PHOSPHORYLATION OF LIVER HISTONE FOLLOWING THE ADMINISTRATION OF GLUCAGON AND INSULIN*

BY THOMAS A. LANGAN

CHARLES F. KETTERING RESEARCH LABORATORY, YELLOW SPRINGS, OHIO

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Abstract.—The administration of glucagon to rats causes a marked increase in the phosphorylation of a specific serine residue in lysine-rich (f1) histone of liver during a one-hour period following the administration of the hormone. It is proposed that histone phosphorylation is the mechanism by which glucagon, and perhaps other hormones whose actions are mediated by adenosine 3′,5′-cyclic phosphate (cyclic AMP), induce RNA synthesis in target tissues. The incorporation of 32P-phosphate into lysine-rich histone is determined by isolation of a tryptic peptide which contains the phosphorylated serine residue. This peptide is identical to the major tryptic phosphopeptide obtained from lysine-rich histone after phosphorylation in vitro by a purified cyclic AMP-dependent liver histone kinase preparation; the partial sequence Lys-Ala-SerPO4(Thr,Ser,Glu,Pro2,Gly, Val,Ile,Leu)Lys has been determined for the peptide. Hydrocortisone and adrenocorticotropic hormone do not cause a detectable increase in histone phosphorylation in liver. However, insulin, which like glucagon induces an actinomycin sensitive synthesis of liver enzymes, also causes increased histone phosphorylation.

Phosphorylation of histones, catalyzed by a specific protein kinase present in liver, is markedly increased by adenosine 3′,5′-cyclic phosphate (cyclic AMP) both in vitro1 and in vivo.2 Cyclic AMP acts as the mediator of many hormone responses3 and, in certain cases, these responses involve the synthesis of new protein4,5 or are blocked by inhibitors of protein1,6–8 and RNA10–12 synthesis. Thus, a role for the cyclic nucleotide in regulating transcription or translation of genetic information is implied.

Histones may act as regulators of transcription from DNA templates in eukaryotic cells.13–16 There is also evidence that modification of histones by acetylation17 or phosphorylation18 may influence DNA-histone interactions and cause derepression of template activity. To test whether histone phosphorylation occurs during the induction of enzyme synthesis by hormones which act via cyclic AMP, the response of liver to glucagon administration was studied. Extensive evidence from the laboratories of Sutherland5,19 and Park20 indicates that cyclic AMP mediates the effects of glucagon in liver. Administration of glucagon to rats causes de novo synthesis of a number of liver enzymes4,5 and the induction of these enzymes is blocked by actinomycin D. It has been demonstrated recently that enzyme synthesis in liver can be induced directly by cyclic AMP.21,22,12

In this paper it is shown that glucagon, given in doses which are effective in inducing enzyme synthesis, causes a 15-to 25-fold increase in phosphorylation of a specific serine residue in rat liver lysine-rich (f1) histone shortly after the ad-
ministration of the hormone. Insulin, which like glucagon induces an actinomycin sensitive synthesis of enzymes in liver, also causes increased histone phosphorylation. On the basis of these results, a mechanism for the induction of RNA synthesis by hormones is proposed. A preliminary report of this work has appeared.

Materials and Methods.—Electrophoresis and chromatography: Electrophoresis was carried out on a water-cooled flat plate; wicks leading to buffer vessels were isolated from electrophoresis papers by four layers of cellophane dialysis membrane. The buffers used were: pH 7.9, 0.06 M NH₄HCO₃; pH 4.7, 25 ml glacial acetic acid plus 25 ml pyridine per liter; pH 1.9, 25 ml 88% formic acid plus 87 ml glacial acetic acid per liter; pH 1.58, 100 ml 88% formic acid per liter, adjusted to pH 1.58 by further addition of formic acid. The chromatographic solvents used were: solvent A, pyridine/n-butanol/glacial acetic acid/H₂O = 10/15/3/12; solvent B, n-butanol/glacial acetic acid/H₂O = 4/1/2. Except where indicated, Eastman no. 6064 cellulose thin layers were used.

