ybicyclo[3.3.1]nonane-1,*exo*-3-dicarboxylic acid on the chair transition state structure shown in Figure 6. Both of these superimpositions provide maximum overlap between corresponding atoms in the two structures (average separation 0.20 Å), particularly in the ring carboxyl and hydroxyl groups. It is clear, however, that good superimposition of the hydrocarbon backbone, as in Figure 6a, is incompatible with reasonable placement of the side-chain carboxyl group, as in Figure 6b. Molecules with better alignment of the side-chain carboxyl group are therefore required to obtain maximum inhibition.

Because of the nature of the partial bonds in the transition state, correct alignment of the side-chain carboxyl group may prove to be impossible. Efforts to design better inhibitors are nevertheless warranted, particularly in view of the recent observation (Danilenko et al., 1975) that adamantane-1-carboxylic acid and adamantane-1-acetic acid display broad spectrum bacteriostatic activities at concentrations similar to those found here to inhibit chorismate mutase. It is likely that 4-hydroxyadamantane-1-carboxylic acid, which combines the best features of the present series of inhibitors, but which lacks the second acidic moiety, would provide a sound starting point in the search for better inhibitors.

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Phosphorylation of Nuclear Proteins in Rat Regenerating Liver†

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ABSTRACT: In studies of the phosphorylated proteins in rat liver and Walker-256, it was established that the ratio of various fractions of P–N linkages to P–O linkages varies from 0.6 to 3.1. In rat regenerating liver nuclei, the ratio of P–N and P–O varies with time after partial hepatectomy. Using [^{3}H]-lysine and $^{32}P_i$, it is shown that phosphoryllysine forms in some new and, presumably, some preexisting H1 molecules. Using [^{3}H]histidine and $^{32}P_i$, it is shown that phosphohistidine forms exclusively in preexisting H4. The half-life of H4 phosphohistidine appears to be about 2 h.

Two types of phosphorylation are known to occur in histones; one on hydroxy amino acids forming P–O linkages (Ord et al., 1975), and the other on basic amino acids forming P–N linkages (Chen et al., 1974). The study of the P–O linkages (phosphoserine and phosphothreonine) in histones, which are commonly extracted under acidic conditions, has been facilitated by the acid-stable nature of these linkages. This is reflected by the large amount of experimental data collected on P–O linkages in histones (Ord et al., 1975). The P–N linkages, phosphohistidine, phospholysine, and phosphoarginine (Smith et al., 1976; Bruegger, 1977), are acid-labile and have been demonstrated in phosphorylated myelin basic protein and from chromosomal proteins isolated under neutral or basic conditions. This paper attempts to determine the relative contribution of P–O and P–N linkages to the phosphorylation of the nuclear proteins extracted at various time intervals from rat regenerating liver. H1 and H4 that have been isolated from rat regenerating liver have been shown to contain P–N linkages (Chen et al., 1974). A determination of whether these P–N linkages are formed on old, preexisting H1 and H4 or on new, de novo synthesized H1 and H4 during rat liver regeneration is presented. In addition, we also examined the turnover of the P–N linkage in H4 during rat liver regeneration.

Materials and Methods

Chemicals. [^{3}H]Lysine, [^{3}H]histidine, and carrier-free $^{32}P_i$ were purchased from ICN Pharmaceuticals. Trypsin and Pronase were purchased from Calbiochem. Whole histone was obtained from Sigma Chemical Co. Phospholysine and phosphohistidine were prepared as described previously (Chen et al., 1974).

Preparation of Regenerating Rat Liver. Female Sprague-Dawley rats, 250–300 g, underwent partial hepatectomy by

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the method of Higgins and Anderson (1931). Sixteen hours after partial hepatectomy, the rats were injected intraperitoneally with 5 mCi of [^{3}H]amino acid in 0.9% NaCl. Two hours later 10 mCi of $^{32}P_i$ in 0.9% NaCl were injected intraperitoneally. Ninety minutes after the second injection, the rats were sacrificed and the livers were removed and immediately frozen on dry ice.

