AN ABSTRACT OF THE THESIS OF

James Kenneth Walker Mardian for the degree of Doctor of Philosophy

in Biochemistry and Biophysics presented on January 20, 1978

Title: PHYSICAL STUDIES OF THE YEAST INNER HISTONES

Abstract approved: _____

Redacted for Privacy

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In this work, the four yeast inner histones were prepared by the use of consecutive slab gel fractionation. The use of this technique allowed the first complete fractionation and rigorous identification of the four yeast inner histones, and made possible a study of their complexing behavior.

Variants and adjuncts of preparative electrophoresis by the method of gel slicing are presented. These include improved methods of locating and eluting protein bands. The yeast histones were located in the gel using the phosphorescence detection technique of Isenberg et al. (1975). Elution was achieved conveniently and efficiently using an all-glass gel eluter of original design. The method permits the separation of closely spaced components and may be used in whole, or in part, to isolate a wide variety of proteins in pure form from crude extracts.

The complexing pattern of the yeast inner histones was

investigated using fluorescence anisotropy and circular dichroism. The pattern was found to be the same as that found previously for calf and pea histones. In particular, strong interactions were observed for the following pairs of yeast histones: H2a-H2b, H2b-H4, and H3-H4.

Interspecies binding constants for calf and yeast histones were measured and found to be close to the intraspecies binding constants. This result indicates a high degree of conservation of binding surfaces.

Physical Studies of the Yeast
Inner Histones

by

James Kenneth Walker Mardian

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed January 20, 1978

Commencement June 1978

APPROVED:

Redacted for Privacy

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Date thesis is presented _____January 20, 1978_____

Typed by A & S Bookkeeping for James Kenneth Walker Mardian

ACKNOWLEDGEMENTS

To my wife and my
parents, who stood behind
every word written herein.

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PHYSICAL STUDIES OF THE YEAST INNER HISTONES

INTRODUCTION

The Histones

Our understanding of the physical, chemical, and molecular basis of biology has made enormous strides in the third quarter of the twentieth century. A basic problem presently under attack is the understanding of the mechanisms of control that govern cell growth and differentiation. Progress in the understanding of the physical processes and structures occurring in living organisms has been intertwined with advances in the understanding of the molecular elements that constitute them, the most spectacular example of this being the impact of the discovery of the structure of DNA on our understanding of the genetic basis of life.

While the general outlines of the regulatory process are becoming clear for bacterial cells, the situation is much different for the nucleated cells of higher organisms, the eucaryotes. Unlike the chromosomes of procaryotes, which are composed mainly of DNA, the genetic material of higher eucaryotes also contains an amount of protein, roughly equal to twice the amount of DNA in weight, and a lesser amount of RNA. During cell division, the genetic material forms itself into chromosomes. At other times of the cell cycle, the material exists in a much more diffuse array.

This genetic material, as isolated from interphase cells, is known as chromatin.

The workings of this genetic complex is the goal of much present research.   An important part of this research is an attempt to define the constituents that make up chromatin, and to determine the structural arrangements of these constituents.   When chromatin is extracted with acid, about half the protein is solubilized.   The acid-extractable, basic proteins obtained in this way are the histones.

Histones have been studied since Kössel, a student of the great biochemist Hoppe-Seyler, isolated a basic substance from goose erythrocytes in 1884 and gave it the name histone.   Lilienfield, a student of Kössel, first isolated histone from calf thymus in 1892 (Kössel, 1928; Luck, 1964).   Thus, before the end of the nineteenth century, histone had been extracted and studied from the two tissues most widely used by present day histone investigators.

In 1950, Stedman and Steadman showed that there were at least two different histones, which they called the main and the subsidiary histones. They also found a variation in histone content from different tissues.   From this they predicted a great many different histones would be found, and that this multiplicity would, in fact, be sufficient to enable the histones to function as specific gene repressors.   By the time a world-wide histone conference was held in Pasadena in 1963, there was a general feeling that a great number of different histone species had already

been characterized, and that more would be found in the future (Murray, 1964).

The well-known propensity of histones to aggregate in neutral solutions, their ability to form complexes with one another, as well as the possibility of inter- and intra-molecular disulfide bond formation by the two sulfhydrils of calf thymus histone H3, all contributed to the spurious multiplicity of histone fractions. However, with the introduction and use of new fractionation and analytical techniques, including ion exchange chromatography (Crampton et al., 1955; Luck et al., 1958; Bonner et al., 1968), starch gel electrophoresis (Neelin and Neelin, 1960; Johns et al., 1961) and polyacrylamide gel electrophoresis (Reisfeld et al., 1962; Cruft, 1964; Fambrough et al., 1968; Panyim and Chalkley, 1969), it soon became apparent that there were only five main histone fractions.

Histone nomenclature has reflected some of the past confusion over the true number and identity of the main histone fractions. Different sets of nomenclature were based on the different methods used to fractionate or analyze the histones. Thus, the roman numeral nomenclature of Luck et al. (1958) was based on the elution order of histone fractions from Amberlite IRC-50. Similarly, the "F" nomenclature of Johns et al. (1960) was based on the elution of three fractions from carboxymethyl cellulose. Further refinements in fractionation led to subfractions denoted by appropriate letters and numbers.

Another nomenclature, based on the three most abundant amino acids

as designated by the single-letter code, was agreed upon at a 1972

Gordon Conference (Huberman, 1973). This most logical designation

turned out to be the shortest lived. Fortunately, a compromise "H"

nomenclature based on those of Johns and Luck was worked out at a 1974

CIBA conference, and has since found widespread acceptance (Bradbury,

1975). I will use the CIBA nomenclature throughout. Table 1 lists

the five main histone fractions and their corresponding names, along

with some elementary properties.

Though the histones have been studied for almost a hundred years,

and the literature is not lacking from numerous proposals and models

describing their function, it was not until very recently that solid evi-

dence was obtained to indicate the true nature of the first level of

chromatin organization.

The Nucleosome

Before 1973, the idea which dominated the thinking about chro-

matin structure was that of the supercoil, based on X-ray diffraction

studies (Richards and Pardon, 1970; Bram and Ris, 1971; Pardon and

Wilkens, 1972). However, since 1973, several lines of evidence have

converged to suggest a different structure. This structure is variously

described as the subunit structure or beads-on-a-string model (Van

Table 1.   Histone nomenclature and elementary properties of calf thymus histones.

Class	CIBA	Luck	Johns	Gordon Conf.	Number of Residues	Molecular Weight
Outer	H1	I	f1	KAP	～215	～21,500
Inner	H2a	IIb1	f2a2	LAK	129	14,004
Inner	H2b	IIb2	f2b	KAS	125	13,774
Inner	H3	III	f3	ARE	135	15,324
Inner	H4	IV	f2a1	GRK	102	11,282

Holde and Isenberg, 1975; Olins et al., 1978) with the subunit or bead

called a ν-body by Olins and Olins (1974) or a nucleosome by Oudet

et al. (1975). In this model, the DNA is wrapped around the outside

(Baldwin et al., 1975; Pardon et al., 1975) of a globular protein core

consisting of two each of the histones H2a, H2b, H3 and H4 (Olins and

Olins, 1974; Kornberg, 1974; Shaw et al., 1976; Olins et al., 1978;

Joffe et al., 1977). This particle with a histone core and DNA wrapping

is the nucleosome. It occurs at a more or less regular spacing along

the chromatin fiber. Evidence for the model comes from direct visuali-

zation of such a structure in the electron microscope (Olins and Olins,

1973; Woodcock, 1973; Olins and Olins, 1974; Oudet et al., 1975),

nuclease digestion studies (Hewish and Burgoyne, 1973; Sahasabuddhe

and Van Holde, 1974; Noll, 1974), and studies of native histone com-

plexes as well as these complexes reconstituted with DNA (Kornberg

and Thomas, 1974; Kornberg, 1974).

The fact that H1 is present in multimers of nucleosomes but

greatly reduced or absent in monomer preparations (Shaw et al., 1976;

Whitlock and Simpson, 1976; Noll and Kornberg, 1977) strongly sug-

gests that it resides outside the nucleosome and is associated with the

linker region of DNA. Besides this distinction, there are other dif-

ferences that set H1 apart from the other histones. H1 may be removed

from chromatin by mild methods (Ilyin et al., 1971). It is not needed to

generate the characteristic X-ray pattern of chromatin (Richards and

Pardon, 1970; Kornberg and Thomas, 1974). In contrast to the other histones, there are several different H1 molecules found in a given organism (Kinkade and Cole, 1966; Kinkade, 1969; DeLange and Smith, 1975). In addition, H1 is much more evolutionarily divergent than the other histones (Elgin and Weintraub, 1975; DeLange and Smith, 1975).

A nomenclature which is useful in recognizing these differences is that proposed by Isenberg (1977). H1 is placed in the class of the outer histones and H2a, H2b, H3, and H4 are collectively referred to as the inner histones.

Properties of the Inner Histones

The complete primary structure of each of the four inner histones of calf thymus has been determined (DeLange et al., 1969; Ogawa et al., 1969; Iwai et al., 1970; DeLange et al., 1972; Yeoman et al., 1972; Sautiere et al., 1974). In contrast to the sequence variations, or heterogeneities found for different H1 subfractions, only limited heterogeneities have been found for the inner histones, although these seem to be functionally significant. Both pea (Patthy et al., 1973) and calf (Marzluff et al., 1972; Patthy and Smith, 1975) H3 have two different forms, differing in the residue at position 96. Similarly, two different forms of H2a have been isolated from rat chloroleukaemia, differing at residues 16 and 99 (Sautiere et al., 1975).

Various inner histones appear at definite times during the embryogenesis of sea urchins and Drosophila (Cohen et al., 1975;

Newrock et al., 1978) and, in addition, mammalian histone subtypes

have been observed in Triton gels and these have been shown to have

different primary structures (Franklin and Zweidler, 1977). It should

be emphasized that these latter show more limited variations than do

the Hl subfractions, although the biological distinctions may be major

ones. It should also be noted that numerous post-synthetic modifica-

tions have been described for the histones (DeLange and Smith, 1975).

With the sequencing of the first inner histone, H4, it was pointed

out that the N-terminal region of the molecule was very basic and may

therefore be the primary site of protein-DNA interaction. It was also

noted that the C-terminal, with its much lower net charge, and rela-

tive concentration of hydrophobic residues, could be capable of assum-

ing a specific conformation and of interacting with other chromosomal

proteins (DeLange et al., 1969). Indeed, now that all of the inner

histones have been sequenced, we know that this assymetry in net

charge between the N- and C-terminal portions exists for all of the

inner histones. Table 2 demonstrates that the first 47 N-terminal

residues of each inner histone is markedly more basic than the rest

of the molecule.

Isenberg (1977) has compared charge diagrams of the C-terminal

portions of the inner histones with those of known globular proteins,

and found them to be quite similar in nature. Thus, at the very least,

it would not be surprising to find that a portion of each of the inner

Table 2. A charge comparison of the amino and carboxyl portions
of the inner histones.[a]

Peptide	lys + arg	glu + asp	Net positive charge	Net positive charge per 100 residues
H2a (1-47)	13	1	12	26
H2a (48-129)	13	8	5	6
H2b (1-47	17	2	15	32
H2b (48-129)	11	7	4	5
H3 (1-47)	14	0	14	30
H3 (48-129	17	11	6	7
H4 (1-47)	16	1	15	32
H4 (48-102)	19	6	3	5

[a]Data taken from Isenberg (1977).

histones was capable of assuming a unique, native conformation under suitable conditions of ionic strength and pH.

That histones aggregate in neutral salt solutions is a well-known and well-studied phenomenon (Cruft et al., 1954, 1957, 1958; Laurence, 1966; Davison and Shooter, 1956; Mauritzen and Stedman, 1959; Phillips, 1965, 1967; Johns, 1968; 1971; Fambrough and Bonner, 1968; Edwards and Shooter, 1969; Boublik et al., 1970a; Diggle and Peacocke, 1971; Bradbury et al., 1973, 1975; Bradbury and Rattle. 1972; Lewis et al., 1975; Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1974d; Sperling and Bustin, 1974, 1975; Smerdon and Isenberg, 1973, 1974). The aggregation results in large but definite structures (Sperling and Bustin, 1974, 1975). From the point of view of one interested in histone-histone interactions, however, aggregation is something to be avoided rather than studied. The strategy for avoiding or controlling aggregation in order to study specific interactions between pairs of histones becomes clear when one understands the changes that occur upon the addition of salt to an aqueous histone solution.

Histones in water have little or no defined secondary structure (Bradbury et al., 1965, 1975a; Jirgensons and Hnilica, 1965; Tuan and Bonner, 1969; Boublik et al., 1970a, b; Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1972; 1974a, c). When salt is added at neutral pH, two changes occur. The individual molecules fold and they form large aggregates.

The changes that occur in dilute (10^{-5} M) solutions of H3 and H4 upon the addition of salt at neutral pH have been studied in great detail

using circular dichroism, fluorescence anistropy, and light scattering
(Li et al., 1972; Wickett et al., 1972; Smerdon and Isenberg, 1973,
1974; D'Anna and Isenberg, 1974c). It was found that upon the addition
of salt, there is an immediate change in the circular dichroism and
fluorescence anisotropy, but not in light scattering. Above a critical
salt concentration, this initial change, called the fast step, is followed
by a slower change in circular dichroism, fluorescence anisotropy, and
now light scattering, called the slow step (Li et al., 1972; D'Anna and
Isenberg, 1974c; Smerdon and Isenberg, 1973; Isenberg, 1977).

At 10^{-5} M histone concentration the rates of these two processes
differ by orders of magnitude. The fast step is too fast to measure
by the techniques used, while the slow step occurs over a period of a
few hours (Li et al., 1972; D'Anna and Isenberg, 1974c). Circular
dichroism measurements show that the fast step is accompanied mainly
by α- helix formation, with little or no β-sheet evident. A test of the
cooperativity of the change that accompanies the fast step has been
made by assuming a two-state system (unfolded-folded) and calculating
the proportion of molecules in each state using both circular dichroism
and fluorescence anistropy measurements. These two measurements
are sensitive to two very different aspects of protein conformation,
the first responding to changes in the secondary structure, and the
second being sensitive to the rotatory diffusion of the tyrosine residues.
The fact that these two measurements indicate the same fraction of
molecules in the two states for varying salt concentrations, as well

as for different salts, argues strongly for the existence of a cooper-
ative transition (Li et al., 1972; Wicket et al., 1972; D'Anna and
Isenberg, 1972, 1974 a, c; Isenberg, 1977; Baldwin, 1975). The
cooperative change accompanied by an increase of secondary structure
that is observed for the fast step is just what one would expect to see
for the renaturation of a globular protein. These studies indicate that
denatured, acid extracted histones refold upon the addition of salt to an
aqueous solution to what may be a native conformation. That the
N-terminal regions of all the histones remain in an extended, flexible
form (Moss et al., 1976 a, b; Lilley et al., 1976, 1977; Clark et al.,
1974; Bohm et al., 1977) agrees with the predictions from sequencing
data that the C-terminal portions of the inner histones are most likely
to assume a globular form.

In contrast to the fast step, the slow step is accompanied mainly
by β-sheet formation with no change or a decrease in α-helix (Li et al.,
1972; Wickett et al., 1972; D'Anna and Isenberg, 1974c). It is also
accompanied by an increase in light scattering (Smerdon and Isenberg,
1973; D'Anna and Isenberg, 1974c), a technique that is sensitive to
molecular weight. The rate of the slow step was found to vary with
histone concentration. All this indicates that homo-aggregation occurs
during the slow step of H3 and H4. At 10^{-5} M concentration, H2a and
H2b show no appreciable slow step, and therefore no aggregation, over
a period of hours (D'Anna and Isenberg, 1972, 1974a). At higher

concentrations, all of the inner histones aggregate in neutral salt solutions (Boublik et al., 1970b; Diggle and Peacocke, 1971; Bradbury et al., 1975a; Sperling and Bustin, 1975).

Complexing of the Inner Histones

The interactions of calf thymus and chicken erythrocyte histones have been studied using a variety of techniques (Skandrani et al., 1972; D'Anna and Isenberg 1973, 1974 b, d; Kelley, 1973; Roark et al., 1974; Kornberg and Thomas, 1974; Sperling and Bustin, 1975; Weintraub et al., 1975; Lewis, 1976a, b, c; Clark et al., 1974; Bohm et al., 1977).

The knowledge of the behavior of histones in dilute aqueous solution upon the addition of salt allows one to design a histone-histone complexing experiment with a minimum of interference from homo-aggregation. Histone stock solutions may be prepared in either water or dilute acid, to prevent aggregation from occurring. Then two histones are added together, an appropriate amount of buffered salt solution is added, and measurements are made immediately after mixing. The folding and complexing will occur before appreciable homo-aggregation takes place (Isenberg, 1978). D'Anna and Isenberg (1973, 1974 b, d) have made a systematic study of all six pair-wise histone interactions. They used two basic types of experiments to determine the presence of a specific histone-histone interaction.

