Brunner, R., Röhr, M., and Zinner, M. Z. (1968), *Physiol. Chem. 349,* 95.
Demain, A. L. (1966), in Biosynthesis of Antibiotics, Vol. I, Snell, J. F., Ed., New York, N.Y., Academic Press, p 29.
Fawcett, P. A., Usher, J. J., and Abraham, E. P. (1975), in The Proceedings of the Second International Symposium on the Genetics of Industrial Microorganisms, Macdonald, K. D., Ed., August 1974, Sheffield, England, Academic Press.
Flynn, E. H., McCormick, M. H., Stamper, M. C., De Valeria, H. and Godzeski, C. W. (1962) *J. Am. Chem. Soc. 84,* 4594.
Gatenbeck, S., and Brunsberg, V. (1968), *Acta Chem. Scand. 22,* 1059.
Goulden, S. A., and Chattaway, F. W. (1968), *Biochem. J. 110,* 55p.
Lemke, P. A., and Nash, C. H. (1972), *Can. J. Microbiol. 18,* 255.
Loder, P. B., and Abraham, E. P. (1971), *Biochem. J. 123,* 471.
Meister, A., Fraser, P. E., and Tice, S. V. (1954), *J. Biol. Chem. 206,* 561.
Newton, G. G. F., and Abraham, E. P. (1953), *Nature (London) 172,* 395.
Smith, B., Warren, S. C., Newton, G. G. F., and Abraham, E. P. (1967), *Biochem. J. 103,* 877.
Somerson, N. L., Demain, A. L., and Nunheimer, T. D. (1961), *Arch. Biochem. Biophys., 93,* 238.
Spencer, B. (1968), *Biochem. Biophys. Res. Commun. 31,* 170.

Chromatin-Bound Protease: Degradation of Chromosomal Proteins under Chromatin Dissociation Conditions†

Donald B. Carter and Chi-Bom Chae*

ABSTRACT: A chromatin-bound protease, active in 2 *M* NaCl–5 *M* urea or 5 *M* urea alone, was demonstrated in rat liver, thymus, kidney, testes, brain, rabbit bone marrow, chicken reticulocyte, and Ehrlich ascites chromatin. Chicken erythrocyte chromatin did not possess any detectable proteolytic activity in salt and urea. The proteolytic activity of rat liver chromatin in salt and urea was found to be independent of the methods of chromatin preparation. The protease can be inhibited by the serine specific reagents phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate and the alkylating reagent, carbobenzoxyphenylalanine chloromethyl ketone, in the presence of organic solvents at 1 m*M* concentration. The inhibitions of chromatin-bound protease in rat liver by these compounds are irreversible. On the other hand, carbobenzoxyphenylalanine and *p*-nitrophenyl acetate were shown to be reversible inhibitors of rat liver chromatin-bound protease. The application of these inhibitors during the dissociation of chromatin by salt and urea may be useful to researchers interested in purifying various chromosomal proteins or to those researchers doing reconstitution studies with labile chromatins.

It has been shown previously that chromatin from calf thymus and rat liver tissue contains a proteolytic activity which degrades histones as well as non-histone proteins (Furlan and Jericijo, 1967a,b; Furlan et al., 1968; Bartley and Chalkley, 1970; Garrels et al., 1972; Kurecki and Toczko, 1972, 1974; Chae and Carter, 1974). Furlan and coinvestigators and Kurecki and Toczko have established that the calf thymus chromatin-bound proteolytic activity resides in a protein which has a molecular weight of between 16000 and 24000, a pH optimum of 7.8–8.5, and which is insensitive to thiol-blocking and chelating reagents. Furlan and Jericijo (1967b) report that the calf thymus protease is active in the presence of high salt concentrations (>1 *M* NaCl). Chong et al. (1974) have isolated a high molecular weight protease from rat liver chromatin prepared from whole tissue which is sensitive to sulfhydryl and chelating reagents and is inactive in salt concentrations greater than 1 *M* NaCl. These data suggest that a chromatin-bound protease may be a normal constituent of animal chromatin.

That a protease does exist in rat liver chromatin which is active in the presence of 2 *M* NaCl–5 *M* urea (pH 6–8) was reported by Chae and Carter (1974). These observations are significant for those investigators interested in studying the structure and reconstitution of chromatin, since chromatin generally has been dissociated in high salt and urea (Bekhor et al., 1969; Huang and Huang, 1969; Gilmour and Paul, 1969; Stein et al., 1972). Other investigators have dissociated chromatin in 2 *M* NaCl–5 *M* urea or 3 *M* NaCl in order to fractionate non-histone proteins (Gilmour and Paul, 1970; MacGillivray et al., 1972; Richter and Sekeris, 1972; van den Broek et al., 1973).

This investigation characterizes the chromatin-bound proteolytic activity of rat liver in the presence of denaturing solvents commonly used for dissociation of chromatin and establishes techniques for inhibition of the protease. Proteolytic activities of chromatin from tissues other than rat liver are also reported.

