Rapid Electrophoretic Analysis for Histone Phosphorylation. A Reinvestigation of Phosphorylation of Lysine-Rich Histone During Rat Liver Regeneration*

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ABSTRACT: Lysine-rich histone isolated by normal techniques is so contaminated with phosphate that previous assessments of the amount of phosphorylation of this histone fraction during liver regeneration have been underestimated. If the lysine-rich histone is rigorously purified the extent of its phosphorylation in control livers is very small, but it increases dramatically following partial hepatectomy and subsequent regeneration. An electrophoretic method for studying histone phosphorylation has been further exploited and its facility for rapid survey studies documented.

It has been suggested that phosphorylation of histones, particularly the lysine-rich or F_1 fraction, is in some way correlated with DNA synthesis and the mitotic activity of the cell. Gutierrez and Hnilica (1967), working with several different tissues, argued that the more mitotically active the cell, the lower the degree of histone phosphorylation. On the other hand, Shepherd *et al.* (1971) have presented evidence that the synthesis and phosphorylation of lysine-rich histone in synchronized Chinese hamster cells begins 6 hr prior to DNA synthesis. Ord and Stocken (1969) and Buckingham and Stocken (1970) have presented evidence that there is an increase in the phosphorylation of histones prior to cell division in regenerating rat liver. They worked only with unfractionated lysine-rich histone and failed to observe any heterogeneity within this fraction. Sherod *et al.* (1970) demonstrated the existence of an electrophoretic microheterogeneity in the lysine-rich fraction of a rapidly dividing ascites tumor and provided evidence that at least part of this microheterogeneity is due to differential phosphorylation of a more rapidly migrating parent molecule.

We were interested in studying the possible ^{32}P labeling of various liver lysine-rich histone electrophoretic bands both before and after partial hepatectomy. However, during the isolation of the lysine-rich histone obtained by the method of Johns (the method utilized by most previous workers in this area) we observed the presence of substantial amounts of contaminating phosphate, mostly in the form of inorganic phosphate, though some RNA or ribonucleotides can also be detected. The extent of this phosphate contamination is such that *in vivo* ^{32}P-labeling studies of histone phosphorylation are almost totally overshadowed by radioactive label which is not covalently bound to histone. The level of contaminating ^{32}P in regenerating rat liver lysine-rich histone is about three times higher than that of control liver and this has previously been interpreted as showing a threefold increase in the level of F_1 phosphorylation upon partial hepatectomy. We will show that if the substantial levels (>90%) of contaminating radioactivity are removed, the effect of partial hepatectomy upon histone phosphorylation is in fact much more dramatic than previously reported, showing a tenfold increase from the very low levels found in the normal, very slowly dividing liver. The problem of contaminating radioactivity is ideally removed by column chromatography followed by electrophoresis. A modified method of high-resolution polyacrylamide gel electrophoresis is described as the most reliable means for studying the extent of lysine-rich histone phosphorylation during rat liver regeneration. We have confirmed earlier reports that phosphorylation is positively correlated with DNA synthesis and that electrophoretic analysis coupled with enzymatic dephosphorylation can be used as a rapid and fairly precise means of determining the extent of lysine-rich histone phosphorylation in other systems.

Materials and Methods

Partial Hepatectomy. Holtzman rats weighing 100–200 g were partially hepatectomized by the method of Higgins and Anderson (1931), removing approximately two-thirds of the liver. All hepatectomies were performed such that the removal of the regenerated liver occurred between 11:00 a.m. and 1:00 p.m., preventing possible diurnal variations in mitotic activity between experiments. The rats were fed *ad libitum* and the remaining liver removed following the appropriate interval of liver regeneration.

Isolation of Histone. Whole histone was isolated from purified nuclei by the method of Panyim and Chalkley (1969). It was necessary to increase the concentration of Triton X-100 to 1% in order to obtain sufficiently clean nuclei.

Lysine-rich (F_1) histone was selectively extracted either from chromatin or from whole histone using the perchloric acid method of Johns (1964). The lysine-rich histone was then dialyzed extensively against 0.4 N sulfuric acid at 4° prior to precipitation by dialysis against 95% ethanol.

Electrophoresis. Electrophoresis of histone dissolved in 15% sucrose–0.9 N acetic acid was performed for 24 hr at 25° and for 40 hr at 4° on 25-cm preelectrophoresed gels (15% acrylamide) containing 2.5 M urea in 0.9 N acetic acid (pH 2.8). After electrophoresis at 200 V, the gels were removed by breaking the tubes. They were stained in Amido-

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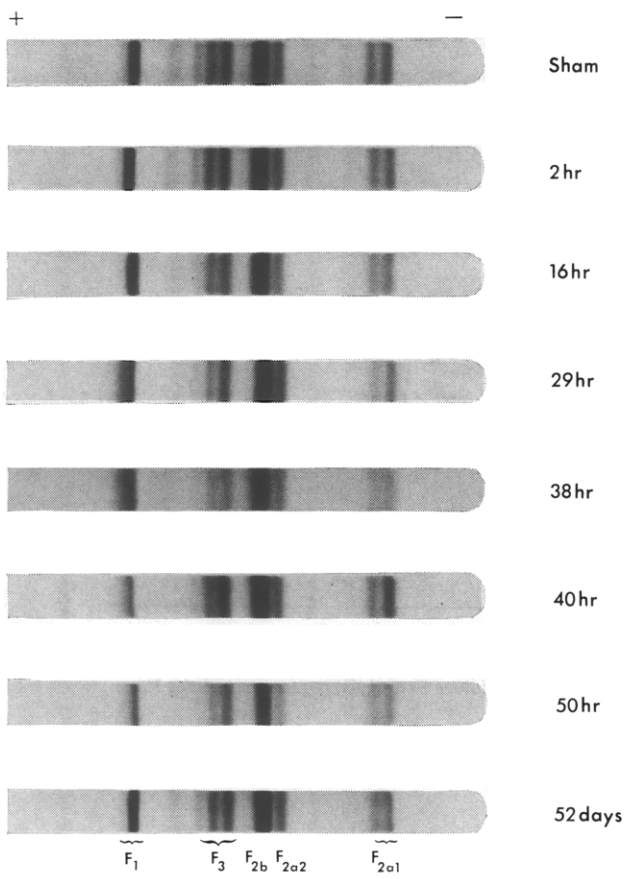

FIGURE 1: Polyacrylamide gel electrophoretic patterns of rat liver histone during regeneration. Electrophoresis was performed for 24 hr at 2 mA/gel and 200 V at 4°.