The first type may be performed when one of the histone pairs has a measurable slow step (H3 or H4 for calf). In this case, a given parameter (anisotropy of fluorescence, light scattering, or ellipticity at 200 nm) is monitored over time for each histone, by itself, at a given concentration (buffer is added at t = 0). The same measurement is then made on a fresh mixture of the two histones together. Any deviation over time from the curve calculated for no interaction indicates complexing. In particular, blocking of the slow step of H3 or H4 indicates a strong interaction.

In the second type of experiment, the method of continuous variations is used (D'Anna and Isenberg, 1973). In this case, solutions of varying molar proportions of two histones are made up, keeping the total molar concentration of protein constant. CD at 200 nm or fluoresence anisotropy is then measured for the various solutions. Subtracting the straight line calculated for non-interacting species from the measured curve gives a curve that is a function of the amount of complex formed. Binding constants may be estimated from such a curve (D'Anna and Isenberg, 1973). If aggregation is slow enough, it will not seriously affect such measurements, provided the solution is measured immediately after the addition of buffer.

When the six possible pair-wise interactions of the four calf inner histones were studied in this way, it was found that three of the pairs (H3-H4, H2b-H4, and H2a-H2b) form strong 1:1 complexes, one

pair (H2a-H3) forms a complex of intermediate strength, and the

remaining two pairs (H2a-H4 and H2b-H3) interact weakly.

The Evolutionary Conservation of the Inner Histones

It is well-known that two of the inner histones, H3 and H4, are

very highly conserved. In fact, they have the two lowest calculated

mutation rates of any proteins yet observed (DeLange and Smith, 1975;

Wilson et al., 1977). H4 has been sequenced from calf (DeLange et al.,

1969; Ogawa et al., 1969) and pea (DeLange et al., 1969). Of 102

residues, only two conservative changes in sequence were found.

These were isoleucine for valine at residue 60 and arginine for lysine

at residue 77 (DeLange et al., 1969). H3 has also been sequenced from

calf (DeLange et al., 1972, 1973) and pea (Patthy et al., 1973).

Only four differences were observed, two or three of which may be

considered conservative (Patthy et al., 1973; Delange and Smith,

1975).

H2a and H2b, while still relatively conserved, nevertheless vary

much more than H3 and H4 (DeLange and Smith, 1975; Dayhoff, 1976;

Wilson et al., 1977). Variations in gel mobilities of H2a and H2b

have been found among vertebrates (Panyim et al., 1971). Differences

in the number of residues have also been reported. The sequence of

trout H2a indicates that it is four residues shorter than calf thymus

H2a (Bailey and Dixon, 1973). Two different H2b's from the sea

urchin <u>Parechinus</u> <u>angulosus</u> have been sequenced. One is 18 residues

longer and the other is 19 residues longer than calf thymus H2b

(Strickland, M., <u>et al</u>., 1977; Strickland, W.N., <u>et al</u>., 1977).

The largest differences, however, have been reported for plant H2a

and H2b.

Plant H2a and H2b differ to such an extent that Nadeau <u>et al</u>.

(1974), in an electrophoretic study of the histones of several plant

species, found no bands which clearly corresponded to H2a and H2b.

They did find two bands of lower mobility than H3 on both acetic

acid-urea and sodium dodecyl sulfate (SDS) gels, which they designated

simply as PH1 and PH2 for plant histone 1 and 2. Spiker and Isenberg

(1977) have since demonstrated which of these pea histones corres-

pond to calf thymus H2a and H2b.

Both pea H2a and H2b differ significantly from the corresponding

calf histones in size, as judged by mobility on SDS gels, and amino

acid composition (Spiker and Isenberg, 1977; Spiker and Isenberg, 1978,

Isenberg, 1978).

Despite these differences, the histone complexing pattern is the

same for the pea as it is for the calf inner histones (Spiker and

Isenberg, 1977). Furthermore, each of the strong interactions has

been found to occur between inter-species pairs of histones (Spiker

and Isenberg, 1978). Measurements of the binding constants

for intra- and inter-species complexes between H2a and H2b

showed a very high degree of conservation of binding energies, and therefore binding surfaces (Spiker and Isenberg, 1978; Isenberg, 1978).

These results have led to the prediction that sequencing studies of H2a and H2b would reveal that these two molecules were evolutionary hybrids, with part of the molecule highly conserved and the rest poorly conserved (Spiker and Isenberg, 1978).

Protein nuclear magnetic resonance (NMR) studies on complex formation between H3 and H4, as well as between each molecule and specific peptides of the other molecule, have indicated which regions are important for complex formation (Bohm et al., 1977). It was concluded that residues 42-120 in H3 and 38-102 in H4, that is, the C-terminal regions, are those regions that are involved in complex formation. In agreement with these results, are studies on the H3-H4 tetramer using ^{13}C NMR which indicate the existence of random coil N-terminal regions, or "tails" (Lilley et al., 1976).

NMR studies of the H2a-H2b complex indicate that residues 31-95 in H2a and 37-114 in H2b are involved in the complex (Moss et al., 1976). While such evidence is lacking for the H2b-H4 complex, there is evidence that core protein extracted from chromatin has such N-terminal tails attached to C-terminal globular regions (Lilley et al., 1977). This core protein contains one each of H2a, H2b, H3, and H4 and is the same as the "heterotypic tetramer" of Weintraub et al. (1975).

In addition to the NMR data, the common feature of all the inner

histones of a basic N-terminal region and a less basic "globular-like" C-terminal region, mentioned above, leads to the expectation that the C-terminal regions of all the histones are involved in highly specific histone-histone interactions. Therefore, it is the C-terminal two-thirds of H2a and H2b that is expected to be highly conserved (Spiker and Isenberg, 1978; Isenberg, 1978).

Evidence from animal sequences known to date tend to confirm this prediction (Bailey and Dixon, 1973; Kootstra and Bailey, 1976; Strickland, M., et al., 1977; Strickland, W., et al., 1977). The sperm of the sea urchin, Parechinus angelosus has two H2b molecules both of which have been sequenced (Strickland, M., et al., 1977; Strickland, W. N., et al., 1977). In each case the first third of the molecule is variable and the remaining, C-terminal portion is highly conserved. A similar result has been found for the sequence of H2b from Drosophila. Elgin, Schilling and Hood (Elgin, personal communication) have found that only the carboxyl two-thirds of the molecule is highly homologous to H2b in calf thymus. The amino terminal was found to be quite variable.

While no plant H2a or H2b has yet been entirely sequenced, preliminary work on pea H2b indicates that most of the differences occur in the N-terminal region and that the remainder at the molecule is relatively conserved (Hayashi et al., 1977).

The Identification of Histones

Several different methods are used to fractionate and analyze

histones. These include ion exchange (Luck et al., 1958; Johns et al.,

1960; Johns, 1971), gel filtration (Bohm et al., 1973, Sommer and

Chalkley, 1974), and affinity (Ruiz-Carrillo and Allfrey, 1973)

chromatrography, chemical fractionation (Johns, 1964; 1967, 1971),

and polyacrylamide gel electrophoresis in the presence of acetic acid

and urea (Panyin and Chalkley, 1969), SDS (laemmli, 1971; Thomas

and Kornberg, 1975), and Triton (Alfageme et al., 1974).

Applied to calf thymus histones, these methods have revealed

the existence of the five major histone classes, H1, H2a, H2b, H3, and

H4. The histones are conserved sufficiently among the vertebrates that

histones corresponding to each of the five calf histones may be readily

identified in a large number of different species (Panyim et al., 1971).

However, as indicated above, the further one moves in evolution-

ary and phylogentic distance from the vertebrates, the more variation

one sees, especially for the less conserved histones H1, H2a, and H2b.

The usual procedure followed in attempting to achieve a thorough char-

acterization of the histones of an organism distantly related to mammals

is first to fractionate the histones by one or more methods until homo-

geneity, as judged by gel electrophoresis, is achieved. Then amino

acid analysis is performed. All of the data, including behavior in any

fractionation system used, gel mobilities, and amino acid analyses, are considered in comparison with the known behavior for each of the calf thymus histones. Often such a characterization will result in an unambiguous identification of the unknown histone fractions.

Sometimes, however, standard techniques will not successfully fractionate or characterize the histones from a species distantly related to mammals. We have already encountered the marked differences that exist in the behavior and properties of pea H2a and H2b and the corresponding calf histones. Johmann and Gorovsky (1976) in characterizing the histones of Tetrahymena pyriformis obtained a fraction they called HX which did not clearly correspond to any calf histone. Fazal and Cole (1977) have detailed the anomalies encountered in attempting to characterize wheat germ histones. They caution against using any single criterion to identify a histone fraction.

The discovery of the nucleosome at least provides a definition of an inner histone. Inner histones are those proteins found to compose the core of the nucleosome. Thus, isolation of nucleosome monomers from a given organism, and the analysis of the proteins by gel electrophoresis, provides a methodology for determining the inner histone complement for that organism. It still does not tell, however, which unknown inner histone corresponds to which calf inner histone. Also a single gel system may not reveal the true heterogeneity of the fractions present. In determining the true number of species, it is

useful to analyze individual fractions with several different gel systems, or to analyze a mixture of histones on two-dimensional gels.

As illustrated by studies on the pea inner histones (Spiker and Isenberg, 1977, 1978; Isenberg, 1978), the intra- and inter-species complexing patterns can serve unambiguously to identify the histones of an organism distantly related to mammals, provided the interaction sites are highly conserved. We have noted above that a study of pea-calf inter-species complexes indicates a very high degree of conservation of the histone-histone binding sites.

Yeast Histones

Studies on the histones of Saccharomyces cervevisiae, the common baker's yeast, began more than ten years ago. Tonino and Rozijn (1966a, b) fractionated yeast histone into two fractions by ion exchange chromatography, and reported that both fractions were much less basic than mammalian histones. They also found that only a small amount of yeast histone was soluble in 5% trichloroacetic acid, and that the protein extracted was acidic and had a low lysine content, indicating that yeast did not contain H1. Wintersberger et al. (1973) found three major bands in the region of the mammalian histones when they analyzed yeast histone on acetic acid-urea gels. They found no major bands in the region of H1.

Franco et al. (1974) separated yeast histone into 3 bands (a, b and

c) on acetic acid gels. They fractionated whole yeast histone according to Johns (1964) and found that band b was made up of two components, b1 and b2. They obtained fractions of a + b1, b2, and c. Solubility properties, amino acid analyses, and gel mobilities indicated that c was H4 and b2 was H2a. They were not able to separate a from b1, though they noted that this fraction together had some properties of H2b. Calf thymus H3 is soluble in ethanol-HCl and precipitates upon dialysis against ethanol (Johns, 1964). Franco et al. (1974) found that no precipitate was formed when ethanol-HCl extracts of yeast chromatin were dialyzed against ethanol. Thus, they reported they were unable to detect H3 in yeast. Similarly, they found that no protein was obtained when yeast chromatin was extracted with 5% perchloric acid, a treatment which removes mammalian H1.

Brandt and Von Holt (1976) resolved yeast histone into four major fractions, two of them doublets, on acetic acid-urea gels. Gel filtration on Biogel P-60 yielded four fractions. Gels stained with Coomasie Blue revealed the presence of a large number of different proteins in small concentration in these fractions. Two of the fractions, containing the two fastest moving major electrophoretic bands, were purified further using preparative electrophoresis. When this was done, there was a close resemblance between the amino and composition of the fastest band with calf H4, as expected. They also found that a highly purified preparation of the second-fastest band closely resembled calf

H3 in composition. That this fraction was indeed H3, was confirmed by sequencing the first 15 N-terminal residues. The sequence was found to be identical to that of the first 15 residues of calf and pea H3.

Moll and Wintersberger (1976) also obtained four major bands from yeast histone on acetic acid-urea gels. They demonstrated that neither of these four proteins contained tryptophan, and that all were synthesized nearly exclusively during S-phase of the cell cycle.

Thomas and Furber (1976) analyzed the proteins from yeast nuclei and chromatin on SDS gels. They found four major yeast histones with mobilities quite similar to the mobilities of the mammalian inner histones. It should be noted that only a single band, that identified as H4 by Brandt and Von Holt (1976) clearly corresponds in gel mobility when yeast and mammalian histones are compared on acetic acid-urea gels (Brandt and Von Holt, 1976; Wintersberger et al., 1973). Nelson et al. (1977) isolated chromatin subunits from baker's yeast and showed that they contained these same four fractions as displayed on SDS gels. Electrophoretic analyses of the monomer and oligomer fractions of Nelson et al. (1977) showed them to be quite free of other constituents, with the exception of three very minor bands whose mobilities are close to those of the chicken erythrocyte outer histones H1 and H5.

The Complexing Pattern of the Inner Histones and Chromatin Structure

Cross-linking experiments have been instrumental in determining

the histone composition of the nucleosome. Many such experiments

have been performed to study the relationship of the histones to each

other in chromatin (Martinson and McCarthy, 1975; Bonner and Pollard,

1975; Van Lente et al., 1975; Thomas and Kornberg, 1975; Chalkley,

1975; Martinson et al., 1976). Cross-linkers have a variety of lengths

and it is important to consider the lengths in interpreting experiments.

Zero length cross-linkers are unique in that they will only cross-link

proteins that are in actual contact rather than merely close to one

another. Therefore, it is of great interest that each of the three strong

pair-wise histone complexes has been found in chromatin using zero-

length cross-linkers (Martinson and McCarthy, 1975; Bonner and

Pollard, 1975; Martinson et al., 1976). Thus it is highly likely that

these strong complexes exist in chromatin and are responsible for hold-

ing together the histone core of the nucleosome.

In the five-kingdom classification proposed by Whittaker (1969),

eucaryotes are divided into four kingdoms: protista, plantae, fungi,

and animalia. The fungi are placed alongside plants and animals and

are thought of as representing a third major line of evolution.

The subunit pattern of chromatin organization, as evidenced by a

repeat distance of nuclease-sensitive sites on chromatin DNA, has been

found in all four eucaryotic kingdoms (Hewish and Burgoyne, 1973;

Sahasrabuddhe and Van Holde, 1974; Noll, 1974; Gorovsky and Keevert,

1975; McGhee and Engle, 1975; Lohr and Van Holde, 1975; Noll, 1976;

Thomas and Furber, 1976; Morris, 1976; Jerzmanowski et al., 1976; Lipps and Morris, 1977).

The histone pair-wise complexing pattern has been shown to be the same for calf and peas (D'Anna and Isenberg, 1974d; Spiker and Isenberg, 1977). Furthermore, it has been shown that the individual binding sites have been conserved (Spiker and Isenberg, 1978). It is clearly of interest to determine the extent of conservation of the histone complexing pattern and binding sites, and in particular to examine whether or not these features are conserved for fungal histones.

As we have seen, evidence has accumulated indicating that yeast contains four histones which correspond to the four inner histones of higher eucaryotes (Wintersberger et al., 1973; Franco et al., 1974; Moll and Wintersberger, 1976; Brandt and Von Holt, 1976; Thomas and Furber, 1976; Nelson et al., 1977). However, the presence of H1 in yeast has not yet been demonstrated. While partial fractionations of the yeast histones have been achieved (Franco et al., 1974; Brandt and Von Holt, 1976), a complete fractionation has not yet been reported.

Although all four inner histones exist in yeast, their separation has presented problems: They do not fractionate well when the methods that have worked with mammalian histones are used. As we have seen, the yeast histones do not separate into individual fractions when the fractionation procedures of Johns are used (Franco et al., 1974). Mammalian histones may also be separated by exclusion

chromatography (Bohm et al. , 1973; Sommer and Chalkley, 1974). Yet

Brandt and Von Holt (1976) reported that yeast histone fractions obtained

by exclusion chromatography still contain significant quantities of

numerous contaminating proteins.

Because of the difficulties with standard techniques for fraction-

ating histones, I have turned to preparative electrophoresis on slab

gels to isolate the yeast inner histones. By using the methods described

below, I have been able to achieve a complete fractionation of the yeast

inner histones, obtaining proteins of relatively high purity.

Since gel criteria alone are not sufficient to identify histones, and

since a complete fractionation of the yeast histones has not hitherto

been made, it has not been possible to identify all of the yeast histones

in a rigorous way. Acid extracts of yeast showed four prominent bands

on acid-urea gels (Moll and Wintersberger, 1976; Brandt and Von Holt,

1976) which migrated similarly to mammalian histones; SDS extracts

showed the existence of four bands on SDS gels (Thomas and Furber,

1976; Nelson et al. , 1976). However, because of the preparative dif-

ficulties, there has not yet been a consistent, definitive identification

of all of the yeast inner histones, although one or another of them have

been identified by separate methods (Franco et al. , 1974; Brandt and

Von Holt, 1976).

A similarity in gel migration alone cannot identify histones (Fazal

and Cole, 1977). Furthermore, marked differences in gel migration

do not demonstrate that the histones are not homologous, for we note

that calf and pea H2a and H2b migrate quite differently on both acid-

urea and SDS gels (Spiker and Isenberg, 1977). Thus a definitive

identification of the yeast histones is still needed. The lack of pure

yeast histones has, of course, also blocked physical and chemical

studies of them.