† From the Department of Biochemistry and Nutrition, University of North Carolina, Chapel Hill, North Carolina 27514. *Received July 25, 1975.* Supported by U.S. Public Health Service General Research Support Award 5 S01-FR-05406, Grant (GM 21846-01), from the National Institute of General Medical Sciences, National Institutes of Health, and Grant (HD05277) awarded to Dr. J. L. Irvin from the National Institute of Child Health and Human Development, National Institutes of Health.

Experimental Section

Materials

Rat brain, liver, kidney, thymus, and testes were obtained from 2 to 3 month old Sprague Dawley rats immediately after decapitation and placed in ice-cold 0.25 *M* sucrose–3 m*M* $MgCl_2$–10 m*M* potassium phosphate (pH 5.8). Rabbit bone marrow was obtained from Pel-Freeze Biologicals, Inc.; chicken erythrocytes were obtained from Leghorn chickens by decapitation and collecting blood in a beaker containing ice-cold 0.15 *M* NaCl–0.015 *M* sodium citrate. Chicken reticulocytes were obtained by the same method from Leghorn chickens made anemic by repeated injections of phenylhydrazine.

Carbobenzoxyphenylalanine chloromethyl ketone, phenylmethanesulfonyl fluoride, tosyllysine chloromethyl ketone, tosylphenylalanine chloromethyl ketone, diisopropyl fluorophosphate, and carbobenzoxyphenylalanine were obtained from Sigma Chemical Co., Saint Louis, Mo.; [^{3}H]acetic anhydride, 400 Ci/mol, was obtained from Schwarz/Mann, Rockville, Md.; *p*-nitrophenyl acetate was the kind gift of Dr. M. Caswell; dimethyl sulfoxide, 2-propanol, *p*-dioxane (all 99 mol % pure), EDTA, sodium dodecyl sulfate, and sucrose (all Fisher certified ACS grade) were obtained from the Fisher Chemical Co., Pittsburgh, Pa.

Methods

Isolation of Chromatin. Chromatin was prepared by five different methods. The predominant method of preparation resulted in what is labeled chromatin A. The alternate methods of preparation were variants of the method of chromatin A except for the method which produced chromatin E.

Purification of Nuclei. Nuclei were prepared by centrifugation through 2.3 *M* sucrose and subsequent washings in 1% Triton X-100 containing 0.25 *M* sucrose–3 m*M* $MgCl_2$–10 m*M* potassium phosphate (pH 6.5) as described by Smith and Chae (1973). All steps were carried out at 4°C.

Chromatin A. Nuclei were washed by homogenization in 0.075 *M* NaCl–0.024 *M* EDTA (pH 7.0). The nuclear pellet was washed in 0.3 *M* NaCl (pH 6.0) and 5 m*M* potassium phosphate (pH 6.5) as described by Smith and Chae (1973). The final chromatin was suspended in cold water (pH 8.0) and sheared at 50 V for 90 sec in a Virtis homogenizer.

Chromatin B. The nuclei were lysed in cold water (pH 8.0) after washing in 0.075 *M* NaCl–0.024 *M* EDTA.

Chromatin C. The nuclei washed with 0.075 *M* NaCl–0.024 *M* EDTA were washed twice in 3 volumes (v/w of original tissue) of 10 m*M* Tris (pH 8.0) and suspended in cold water (pH 8.0).

Chromatin D. The nuclei washed with 0.075 *M* NaCl–0.024 *M* EDTA were washed successively (twice each) with three volumes of 50 m*M* Tris (pH 8.0) and three volumes of 10 m*M* Tris (pH 8.0). The final pellet was suspended in cold water as described by Huang and Huang (1969).

Chromatin E. Chromatin was prepared from whole tissue by the method of Elgin and Bonner (1970).

Labeled Substrate. Lysine-rich histones from calf thymus were prepared as described by Johns (1964). Labeling of a 1:1 mixture of F1 and F2B histones was carried out by a modification of the method of Hillie et al. (1970). Incubation of 2 mCi of tritium-labeled acetic anhydride with 100 mg of F1–F2B mixture in 2 ml of 10 m*M* potassium phosphate (pH 7.0) for 2 hr at 4°C was followed by exhaustive dialysis against 2 *M* NaCl–5 *M* urea–10 m*M* potassium phosphate (pH 7.0) at 4°C. The specific radioactivity of the labeled histones was 58000 cpm/mg. Radioactivity was determined in a scintillation fluid consisting of 2.5 g of 2,5-diphenyloxazole, 0.15 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene, 500 ml of Triton X-100, and 500 ml of toluene. Label was incorporated into F2B three times more effectively than into F1.

Disc Gel Electrophoresis. Histones were analyzed on 15% polyacrylamide gels (pH 3.2) according to the method of Panyim and Chalkley (1969). Sodium dodecyl sulfate gel electrophoresis was carried out as described before (Chae, 1975).