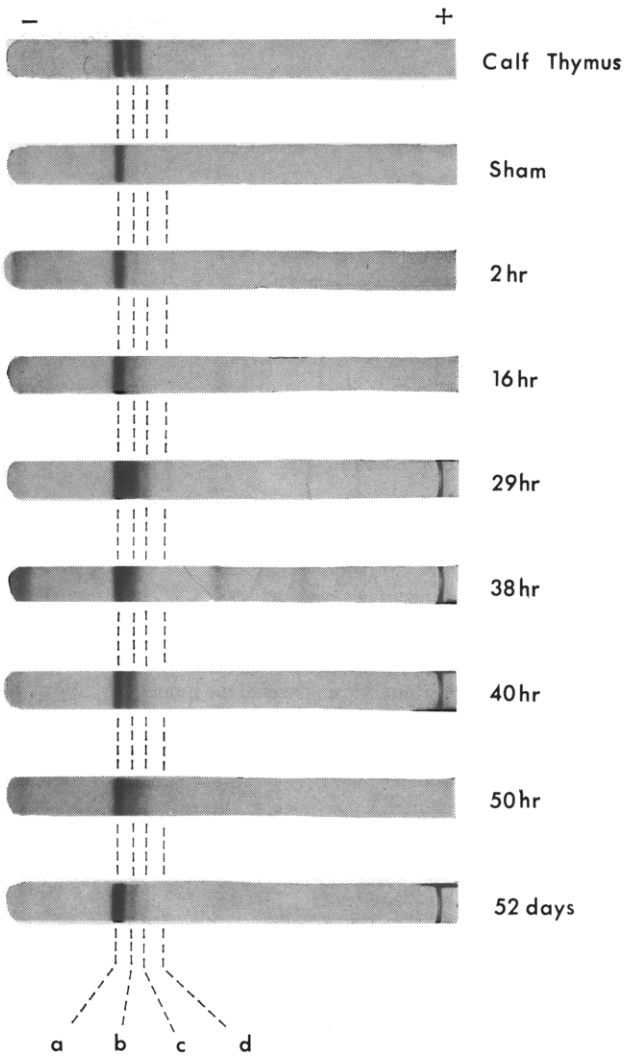

FIGURE 2: Changes in the electrophoretic pattern of lysine-rich rat liver histone during regeneration. Electrophoresis was performed at 4° for 40 hr at 2 mA/gel and 200 V.

Schwarz (0.1% Amido-Schwarz, 7% acetic acid, and 20% ethanol). After destaining, the gels were scanned at 600 mμ in a Gilford microdensitometer, Model 2000, and the scans analyzed on a DuPont curve resolver.

Radioactive Labeling of Histones. Phosphorylated histones were labeled by injecting partially hepatectomized rats intraperitoneally with 300 μCi of ^{32}P (phosphoric acid, New England Nuclear Corp., neutralized in 0.01 M Tris buffer, pH 8.0) at 18, 12, and 6 hr prior to removal of the regenerating liver. After electrophoresis for 24 hr on 25-cm gels at 25°, 3-mm sections of the gels containing the lysine-rich histone subfractions were dried and counted in a Nuclear-Chicago biospan planchet counter, Model 4338 (40% efficiency). The method used to determine the amount of protein in each subfraction has been described previously (Sherod *et al.*, 1970).

Dephosphorylation of Histones. Incubation of lysine-rich histone with *Escherichia coli* alkaline phosphatase (Worthington Biochemical Co.) was performed in 0.01 M Tris-HCl buffer (pH 8.2). Approximately 1 mg of enzyme was used per 15–25 mg of histone of which half the enzyme was added initially and the remainder 12 hr later. The reaction was terminated after 24 hr by the addition of glacial acetic acid and sucrose to final concentrations of 0.9 M and 15%, respectively.

Purification of Lysine-Rich Histone. Chromatographic purification of lysine-rich histone was carried out on Amberlite IRC-50 (1 × 20 cm, preequilibrated with 3% sodium chloride–0.1 M Tris-HCl, pH 8.2). The runoff impurity peak was eluted with 3% sodium chloride–0.1 M Tris (pH 8.2) prior to elution of the lysine-rich histone with 9% sodium chloride. Protein elution was monitored at 230 mμ. Fractions containing purified lysine-rich histone were pooled and precipitated with 20% trichloroacetic acid.

