Jockusch, B. M., & Walker, I. O. (1974) *Eur. J. Biochem. 48*, 417–425.
Keller, W., Muller, U., Eicken, I., Wendel, I., & Zentgraf, H. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 227–244.
Knippers, R., Otto, B., & Bohme, R. (1978) *Nucleic Acids Res. 5*, 2113–2131.
Kornberg, R. (1974) *Science 184*, 868–871.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laemmli, U. K., Cheng, S. M., Adolph, K. W., Paulson, J. R., Brown, J. A., & Baumbach, W. R. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 351–360.
LeStourgeon, W. M., & Rusch, H. P. (1973) *Arch. Biochem. Biophys. 155*, 144–158.
Marks, D. B., Park, W. K., & Borun, T. W. (1973) *J. Biol. Chem. 248*, 5660–5667.
Mittermayer, C., Braun, R., & Rusch, H. P. (1965) *Exp. Cell Res. 38*, 33–41.
Mohberg, J. (1975) in *The Cell Nucleus* (Busch, H., Ed.) Academic Press, New York.
Mohberg, J., & Rusch, H. P. (1969) *Arch. Biochem. Biophys. 134*, 577–589.
Mohberg, J., & Rusch, H. P. (1971) *Exp. Cell Res. 66*, 305–316.
Noll, M., & Kornberg, R. D. (1977) *J. Mol. Biol. 109*, 393–404.
Olins, A., & Olins, D. E. (1974) *Science 183*, 330–332.
Panyim, S., & Chalkley, R. (1971) *J. Biol. Chem. 246*, 7557–7560.
Paulson, J. R., & Laemmli, U. K. (1977) *Cell 12*, 817–828.
Renz, M., Nehls, P., & Hozier, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1879–1883.
Tanphaichitr, N., Moore, K. C., Granner, D. K., & Chalkley, R. (1976) *J. Cell Biol. 69*, 43–50.
Varshavsky, A. J., Bakayev, V. V., & Georgiev, G. P. (1976) *Nucleic Acids Res. 3*, 477–492.
Weintraub, H., & VanLente, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4249–4253.
Woodcock, C. L. E. (1973) *J. Cell Biol. 59*, 368a.

Antibodies Specific for Histone H2b Fragments 1–58 and 63–125 in Antisera to H2b and to the Fragments: Probes for Histone Evolution†

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ABSTRACT: Rabbit antibodies induced by H2b–RNA complexes reacted with intact H2b and with fragments 1–58 and 63–125 produced by cleavage of H2b with cyanogen bromide. Complement fixation titers for a typical serum were 5400 with H2b, 4300 with 63–125, and 2300 with 1–58. Affinity chromatography revealed that 0.9 as much anti-H2b antibody was isolated with (63–125)–Sepharose as with H2b–Sepharose, whereas only 0.1 as much was isolated with (1–58)–Sepharose. The antibodies isolated from the fragment–Sepharose columns reacted with the corresponding fragment and intact H2b in both complement fixation assays and in a solid-phase radioimmunoassay. The distribution of anti-H2b antibodies primarily to the 63–125 region may explain the inability of anti-H2b antisera to distinguish between *Drosophila* and calf H2b, which differ mainly in the amino-terminal portion of the molecule. Antibodies were then induced by mixtures of RNA with the separated fragments. Early anti-(1–58) antisera gave complement fixation reactions with H2b but stronger reactions with the fragment; late sera fixed complement only with the fragment. Both early and late anti-(63–125) antisera reacted with H2b better than with the immunogen 63–125 itself. Like anti-H2b, the anti-(63–125) antiserum measured little or no difference between *Drosophila* and calf H2b. Anti-(1–58) antiserum detected a large difference between these histones. Cross-reactions of H2b from other species with the antifragment antisera were also consistent with degrees of sequence homology in the corresponding regions of the molecule.