Proteolytic Assay with Labeled Substrate. Chromatin having a concentration of 2 mg/ml of DNA was diluted to 1 mg/ml of DNA in the desired reaction mixture. The ratio of [^{3}H]histones (F1 + F2B) added to the reaction mixture was 0.1 mg/mg of chromatin DNA with chromatin DNA at 20 A_{260} units. To determine the acid-soluble radioactive peptides released during an incubation period, an equal volume of cold 50% Cl_3CCOOH was added to the reaction mixture, and after 15 min in ice, the acid-insoluble material was removed by centrifugation for 20 min at 20000*g*. An aliquot of the supernatant was counted for radioactivity.

Inhibition Studies. Inhibition of chromatin-bound protease in salt and urea was carried out in the presence of 10% *p*-dioxane, 10% isopropyl alcohol, or 1% dimethyl sulfoxide (Me_2SO) for the inhibitors, phenylmethanesulfonyl fluoride, carbobenzoxyphenylalanine chloromethyl ketone, and *p*-nitrophenyl acetate. The inhibitors, dissolved in any of the three solvents at a concentration of 0.1 *M*, were added to chromatin in water and incubated at 25°C for 10 min. The reacted chromatin was subsequently made 2 *M* NaCl–5 *M* urea–10 m*M* potassium phosphate (pH 7.0) by addition of 3.6 *M* NaCl–9 *M* urea–18 m*M* potassium phosphate (pH 7.0) and incubated 16 hr prior to assay for inhibition of proteolytic activity.

For reversibility studies, the reacted chromatin was dialyzed exhaustively against 2 *M* NaCl–5 *M* urea–10 m*M* potassium phosphate (pH 7.0) with or without 10% *p*-dioxane or 1% Me_2SO before assaying for proteolytic activity. The results of the assay were independent of whether or not *p*-dioxane or Me_2SO were initially present in the dialysate.

For the water-soluble inhibitor carbobenzoxy-L- or -D-phenylalanine, the chromatin in 10 m*M* Tris (pH 8.0) was adjusted to 20 m*M* carbobenzoxy-L- or -D-phenylalanine with 0.1 *M* carbobenzoxy-L- or -D-phenylalanine (pH 8.0) in water. Inhibition with diisopropyl fluorophosphate (Dip-F)[1] was accomplished by adjusting chromatin in 10 m*M* Tris (pH 8.0) to 1 m*M* Dip-F from a stock solution of 0.1 *M* Dip-F in propylene glycol.

Results

Independence of Proteolytic Activity of Chromatin and Method of Preparation. Chromatin isolated by the procedures given in the Experimental Section was examined by sodium dodecyl sulfate gel electrophoresis in order to examine the possibility that extensive washing of chromatin either causes the loss of natural protease inhibitors or alters the pattern of proteolytic degradation of chromatin in the

[1] Abbreviation used is: Dip-F, diisopropyl fluorophosphate.

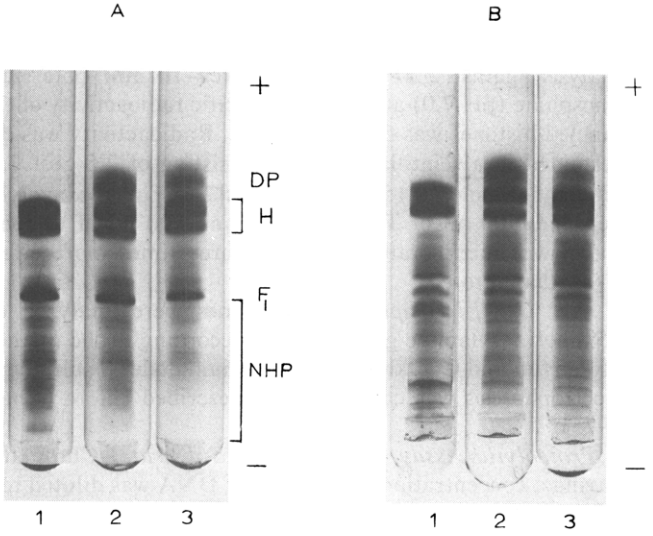

FIGURE 1: Degradation of rat liver chromatin in the presence of 2 *M* NaCl–5 *M* urea or 5 *M* urea alone. Rat liver chromatin, prepared as described in the Experimental Section, was incubated in either 2 *M* NaCl–5 *M* urea–10 m*M* KPO_4 (pH 7.0) or 5 *M* urea–10 m*M* KPO_4 (pH 7.0). (A) Chromatin A dissolved in 1% sodium dodecyl sulfate as a control (1); chromatin A incubated in salt and urea for 16 hr at 4°C (2); chromatin A incubated in urea for 16 hr at 4°C (3). (B) Chromatin E control (1); chromatin E incubated in salt and urea (2); chromatin E incubated in urea alone (3). Migration is from the bottom (−) to the top (+) in 7.5% polyacrylamide gels. DP refers to degraded protein and NHP refers to non-histone proteins. Histones are indicated by the nomenclature of Johns (1964) and the letter H represents the histones F2A1, F2A2, and F3 + F2B in order of decreasing electrophoretic activity.