Hydrolysis of Lysine-Rich Histone and Isolation of Phosphorylated Amino Acids. Purified ^{32}P-labeled lysine-rich histone was hydrolyzed enzymatically using a combination of pronase (Calbiochem) and hog kidney leucine aminopeptidase (Worthington Biochemical Co.) following a modified method of that described by Hill and Schmidt (1962). Lysine-rich histone (1 mg) was digested for 24 hr at 37° in 0.25 M Tris (pH 7.8) and 9×10^{-4} M calcium chloride with 0.25 mg of Pronase. After heat denaturation of the Pronase at 100° for 15 min and subsequent cooling to room temperature, the enzymatic digestion was continued by adding a solution of activated leucine aminopeptidase (0.4 ml, 0.5 mg/ml) which in addition contained Tris-HCl buffer (0.5 M, pH 8.5), 0.10 ml; manganese chloride (0.025 M), 0.10 ml; and water, 2.00 ml. Activation was carried out at 40° for 3 hr. The mixture was incubated at 37° for 96 hr. The enzymes themselves contain no serine or threonine phosphate, nor was any phosphatase activity present in control studies. Control experiments with serine phosphate indicated that the ester is stable to this treatment.

After enzymatic digestion was complete, 2.5 μmoles of

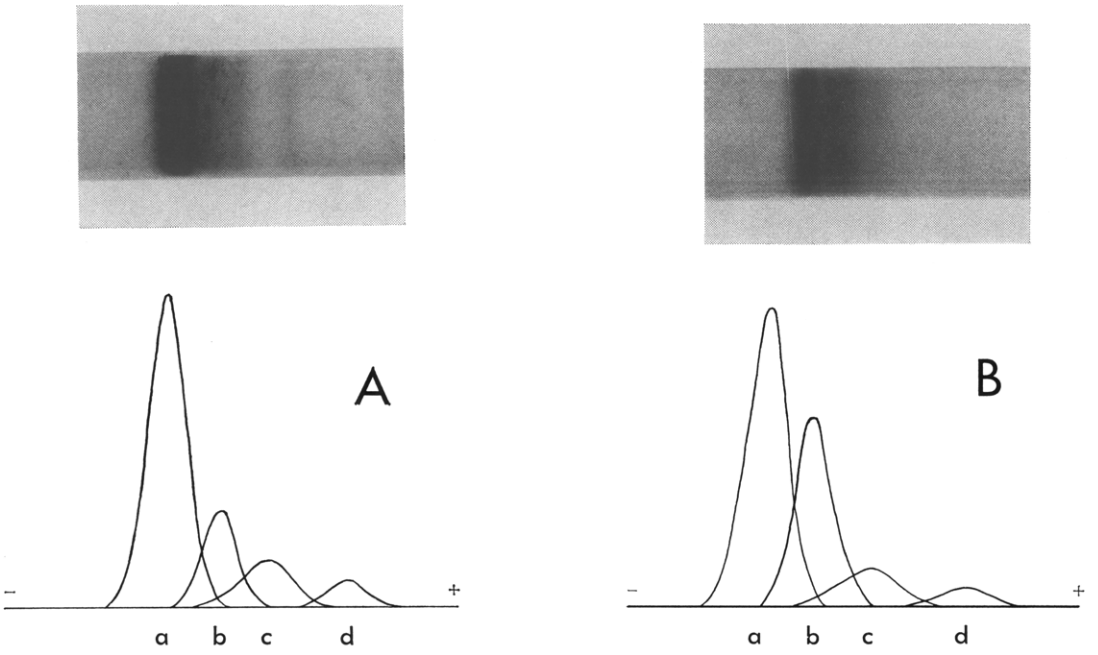

FIGURE 3: Microdensitometric analysis of the lysine-rich subfractions. (A) Sham operated liver F_1 and (B) regenerating liver F_1.

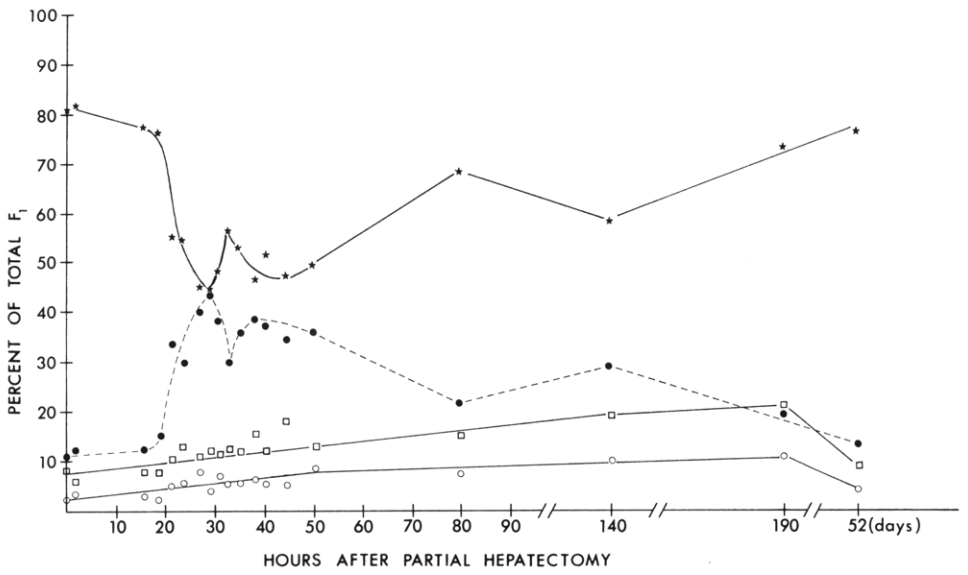

FIGURE 4: Changes in the lysine-rich histone subfractions during rat liver regeneration. Subfractions plotted are a (*), b (●), c (□), and d (○).

unlabeled *dl*-phosphoserine was added and the mixture chromatographed on a Dowex 50-8X column (1 × 41 cm, preequilibrated with 0.05 N HCl), eluting with 0.05 N hydrochloric acid. Samples (100 μl) of the neutralized effluent fractions were added to Bray's solution (10 ml) and the ^{32}P counted at 93% efficiency in a Unilux III Nuclear-Chicago scintillation counter. Total organic phosphate determinations were performed on a 1-ml aliquots of every other Dowex 50 fraction by the method of Bartlett (1959) and inorganic phosphate assayed by the method of Lowry and Lopez (1946). Ninhydrin assays for amino acids were performed on 0.5-ml aliquots of the column fractions using the method of Rosen (1957). DNA was determined by reaction with diphenylamine (Burton, 1956), and RNA by reaction with orcinol (Schneider, 1957).