Antihistone antibodies have been useful for comparative studies of histones and for exploring the organization of histones in chromatin (Stollar, 1978; Bustin, 1978). In comparative studies, serological cross-reactions have usually been consistent with the extent of evolutionary change in amino acid sequences. Anti-H4 antibodies gave nearly identical complement fixation reactions with H4 samples from a wide range of vertebrate and invertebrate species (Stollar & Ward, 1970), whereas anti-H1 antisera revealed differences among H1 subfractions from one tissue and among H1 samples from much less divergent species (Bustin & Stollar, 1973a). With surprising specificity, anti-H2a sera were able to distinguish between two mouse H2a variants that differ in only two amino acids (Blankstein et al., 1977). In the case of H2b, on the other hand, specific antibodies showed little or no difference between H2b's as different as those of calf and *Drosophila* (Bustin et al., 1977) even though there are extensive sequence differences in the amino-terminal regions of these molecules (Elgin et al., 1979).

For both comparative studies and for the use of antibodies as probes for chromatin organization, it is important to know whether the recognition of different parts of the molecule is random and uniform or whether certain parts of the molecule are preferentially detected by the antibodies. This article describes experiments that approach this question with anti-H2b antibodies. Calf H2b has two methionines, at positions 59 and 62. This allows cleavage of the molecule into almost equal halves by cyanogen bromide (Johns et al., 1972). With

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the resulting fragments 1–58 and 63–125, used as soluble antigens and as insoluble affinity reagents, we have found that anti-H2b serum contains 9 times as much anti-(63–125) antibody as anti-(1–58) antibody. Since these findings could explain the inability of anti-H2b antibodies to distinguish between *Drosophila* and calf H2b, antibodies were then prepared to the 1–58 and 63–125 fragments separately. Comparative studies with these reagents were consistent with amino acid differences among H2b's of several species.

Materials and Methods

Preparation of Antigens: Calf H2b Histone and Cyanogen Bromide Fragmentation of H2b. H2b was prepared by acid extraction from calf thymus according to method 2 of Johns (1964). The acetone-precipitated protein obtained by this procedure was purified further by gel filtration; 100 mg of H2b in 5 mL of freshly prepared 8 M urea with 0.14 M 2-mercaptoethanol was applied to a 2.5 × 100 cm column of Bio-Gel P-60 and washed through with a solution of 0.02 N HCl, 0.05 M NaCl, and 0.02% sodium azide. H2b-containing fractions were identified by complement fixation assays with anti-H2b antiserum (Stollar & Ward, 1970). H2b-containing fractions were pooled, dialyzed against water, and lyophilized.

H2b was cleaved by reaction with cyanogen bromide (Johns et al., 1972); 50 mg of CNBr was added to 125 mg of H2b in 4.2 mL of water and 9 mL of 99% formic acid. Crude fractions containing the amino-terminal and carboxyl-terminal fragments were obtained by fractional acetone precipitation as described (Johns et al., 1972). Each acetone-precipitated fraction was dissolved in 2 mL of 0.1 N HCl and passed through a 2.5 × 30 cm column of Bio-Gel P-10 with 0.1 N HCl. In each case the major protein peak was collected. Yields of purified peptides from 125 mg of H2b were 30 mg of 1–58 and 20 mg of 63–125. The 1–58 still had some minor contamination with intact H2b, detected by analytical gel electrophoresis, and was purified further by preparative gel electrophoresis, as described below.

H2b from Other Species. Electrophoretically purified trout testis H2b was kindly provided by G. Dixon, *Drosophila* H2b by S. Elgin, mouse erythroleukemia cell H2b by L. Blankstein, and sea urchin (*Strongylocentrotus purpuratus*) by M. McClure and A. Johnson. Chick and frog H2b were prepared previously (Stollar & Ward, 1970). All samples were repurified by preparative gel electrophoresis as described below.

Protein–Sepharose Conjugates for Affinity Chromatography. Sepharose 4B was activated with cyanogen bromide as described by Porath et al. (1973). For intact H2b, 10 g of Sepharose was activated and added to 20 mg of protein; for 1–58 and 63–125, 5 g of activated Sepharose was added to 10 mg of each polypeptide. The suspensions were incubated at 25 °C for 4 h and at 4 °C overnight, washed with PBS, incubated with 1 M ethanolamine, pH 8.5, for 2 h, and rewashed with PBS. Eighty-five to ninety percent of the protein was covalently conjugated to the Sepharose.