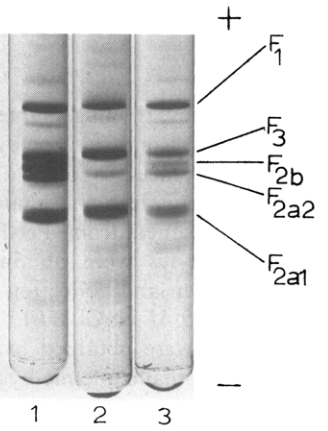

FIGURE 2: Degradation of histones in rat liver chromatin, prepared by various methods, in the presence of 2 *M* NaCl–5 *M* urea and 5 *M* urea alone. Rat liver chromatin prepared as described in the Experimental Section was incubated in either 2 *M* NaCl–5 *M* urea–10 m*M* $K\text{-}PO_4$ (pH 7.0) or 5 *M* urea–10 m*M* $K\text{-}PO_4$ (pH 7.0), 16 hr at 4°C. Histones were extracted with 0.4 *N* H_2SO_4 and dialyzed against 7 *M* urea–5% acetic acid–0.1% β-mercaptoethanol all at 4°C. Electrophoresis was carried out on 15% acid–urea polyacrylamide gels as described in the Experimental Section. Control histones (1); chromatin B incubated in urea alone (2); and salt and urea (3). Migration is from top (+) to bottom (−).

presence of 2 *M* NaCl–5 *M* urea. When chromatin A is prepared from rat liver, the pattern of chromatin autolysis in the presence of 2 *M* NaCl–5 *M* urea and 5 *M* urea is shown in Figure 1. Dissolving chromatin in 1% sodium dodecyl sulfate or heating chromatin to 100°C for 2 min completely inhibits the proteolytic activity. Routinely a band of degraded protein (DP in Figure 1A) migrates with an electrophoretic mobility greater than the smallest of the histones, F2A1, after autolysis. Concurrently, there is a loss of high molecular weight non-histone proteins. Similar patterns of autolysis occur for each method of chromatin preparation studied. The overall electrophoretic pattern of chromosomal proteins for chromatin isolated from rat liver nuclei by different methods is qualitatively the same as shown by the control gel in Figure 1. However, there appears to be more high-molecular weight proteins in evidence when chromatin is prepared from whole tissue (Figure 1B). Histone degradation is also independent of the method of chromatin preparation when chromatin is incubated in 2 *M* NaCl–5 *M* urea or 5 *M* urea (Figure 2). Chromatin prepared from rat liver according to Stein et al. (1975) also possesses proteolytic activity in 3 *M* NaCl–5 *M* urea and 5 *M* urea (not shown here).

Chromatin in 2 *M* NaCl–5 *M* urea (pH 7.0) shows a loss of all histone species except F1 for each chromatin preparation. On the other hand, Bartley and Chalkley (1970) observed that when calf thymus chromatin is incubated 120 hr at 2°C at an ionic strength of 5×10^{-3} *M* at pH 8.0, F1 and F3 were the first histones to degrade. These results were also obtained by Garrels et al. (1972) for rat liver chromatin E incubated in 5 m*M* Tris (pH 8.0) at 37°C for 4 hr. At ionic strengths less than 0.3 *M* NaCl, F1 histone remains bound to DNA in chromatin whereas F1 histone is completely dissociated from chromatin in 2 *M* NaCl–5 *M* urea and may possess a different conformation (less vulnerable to proteolytic attack) than when complexed with DNA in chromatin. These contentions will be discussed later.

Properties of Rat Liver Chromatin Bound Protease. Acetylated histones ([^{3}H]F1 + F2B) are progressively degraded by rat liver chromatin A in 2 *M* NaCl–5 *M* urea for 6 hr at 25°C, and the degradation reaches a constant level, that is, up to 30% of histones are degraded (Figure 3). However, in the case of bone marrow chromatin A, up to 50% of histones are degraded during 24 hr of incubation at 25°C. There is also a concurrent progressive degradation of non-histone proteins as judged by sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns (not shown here).

Chromatin A prepared from other tissues also shows proteolytic activity in the presence of 2 *M* NaCl and 5 *M* urea or 5 *M* urea alone. The tissues examined were rat brain, kidney, thymus, testes, Morris hepatoma, chicken bone marrow, chicken reticulocyte, calf thymus, and Ehrlich ascites carcinoma. The only exception is chicken erythrocyte chromatin, that is, the chromatin shows no apparent proteolytic activity in salt and urea (Figure 3) or 5 *M* urea alone (not shown).

pH Dependence. Chromatin A prepared from rat liver shows a rather broad dependence of proteolytic activity on pH in the presence of 2 *M* NaCl–5 *M* urea. The pH optimum for protease activity lies between pH 8.0 and pH 9.0 as displayed in Figure 4.

