The Functioning of Histidine Residues of Sarcoplasmic Reticulum in Ca^{2+} Transport and Related Activities†

Byung P. Yu,*‡ E. J. Masoro,‡ and H. A. Bertrand

ABSTRACT: Irradiation, in the presence of Rose Bengal, of a suspension of sarcoplasmic reticulum vesicles isolated from rat skeletal muscle rapidly causes a loss of the following four functional activities of these membranes: (a) ATP-dependent, oxalate-promoted Ca^{2+} transport; (b) the ATPase energizing this Ca^{2+} transport; (c) the steady-state level of the phosphorylated intermediate formed during the ATPase reaction; and (d) ATP-dependent Ca^{2+} binding. Although irradiation in the presence of Methylene Blue had the same qualitative actions it was quantitatively less effective than Rose Bengal. Experiments in which the pH of the irradiation was systematically modified and in which the destruction of amino acid residues of sarcoplasmic reticulum proteins was monitored and correlated with loss of function indicate that it is the photooxidation of histidine residues that is responsible for the loss of sarcoplasmic reticulum functional activity during photoirradiation in the presence of Rose Bengal. Kinetic analysis of the inhibition of these functional activities indicates that the ATPase and the formation of its phosphorylated intermediate as well as the ATP-dependent Ca^{2+} binding activity involve the same active functional group, a histidine residue. This analysis reveals, however, that the Ca^{2+} transport function is more sensitive to photoinactivation than the other three processes and that its inactivation may involve other components of the system than this histidine residue. Evidence is presented to establish that the irradiation procedure in the presence of Rose Bengal does not induce peroxidation of the lipids of the sarcoplasmic reticulum membrane.

In recent years (Duncan and Bowler, 1969; Feldman and Butler, 1972), investigators have used irradiation with visible light in the presence of photosensitizable dyes to selectively oxidize amino acid residues in proteins. The data obtained from these studies have made evident correlative relationships between the functional activity of enzymes and other biologically active peptides and particular amino acid residues contained in the proteins in question (Freude, 1968; Ray and Koshland, 1962). In an earlier paper (Yu *et al.*, 1967), we utilized this technique to explore the function of sarcoplasmic reticulum (SR)[1] membranes and found that the Ca^{2+} transport function and its associated ATPase were inactivated when SR membranes were irradiated with visible light in the presence of Methylene Blue. Moreover, the V_{max} of this ATPase was found to abruptly change at pH 6.5. On the basis of these data, we tentatively concluded that the imidazole ring of a histidine residue of SR protein is involved in both functional activities.

In the present paper, more support for this conclusion is presented. Rose Bengal, which proved to be a more effective dye than Methylene Blue, was the primary catalyst used for the photooxidation work. Moreover, SR functions in addition to Ca^{2+} transport and its associated ATPase were studied, namely the steady-state concentration of the phosphorylated intermediate of this ATPase, and ATP-dependent Ca^{2+} binding by SR membranes. In addition, the changes noted in functional activity were correlated with the extent of destruction of amino acid residues in SR membrane protein.

Experimental Section

Materials. Rose Bengal was purchased from the Fisher Scientific Co. and [γ-^{32}P]ATP from Searle Radiochemical Co. (Chicago, Ill.). All chemicals used for amino acid analysis were obtained from Pierce Chemical Co. Male rats (retired breeders) of the Sprague-Dawley strain were purchased from Charles River Laboratories.

Preparation of SR Membranes. Sarcoplasmic reticulum membranes were prepared from rat skeletal muscle by a previously published procedure (Yu *et al.*, 1968a).

ATPase Assay. The following incubation system for the assay of SR ATPase was used: 40 μmol of Tris-maleate (pH 7.3), 6 μmol of $MgCl_2$, and 6 μmol of ATP for a total volume of 2.0 ml which contained approximately 50 μg of SR protein. The incubation was carried out in the semidark at 37° and the reaction was terminated at the end of 5 min by addition of 0.2 ml of 50% trichloroacetic acid. The P_i liberated was determined as described previously (Yu *et al.*, 1968a). Addition of micromolar concentrations of $CaCl_2$ to the incubation system did not significantly change the ATPase activity but this activity is almost totally suppressed by 0.25 mM EGTA. Clearly, the components of the incubation system contained sufficient contaminating Ca^{2+} to fully activate the SR (Ca^{2+},Mg^{2+})-ATPase.