In the study of the P–O linkages in nuclear proteins, $^{32}P_i$ at a dose of 2 mCi/100 g of body weight was injected intraperitoneally and also into the portal vein 1.5 h before the regenerating livers were collected. To study the turnover of P–N linkages in H4 18 h after partial hepatectomy, the rats were injected intraperitoneally with $^{32}P_i$ at a dose of 2 mCi/100 g of body weight. Livers were removed from groups of four rats, at 2-h intervals, beginning 19.5 h after partial hepatectomy.

Analysis of the P–O and P–N Linkages in Nuclear Proteins. Two methods were used to determine the P–O and P–N linkages in nuclear proteins.

(1) Two-phase Aqueous Polymer Extraction (Smith, 1974; Simon and Becker, 1976). A two-phase polymer solution called polymer I was prepared by mixing 30 g of 20% (w/w) dextran with 30 g of 40% (w/w) polyethylene glycol-6000 and 40 g of 0.01 M sodium phosphate (pH 7). Each tissue fraction to be tested was mixed well with polymer I and enough NaCl to give a final concentration of 4.5 M. The top phase was removed and set aside and the bottom phase was reextracted with an equivalent volume of fresh top phase. After complete mixing and centrifugation, the top phase was removed and pooled with the first top phase. The pooled top phase solution was dialyzed twice against 100 volumes of distilled water (to remove inorganic phosphate) and treated with hot 5% trichloroacetic acid to remove acid-labile phosphates. The solution was then subjected to a phosphate assay by the method of Sugino and Miyoshi (1964). The inorganic phosphate, precipitated as a phosphomolybdate complex, was used as a measure of acid-labile phosphate, while the supernatant contained the acid-stable phosphate still attached to proteins.

(2) Phenol Extraction Method. Using the Boyer and Bieber (1967) method, 2 mL of the nuclear protein fraction, which was prepared as described previously (Chen et al., 1974; Chauveau et al., 1956), were mixed with 10 mL of phenol solution. Ten milliliters of a wash buffer, 0.01 M Na_2EDTA, 0.01 M $Na_4P_2O_7$, 0.01 M sodium phosphate buffer, pH 8.3, were added to the phenol–nuclear fraction solution, and the mixture was shaken and then centrifuged at 2500 rpm in a swinging bucket centrifuge to facilitate separation of the phases which consisted of a lower phenol layer containing protein and an upper aqueous phase containing some of the DNA (the rest of the DNA appeared as insoluble debris at the interface). The aqueous layer and interface material were discarded, and the extraction of the phenol layer with the wash buffer was repeated once more. Finally, the proteins were precipitated from an aliquot of the phenol layer using 5 volumes of acetone, and the precipitate was washed once with acetone. The dried precipitate was treated with 5 mL of a 0.3 N Cl_3CCOOH–0.001 M Na_2HPO_4 solution, heated at 60 °C for 5 min, cooled, and centrifuged at 6000*g* for 10 min. The $^{32}P_i$ in the supernatant represented the contribution of the P–N linkages. The contribution from P–O linkages was measured as follows. Acetone was used as before to precipitate the protein from another aliquot of the phenol layer. The dried precipitate was treated with 5 mL of a 0.5 N NaOH solution, heated at 60 °C for 5 min, cooled, and centrifuged at 6000*g* for 10 min. The ^{32}P in the supernatant represented the contribution of the P–O linkages.

Isolation of the Amino Acids from the Phosphorylated Histones of Rat Regenerating Liver. From the excised livers, nuclei were isolated, the base-treated chromosomal proteins fractionated, and phosphorylated H1 and H4 isolated on a Bio-Rex 70 column as described earlier by Chen et al. (1974). The isolated histones were suspended in 0.1 M Tris[1]-HCl, pH 8.0, and incubated at 37 °C for 2 h with trypsin at a weight ratio of 100:1 (histone to enzyme). After the sample was heated at 60 °C for 15 min to denature the trypsin, the sample was cooled and incubated with 25 units of Pronase for 24 h at 37 °C.