Effect of Ionic Strength. Chromatin A isolated from rat liver shows progressively more proteolytic activity as ionic strenth is increased (results of sodium dodecyl sulfate gels not shown). The high molecular weight proteins of chromatin disappear and two major degradation bands accumulate ahead of F2A1, the fastest migrating protein of chromatin in this gel system. The effect of ionic strength on histones is shown in Figure 5. It is clear that there is a differential susceptibility of histones as ionic strength is increased. The progressive disappearance of F1 as ionic strength increases

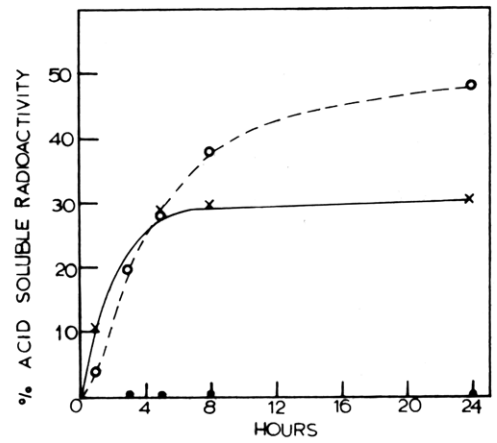

FIGURE 3: Time course degradation of [^{3}H]F1 + F2B histones by chromatin A in the presence of 2 *M* NaCl–5 *M* urea–10 m*M* $K\text{-}PO_4$ (pH 7.0) at 25°C. Rabbit bone marrow (O), rat liver (x), and chicken erythrocyte (●).

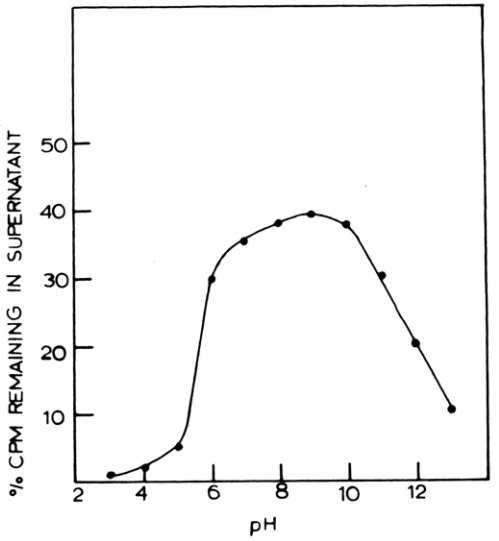

FIGURE 4: The pH dependence of proteolytic activity on rat liver chromatin A in the presence of 2 *M* NaCl–5 *M* urea–10 m*M* sodium acetate (pH 3–6) or 20 m*M* Tris (pH 7–13). Proteolytic activity was assayed by the release of trichloroacetic acid soluble degradation products of [^{3}H]F1 + F2B as described in the Experimental Section. The reactions were carried out with 0.5 mg/ml of chromatin each for 8 hr at 25°C.

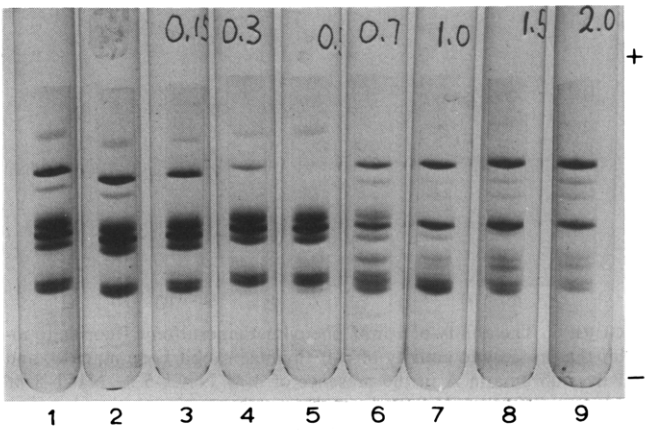

FIGURE 5: The effect of ionic strength on the degradation of histones in rat liver chromatin A. The chromatin was incubated for 2 hr at 37°C in 10 m*M* Tris (pH 8.0) containing the various concentrations of NaCl as follows: control (1); 0.0 *M* (2); 0.15 *M* (3); 0.3 *M* (4); 0.5 *M* (5); 0.7 *M* (6); 1.0 *M* (7); 1.5 *M* (8); 2.0 *M* (9). Migration is from top (+) to bottom (−) on 15% polyacrylamide acid–urea gels.

Table I: Proteolytic Activity of Rat Liver Chromatin A in the Presence of Various Inhibitors.[a]

Inhibitor	Acid Soluble Radioactivity (% of Control)
Control	100
$PhCH_2SO_2F$ (0.1 m*M*)	85
$PhCH_2SO_2F$ (1 m*M*)	0
Z-PheCH_2Cl (1 m*M*)	10
Tos-LysCH_2Cl (1 m*M*)	100
Tos-PheCH_2Cl (1 m*M*)	74
NphOAc (0.1 m*M*)	100
NphOAc (1 m*M*)	90
NphOAc (10 m*M*)	0
Z-L-Phe (1 m*M*)	100
Z-L-Phe (5 m*M*)	85
Z-L-Phe (10 m*M*)	38
Z-L-Phe (20 m*M*)	0
Dip-F (1 m*M*)	0