In what follows, I describe a method of preparing the yeast inner

histones which makes possible a definitive identification based on amino

acid analyses. The elementary properties of the yeast inner histones

are described, as well as their pair-wise complexing behavior. Inter-

species complexes between calf and yeast histones are also investigated.

Preparative Gel Electrophoresis

The high resolving power of electrophoresis carried out in

polyacrylamide gels has resulted in its widespread use as a diagnostic

both for the purityof fractionated histone samples, as well as for the

components present in a given histone extraction. As mentioned above,

the introduction of the use of polyacrylamide gels, with subsequent

refinements in technique, into the histone field was instrumental in

elucidating the question of histone heterogeneity (Hnilica, 1971).

Polyacrylamide gel electrophoresis has also been used as a pre-

parative method. Two different techniques have been used. In the first,

for which commercial apparatuses are available, the protein fractions

are collected as they are eluted off the end of a separating gel (Jovin,
et al., 1964; Gordon and Louis, 1967; Schenkein et al., 1968;
Brownstone, 1969; Hjerten et al., 1969; Shuster, 1971; Ryan et al.,
1976; van Jaarsveld et al., 1976; Chrambach and Rodbard, 1971).
Problems with this method include low recoveries, the continuous elu-
tion of a nondialyzable impurity given off continuously during electro-
phoresis, mixing of components as they are eluted, and cumbersome
concentration steps necessitated by excessive dilution (Chrambach and
Rodbard, 1971).

In the second method, a separation is made on a cylindrical or
slab gel as in analytical electrophoresis. Individual protein bands are
then located, sliced from the gel, and eluted either by chemical extra-
ction or electrophoretic elution (Lewis and Clark, 1963; Shuster, 1971;
Weliky et al., 1976; Fries, 1976; Posner, 1976). This method has been
used very successfully in isolated instances (Brandt and Von Holt, 1976;
Goldknopf et al., 1975; Martinage et al., 1976) though it has not gener-
ally been exploited to its full potential due to difficulties in accurately
detecting protein bands by non-destructive means, and in collecting the
protein conveniently in a clean form and in high yield.

Protein bands may be located in an imprecise manner by staining
representative vertical gel slices. Other methods that have been
reported include ultraviolet shadowing (Eisinger, 1971; Hassur and
Whitlock, 1974a, b), light refraction techniques (Fries, 1976), and

detection by intrinsic phosphorescence (Isenberg et al., 1975).

I have developed variants and adjuncts of gel-slicing which I believe are superior to existing procedures both for the detection of the position of the protein in the gel, and for its elution. For detection, when possible, I used an extension of the phosphorescence detection method described by Isenberg et al. (1975). For elution an all-glass gel elutor of original design was used.

Phosphorescence detection permits the localization of most proteins. It uses the intrinsic phosphorescence of tryptophan and tyrosine, and requires no stain. In this way, the actual position of the protein may be observed across the entire gel rather than at only a few positions where strips have been stained. By its use, closely spaced protein bands may be separated. Many gel systems can be used for phosphorescence: acid-urea gels, SDS gels and neutral gels. However, phosphorescence detection cannot be used for gels containing Triton X-100, since Triton X-100 itself phosphoresces brilliantly. In such a case, one is forced to stain strips, or use another technique for detection. However, Triton DF-16 has only a weak phosphorescence and is therefore preferable if it can be used in place of Triton X-100.

The elutor, which shall be described in detail, gives high yields and does not dilute the protein. It can, in fact, be used to concentrate the sample.

These methods were developed in the process of preparing yeast histones. However, I feel that each step of this procedure will be useful in preparing many and various types of proteins. The preparation of histones provides a severe test of the procedure; the preparation of most other proteins will be easier. For one thing, histones have no tryptophan, and their phosphorescence is much lower than proteins that do. Furthermore, the histone bands are closely spaced in the gel. It follows, therefore, that these procedures should be generally useful.

In addition to describing these techniques, I also shall describe ways of avoiding the oxidation artifacts which can occur in gels, particularly Triton gels.

MATERIALS AND METHODS

Preparation of Whole Histone

Crude whole yeast histone was prepared from commercial pressed baker's yeast (Red Star) according to a scaled-up version of the method of Tonino and Rozijn (1966b). Buffer A is 5 mM phosphate, pH 6.5, containing 1 mM $MgSO_4$. Buffer B is 5 mM tris-HCl, pH 8.0, containing 1 mM $MgSO_4$. A "+" sign here indicates that I have added 1 ml of 0.1 M phenylmethylsulfonyl fluoride (PMSF) in ispropanol per 1000 ml of buffer (Nooden et al., 1973; Ballal et al., 1975). The PMSF was always added to the buffer shortly before use, to minimize hydrolysis by hydroxide ion (Gold, 1967). All operations were carried out in the cold ($0-4^{\circ}C$).

The yeast (600 grams) was suspended in A+ buffer, to 1200 ml. This suspension (35 ml at a time) was then shaken for 45 seconds with 20 ml of beads in a 75 ml duran sample flask in a Bronwill model MSK cell homogenizer, using CO_2 cooling. Then fresh A+ buffer was added to a total volume of 3000 ml. This suspension was centrifuged in six 500 ml bottles at 12,000 x g for 1 hour. The supernatant was aspirated off. The resulting sediment consisted of three layers, with unbroken cells on the bottom, white cell walls in the middle, and a brownish layer of nuclear material on top. The supernatant was aspirated off, and the nuclear layer was shaken up in 1500 ml buffer A+.

This suspension was then centrifuged in six 250 ml bottles for 30 minutes at 12,000 x g and the resulting sediment was washed twice with 1500 ml A+ buffer (30 minutes at 12,000 x g) and once with 1500 ml B+ buffer (30 minutes at 25,000 x g).

The crude chromatin was resuspended in 288 ml B+ buffer and homogenized with 3 up and down strokes in a Potter-Elvehjem homogenizer with a motor-driven tight-fitting pestle. The homogenized suspension (12 ml) was layered on each of 24 sucrose step gradients consisting of 12 ml of 2.0 M sucrose and 12 ml of 1.5 M sucrose, both in B buffer. Centrifugation was for 1 hour at 24,000 rpm in an SW 27 rotor. The supernatant was then poured off, the sides of the tube wiped with a Kim wipe, and the pellet washed twice with .15 M NaCl (10 minutes at 10,000 x g). The resulting salt-washed purified chromatin was then resuspended in 50 ml H_2O using a Potter-Elvehjem homogenizer, and 50 ml of .5 N HCl was added while stirring. Histones were extracted in the cold for 1 hour. After centrifugation for 20 minutes at 10,000 x g, 800 ml cold acetone was added to the supernatant, and it was left at -20° overnight. The next day the histone precipitate was collected by centrifugation in a bench-top centrifuge, and was washed three times with acetone. It was then allowed to dry under vacuum.

Histone was also prepared by first isolating yeast nuclei. Using a pure culture derived from a single colony of Red Star yeast, histone

was prepared according to the method of Wintersberger et al. (1973),

with the following exceptions: For pre-incubation per 1 gram of yeast,

1.4 ml of 0.1 M EDTA and 0.1 M tris, pH 8.0 was added, instead of

the EDTA alone (Schwencke et al., 1968). Pre-incubation was for 15

minutes, followed by incubation with glusulase for 15 minutes. Extrac-

tion of histones was according to Figure 1c of Wintersberger et al.

(1973).

Preparative Electrophoresis

The individual yeast inner histones were isolated using prepara-

tive electrophoresis on polyacrylamide slab gels 3 mm x 140 mm x

102 mm (Biorad Model 220). A preliminary separation of H2a from

H2b, H3, and H4 was accomplished on an acetic acid-urea slab contain-

ing Triton X-100. This step also served to separate out a very large

number of proteins other than histones that are present in acid

extracts of yeast chromatin. An acetic acid-urea slab without Triton

was used to separate the individual inner histones. These steps are

outlined in Figure 1.

Slabs without Triton were made according to Panyim and Chalkley

(1969) with minor modifications. Slabs contained 15% (w/v) acrylamide,

0.1% (w/v) bis, 0.05% (w/v) ammonium persulfate, 5% (w/v) acetic acid

0.5% (w/v) TEMED, and 2.5 M urea. Triton slabs were identical to

the above with the exception of an additional 1% (w/v) Triton X-100

a)　Crude Histone

Triton Slab

H2a
+ Other Proteins

Acetic Acid – Urea Slab

H2a

H2b, H3, H4
+ Other Proteins

Acetic Acid – Urea Slab

H2b　H3　H4

b) Triton Slab

Pre-Electrophorese
Scavange
Electrophorese
Stain-destain strip
Draw cutting diagram
Cut out bands
Elute
Remove Triton
Desalt
Lyophilize

c) Acetic Acid-Urea Slab

Pre-Electrophorese
Scavange
Electrophorese
Cool in liquid N_2
Photograph
Draw cutting diagram
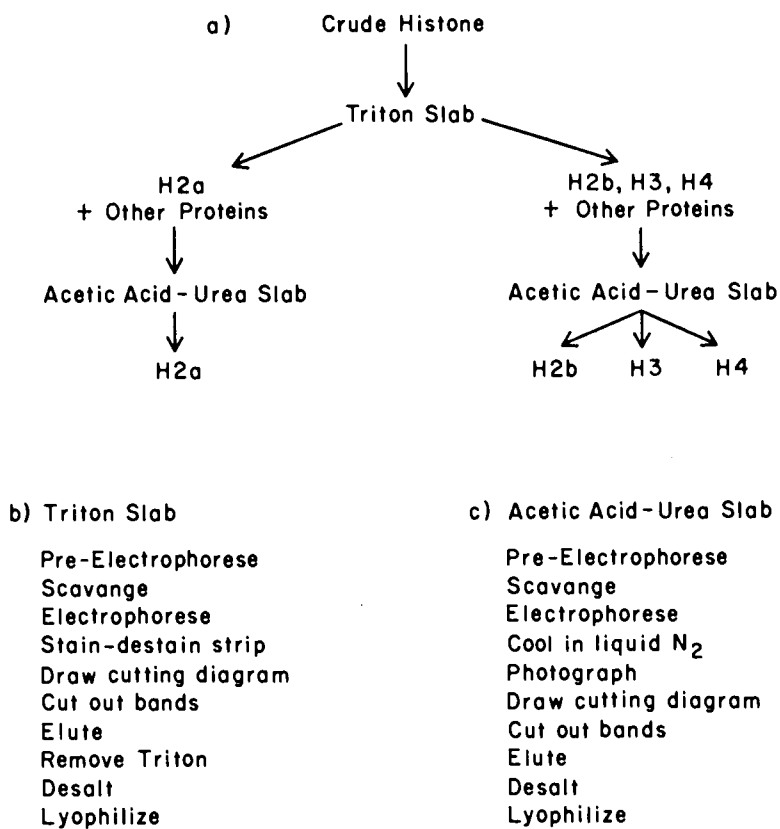Cut out bands
Elute
Desalt
Lyophilize

Figure 1.　(a)　Outline of the preparation of yeast inner histones by consecutive slab gel fractionation. (b) Sequence of steps for Triton slabs. (c) Sequence for acid-urea slabs.

(Alfageme et al., 1974). For Triton slabs, a 16 ml plug without Triton was poured first, and allowed to polymerize, before pouring the remainder of the slab with Triton. This was necessary to prevent the gel from slipping out during pre-electrophoresis.

Pre-electrophoresis was for 72 hours with 5% (w/v) acetic acid in the upper and lower buffer chambers, and an additional 48 hours with acetic acid in the upper and 6% (v/v) mercaptoacetic acid in the lower chamber. The upper buffer was changed twice daily throughout pre-electrophoresis, and the lower buffer changed once a day during the first 72 hours. The mercaptoacetic acid in the lower buffer chamber was unchanged throughout the remaining time of pre-electrophoresis and electrophoresis. For Triton slabs 4 ml of 2.5 M urea, 5% (w/v) acetic acid, and 1% (w/v) Triton X-100 was layered on the top of the gel after each change of the upper buffer, to maintain the concentration of Triton in the upper part of the gel. Adding Triton to the entire upper buffer resulted in the appearance of a yellow color, and so was avoided. Otherwise, pre-electrophoresis and electrophoresis procedures were the same for the two types of gels. Pre-electrophoresis was carried out at 80 volts. Immediately prior to electrophoresis, 2.5 ml of 5% (w/v) acetic acid, 1.0 M cysteamine was loaded on each gel and run at 80 volts for one hour, as an additional scavenger for free radicals (Alfageme et al., 1974).

For electrophoresis, protein (20 mg crude histone) was dissolved

in 0.5 ml 8 M urea, 10% (w/v) acetic acid. To this was added 0.1 ml

1M cysteamine, 5% (w/v) acetic acid (scavenging solution), and 0.4

ml H_2O. The sample (1.0 ml) was applied to the gel and electro-

phoresed at room temperature for 20 hours at 20 ma constant current.

Visualization of Protein Bands

It was not possible to visualize protein bands using phosphore-

scence (see below) in gels containing Triton X-100 due to a bright

background phosphorescence contributed by the Triton. Because of

this, the less accurate method of staining a strip from the gel was

used. A 3 mm vertical strip was cut from the center of the gel and

stained for 15 minutes in .5% (w/v) Amido Black, 7% (w/v) acetic

acid, 20% (v/v) ethanol. The strip was then destained electrophoretic-

ally in distilled water for 15 minutes at 75-100 ma.

Measurements of the boundaries of the desired protein bands were

made on the strip. These measurements were all multiplied by the

ratio of the height of the unstained slab to the length of the strip to allow

for swelling of the strip. A diagram was drawn on a piece of paper

indicating the top and bottom of the slab as well as the bands to be cut

out for elution of protein. The gels were cut using commercially-

available microtome blades in a simple holder. Due to the tendency of

the protein bands to curve at the extreme edges of the gel, a 3 mm

strip was cut off each side and either stained or discarded. In addition,

another 3 mm strip was cut off each side and stained for future refer-

ence.

For gels containing no Triton, proteins were visualized using the

phosphorescence technique of Isenberg et al. (1975). As for the Triton

gels, a 3 mm strip was cut from each side of the gel and discarded.

Another 3 mm strip was cut from each side and stained for future

reference.

Cooling Slabs for Phosphorescence Detection of Bands

Phosphorescence detection requires low temperatures (Isenberg

et al., 1975) and it is convenient to use liquid nitrogen as a coolant.

The chilling may be carried out in a styrofoam box which has been

sprayed with black paint. The slab may rest directly on an aluminum

plate which stands on aluminum legs in the box. The nitrogen is then

slowly poured into the box. However, chilling in this way will often

cause the gel to crack and it is difficult to handle the gel in subsequent

operations.

It was found that freezing a slab gel on a pyrex plate facilitated

handling of the gel after visualization of bands by phosphorescence and

did not increase the likelihood of the gel cracking during cooling. It

was necessary to use a layer of plastic wrap between the glass plate

and the slab to avoid cracks in the slab. It appears that, upon cooling,

the slab contracts more than the glass, and also sticks to the glass.

The plastic wrap enables the slab to slide over the glass and avoid cracking.

To prevent cracking, slabs, with the extreme right and left edges removed as described above, were soaked in glycerol (Matheson, Coleman and Bell #GX 190 was found to contribute a minimum of background phosphorescence) for 45 minutes. They were then cut into three pieces to facilitate the final cutting operation. A 2.5 cm piece was cut off each side, leaving a large center portion. In this way, if there was curvature of the bands toward the edges, it was still possible to use straight-line cuts to excise the protein bands.

To aid in the alignment of the slab for cutting, it was helpful to place marker pins topped with tryptophan in each gel piece. The marker pins were prepared by coating straight pins with a 1:1:1 mixture of tryptophan, ethyl acetate and Duco cement and permitting the mixture to dry. The pins were then clipped to a length of 3-4 mm and inserted into the regions of the gel pieces which did not contain protein. The final configuration of the gel pieces with the inserted marker pins may be seen in Figure 2, which shows two complete slabs prepared in this way.

