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ISOLATION AND CHARACTERIZATION OF A
PHOSPHATASE SPECIFIC FOR PHOSPHORYLATED
HISTONES AND PROTAMINES.

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ISOLATION AND CHARACTERIZATION OF A PHOSPHATASE
SPECIFIC FOR PHOSPHORYLATED HISTONES
AND PROTAMINES

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
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School of the Ohio State University

By
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The Ohio State University
1968

Approved by

Adviser
C. F. Kettering Research Laboratory

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PUBLICATIONS


TABLE OF CONTENTS

- ACKNOWLEDGMENTS ........................................ ii
- VITA ................................................ iii
- LIST OF TABLES ........................................ vi
- LIST OF ILLUSTRATIONS .............................. vii

PART I

INTRODUCTION .............................................. 1

HISTORICAL

  Structure and Metabolism of Histones
      and Protamines .................................. 3
  Function of Histones ............................. 7
  Enzymatic Modifications of Histones .......... 16
  Phosphoprotein Phosphatases ................... 29

OBJECTIVES OF THIS WORK ............................... 32

PART II

MATERIALS AND METHODS ................................. 33

  Preparation of Substrates ......................... 36
  Assay of Histone Phosphatase Activity .......... 39

EXPERIMENTAL RESULTS AND DISCUSSION

  Subcellular Distribution of Histone
      Phosphatase Activity .......................... 42
  Purification of Histone Phosphatase .......... 57
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Amino acid composition of histone fractions</td>
<td>5</td>
</tr>
<tr>
<td>2. Enzymatic Modifications of Histones</td>
<td>17</td>
</tr>
<tr>
<td>3. Properties of Phosphoprotein Phosphatases</td>
<td>30</td>
</tr>
<tr>
<td>4. Subcellular Distribution of Histone Phosphatase</td>
<td>44</td>
</tr>
<tr>
<td>5. Histone Phosphatase Activity of Purified Nuclei</td>
<td>46</td>
</tr>
<tr>
<td>6. Subnuclear Localization of Histone Phosphatase</td>
<td>48</td>
</tr>
<tr>
<td>7. Comparison of Nuclear and Cytoplasmic Activity</td>
<td>49</td>
</tr>
<tr>
<td>8. Absence of Histone Phosphatase Activity in Purified Lysosomes</td>
<td>54</td>
</tr>
<tr>
<td>9. Purification of Histone Phosphatase</td>
<td>63</td>
</tr>
<tr>
<td>10. Effect of Various Compounds on Histone Phosphatase Activity</td>
<td>75</td>
</tr>
<tr>
<td>11. $K_m$ and $V_{max}$ for known Substrates</td>
<td>77</td>
</tr>
<tr>
<td>12. Substrate Activity of Phosphoproteins</td>
<td>78</td>
</tr>
<tr>
<td>13. Substrate Activity of Phosphopeptides</td>
<td>80</td>
</tr>
<tr>
<td>14. Substrate Specificity of Histone Phosphatase</td>
<td>82</td>
</tr>
<tr>
<td>15. Inactivity of Alkaline Phosphatase towards Phosphorylated Protamine</td>
<td>84</td>
</tr>
<tr>
<td>16. Inactivity of Diesterase towards Phosphorylated Protamine</td>
<td>85</td>
</tr>
<tr>
<td>17. Tissue Distribution of Histone Phosphatase Activity</td>
<td>100</td>
</tr>
<tr>
<td>18. Species Distribution of Histone Phosphatase Activity</td>
<td>102</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Linearity of Histone Phosphatase Reaction with Time</td>
<td>41</td>
</tr>
<tr>
<td>2. Linearity of Reaction with Enzyme Concentration</td>
<td>41</td>
</tr>
<tr>
<td>3. Subnuclear Fractionation Procedure</td>
<td>46</td>
</tr>
<tr>
<td>4. Comparison of Lysosomal beta-Glucuronidase and Histone Phosphatase</td>
<td>55</td>
</tr>
<tr>
<td>5. DEAE Cellulose Chromatography of Calcium Phosphate Fraction</td>
<td>60</td>
</tr>
<tr>
<td>6. Chromatography of DEAE Fraction on Sephadex G-200</td>
<td>62</td>
</tr>
<tr>
<td>7. DEAE Cellulose Chromatography of Fresh Soluble Fraction</td>
<td>65</td>
</tr>
<tr>
<td>8. Separation of p-Nitrophenyl Phosphatase from Histone Phosphatase on DEAE Cellulose</td>
<td>67</td>
</tr>
<tr>
<td>9. pH Dependence of Histone Phosphatase</td>
<td>69</td>
</tr>
<tr>
<td>10. Ionic Strength Dependence of Histone Phosphatase</td>
<td>71</td>
</tr>
<tr>
<td>11. Denaturation of Histone Phosphatase at 52° C</td>
<td>74</td>
</tr>
<tr>
<td>12. Apparent Km for Dephosphorylation of Histone fl and Protamine</td>
<td>87</td>
</tr>
<tr>
<td>13. Molecular Weight Estimation on Sephadex G-200</td>
<td>89</td>
</tr>
<tr>
<td>14. Disc Electrophoresis of Dephosphorylated Protamine</td>
<td>92</td>
</tr>
<tr>
<td>15. Electrophoresis of Dephosphorylated Protamine on Polyacrylamide Gel</td>
<td>93</td>
</tr>
<tr>
<td>16. Gel Electrophoresis of Dephosphorylated Histone fl</td>
<td>95</td>
</tr>
</tbody>
</table>
17. Effect of Phosphorylation on Tm of Nucleohistones ................................... 98
INTRODUCTION

In spite of a century of active study of histones and protamines, the biological function of these ubiquitous nuclear proteins has remained an enigma. As specific components of chromatin in close association with DNA, these proteins may be involved in the regulation of genetic activity in eukaryotic cells. For this reason, there is considerable current interest in histone metabolism and function.

Several enzymatic reactions in which specific amino acid residues of histone molecules are reversibly modified have recently been discovered. These provide a mechanism for dynamic control of histone structure, with interesting potential for regulation of histone function. Methylation, acetylation and phosphorylation of histones have been described; this thesis is concerned with histone phosphorylation.

Recent evidence for the rapid phosphorylation of histones and protamines independently of protein synthesis led us to postulate the existence of a specific histone phosphatase. We have succeeded in demonstrating the presence of such an enzyme in rat liver. The enzyme has been prepared free from contaminating phosphatase activity; it catalyzes the release of orthophosphate from
phosphorylated histones and protamines without degrada-
tion of the substrate protein. Histone phosphatase is
highly specific; it is not active towards other phospho-
proteins or low molecular weight phosphates. The
enzyme may function in a cycle of histone phosphorylation
and dephosphorylation in vivo.
HISTORICAL

The study of histones and protamines began almost one hundred years ago, with their description by Miescher in 1871. Their close association with cellular DNA was first demonstrated histochemically and later by analysis of isolated nucleoprotein and chromatin. The histones are universal components of the nuclei of eukaryotic cells, but are not found in prokaryotes. Their evolutionary appearance thus coincides with the transition to complex organization of the genetic material within the nucleus. As a result of these indications that histones are involved in some aspect of nuclear function, they are central to the current effort to understand the process of differential gene activation in higher organisms.

Structure and Metabolism of Histones and Protamines

There have been several recent reviews of histone structure (Murray, 1965; Busch, 1965; deRueck, 1966). Histones are a heterogeneous class of proteins of average molecular weight 20,000 to 30,000 daltons. The basic amino acids, arginine, lysine and histidine, account for 25 - 30 % of the total residues. As a result of their basicity, the histones are soluble in dilute acids. Since there is no known functional assay, identification of
isolated proteins as histones requires the demonstration that extractability, electrophoretic mobility and amino acid composition correspond to those of previously-described histones.

Several procedures for the fractionation of histones have been employed, based upon acid extraction or extraction by high concentrations of salt which dissociate the ionic bonds between histones and DNA (Murray, 1965). A method in widespread use for the fractionation of calf thymus histones by sequential acid extraction of nuclei was described by Johns in 1964. Four histone subfractions result from the fractionation procedure of Johns, in relative yields, of 1:2:1:1; they are fraction f1 ("lysine-rich"), fractions f2a and f2b ("moderately lysine-rich") and fraction f3 ("arginine-rich"). Typical values for the amino acid composition of these fractions is presented in Table 1. Some heterogeneity within these fractions is revealed by gel electrophoresis, and further subfractionations have been described (Murray, 1968; Starbuck, 1968).

The protamines are smaller proteins than histones, with average molecular weight approximately 5,000 daltons. Protamines are found exclusively in the sperm of fish, where they replace histones as the protein component of nucleoprotein during the course of spermatogenesis. They are chemically less complex than histones; they typically
Table 1. Amino Acid Composition of Representative Histone Subfractions

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Very lysine-rich F1</th>
<th>Slightly lysine-rich N-acetylated alanine F2a</th>
<th>Slightly lysine-rich N-proline F2b</th>
<th>&quot;Arginine-rich&quot; F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>23.5</td>
<td>10.5</td>
<td>10.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.5</td>
<td>11.5</td>
<td>7.5</td>
<td>13.0</td>
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<tr>
<td>Aspartic acid</td>
<td>2.8</td>
<td>6.0</td>
<td>5.5</td>
<td>5.0</td>
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<tr>
<td>Glutamic acid</td>
<td>6.0</td>
<td>8.5</td>
<td>9.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.8</td>
<td>12.5</td>
<td>7.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.5</td>
<td>2.0</td>
<td>2.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.6</td>
<td>4.5</td>
<td>5.0</td>
<td>5.0</td>
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<tr>
<td>Leucine</td>
<td>4.4</td>
<td>10.5</td>
<td>6.0</td>
<td>8.5</td>
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<tr>
<td>Lysine</td>
<td>26.3</td>
<td>10.5</td>
<td>14.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>—</td>
<td>—</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.8</td>
<td>1.6</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Proline</td>
<td>7.9</td>
<td>3.0</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Serine</td>
<td>6.2</td>
<td>3.1</td>
<td>9.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.5</td>
<td>5.6</td>
<td>6.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.5</td>
<td>3.0</td>
<td>3.1</td>
<td>2.0</td>
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<tr>
<td>Valine</td>
<td>5.0</td>
<td>7.0</td>
<td>6.8</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* The values are percentages of total moles of amino acids recovered in the particular amino acid. The data are averages from representative studies (21, 22, 25-28).

It from Busch (1965), page 45.

contain only five or six neutral amino acids in addition to large amounts of arginine, which can account for as much as 70% of the total amino acid residues. Histones and protamines are regarded as homologous proteins.

Two unusual aspects of histone metabolism have been the subject of recent research. The exclusive localization of histones in the cell nucleus had led to the suggestion that their synthesis might take place in the nucleus.

It was observed that isolated thymus nuclei can incorporate labeled amino acids into histone F1 (Reid and Cole, 1964).

However, Bloch in 1964 detected by radioautography the cytoplasmic synthesis of histones in grasshopper spermatids.
More recently, Borun, Scharff and Robbins (1967) presented convincing evidence for the presence of histone-like material on cytoplasmic polysomes in Hela cells, as indicated by differential labeling with radioactive lysine and tryptophan. In a related paper (Robbins and Borun, 1967) the movement of labeled histone precursors from the cytoplasm into the nucleus was demonstrated. It is now generally accepted that the major portion of histone biosynthesis proceeds by the standard cytoplasmic pathways. Whether lymphocyte or other nuclei can also carry out protein synthesis remains unresolved.

A unique feature of histone synthesis is its temporal association with the period of DNA synthesis in the cell cycle. There is considerable histochemical evidence of this relationship; it was recently demonstrated biochemically by Robbins and Borun (1967) by their technique for the identification of histone-bearing polysomes. This class of polysomes could only be detected in Hela cells during the DNA-synthetic phase of the cell cycle. The sensitivity of the coordination was demonstrated by the rapid decline of histone synthesis which followed the addition of inhibitors of DNA synthesis. The control of histone biosynthesis to coincide with DNA replication assures a constant ratio of histone to DNA in the nucleus, and is indicative of the importance of these proteins to nuclear function.
Function of Histones

There have been two major hypotheses as to the role of histones in nuclear function. One is that they are critical structural components of chromatin, active in the coiling of nucleohistone into compact chromosomes during cell division. The other is that they are involved in regulation of the genetic activity of the DNA template. It may be misleading to create a dichotomy between these alternatives, since a change in state of genetic activity would seem to require a concomitant structural change, and likewise a gross alteration in structure should have consequences for genetic activity. Histones may well be important in both aspects of nuclear activity.

a. Nucleohistone Structure. It has been demonstrated by X-ray crystallography that the Watson - Crick double-helical structure of DNA is retained in nucleoprotein complexes (Fuegelman et al., 1955). The relationship of protein molecules to the DNA in nucleoprotein has not been resolved by crystallography, and ideas in this field are still speculative. The three major components of chromatin are DNA, histone and "non-histone protein". The weight ratio of histone to DNA is fairly constant at approximately 1:1, while the content of non-histone protein is variable.
In the interphase cell, nucleoprotein is present in two forms: as dispersed "euchromatin" whose DNA is available for transcription, and as condensed, inactive "heterochromatin." During cell division, the nucleoprotein becomes tightly coiled into discrete chromosomes. The molecular basis for these dramatic alterations in structure is not known.

Association with histones has been shown to bring about shortening of DNA fibers (Zubay and Doty, 1959; Ohba, 1966) and results in distortion of 60% of the bases from their position perpendicular to the axis of the helix. From physical studies of nucleohistone structure the general conclusion has emerged that the lysine-rich and arginine-rich histones have very different structural relationships to the DNA. This distinction was first suggested by Zubay and Doty (1959), who proposed a model in which the helical, non-lysine-rich histones (representing 70% of total histones) lie within the large groove of the DNA helix, wound along the molecule, while the lysine-rich histones in extended beta-configuration form cross-linkages between nucleohistone molecules. Subsequent studies have provided some support for the basic concept of this model. Littau, Allfrey and Mirsky (1965) by selective extraction and re-addition of histone subfractions to lymphocyte nuclei, found that the lysine-rich histones
were responsible for the reversible aggregation of disperse euchromatin into dense heterochromatin. Izawa, Allfrey and Mirsky (1963) found that only arginine-rich histones cause retraction of the intra-molecular loops of the lampbrush chromosomes. Ohba (1966) reported that the tightly-bound arginine-rich histones are responsible for the hydrodynamic properties associated with shortening of the DNA molecules.