Purification and characterization of the major tryptic phosphopeptide of enzymatically phosphorylated lysine-rich histone: Calf thymus lysine-rich (fl) histone prepared by the methods of Johns was phosphorylated by incubation with purified liver histone kinase and ³²P-ATP as previously described. Tryptic digestion and preparative scale paper electrophoresis were carried out as described in the legend to Figure 1. The major radio-

![Figure 1](https://example.com/figure1.png)

active band was eluted and further purified by chromatography with solvent A on cellulose thin layers, or by treatment with E. coli alkaline phosphatase (0.05 mg/ml in 0.04 M NH₄HCO₃) followed by another electrophoresis at pH 7.9. Samples purified by both methods had closely similar amino acid compositions, as determined with an automatic analyzer. (These analyses were kindly performed by Dr. K. Fry.) Conditions for the enzymatic digestions used to establish the partial sequence of the peptide were: leucine amino peptidase (Worthington) 0.5 mg/ml in 0.025 M NH₄HCO₃, 2.5 mM MgCl₂; trypsin (Sigma, twice crystallized), 0.15 mg/ml in 0.02 M NH₄HCO₃. Amino acids released were identified by thin-layer electrophoresis for 40 min at 75 v/cm on 10 X 40 cm cellulose thin layers (Brinkmann) in formic acid buffer, pH 1.58, followed by chromatography in the second dimension with solvent B.

Hormones: Crystalline glucagon and bovine insulin, 25 units/mg, containing 0.024% glucagon, were gifts from Drs. B. H. Frank and W. Bromer, Eli Lilly Co. ACTH (Ach) was purchased from Armour Company and hydrocortisone from Calbiochem.

Determination of liver lysine-rich histone phosphorylation in vivo: Rats, 180 to 250 gm,
obtained from Holtzman Co. or Charles River Laboratories, were fasted overnight and 
ijected intraperitoneally with the indicated dose of hormone in 0.5 ml of 0.14 M NaCl. 
Except where indicated, 2 mCi of $^{32}$P-phosphate (New England Nuclear) in 0.5 ml of 
0.14 M NaCl containing $10^{-4} M$ carrier inorganic phosphate was injected immediately 
after hormone administration. Control animals received $^{32}$P-phosphate only. At 
the designated times, animals were killed and the livers quickly frozen in liquid nitrogen. 
The method of de Nooij and Westenbrink, 28 slightly modified, was used to prepare lysine-
rich histone. Pooled livers from two rats were homogenized at 4°C in a Waring blender 
for 1 min. at 85 v and 2 min at 45 v in 325 ml of 0.14 M NaCl, 0.01 M sodium citrate, 
pH 6.0. A 1-ml aliquot of the homogenate was frozen immediately for subsequent deter-
mination of the specific activity of liver phosphate pools by the method of Martin and 
Doty. 29 The homogenate was filtered through a single layer of Miracloth (Chieopee 
Mills, Inc.) and centrifuged 15 min at 3300 g. The sediment was suspended in 280 ml 
of homogenizing medium and recentrifuged. To extract lysine-rich histone, the com-
bined pellets were blended in 47.5 ml of H$_2$O for 1 min at top speed and 2.5 ml of 100% 
(1 gm/ml) trichloroacetic acid was added during the next minute of blending. Blending 
was continued for an additional 30 sec and the suspension centrifuged 5 min at 3300g. 
The trichloroacetic acid concentration of the supernatant was adjusted to 18% and the 
suspension allowed to stand in ice for 20 min before centrifuging for 20 min at 13,000 g. 
The precipitated lysine-rich histone was dissolved in water and reprecipitated twice with 
25% trichloroacetic acid. It was then washed once with acetone containing 0.5 ml HCl/ 
100 ml, once with acetone, and dried in vacuo. The dry histone was dissolved in 0.15 ml 
of H$_2$O and insoluble material was removed by centrifugation. An aliquot was removed 
for protein determination by the biuret method 30 in a final volume of 0.25 ml. The yield 
from 10 gm of liver was approximately 2 mg. Unlabeled enzymatically phosphorylated 
calf thymus lysine-rich histone containing 10 to 15 mmoles of phosphate was then added 
as carrier, followed by 10 mmoles of NH$_4$HCO$_3$ and 50 $\mu$g of trypsin. After incubation for 
1 hr at 37°C, the digest was subjected to electrophoresis on Schleicher and Schuell no. 
589 green ribbon paper in pH 7.9 buffer for 45 min at 100 v/cm. In order to remove ma-
terial that is frequently present in this paper and that interferes with subsequent thin-
layer chromatography of eluates, it was autoclaved before use for 90 min in 0.05 M 
NH$_4$OH, washed thoroughly with water, and soaked overnight in 95% ethanol. Tryptic 
digests of $^{32}$P-labeled enzymatically phosphorylated thymus lysine-rich histone were 
used as markers in the electrophoresis. Radioactive bands were located by radioautog-
raphy (60 hr exposure to Kodak blue brand X-ray film) and eluted. The eluates were 
spotted on cellulose thin layers and chromatographed in solvent A. Radioactive peptides 
were located by radioautography for 12 to 16 hr, cut out, and counted in a low background 
GM counter. The observed counts were corrected to account for individual variations 
in the labeling of phosphate pools with the following formula: Relative cpm = observed 
cpm in phosphopeptide/cpm per pmole in liver inorganic phosphate. In some experi-
ments, the specific activity of acid-labile phosphate in liver nucleotides adsorbable on char-
coal 31 was also measured and found not to differ significantly from the values for inorganic 
phosphate. None of the hormone treatments produced significant changes in the specific 
activity of liver phosphate pools.