Gels were cooled on a 1/8 inch thick pyrex plate clamped to an aluminum heat sink with two spring-loaded clamps. The heat sink consisted of a 9 x 10 x 1/16 inch thick aluminum plate with several 1/4 x 1 inch aluminum bolts spaced at even intervals around the edges.
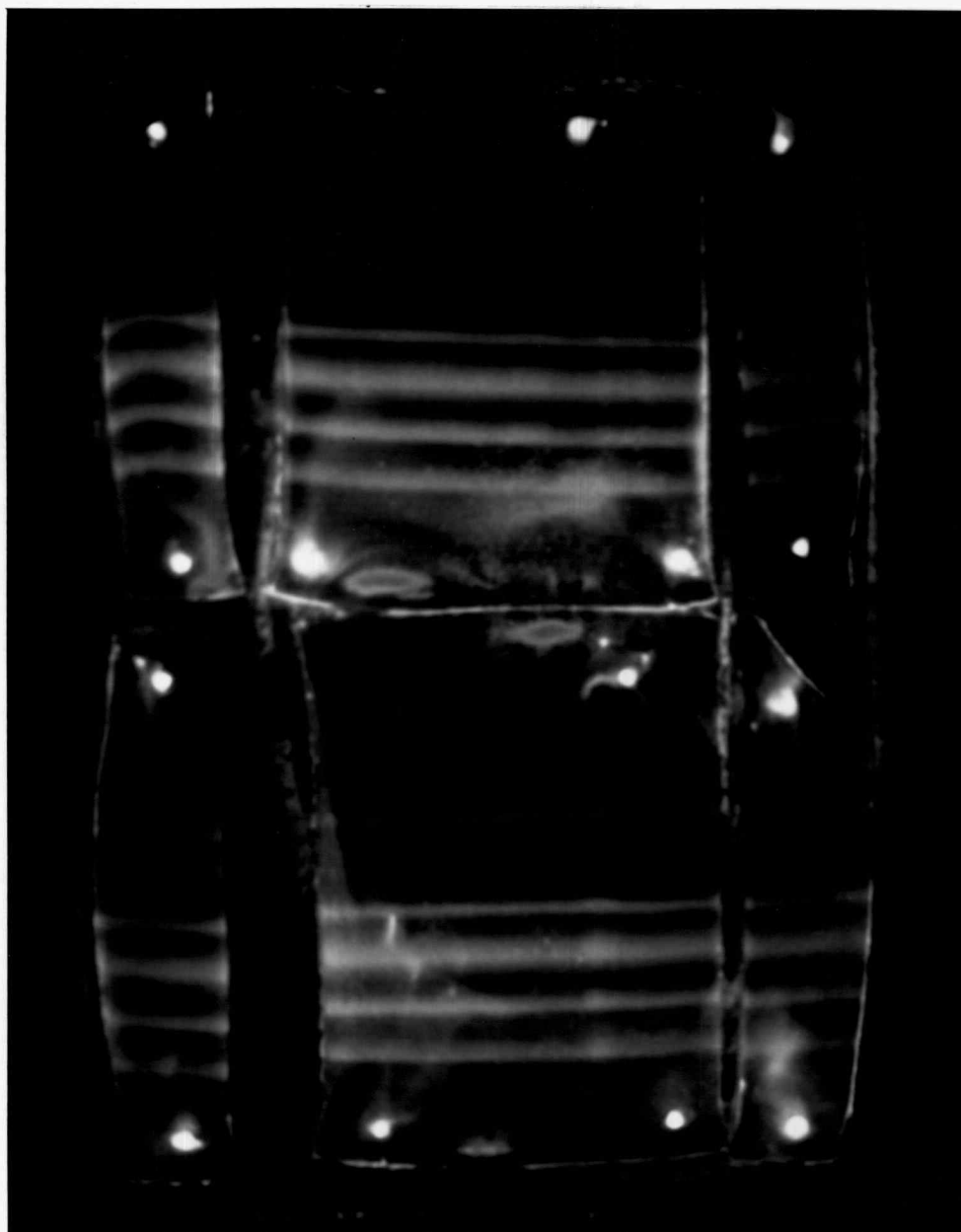
Figure 2. Phosphorescence of two acid-urea slab gels containing, from top to bottom, a non-histone protein, H2b, H3, and H4.

To insure efficient heat flow from the glass plate to the aluminum, a layer of glycerol was placed between the two as follows. A puddle of glycerol was first placed in the middle of the aluminum plate. The pyrex plate was placed on top of the glycerol and held in place with the two clamps. After a few minutes, to allow for the spreading of the puddle, the excess glycerol was removed from around the edges of the glass. A piece of clear polyethylene (Glad Wrap) was stretched over the glass as smoothly as possible. The gel pieces were placed on the plastic wrap and any air bubbles were gently squeezed out. Plastic gloves were used throughout in handling the slabs, as finger-prints are visible by phosphorescence (Isenberg et al., 1975). The gel was now ready to be cooled down, and the entire assembly was placed in the blackened styrofoam box. The liquid nitrogen was poured in, in stages, using a plastic funnel and a piece of large size vacuum tubing. The entire cooling-down procedure took about 40 minutes, from the first addition of liquid nitrogen to complete immersion. Phosphorescing gels were photographed as in Isenberg et al. (1975) using a 1 second exposure at f/1.2 with Tri-X film (Figure 2). Adequate irradiation was obtained without removing the filter from the ultraviolet lamp.

The film was developed and when it was determined that a satis-factory negative had been obtained, the gel was removed from the liquid nitrogen and allowed to thaw. The negative was placed in an enlarger

and projected onto a piece of white paper. The pyrex plate with the

gel on it was then placed over the white paper so that the image could

be adjusted to the proper size by aligning it to the marker pins. When

the correct size was determined, the plate and gel were removed and

a diagram was drawn (Figure 3) using the image projected from the

negative. This cutting diagram indicated the outline of the gel pieces,

the location of the marker pins, and the straight line cuts to be made

to cut out the desired protein fractions.

Precise Cutting of Bands

For cutting, the pyrex plate with the gel still on it was placed

over the cutting diagram (Figure 3). It was aligned as precisely as

possible using both the outline of the particular gel piece being cut and

the marker pins in that piece. To make a cut, a microtome blade was

placed as accurately as possible over a straight line on the diagram

and pressed down on the gel. In most cases, a rocking motion was

sufficient to cut the gel. However, in some cases, it was necessary

to scribe along the microtome blade with a small razor blade.

The first cut made was that below the lowest band. The lower

part of the gel was then removed. If the remaining gel piece had

shifted during the execution of this cut, it was realigned on the cutting

diagram. Then the cut above the uppermost band was made, and the

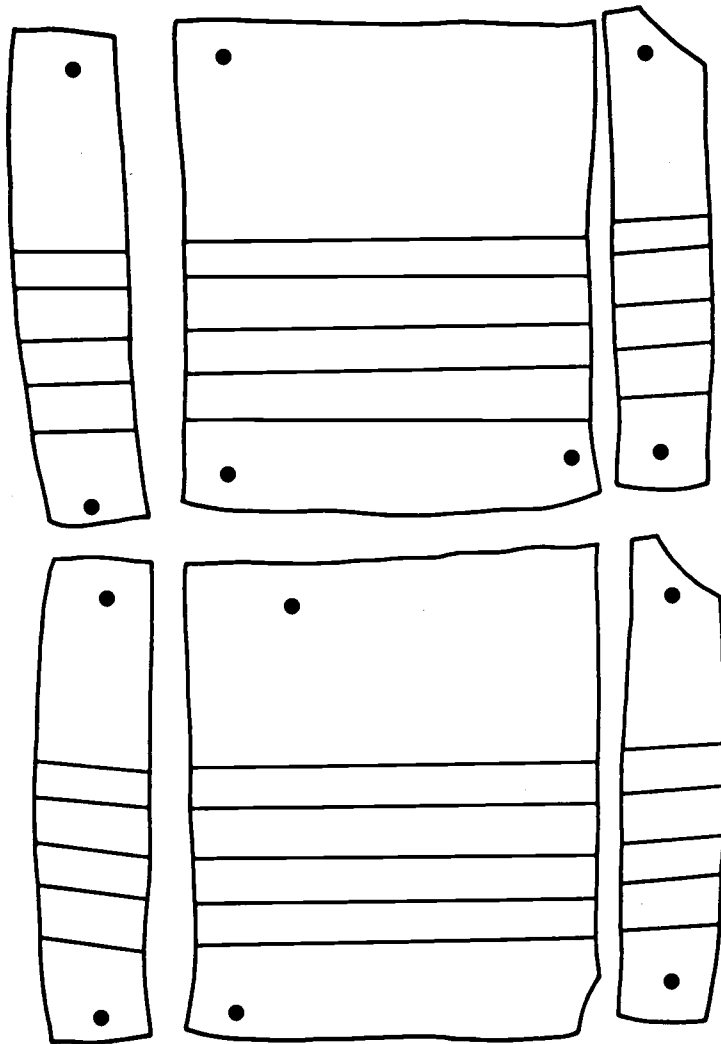top part of the gel removed. After each subsequent cut, the gel portion

Figure 3.   A sample of a cutting diagram.

remaining was realigned to compensate for minor shifts of the gel

during the actual cutting operation. If the protein was not immediately

eluted, the gel pieces were stored at $-80^{\circ}C$ in small glass beakers

sealed with parafilm.

Elution of Protein

A key step in this method of preparative electrophoretic frac-

tionation of protein was the efficient recovery of protein from gel pieces

in high yield with a minimum of contamination from components of the

gel matrix itself. To this end, an apparatus was designed that electro-

phoretically eluted protein from diced gel pieces. In experiments with

the eluter in which a known amount of calf thymus H2b was electro-

phoresed into a preparative Triton gel and then eluted from it, overall

recoveries, from the original loading to the final lyophilization of the

recovered H2b, of 77-87% were measured. Taking into account losses

in handling from the application of the protein to the gel to the assay

after recovery, the true efficiency of the elutor itself in

recovering protein from gel pieces may be much higher. Typically,

the amount of protein in the collecting chamber built up during the first

24-48 hours of elution and thereafter decreased at a rate of about

5-10% per 24 hours.

A diagram of the apparatus is given in Figure 4. It was made of

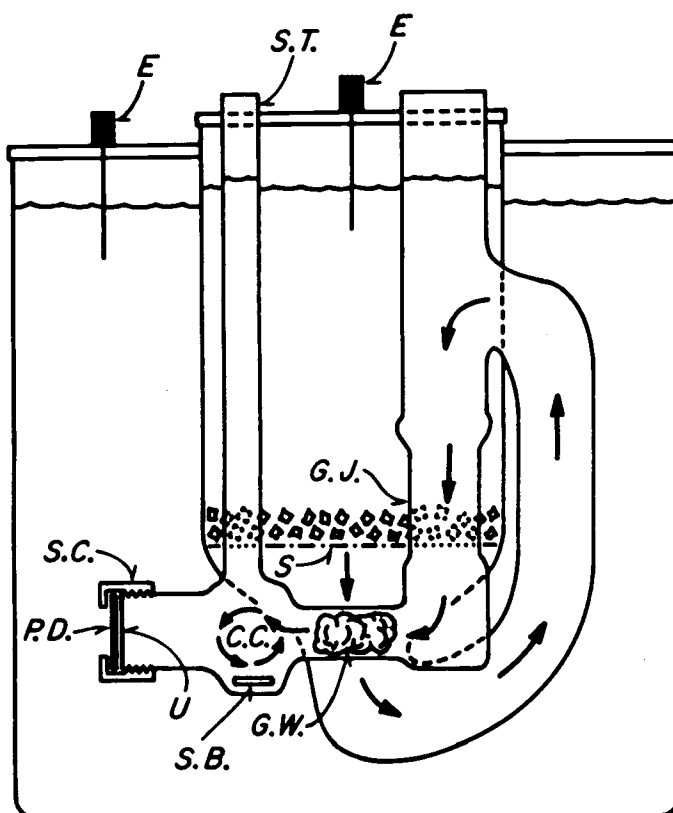hard glass and had lucite fittings. It was entirely contained in a

Figure 4.   Diagram of gel eluter.   See text for description.

beaker 10 cm high and 10 cm in diameter. To assemble the apparatus, a piece of washed and dried glass wool (G. W.) was placed in the narrow tube connecting the collecting chamber to the rest of the apparatus. An Amicon UM 2 ultrafiltration membrane (U) and a porous polyethylene disc (P. D.), both cut to a diameter of 13.5 mm with a sharp borer, were assembled as shown in the diagram (Figure 4) by means of a lucite screw cap (S. C.). A magnetic stir bar (S. B.) was dropped into the collecting chamber (C. C.). The inner assembly was placed inside the outer buffer chamber, and held in place by the lucite top, which contained the negative electrode (E). The elution buffer, which consisted of 5% (w/v) acetic acid and 2% (w/v) thiodiglycol, was placed in the two separate chambers to the levels indicated in Figure 4. The teflon screen (S) was dropped into place, and the gel pieces containing protein were diced into small pieces with a pair of stainless steel scissors and allowed to fall into the teflon screen. Then the top to the inner buffer chamber containing the positive electrode was put in place and the apparatus placed on a magnetic stirrer. With the stir bar rotating at a moderate rate, the elution was run at 250 volts. This voltage gave a steady current of about 7 ma.

The progress of the elution was monitored by first gently inserting a plug into the ground-glass joint (G. J.) and then withdrawing a sample with a pastuer pipette inserted into the collecting chamber through the sampling tube (S. T.). A U. V. absorbance spectrum was

used to determine the amount of protein eluted at various time intervals
(Figure 5). After taking a reading the sample was replaced and the
plug carefully removed. Total elution time ranged from 36-48 hours
at room temperature.

Removal of Triton X-100 From Eluted
Protein Samples

Triton X-100 (octyl phenoxy polyethoxyethanol) has an absorbance
spectrum that looks identical to that of tyrosine. Thus in looking at the
absorbance spectrum of a solution containing both protein and Triton,
one sees what appears to be more protein than is really present. In
eluting protein from Triton gels, it became apparent that Triton was
entering the collecting chamber with the protein. To remove the Triton
before desalting and lyophilization, protein solutions eluted from Triton
gels were stirred with SM-2 bio-beads (Holloway, 1973). An experi-
ment with calf thymus whole histone indicated that the biobeads were
very efficient in removing the levels of Triton we encountered, without
binding appreciable amounts of histone (Figure 6).

In an initial elution from a Triton gel, the solution in the collect-
ing chamber was removed at 26, 48, and 72 hours. After stirring with
biobeads, an absorbance spectrum of each sample revealed that most
of the protein had been collected in the first 26 hours, with a lesser,
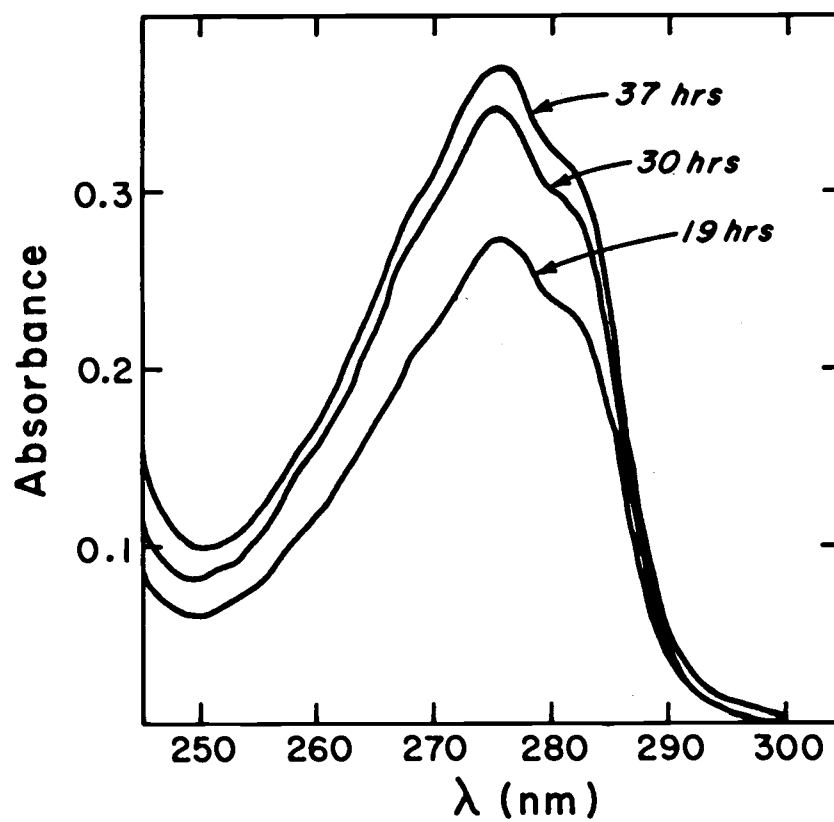but appreciable, amount coming off in the next 22 hours and little or

Figure 5. Absorbance monitoring of H2b elution. The solution in the collecting chamber was removed and the absorbance measured at the times shown. The solution was replaced after the first two measurements.
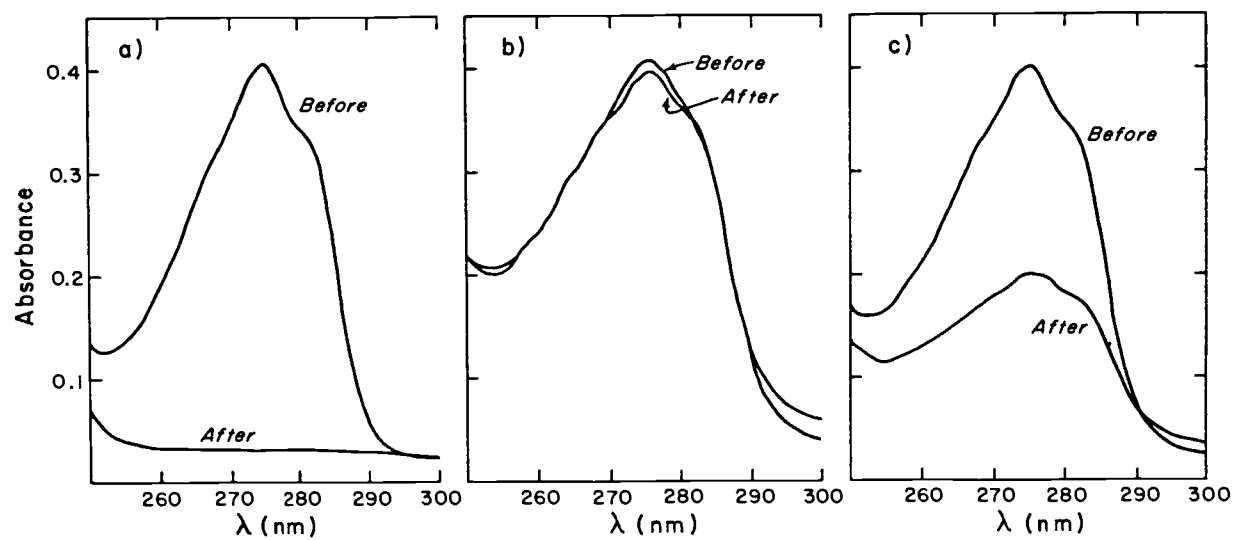
Figure 6.   An experiment to determine the relative uptake of Triton X-100 and calf thymus whole
histone by SM-2 Bio-Beads. Solutions were prepared to have the same absorbance.
These solutions, and a 1:1 mixture of the two, were then stirred for 1 hour with a
0.1 x volume of 50% (v/v) beads. All solutions had 5% acetic acid. (a) Triton alone.
(b) Histone alone. (c) 1:1 mixture of the two.

none in the next 24 hours. In this way it was decided to elute the

Triton gels for 48 hours as a standard procedure.