This evidence on the relationship of histones to DNA is not yet sufficient to permit a detailed description of nucleohistone structure; consequently it is difficult to suggest a precise mechanism by which histones could function as regulators of this structure.

b. Histones as Genetic Regulators. The close association of histones with DNA in the cells prompted the original suggestion by Stedman and Stedman (1950) "that one of their physiological functions is to act as gene suppressors." In the intervening 18 years, we have achieved an impressive understanding of the mechanism of genetic control in bacterial cells, where the continuous transcription of messenger RNA with a half-life of several minutes is regulated by specific protein repressors. Although our understanding of regulation in nucleated cells is much more limited, the bacterial model has dominated most thought on the subject. However, the
evolution of nuclear organization of the genetic material may have necessitated the development of more complex regulatory mechanisms. "Induction" and "repression" as understood in bacteria have not been demonstrated in higher organisms. The observation that messenger RNA in mammalian cells may persist for as long as 40 hours has turned attention towards the possibilities of translational control, which have recently received experimental support. Although transcriptional control is not "all we know nor all we need to know," it is still considered probable that it does contribute to differential gene activity in the cells of higher organisms. Histones have been considered as possible repressors of DNA transcription, and the past five years has seen a stimulated interest in this possibility.

The source of recent interest in histones as gene regulators is found in studies published by Huang and Bonner in 1962, and by Allfrey, Littau and Mirsky in 1963, demonstrating the inhibition of RNA synthesis by histones in cell-free systems.

Huang and Bonner (1962) studied the template activity of isolated chromatin prepared from pea embryos. Chromatin was dissociated, by centrifugation in 4 molar cesium chloride, into DNA and protein components and then reconstituted by recombination of the components. When histone
was omitted during reconstitution, the resultant nucleo­histone was five times more active as a template for RNA synthesis by endogenous RNA polymerase.

Allfrey, Littau and Mirsky (1963) studied the incorpor­oration of guanosine-8-14C and adenosine-8-14C by suspen­sions of thymus nuclei. A marked inhibition of RNA synthesis was caused by the addition of lysine-rich or arginine-rich histone to the nuclear suspensions. If nuclei were treated with trypsin to remove histones, RNA synthesis was greatly enhanced; the re-addition of histones again inhibited the reaction.

A great deal of research has followed these two demonstrations that histones can repress genetic activity. One objection which was raised was the possibility that histone inhibition might simply result from precipitation of the DNA template. In a careful study, Butler and Chipperfield (1967) have demonstrated that loss of solubil­ity and loss of template activity do not show the same dependence on histone concentration; that is, there is a true inhibitory effect of histones on the RNA polymerase reaction, independent of the effect on DNA solubility.

Recent work has demonstrated not only an inhibition of the rate of RNA synthesis, but a change in the nature of RNA synthesized when the histone component of the template is altered. Georgiev (1966, 1968) has studied
the effect of removal of histone fl upon the template activity of isolated chromatin. Extraction of the deoxy-nucleoprotein with 0.6M NaCl selectively removes histone fl, leaving 80% of the protein in the complex. The effect of this extraction is a four-fold increase in the rate of RNA synthesis from the DNA template. In addition, the percentage of DNA with which the product RNA will hybridize is increased from 3.7% to 15.3%. (RNA synthesized from free DNA hybridizes with 17% of the DNA.) Thus, the specific removal of the histone fl component of chromatin de-represses the template activity of the DNA practically to the level of free DNA. If histone is added back to the nucleoprotein, the rate of RNA synthesis is reduced almost to its original level, but the hybridizability remains high, indicating that the specificity of association of histone with DNA in the original nucleoprotein cannot be recovered by simple combination of the components.

Hurwitz et al. (1963) and Skalka (1966) have also found qualitative differences between RNA synthesized in the presence and absence of histones. The effect of histones and protamine in vitro on the products of the reactions catalyzed by DNA polymerase, RNA polymerase, and DNA methylase were studied. Only in the case of RNA polymerase was there a change in the reaction product.
The RNA synthesized in the presence of histones was smaller and had a base ratio and sequence different from the normal product.

Bonner, Huang and Gilden (1963) coupled RNA synthesis and protein synthesis in vitro and studied the synthesis of a pea globulin characteristic of pea cotyledons. Synthesis of the protein was detected by immunochromological assay. Isolated chromatin from pea cotyledon supported the synthesis of the globulin in vitro, but chromatin from pea buds was inactive. Deproteinization of the two types of chromatin resulted in loss of specificity; both types of deproteinized DNA were active templates for globulin synthesis. These experiments do not indicate the nature of the component responsible for the apparent tissue-specificity of isolated chromatin.

The in vitro evidence for a controlling effect of histone on RNA synthesis is the basis for considering them as possible genetic repressors. Arguing against a repressor role is the observation that histones do not possess the degree of specificity expected of bacterial-type repressors. There is a remarkable constancy in the kind and amount of histones isolated from disparate sources. To cite a few of many comparative studies, the electrophoretic patterns of histones from normal and cancer cells have been compared by Butler (1963), plant
and animal histones by Fambrough and Bonner (1966),
histones of various tissues during embryonic development
by Kischer and Hnilica (1967), histones of genetically
"active" and "inactive" chromatin by Comings (1967), and
histones of erythrocytes from different species by
Vendreley and Vendreley (1968). In all cases, only minor
differences in electrophoretic patterns were observed.
(Authors do not agree as to the significance of the minor
variations, and no functional assay is available with
which to compare the histones from different sources.)

With the preparation of homogeneous purified histone
fractions, sequence determination on histones has recently
become possible. DeLange and Fambrough (1968) have
reported that the 18-residue carboxy-terminal sequence
is identical in the histone IV isolated from pea and from
calf thymus.

In addition to this extreme lack of specificity, the
histones are characterized by a low rate of metabolic
turnover in comparison with other nuclear and cytoplasmic
proteins (Piha, 1966). Laurance and Butler (1965)
measured the rate of synthesis and turnover of histones
in normal and malignant tissues, and obtained results
which indicated that histone synthesis occurs mainly as
required for cell division. An essentially constant
quantitative ratio of histone protein to DNA, equal to
0.8 - 1.0, was found in the isolated chromatin of all tissues examined by Dingman and Sporn (1966), regardless of the state of genetic activity of the tissue. These observations have raised doubts that histones can function in the same manner as the protein repressors which have been found in bacterial systems.

Under normal conditions, the major portion of the genome of a particular cell type in a higher organism becomes "permanently" repressed during differentiation. It has been suggested that there may be two types of repression in higher organisms; "unconditional repression" of the portion of the genome which is never expressed, and "conditional repression" of inducible genes (Bonner, 1967). Histones, with their lack of specificity and their inhibitory effect on RNA synthesis, might represent the "unconditional" repressors, while another class of repressors similar to the specific bacterial proteins might function in the dynamic regulation of expressible genes.

Huang and Bonner have found, in association with histone, a small RNA fragment of 40 to 60 residues with a high content of dihydrourylidic acid (Huang and Bonner, 1964; Huang, 1967). They suggest that the base sequence of the associated RNA might confer specificity on the interaction of histones with DNA (Bonner, et al., 1968).

An interesting discussion of the possibilities of histone repression is available (Ciba Symposium, 1966).
Enzymatic Modifications of Histone Structure

A recent development has been the discovery of several enzymatic reactions which could provide a mechanism for control of DNA–histone interaction. These reactions modify a particular amino acid residue of intact histone molecules. They therefore afford dynamic control of histone structure which could be significant for either of the functional roles suggested above.

Methylation, acetylation, and phosphorylation of histones have been studied. These histone modifications are summarized in Table 2. Acetylation and phosphorylation reduce the net positive charge of the histones; methylation increases the basicity of the lysine residues.

Preliminary description of the enzymatic transfer of the adenosine diphosphate moiety from nicotinamide adenine dinucleotide to histones has been reported (Nishizuka, Ueda, Honjo and Hayaishi, 1968). This enzymatic activity is strictly associated with the chromatin of mammalian nuclei.

A. Histone Acetylation

The presence of acetyl groups in histones was first detected by Phillips in 1963. He has recently reported the isolation of the N-terminal peptides from tryptic
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A. Histone Acetylation

The presence of acetyl groups in histones was first detected by Phillips in 1963. He has recently reported the isolation of the N-terminal peptides from tryptic
### Table 2.—Enzymatic Modification of Histones

<table>
<thead>
<tr>
<th>Source</th>
<th>Precursor</th>
<th>Amino acid Modified</th>
<th>moles per 20,000 g protein</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus nuclei; Regenerating rat liver.</td>
<td>$^{14}$C-acetate</td>
<td>1. N-terminus; 2. epsilon-N-lysine</td>
<td>----</td>
<td>Pogo, 1966; Allfrey, 1968</td>
</tr>
<tr>
<td>Same</td>
<td>$^{14}$C-Me-Met; S-adenosyl-Met</td>
<td>epsilon-N-lysine</td>
<td>1.8 (f3)</td>
<td>Tidwell, 1968</td>
</tr>
<tr>
<td>Thymus nuclei</td>
<td>$^{32}$P-Pi</td>
<td>Serine</td>
<td>1.0 (f1)</td>
<td>Ord and Stocken, 1966</td>
</tr>
<tr>
<td>Histone kinase from rat liver</td>
<td>$^{32}$P-ATP</td>
<td>Serine</td>
<td>1.0 (f1)</td>
<td>Langan, 1967 a</td>
</tr>
</tbody>
</table>
digests of calf thymus histone fractions f2al and f2a2 (Phillips, 1968). Both fractions, which together comprise 40% of the total histone, have the same N-terminal sequence: N-Acetyl-Ser-Gly-Arg. The N-acetyl group accounts for all the acetate previously observed in these purified fractions.

Allfrey, Faulkner and Mirsky (1964) studied the uptake of acetate-$^{14}$C into histones by isolated thymocyte nuclei. The acetate label migrated with the histone fractions on carboxy methyl cellulose columns and during electrophoresis on cellulose polyacetate strips, and was resistant to hydrolysis by trichloracetic acid at 90°C for 15 minutes. Acetate incorporation was not inhibited by levels of puromycin which prevented incorporation of amino acids, indicating that acetylation occurs after completion of the polypeptide chain. The interesting observation was made that chemically-acetylated histones were much less inhibitory than non-acetylated histones when added to an in vitro RNA polymerase system. At the same time, the acetylated histones retained their effectiveness in stabilizing the DNA against thermal denaturation. Apparently, acetylation can influence the degree of repression exerted by DNA-bound histone.

The response of histone acetylation to gene activation in phytohemagglutinin-stimulated lymphocytes and in
regenerating liver has been studied (Pogo, 1966; Allfrey, 1968). In both cases, increased acetylation of histones preceeded the stimulation of RNA synthesis, as would be expected if acetylation is part of the de-repression mechanism. Sixty one percent of the acetate incorporated in these experiments was recovered in histone fraction f2a1. An increased incorporation of acetate into histones was also observed after administration of hydrocortisone to rats. Although no alteration in the characteristics of newly-synthesized RNA has been directly attributed to histone acetylation, the accumulation of correlations with gene activation are indicative of a possible functional relationship.

B. Histone Methylation

The presence of epsilon-N-methyl lysine in calf thymus histones was detected by K. Murray in 1964. Kim and Paik (1965) have shown that epsilon-N-dimethyl lysine is present in three-fold excess over the monomethyl form in rat histones. The methylation reaction has been studied by Allfrey and co-workers. Incorporation of $^{14}$C-methyl-methionine label into histones by thymus nuclei was not inhibited by puromycin (Allfrey, 1964). The relationship of methylation to histone and DNA synthesis was studied in regenerating rat liver (Tidwell, 1968) by measurement of the incorporation of intraperitoneally injected
$^{14}$C-methyl-methionine, $^{14}$C-arginine, and $^{14}$C-thymidine into histones and DNA extracted from purified nuclei. Methylation of histones was clearly a later event than histone synthesis, and was also delayed with respect to DNA synthesis. The peak in rate of methylation was observed at a time when the rates of DNA and histone synthesis are declining. Methylation occurs much later than the increased RNA synthesis, in sharp contrast with histone acetylation. It is concluded that histone methylation is not related to de-repression, but may accompany the structural changes in nucleohistone structure which occur prior to cell division.

C. Histone Phosphorylation

The presence of phosphate in purified histone and protamine fractions has been observed in several laboratories. K. Murray (1966) reported the presence of phosphoserine in partial acid hydrolysates of commercial protamine. Murray (1968) employed two-dimensional electrophoresis of acid hydrolysates of histones purified from chick erythrocytes for qualitative analysis of phosphoserine and phosphothreonine. Phosphoserine was detected in histone f1, but not in f2a, f2b or f3. Phosphothreonine was not detected.

In 1966, Ord and Stocken injected $^{32}$P-orthophosphate intramuscularly into rats. Non-dialyzable $^{32}$P was detected
in extracted histones, and migrated with the protein on electrophoresis. Histone fl contained 20.4 to 24.8 micromoles of phosphate per gram. The time course of $^{32}\mathrm{P}$ uptake into histones could be distinguished from uptake into acid-labile phosphate, RNA, and DNA. Phosphoserine was identified in hydrolysates of histone fl by paper chromatography. $^{32}\mathrm{P}$ could not be removed from histones by ribonuclease, phenol, or chromatography on Dowex-1, and was considered to be incorporated into the histone protein.

These authors have studied the incorporation of phosphate into histones under a variety of conditions. Rat thymus nuclei incubated with $^{32}\mathrm{P}$-orthophosphate incorporated 25 to 45 micromoles of phosphate per gram of histone fl, in a reaction which was insensitive to actinomycin and puromycin at 100 ug/ml (Steveley and Stocken, 1966). When the phosphate content of histones fl and f3 during liver regeneration was examined (Ord and Stocken, 1968), a two-fold increase in the phosphate content of histone fl was observed 16 to 29 hours after partial hepatectomy. No change in phosphate content or specific activity was observed 4 to 5 hours postoperatively, during the period of enhanced RNA and protein synthesis. There was no variation in the phosphate content of histone f3 at any time. Irradiation of the rats during the period of DNA synthesis caused a decrease in the amount of phosphate
subsequently incorporated into histone fl, suggesting a possible relationship between histone synthesis and phosphorylation.