[a] The proteolytic assay was performed as described in Materials and Methods with [^{3}H]F1 + F2B as substrate. Chromatin was prepared by method A and each sample was incubated 16 hr in 2 *M* NaCl–5 *M* urea–20 m*M* Tris (pH 8.0) at 25°C. $PhCH_2SO_2F$, ZPCK, TPCK, and PNPA were dissolved in *p*-dioxane or Me_2SO, and the final effective concentration of *p*-dioxane in chromatin was 10 or 1% for Me_2SO. Abreviations used are: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Z-PheCH_2Cl, carbobenzoxyphenylalanine chloromethyl ketone; Tos-LysCH_2Cl, tosyllysine chloromethyl ketone; Tos-PheCH_2Cl, tosylphenylalanine chloromethyl ketone; NphOAc, *p*-nitrophenyl acetate; Z-L-Phe, carbobenzoxy-L-phenylalanine.

from 0.1 *M* NaCl to 0.5 *M* NaCl suggests a remarkable interaction between F1 as the substrate and the proteolytic activity endogenous to rat liver chromatin. The maximum rate of F1 degradation occurs at the ionic strength found to release F1 from chromatin (Ohlenbush et al., 1967).

Effect of Various Inhibitors. The presence of reducing agents at relatively high concentrations (0.2 *M* β-mercaptoethanol) does not inhibit the chromatin-bound proteolytic activity in 2 *M* NaCl–5 *M* urea (pH 7.0). Sulfhydryl reagents such as Hg^{2+}, iodoacetamide, and *p*-hydroxymercuribenzoate at 1 m*M* concentration also showed no inhibitory effects on the proteolytic activity of chromatin in 2 *M* NaCl–5 *M* urea (pH 7.0). Sodium bisulfite which has been reported to be an inhibitor of endogenous chromatin proteolytic activity (Panyim and Chalkley, 1969; Garrels et al., 1972) at a concentration of 50 m*M* was found to be only partially effective at low pH and ineffective at higher pH. Sodium bisulfite at 50 m*M* pH 7.0 or 8.0 was found to be ineffective in the protection of histones when chromatin is disassociated in 2 *M* NaCl–5 *M* urea (pH 7.0 or 8.0). Nonhistone chromatin proteins are degraded at pH's 6, 7, and 8 in the presence of 2 *M* NaCl–5 *M* urea–50 m*M* $NaHSO_3$ (results not shown). It is concluded that caution must be observed when sodium bisulfite is used with the intent of inhibiting rat liver or calf thymus endogenous chromatin protease in the presence of salt and/or urea.

The trypsin inhibitor, tosyllysine chloromethyl ketone, at 1 m*M* concentration and the chymotrypsin inhibitor, tosylphenylalanine chloromethyl ketone, also at 1 m*M* concentration are not effective in suppressing chromatin proteolytic activity. However, the water-insoluble compound, carbobenzoxy chloromethyl ketone, at 1 m*M* concentration does inhibit chromatin protease activity (Table I). Phenylmeth-

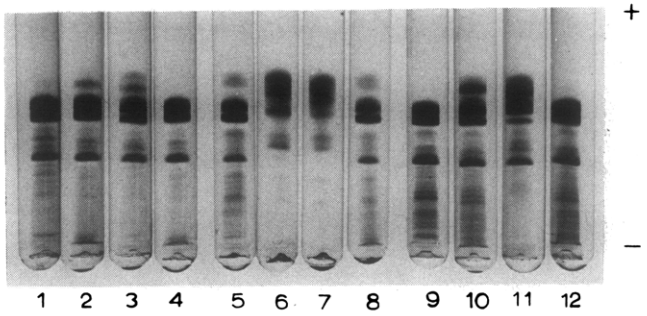

FIGURE 6: The ability of 1 m*M* phenylmethanesulfonyl fluoride to inhibit the proteolytic activity of calf thymus, rabbit bone marrow, and rat liver chromatin A in the presence of 2 *M* NaCl–5 *M* NaCl–5 *M* urea–10 m*M* Tris (pH 8.0). In each case chromatin was incubated at 25°C for 16 hr. Gel 1 is the calf thymus control chromatin A; calf thymus chromatin A in 10% *p*-dioxane–salt and urea (2); salt and urea alone (no *p*-dioxane) (3); salt and urea–10% *p*-dioxane–1 m*M* $PhCH_2SO_2F$ (4). Gel 5 is the rabbit bone marrow control chromatin A; rabbit bone marrow chromatin A in 10% *p*-dioxane–salt and urea (6); salt and urea (7); salt and urea–10% *p*-dioxane–1 m*M* $PhCH_2SO_2F$ (8); gel 9 is the rat liver control chromatin A; rat liver chromatin A in 10% *p*-dioxane–salt and urea (10); salt and urea (11); salt and urea–10% *p*-dioxane–1 m*M* $PhCH_2SO_2F$ (12). Migration is from bottom (−) to top (+) on 7.5% sodium dodecyl sulfate polyacrylamide gels.