Triton X-100 was removed from protein samples eluted from

Triton gels using SM-2 biobeads as follows. The biobeads were

washed (Holloway, 1973) and then placed in 5% (w/v) acetic acid to

an amount such that the settled volume of beads was half the total

volume. An amount of this solution equal to one-tenth volume of the

protein solution was then added to the protein solution and stirred

for one hour in a small beaker. The protein solution was then trans-

ferred to a conical centrifuge tube and the beads allowed to settle.

The supernatant was pipetted off. The procedure was then repeated

a second time.

Desalting and Lyophilization

After elution (or after Triton removal, for protein eluted from

Triton gels) the protein solution was desalted using a Sephadex G-25

column equilibrated with .01 N HCl. Column fractions were monitored

by absorbance at 230 nm. The elution apparatus recovered the protein

from a gel in a volume of 5-6 ml. This volume was applied directly

to a 1.2 x 26 cm G-25 column and the protein collected in 3-5 fractions

of 2 ml each. Column fractions were pooled and lyophilized. This

procedure of desalting followed by lyophilization gave an exceptionally

clean and easily dissolved histone preparation.

Analytical Techniques

Acetic acid-urea gels for analytical work were according to

Panyim and Chalkley (1969), except that they contained 5% (w/v) acetic

acid (Alfageme et al., 1974). Tray buffer was also 5% (w/v) acetic

acid, and the gels contained 2.5 M urea. Acetic acid-urea gels with

Triton X-100 or DF-16 were identical in composition to the above,

except that they contained an additional 1% Triton (Alfageme et al.,

1974). SDS gels (15% acrylamide separating gel, 6% stacking gel) were

according to Laemmli (1971). They were run on a microslab apparatus

(Matsudaira and Burgess, 1978). Two dimensional gels with acid-

urea in the first and Triton in the second dimension were according to

Spiker (1976a, b).

SDS gels were stained with Coomassie Brilliant Blue R-250

(Matsudaira and Burgess, 1978). Other gels were stained with Amido

Black (Panyim and Chalkley, 1969) or Coomassie Brilliant Blue

G-250 (Reisner et al., 1975).

Concentrations of histone stock solutions in water were deter-

mined from the absorbance at 275.5 nm (D'Anna and Isenberg, 1974d).

Molar extinction coefficients, assuming 3, 5, 2, and 4 tyrosines, of

4.0×10^3, 6.7×10^3, 2.7×10^3, and 5.4×10^3 cm^{-1} M^{-1} were used

for H2a, H2b, H3, and H4, respectively. The contribution due to

scatter was estimated by extending a straight line to 275 nm according

to the absorbance from 350 to 300 nm. All stock solutions were in the neighborhood of 1 mg/ml in concentration. Absorption spectra were measured with a Cary 14 spectrophotometer.

Final solutions were composed by first pipetting the necessary amount of protein stock solution(s), then water sufficient to attain 0.9 x the final volume, and finally 0.1 x volume of 160 mM phosphate buffer (pH 7.0 upon 10 x dilution). All pipetting was done with Pipetman adjustable hand-held pipettes. Solutions were mixed in small hard-glass culture tubes, or where possible, directly in the curvettes themselves. Mixing was achieved by pumping up and down five times with a 200 μl pipette.

Fluorescence anisotropy, $r = (I_{11} - I_{\perp})/(I_{11} + 2 I_{\perp})$, and fluorescence intensity, were measured on a computerized fluorescence anisotropy spectrophotometer designed and constructed in this laboratory (Ayres et al., 1974). Samples were measured in a 0.5 x 0.5 cm curvette in a final sample volume of 600 μl. Excitation was at 279 nm and the emission was monitored at 325 nm. Corning CS-054 filters were used in the emission beam. All samples were measured at 22°C.

Circular dichroism (CD) measurements were made with a Durrum-Jasco Model J-10 CD recorder. Sample temperature was 22°C. Two different cells were used, one with a path length of 1mm and volume of 200 μl, and another with a path length of 1 cm and volume of 600 μl. CD measurements were calibrated using d-10 camphorsulfonic

acid. CD measurements were reported as $\Delta \varepsilon = \varepsilon$ (left) $- \varepsilon$ (right) in units of cm^{-1} 1 (mole of protein)$^{-1}$. The term $\Delta \varepsilon_i'$ is calculated assuming noninteracting mixtures.

Amino acid analyses were performed as described in Spiker and Isenberg (1977). The samples for analyses were hydrolyzed for 20 hours. Proline was determined separately on a Beckman Model 120B analyzer.

RESULTS

Analytical Electrophoresis of Whole Histone

Figure 7 shows an acetic acid-urea gel of whole yeast histone compared with calf thymus histone. The pattern observed is in agreement with earlier investigations (Wintersberger et al., 1973; Moll and Wintersberger, 1976; Brandt and von Holt, 1976) using the same gel system. Doublet bands are observed for both H2b and H4.

Figure 8 shows two-dimensional gels of yeast histones prepared by two different methods and calf thymus histones. The first dimension (horizontal) contains acetic acid and urea, and the second (vertical) contains Triton X-100. The chromatin isolation procedure of Tonino and Rozijn (1966b) was used to obtain preparative amounts of crude histone. In their procedure, cells are fragmented, and the nucleus material is collected and washed by centrifugation. Commerical pressed baker's yeast is the starting material. As a control, a small amount of crude histone was prepared from a pure culture of the same yeast (cells were collected in late log phase) by first isolating nuclei according to Wintersberger et al. (1973). The results of the two methods are compared in Figures 8b and 8c. The two methods of histone isolation resulted in remarkably similar two-dimensional gel patterns. This result demonstrates two things: first, that the commercial pressed baker's yeast used was not contaminated
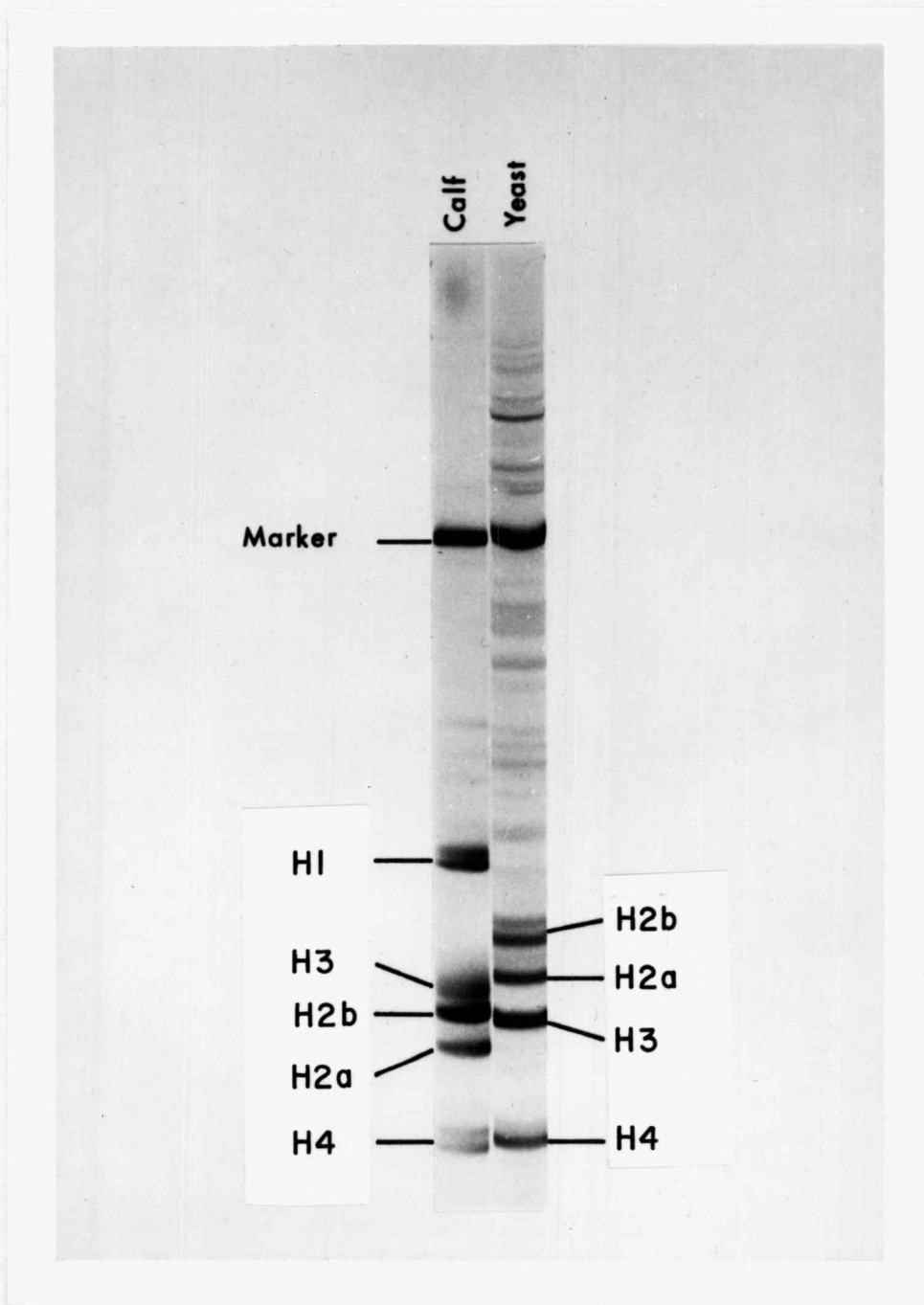
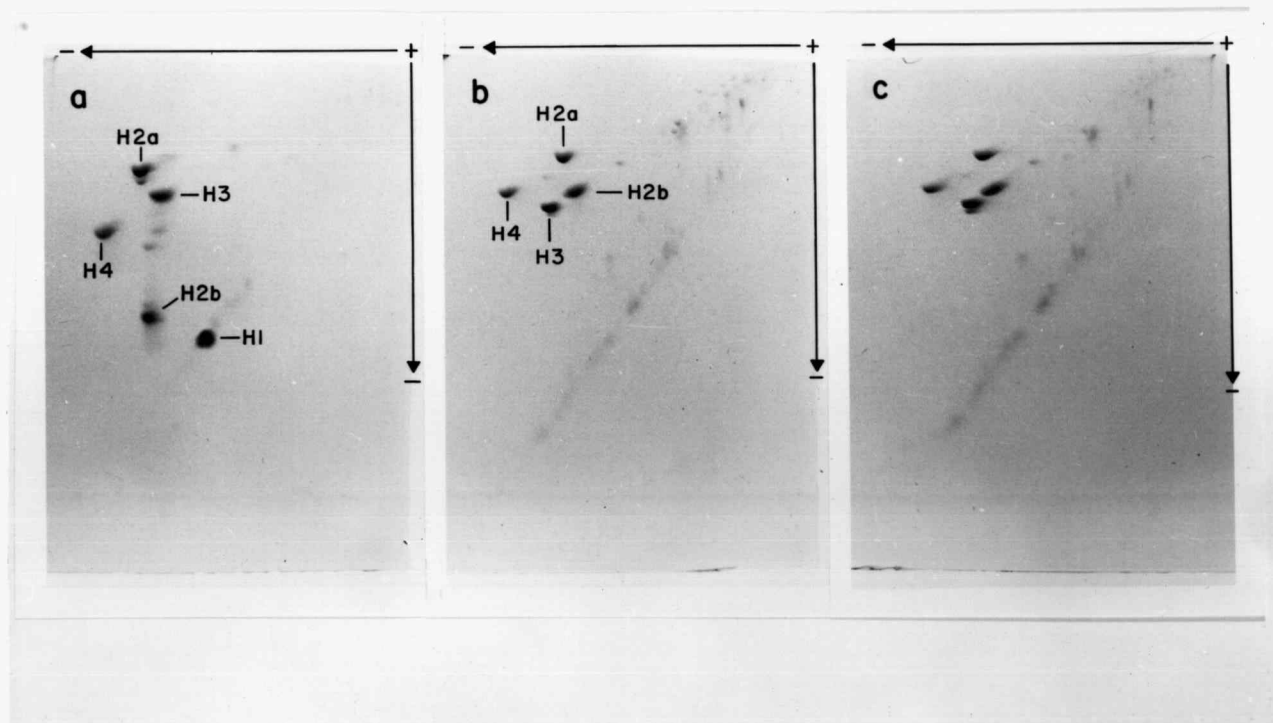Figure 7. A comparison of calf thymus and yeast histones in an acid-urea gel.

Figure 8.   Two dimensional gels of (a) calf thymus histones, (b) yeast histones prepared via chromatin from broken cells, and (c) yeast histones prepared via chromatin from nuclei according to Wintersberger et al. (1973).

with bacterial or other contaminants that would interfere with the

isolation of yeast histone; and second, that the use of disintegrated

whole cells as a source of histone did not result in an increased

cytoplasmic contribution of proteins to the histone fraction, as com-

pared to a nuclear preparation.

<u>The Prevention of Oxidation of Proteins</u>
<u>During Preparative Electrophoresis</u>

Alfageme <u>et</u> <u>al</u>. (1974) have reported that proteins can be oxidized

during preparative electrophoresis in Triton gels. This modification,

which is believed to be due to methionine oxidation (Alfageme <u>et</u> <u>al</u>.,

1974), can often be reversed by mercaptoacetic acid. However, in

some cases, partial degradation occurs before the protein is completely

reduced back to its original form (A. Zweidler, personal communica-

tion). It is therefore important to avoid the damage rather than

reverse it.

Calf thymus H2b is a convenient test substance to determine

the conditions under which the oxidation does not occur. Furthermore,

whether H2b is reduced or oxidized can be easily monitored by

analytical electrophoresis on Triton gels (Alfageme <u>et</u> <u>al</u>., 1974). As

seen in Figure 9, the calf thymus H2b which was subjected to pre-

parative electrophoresis and subsequent elution as described in

Materials and Methods showed no trace of oxidation. Calf H2b which

Figure 9.   Triton X-100 gels of calf thymus H2b used to monitor the oxidation of methionine residues (Alfageme et al., 1974) during preparative electrophoresis and elution. (1) H2b before preparative electrophoresis. (2) H2b recovered from a preparative gel, with pre-electrophoresis, electrophoresis, and elution as described in Materials and Methods. (3) H2b recovered from a preparative gel, with pre-electrophoresis for only 48 hours in acetic acid, scavenging according to Materials and Methods, and elution in 5% (w/v) acetic acid only. (4) H2b oxidized for 10 minutes with 1.5% H_2O_2 (Alfageme et al., 1974).