An effect of phosphate content on the extent of inhibition of RNA synthesis by histones was also observed (Steveley and Stocken, 1966). Two preparations of histone fl were compared as to their effect on the incorporation of $^{14}$C-ATP by RNA polymerase in vitro. The fl preparations contained 34.2 and 22.4 micromoles of phosphate per gram of protein; ATP was incorporated to the extent of 900 cpm and 665 cpm respectively. A higher level of phosphorylation thus appears to reduce the inhibitory effect of histones. This is the only report that phosphorylation can influence histone activity; a similar result has been observed with DNA polymerase (Ord and Stocken, 1968).

Kleinsmith, Allfrey and Mirsky (1966) studied protein phosphorylation by isolated thymocyte nuclei incubated with AT$^{32}$P. Approximately 14% of the $^{32}$P-labeled protein was extracted from the nuclei by 0.2 N HCl, and co-chromatographed with the histones on carboxy methyl cellulose columns. Protein phosphorylation in these experiments was also found to be independent of protein synthesis.

Gutierrez and Hnilica (1967) observed incorporation of $^{32}$P-orthophosphate into rat histones, as evidenced
by the association of radioactivity with histone bands on starch gel electrophoresis. Some differences in the pattern of histone phosphorylation of different tissues was observed.

The phosphorylation of protamine during spermatogenesis in trout testis has been studied in detail by Dixon and coworkers (Ingles and Dixon, 1967). Cell suspensions from trout testes (induced to develop by injections of salmon pituitary extract) were incubated with $^{32}$P-orthophosphate or $^{14}$C-serine for 1-3 hours. Washed cells were then extracted with 0.2 N HCl, and the extracted protein subjected to a five-step chromatographic purification. Both histone and protamine were found to be labeled. The phosphate linkage was stable to acid (0.1 N, 100°C, 15 minutes) but labile to alkali, suggesting the presence of O-phosphoserine. Phosphopeptides were isolated after tryptic digestion of the purified protamine. Three distinct serine-containing sequences were recovered: Val-PSer-Arg, PSer-PSer-Arg, and (Ala,PSer,Arg,)Arg. Comparison of total phosphorous (which was equal to alkali-labile phosphate) with total serine indicated that 74% of the serine residues are phosphorylated in immature testes, while in mature testes only 9% of the serine is phosphorylated. The phosphate content of protamine is thus related to the functional state of the tissue. In
immature spermatid cells there is some RNA and protein synthesis, whereas after sperm maturation the genome is essentially inactive. It is suggested that the addition of phosphate groups may reduce the interaction of protamine with DNA and other cell components until the tight association with nuclear DNA is required.

Protamine synthesis and phosphorylation both take place in the cytoplasm of the spermatid cells, followed by transport of the phosphorylated protamine into the nucleus (Marishuge, 1968).

An independent line of evidence for histone phosphorylation is the discovery by Langan (1967 a) of a specific histone phosphokinase. This enzyme catalyzes the transfer of phosphate from ATP to serine residues of histone and protamine. Phosphoserine has been recovered from enzymatically phosphorylated histone and demonstrated to account for essentially all of the protein-bound phosphate. Histone phosphokinase has been purified four hundred fold from calf liver (Langan, 1968 b). It is practically inactive in the phosphorylation of casein and phosvitin. All histone fractions can be phosphorylated to some extent, but the enzyme is considerably more active in phosphorylating histones f1 and f2b, as compared to f2a or f3. There are indications of the presence of more than one enzyme, with differing specificies for histone subfractions.
A phosphopeptide has been isolated from histone fl after enzymatic phosphorylation by histone kinase (Langan, 1968 a). This single peptide accounts for up to 80% of the phosphate incorporated into histone fl. The composition of the peptide is: Lys-Ala-PSer(Thr,Ser,Glu,Pro₂,Gly,Val, Ilu,Lue)-Lys. Isotope dilution experiments in which the labeled phosphopeptide was diluted with tryptic digests of isolated histone fl indicated that at least 75% of the endogenous phosphate in isolated histone fl is not identical to the phosphopeptide, and may represent contamination by non-histone material.

It has recently been observed that phosphorylation of histone fl by histone phosphokinase is stimulated four-fold in the presence of $10^{-7}$ M 3'5'AMP (Langan, 1968 b). This observation suggests a possible mechanism for hormone-initiated gene activation, mediated by the synthesis of cyclic AMP and the resultant histone phosphorylation.

The in vivo studies of histone phosphorylation described earlier are impaired by the absence of a definitive criterion for histone-bound phosphate. In particular, one must consider the possibility of contamination of the isolated histones by other phosphate-containing proteins. Langan and Lipmann (manuscript in preparation; Langan, 1967 b) have described a class of highly-phosphorylated nuclear proteins. These acidic phosphoproteins form
complexes with histones in vitro. Minor contamination with these rapidly-labeled phosphoproteins could account for the phosphate detected in purified histone preparations. In future studies, isolation of the histone fl phosphopeptide mentioned above might make possible a more rigorous determination of histone phosphate.

The level of phosphate detected in isolated histones (10 micromoles/gram) is considerably lower than can be introduced by enzymatic phosphorylation (40 micromoles/gram into histone fl). In view of the widespread presence of histone phosphatase activity, as described in this thesis, it seems possible that the endogenous phosphate is enzymatically cleaved during the early stages of histone purification.

The phosphate content of histone fl phosphorylated in vitro is approximately one phosphate moiety per molecular weight of 20-25,000 daltons. This represents an addition of two negative charges to a molecule containing an average of 50 basic and 25 acidic amino acids, an 8% reduction in net positive charge. This change might be expected to reduce the ionic interaction between histone and DNA in chromatin. In addition, configurational changes may be produced by phosphorylation. When considering the possible contribution of one phosphate group to the conformation of a histone molecule, the precedent of the phosphorylase "b"
to "a" conversion may be taken as evidence that the structural effect of a low level of phosphorylation may be significant.

To summarize, the presence of phosphate in histones and protamines has been reported by several laboratories. Phosphorylation occurs on intact proteins, and does not require de novo protein synthesis. Histone fl appears to be more highly phosphorylated than the other fractions. Purified fl histones contain less endogenous phosphate (10 micromoles/gram) than can be introduced enzymatically by histone kinase (40 micromoles/gram); the difference may reflect loss of histone phosphate during purification. Protamine is phosphorylated and subsequently dephosphorylated during the process of sperm maturation. There is some suggestion that histone phosphorylation can reduce the extent of repression of RNA synthesis by histones, or that it may accompany the process of histone synthesis, but our understanding of the functional role of phosphorylation is very limited.

Histone phosphorylation must be considered within the context of the unresolved question, discussed above, of the role of histones themselves. An understanding of the phosphorylation phenomenon may contribute to a resolution of the larger question, but it cannot be treated independently. The eventual clarification of the enigmas
surrounding this major component of chromatin cannot fail to be of major biochemical significance.
Dephosphorylation of Phosphoproteins by Phosphatases

Histone phosphatase is a member of the class of phosphoprotein phosphatases, which catalyze the hydrolysis of phosphoesters at the hydroxyl groups of serine and threonine residues of protein molecules. A summary of some properties of the known mammalian phosphoprotein phosphatases, including histone phosphatase, is presented in Table 3.

The phosphatases in the first section of the table are active against the classical, highly-phosphorylated phosphoproteins such as phosvitin (10% phosphorus) and casein (0.9% phosphorus). Even when highly purified, these enzymes retain activity towards certain low molecular weight compounds, such as aromatic phosphates and pyrophosphates.

The three enzymes of the second type are quite different in that each is active towards one specific protein, whose phosphate content is very low (i.e. 1 to 4 phosphate/molecule). These specific phosphoprotein phosphatases are inactive towards the classical phosphoproteins as well as towards low molecular weight phosphates. Phosphorylase phosphatase is the most well studied enzyme of this type; it catalyzes the release of four moles of orthophosphate from phosphorylase "a", converting it to
### TABLE 3.— Phosphoprotein Phosphatases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Substrate</th>
<th>Relative Activity</th>
<th>Km</th>
<th>pH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Other&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 1.</td>
<td>Phosphoprotein phosphatase</td>
<td>Beef spleen, rat spleen</td>
<td>Casein, pNpp, pyrophosphate, betaGPE</td>
<td>100</td>
<td>6 x 10&lt;sup&gt;-4&lt;/sup&gt;M</td>
<td>6.0</td>
<td>no Heat stable, -100%, 10&lt;sup&gt;-4&lt;/sup&gt;Mg</td>
<td>Revel (1963)</td>
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<td>100</td>
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<td>150</td>
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<td>0</td>
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<tr>
<td>2.</td>
<td>Phosphoprotein phosphatase</td>
<td>Mouse liver</td>
<td>Casein</td>
<td>100</td>
<td>8 x 10&lt;sup&gt;-4&lt;/sup&gt;M</td>
<td>&lt;6.0</td>
<td>no Particulate + ferrous ion -50%, 10&lt;sup&gt;-5&lt;/sup&gt;Mg</td>
<td>Paigen (1958)</td>
</tr>
<tr>
<td>3.</td>
<td>Phosphoprotein phosphatase</td>
<td>Ox brain</td>
<td>Casein, Phosvitin, betaGPE</td>
<td>yes, yes, no</td>
<td></td>
<td></td>
<td></td>
<td>Rose (1961)</td>
</tr>
<tr>
<td>II 1.</td>
<td>Phosphorylase phosphatase</td>
<td>Dog liver</td>
<td>Phosphorylase &quot;a&quot;, Phosvitin</td>
<td>yes, yes, no</td>
<td>&gt; 10&lt;sup&gt;-6&lt;/sup&gt;M</td>
<td>8.0</td>
<td>no -80%, 0.15M NaCl, -100%, 10&lt;sup&gt;-2&lt;/sup&gt;M NaF</td>
<td>Wosilait (1956)</td>
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<tr>
<td>2.</td>
<td>Phosphorylase phosphatase</td>
<td>Rabbit muscle</td>
<td>Phosphorylase &quot;a&quot;, Phosvitin Casein, Low M.W.</td>
<td>100</td>
<td>- - - - -</td>
<td>6.8</td>
<td>no</td>
<td>Graves (1959)</td>
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<tr>
<td>3.</td>
<td>Phosphorylase kinase phosphatase</td>
<td>Rabbit muscle</td>
<td>Phos.kinase&lt;sup&gt;f&lt;/sup&gt;, pNpp</td>
<td>100</td>
<td>- - - - -</td>
<td>6.8</td>
<td>Yes -50%, 10&lt;sup&gt;-2&lt;/sup&gt;M NaF</td>
<td>Riley (1968)</td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>4.</td>
<td>Histone phosphatase</td>
<td>Rat liver</td>
<td>P-Protamine, P-Histone, Phosvitin</td>
<td>100</td>
<td>2 x 10&lt;sup&gt;-5&lt;/sup&gt;M</td>
<td>7.5</td>
<td>no -15%, 10&lt;sup&gt;-3&lt;/sup&gt;M Mg&lt;sup&gt;9&lt;/sup&gt;, -15, 10&lt;sup&gt;-2&lt;/sup&gt;M NaF, -0%, 10&lt;sup&gt;-3&lt;/sup&gt; NaCN, +100%, 0.2M NaCl</td>
<td>Part II</td>
</tr>
</tbody>
</table>

<sup>a</sup> pH at which optimal activity is observed.
<sup>b</sup> Reaction stimulated by millimolar concentrations of magnesium ions.
<sup>c</sup> -X% represents % inhibition; +X% represents degree of stimulation.
<sup>d</sup> p-nitrophenyl phosphate.
<sup>e</sup> beta-glycerol phosphate.
<sup>f</sup> Activated phosphorylase kinase.
<sup>g</sup> Molybdate ion.
the less active form, phosphorylase "b". The second example, recently described by E. G. Krebs and co-workers, is an enzyme which reverses the activation of phosphorylase kinase, which is itself activated by phosphorylation (Riley, et al., 1968; DeLange, et al., 1968).

Histone phosphatase is readily distinguished from the enzymes of the first type by its low level of activity towards casein and phosvitin. It is more difficult to distinguish between the specific phosphoprotein phosphatases, since their substrates are not readily available for cross-testing. Even where substrates are available, it is difficult to extrapolate from experimental assays to physiological activities. Since these enzymes have been isolated from different tissues and species, direct comparison of their properties cannot unambiguously distinguish between them.
OBJECTIVES OF THIS WORK

The object of this work was to investigate the poorly-understood phenomenon of histone phosphorylation. Attention was directed towards the previously unstudied dephosphorylation reaction. Histone phosphate undergoes rapid turnover. Is the dephosphorylation catalyzed by a specific enzyme? If so, when and where does the reaction occur, and how does the addition or removal of phosphate from histones influence their biological activity?

We have succeeded in demonstrating the presence in rat liver of a highly specific phosphatase which removes phosphate from histones and protamines. The enzyme has been prepared free from contaminating phosphatase and protease activities. Most of the work reported here is concerned with purification and characterization of this new enzyme. Some information relevant to considerations of function has been obtained. Purified histone phosphatase is a potentially useful tool for future investigation of the biological questions raised above.
MATERIALS AND METHODS

Materials.

Histone fractions f1 and f2b were prepared from calf thymus by the methods of Johns (1964). Thymus glands were obtained at a local slaughterhouse, transported to the laboratory on ice, and frozen in liquid nitrogen for storage.

Protamine was isolated from salmon sperm milt by the method of Callanan (1957).

Histone kinase was prepared according to the method of Langan (1968 b). Enzyme purified to the ammonium sulfate stage was employed in the preparation of phosphorylated protamine, and to the DEAE stage for phosphorylation of histone f1. One unit of histone kinase catalyzes the transfer of one micromole of phosphate per hour from ATP to histone.