Ca Uptake by SR. The method of Yu *et al.* (1968a) was used for the measurement of Ca^{2+} uptake by the SR; this method utilizes initial concentrations of ATP and oxalate of 1 and 6 mM, respectively, and involves measuring the retention of ^{45}Ca by the SR after separation from the incubation by Millipore filtration.

ATP-Dependent Ca^{2+} Binding by SR. To measure ATP-dependent Ca^{2+} binding, the technique of Ohnishi and Ebashi (1963) was used.

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[1] Abbreviation used is: SR, sarcoplasmic reticulum.

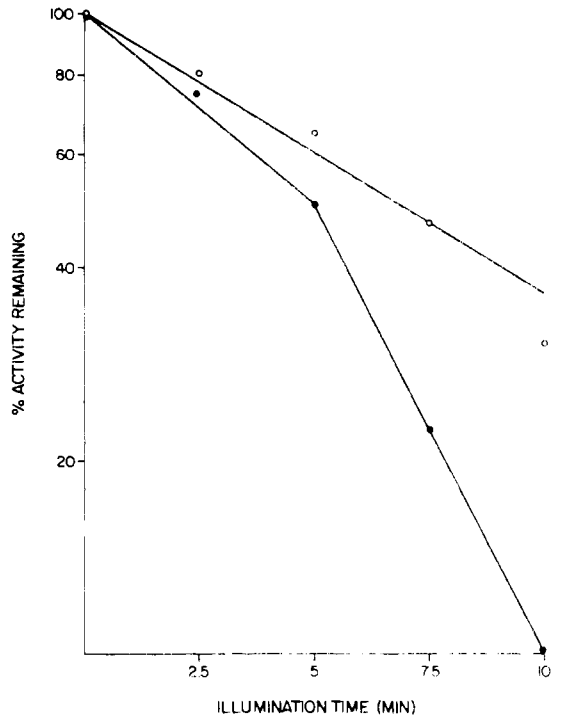

FIGURE 1: Effect of photooxidation on SR ATPase and Ca^{2+} transport activities. The samples were irradiated for 10 min. Aliquots were taken at timed intervals for assay of the activities (ATPase [○] and Ca^{2+} uptake [●]). The concentration of Rose Bengal was 5×10^{-6} M; average values from ten experiments. The specific activity of ATPase at zero time was 140 ± 10.2 μg of P_i/mg of SR per min. Ca^{2+} uptake activity was 3.5 ± 0.4 μmol of Ca^{2+}/mg of SR.

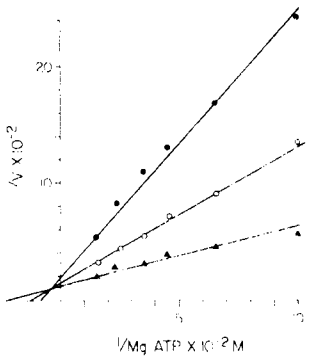

FIGURE 2: Comparison of effects of irradiation in the presence of Rose Bengal and of Methylene Blue on ATPase activity. In these experiments, the irradiation was carried out in the usual medium but in addition containing Mg and ATP in the concentrations noted in the figure. Aliquots were removed from the irradiating flask after 10 min of irradiation for assay for ATPase activity: control (▲), 5×10^{-7} M Rose Bengal (●), and 5×10^{-5} M Methylene Blue (○); average values from six experiments.

Lipid Peroxidation of SR Membrane. Detection of peroxidized lipid was carried out with thiobarbituric acid test as described by Zalkin and Tappel (1960).

Steady-State Concentration of the SR Phosphorylated Intermediate. The method described by Meissner and Fleischer (1971) was used to measure the steady-state concentration of phosphorylated intermediate in SR with the exception that the radioassay of ^{32}P was made by the method of Patterson and Greene (1965).

Photoirradiation of SR Membranes. Irradiation was carried out in a dark cold room (4°) equipped with circulating fan. The basic protocol was as follows. SR membranes suspended in 50-ml erlenmeyer flasks in 20 ml of a medium containing Tris buffer (20 mM, pH 7.2) in 0.6 M sucrose were irradiated with a 75-W incandescent lamp at a distance of 30 cm from the bottom of the flask. The concentration of SR membrane protein was 1-2 mg/ml and the concentration of Rose Bengal ranged from 5×10^{-5} to 5×10^{-8} M depending on the conditions desired. Aliquots were taken at various time intervals for assay of the SR functional activities to be tested and for amino acid analysis. During irradiation the volume of the SR suspension ranged between 20 and 15 ml. All experiments were carried out with proper controls which consisted of an SR suspension irradiated in a medium flask containing no dye and an SR suspension in a dye containing medium without irradiation. All biological assays carried out subsequently were executed in the semidark.