After the proteolytic enzyme treatment of the phosphorylated H4, the amino acids released were isolated by ascending paper chromatography (Zetterqvist and Engstrom, 1966) on Whatman 3MM paper in ethanol–2-propanol–water–triethylamine (30:30:39:1). The amino acids resulting from the proteolytic treatment of phosphorylated H1 were isolated by high-voltage paper electrophoresis on Whatman 3MM paper with 0.001 M EDTA, 0.1 M ammonium acetate, pH 8.25, for 30 min at 57 V/cm in a water-cooled apparatus. The paper chromatograms were cut into strips which were analyzed for their ^{3}H and ^{32}P content in a Beckman LS-100 liquid scintillation counter.

TABLE I: Ratio of Acid-Labile Phosphate to Acid-Stable Phosphate in Nuclei and Nuclear Protein Fractions.

	Ratio of acid-labile phosphate to acid-stable phosphate[a]			
Tissue	Nuclei	Acidic protein	Deoxynucleoprotein complex	Nucleoplasm
Walker-256	0.6 ± 0.1	1.2 ± 0.5	1.0 ± 0.5	1.3 ± 0.1
Rat liver	1.7 ± 0.4	3.1 ± 0.8	1.4 ± 0.4	0.6 ± 0.1

[a] The values include the square root of the variance using determinations from each of the two methods (two-phase system and phenol extraction).

Results

Comparison of Acid-Labile and Acid-Stable Phosphoprotein Phosphates in Various Cell Fractions of Rat Liver and Walker-256 Carcinosarcoma. The nuclei were isolated from liver and Walker-256 tumor of rats that had been injected with ^{32}P inorganic phosphate. The nuclei were fractionated by the method of Wang (1967) into three fractions. Each of these fractions and whole nuclei were examined for the presence of acid-labile and acid-stable phosphoprotein phosphates by two independent phosphoprotein isolation procedures: a phenol extraction procedure and a two-phase aqueous polymer extraction procedure. The results of these experiments are shown in Table I.

Relative Contribution of P–O and P–N Linkages to Phosphorylation of Nuclear Proteins from Rat Regenerating Liver. The results shown in Figure 1 reveal that total phosphorylation of nuclear proteins in rat regenerating liver changes little up to 18 h and then decreases to 24 h approximately coincident with the first wave of cell division, and then a marked increase up to 42 h is seen. Further analysis of the phosphoryl groups shows that the ratio of acid-labile (P–N) to acid-stable (P–O) linkages ranges from 0.5 to 4.5.

The Formation of P–N Linkages in H1 and H4 during de Novo Synthesis of Histones. Histone synthesis in rat regenerating liver and in other systems has been shown to be tightly

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

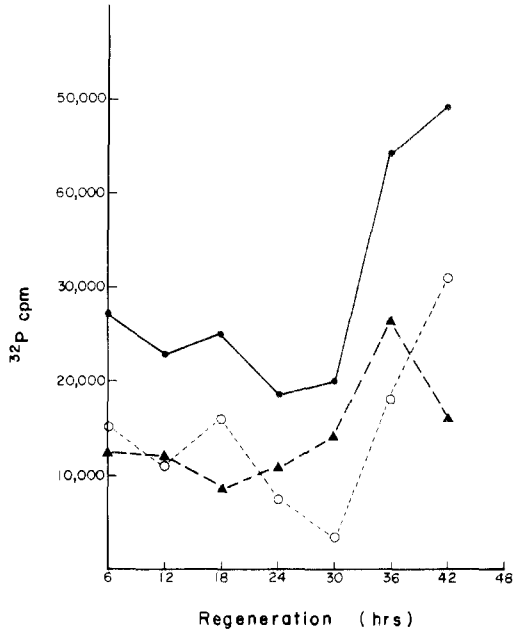

FIGURE 1: The ^{32}P content of the nuclear proteins from rat livers removed at various time intervals after partial hepatectomy. The total ^{32}P content of the nuclear proteins is plotted with the solid line connecting the solid circles. The ^{32}P content of P–N linkages is plotted with the dashed line connecting the ▲. The ^{32}P content of the P–O linkages is plotted with the dotted line connecting the open circles.