Results

Variations in Histone from Regenerating Rat Liver Following Partial Hepatectomy. The electrophoretic patterns of whole histone extracted from sham operated and partially hepatectomized rats at various time intervals following hepatectomy are shown in Figure 1. These gels, run for 24 hr, all show similar patterns and in general histone fractions other than the lysine-rich fraction do not show variations as a function of the time after partial hepatectomy. The acetylation-induced microheterogeneity of fractions F_3 and F_{2a1} seems to be fairly constant, though of course any changes in the rate of turnover of acetate groups on a parent molecule would not be detected in this system and we can only comment on the absolute amounts of a histone and its acetylated deriva-

TABLE I: F_1 Histone During Rat Liver Regeneration.[a]

Hr after Partial Hepatectomy	% of Whole Histone[b]
Sham	20.8
38	19.2
50	22.5
142	22.3

[a] Quantitation for samples electrophoresed 16 hr. [b] Calculated using DuPont curve resolver.

tives. In contrast, it is clear that changes are occurring in the F_1 band. In the sham-operated control and also 52 days after partial hepatectomy the lysine-rich histone consists of three minor bands and a major faster moving band. However, beginning at 16 hr and more obviously in the period 29–40 hr after hepatectomy the faster moving band becomes much less sharply resolved and, in these gels, appears to overlap the minor bands.

Heterogeneity within the Lysine-Rich Fraction During Regeneration. To improve the resolution of the F_1 fraction, the samples were electrophoresed for 40 hr. It is apparent from the electrophoretic patterns of the lysine-rich histone fractions, shown in Figure 2, that the amount of histone present in the various subfractions changes during the course of regeneration. This shift is largest at 29 hr after operation and is shown in Figure 3 along with microdensitometric quantitation of the various lysine-rich bands. The effect of partial hepatectomy upon the electrophoretic pattern of liver lysine-rich histone is to increase the amount of subfraction b relative to the faster moving subfraction a. Quantitation of the amount of protein present in each of the resolved subfractions of the lysine-rich histone at frequent time intervals during regeneration was performed in the same way and the results are documented in Figure 4. Subfraction d appears to remain relatively constant in amount throughout the period of regeneration. The next more rapidly migrating subfraction, c, also appears to change little, possibly increasing only slightly during the same interval in which the next, b, increases substantially. The increase in the amount of subfraction b occurs approximately 18 or 19 hr after partial hepatectomy, reaching a first maximum near 29 hr. The amount of histone in subfraction b then appears to decrease rapidly until 33 hr when it begins to increase once more, reaching a second maximum near 38 hr, after which time it again decreases. Subfraction a, in a manner complementary to subfraction b, decreased during the same time intervals when subfraction b increased. Each maximum and minimum point in the graph represents an average of at least three experiments, and in all cases the standard deviation was of the order of ±2%, indicating that the changes observed are indeed significant. That fractions a–d are all members of the lysine-rich histone group is deduced from their electrophoretic mobility and confirmed by the observation that each fraction is quantitatively extracted into 5% perchloric acid, a characteristic of the lysine-rich group of histones.

Total Amount of the Lysine-Rich Histone Is Constant During Regeneration. In order to accurately assess any change within subfractions of the lysine-rich fraction, F_1, we wondered if the total amount of lysine-rich histone varies during the course of regeneration. Electrophoretic patterns of histone

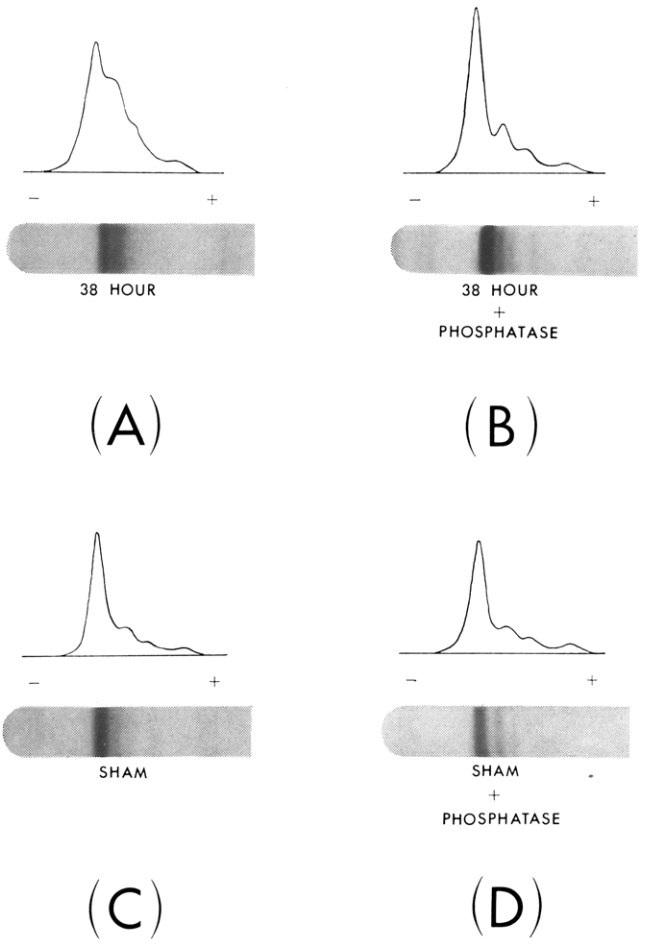

FIGURE 5: Phosphatase treatment of lysine-rich histones. Phosphatase treatment was performed as described in Materials and Methods. (A) Untreated 38-hr regenerating liver F_1; (B) phosphatase treated 38-hr regenerating liver F_1; (C) untreated sham-operated liver F_1; and (D) phosphatase-treated, sham-operated liver F_1.

on polyacrylamide gels from sham-operated and partially hepatectomized rats at various time intervals during regeneration were scanned and the curves electronically analyzed. The results are shown in Table I. The percentage of F_1 relative to the rest of the histone complement varies from 19.2% at 38 hr after partial hepatectomy to 22.5% in the 50-hr systems. This represents a difference of only 3% of total histone and it is unlikely that this change is significant.