anesulfonyl fluoride as previously reported (Nooden et al., 1973) is also effective in blocking the chromatin-bound proteolytic activity. The ability of phenylmethanesulfonyl fluoride to inhibit not only rat liver chromatin proteolysis in salt and urea but calf thymus and rabbit bone marrow chromatin proteolytic activities is demonstrated in Figure 6. The conditions of exposure of chromatin to phenylmethanesulfonyl fluoride in the presence of organic solvents are important in order to achieve complete inhibition. Although phenylmethanesulfonyl fluoride will dissolve sparingly in 2 *M* NaCl–5 *M* urea (pH 8.0), the inhibition obtained is incomplete. Chromatin A, which has been reacted with either phenylmethanesulfonyl fluoride or carbobenzoxyphenylalanine chloromethyl ketone in the presence of 10% *p*-dioxane, and dialyzed exhaustively against 2 *M* NaCl–5 *M* urea (pH 7.0), as described in the Experimental Section, shows no degradation (results not shown), even after the extensive exposure of chromatin to salt and urea. It is concluded that the inhibition of proteolytic activity by phenylmethanesulfonyl fluoride and carbobenzoxyphenylalanine chloromethyl ketone is irreversible. Dip-F at 1 m*M* concentration also is a potent inhibitor of the endogenous chromatin protease activity. The investigation of Dip-F inhibition of hydrolytic enzymes has shown that in every instance the exclusive phosphorylation of a single serine residue per catalytic site without reaction with other residues is concurrent with loss of enzyme activity (Cohen et al., 1967). The phosphorylated serine hydroxyl intermediate hydrolyzes very slowly, thus the inhibition produced by Dip-F is essentially irreversible.

Reversible inhibitors of the chromatin-bound protease in rat liver were also discovered. Carbobenzoxy-L- or -D-phenylalanine are equally effective in the inhibition of chromatin proteolytic activity in salt and urea (Table I). At a concentration 20 m*M*, carbobenzoxy-L- or -D-phenylalanine inhibits the proteolytic activities of rat testes, rat thymus, and calf thymus, but is not effective against rabbit bone marrow chromatin proteolytic activity in 2 *M* NaCl–5 *M* urea (pH 8.0). Chromatin treated with carbobenzoxy-L- or -D-phenylalanine, extensively dialyzed against 2 *M* NaCl–5 *M* urea, and then assayed for proteolytic activity either by polyacrylamide gel electrophoresis or by capacity for hydrolysis of [^{3}H]F1 + F2B retained proteolytic activity in salt and urea. However, carbobenzoxy-L- or -D-phenylalanine apparently alters the extractability of histones from chromatin since 0.4 *N* H_2SO_4 extraction of chromatin which had been treated with 20 m*M* carbobenzoxy-L- or -D-phenylalanine yielded only a small quantity of F1 and no other histones as determined by acid–urea gel electrophoresis (not shown here).

Reversible inhibition of chromatin-bound protease is also achieved with *p*-nitrophenyl acetate. *p*-Nitrophenyl acetate dissolved in Me_2SO and added to chromatin to give a final concentration of 10 m*M* *p*-nitrophenyl acetate in 10% Me_2SO effected complete inhibition of chromatin proteolytic activity of rat liver in the presence of 2 *M* NaCl–5 *M* urea (pH 8.0). However, under the same conditions, *p*-nitrophenol did not produce any inhibition; hence, although some hydrolysis of *p*-nitrophenyl acetate takes place in 2 *M* NaCl–5 *M* urea (pH 8.0) it is the acetate form, *p*-nitrophenyl acetate, which is the inhibitor.

Discussion

Chromatin prepared by any of the tested methods from cells except chicken erythrocyte undergoes extensive proteolytic degradation during dissociation and reconstitution in salt and urea at 4°C. Only two of the published methods of inhibition of the chromatin-bound protease, inhibition by 1 m*M* Dip-F (Furlan and Jericijo, 1968) and inhibition by 1 m*M* phenylmethanesulfonyl fluoride in the presence of an organic solvent (Nooden et al., 1973), are effective in salt and urea. Although others (Bekhor et al., 1973) have attempted to inhibit endogenous chromatin proteolytic activity by dissolution of phenylmethanesulfonyl fluoride in 2 *M* NaCl–5 *M* urea prior to dissociation of chromatin in salt and urea, this technique was found to give only partial protection of chromatin from proteolytic digestion in this laboratory (results not shown). We have established that carbobenzoxyphenylalanine chloromethyl ketone and *p*-nitrophenyl acetate in the presence of organic solvents also provide complete protection of chromatin proteins in rat liver chromatin in the presence of salt and urea. Moreover, carbobenzoxy-L- or -D-phenylalanine, water-soluble compounds, at 20 m*M* concentration effectively inhibit proteolysis of rat liver chromatin in salt and urea. These compounds may be useful in future studies when it is necessary to minimize the degradation of chromatin in salt and/or urea.

The fact that *p*-nitrophenyl acetate and carbobenzoxyphenylalanine chloromethyl ketone, both inhibitors of chymotrypsin, also inhibit the rat liver chromatin-bound protease suggests that the substrate specificity of the enzyme is similar to that of chymotrypsin. Interestingly, tosylphenylalanine chloromethyl ketone, a potent inhibitor of chymotrypsin, does not inhibit the chromatin protease. The design of phenylalanine derivatives would appear to be a productive avenue in any investigation of the substrate specificity of the chromatin protease.