Beta, gamma-labeled AT^{32}P was prepared by photophosphorylation carried out by spinach chloroplasts, by a procedure (Langan, unpublished) based on the method of Petrack et al. (1965). The photophosphorylation reaction was stopped by heating for two minutes at 100°C. AT^{32}P was purified by chromatography on DEAE-cellulose, eluting with (NH$_4$)$_2$CO$_3$ buffers, pH 8.6. In some cases, AT^{32}P was used without purification from chloroplast supernate.
Calcium phosphate gel was prepared from calcium chloride and trisodium phosphate by the method of Keilin and Hartree (Colowick, 1955).

Preparation of phosphorylated histones and protamines is described below. Other compounds and enzymes were obtained commercially.

I thank Mr. John Koontz for providing the histone, histone kinase and ATP32P preparations used in this study, and Mr. T. Sewell for protamine preparations.

Methods.

Protein was determined by the biuret method (Layne, 1957), with bovine serum albumin as a standard.

The chemical determination of inorganic phosphate was performed by a modification of the method of Martin and Doty (1949). To 2 ml of the deproteinized sample is added 0.5 ml of 5% ammonium molybdate in 4N sulfuric acid. The phosphomolybdate complex is extracted by emulsification with an equal volume of isobutanol/benzene, 1/1. An aliquot of the solvent layer is reduced by the addition of stannous chloride. The absorption due to reduced molybdate is measured in a Klett colorimeter with filter #66, or at 660 millimicrons with the Zeiss spectrophotometer. The method is accurate down to 10−8 moles of inorganic phosphate. When radioactive substrates were
employed, an aliquot of the isobutanol/benzene extract was plated on stainless steel planchets, dried and radioactivity measured in a Nuclear Chicago gas flow counter.

**Electrophoresis** of histones and protamines on poly­acrylamide gels was performed in an E.C. Corporation preparative/analytical cell, and in the Canalco apparatus for disc electrophoresis. Ten percent polyacrylamide gels containing 5% NN'methylene bis acrylamide and 1% NNN'N' tetramethyl ethylene diamine were prepared in 0.04 M beta-alanine, acetic acid buffer at pH 4.5. Polymerization was initiated by ammonium persulfate. Gels were stained overnight with amido black, and destained electrophoretically.
Preparation of Substrates

$^{32}$P-labeled protamine and histone were phosphorylated enzymatically with histone kinase, and isolated from the reaction mixture by differential precipitation with TCA (Langan, unpublished results). The components of a typical preparative incubation mixture are listed below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protamine or histone</td>
<td>25 mg</td>
</tr>
<tr>
<td>Histone kinase (2 units)</td>
<td>ca. 3 mg</td>
</tr>
<tr>
<td>beta-gamma-AT$^{32}$P</td>
<td>5 micromoles</td>
</tr>
<tr>
<td>(ca. 80 x $10^6$ cpm)</td>
<td></td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>5 micromoles</td>
</tr>
<tr>
<td>Tris HCl, pH 7.5</td>
<td>250 micromoles</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>25 micromoles</td>
</tr>
</tbody>
</table>

in 5 ml reaction volume

After incubation for five hours at 37°C, the reaction was stopped by the addition of trichloracetic acid (TCA) to a final concentration of 2%. This is sufficient to precipitate the histone kinase, while leaving the histone or protamine in solution. After washing the precipitated protein once with 2% TCA, the combined supernates are brought to 25% TCA by the addition of an equal volume of 50% TCA. This results in precipitation of the histone or
protamine, which is washed three times by suspension in water and reprecipitation with TCA, in order to remove all contaminating AT$^{32}$P from the protein. The reprecipitated histone is washed twice with ethanol/ether, 20/80, to remove TCA, and twice with 0.1 N HCl in ethanol/ether, 20/80. The washed protein is dried overnight in a vacuum dessicator, then dissolved in water and assayed.

The content of alkali labile phosphate (1 N NaOH, 100°C, 15 minutes) and acid labile phosphate (0.8 N HCl, 0.2 N H$_2$SO$_4$, 100°C, 15 minutes) are determined chemically as described above. The esterified phosphate incorporated into histone as phosphoserine is released by the alkali treatment. Acid labile phosphate represents contaminating AT$^{32}$P, AD$^{32}$P, and inorganic phosphate adhering to the washed protein, as well as some hydrolysis of phosphoserine. Acid labile radioactivity was always less than 1% of the total.

A typical preparation resulted in a 75 to 85% yield of protein. Protamine was phosphorylated to the extent of ca. 250 micromoles of phosphate per gram, and histone $f_1$ to 50 micromoles per gram. The level of phosphorylation in both cases corresponds to approximately one phosphate moiety per protein molecule (one mole phosphate per 5,000 grams of protamine or 20,000 grams of histone $f_1$). However, we do not know the actual distribution of phosphate among protein molecules. Electrophoresis of protamine on
polyacrylamide gels demonstrates the appearance of at least two new bands of phosphorylated protamine (Langan, unpublished observation) which disappear as a result of dephosphorylation (Figure 15). These phosphorylated bands presumably differ with respect to their phosphate content, but the absolute values are not known.
Assay of Histone Phosphatase Activity

Release of phosphate from the phosphorylated substrates described above was assayed either by chemical determination of inorganic phosphate released or by determination of the $^{32}$P radioactivity present as inorganic phosphate. When the radioactivity assay was used, samples were counted and compared with standards prepared during the analysis of phosphorylated substrates. The specific activity of substrates varied from 1,000 to 6,000 cpm/nanomole phosphate, making possible the accurate determination of as little as $10^{-10}$ moles of phosphate, a sensitivity one hundred fold greater than that of the chemical assay. The radioactive assay procedure was routinely employed; chemical phosphate determinations were used only for the specificity studies. All assays were performed in duplicate; less than 5% difference between duplicates was accepted.
Incubation medium:

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylated histone (1.2 mg/ml) or protamine (0.3 mg/ml)</td>
<td>$6 \times 10^{-5}$ M phosphate</td>
</tr>
<tr>
<td>Enzyme, to release less than 25% of total phosphate</td>
<td>-----------</td>
</tr>
<tr>
<td>Tris HCl, pH 7.5</td>
<td>0.05 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.20 M</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.001 M</td>
</tr>
</tbody>
</table>

Reaction volume, 0.10 ml or 0.25 ml.

Assay Procedure

1. Reaction started by addition of substrate.
2. Incubate 5 minutes at 37.5°C.
3. Reaction stopped by addition of 2 ml of silicotungstate/sulfuric acid reagent, $5 \times 10^{-3}$ N in each component.
4. Centrifuge at 1000 X g for 5 minutes to remove precipitated protein.
5. Aliquot 2 ml of supernatant solution for phosphate determination by either of the methods described above.

Under these conditions, the histone phosphatase-catalyzed reaction is linear with respect to time and enzyme concentration within the ranges employed in these studies, as demonstrated in Figures 1 and 2.
Figure 1.—Linearity of Histone Phosphatase Reaction with Time. Enzyme = 7 micrograms of calcium phosphate fraction. Reaction conditions in text.

Figure 2.—Linearity of Histone Phosphatase Reaction with Enzyme Concentration. Enzyme = ammonium sulfate fraction. Reaction time, 5 minutes.
EXPERIMENTAL RESULTS

Subcellular Distribution of Histone Phosphatase Activity

The subcellular localization of histone phosphatase activity received considerable attention because of its functional implications. If histone phosphorylation and dephosphorylation are related to genetic control, then we would predict that the phosphatase would be found in the nucleus. If phosphorylation accompanies synthesis and/or transport of histones across the nuclear membrane, as suggested by Dixon for protamine, then histone phosphatase activity might be cytoplasmic, or associated with the nuclear membrane. Unfortunately, the technique of cell fractionation applied here was not adequate for the unambiguous resolution of the subcellular localization of histone phosphatase.

A. Cell Fractionation in 0.25 M Sucrose.

Subcellular fractions were prepared from rat liver by standard procedures. Rats were stunned, sacrificed by decapitation and bled. Livers were removed to ice cold 0.25 M sucrose, washed and homogenized in 30 ml of sucrose solution per 10 grams wet weight, in a glass homogenizer with a rotating teflon pestle (940 rpm). The enzyme distribution was not altered by varying the
duration of homogenization; 8 strokes were routinely used. All subsequent steps were performed at 0° - 4°C. The homogenate was diluted to 60 ml/10 grams of liver with sucrose, filtered through four layers of gauze, and subjected to differential centrifugation. Centrifugation at 700 x g for ten minutes brought down the crude nuclear pellet. Mitochondria were collected by centrifugation at 10,000 x g for ten minutes. Further centrifugation of the mitochondrial supernatant in the #40 rotor, Spinco Model L ultracentrifuge, for one hour at 40,000 rpm (105,000 x g) resulted in separation of the microsomal pellet from the soluble fraction of the cell.

The results of a typical preparation from one rat liver are presented in Table 4.

The major portion of the activity (more than 80%) is recovered in the high-speed supernatant fraction, which has been used as the starting material for enzyme purification. The activity of the mitochondrial and microsomal fractions was negligible. The crude nuclear fraction contained approximately 6% of the cellular activity; its specific activity varied from 40 - 70% of that of the total homogenate.

1 Purified lysosomal preparations were also inactive - (Section F).
Table 4.—Distribution of Histone Phosphatase Activity in Subcellular Fractions Prepared in 0.25 M Sucrose.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg</th>
<th>Protein %</th>
<th>Specific Activity μM/hr/mg</th>
<th>Units</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homogenate</td>
<td>1,865</td>
<td>100%</td>
<td>133</td>
<td>248,000</td>
<td>100%</td>
</tr>
<tr>
<td>Crude nuclear (700 x g)</td>
<td>261</td>
<td>14</td>
<td>56</td>
<td>14,600</td>
<td>6</td>
</tr>
<tr>
<td>Mitochondrial (10,000 x g)</td>
<td>435</td>
<td>23</td>
<td>3</td>
<td>130</td>
<td>0.5</td>
</tr>
<tr>
<td>Microsomal (105,000 x g)</td>
<td>394</td>
<td>21</td>
<td>13</td>
<td>512</td>
<td>0.2</td>
</tr>
<tr>
<td>Soluble Recovery</td>
<td>645</td>
<td>35</td>
<td>313</td>
<td>202,000</td>
<td>81</td>
</tr>
</tbody>
</table>

B. Preparation of Purified Nuclei.

The considerable specific activity of the crude nuclear fraction, coupled with the theoretical issues discussed earlier, prompted us to further investigation of the nuclear activity. First we tried to minimize possible enzyme leakage by stabilization of the nuclear membrane through the addition of 3 mM magnesium chloride to the homogenization medium (Hogebloom, Schneider and Striebich, 1952). The presence of magnesium ions had no effect on the total activity of the crude nuclear fraction, although it did bring about redistribution of total protein away from mitochondria and microsomes and into the crude nuclear pellet.
We then reasoned that if the activity of the crude nuclear fraction reflected contamination by cytoplasmic components, further purification of the nuclei should reduce their contaminating activity. Accordingly, nuclei were purified by the method of Chauveau (1956). Livers were homogenized in 0.88 M sucrose, 3 mM magnesium chloride. The homogenate was centrifuged at 1600 x g for ten minutes. The crude nuclear pellet was resuspended in 2.2 M sucrose, 3 mM magnesium chloride and centrifuged at 28,000 rpm for forty-five minutes in the Model L ultracentrifuge, #30 rotor. In this dense sucrose medium, the nuclei are pelleted while erythrocytes, whole cells and cytoplasmic debris float to the surface. The supernate was discarded and the purified nuclei resuspended in 0.88 M sucrose for assay. In one experiment, the recovery of DNA in the purified nuclei was 70% that of total DNA, and the ratio of DNA to protein was 0.36. The results are presented in Table 5. As indicated, the specific activity of the nuclei was not decreased by the removal of cytoplasmic contamination; rather, the activity appeared to actually be associated with the nuclei.

C. Subnuclear Localization of Histone Phosphatase Activity.

Having demonstrated that there is histone phosphatase activity in purified nuclei, we were interested in possible compartmentalization of the enzyme within the nucleus.
Table 5.—Histone Phosphatase Activity of Liver Nuclei Purified by the Chauveau Method

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Specific Activity, μM/hr/mg</th>
<th>% of Total Activity in Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Nuclei · Homogenate</td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>115</td>
<td>132</td>
</tr>
<tr>
<td>2</td>
<td>196</td>
<td>212</td>
</tr>
<tr>
<td>3</td>
<td>213</td>
<td>213</td>
</tr>
<tr>
<td>4</td>
<td>148</td>
<td>205</td>
</tr>
</tbody>
</table>

Purified nuclei were subjected to fractionation by a procedure previously employed by Langan and Lipmann (manuscript in preparation; Langan, 1967b). The enzymatic activity of each fraction was determined. The procedure employed is outlined in Figure 3 and described below.

Figure 3.—Subnuclear fractionation procedure.
The purified nuclear pellet is suspended in 0.14 M NaCl, 0.04M Tris pH 7.5, and centrifuged at 1600 x g for ten minutes. The supernate is the "0.14M extract". The residue is resuspended in 0.14 M NaCl, 0.04 M Tris, to which is added an equal volume of 2 M NaCl, bringing the final concentration to 1 M. The 1 M NaCl suspension is treated with the Polytron high frequency homogenizer (Kinematica GMBH, Lucerne, Switzerland) for 20 seconds, followed by centrifugation at 33,000 x g for fifteen minutes. This step solubilizes the nucleoprotein, which is next removed from the 1 M extract by dilution of the extract to a final salt concentration of 0.4 M. The nucleohistone gel is removed by centrifugation at 78,000 x g for 75 minutes; the supernate from the final centrifugation is the "0.4 M extract". The distribution of histone phosphatase activity among these fractions is presented in Table 6. The activity was distributed between the 0.14 M and 0.4 M NaCl extracts. This division of activity between the two fractions is somewhat unusual. The result was quite reproducible, and repeated washing with 0.14 M NaCl at step one released only 2% more of the activity. Siebert (1965) has classified nuclear enzymes either as "soluble" in 0.14 M NaCl (glycolytic enzymes and DNA polymerase), extractable by 1 M NaCl (DPN pyrophosphorylase)
Table 6.—Subnuclear Localization of Histone Phosphatase Activity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein %</th>
<th>Specific Activity (µM/hr/mg)</th>
<th>Relative Specific Activity</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chauveau Nuclei</td>
<td>100%</td>
<td>148</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100%</td>
</tr>
<tr>
<td>0.14 M Extract</td>
<td>15</td>
<td>338</td>
<td>230</td>
<td>33</td>
</tr>
<tr>
<td>0.4 M Supernate</td>
<td>27</td>
<td>250</td>
<td>170</td>
<td>47</td>
</tr>
<tr>
<td>Nucleoprotein</td>
<td>34</td>
<td>14</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Residual protein</td>
<td>18</td>
<td>62</td>
<td>40</td>
<td>8</td>
</tr>
<tr>
<td>Recovery:</td>
<td>94%</td>
<td></td>
<td></td>
<td>91%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total homogenate = 100 to 140.

or insoluble in 1 M NaCl. The behavior of histone phosphatase is intermediate between the first two categories.

