *et al.*, 1972), or mitosis (Marks *et al.*, 1973). Questions as to the roles of acid-labile phosphorylation of histones and the coupling with template activity of DNA, as well as its relationship to other cellular events, are currently being studied. In regenerating rat liver, the histone kinase activity at pH 9.5 correlated

well with the onset of DNA synthesis after partial hepatectomy. This result, together with the observation that the protein kinases with high pH optima are extremely active in rapidly dividing tissues and inactive in nondividing tissues such as brain (Smith *et al.*, 1973), suggests that this histone kinase might play a role in replication of DNA.

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