^{32}P Incorporation into the Lysine-Rich Subfractions. We were curious as to the nature of the increases in amount of the subfractions b and c and we decided to test whether they were due to the phosphorylation of a parent lysine-rich histone subfraction a. [^{32}P]Phosphoric acid was injected into both sham-operated and partially hepatectomized rats (29 hr) as described in Materials and Methods. The lysine-rich histone subfractions after electrophoresis for 24 hr at 25° were cut out of the gel and counted. The specific activity of each subfraction is presented in Table II. Subfractions a, b, and c in the sham-operated animals have very low specific activities, the more slowly migrating subfractions, in general, having higher specific activity. Dramatically increased specific activities for subfractions b, c, and d were observed in the 29-hr regenerating system. Again, the more slowly migrating subfractions had the highest specific activities.

Enzymatic Dephosphorylation. Incorporation of [^{32}P]phos-

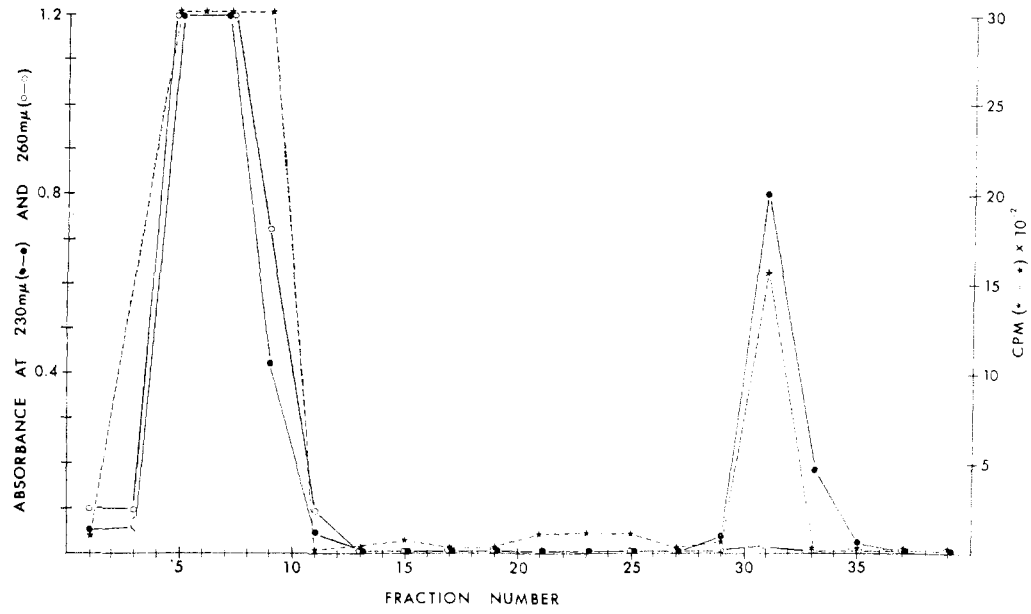

FIGURE 6: Purification of lysine-rich histone. Regenerating rat liver lysine-rich histone (38 hr) was chromatographed on IRC-50 resin as described in Materials and Methods. The runoff peak was eluted with 3% sodium chloride–0.1 M Tris (pH 8.5) prior to elution of the histone with 9% sodium chloride–0.1 M Tris (pH 8.5.)

phate into the lysine-rich histone subfractions does not unequivocally demonstrate phosphorylation of serine or threonine residues within the protein, since it has been shown that phosphate binds quite tightly to the lysine-rich histone (D. Sherod, J. Bartley, and R. Chalkley, unpublished results). It was necessary, therefore, to demonstrate a covalent binding, and we have exploited enzymatic dephosphorylation with *E. coli* alkaline phosphate as described by Sherod *et al.* (1970).

The gel patterns obtained following treatment of the lysine-rich histone from 38-hr regenerating rat liver and control liver with alkaline phosphatase are shown in Figure 5. Microdensitometric scans of these gels demonstrate a dramatic decrease in subfraction b and a concomitant increase in subfraction a in the 38-hr regenerating liver lysine-rich histone after treatment of the labeled lysine-rich histone with phosphatase resulted in the complete removal of label from all of the subfractions.