The enzyme is probably not associated with chromatin in vivo, since it does not come out of solution when the nucleoprotein is precipitated with 0.4 M NaCl. We have also observed that the specific activity of purified chromatin is very low (25 nanomoles/hr/mg).

D. Comparison of Nuclear and Cytoplasmic Histone Phosphatase Activities.

The question arose as to whether the dephosphorylation of histones by the cytoplasmic and nuclear fractions might
be catalyzed by two different enzymes. Therefore, the properties of the nuclear extracts (0.14 M and 0.4 M NaCl) were both studied, and compared with the cytoplasmic enzyme. No major differences were observed, as summarized in Table 7.

Table 7.—Comparison of Cytoplasmic and Nuclear Histone Phosphatase

<table>
<thead>
<tr>
<th>Item</th>
<th>Nuclear(^a)</th>
<th>Cytoplasmic(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of Total Activity</td>
<td>2%</td>
<td>80%</td>
</tr>
<tr>
<td>pH Optimum</td>
<td>7 - 8</td>
<td>7 - 8</td>
</tr>
<tr>
<td>Apparent Km, Protamine</td>
<td>(12 \times 10^{-6} M)</td>
<td>(5 \times 10^{-6} M)</td>
</tr>
<tr>
<td>Relative Activity, Protamine : Histone fl</td>
<td>1.8 : 1</td>
<td>2.5 : 1</td>
</tr>
<tr>
<td>Activation by 1 mM DTT</td>
<td>+ 15%</td>
<td>+ 15%</td>
</tr>
</tbody>
</table>

\(^a\) Combined 0.14 M and 0.4 M NaCl Extract  
\(^b\) 78,000 x g supernatant

The pH dependency of the two fractions is superimposable. The values for apparent Km agree within a factor of two, which is not regarded as a significant difference. The relative rates of hydrolysis of protamine and histone fl are similar. The nuclear enzyme was less sensitive to changes in ionic strength than the cytoplasmic activity.
This evidence does not establish that the nuclear and cytoplasmic enzymes are different; it does suggest that the same or similar enzymes may be responsible for the observed activities.

E. Histone Phosphatase Activity of Nonaqueous Fractions.

Our observations of activity associated with the nuclear and the soluble fractions of the cell might be explained if histone phosphatase is a soluble nuclear enzyme which is released into the cytoplasm during homogenization of the tissue in aqueous solvents. DNA polymerase is one example of an enzyme which exhibits this behavior, as discussed below. Nonaqueous fractionation techniques would be expected to reduce nuclear leakage, and therefore to result in nuclear preparations with a higher specific activity than that observed after aqueous isolations. We investigated this possibility by determinations of the specific activity of nuclear and cytoplasmic fractions prepared under nonaqueous conditions.

We are grateful to Dr. LeRoy Kuehl, of the University of Utah School of Medicine, for providing us with nonaqueous fractions prepared by modifications of the method introduced by Behrens (Kuehl, 1967). In this type of preparation, the fresh tissue is immediately frozen and lyophilized. The dry powder is ground with petroleum ether in a ball mill to fragment the cells. The nuclei are then separated
from both lighter and heavier tissue components by alternate sedimentations and flotations in cyclohexane/carbon tetrachloride mixtures of varying specific gravity. After the final centrifugation, the fractions are dried by washing with petroleum ether and stored in a vacuum dessicator.

Dry nuclear and cytoplasmic fractions, as received from Dr. Kuehl, were extracted with 0.4 M NaCl, 0.02 M Tris pH 7.5, or with 1 M NaCl, by homogenization by hand in a glass/glass homogenizer. Insoluble protein was removed by centrifugation at 1600 x g for ten minutes. Two independent extracts were prepared from each cell fraction by slightly different procedures. The observed values for histone phosphatase specific activity were 147 and 178 muM/hr/mg for the cytoplasmic fractions, 354 and 280 for the nuclear fraction. These values are very similar to our results on aqueous fractions, i.e., a specific activity of 210 muM/hr/mg for the 10,000 x g supernate of Table 4, and 250 muM/hr/mg for the 0.4 M nuclear extract in Table 6. Fractionation in organic solvents evidently has little effect on the absolute value or on the subcellular distribution of histone phosphatase activity.

It is relevant to compare this result with the observations which have been made with DNA polymerase.
The nuclear site of the major portion of cellular DNA synthesis, presumably catalyzed by DNA polymerase, has been established by radioautography. However, when subcellular fractions prepared in aqueous sucrose are assayed, essentially no DNA polymerase activity is detected in the low speed pellets. The development of nonaqueous fractionation procedures made possible the demonstration of measurable DNA polymerase activity in nuclei. But even in nonaqueous preparations, cytoplasmic activity is considerable. For example, Kier (1962) found essentially equivalent specific activities in nonaqueous cytoplasm, nuclei and solvent-treated whole tissue. Behki and Schneider (1963) reported these values for the percentage of total activity of lyophilized tissue recovered in nonaqueous nuclear fractions: normal liver nuclei - 9.4%, regenerating liver nuclei - 25%, hepatoma nuclei - 54%. Smith and Kier (1963) obtained values for DNA polymerase specific activity of 2.7 and 3.9 μM/hr/mg respectively in nonaqueous cytoplasmic and nuclear preparations from rat thymus. In spite of this observation of activity in nonaqueous cytoplasmic fraction, it is usually assumed that DNA polymerase is a nuclear enzyme, due to the histochemical and radioautographic evidence.

We are left with an unresolved situation. We might conclude that histone phosphatase activity is distributed
throughout the soluble space of the cell as is the case for the glycolytic enzymes (Siebert, 1965). However, our data is not incompatible with a nuclear source of the histone phosphatase. Histological studies would be desirable to obtain more evidence, since this point is not resolved by cell fractionation techniques.

F. Absence of Histone Phosphatase in Purified Lysosomes.

It is well known that many hydrolytic enzymes of the cell are localized in the lysosomes. Since release of an enzyme due to rupture of lysosomes during homogenization might produce a subcellular distribution pattern similar to that observed, we undertook a study of the histone phosphatase content of purified lysosomes. We were able to demonstrate the absence of histone phosphatase in purified, intact lysosomes.

Purified lysosomes were prepared by the method of deDuve (1955). Rat liver was gently homogenized in 0.25 M sucrose, and the lysosomes isolated by differential centrifugation. Characteristics of our lysosomal preparations were comparable to those reported by deDuve. Beta-glucuronidase was employed to demonstrate the behavior of a true lysosomal enzyme. In a preparation of purified intact lysosomes beta-glucuronidase activity is low, but if the lysosomes are disrupted, the "latent" activity is released and becomes measurable. Purified lysosomes were
disrupted either by preincubation at 37°C or by exposure to 0.1% Triton X-100.

beta-Glucuronidase activity was assayed by spectro-photometric determination of phenolphthalein released from phenolphthalein glucuronide. Incubation was performed for fifteen minutes at 37°C in acetate buffer, pH 5.5, with one millimolar substrate concentration. The reaction was stopped by addition of glycine buffer, pH 10.7, and the optical density at 545 millimicrons was measured\(^1\). Histone phosphatase was assayed by the usual procedure. The results of these experiments are presented in Figure 4 and Table 8.

Table 8. Effect of 0.1% Triton X-100 on Lysosomal beta-Glucuronidase and Histone Phosphatase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg</th>
<th>beta-Glucuronidase Total Units(^a)</th>
<th>Histone Phosphatase Total Units(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm</td>
<td>1140</td>
<td>132</td>
<td>386</td>
</tr>
<tr>
<td>Cytoplasm + Triton</td>
<td>&quot;</td>
<td>558</td>
<td>285</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>208</td>
<td>16</td>
<td>1.2</td>
</tr>
<tr>
<td>Lysosomes + Triton</td>
<td>&quot;</td>
<td>342</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\(^a\) One unit hydrolyzes one micromole of substrate per hour.

\(^1\) The extinction coefficient of phenolphthalein under these conditions was found to be 2.9 x 10\(^4\).
Figure 4.—Comparison of Lysosomal beta-Glucuronidase with Histone Phosphatase: Effect of Preincubation at 37°C.
As expected for a lysosomal enzyme, both treatments resulted in marked activation of beta-Glucuronidase activity. In Figure 4, the slight decrease in histone phosphatase activity during a two hour incubation is contrasted with the striking release of beta-glucuronidase from the same lysosomes. In Table 8, the effect of 0.1% Triton X-100 (five minutes exposure at 0°C) on the two enzymes is compared. Again, the activity of the lysosomal enzyme is markedly elevated by the detergent, while the histone phosphatase is essentially unchanged from its initial very low value.

We feel that these experiments have excluded the possibility that histone phosphatase is localized in lysosomal particles.
Purification of Histone Phosphatase

The enzyme was purified from soluble fraction of rat liver homogenates. The amount of starting material varied from 10 to 100 grams wet weight; a preparation from 100 grams of liver will be described.

Fasted male rats (ca. 250 grams) were sacrificed by decapitation and bled. Livers were removed, placed in ice-cold 0.25 M sucrose, and washed. All subsequent steps were performed at 0-4°C. Ten gram portions of liver were homogenized in 30 ml of 0.25 M sucrose with 8 passes of a loose fitting teflon pestle revolving at 940 rpm. The volume was brought to 60 ml and the crude homogenate filtered through four layers of gauze. The filtered homogenates were centrifuged at 3,000 x g. The floating lipid layer was removed by aspiration and the remaining supernate transferred to a #30 rotor for centrifugation at 30,000 rpm (78,000 x g) for one hour in the Spinco Model L ultracentrifuge.

The clear 78,000 x g supernate was fractionated with ammonium sulfate. Solid ammonium sulfate (182 grams per liter of supernatant) was added to bring the ammonium sulfate concentration to 31% of saturation. The precipitated protein was discarded after centrifugation at 30,000 x g for fifteen minutes. An additional 100 grams per
liter of ammonium sulfate was added to bring the concentration to 47% of saturation. The precipitated protein was collected by centrifugation and dissolved in a sufficient volume of 0.05 M Tris pH 7.5 to give a protein concentration of 5 mg/ml ("ammonium sulfate 31/47" fraction).

A suspension of calcium phosphate was added dropwise to the ammonium sulfate fraction, to a final weight ratio, gel to protein, of 1.2 to 1. Centrifugation at 3,000 x g for 10 minutes results in collection of the gel at the bottom of the tubes, with all of the enzyme activity absorbed to the gel along with 2/3 of the non-enzyme protein. The gel was resuspended first in 0.08 M ammonium sulfate, containing 0.001 M dithiothreitol and 0.05 M Tris pH 7.5, in a volume equal to that of the original protein solution. After recentrifugation, the supernatant solution containing 10% of the non-enzyme protein is discarded. Further elution of the gel with 0.22 M ammonium sulfate, 0.001 M dithiothreitol, 0.05 M Tris pH 7.5, releases the enzyme into solution, with 2.5 to 3 fold purification and a yield of approximately 45% of the original activity in 300 mg of protein. (The calcium phosphate step was most successful when care was taken to keep the protein concentration below 5 mg/ml throughout).

The calcium phosphate eluate was concentrated five-fold by precipitation with 55% saturated ammonium sulfate.
The precipitate was dissolved in 0.05 M Tris, pH 7.5, at a protein concentration of approximately 5 mg/ml. Portions of this concentrated calcium phosphate fraction were subjected to chromatography on DEAE cellulose columns. For chromatography of 100 mg of protein, a column of dimensions 4 cm X 20 cm was employed. This column was eluted with a two liter linear gradient of sodium chloride, 0.01 M to 0.45 M, containing 0.02 M Tris pH 7.5 and 0.001 M dithiothreitol. Fractions were collected at a flow rate of 120 ml/hr. The salt concentration of each fraction was determined by measurement of conductivity with a Radiometer conductivity meter, type CDM 2e. Histone phosphatase activity was assayed using 0.1 ml aliquots of column fractions.

Histone phosphatase was resolved in DEAE into two approximately equal active fractions, one eluting at 0.18 M NaCl and the other at 0.25 M NaCl, as demonstrated in Figure 5a. From 100 mg of calcium phosphate fraction applied, approximately 15 mg of Fraction I and 10 mg of Fraction II were recovered.

For the final purification step, DEAE fractions I and II were each pooled, collected by precipitation with 55% ammonium sulfate, and subjected to chromatography on Sephadex G-200. Two columns, 2.5 cm x 45 cm, were connected so that the effluent from the first flowed directly into
Figure 5.—DEAE Cellulose Chromatography of Calcium Phosphate Fraction. Histone Phosphatase: cpm/0.1 ml/5 minutes; 2800 cpm/nm phosphate. Columns aligned according to NaCl concentration.

a. Sample applied = 21 mg of calcium phosphate fraction. Peaks I and II pooled as indicated.

b. Re-chromatography of Peak I.

c. Re-chromatography of Peak II.
the second; the net dimensions were 2.5 cm X 90 cm. Samples of up to 10 mg of protein were applied in a 2 ml volume. Sephadex Flow Adaptors from Pharmacia were used to permit upward flow elution. Columns were operated with hydrostatic pressure of 17 cm of water, at a flow rate of 20 ml per hour. The composition of the eluting buffer was 0.05 M NaCl, 0.02 M Tris-HCl, pH 7.5, 0.001 M dithiothreitol. The elution volume of blue dextran from the combined columns was 137 ml; NaCl was eluted at 395 ml. The results of chromatography of DEAE Fraction I are presented in Figure 6. Both DEAE Fractions I and II were eluted with peak activity at 177 ml, +/-2%. The p-nitrophenyl phosphatase activity (substrate concentration, 10 mM) was less than 1% of the histone phosphatase activity of both fractions after chromatography on sephadex.