Amino Acid Analysis. An automated Phoenix analyzer Model M-7800 fitted with a short column containing Phoenix-Spherix resin type XX8-10-0 and a long column containing Phoenix-Spherix resin XX8-60-0 was used for the analysis of most of the amino acids. The amount of each amino acid was estimated on the basis of a factor obtained from an internal reference standard (Ray and Koshland, 1962). All procedures required in preparing the SR sample for amino acid analysis were carried out in the semidark and usually prior to these procedures the sample was extracted with 95% ethanol to remove excess Rose Bengal or Methylene Blue (Ray, 1967). Either of the following procedures was used to prepare the sample for the amino acid analyzer.

ACID HYDROLYSIS. To 1.5 mg of SR membrane protein is added 1.0 ml of 6 N HCl and the material is sealed in evacuated hydrolysis tubes which are then heated at 105° for 18 hr. The sample is evaporated to dryness and dissolved in 0.2 M citrate buffer (pH 2.2) for analysis. Neutral and acidic amino acids as well as histidine, cysteic acid, and methionine sulfone were assayed in this manner.

BASIC HYDROLYSIS. To 1.5 mg of SR membrane protein is added 1.0 ml of 3.6 N NaOH and the material is heated in evacuated, sealed tubes. After 20-hr heating at 105°, the samples were cooled, acidified to pH 1.5 with 6 N HCl, evaporated to dryness, and dissolved in 0.2 M citrate buffer for immediate analysis of amino acid composition. Tyrosine, tryptophan, and methionine sulfoxide (the latter being produced by the photooxidation of methionine) were assayed in this manner.

To convert the SR protein cystine (or cysteine) and methionine residues to cysteic acid and methionine sulfone, respectively, the protein is subjected to performic acid oxidation by the method of Moore (1963).

In addition to assays with the amino acid analyzer, tryptophan content of the protein was also measured spectrophotometrically by the method of Spande and Witkop (1967). Moreover, the sulfhydryl content of the SR protein was measured by a modification of the method of Grassetti and Murray (1967) in which the SR membranes are completely solubilized by 0.3% sodium dodecyl sulfate prior to assay; presumably this is a measure of the cysteine content of the protein.

Results

Effect of Photooxidation on SR Ca^{2+} Transport and ATPase. In agreement with data reported previously with photoactivated Methylene Blue (Yu *et al.*, 1967), photoactivated Rose Bengal rapidly causes inactivation of SR Ca^{2+}

TABLE I: Effect of Irradiation on Amino Acid Destruction of SR Membrane Protein.[a]

	Time (min)				
	0	2.5	5.0	7.5	10
Histidine	13.0	11.7	10.1	9.5	9.0
Methionine	11.6	11.6	11.3	11.0	10.5
Tryptophan	10.5	10.5	10.3	10.1	9.6
Tyrosine	12.5	12.5	12.5	12.4	12.3
Sulfhydryl	14.0	14.0	14.0	14.0	13.9

[a] Irradiation was carried out under the same conditions described in the Experimental Section. The data are average values from 12 experiments. In the case of ten experiments the Rose Bengal concentration was 5×10^{-6} M and in the case of two experiments the concentration was 5×10^{-5} M. Each amino acid content was expressed as moles of amino acid residue/10^5 g of SR protein.