Analytical Polyacrylamide Gel Electrophoresis. Samples of 20–50 μg of protein were applied to 12% polyacrylamide gels containing 8 M urea and 6 mM Triton X-100 as described by Alfageme et al. (1974). The 0.7 × 13 cm cylindrical gels were run at 120 V for 5 to 6 h until a pyronin Y dye marker ran through the gel; the running solution was 5% acetic acid. Gels were stained in 0.1% amido black for 20 min and destained in 7% acetic acid–40% ethanol in a Bio-Rad diffusion destainer.

Preparative Polyacrylamide Gel Electrophoresis. Whole H2b and the 1–58 fragments were purified in large diameter (1.2 × 14 cm) gels of 7.5% polyacrylamide–0.5% bis(acrylamide) in 8 M urea and 6 mM Triton X-100 (Zweidler, 1978). Up to 2 mg of protein dissolved in 0.25 mL of sample solution (8 M urea, 4% 2-mercaptoethanol, 5% acetic acid, 6 mM Triton X-100, and 12% sucrose) was applied per gel. Electrophoresis was run in a Bio-Rad Model 155 unit at 150 V until a pyronin Y dye marker ran through the gel; the running solution was 5% acetic acid. After electrophoresis, the gel was frozen and a small longitudinal section was sliced, stained with amido black, and destained. After correction was made for the increase in length of the stained gel, the region in the unstained portion corresponding to H2b or the 1–58 fragment was located and sliced. This slice was placed above a 1.4 × 3 cm plug of 7.5% polyacrylamide in urea–Triton in the bottom of another preparative gel tube. The protein was electrophoresed through the plug into a dialysis sac secured with a rubber band to the bottom of this elution tube; the dialysis sac contained 3 mL of 5% acetic acid. Dialysis tubing with a 6000–8000 molecular weight cutoff was used for elution of H2b, and tubing with a 3500 molecular weight cutoff was used for the 1–58 fragment. After elution, the contents of the dialysis sac were dialyzed against distilled water and lyophilized. Recovery was 60–80% of that expected on the basis of starting load and the appearance of the stained gel sample.

Immunization and Collection of Antihistone Antisera. Rabbits were immunized with complexes of yeast RNA and H2b, 1–58, or 63–125 mixed in a 1:3 ratio of RNA to protein (Stollar & Ward, 1970). A fine precipitate formed when RNA was mixed with H2b or 63–125, but no precipitation occurred with the RNA–(1–58) mixture. The mixtures were emulsified with an equal volume of complete Freund's adjuvant for the first injection and with incomplete adjuvant for subsequent injections. New Zealand white female rabbits were immunized with complexes containing 100 μg of H2b or 50 μg of each fragment, given intradermally at multiple sites along the back. The same dose was given 2 and 4 weeks later for H2b and 4 and 6 weeks later for the fragments. Rabbits were bled 1 week after the last injection and at weekly intervals; if necessary, additional intradermal immunizations were given to raise or maintain serum titers. Sera were heated at 56 °C to inactivate complement and were stored at −20 °C. IgG was obtained from each serum by DEAE-cellulose chromatography as described previously (Stollar & Rezuke, 1978).

Serological Assays. Quantitative microcomplement fixation was performed as described (Stollar, 1978) with a buffer containing 0.1% gelatin in place of bovine serum albumin. A solid-phase radioimmunoassay (Blankstein et al., 1980) was also used to measure histone–antihistone reactions. Disposable polystyrene tubes (12 × 75 mm) were coated with 1 mL of H2b histone (2 μg/mL in PBS) or histone fragment. Washed antigen-coated tubes were incubated with IgG of antiserum or normal serum, washed with 0.1 M borate–1 M NaCl, pH 8.5, containing 0.1% Tween-20 (BBS–Tween) (Smith et al., 1978), and then incubated with ^{125}I-labeled purified goat antirabbit IgG. The tubes were washed again and counted in a γ scintillation counter.