Typical results from the complete purification procedure are presented in Table 9.
Figure 6.—Chromatography of DEAE Fraction I on Sephadex G-200. Sample applied = 4 mg in 2 ml volume. Six ml fractions collected. Conditions described in text. Histone Phosphatase, cpm/3 min/0.2 ml; 1300 cpm/nm phosphate.
Table 9.—Results of Purification of Histone Phosphatase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg</th>
<th>Specific Activity muM/hr/mg</th>
<th>Total Units</th>
<th>Purification</th>
<th>Yield of Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Homogenate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12,400</td>
<td>150</td>
<td>1,860,000</td>
<td>1.0x</td>
<td>100%</td>
</tr>
<tr>
<td>78,000 x g Supernate</td>
<td>4,800</td>
<td>310</td>
<td>1,490,000</td>
<td>2.1x</td>
<td>80</td>
</tr>
<tr>
<td>Ammonium Sulfate, 31/47%</td>
<td>1,400</td>
<td>700</td>
<td>980,000</td>
<td>4.7x</td>
<td>53</td>
</tr>
<tr>
<td>Calcium Phosphate</td>
<td>320</td>
<td>2,000</td>
<td>640,000</td>
<td>13.3x</td>
<td>34</td>
</tr>
<tr>
<td>DEAE I</td>
<td>51</td>
<td>3,500</td>
<td>180,000</td>
<td>23 x</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>36</td>
<td>5,500</td>
<td>200,000</td>
<td>37 x</td>
<td>+ 21%</td>
</tr>
<tr>
<td>DEAE II</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ 11%</td>
</tr>
<tr>
<td>Sephadex I</td>
<td>32</td>
<td>5,000</td>
<td>160,000</td>
<td>33 x</td>
<td>9</td>
</tr>
<tr>
<td>+</td>
<td>20</td>
<td>9,000</td>
<td>180,000</td>
<td>60 x</td>
<td>+ 19%</td>
</tr>
</tbody>
</table>

<sup>a</sup> From 100 grams wet weight rat liver. DEAE and Sephadex steps were performed on portions corresponding to 20 to 40 grams wet weight, and values for mg Protein, Total Units, and yield were calculated.
The resolution of histone phosphatase activity into two fractions on DEAE cellulose was further investigated. When the tubes in each peak were pooled and concentrated, and the fraction reapplied to the column, each fraction rechromatographed as a single peak which approximated the original chromatographic behavior (Figure 5b and c). In order to determine whether the resolution of activity into two fractions on DEAE reflected degradation of the enzyme during the early stages of purification or during frozen storage, a fresh 78,000 x g supernatant fraction from liver homogenates was applied to a column within two hours after the death of the animal. As shown in Figure 7, the same resolution into two active fractions was observed. A third, minor peak eluting at 0.35 M NaCl was sometimes observed (Figure 8).

The two DEAE fractions were characterized by a reproducible difference in the relative rates of dephosphorylation of protamine as compared with histone H1. Fraction I typically demonstrated a relative activity towards the two substrates of 4/1, while the corresponding value for Fraction II was 2/1. No difference in $K_m$ for dephosphorylation of protamine was observed (Table 11) nor in the rate of heat denaturation at 52°C (Figure 11). The difference in relative rates with histone and protamine is probably a difference in $V_{max}$, since assays were performed at substrate concentrations well above observed values of $K_m$. To summarize, these two fractions differ
Figure 7.—DEAE Chromatography of Fresh High Speed Supernate from Rat Liver Homogenate. Sample applied = 29 mg. Five ml fractions collected.
in their chromatographic behavior in DEAE cellulose and in the relative rates of activity toward two substrates. They exhibit similar behavior on Sephadex G-200, heat denaturation at one temperature, and apparent Km for protamine. The fractions may reflect the presence of two enzymes catalyzing the histone phosphatase reaction in rat liver. On the other hand, minor modifications of a single protein, such as change in degree of amidation or carbohydrate content, might produce two fractions which differ in their net charge and could, therefore, be separated in DEAE cellulose. More information is necessary to explain this observation at the molecular level.

Chromatography on DEAE was effective in removing nonspecific phosphatase activity from the histone phosphatase fraction, as shown in Figure 8. P-nitrophenyl phosphatase assays were performed directly on 0.5 ml aliquots of column fractions. P-nitrophenyl phosphate as substrate was required at 10 mM concentration; the reaction was barely detectable at 1 mM substrate concentration. Phosphatase activity was measured after a 30 minute incubation at 37°C by determination of the absorption at 410 millimicrons, after the addition of 0.5 ml of 0.5 M NaOH to the reaction mixture. P-nitrophenyl phosphatase activity is clearly distinguished from histone phosphatase in these experiments.
Figure 8.--Resolution of p-Nitrophenyl Phosphatase from Histone Phosphatase on DEAE Cellulose. Sample applied = 55 mg of calcium phosphate fraction. Twenty ml fractions collected.
Some Requirements of Histone Phosphatase Activity

The pH dependency of histone phosphatase, its ionic strength requirements, stability, and response to activators and inhibitors will be described in this section.

The pH optimum for protamine dephosphorylation was determined with three different buffers, in order to control the effects of varying ionic strength. Tris-HCl was used initially to study the range pH 7.0 to 8.8. Two three-component buffers were used to study pH dependence from pH = 3.0 to pH = 10. Buffer "MAT" was prepared by titrating a mixture of 0.12 M Maleate, 0.12 M Acetate with 2M Tris, with aliquots taken for use at half unit intervals between pH 3 and 8.5. Buffer "TAM" was prepared by titration of 0.12 M Tris with the mixture 0.12 M maleate plus 0.12 M acetate. The absolute values of reaction rate at optimal pH were influenced by the composition of the buffer; the relative rates with Tris: "TAM" : "MAT" were 100:80:47. When the results were normalized for comparison, a similar pH dependence was observed, as indicated in Figure 9. There is a sharper decline in activity above pH 8 when Tris-HCl is present, possibly due to the lower ionic strength.

Histone phosphatase is a neutral phosphatase, with a broad pH optimum between pH 7-8. It is distinguished
Figure 9.—pH Dependence of Histone Phosphatase Reaction. Enzyme, ammonium sulfate fraction. Substrate, phosphorylated protamine. Buffers: ■ "MAT", Δ "TAM" (see text).
in this respect from most nonspecific phosphatases, which hydrolyze low molecular weight phosphates at either acid or alkaline pH. Most of the other phosphoprotein phosphatases exhibit maximal activity in the neutral range (Table 3); this probably reflects the sensitivity of these reactions to conformational changes in substrate proteins as well as enzyme molecules. Histone phosphatase assays were routinely performed at pH 7.5 in Tris-HCl buffer.

Histone phosphatase activity is markedly dependent on the ionic strength of the reaction medium, as shown in Figure 10. A two-fold stimulation of protamine dephosphorylation was brought about by 0.2 M concentrations of monovalent cations (Na$^+$, K$^+$ and NH$_4^+$, Figure 10a). Reactions were normally performed in the presence of 0.2 M NaCl, although this requirement was always checked on new enzyme preparations.

Divalent cations were inhibitory to the reaction at concentrations required to reach ionic strength of 0.2, although they did not inhibit at concentrations below 10 mM (Figure 10c). Polyvalent anions were also inhibitory; sulfate ions inhibited at high concentrations (Figure 10b), and phosphate inhibited the reaction at all concentrations studied (Figure 10d).

There is some evidence that the ionic strength is affecting the conformation of the substrate rather than
Figure 10.--Ionic Strength Dependence of Histone Phosphatase Reaction. Enzyme, ammonium sulfate fraction. Substrate, phosphorylated protamine. pH = 7.5.

a. NaCl, KCl, NH₄Cl
b. Na₂SO₄, (NH₄)₂SO₄
c. MgCl₂, CaCl₂
d. K₂HPO₄
the enzyme; dephosphorylation of protamine and histone f2b are maximal at ionic strength of 0.2, but histone f1 is more active at ionic strength of 0.1.

It has been demonstrated by optical rotary dispersion that the alpha-helical content of histones is increased sharply by raising the salt concentration in the range 0.01 - 0.25 M NaCl (Bradbury et al., 1965; Jirgensons and Hnilica, 1965; Bradbury et al., 1967a). This conformational change may contribute to the increased substrate activity observed at salt concentrations in this range. Protamine and histone f1 are similar in possessing a low helical content at 0.25 M NaCl (reported values for $b_0 = +25$ and $-40$, respectively) in contrast to the other histones, including f2b, whose helical content is much greater ($b_0 = -127$). Since the observed ionic strength optima are 0.2 for protamine and f2b and 0.1 for histone f1, while the alpha-helical content continues to increase for all substrates in this range, this cannot be the only factor responsible for the ionic strength dependence of the histone phosphatase reaction.

Since both substrate and enzyme in this reaction are proteins, the direct effect of ionic strength on protein-protein interactions should influence the reaction rate. Formation of the enzyme-substrate complex will be favored by low ionic strength, while dissociation of the enzyme-
product complex will be favored at high ionic strength; the net consequence of these opposing dependencies would produce a rate dependence with a maximum at some intermediate value of the ionic strength, such as was observed experimentally.

Histone phosphatase is a rather stable enzyme. It can be stored frozen without loss of activity for at least six months, and is not damaged by repeated freezing and thawing. The enzyme can be dialyzed for 12 hours at 0°C against dilute salt solutions. Incubation at pH 8.8 for three hours did not destroy activity. The enzyme is stable to the organic solvents employed in the non-aqueous methods of cell fractionation; i.e., petroleum ether, carbon tetrachloride, and cyclohexane. Activity is lost after exposure to 10-20% ethanol at 4°C.

Histone phosphatase is stable at 37°C for at least one hour, but it is rapidly denatured at 52°C (Figure 11). The biphasic denaturation may indicate that denaturation proceeds by at least two independent processes.

Chromatography on DEAE cellulose has a destabilizing effect; enzyme activity decays to approximately 65% of its initial value in the first week after DEAE exposure, but thereafter remains constant for at least one month.

The effects of several activators and inhibitors of histone phosphatase activity are presented in Table 10.
Figure 11.—Inactivation of Histone Phosphatase at 52°C.

○ ○ DEAE Fraction I, ■ DEAE Fraction II.
Table 10.--Effect of Various Compounds on Histone Phosphatase Activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration mM</th>
<th>Effect %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dithiothreitol</td>
<td>1</td>
<td>+ 15%</td>
</tr>
<tr>
<td>1. p-CMS</td>
<td>1</td>
<td>- 85%</td>
</tr>
<tr>
<td>2. N-ethyl maleimide</td>
<td>10</td>
<td>- 80</td>
</tr>
<tr>
<td>3. Iodoacetate</td>
<td>4</td>
<td>- 30</td>
</tr>
<tr>
<td>4. Molybdate</td>
<td>1</td>
<td>- 15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>- 100</td>
</tr>
<tr>
<td>5. Ferric chloride</td>
<td>1</td>
<td>- 50</td>
</tr>
<tr>
<td>6. Orthophosphate</td>
<td>10</td>
<td>- 20</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>- 60</td>
</tr>
<tr>
<td>7. Pyrophosphate</td>
<td>1</td>
<td>- 20</td>
</tr>
<tr>
<td>8. Sodium fluoride</td>
<td>10</td>
<td>- 10</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>- 60</td>
</tr>
<tr>
<td>1. Sodium arsenite</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2. Potassium cyanide</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3. EDTA</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4. Calcium chloride</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>5. 3'5'AMP</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>6. Sucrose</td>
<td>250</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme is moderately sulfhydryl-dependent. Activity is increased 15% by the addition of 1 mM dithiothreitol, which was present during all purification steps and during assays. Inhibition by sulfhydryl reagents is observed at relatively high inhibitor concentrations. Orthophosphate, a product of the reaction, is inhibitory, and pyrophosphate even more so. Magnesium ions are not required for reaction, as demonstrated by the lack of inhibition by 2 mM EDTA.
Substrate Specificity of Histone Phosphatase

A considerable number of phosphoproteins and low molecular weight compounds have been tested as substrates with purified preparations of histone phosphatase. Of these, only phosphorylated histones and protamines were effective substrates.

A. Relative Activity of Histones and Protamines.

Protamine, histone fl and histone f2b are the three known substrates for histone phosphatase. (Other histone fractions have not been tested.) The three substrates are compared with respect to their apparent $K_m$ and rate of dephosphorylation at saturating substrate concentrations ($V_{max}$) in Table 11.

The observed values for $K_m$ are all within the range of 5 to 25 micromolar concentrations of the phosphate moiety. A higher affinity for protamine was observed, as well as a greater $V_{max}$. Since protamine is not a normal constituent of liver cells, its greater activity as substrate cannot be taken as evidence that it is the preferred

1 Preparation of phosphorylated substrates is described under Methods.

2 Measurement of $K_m$ is discussed on page 86.
substrate in vivo. In view of the chemical and biological relationship between histone and protamine, the observed activity is not surprising.

(Protamine has been employed in these studies for reasons of convenience; it is more easily prepared than purified histone fractions, and can be saturated with a higher phosphate content per mg of protein.)

Table 11.--Apparent Km and Vmax of Known Histone Phosphatase Substrates.

<table>
<thead>
<tr>
<th>Enzyme Preparation</th>
<th>Substrate</th>
<th>Apparent Km x 10^5 M</th>
<th>Relative Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td>Protamine</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>f1</td>
<td>2.0</td>
<td>32, 40^a</td>
</tr>
<tr>
<td></td>
<td>f2b</td>
<td>1.5</td>
<td>21, 23^a</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>Protamine</td>
<td>---</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>f1</td>
<td>---</td>
<td>26, 31^a</td>
</tr>
<tr>
<td></td>
<td>f2b</td>
<td>---</td>
<td>20</td>
</tr>
<tr>
<td>DEAE I</td>
<td>Protamine</td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>f1</td>
<td>---</td>
<td>22, 26^a</td>
</tr>
<tr>
<td>DEAE II</td>
<td>Protamine</td>
<td>2.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>f1</td>
<td>---</td>
<td>46, 43^a</td>
</tr>
</tbody>
</table>

^a Determinations on independent enzyme preparations.
B. Activity with Other Phosphoproteins.