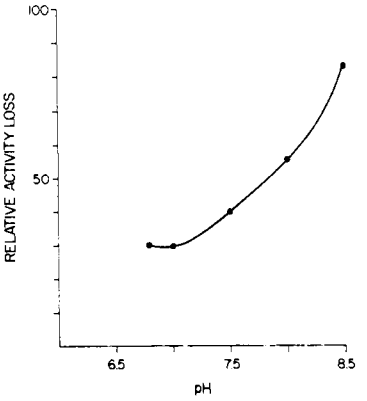

FIGURE 3: Relative loss of ATPase activity by irradiation in the presence of Rose Bengal (5×10^{-6} M) at various pH values. SR membranes were suspended in a flask containing 20 mM Tris-maleate and adjusted to the desired pH with either addition of HCl or Tris base. Changes in the final volume were negligible. Photoirradiation was terminated after 15 min; average values from four experiments.

transport and ATPase activities (Figure 1). Moreover, photoillumination has a much greater inhibitory effect when Rose Bengal is present at 5×10^{-7} M than when Methylene Blue is present at a 100 times greater concentration at all Mg-ATP concentrations studied (Figure 2). Also the inhibition of Ca^{2+} transport by photoactivated Rose Bengal is greater at any time interval studied than is the inhibition of the ATPase activity (Figure 1). The K constant of Ray and Koshland (1961), which was developed for the kinetic analysis of the relation between amino acid destruction by photoirradiation and enzymatic activity, is 0.106 for the ATPase and 0.190 for Ca^{2+} transport (based on the initial slope). The relative inhibition of Ca^{2+} transport compared to that of the ATPase shows a similar pattern with photoactivated Methylene Blue (data not presented).

The phosphorylation of SR protein is reported to be an intermediate step in the SR ATPase reactions. The steady-state concentration of this intermediate can be assayed as described in the Experimental Section. Irradiation in the presence of Rose Bengal caused a loss in the steady-state concentration of the phosphorylated intermediate in SR with a kinetic characteristic similar to that shown in Figure 1 for ATPase inactivation. By 10 min of irradiation the concentration of intermediate fell to 40% of control value. The K constant of Ray and Koshland (1961) for this inactivation process is 0.093. Martonosi *et al.* (1972) reported an initial rise in steady-state concentration of phosphorylated intermediate with irradiation in the presence of Rose Bengal in SR membrane containing low steady-state concentration of it, but in agreement with our results no such increase was obtained in SR membrane containi g high concentrations of the intermediate.

Effect of Photooxidation on ATP-Dependent Ca^{2+} Binding by SR. The effect of photoactivated Rose Bengal on the ATP-dependent Ca^{2+} binding activity of SR as defined by Ohnishi and Ebashi (1963) was measured. Irradiation resulted in a progressive loss of Ca^{2+} binding capacity, with a kinetic characteristic similar to that shown in Figure 1 for ATPase inactivation, with a 55% loss occurring by 10 min of irradiation. The K constant of Ray and Koshland (1961) is 0.092 for this process. Moreover, the initial rate of the Ca^{2+} binding process is decreased by this photooxidation procedure—13 nmol/sec per mg of SR prior to irradiation and 6.9 nmol/sec per mg of SR after 10 min of irradiation (average values from two experiments).

pH Sensitivity of the SR ATPase to Photooxidation. The photooxidative destruction of histidine, unlike other amino acids, is sensitive to pH; presumably it is less inactivated by this irradiation when its imidazole ring is protonated (Freude, 1968; Irie, 1970; Takahashi, 1970; Westhead, 1965). Therefore, the effect of the pH of the medium in which the SR is suspended during irradiation was varied from 6.5 to 8.5 by appropriate mixtures of Tris base and HCl. The pH after irradiation was found to be within 0.2 unit of the initial pH. To rule out any effect of pH *per se*, appropriate nonirradiated controls were run. The results are plotted in Figure 3 and indicate a more rapid loss in ATPase activity when irradiation was carried out at higher pH values. The shape of this curve is compatible with the ionization characteristics of the imidazole ring of histidine residues.

Destruction of Amino Acid Residues in SR Membrane Protein by Photooxidation. For amino acid residues other than those listed in Table I, photooxidation at a level that causes the loss of SR function did not cause destruction of amino acid residues. In contrast, there was destruction of the histidine, methionine, tryptophan, tyrosine, and presumably cysteine residues (Table I). Moreover, the most sensitive residue was histidine. Irradiation for as short a period as 5 min in the presence of 5×10^{-6} M Rose Bengal caused as much as 20% destruction of histidine residues without destroying any other amino acid residue; it should be noted that at this time interval of irradiation 30% of the SR ATPase activity is lost.