Purification of the Lysine-Rich Histone. The final proof for phosphorylation of an amino acid residue in the F_1 subfractions and a demonstration of the validity of the electrophoretic analysis coupled with dephosphorylation, as a tool for analyzing for histone phosphorylation, demands the isolation and identification of the phosphorylated amino acid. Frequently the identification of phosphate or ^{32}P radioactivity in 5% perchloric or trichloroacetic acid extracted lysine-rich histone has been used as an indication of phosphorylation, assuming that the specificity of extraction would yield a pure protein fraction. Shepherd *et al.* (1970), on the other hand, proposed that some of the phosphate found in histones might be due to nonspecific binding of nucleotides or oligonucleotides extracted from RNA. It is extremely important, therefore, that lysine-rich histone used in phosphate determinations as well as for serine or threonine phosphate identification be pure. Since the most likely ^{32}P-containing contaminant is negatively charged, we have exploited chromatography on a cation-exchange resin, IRC-50. The specific activities of control and regenerating rat liver lysine-rich histone as directly isolated are in the ratio 1:4 as shown in Table 3 (Ord and Stocken (1969), report 1:2.5). However, after chromatography on IRC-50 (Figure 6) it is seen that the large bulk of the radioactivity is not histone bound, but rather associated with the runoff peak. The specific activity ratios of the purified lysine-rich fractions are in the ratio of 1:7. Analysis of the highly labeled runoff peak confirmed the presence of inorganic phosphate, ribonucleotides, and a small amount of deoxyribonucleotides. Thus the extent of lysine-rich histone phosphorylation using histone isolated by the method of Johns (1964) cannot be computed without employing additional purification procedures. That the specific activity of the impure histone from regenerating liver is fourfold higher than that of control is presumably a reflection of

TABLE II: ^{32}P Incorporation into F_1 Histone Fractions.

Hr after Partial Hepatectomy	Specific Radioactivity[a]			
	a	b	c	d
Sham	75	199	230	*b*
29	12	576	2100	4275
29 + phosphatase	*b*	*b*	*b*	*b*

[a] Counts per minute per milligram of protein. [b] Not detectable.

TABLE III: Specific Activity of ^{32}P-Labeled F_1 Histone (cpm/mg).

Unpurified sham F_1	39,600
Column purified sham F_1	1,642
Unpurified 29-hr F_1	139,000
Column purified 29-hr F_1	11,357

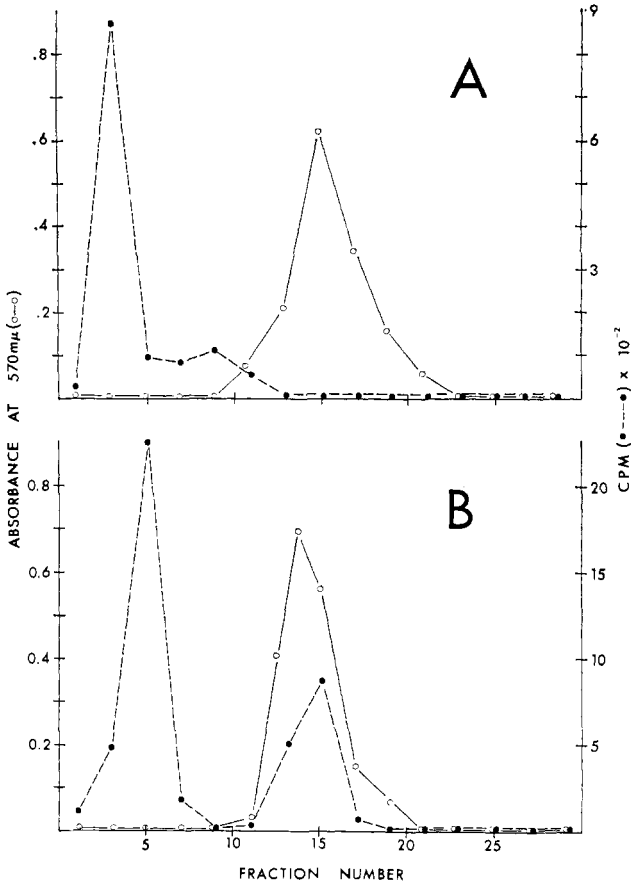

FIGURE 7: Isolation of amino acid–phosphate. The enzymatic hydrolysates of lysine-rich histone were chromatographed on Dowex 50 as described in Materials and Methods. Serine phosphate was detected by ninhydrin assay. The eluent was 0.05 N hydrochloric acid. (A) Sham-operated F_1 and (B) 38-hr F_1.

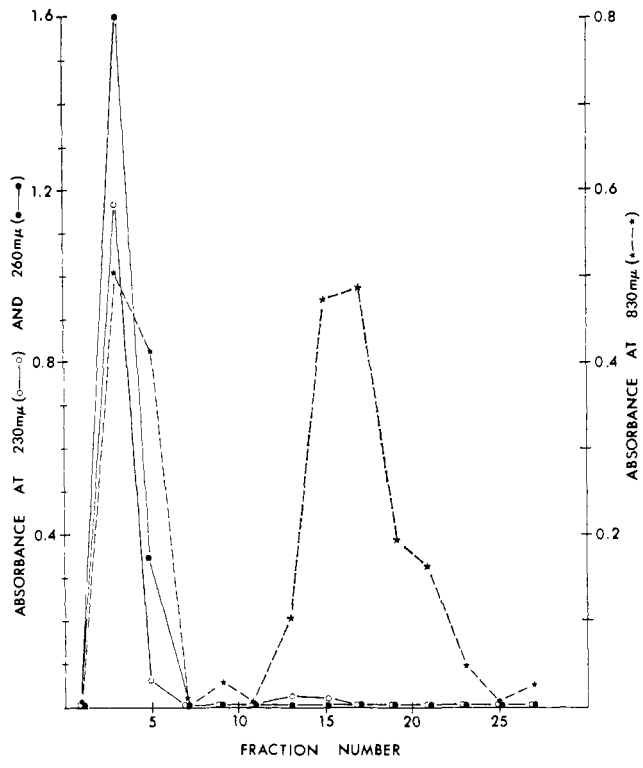

FIGURE 8: Chromatography of serine phosphate and nucleotides on Dowex 50. Total phosphate was determined by the Fisher–Subbarow assay (*). Nucleotides were detected by absorbance at 230 mμ (○) and 260 mμ (●). The eluent was 0.05 N hydrochloric acid.

increased synthesis during regeneration of the RNA and DNA which contaminate histone; and the earlier, correct conclusion that the lysine-rich histone itself is more highly phosphorylated in the regenerating liver (*in vivo*) appears to have been purely fortuitous.