Histone phosphatase does not catalyze the release of phosphate from three phosphoproteins studied: casein, phosvitin, and the nuclear phosphoprotein preparations described by Langan and Lipmann (in preparation; Langan, 1967b). Their higher phosphate content is one way in which these phosphoproteins differ from phosphorylated histones and protamines (Table 12). In these studies, chemical assays of histone phosphatase were performed as described under Methods. Unlabeled phosphorylated protamine assayed by the same procedure served as the control. The results are presented in Table 12.

Table 12.---Activity of Histone Phosphatase Against Phosphoproteins

<table>
<thead>
<tr>
<th>Phosphoprotein</th>
<th>Phosphate Content uM/gram</th>
<th>Concentration mM</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protamine</td>
<td>200 uM/g</td>
<td>1.0, 0.12</td>
<td>100</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>3100</td>
<td>1.3, 7.0</td>
<td>5</td>
</tr>
<tr>
<td>Casein</td>
<td>250</td>
<td>0.3</td>
<td>8</td>
</tr>
<tr>
<td>Nuclear Phosphoprotein</td>
<td>400</td>
<td>0.1</td>
<td>---</td>
</tr>
</tbody>
</table>

a Concentration expressed as molarity of phosphate moiety.

b Prepared from rat liver nuclei by method of Langan and Lipmann.
While there was minor phosphoprotein phosphatase activity in the ammonium sulfate fraction, this was removed after purification with calcium phosphate. Histone phosphatase is thus clearly distinguished from the phosphoprotein phosphatases discussed in Part I.

C. Substrate Activity of Phosphopeptides from Histone fl and Protamine.

Phosphorylated peptides have been purified in the laboratory from tryptic hydrolysates of \( ^{32}P \)-labeled phosphorylated histone fl and protamine (Langan, 1968a). These peptides were examined for substrate activity at phosphate concentrations which were saturating for the proteins from which they were prepared. Assays were also performed on mixtures of peptide plus macromolecule, to control for inhibition by residual trypsin in the peptide preparations. The results of these experiments are presented in Table 13. Under these conditions, the peptides are dephosphorylated at less than 2% of the rate of dephosphorylation of the parent proteins.

D. Substrate Activity of Low Molecular Weight Phosphates.

The activity of histone phosphatase with a variety of low molecular weight compounds was investigated. Unlabeled protamine served as control substrate; assays were performed by chemical determination of phosphate released. The
Table 13.—Substrate Activity of Phosphopeptides Prepared from Histone fl and Protamine.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Concentration</th>
<th>cpm$^a$</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Histone fl</td>
<td>$6 \times 10^{-5}$ M</td>
<td>1520</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>fl peptide</td>
<td>$3 \times 10^{-5}$</td>
<td>24</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>fl peptide</td>
<td>$7 \times 10^{-5}$</td>
<td>23</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>Histone plus peptide</td>
<td>$6+3 \times 10^{-5}$</td>
<td>1515</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Protamine</td>
<td>$6 \times 10^{-5}$</td>
<td>3770</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Protamine peptide</td>
<td>$6 \times 10^{-5}$</td>
<td>6</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Protamine plus peptide</td>
<td>$6+6 \times 10^{-5}$</td>
<td>3580</td>
<td>95</td>
</tr>
</tbody>
</table>

$^a$ Specific activity of histone and histone peptide = 1825 cpm/nanomole phosphate.
Specific activity of protamine and protamine peptide = 980 cpm/nanomole phosphate.

activity of histone phosphatase purified through the ammonium sulfate step and through the calcium phosphate fractionation were compared. The results of these experiments are presented in Table 14.

Although several substrates were dephosphorylated by the ammonium sulfate preparation, this nonspecific activity was considerably reduced by the calcium phosphate fractionation in all cases. The residual p-nitrophenyl
phosphatase activity of the calcium phosphate fraction was subsequently separated from histone phosphatase by chromatography on DEAE cellulose (Figure 7). Of particular interest are phosphoserine and phosphothreonine, which are not active substrates for the enzyme.

In order to demonstrate that the histone phosphatase was active under all assay conditions, internal controls containing test substrate in the presence of radioactive protamine were included in these experiments. In no case was major inhibition of protamine dephosphorylation by the test substrate observed.

We have demonstrated that the enzyme which is responsible for histone dephosphorylation is not active with these other compounds at one millimolar concentrations, which is far in excess of the observed Km for dephosphorylation of histones (0.02 mM).
Table 14.—Substrate Specificity of Histone Phosphatase Preparations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ammonium Sulfate Fraction</td>
</tr>
<tr>
<td>Phosphorylated Protamine</td>
<td>100</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>3</td>
</tr>
<tr>
<td>Phosphothreonine</td>
<td>4</td>
</tr>
<tr>
<td>beta-Glycerol phosphate</td>
<td>3</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>24</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>2</td>
</tr>
<tr>
<td>Ribose-5-phosphate</td>
<td>4</td>
</tr>
<tr>
<td>5'AMP</td>
<td>70</td>
</tr>
<tr>
<td>2'3'AMP</td>
<td>3</td>
</tr>
<tr>
<td>dAMP</td>
<td>22</td>
</tr>
<tr>
<td>ADP</td>
<td>9</td>
</tr>
<tr>
<td>ATP</td>
<td>22</td>
</tr>
<tr>
<td>Na₄P₂O₇</td>
<td>3</td>
</tr>
</tbody>
</table>

a Substrate concentration, 1 mM in all cases. Reaction volume 1 ml; 0.05 M Tris pH 7.5, 0.001 M dithiothreitol. +/-0.2 M NaCl, 15 minute incubation at 37°C. Phosphate release determined chemically.

b p-Nitrophenyl phosphatase activity is separated from histone phosphatase by subsequent chromatography on DEAE cellulose (Figure 7).
Action of Nonspecific Phosphatases on Phosphorylated Protamine

In view of the great substrate specificity of histone phosphatase, we were interested in knowing whether the dephosphorylation of histones is enzyme specific, that is, whether other nonspecific phosphatases can catalyze the reaction. We investigated the activity of alkaline phosphatase from calf mucosa (Sigma Type I) as a representative mammalian nonspecific phosphatase. Unlabeled phosphorylated protamine was employed as substrate, at concentrations comparable to those employed in the assay of histone phosphatase. We found that the release of inorganic phosphate from protamine was catalyzed at less than 0.1% of the rate of dephosphorylation of beta-glycerol phosphate (Table 15).

As additional evidence that nonspecific phosphatases are inactive in the dephosphorylation of histones and protamine we can mention the clear distinction between p-nitrophenyl phosphatase and histone phosphatase activities obtained by DEAE cellulose chromatography (Figure 7), as well as the inactivity of plasma in the dephosphorylation of protamine, in spite of its considerable activity in dephosphorylation of p-nitrophenyl phosphate (Table 17).
### Table 15.—Relative Activities of Calf Intestine Alkaline Phosphatase and Histone Phosphatase Towards Protamine and beta-Glycerol Phosphate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Concentration mM</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone Phosphatase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Protamine</td>
<td>0.06</td>
<td>100</td>
</tr>
<tr>
<td>Histone Phosphatase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Protamine</td>
<td>1.00</td>
<td>86</td>
</tr>
<tr>
<td>Histone Phosphatase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>beta-GlycerolP</td>
<td>1.00</td>
<td>0.5</td>
</tr>
<tr>
<td>Alkaline Phosphatase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Protamine</td>
<td>1.00</td>
<td>0.1</td>
</tr>
<tr>
<td>Alkaline Phosphatase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>beta-GlycerolP</td>
<td>1.00</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fifty micrograms of Histone Phosphatase, calcium phosphate fraction. Tris, pH 7.5

<sup>b</sup> One mg of Histone Phosphatase, calcium phosphate phosphate fraction. Tris, pH 7.5

<sup>c</sup> Five micrograms of enzyme (Sigma Type I); Ethanolamine: HCl, pH 9.5; 10⁻³ M magnesium chloride.

A possible explanation of the inactivity of nonspecific monosterases is that the phosphate in protamine might be present in an internal diester linkage. Evidence for the presence of internal diester linkage in casein, based on nuclear magnetic resonance, has been reported (Ho, 1966). If this were the case, the observed specificity of histone phosphatase could result from its diesterase activity. This
possibility was tested by exposing phosphorylated protamine to the combined action of venom phosphodiesterase (Worthington) and calf alkaline phosphatase. In this experiment, the release of inorganic phosphate from protamine was less than 1% of that observed with the model substrate bis(p-nitrophenyl) phosphate (Table 16). Both phosphatases in combination demonstrate only minor activity towards phosphorylated protamine.

Table 16.—Inactivity of Alkaline Phosphatase plus Diesterase towards Phosphorylated Protamine.

<table>
<thead>
<tr>
<th>Substratea</th>
<th>Concentration mM</th>
<th>Optical Density 660 millimicrons</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis p-nitrophenyl Phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>2.0</td>
<td>0.144</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.5</td>
<td>0.090</td>
<td>62</td>
</tr>
<tr>
<td>Phosphorylated protamine</td>
<td>0.5</td>
<td>0.002</td>
<td>1</td>
</tr>
</tbody>
</table>

a Reaction conditions: 0.03 M Tris pH 8.5; 0.01 M MgCl₂ 25 μg of venom phosphodiesterase + 5 μg alkaline phosphatase. Reaction volume, 2 ml; reaction time 15 minutes.
Apparent Km of Histone Phosphatase Reaction

When the rate of dephosphorylation of histone is measured at increasing substrate concentrations a saturation effect is observed which can be described by Michaelis-Menten kinetics. For this analysis, we have employed the concentration of the phosphate moiety as the substrate concentration. This was necessary since precise values for the molecular weight of the histone fractions are not available and because we do not know how the phosphate is distributed among histone molecules. The success of this treatment indicates that the available phosphate groups are independently and equivalently accessible to the enzyme.

The results of these determinations for the substrates protamine and histone fl are presented in Figure 12. From the reciprocal Lineweaver-Burke plot values for the apparent Km of $5 \times 10^{-6}$ M and $25 \times 10^{-6}$ M were observed repeatedly with several different enzyme preparations. These values are quite low, and indicate a high affinity of the enzyme for both substrates.
Figure 12.—Apparent Km for Dephosphorylation of Histone fl and Protamine.

**Histone fl:** Enzyme = 50 micrograms of ammonium sulfate fraction. Reaction time = 2 minutes. 1/V, 2 min/nm phosphate.

**Protamine:** Enzyme = 8 micrograms of ammonium sulfate fraction. Reaction time = 5 minutes. 1/V, 5 min/nm phosphate.
Estimation of Molecular Weight

The molecular weight of histone phosphatase was estimated from its behavior during gel filtration on Sephadex G-200. Two columns of dimensions 2.5 cm x 45 cm, equipped with flow adaptors, were connected and the sample passed through both by ascending chromatography. Under hydrostatic pressure of 17 cm of water, the flow rate was 20 ml/hr. Samples were applied in a 2 ml volume, and 3 ml fractions were collected. The void volume, $V_0$, was determined to be 137 ml, the elution volume of blue dextran. The column was calibrated by the use of non-enzymatic protein molecular weight markers purchased from Mann Research Laboratories. The observed relationship between the elution volume, $V_e$, and the log of the molecular weight of the protein (Determann, 1968) is presented in Figure 13. (Duplicate determinations of elution volumes agreed within 2%). The relationship is linear within the molecular weight range 17,800 (myoglobin) to 160,000 (gamma-globulin). The elution volume of histone phosphatase activity is just outside this range; assuming that linearity holds, the approximate molecular weight is 186,000 to 195,000.

Due to the influence of tertiary structure and gel-interaction on the elution of proteins from sephadex columns, this value must be taken as an approximation.
Figure 13.—Estimation of Molecular Weight by Chromatography on Sephadex G-200. $V_e =$ elution volume; $V_o =$ void volume (137 ml).

1. Myoglobin 17,000 daltons = molecular weight
2. Ovalbumin 45,000
3. Albumin (bov) 67,000
4. $\gamma$ Globulin (hum) 160,000
5. Apoferritin 480,000
6. Histone Phosphatase 186,000-195,000
Products of the Histone Phosphatase-Catalyzed Reaction

A. Inorganic Orthophosphate.

The procedure employed for the assay of histone phosphatase is specific for orthophosphate, as distinguished from any other low molecular weight phosphate which might be released from the protein (Behrenbloom and Chain, 1938; Martin and Doty, 1949). Although 100% of the radioactivity of \(^{32}\text{P}\)-labeled histones and protamine is alkali-labile, and has been shown to be present as phosphoserine (Langan, unpublished results), we have only been able to recover 80% as orthophosphate after extensive incubation with purified histone phosphatase. We do not have an explanation for the resistance of 20% of the phosphate; it may be that there are two distinct classes of alkali-labile phosphate, only one of which is an active substrate.

(The fact that the endogenous phosphate content of purified histones is approximately 20% that of enzymatically phosphorylated histones may also reflect the insensitivity of a portion of the endogenous phosphate to the action of histone phosphatase during purification.)

B. The Protein Product.

Polyacrylamide gel electrophoresis of histones and
protamine before and after incubation with histone phosphatase demonstrates that the major portion of the protein does not undergo degradation during dephosphorylation. Figure 14 shows the disc electrophoresis of original, unphosphorylated protamine (gel 1), phosphorylated protamine (gel 2) and protamine dephosphorylated by incubation for 60 minutes with histone phosphatase calcium phosphate fraction, 2 ug/disc (gel 3). During this incubation, 78% of the radioactivity of the phosphorylated protamine was released as orthophosphate. It is clear, from comparison of the staining densities of gels containing less protein, that the major band in gel 3 contains most of the total protamine in un-degraded form. The intensification of the leading band in gel 3 is evidence for some proteolytic degradation.

Figure 15 shows the results of a similar experiment in which the samples have been electrophoresed on polyacrylamide gel slabs. In this experiment, 74% of the radioactivity of the protamine was released by dephosphorylation with calcium phosphate fraction enzyme. The improved resolution obtained with gel slabs makes visible the appearance of new protamine species as a result of phosphorylation (band 5), and their disappearance as a result of dephosphorylation (band 6). These new protamine species migrate more slowly than the original protamine

\(^1\) Langan, unpublished results.
Figure 14.—Disc Gel Electrophoresis of Dephosphorylated Protamine. Electrophoresis performed at pH 4.5 for five hours, at 4 ma/tube. Migration towards the negative pole. Major band migrated 7.4 cm.