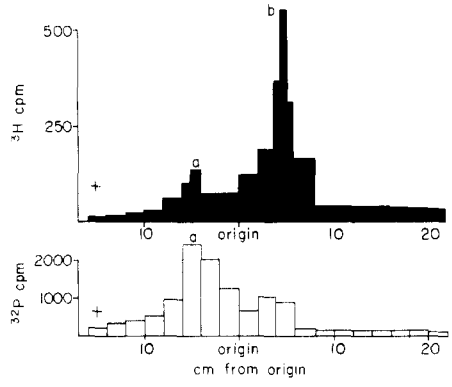

FIGURE 2: Paper electrophoresis of the amino acids of H1 isolated from rat regenerating liver nuclei. The paper electrophoresis was performed on Whatman 3MM paper in 0.001 M EDTA, 0.1 M ammonium acetate, pH 8.25, at 57 V/cm for 30 min. The chromatogram was cut into strips which were analyzed for ^{3}H content (black bars) and ^{32}P content (white bars). The phospholysine peak (a) and lysine peak (b) were identified by the comigration of their ninhydrin-staining standards.

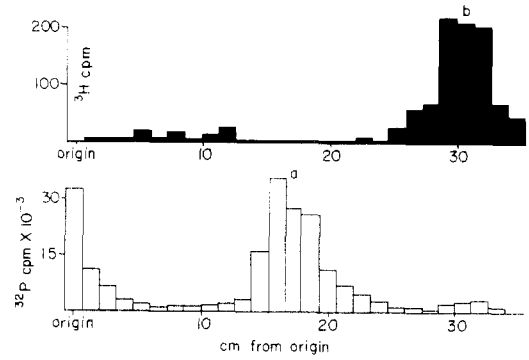

FIGURE 3: Ascending paper chromatography of the amino acids of H4 from rat regenerating liver. The chromatography was performed on Whatman 3MM paper in ethanol–2-propanol–water–triethylamine (30:30:39:1). The chromatogram was cut into strips which were analyzed for ^{3}H content (black bars) and ^{32}P content (white bars). The phosphohistidine peak (a) and histidine peak (b) were identified by the comigration of their ninhydrin-staining standards.

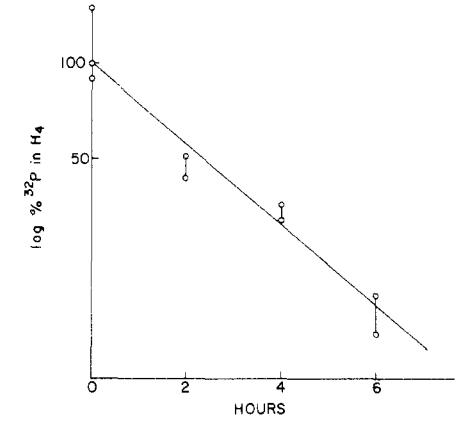

FIGURE 4: Turnover of the phosphoryl–nitrogen linkages in H4 isolated from rat regenerating liver. Time zero is 19.5 h after partial hepatectomy. The % activity is relative to the [^{32}P]phosphoryl–nitrogen content of H4 at time zero, which is represented as 100% activity. The % activity at each 2-h interval from time zero was determined from two regenerating livers, represented by the open and closed circles at each time interval.

coupled to DNA synthesis which we (Chen et al., 1974) and others have shown to begin at about 15 h and to peak at 18 to 19 h after partial hepatectomy. Thus it seemed possible that we might learn whether P–N linkages formed on newly synthesized or preexisting H1 and H4 in rat regenerating liver by appropriate administration of [^{3}H]lysine or [^{3}H]histidine and $^{32}P_i$. As outlined in the Materials and Methods section, phosphorylated H1 was isolated from regenerating livers that were removed from rats injected with [^{3}H]lysine and $^{32}P_i$. The amino acids resulting from proteolytic treatment of the phosphorylated H1 were applied to paper electrophoresis as shown in Figure 2. The lysine and phospholysine peaks were each identified by the comigration of ninhydrin-staining standards of lysine and phospholysine. The small amount of ^{32}P that has migrated toward the cathode is unidentified. In the phospholysine peak both the ^{3}H and ^{32}P labels are found, indicating that the de novo synthesized H1 molecules which incorporated [^{3}H]lysine were phosphorylated. This result, however, does not exclude the formation of *N*-phosphoryllysine in preexisting H1 molecules.