1 2 3 4
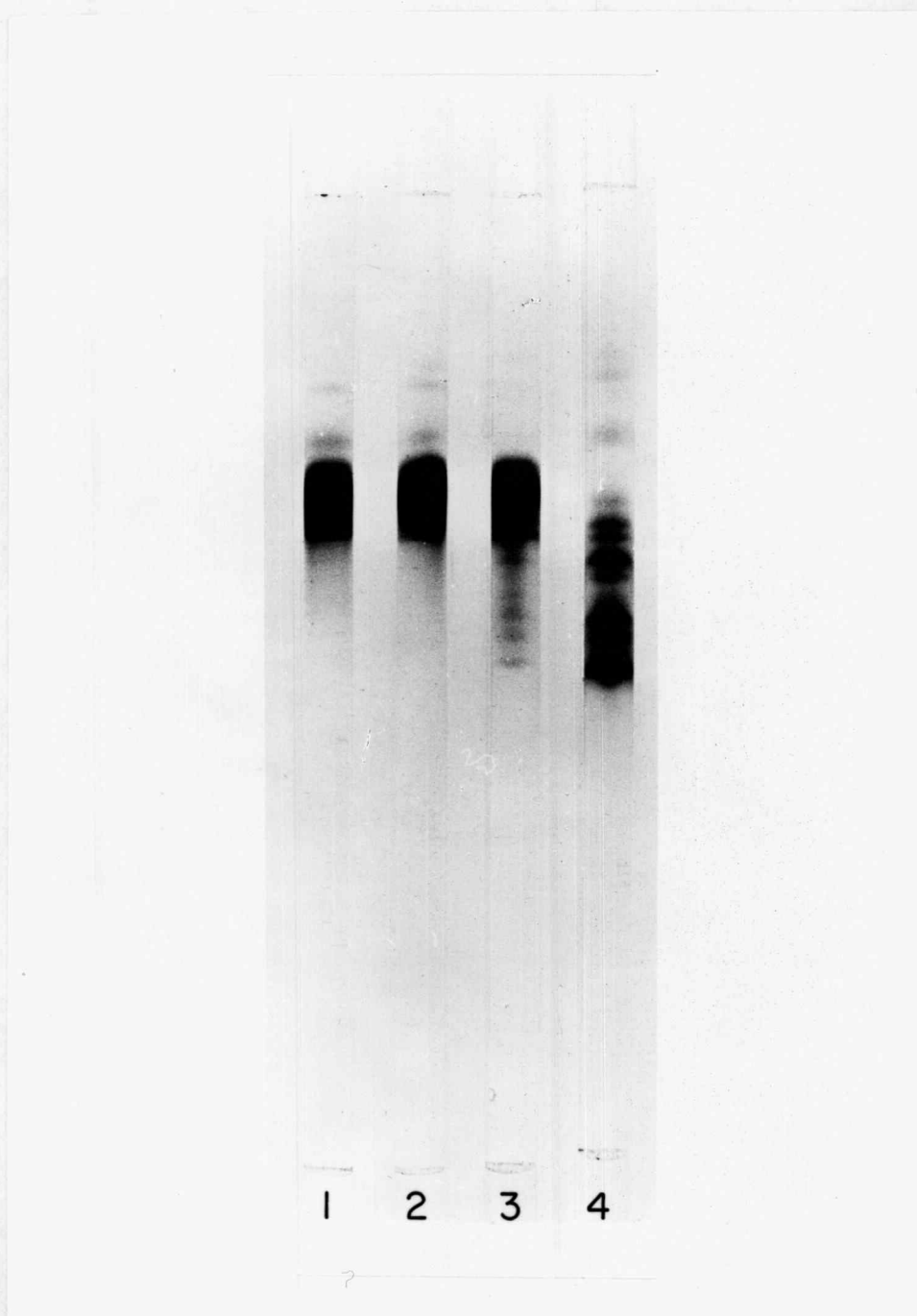
was subjected to a less rigorous procedure did show some oxidation

products (Figure 7).

No attempt was made to determine which elements of the rigor-

ous precautionary scheme used (extensive (five day total) pre-

electrophoresis time, two days of infiltration of the gel from below

with mercaptoacetic acid, an additional scavenging from above by

cysteamine, and thiodiglycol in the elution buffer) were necessary to

prevent methionine oxidation. However, since no UV-absorbing or

scattering impurities were evident during the elution of protein from

the gels, it appears that the extensive period of pre-electrophoresis

was very effective in completely removing such impurities from the

gels. This is indicated in Figure 5, which demonstrates the monitoring

of eluted protein with time, and Figure 10, which shows the absorbance

of purified H2b in water. In both instances there is very low absorbance

from 340 to 300 nm, a region where light scattering impurities, or

aggregation, is observed in histone solutions. Also the depth of the

valley at about 250 nm in these figures, relative to the peak at 275 nm,

indicates very low absorbance in this region from any non-protein

substances.

Fractionation and Characterization of the Yeast Inner Histones

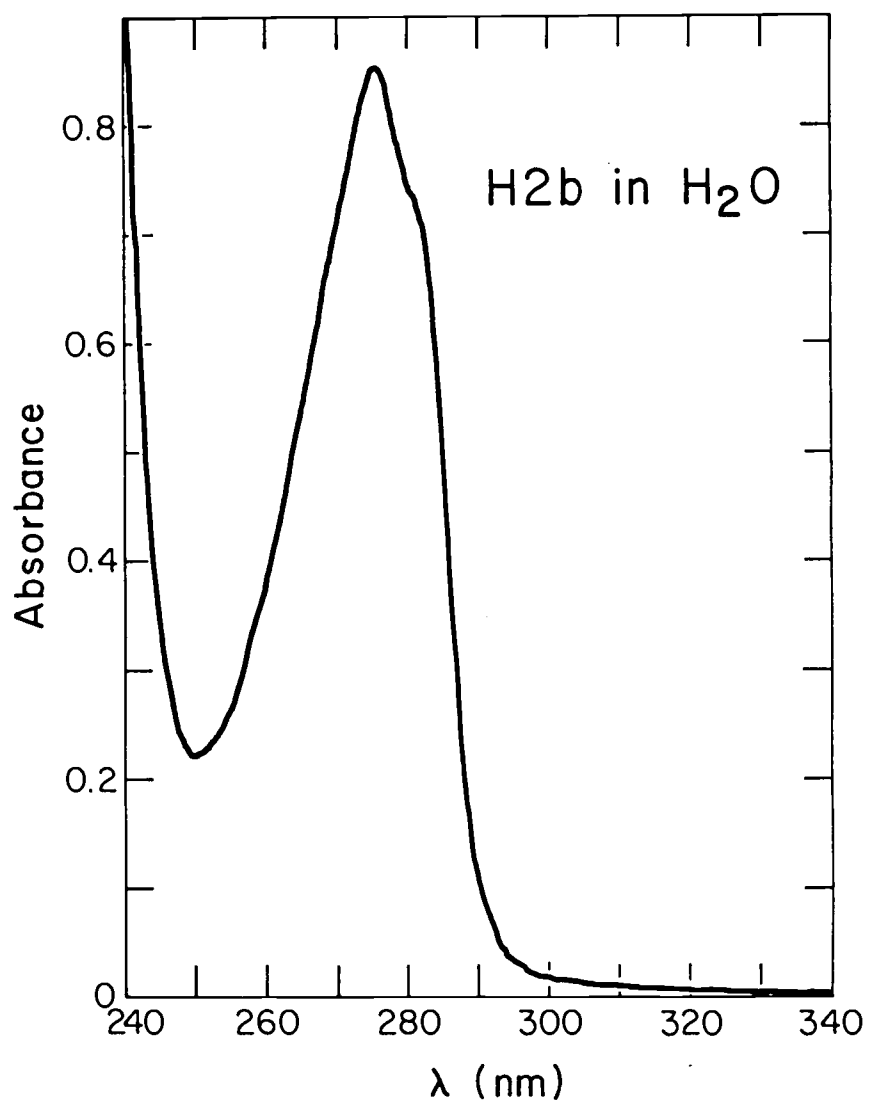Figure 11 shows the results of the preliminary fractionation of

Figure 10. Absorbance of yeast H2b in water, after final lyophiliza-
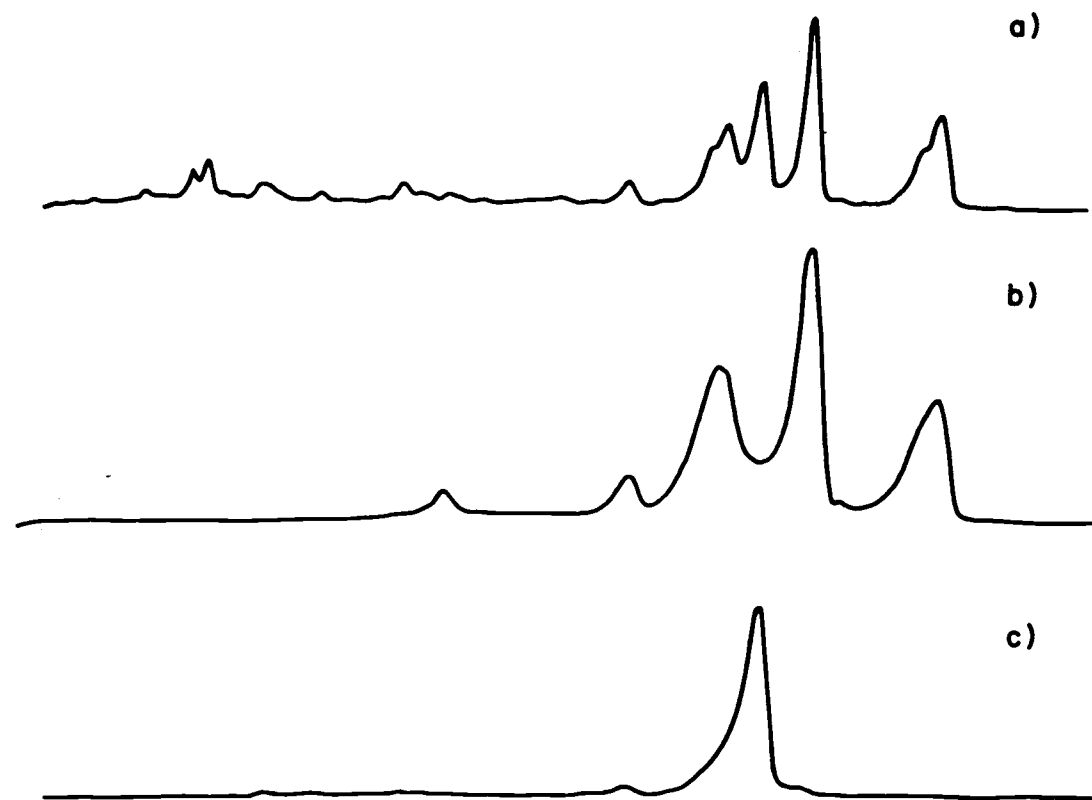tion (see Figure 1).

Figure 11. Scans of acid urea gels before and after the first slab gel fractionation. (a) Before. (b) Fraction containing H2b, H3, and H4. (c) Fraction containing H2a.

crude yeast histone on Triton X-100 slab gels, as outlined in Figure 1. This step separated H2a from H2b, H3, and H4 and also removed a large number of other proteins from both fractions. As may be seen in the two-dimensional gel in Figure 8b, there is a group of proteins which run well ahead of the histones on a Triton gel, but which migrate in the region of the histones in an acetic acid-urea slab. These proteins were among those removed in the first fractionation step. In a typical run, 22 mg of crude whole histone was fractionated to yield 2.2 mg of the fraction containing H2b, H3, and H4, and 0.8 mg of the fraction containing H2a. A total of eight Triton slabs were run. The results of further purifying the resulting two fractions (Figure 11b and c) on acetic acid-urea gels are shown in Figure 12. Gels 1 and 3 in Figure 12 were loaded with 15 µg of protein. This overloading was used to search for contaminants. The gels revealed two very minor impurities in the H3 fraction, and some H2a in the H2b fraction. Gel 7 in Figure 12 contained Triton DF-16, which gave a good separation of H2a and H2b. A scan of this gel indicated that the amount of H2a present in the H2b fraction was about 4%. The H2a and H4 had no detectable impurities. Each of the two fractions from the first step was applied to four slabs in the second step, for a total of eight.

A loading of 4.4 mg of the fraction containing H2b, H3, and H4 on a single slab yielded 0.9 mg of H2b, 0.8 mg of H3, and 0.3 mg H4.
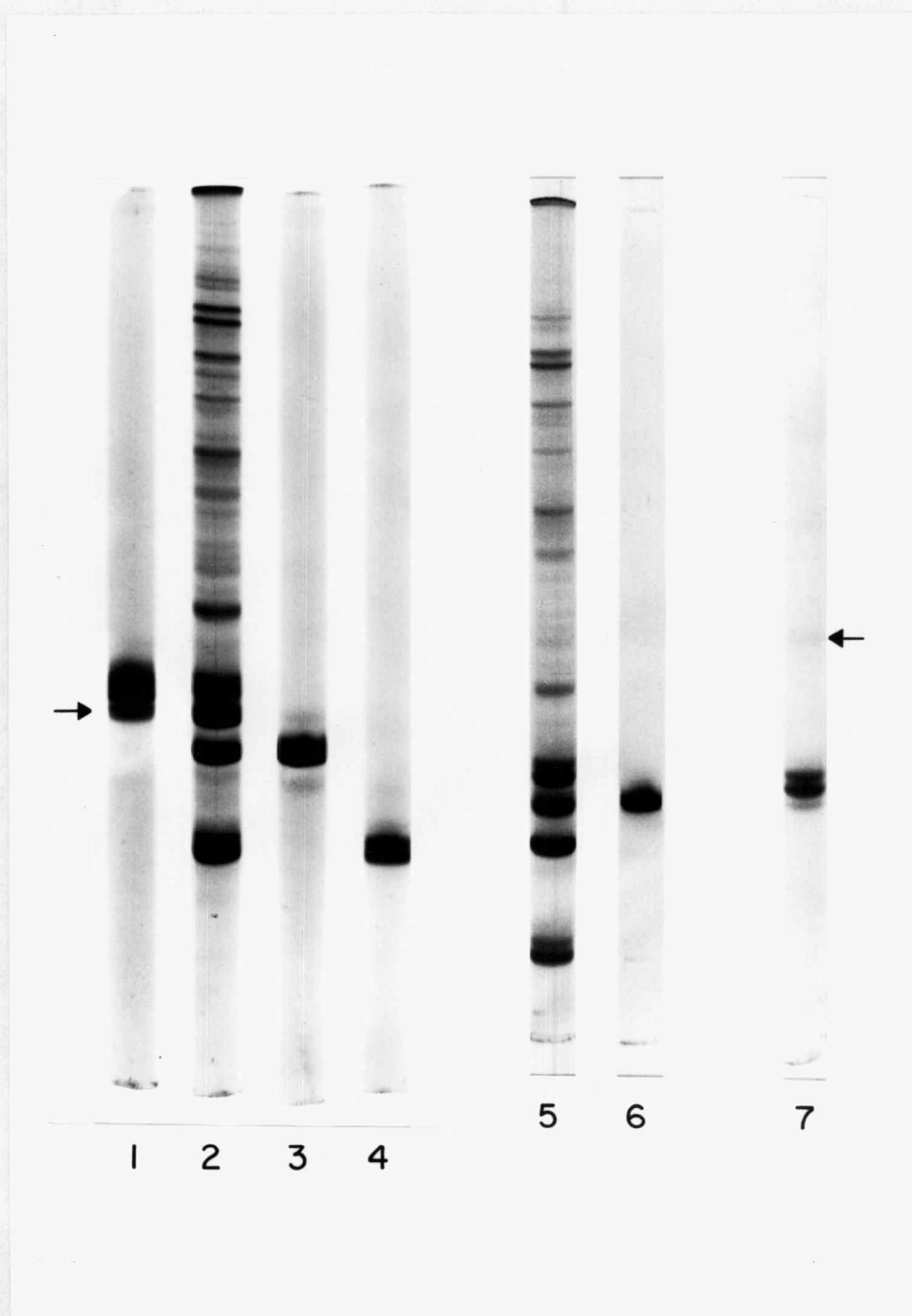
Figure 12.   Results of the second slab gel fractionation.   All gels are
acetic acid-urea; gel 7 also contains Triton DF-16.   Gels
1-6 were overloaded to show contaminants.   1:   Gel of
H2b.   Arrow indicates an H2a contamination of 4%; 2, 5:
Crude yeast histone preparations; 3: H3; 4: H4; 6:H2a;
7: H2b.   Subfractions may be seen.   The arrow shows the
4% H2a contaminant.

A loading of 1.6 mg of the fraction containing H2a yielded 0.5 mg per slab.

Table 3 presents amino acid analyses of the four yeast inner histones and a comparison with the calf thymus histones. Figure 13 shows the data plotted as residue profiles. These profiles permit an easy identification of the four histones. This identification was verified by the complexing results, as described below. Figure 14 gives an example of a non-homologous comparison as a contrast to the homologies demonstrated in Figure 13.

Figure 15 shows an SDS gel of the yeast histones. The yeast histones, identified by amino acid analysis as H2a, H2b, H3, and H4 are seen to correspond quite closely in mobility, and therefore in molecular weight, to their calf counterparts. It may be seen that yeast H3 and H4 migrate slightly slower and yeast H2a slightly faster than the corresponding calf histones. If these differences are due to molecular weight alone, then the inner histones of the two species have molecular weights differing by 2-3%.