Results.—Characterization of phosphorylation site in lysine-rich histone: Previous 
studies 32 of the phosphorylation of lysine-rich (fl) histone by liver histone 
kinase indicated that the maximum amount of phosphate which can be transferred 
to this histone is close to 1 mole per mole of protein. In order to characterize 
the phosphorylation site in lysine-rich histone, the distribution of phosphate in 
tryptic peptides of enzymatically phosphorylated, $^{32}$P-labeled calf thymus lysine-
rich histone was examined by paper electrophoresis at pH 7.9 (Fig. 1). One 
major radioactive peptide was found which represented 65 per cent of the 
total $^{32}$P in the digest shown, but which has been obtained in yields up to 80 per
A representative analysis of the major phosphopeptide, determined after purification from nonradioactive peptides which migrate with it at pH 7.9, is shown in Table 1. The analyses indicate that the peptide contains 13 amino acid residues, many of them neutral or hydrophobic. There are two serine residues and a single phosphate group. The amino acid sequence at the N-terminal end of the peptide has been investigated by enzymatic degradation (see Materials and Methods). Treatment with E. coli alkaline phosphatase followed by leucine aminopeptidase releases 32P-inorganic phosphate, lysine, alanine, and serine, plus a peptide which is not degraded further. Treatment with leucine aminopeptidase alone releases lysine and alanine plus a radioactive peptide. Phosphoserine and a nonradioactive peptide corresponding to the previous peptide product are then released slowly. Finally, treatment with high concentrations of trypsin releases free lysine, demonstrating the N-terminal location of one lysine residue. The partial sequence: Lys-Ala-SerPO$_4$(Thr,Ser,Glu,Pro$_2$,Gly,Val,Ile,Leu)Lys is indicated for the peptide by these data. In contrast, Dixon et al. have found that phosphorylation of trout testis histones II and IV occurs in the N-terminal sequence N-acetyl-SerPO$_4$-Gly-Arg. The isolation in good yield of a single major tryptic phosphopeptide from enzymatically phosphorylated lysine-rich histone provides conclusive evidence that liver histone kinase acts mainly on a single specific serine residue in the histone molecule, and not at many sites in a minor component or contaminant of the preparation. The isolation of this peptide also provided a specific method for demonstrating the action of histone kinase in vivo and for studying the effects of hormones on histone phosphorylation, as described below.

Phosphorylation of lysine-rich histone after hormone administration: Rats were injected with $^{32}$P-phosphate or $^{32}$P-phosphate plus glucagon and liver lysine-rich histone isolated, digested with trypsin, and subjected to paper electrophoresis as described in Materials and Methods. The tryptic digest of lysine-rich histone from glucagon treated animals showed a clear band of radioactivity corresponding to the major tryptic phosphopeptide from enzymatically phosphorylated lysine-rich histone (Fig. 2a). This band and the corresponding area from the control sample were eluted and chromatographed on cellulose thin layers, de-
(Left) Fig. 2.—Radioactive phosphopeptides in tryptic digests of liver lysine-rich histone from control and hormone-treated rats. Paper electrophoresis was carried out at pH 7.9 and bands revealed by radioautography. Marker digests of enzymatically phosphorylated thymus lysine-rich histone are designated by the letter m. Glucagon (300 μg/rat) was given 60 min- and insulin (1.25 units/rat) and ACTH (5 units/rat) 70 min before animals were killed. 32P-phosphate was given at the time of hormone administration, or 60 min-before killing control animals.