Separation and Identification of the Phosphorylated Amino Acids. Column purified liver lysine-rich histones were enzymatically hydrolyzed as described in Materials and Methods and subsequently chromatographed on Dowex 50 with unlabeled serine phosphate. A runoff peak containing a substantial amount of the ^{32}P label emerged in fractions 4–8 from both sham-operated and regenerating liver (Figure 7A,B). This peak contained inorganic phosphate but no detectable ribo- or deoxyribonucleotides, it did not react with ninhydrin, nor did it contain serine phosphate. The remaining label elutes with serine phosphate in fractions 12–21 in the regenerating liver F_1 hydrolysate, indicating the presence of serine or threonine [^{32}P]phosphate (the only amino acids that elute at this point (Engström, 1961). However, no detectable radioactivity was eluted at this point for the sham operated F_1 hydrolysate.

To demonstrate that the label eluting with serine phosphate could not be labeled nucleotides (Shepherd *et al.*, 1970), a mixture of serine phosphate and several nucleotides was chromatographed on the Dowex 50 column. Two phosphate peaks eluted (Figure 8). One, the runoff peak, contained all the nucleotides while the other contained only serine phosphate.

Discussion

The phosphorylation of lysine-rich histone from regenerating rat liver has been demonstrated by two independent means. The more direct approach consisted of the isolation of ^{32}P-labeled phosphorylated amino acids from the lysine-rich fraction. The other approach exploited the decrease in overall positive charge on a histone following phosphorylation, the ability to detect the decrease in electrophoretic mobility of the new species, and the observation that the shift in mobility due to phosphorylation could be abolished by a prior incubation with alkaline phosphatase. The latter approach permits us to quantitate the net shift from unphosphorylated histone to the phosphorylated form, whereas the former confirms that the shifts we observe in the gels are indeed due to phosphorylation of amino acids in the lysine-rich histone.

Thus, exploiting both approaches permits us to arrive at an estimate of the extent of the increase in phosphorylation of rat liver lysine-rich histone following partial hepatectomy. It is clear that the amount of phosphorylated histone in the normal liver is very low (Figure 6 and Table IV). Apparently turnover of histone phosphate in normal liver (if it occurs at all) is also low (Langan, 1969) as essentially no radioactivity was found in serine or threonine phosphate isolated from purified lysine-rich histone of control livers even 18 hours after a repeated injection of radioactive ^{32}P. This result agrees well with the direct electrophoretic data which shows that very little of the lysine-rich histone (<5%) moves with mobility less than the major band, and further, that incubation with phosphatase did not effect the electrophoretic pattern (Figure 6). Presumably the heterogeneity of the F_1 histone in normal liver or in regenerating liver after phosphatase

TABLE IV: Phosphate Associated with Lysine-Rich Histone.

Sample	Moles of Phosphate/ Mole of F_1
Unpurified sham-operated liver F_1	2.7
Purified sham-operated liver F_1	0.11
Unpurified 29.5-hr regenerating liver F_1	4.5
Purified 29.5-hr regenerating liver F_1	0.77

treatment is a reflection of the presence of several different lysine-rich histone molecules of slightly different sequence in agreement with the observations of Bustin and Cole (1968). About 20 hr after partial hepatectomy both methods indicate that a dramatic increase in phosphorylation has begun. Because of the low level of phosphorylation in normal liver, it is difficult to assay the precise extent of the increase, but after consideration of the sensitivity of the two methods, we estimate that the increase is greater than tenfold (Figures 4 and 6, and Table II). This result is not in full agreement with previous reports which have indicated that hepatectomy induces a 2.5-fold increase in the phosphorylation of lysine-rich histone from an already substantial level existing in control liver. However, we have documented that this conclusion was mostly due to the substantial contamination of the lysine-rich histone by nucleotides and inorganic phosphate when isolated by standard methods (Johns, 1964). Furthermore, we have recently found that the lysine-rich histone binds inorganic phosphate more tightly than any other histone, although it can be removed by repeated chromatography or electrophoresis.

The onset of phosphorylation is not accompanied by a net increase in the amount of lysine-rich histone relative to the other four histone fractions. It appears, therefore, that the phenomenon involves simply a shift in the amounts of the various types of lysine-rich histone (phosphorylated or otherwise) while the amount of the whole is constant. Evidently the factors which regulate lysine-rich histone synthesis are not sensible to the subsequent phosphorylated state of the molecule.

Examination and quantitation of changes in the F_1 subfractions throughout regeneration has revealed several points. A large, sudden increase in phosphorylation of the lysine-rich histone occurs approximately 18–19 hr after partial hepatectomy, as demonstrated by the uptake of ^{32}P label and the increase in protein in subfraction b. This corresponds quite closely with the onset of DNA synthesis (Cater *et al.*, 1956a; Holbrook *et al.*, 1962; Hecht and Potter, 1956; Bucher *et al.*, 1964). A phosphorylation peak is reached at 29 hr, coincident with the first wave of mitosis. In the next four hours there is a sharp decrease in phosphorylation which is followed by a second rapid increase in phosphorylation at approximately 33 hr, synchronous with a second round of DNA synthesis (Cater *et al.*, 1956a,b; Holbrook *et al.*, 1962; Verbin *et al.*, 1969; Bucher, 1963), which peaks at 38–40 hr, just prior to a second, smaller wave of mitosis (Cater *et al.*, 1956a,b; Holbrook *et al.*, 1962; Verbin *et al.*, 1969; Bucher, 1963). The second peak in phosphorylation is smaller and broader than the first. This is a typical characteristic of mitotic activity plots observed in cell systems immediately following synchronization. The electrophoretic pattern of the lysine-rich histone returned to normal 52 days after hepatectomy, at which time the more usual low mitotic and DNA synthetic activity of the liver was observed. Thus, it appears as though both DNA synthesis and phosphorylation of the lysine-rich histone occur at approximately the same time. We have recently examined the lysine-rich histone from a rapidly growing rat hepatoma and have found a substantial increase in phosphorylation over that found in normal liver. We are currently examining the degree of F_1 phosphorylation in a collection of rat and mouse tumors of different types and growth rates to assay further the correlation between the rate of DNA synthesis and lysine-rich histone phosphorylation.