All of the yeast inner histones showed immediate changes, in both anisotropy and intensity of emission, upon the addition of phosphate buffer. The values observed are presented in Table 4. In addition to a fast step, yeast H3 and H4 showed a slow change in anisotropy over time. H2a and H2b did not have an appreciable slow step. That H3 and H4 had, and H2a and H2b did not have, an

Table 3. Amino acid composition[a] of yeast and calf thymus histones.

| | Mole % | | | | | | | | |
| | H4 | | H3 | | H2a | | H2b | | |
	Yeast	Calf[b]	Yeast	Calf[c]	Yeast	Calf[d]	Yeast	Calf[e]	Yeast[f]
asx	4.1	4.9	3.9	3.7	8.3	6.2	4.6	4.8	4.4
thr	5.5	6.9	6.3	7.4	3.6	3.9	8.6	6.4	8.8
ser	5.4	2.0	7.1	3.7	5.9	3.1	12.3	11.2	12.6
glx	6.6	5.9	11.6	11.1	8.9	9.3	10.0	8.0	10.0
pro	1.5	1.0	3.7	4.4	4.0	3.9	3.9	4.8	3.9
gly	15.0	16.7	5.6	5.2	10.5	10.9	3.7	5.6	3.4
ala	6.6	6.9	12.4	13.3	15.2	13.2	13.9	10.4	13.8
cys	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0
val	7.1	8.8	5.1	4.4	4.6	6.2	4.2	7.2	4.2
met	0.0	1.0	0.0	1.5	0.0	0.0	0.0	1.6	0.0
ile	6.5	5.9	5.5	5.2	4.7	4.7	6.3	4.8	6.4
leu	9.1	7.8	8.8	8.9	12.8	12.4	5.4	4.8	5.1
tyr	3.4	3.9	1.5	2.2	1.9	2.3	3.6	4.0	3.7
phe	2.3	2.0	3.5	3.0	0.8	0.8	2.4	1.6	2.5
lys	10.7	10.8	10.4	9.6	8.8	10.9	14.6	16.0	14.8
his	1.9	2.0	1.5	1.5	2.1	3.1	1.3	2.4	1.3
arg	14.5	13.7	13.2	13.3	8.1	9.3	5.1	6.4	5.0
B/A[g]	2.53	2.45	1.62	1.65	1.10	1.50	1.44	1.94	1.47

[a] No corrections have been made for destruction of amino acids or incomplete hydrolysis.

[b] Based on sequence (DeLange et al., 1969).

[c] Based on sequence (DeLange et al., 1972).

[d] Based on sequence (Yeoman et al., 1972).

[e] Based on sequence (Iwai et al., 1970).

[f] Corrected for the presence of 4% (mole/mole) H2a.
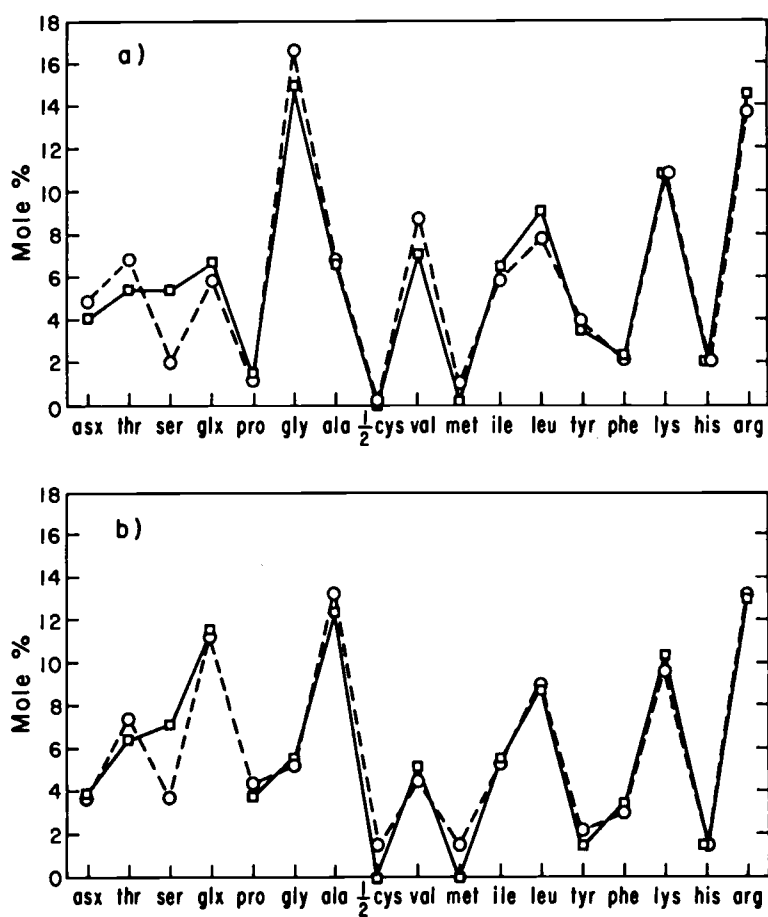
[g] (lys + his + arg)/(asx + glx).

Figure 13 a and b.   Amino acid analyses of yeast (solid line) and calf (broken line). (a) H4; (b) H3.
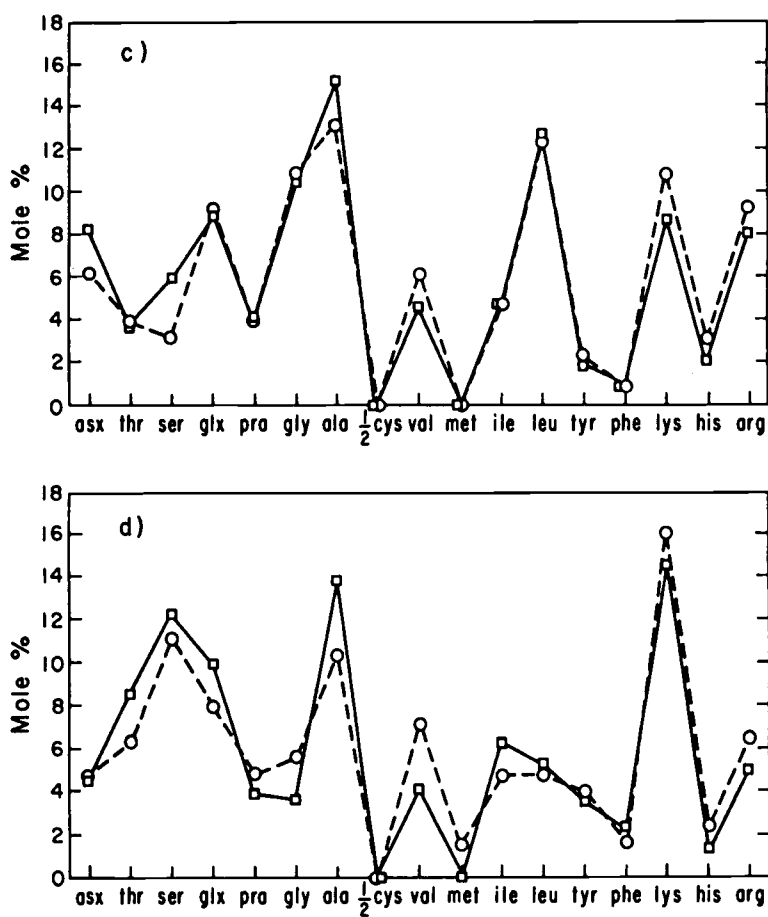
Figure 13 c and d. Amino acid analyses of yeast (solid line) and calf (broken line). (c) H2a; (d) H2b.
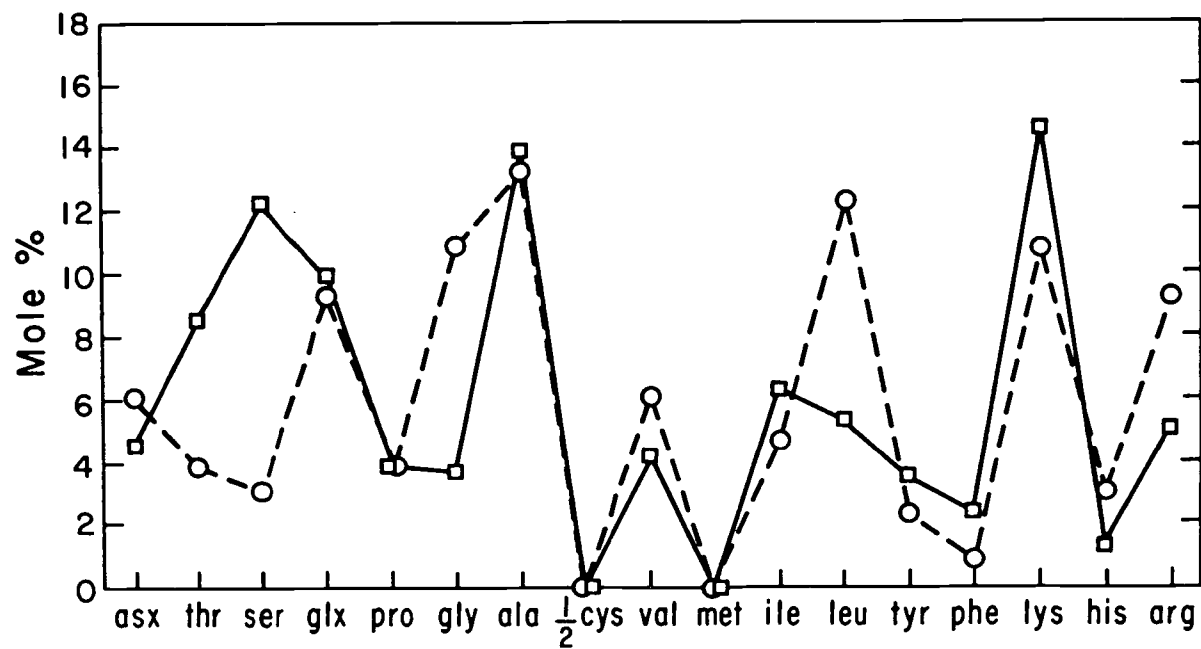
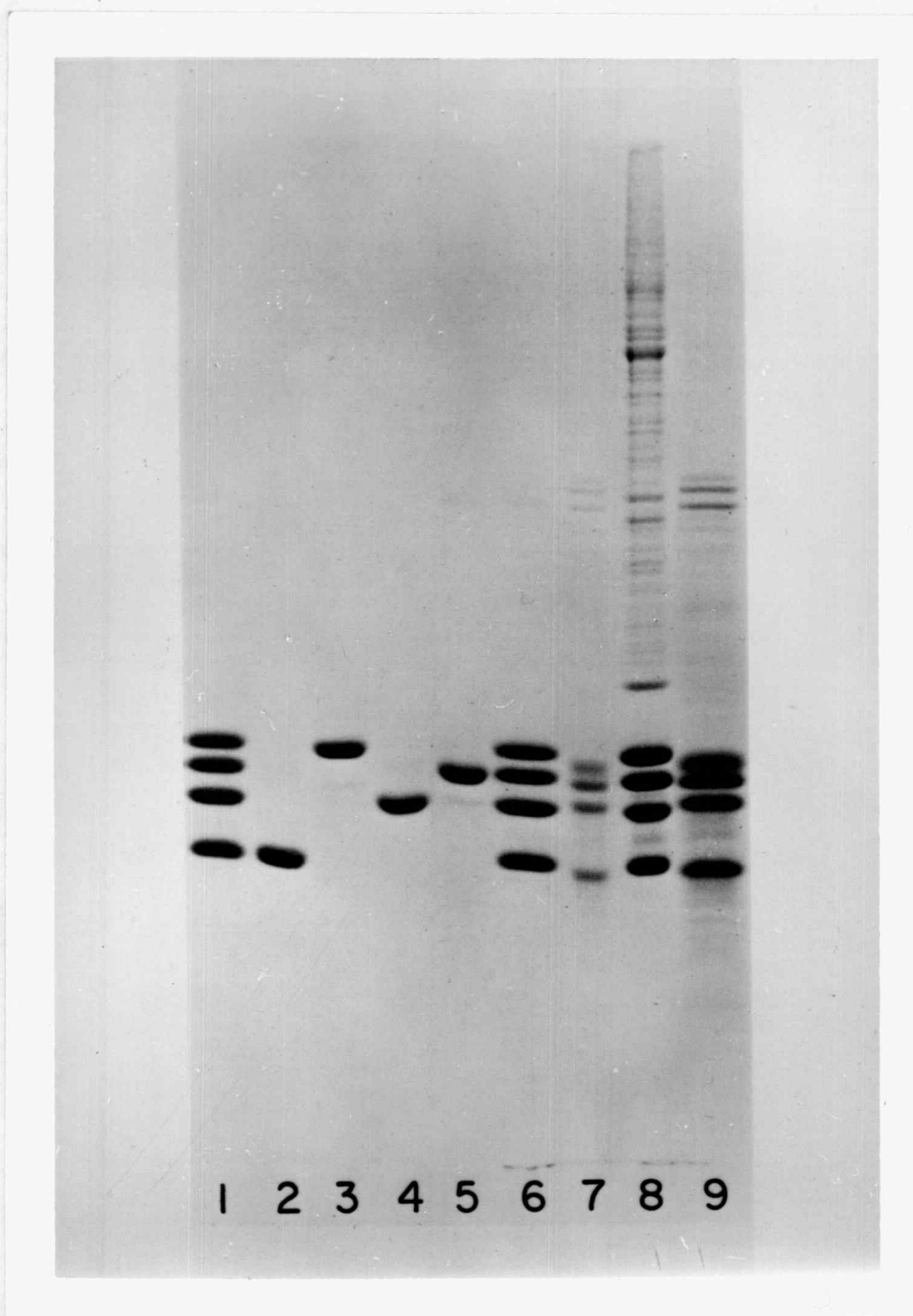Figure 14. A non-homologous comparison of yeast H2b (solid line) and calf H2a (broken line).

Figure 15. SDS gel of yeast histones and calf histones. (1 and 6)
Mixtures of the four yeast inner histones. (8) Crude
yeast fraction. (7 and 9) Calf thymus histone. (2)
H4. (3) H3. (4) H2a. (5) H2b.

Table 4. Changes in fluorescence intensity (F) and anisotropy (r) for the yeast inner histones upon the addition of sodium phosphate, pH 7.0.

	r		F	
	H_2O	16mMPO$_4$	H_2O	16 mMPO$_4$
H2a[a]	.078	.133	1.34	1.07
H2b[a]	.068	.111	1.94	1.89
H3[b]	.086	.150	1.14	1.16
H4[a]	.061	.106	1.87	1.43

[a]Concentration, 5×10^{-6} M.

[b]Concentration, 7.5×10^{-6} M.

appreciable slow step at dilute concentrations, was also the case for both the pea and calf inner histones (Li et al., 1972; D'Anna and Isenberg, 1972; 1974 a, c; Smerdon and Isenberg, 1973; Spiker and Isenberg, 1977).

Complexing of the Yeast Inner Histones

The five possible yeast inner histone pair-wise interactions involving H3 and H4 were studied by monitoring the fluorescence anisotropy of each histone pair together in solution over time and comparing the observed results to those expected for no interaction.

As observed for both calf and pea (D'Anna and Isenberg, 1974d; Spiker and Isenberg, 1977), H3 and H4 blocked each other's slow step, indicating a strong interaction. A strong interaction was further indicated by the fact that the CD spectra of H3 and H4 alone were found to change over time, with additional β-sheet formation, while the spectrum of the two together did not change with time. As also observed for calf and pea (D'Anna and Isenberg, 1973; Spiker and Isenberg, 1977), H2b stopped the slow step of H4, indicating a strong interaction.

H2a did not stop the slow step of H3, though the values measured for the two together were somewhat larger than those calculated for non-interacting species. This indicates a weak interaction. The anisotropy of H2b and H3 together was indistinguishable from that

calculated for non-interaction, indicating weakly or non-interacting

species. H2a did not stop the slow step of H4, though it shifted the

value at zero time and slowed the rate of increase at early times,

indicating a weak interaction.

The method of continuous variations, using both fluorescence

anisotropy and CD, was used to investigate the strong complexes.

For the H3-H4 complex, it was found that neither the CD nor the

fluorescence anisotropy of the two together at zero time differed from

the calculated value. Thus a binding constant could not be estimated.

That the CD did not change indicates little or no change in secondary

structure of the two upon complexing, a result that differs from that

found for calf histones (D'Anna and Isenberg, 1974d).

A CD continuous variation curve for yeast H2b and H4 is given

in Figure 16. The stoichiometry appears to be 1:1 and a binding

constant of $1 \times 10^6 M^{-1}$ was estimated for dimer formation (D'Anna

and Isenberg, 1973).

That H2a and H2b interact strongly at a stoichiometry of 1:1

may be seen from the CD continuous variation curve in Figure 17.

From this data a binding constant for dimer formation of $4 \times 10^6 M^{-1}$

was estimated. A similar measurement using a fluorescence aniso-

tropy continuous variation curve gave a value of $9 \times 10^6 M^{-1}$.