The differential specificity of proteolytic digestion of histones in solutions of increasing ionic strength may be explained in several ways. Since it has been reported that rat liver chromatin contains a high molecular weight protease molecule (Chong et al., 1974) which is active in concentrations of salt less than 1 *M* and concentrations of urea less than 2 *M*, more than one proteolytic activity residing in different molecular species may be endogenous to rat liver

chromatin. The differential degradation of histones in varying salt solutions may express the differential activities of the different protease molecules in varying salt concentrations. One may also expect that the conformation of histone molecules in various salt concentrations and the degree of complexation with DNA will influence the susceptibility of histones to proteolytic attack. The greatly enhanced susceptibility of F1 histone in 0.5 *M* NaCl relative to either higher or lower ionic strengths suggests that a particularly vulnerable structure of F1 occurs at this ionic strength; that one of the putative chromatin-bound proteolytic activities assumes a particularly voracious appetite for F1 in 0.5 *M* NaCl; or that some combination of the two possibilities pertains. Ohlenbush et al. (1967) have reported that the lysine-rich histones are released from calf thymus chromatin in the range 0.4–0.5 *M* NaCl. Thus, one may hypothesize that F1's incipient release from DNA renders F1 most susceptible to the protease or proteases. However, at higher ionic strengths, from 0.7 to 2 *M* NaCl F1 is virtually impervious to proteolytic digestion. Possibly the proteases relinquish their ability to attack F1 because of conformational changes induced in the protease by the higher ionic strengths above 0.7 *M* NaCl.

It is highly unlikely that the protease activity associated with chromatin is derived from nucleoplasm. Chromatin sheared by sonication or by mechanical shearing in a Sorvall Omni mixer was centrifuged through a gradient of glycerol or sucrose, and it was found that the protease activity was still associated with chromatin (not shown here).

References

Bartley, J., and Chalkley, R. (1968), *Biochim. Biophys. Acta 160,* 224.

Bartley, J., and Chalkley, R. (1970), *J. Biol. Chem. 246,* 4286.

Bekhor, I., Kung, G. M., and Bonner, J. (1969), *J. Mol. Biol. 39,* 351.

Burton, K. (1956), *Biochem. J. 62,* 315.

Chae, C.-B. (1975), *Biochemistry 14,* 900.

Chae, C.-B., and Carter, D. B. (1974), *Biochem. Biophys. Res. Commun. 57,* 740.

Chong, M. T., Garrard, W. T., and Bonner, J. (1974), *Biochemistry 13,* 5178.

Cohen, J. A., Osterbaan, R. A., and Berends, F. (1967), *Methods Enzymol. 11,* 686.

Elgin, S. C. R., and Bonner, J. (1970), *Biochemistry 9,* 4440.

Furlan, M., and Jericijo, M. (1967a), *Biochim. Biophys. Acta 147,* 135.

Furlan, M., and Jericijo, M. (1967b), *Biochim. Biophys. Acta 147,* 145.

Furlan, M., Jericijo, M., and Suhar, A. (1968), *Biochim. Biophys. Acta 167,* 154.

Garrels, J. I., Elgin, S. C. R., and Bonner, J. (1972), *Biochem. Biophys. Res. Commun. 46,* 545.

Gilmour, R. S., and Paul, J. (1969), *J. Mol Biol. 40,* 137.

Gilmour, R. S., and Paul, J. (1970), *FEBS Lett. 9,* 242.

Hillie, M. B., Barrett, A. J., Dingle, J. T., and Fell, H. B. (1970), *Exp. Cell Res. 61,* 470.

Huang, R. C. C., and Huang, P. C. (1969), *J. Mol. Biol. 39,* 365.

Johns, E. W. (1964), *Biochem. J. 92,* 55.

Kurecki, T., and Toczko, K. (1972), *Bull. Acad. Pol. Sci. 20,* 543.

Kurecki, T., and Toczko, K. (1974), *Acta Biochim. Pol. 21,* 225.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

MacGillivray, A. J., Cameron, A., Krauze, R. J., Richwood, D., and Paul, J. (1972), *Biochim. Biophys. Acta 277,* 384.

Nooden, L. D., van den Broek, H. W. J., and Sevall, J. S. (1973), *FEBS Lett. 29,* 326.

Ohlenbush, H. H., Olivera, B. M., Tuan, D., and Davidson, N. (1967), *J. Mol. Biol. 25,* 299.

Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130,* 337.

Richter, K. H., and Sekeris, C. E. (1972), *Arch. Biochem. Biophys. 148,* 44.

Smith, M. C., and Chae, C.-B. (1973), *Biochim. Biophys. Acta 317,* 10.

Stein, G., Chaudhuri, S., and Baserga, R. (1972), *J. Biol. Chem. 247,* 3918.

Stein, G. S., Mans, R. J., Gabbay, E. J., Stein, J. L., David, J., and Adawadkan, P. D. (1975), *Biochemistry 14,* 1859.

van den Broek, H. W. J., Nooden, L. D., Sevall, J. S., and Bonner, J. (1973), *Biochemistry 12,* 229.