1. Original protamine, 14 µg.
2. Phosphorylated protamine, 13 µg.
3. Dephosphorylated protamine, 17 µg.
Figure 15.—Electrophoresis of Dephosphorylated Protamine.
Electrophoresis performed at pH 4.5 for 2 1/2
hours, at 350 ma, 400 volts. Migration towards
negative pole. Major band migrated 10 cm.

1. Original protamine, 10 \( \mu g \).
2. Phosphorylated protamine, 25 \( \mu g \).
3. Dephosphorylated protamine, 20 \( \mu g \).
4. Original protamine, 50 \( \mu g \).
5. Phosphorylated protamine, 50 \( \mu g \).
6. Dephosphorylated protamine, 40 \( \mu g \).
7. Phosphorylated protamine, No Enzyme Control,
   35 \( \mu g \).
8. Phosphorylated protamine, 25 \( \mu g \).
(band 4), as would be expected due to the reduction in their net positive charge by addition of phosphate groups. (Electrophoresis is carried out at pH 4.5.) It is evident again that the bulk of the protamine is not degraded during phosphorylation, although small amounts of degradation products are visible. Controls incubated in the absence of histone phosphatase were identical to phosphorylated protamine (Band 7).

We attempted to determine whether the \( ^{32}P \)-radioactivity of band 2 in Figure 15 was localized in the slower-moving bands, by radioautography of the gel and by manual slicing and counting of the band area. Both methods indicated that labeling was greatest in the slowest band. Resolution was not adequate to determine whether the major band was also labeled.

It appears from this result that protamine phosphorylation is not limited to one site on the protein, but rather than some molecules contain more than 1 phosphate while others may contain none. (The average phosphate content is one phosphate per 5,000 grams of protein, or nearly one phosphate per molecule.) Dixon et al. (1967) have found that all three serine residues of protamine can be phosphorylated \textit{in vivo}.

Examination of dephosphorylated histones by gel electrophoresis was also carried out (Figure 16). In
Figure 16.—Gel Electrophoresis of Dephosphorylated Histone fl. Electrophoresis performed at pH 4.5, for 1 1/4 hours at 300 mA, 500 volts. Migration towards negative pole. Major band migrated 3 cm. Enzyme fraction DEAE II, 3 µg, employed for dephosphorylation.

1, 2 Initial histone fl, 86 µg.
3 Phosphorylated histone fl, 67 µg.
4, 5 Dephosphorylated histone fl, 54 µg.
this experiment, 50% dephosphorylation was brought about by incubation with histone phosphatase, DEAE II fraction. There is no change in migration of the major peak after phosphorylation.\textsuperscript{1}

It is important to note that the DEAE enzyme fraction employed in Figure 16 is also free of protease activity, as indicated by the absence of a leading band of degraded material. This has been confirmed by electrophoresis of protamine dephosphorylated by DEAE fraction.

This evidence indicates that the reaction catalyzed by histones phosphatase can be written:

\[
\text{histone phosphatase} \\
\text{Phosphorylated histone} \xrightarrow{\text{---}} \text{Orthophosphate + Histone (protamine)}
\]

This is consistent with the evidence discussed in Part I for the turnover of histone phosphate independently of \textit{de novo} biosynthesis of the protein.

\textsuperscript{1} Langan, unpublished observations.
MISCELLANEOUS EXPERIMENTS

The Effect of Phosphorylation of Histones on Nucleoprotein Stability

The influence of phosphorylation on the interaction of histone with DNA was investigated by studying the temperature-induced helix-coil transition of reconstituted nucleohistones. Nucleohistone was prepared in dilute saline-citrate (2.8 mM NaCl, 3 mM Na Citrate, pH 7.3), by the combination of calf thymus DNA with calf thymus histone fl in a weight ratio of 1.1 to 1.0, DNA to histone. The hyperchromic transition was measured in a Beckman DK 2 Recording Spectrophotometer while samples were heated stepwise by an aluminum heating block. Enzymatically phosphorylated histone fl containing 45 micromoles of phosphate per gram was compared with native histone fl containing 9 micromoles of phosphate per gram. The native fl was incubated with histone kinase under the conditions of phosphorylation except that ATP was absent from the incubation medium. The results of a typical experiment are presented in Figure 17.

A minor difference in Tm of one to three degrees was

\(^1\) Tm is defined as the temperature at which 50% of total hyperchromicity is observed.
Figure 17.—Effect of Histone Phosphorylation on Tm of Nucleohistone. See text for phosphate content of histones.

A. DNA, Tm = 67°C.
B. DNA plus phosphorylated histone fl, Tm = 76°C.
C. DNA plus control histone fl, Tm = 76°C.
observed. The shift was in the direction predicted from consideration of the fact that the introduction of negatively charged phosphate groups will reduce the ionic attraction between the polycationic histone and the polyanionic DNA.

These results indicate that the effect of phosphorylation of the interaction between histone and DNA is small. Whether the minor differences observed reflect functional alteration of the nucleohistone is not known.

Allfrey, Faulkner and Mirsky (1964) observed that acetylation of histones could result in a major reduction of inhibition of RNA polymerase activity while exerting only a slight effect on the Tm of nucleohistone complexes (minus 0.5°C). Apparently, the Tm is insensitive to histone modifications which may be of functional significance.
Tissue Distribution of Histone Phosphatase Activity

Total homogenates prepared from various rat tissues were assayed for histone phosphatase activity. The specificity of the enzyme responsible for phosphatase activity was not determined. The results of these experiments are presented in Table 17.

Table 17.—Tissue Distribution of Histone Phosphatase Activity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Histone Phosphatase</th>
<th>p-nitrophenyl phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>100^a</td>
<td>100^b</td>
</tr>
<tr>
<td>Brain</td>
<td>252</td>
<td>100</td>
</tr>
<tr>
<td>Kidney</td>
<td>141</td>
<td>152</td>
</tr>
<tr>
<td>Lung</td>
<td>214</td>
<td>143</td>
</tr>
<tr>
<td>Spleen</td>
<td>191</td>
<td>278</td>
</tr>
<tr>
<td>Testis</td>
<td>185</td>
<td>162</td>
</tr>
<tr>
<td>Thymus</td>
<td>290</td>
<td>207</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.005</td>
<td>14</td>
</tr>
</tbody>
</table>

^a Specific activity = 195 nanomoles/hr/mg.
^b Specific activity = 1000 nanomoles/hr/mg.
^c Assayed at 10 mM p-nitrophenyl phosphate, 0.05 M Tris pH 7.5.
Histone phosphatase activity was detected in all tissues examined. There was no more than three-fold variation in specific activity among tissues. This distribution pattern is not surprising, since histones are found, in comparable amounts, in all mammalian cells.

The distribution of histone phosphatase activity is distinctly different from that of p-nitrophenyl phosphatase.
Species Distribution of Histone Phosphatase Activity

Hydrolysis of phosphorylated protamine was observed with extracts from a variety of organisms, as indicated in Table 18.

Table 18.—Species Distribution of Histone Phosphatase Activity

<table>
<thead>
<tr>
<th>Species</th>
<th>Specific Activity, nanomoles/hr/mg</th>
<th>Histone Phosphatase</th>
<th>p-nitrophenyl phosphatase&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat, liver</td>
<td>150</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>Chick embryo, five day</td>
<td>55</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Pea seedling</td>
<td>46</td>
<td>---&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Neurospora</td>
<td>186</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Euglena</td>
<td>38</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Chlorella</td>
<td>50</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Yeast (S. cerevisiae)</td>
<td>132</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Amoeba proteus</td>
<td>90</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>E. Coli</td>
<td>&lt;0.10</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Azotobacter</td>
<td>&lt;0.05</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Phormidium luridum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.5%&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100%&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Assayed at 10 mM p-nitrophenyl phosphate, 0.05 M Tris pH 7.5.

<sup>b</sup> Not done.

<sup>c</sup> Protein concentration not determined; p-nitrophenyl phosphatase activity taken as 100%.
Since the specificity of the phosphatase activity in these extracts was not determined, we cannot draw conclusions about the presence of histone phosphatase *per se* in these organisms. However, it is interesting to note that measurable histone phosphatase activity was observed in all eukaryotic cells examined. There was no activity in the three types of prokaryotes assayed (E. Coli, Azotobacter, and P. luridum), which did however hydrolyze p-nitrophenyl phosphate. This negative result may be artifactual, but it is tempting to relate the absence of histone phosphatase activity in prokaryotes to the fact that histones are found exclusively in the nucleus of eukaryotic cells.
SUMMARY

1. A phosphatase specific for the dephosphorylation of histones and protamines has been purified sixty-fold from rat liver. The enzyme is inactive towards casein, phosvitin, phosphopeptides and low molecular weight compounds.

2. Histone phosphatase catalyzes the release of orthophosphate from phosphorylated histones, without degradation of the substrate protein.

3. The reaction is characterized by a broad pH optimum in the neutral range. The apparent Km for dephosphorylation of histones and protamines is approximately $2 \times 10^{-5}$ M. The molecular weight of histone phosphatase is estimated to be 190,000 daltons, by gel fractionation on Sephadex G-200.

4. The enzyme is isolated from the high speed supernate of rat liver homogenate. The possibility of a nuclear localization in vivo has not been eliminated; the enzyme has been demonstrated to be non-lysosomal.

5. Histone phosphatase activity was found in all eukaryotic cells examined, but was absent in the prokaryotes which were studied.
CONCLUSIONS

At the present time, the phenomenon of histone phosphorylation as a biological process is far from well understood. It may be worthwhile to reconsider the status of the field, emphasizing the relevance of the observations reported here.

Histone Phosphorylation in vivo.

The existence of a phosphatase with the great specificity for histones demonstrated here is consistent with the presence of phosphorylated histones in the cell. The description of this phosphatase, together with the known properties of histone kinase, makes it possible to write the equations for a cycle of histone phosphorylation.

It must be noted that histone phosphatase has been operationally defined by an assay employing a synthetic substrate, the product of the in vitro phosphorylation of histones by histone kinase. The possibility exists that, in spite of their activity towards histones in vitro, both enzymes may catalyze a different reaction in vivo.

It would be desirable to demonstrate the presence of the product of histone kinase as a component of living cells. The recent characterization of the specific phosphopeptide of histone fl phosphorylated in vitro makes it
possible to obtain this definitive evidence by examining cell extracts for this phosphopeptide. When this is accomplished, the biological significance of histone phosphorylation will be more firmly established. (Protamine phosphorylation has been well demonstrated by Ingles and Dixon.)

We have mentioned the contrast between the low endogenous phosphate content of isolated histones, as compared with the \textit{in vitro} product of histone kinase. In view of the presence of histone phosphatase, we might attribute the low phosphate content to enzymatic dephosphorylation during the early stages of histone preparation. Future efforts to isolate phosphorylated histones should minimize the opportunity for enzymatic dephosphorylation.

**Possible Functional Roles of Histone Phosphorylation.**

The enzymatic regulation of histone conformation by phosphorylation might be important to any of the models of histone function discussed in Part I.

If histones function in the maintenance of chromosomal conformation, phosphorylation might be related to the differentiation between euchromatin and heterochromatin, or to the reversible condensation and dispersion of the nucleohistone in the course of cell division. In view of the evidence discussed earlier, phosphorylation of the lysine-rich histones might be expected to influence the state of
aggregation of nucleohistone molecules, while phosphorylation of arginine-rich histones could be related to the degree of intra-molecular condensation. The histone kinase employed in our studies preferentially phosphorylates the lysine-rich histones f1 and f2b. However, we do not know the relative extent of phosphorylation of the histone fractions \textit{in vivo}. We cannot, therefore, predict the probable effects of histone phosphorylation on nucleohistone structure, except for the general observation that phosphorylation should reduce the ionic interaction between histones and DNA.

As a modifier of repressor molecules, phosphorylation could provide a mechanism for dynamic control of the degree of genetic activity. At the present, it does not appear that phosphorylation introduces a significant degree of chemical heterogeneity into the histones, since histone f1 appears to be phosphorylated at a single site by liver histone kinase. It is possible that tissue-specific histone kinase enzymes might produce a unique spectrum of phosphorylated histones in each tissue, and thereby confer a degree of specificity on the interaction of histones with the genome. It seems \textit{more} likely, however, that phosphorylation may be a part of the nonspecific machinery which responds to some other specific information by altering the state of activity of a particular portion of the genome.
It is alternatively possible that phosphorylation is not related directly to histone function, but rather represents a transient stage in the maturation of histone molecules. We know that the completed protein molecule is the substrate for phosphorylation and for dephosphorylation. It may be that the newly synthesized histone molecules are "packaged" by phosphorylation in a process analogous to the addition of the carbohydrate component to glycoproteins in the golgi apparatus. In the later case, it has been postulated that the contribution of the carbohydrate moiety to the hydrophilicity of the protein facilitates its transport across the cell membrane. In a similar manner, we might speculate that phosphorylation of histones, by reducing their net positive charge, might facilitate their transport from the cytoplasmic site of synthesis across the nuclear membrane. Phosphorylation might also reduce interaction of the histone with cytoplasmic components. In the case of protamine, there is evidence that newly-synthesized protein is highly phosphorylated, while the protamine of mature sperm has lost its phosphate. We do not know whether this temporal pattern relating phosphorylation to protein synthesis reflects the special requirements of spermatogenesis, or whether it is characteristic of the general process.

At this primitive stage of our understanding of the biological role of histone phosphorylation we cannot
eliminate any of the possibilities suggested here. Further progress will be closely related to developments in the study of histone function. The isolation of the enzymes histone kinase and histone phosphatase represent a basic step in the definition of the process of phosphorylation. Their availability for the preparation of phosphorylated and unphosphorylated histones makes possible further studies of the effect of phosphate content on the chemical and physical properties of histones and nucleohistone. The enzymes are also potentially applicable to the investigation of the functional issues discussed here. Considerable work will be required for the elucidation of the role of histones and histone phosphorylation in the function of the nucleus.
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