Figures 18 and 19 show CD continuous variation curves for

interspecies complexes between calf and yeast H2a and H2b. The

Figure 16. CD (200 nm) continuous variation curve of yeast H2b plus yeast H4 at 16 mM phosphate, pH 7.0. Co = 1.0 x 10^{-5} M.
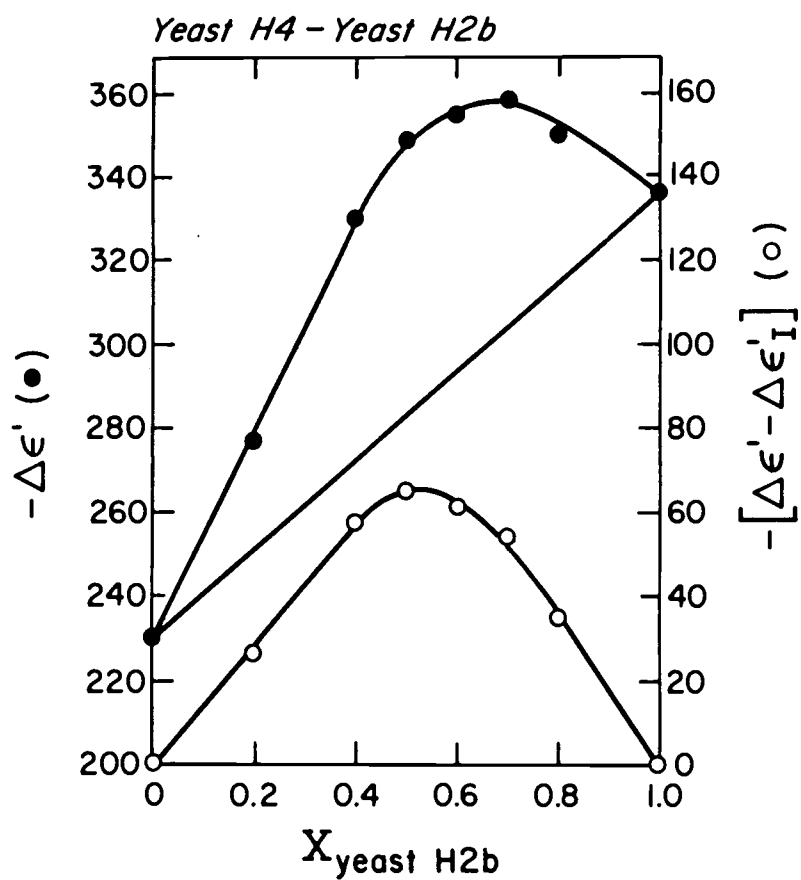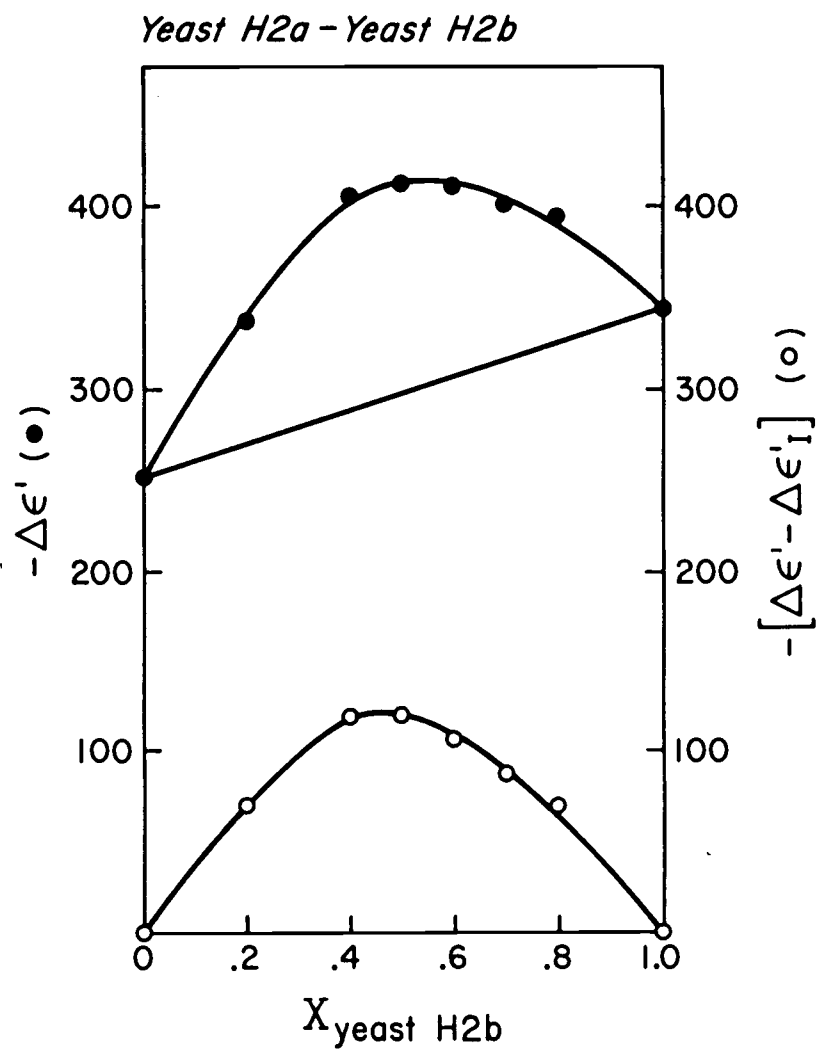
Figure 17. CD (200 nm) continuous variation curve of yeast H2a plus
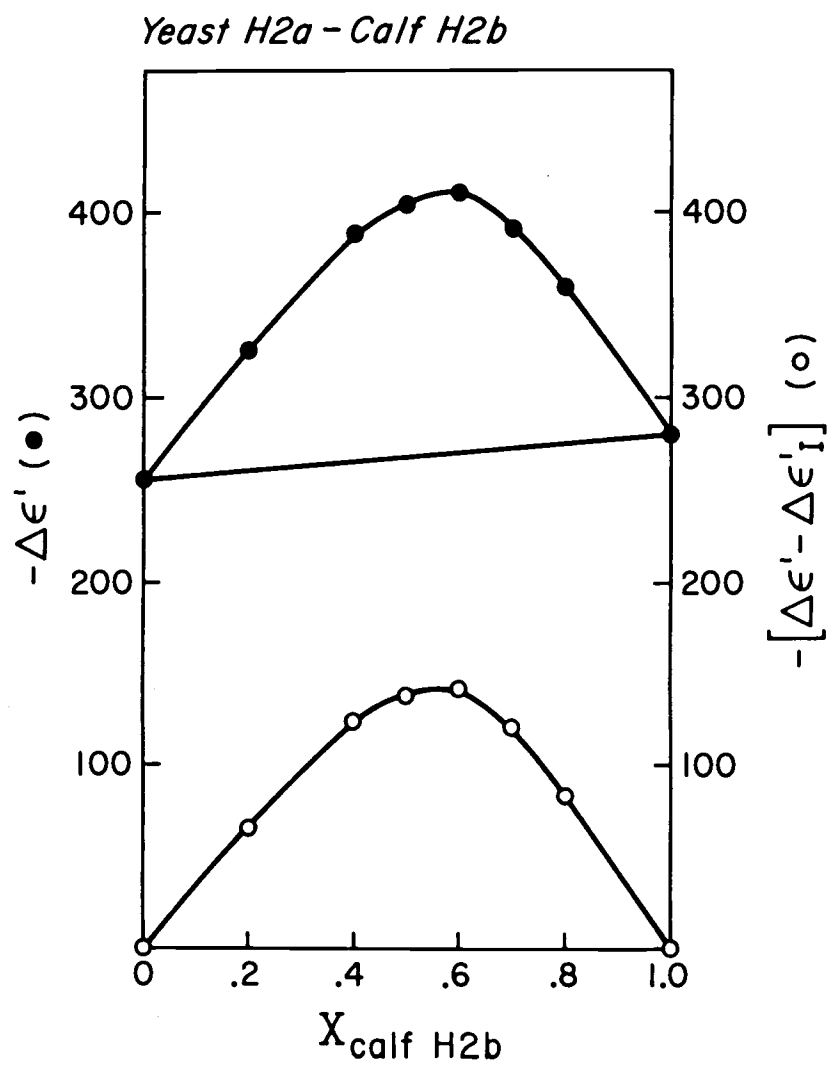yeast H2b at 16 mM phosphate, pH 7.0. Co = 2.5 x 10^{-6} M.

Figure 18. CD (200 nm) continuous variation curve of yeast H2a plus calf H2b at 16 mM phosphate, pH 7.0. Co = 2.5 x 10^{16} M.

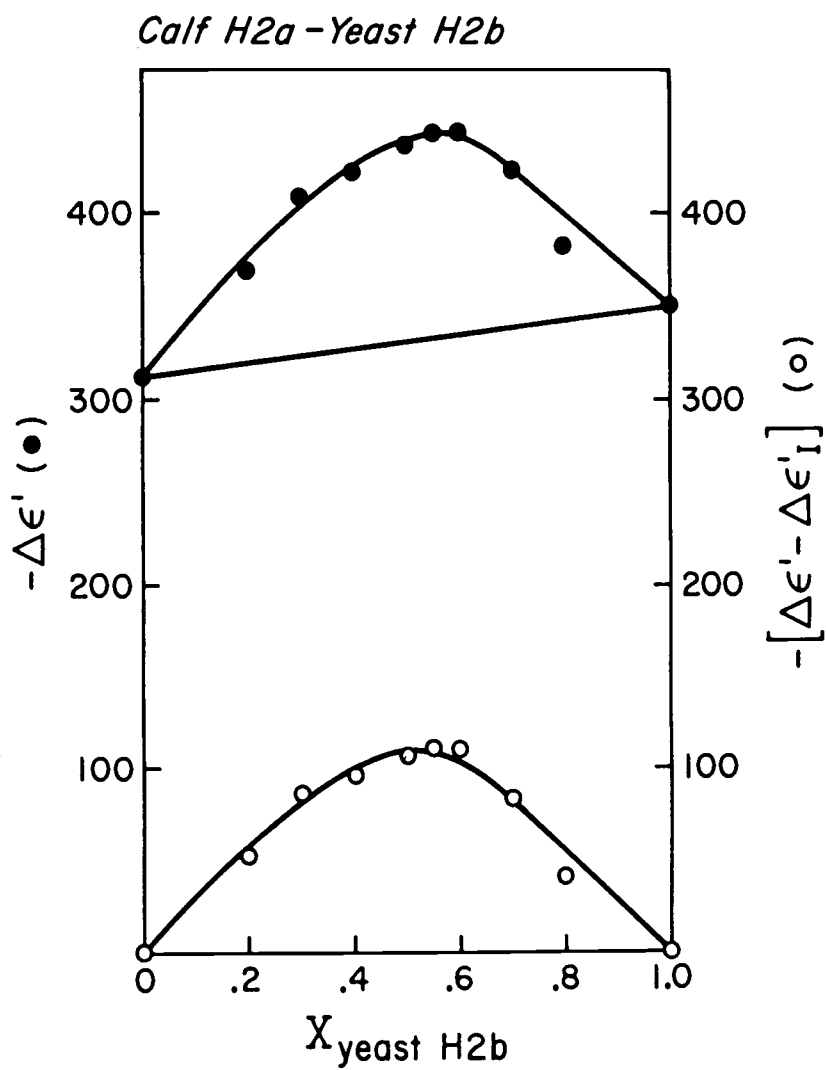Figure 19. CD (200 nm) continuous variation curve of calf H2a plus yeast H2b at 16 mM phosphate, pH 7.0. Co = 2.5 x 10^{-6} M.

continuous variation curves for the interspecies complexes are very similar to those for yeast-yeast (Figure 17) or calf-calf (D'Anna and Isenberg, 1974b) complexes. Table 5 shows the estimated binding constants for the various H2a-H2b complexes, obtained by methods described elsewhere (D'Anna and Isenberg, 1973).

From these values, as was done for pea and calf histones (Spiker and Isenberg, 1978), a difference in the standard free energy to account for such variations in K may be estimated. This value is about 1.2 Kcal/mole. This was about the value estimated for pea and calf histones. Such a small value indicates that the actual histone interacting surface cannot have changed much during the evolutionary divergence of yeast and mammals.

The net result of all of the above studies is that the yeast histones complex with one another in a way which is analogous to that of calf histones (D'Anna and Isenberg, 1974). Furthermore, yeast H2a, calf H2b and calf H2a and yeast H2b interact strongly with one another, as strong, and if anything stronger, than intra-species interactions. However, experimental error limits my conclusions and I can state only that the interactions are not appreciably different and may be the same.

Table 5. Association constants for intra- and inter-species dimer formation of H2a and H2b.

Complex	$KX10^{-6} (M^{-1})$
Yeast H2a-Yeast H2b	3.8
Yeast H2a-Calf H2b	14.0
Calf H2a-Yeast H2b	5.2
Calf H2a-Calf H2b[a]	1.8

[a] D'Anna and Isenberg, 1974.

DISCUSSION

The methods of preparative electrophoresis described here, and illustrated by the isolation of pure yeast inner histones, could be used in part, or in whole, to isolate a wide variety of proteins in pure form from a crude extract. An estimation of whether or not the method will work is easily arrived at by running a series of analytical gels to see which system will give the necessary resolution. Gel electrophoresis, and allied techniques such as isoelectric focusing or isotachophoresis, generally have the highest resolving power of all analytical techniques used to investigate protein heterogeneity. It follows that a refined technique of carrying out preparative gel electrophoresis, such as the one described here, will have a wide applicability in the straightforward isolation of hard-to-separate components.

The preparation of histones was a severe test of the procedure; the preparation of many other proteins will be easier. For one thing, histones have no tryphophan, and their phosphorescence is much lower than proteins that contain tryptophan. Furthermore, the histone bands are closely spaced in the gel. It follows, therefore, that the procedures described above should be generally useful.

Yeast H3, H4, and H2a are all quite close to their calf thymus counterparts in amino acid composition (Table 3 and Figure 13); H2b shows more variation. An identification of the yeast histones may be

made on the basis of their compositions and this is, in fact, the usual

method for identifying histones. This identification was shown to be

correct by the complexing studies.

Yeast H3 contains no cysteine, and none of the yeast histones

contain methionine. In SDS gels, the migration of each yeast histone

is close to that of the corresponding calf histone, with the order of

migration the same for yeast and calf (Figure 15). However, in acetic

acid-urea gels, the order is different (Figure 7). It is H3, H2b, H2a,

H4 for mammals, but H2b, H2a, H3, H4 for yeast. This is not a

simple shift: H2a and H2b move up and H3 moves down.

Given the fact that studies of plant and animal H2a and H2b have

shown them to be only moderately conserved histones, as described

above, it is somewhat surprising that yeast H2a and H2b (especially

H2a) are so close in amino acid composition and molecular weight

(i. e. SDS gel mobility) to their calf thymus counterparts. However,

two things should be noted in this regard. The first is that the sequence

of Drosophila H2b was found to fit the prediction of a variable

N-terminal and conserved C-terminal region even though the overall

amino acid composition was very similar to that of calf thymus H2b

(Elgin, private communication). The second is that histones are well

known to run anomalously on SDS gels (Hamana and Iwai, 1974). For

example, despite the fact that calf H2b is four residues shorter than

calf H2a, and is smaller in molecular weight, it has a lower mobility

on SDS gels, presumably due to charge effects. Both yeast H2a and H2b are less basic than the corresponding calf histones. Thus, the high degree of similarity in mobility in SDS gels (Figure 15) does not necessarily indicate a high degree of similarity in molecular weights.

There is still the question, given the remarkable degree of similarity that does exist between the yeast and calf inner histones, of why the fractionation procedure of Johns failed to separate yeast H3. One possibility is that the difficulty in preparing yeast H3 is not due to H3 itself, but to the presence of other yeast constituents that bind to it and alter its solubility properties. In this connection it should be noted that, in preparations from wheat germ, there are lipids which bind to H3 and H4 and alter their chromatographic behavior (Fazal and Cole, 1977). Another possibility is simply that whatever differences do exist in composition and sequence between yeast and calf H3 are enough in themselves to alter the solubility properties of this yeast histone.

I have shown that the yeast inner histones interact with the same overall pair-wise interaction pattern that has been previously demonstrated for animal (D'Anna and Isenberg, 1974d) and plant (Spiker and Isenberg, 1977) histones. In particular, I have shown the existence of the three strong complexes, H2a-H2b, H2b-H4 and H3-H4. These are the same ones found in calf and pea histones and are considered to be vital in maintaining the nucleosome (Van Holde and

Isenberg, 1975; Isenberg, 1977, 1978). They have now been found in

three eucaryotic kingdoms. This is a strong verification that they

are vital to nucleosomal structure and functioning, since the nucleosome

has been found in all four eucaryotic kingdoms.

Figures 18 and 19 demonstrate that strong interspecies com-

plexes between calf and yeast histones exist. Within experimental

error, these appear to have, at least approximately, the same strength

as intraspecies interactions. Interkingdom complexes between calf

and pea histones have previously been reported (Spiker and Isenberg,

1978). Estimations of binding constants for such interkingdom com-

plexes imply that the interacting surfaces between the histones are

highly conserved. This is because an equilibrium constant goes

exponentially with the free energy of interaction. Consequently even

small changes in an interacting surface, which lead to changes of the

order of 1-2 Kcal/mole in the free energy, will change the binding

constant by an order of magnitude. Yet Table 5 shows that the binding

between histones of different species is of the same order of magnitude

as binding between histones of the same species. (The deviations

shown in the table are to be ascribed to experimental error; no claim

is made that these differences are real. Even if part of the difference

is real, it still represents only minor changes in the binding surface.)

In summary, the complexing pattern of the inner histones of S.

cerevisiae is the same as that found previously for animal and plant

histones. The pattern is conserved over three eucaryotic kingdoms (fungi, plants, and animals). In addition, the binding surfaces themselves are highly conserved. This high degree of conservation strongly supports the idea that specific inner histone complexes are essential to nucleosomal structure and function.

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