Possible Peroxidation of SR Lipids by the Photooxidation Procedure. Since SR ATPase was shown to be a lipid-requiring system (Martonosi *et al.*, 1968; Yu *et al.*, 1968b), alteration of its lipid structure (*e.g.*, by peroxidation of fatty acid moieties by photooxidation) could be the primary cause for the inactivation of the Ca^{2+} transport and ATPase activities. Therefore, an attempt was made to learn if the photooxidation procedure used in these studies caused SR lipid to undergo peroxidation.

Several methods (suggested by Dr. A. L. Tappel, personal communication) were used to determine the extent of lipid peroxidation; all techniques failed to detect such an event in irradiated SR; *e.g.*, after an extended 20-min irra-

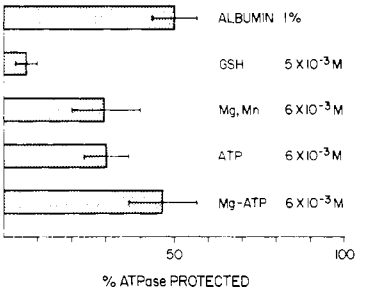

FIGURE 4: Protective action of various agents on the response of SR ATPase to irradiation in the presence of Rose Bengal; average values from four experiments. The irradiation conditions were those described in the Experimental Section. Each experiment was carried out by irradiating SR in the presence of 5×10^{-6} M Rose Bengal with varying concentrations of each protective agent. The results presented graphically are for the concentration at which maximum protection was obtained. After 10-min irradiation, aliquots were taken for the determination of ATPase activity. The specific activities for the nonirradiated control and the control with no protective agent were 137 ± 9.5 and 38 ± 8.2, respectively. The Y axis expresses as 100% that ATPase activity occurring without photoirradiation and 0% that ATPase activity occurring with photoirradiation when no protective agent is added.

diation, there is no evidence of irradiation-induced peroxidation on the basis of the thiobarbituric acid test (Zalkin and Tappel, 1960). Martonosi *et al.* (1972) also found no evidence of lipid peroxidation due to photooxidation.

Compounds Protecting SR ATPase from Inactivation by Photooxidation. Substantial protection from the inactivating effects on SR ATPase of irradiation in the presence of Rose Bengal was afforded by albumin, Mg^{2+}, Mn^{2+}, ATP, and Mg^{2+} + ATP (Figure 4). Conditions were not found, however, in which complete protection occurred. In each case, the protection was dependent on the concentration of the particular compound. For example, the most protection by Mg-ATP ocurrred at concentrations ranging from 6 to 12 mM.

Discussion

The Ca^{2+} transport activity of SR and the ATPase energizing it have been the subject of intensive biochemical investigation in recent years (Meissner and Fleischer, 1971; Hasselbach and Seraydarian, 1966; Martonosi *et al.*, 1971). For example, the use of alkylating reagents provides evidence that a sulfhydryl group of SR protein is involved in these processes (Hasselbach and Seraydarian, 1966). Also the use of lipid-modifying enzymes such as phospholipase C suggests a role for lipids in both Ca^{2+} transport and the ATPase functions (Martonosi *et al.*, 1968; Yu *et al.*, 1968b).

Photooxidation has been used to selectively modify the imidazole rings of histidine residues (Takahashi, 1970; Scoffone *et al.*, 1970), a procedure which has aided in delineating the active site (Takahashi, 1970; Westhead, 1965; Scoffone *et al.*, 1970) of many soluble enzymes. Recently, this approach has been extended to probe membrane bound enzymes as well (Duncan and Bowler, 1969; Feldman and Butler, 1972; Freude, 1968; Grassetti and Murray, 1967; Robinson, 1971). In 1967, Yu *et al.* showed that both the SR Ca^{2+} transport function and the associated ATPase were destroyed by irradiation of an SR suspension in the presence of Methylene Blue and, in a preliminary report in 1971, Yu and Masoro correlated these losses of SR function to destruction of histidine residues. All of these findings have recently been confirmed by Martonosi *et al.* (1972).