(Right) Fig. 3.—Thin-layer chromatography of tryptic phosphopeptides isolated from liver lysine-rich histone of control and glucagon-treated rats. Lane 1, control; lane 2, glucagon; m designates marker peptide isolated from enzymatically phosphorylated lysine-rich histone. 32P-phosphate and glucagon (400 μg) injections were given 2 hr before animals were killed. Tryptic digests were subjected to electrophoresis at pH 7.9 and radioactive bands with mobilities corresponding to the major tryptic phosphopeptide of enzymatically phosphorylated lysine-rich histone (cf. Fig. 2) were eluted and chromatographed on cellulose with solvent A. Radioautographic exposure was continued for four days to visualize minor components.

veloping with solvent A. The migration of the band from hormone-treated animals again matched that of the marker peptide from enzymatically phosphorylated histone, and a faint matching band in the control sample was also visible (Fig. 3). Note that glucagon causes a specific increase in the phosphorylation of the site contained in this peptide; whether other radioactive bands represent phosphorylation sites not affected by glucagon or contaminants of the histone preparation has not been determined. The identity of the major phosphopeptides isolated from liver lysine-rich histone of hormone-treated animals and from enzymatically phosphorylated lysine-rich histone was confirmed by further comparisons of chromatographic mobility in solvent B and electrophoretic migration at pH 4.7 and 1.9. In each of these systems, the mobility of the phosphopeptides derived from histone phosphorylated in vivo and in vitro was identical. In addition, radioactive phosphoserine was identified by electrophoresis at pH 1.9 as the sole phosphoamino acid in partial acid hydrolysates (2 N HCl, 16 hr, 100°)34 of phosphopeptide derived from histone phosphorylated in vivo.
TABLE 2. Phosphorylation of liver lysine-rich histone following administration of glucagon.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Time after glucagon administration</th>
<th>Hormone and antibiotic injections</th>
<th>Relative cpm in phosphopeptide/mg lysine-rich histone</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15 min</td>
<td>Glucagon</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td>Glucagon + cycloheximide</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>26</td>
</tr>
<tr>
<td>B</td>
<td>1 hr</td>
<td>Glucagon</td>
<td>786</td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
<td>Glucagon + actinomycin D +</td>
<td>1296</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cycloheximide</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2 hr</td>
<td>Glucagon</td>
<td>960</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>46</td>
</tr>
</tbody>
</table>

Expt. A—$^{32}$P-phosphate and cycloheximide (10 mg) injections were given 1 hr and glucagon (300 μg) 15 min before animals were killed. Rats weighed 180 to 200 gm.

Expt. B—$^{32}$P-phosphate and glucagon (400 μg) injections were given at the indicated times before animals were killed. Actinomycin D (500 μg in 0.25 ml of propylene glycol) and cycloheximide (10 mg) were given 30 min before glucagon. Rats weighed 230 to 250 gm.

For quantitative assay of histone phosphorylation in vivo, the peptide spots obtained after electrophoresis at pH 7.9 and subsequent thin-layer chromatography were cut out and counted. As shown in Table 2 (expt. A), the response to glucagon is readily detectable 15 minutes after intraperitoneal injection of the hormone. During the next two hours (expt. B), the level of histone phosphorylation rises to a level approximately 20-fold higher than that found in control animals. Phosphorylation is not blocked by actinomycin D or cycloheximide, indicating (1) that phosphorylation takes place on intact histone molecules and (2) that it is a primary effect of the hormone and not dependent on increased synthesis of histone kinase or other enzymes. The increased phosphorylation in response to glucagon administration is consistent with the known ability of glucagon to increase the concentration of cyclic AMP in liver; this and with the previously observed effects of cyclic AMP on the activity of purified liver histone kinase, and on the phosphorylation of liver histone in vivo.

Histone phosphorylation in response to various doses of glucagon was studied. Clear increases in phosphorylation occur after administration of 10 μg per rat, and the maximum response is obtained with doses of 100 μg or larger. Holten and Kenney have shown that doses of glucagon from 100 to 600 μg per rat produce four-to sixfold elevations in the level of liver tyrosine transaminase. Thus, the doses of glucagon which induce enzyme synthesis correspond to those which cause increased histone phosphorylation.

The maximum levels of histone phosphorylation reached in the dose and time studies indicate that glucagon causes the phosphorylation of only a limited fraction of liver lysine-rich histone. The size of this fraction is difficult to determine accurately, but rough estimates indicate that it is on the order of 1 per cent of the total lysine-rich histone.