During the preparation of this manuscript Sung, Smithies, and Dixon reported that during the regeneration of rat liver there is a substantial phosphorylation of histone F_{2b}. We have not assayed other fractions in this study, however, it is of interest that in a study of phosphorylation of histones in a rapidly dividing ascites tumor cell line (Sherod *et al.*, 1970) we have reported phosphorylation of fraction F_{2a2} which has chromatographic properties on an ion-exchange resin (GC-50) very similar to those of F_{2b}. Furthermore, the phosphorylated form of F_{2a2} coelectrophoreses with F_{2b}. Gurley and Walters (1971) also report a specific phosphorylation of F_1 and F_{2a2} in a Chinese hamster tumor cell line.

Acknowledgments

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Degradation of 5′-Deoxyadenosylcobalamin by Ribonucleoside Triphosphate Reductase and Binding of Degradation Products to the Active Center*

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ABSTRACT: In the presence of ribonucleotide reductase, dihydrolipoate, and dGTP, deoxyadenosylcobalamin undergoes a spectrum change consistent with formation of cob(II)alamin (B_{12r}). No other cobamide accumulates in significant amounts. After removal of dihydrolipoate and exposure to air, aquocobalamin and 5′-deoxyadenosine were identified as reaction products. Treatment of the reaction mixture with iodoacetamide, methyl iodide, and acetylene yielded carboxamidomethylcobalamin, methylcobalamin, and vinylcobalamin, respectively. Yields were never greater than expected from reaction with cob(I)alamin formed from accumulated cob(II)alamin. Iodoacetamide also yielded 3-carboxamidomethyl-5,6-dimethylbenzimidazolyl-*Co*-5′-deoxyadenosylcobamide. Cob(II)alamin formed from aquocobalamin and dihydrolipoate was poorly bound to ribonucleotide reductase except in the presence of both dGTP and 5′-deoxyadenosine (dissociation constant 10.5 ± 1.9 μM). This accounts in part for the high resolution of the electron spin resonance spectrum of cob(II)alamin observed only in presence of enzyme, 5′-deoxyadenosine, and dGTP. dGTP and cob(II)alamin enhance the binding of 5′-deoxyadenosine to the enzyme severalfold. Binding of both cob(II)alamin and 5′-deoxyadenosine to the site for 5′-deoxyadenosylcobalamin is indicated by linear competitive inhibition of ribonucleotide reduction by 5′-deoxyadenosine and cob(II)alamin *vs.* 5′-deoxyadenosylcobalamin. $K_{i,app}$ for 5′-deoxyadenosine in the presence of 10 μM cob(II)alamin is 14 μM; $K_{i,app}$ for cob(II)alamin in the presence of 50 μM 5′-deoxyadenosine is 3 μM.

Recently it has been shown (Hamilton *et al.*, 1971) that 5′-deoxyadenosylcobalamin is slowly degraded by the ribonucleoside triphosphate reductase of *Lactobacillus leichmannii* in the presence of the reducing substrate (*e.g.*, dihydrolipoate) and a nucleoside triphosphate activator such as dGTP. One of the degradation products is a paramagnetic cobamide with an electron spin resonance (esr) spectrum identical with that of cob(II)alamin, except that it shows unique resolution. On this evidence the cobamide degradation product was tentatively identified as enzyme-bound cob(II)alamin, presumably formed by degradation of a reactive cobamide intermediate. It is postulated that this intermediate is formed by the interaction of the reducing substrate and deoxyadenosylcobalamin at the active center of the enzyme.

The unique resolution of the cob(II)alamin esr spectrum is interpreted to mean that the unpaired electron on the cobalt atom experiences a relatively constant magnetic field, firstly because the cob(II)alamin is bound to the active center, and secondly because the conformation of the active center is determined by the nucleoside triphosphate activator. Supporting evidence was provided by the observation that although all nucleoside triphosphates determined a similar highly resolved high-field part of the cob(II)alamin spectrum, different nucleotides determined somewhat different spectra in the low-field region. Furthermore, cob(II)alamin formed nonenzymically gave the high-resolution esr spectrum when added to the reductase provided dihydrolipoate, a nucleoside triphosphate, and 5′-deoxyadenosine were also present. It is assumed the 5′-deoxyadenosine causes the appearance of the highly resolved cob(II)alamin spectrum because it binds to the active center with consequent determination of the conformation of that part of the protein. The requirement for added 5′-deoxyadenosine for high resolution with nonenzymically formed cob(II)alamin but not with cob(II)alamin formed by degradation of deoxyadenosylcobalamin suggests that 5′-deoxyadenosine is formed in the latter degradation.

In this report further evidence is presented that the paramagnetic cobamide is indeed cob(II)alamin, that 5′-deoxyadenosine is the other degradation product, and that dGTP and 5′-deoxyadenosine increase binding of cob(II)alamin to the enzyme but probably also determine the conformation of the active site.

Materials

Most of the materials were the same as those previously described (Hamilton *et al.*, 1971). *Lactobacillus leichmannii* was obtained as a frozen paste from Grain Processing Corp., Muscatine, Iowa, and early stages of the preparation of ribonucleotide reductase were carried out on a large scale

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