Phosphorylated H4 was isolated from regenerating livers that were excised from rats injected with [^{3}H]histidine and $^{32}P_i$ as described in Materials and Methods. The amino acids resulting from proteolytic treatment of the phosphorylated H4 were separated by ascending paper chromatography as shown in Figure 3. Standards of phosphohistidine and histidine were cochromatographed and stained with ninhydrin to identify the peaks in Figure 3. The ^{32}P at the origin corresponds to the known position of P_i in this system. The small amount of ^{32}P in the histidine peak is unidentified, but could be residual phosphoserine which in this chromatographic system has a R_f value very near to that of free histidine. No [^{3}H]histidine is detected in the [^{32}P]phosphohistidine peak, indicating that the de novo synthesized H4 molecules, which would incorporate the [^{3}H]histidine, were not phosphorylated, but, instead, preexisting H4 molecules were exclusively phosphorylated 18 h after partial hepatectomy.

Turnover of the P–N Linkages in H4. Livers from rats injected with $^{32}P_i$ 18 h after partial hepatectomy were excised at 2-h intervals 19.5 h after partial hepatectomy. Phosphorylated H4 was isolated from these livers as described in Materials and Methods and the ^{32}P content measured (Figure 4

represents the change in ^{32}P content of phosphorylated H4 during the liver regeneration). The turnover is apparently rapid with an approximate half-life of 2 h.

Discussion

Our results clearly show that the formation of acid-labile (P-N) linkages in nuclear proteins is a process of equal quantitative significance to that of formation of acid-stable (P-O) phosphoryl bonds. In almost every investigation involving protein phosphorylation, reaction termination and isolation procedures involve acidic pH values which clearly preclude the demonstration of P-N linkages. Our previous studies (Chen et al., 1974) have shown that the nonhistone chromosomal proteins are highly phosphorylated with both P-O and P-N linkages, but among the histones in rat regenerating liver, it is H1 and H4 in which P-N linkages chiefly can be found. Garrard et al. (1976) have studied the formation of acid-stable P-O bonds on histones during rat liver regeneration, and their results and our Figure 1 concerning P-O bond formation are approximately in agreement. In order to more accurately assess the extent and role of total phosphorylation on chromosomal proteins, it will become important to explore and develop methods which will permit the isolation of histones rapidly, under conditions in which both P-O and P-N linkages may be expected to survive.

Since our previous investigation (Chen et al., 1974) had shown that the measurable level of the pH 6.5 (H1) kinase remained virtually constant over the period up to the first cell division in rat regenerating liver while the activity of pH 9 (H4) kinase increased concomitantly with DNA synthesis, it was of interest to examine the question of whether it was new or old preexisting histone molecules which were phosphorylated. Tanphaichitr et al. (1974) have shown that P-O linkages are formed immediately in de novo synthesized H1 molecules, and that preexisting H1 molecules are phosphorylated at a much slower rate. The results in the present work clearly suggest that phosphoryllysine is formed in new and very likely a mixture of new and old preexisting H1 molecules. The electrophoretic analysis used (Figure 2) does not discriminate between phospholysine and phosphoarginine, and thus a different analytical method would be required to examine the question whether these two phosphoamino acids are formed at similar or at different rates.

The situation for H4 is, in contrast to that for H1, quite interesting. Clearly it is exclusively preexisting H4 molecules which are phosphorylated in rat regenerating liver at the peak (18 h) of DNA synthesis. This observation, coupled with the increase in H4 kinase activity coincident with DNA synthesis (Chen et al., 1974) in rat regenerating liver, suggests an important role for H4 histidine phosphorylation during DNA replication. It may be that phosphorylation on histidine in H4 loosens the interaction among the histones of the nucleosome in such a way that DNA replication may proceed. We are, at present, investigating possible changes in the binding of H3 and H4 due to histidine phosphorylation on H4.

The first wave of DNA synthesis appears to span about 10 h in rat regenerating liver (Chen et al., 1974). Our measurement of the half-life of H4 histidine phosphate of about 2 h fits very well with the possible functional role of this phosphorylation in DNA replication. Thus far, we have not measured the turnover of P-N bonds in H1.

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