The effects of ACTH, hydrocortisone, and insulin on liver histone phosphorylation were also examined (Fig. 2b, Table 3). ACTH, which acts via cyclic AMP to induce protein synthesis in adrenal cortex, is without effect on histone phosphorylation in liver. Hydrocortisone, which induces enzyme synthesis in liver by a mechanism which does not involve cyclic AMP, also has no effect on liver histone phosphorylation. However, insulin does cause increased histone
phosphorylation, as is clearly shown by the band of radioactive peptide in Figure 2b, and by the data presented in Table 3. Phosphorylation induced by insulin is also insensitive to actinomycin D and cycloheximide. The effect of insulin is not readily explained, since insulin appears to lower slightly the level of cyclic AMP in liver.38 However, as shown in Kenney’s laboratory, insulin induces an actinomycin-sensitive synthesis of tyrosine transaminase in rat liver which is identical in all respects examined to the synthesis induced by glucagon.4 39 In both cases then, hormonal induction of enzyme synthesis is associated with an increase in histone phosphorylation. However, experiments with simpler systems, such as perfused livers, are needed to establish whether insulin acts directly on the liver to cause histone phosphorylation.

Discussion.—Together with the evidence that histones probably function in some way in regulating the activity of genes,13–16 the present findings make it possible to formulate a mechanism for the induction of RNA synthesis by glucagon, and perhaps by other hormones whose actions are mediated by cyclic AMP, which has some detail at the molecular level. The primary site of action of this type of hormone, as shown by the extensive studies of Sutherland and collaborators,8 is the enzyme adenyl cyclase. This is activated by the hormone to catalyze the formation of cyclic AMP which in turn activates histone kinase, resulting in the phosphorylation of histone. It is proposed that the induction of RNA synthesis might be brought about by a change in DNA-histone interaction resulting from histone phosphorylation; presumably this would involve a change in configuration of the histone with consequent derepression of the DNA template, followed by RNA and protein synthesis. The limited amount of histone phosphorylation observed in our studies is consistent with the specific effect of glucagon on the synthesis of a small number of enzymes in liver, but the basis for this specificity is not known. In addition, the role of cyclic AMP in regulating protein synthesis may be more complex than indicated here, since there is evidence that cyclic AMP stimulates the translation46 as well as the synthesis41 of RNA in bacteria and that glucagon can act at a post-transcriptional level in cultured liver cells.42

Note added in proof: Protein phosphorylation in response to insulin treatment has also been observed recently by A. E. Voytovitch, I. S. Owens, and Y. J. Topper (these Proceedings, 63, 215 (1969)) in mammary tissue. In these experiments, isolated explants of mammary gland were used, excluding the possibility that insulin acted indirectly.

Table 3. *Phosphorylation of liver lysine-rich histone following administration of various hormones.*

![Table 3](image-url)

Hydrocortisone (10 mg) was given 30 min before 32P-phosphate and the animals killed 70 min later. Actinomycin D (400 μg) and cycloheximide (10 mg) were given 30 min before insulin. Other injections given as described in Figure 2. Rats weighed 180 to 200 gm.

Discussion.—Together with the evidence that histones probably function in some way in regulating the activity of genes,13–16 the present findings make it possible to formulate a mechanism for the induction of RNA synthesis by glucagon, and perhaps by other hormones whose actions are mediated by cyclic AMP, which has some detail at the molecular level. The primary site of action of this type of hormone, as shown by the extensive studies of Sutherland and collaborators,8 is the enzyme adenyl cyclase. This is activated by the hormone to catalyze the formation of cyclic AMP which in turn activates histone kinase, resulting in the phosphorylation of histone. It is proposed that the induction of RNA synthesis might be brought about by a change in DNA-histone interaction resulting from histone phosphorylation; presumably this would involve a change in configuration of the histone with consequent derepression of the DNA template, followed by RNA and protein synthesis. The limited amount of histone phosphorylation observed in our studies is consistent with the specific effect of glucagon on the synthesis of a small number of enzymes in liver, but the basis for this specificity is not known. In addition, the role of cyclic AMP in regulating protein synthesis may be more complex than indicated here, since there is evidence that cyclic AMP stimulates the translation46 as well as the synthesis41 of RNA in bacteria and that glucagon can act at a post-transcriptional level in cultured liver cells.42

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14. Huang, R. C., and J. Bonner, these PROCEEDINGS, 48, 1216 (1962).