In the research presented in the present paper, Rose Bengal, a more sensitive tool than Methylene Blue, was used in the photooxidation studies and a detailed kinetic analysis is presented on the relationship between the destruction of amino acid residues of SR membrane protein and the major functional activities of the SR. The K constants of Ray and Koshland (1961) for the photooxidation induced loss of the ATPase, of the ATP-dependent Ca^{2+} binding, and of the steady-state concentration of the phosphorylated intermediate are 0.106, 0.092, and 0.093, respectively, indicating a similar kinetic behavior on photooxidation for each of these three processes involving the interaction of ATP molecules with SR membranes. Indeed, these results imply that these three activities share a common reaction step probably involving the same active functional group, presumably a histidine residue. Surprisingly, the K constant of Ray and Koshland (1961) for the Ca^{2+} transport process is 0.190. The reason that the Ca^{2+} transport process is more sensitive to photoinactivation than the other three processes is not clear. However, one possible reason, as proposed by Ray and Koshland (1961) in their theoretical consideration of enzymes, could be that Ca^{2+} transport requires another active group in addition to the histidine groups involved in the three other functions and that both active groups are concomitantly destroyed. The failure to find the destruction of amino acid residues other than histidine in the early irradiation period mitigates against this but does not unequivocally deny it since either a different histidine residue or phospholipid may be the second group involved in Ca^{2+} transport.

Nevertheless, the data reported in the present study almost unequivocally establish the role of a histidine residue in the four SR functions studied. Probably the strongest support for this is the fact that at 2.5 min of irradiation in the presence of Rose Bengal, 10% of the SR membrane histidine residues are destroyed and no other amino acid residue is affected. Since each of the four SR functions is significantly inhibited by this 2.5 min of irradiation, only the histidine residue of the protein bears any relationship to the loss of these functions. Also, the relationship between photoinactivation of these functions and the pH at which the irradiation took place supports this involvement of histidine residues and not of other residues. In addition to the data presented in this paper, our earlier study (Yu *et al.*, 1967) on the relationship of pH to the V_{max} of the SR ATPase strongly implicates the involvement of histidine residue in this enzymatic event. The studies by MacLennan (1970) on the effect of pH on the purified SR ATPase are also consistent with a histidine residue involvement. Carvalho (1972) and Meissner (1973) reported a similar relationship between SR ATP-dependent Ca^{2+} binding and pH. The research of Sreter (1969) found a relationship between pH and SR Ca^{2+} uptake which is also consistent with the functioning of histidine residues in this process.

Although a histidine residue or residues is or are almost certainly involved in these SR functions, the molecular nature of this functional role is far from clear. The competitive nature of photoinactivation (Figure 2) suggests that a histidine residue may be at the active site as does the protective action of Mg-ATP. However, the exploration of molecular mechanisms involving the histidine residue or residues is at such a preliminary stage that the hypothesizing of a possible model of action is probably premature; nevertheless, the hypothesis for the molecular mechanism of histidine residue involvement in the (Na^+,K^+)-ATPase proposed by Robinson (1971) is an attractive one on which to

base further experimentation in this field.

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The Role of the Arginine-Rich Histones in the Maintenance of DNA Conformation in Chromatin†

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ABSTRACT: The selective extraction of all histone proteins, except the arginine-rich histones III and IV, from calf thymus chromatin, is accomplished by a procedure which has been shown not to allow the redistribution of histones between different sites along the DNA during extraction. The resulting arginine-rich nucleohistone has been compared to native chromatin and a reconstituted Ca^{2+} nucleohistone IV complex using circular dichroism spectroscopy. In the presence of 2.5 mM Ca^{2+} these three preparations display almost identical DNA circular dichroism spectra. The role of histone IV and Ca^{2+} in the maintenance of DNA conformation in chromatin is discussed. Theoretical calculations are presented which interpret the circular dichroism of chromatin and arginine-rich nucleohistone in terms of a superhelical tertiary structure for the DNA component of these systems.

Chromatin, the interphase chromosomal material from eukaryote cells, is a complex macromolecular association of DNA and protein. The proteins associated with this chromatin complex may be divided into two groups: non-histone chromosomal proteins and histone chromosomal proteins. Although evidence has been presented for the involvement of both histone (see Wilhelm *et al.,* 1971) and non-histone (see Spelsberg *et al.,* 1972) chromosomal proteins in template restriction, the precise role of these chromosomal protein classes in genetic regulation remains uncertain. It is clear, however, that the histone chromosomal proteins are, in some way, responsible for the structural integrity of chromatin. The fiber X-ray diffraction pattern (Garrett, 1968; Richards and Pardon, 1970), circular dichroism spectrum (Shih and Fasman, 1970; Simpson and Sober, 1970; Wilhelm *et al.,* 1970; Henson and Walker, 1971; Permogorov *et al.,* 1970; Wagner and Spelsberg, 1971), hydrodynamic

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