Antibody Purification by Affinity Chromatography. Washed histone–Sepharose was added to DEAE-purified IgG fractions of anti-H2b or normal rabbit serum and incubated for 2 h at 25 °C and overnight at 4 °C on a clinical rotator. The mixture was then poured into a column and washed with PBS–0.5 M NaCl until the A_{280} was less than 0.05 and then with BBS–Tween and again with PBS. Bound antibody was eluted with 0.5 M ammonia and 0.5 M NaCl, pH 11 (Simpson & Bustin, 1976), and collected into 0.5 M Tris, pH 8. Purified antibodies were dialyzed against PBS and stored frozen.

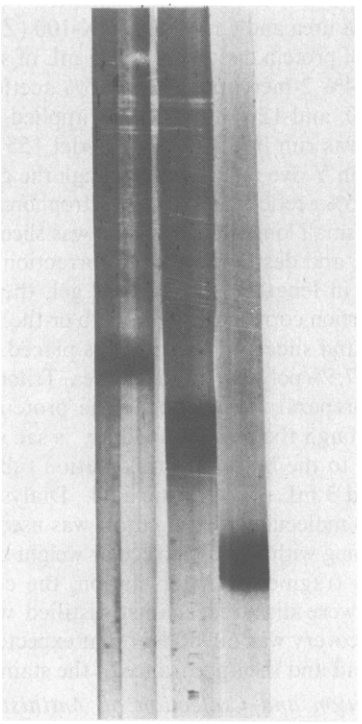

FIGURE 1: Polyacrylamide gel electrophoresis of H2b, 63–125, and 1–58 (from left to right). Gels of 12% polyacrylamide in Triton–urea were prepared according to Alfageme et al. (1974). Samples of 50 μg of protein were applied in 8 M urea, 4% 2-mercaptoethanol, 5% acetic acid, 6 mM Triton X-100, and 12% sucrose. Electrophoresis was run at 120 V for 5 h.

Amino Acid Analysis. H2b histone and its cyanogen bromide fragments (5–10 nmol) were hydrolyzed in 5.7 N constant boiling HCl in sealed evacuated tubes at 110 °C for 24, 48, and 72 h. Analyses were performed with a Durrum D-500 analyzer. Values for serine, threonine, and tyrosine were obtained by extrapolation to zero time; valine and isoleucine contents were obtained from the 72-h hydrolysis.

Analytical Ultracentrifugation. Velocity sedimentation experiments were performed with protein samples of 2–2.7 mg/mL in 0.14 M NaCl and 0.01 M Tris, pH 7.4, run at 48 000 rpm for 64 min at 20 °C in a Beckman Model E centrifuge with Schlieren optics. Photographs were made at 16-min intervals. A rectangular approximation method was used to estimate peak areas.

Results

Characterization of the Antigens. The purity of H2b and the isolated cyanogen bromide fragments was tested by analytical polyacrylamide gel electrophoresis run with the acid–urea–Triton system described by Alfageme et al. (1974). Single bands were observed with heavily loaded gels, and there was no cross-contamination among the intact H2b and separated fragments (Figure 1). With smaller amounts of protein, H2b and 63–125 ran as two closely spaced bands. These may represent two known variants which differ at positions 75 and 76 and can be resolved in the acid–urea–Triton gel system (Zweidler, 1978).

Amino acid analysis confirmed that the isolated fragments were the expected cleavage products (Table I). Small amounts of residual methionine (0.1 residue/fragment) may reflect incomplete cleavage and the presence of small amounts of pieces such as 1–62 and 59–125.

The extent of aggregation, which may affect serological reactivity, was tested by analytical ultracentrifugation. Unaggregated monomer accounted for at least 75% of the H2b,

Table I: Amino Acid Analysis of Cyanogen Bromide Fragments Prepared from Calf Thymus H2b

	mol/mol of protein			
	1–58 found	1–58 expected	63–125 found	63–125 expected
Asp	2.0	2.0	4.0	4.0
Thr	2.0[a]	2.0	5.9[a]	6.0
Ser	7.0[a]	7.0	6.7[a]	7.0
Glu	3.9	4.0	6.0	6.0
Pro	4.8	5.0	1.1	1.0
Gly	3.4	3.0	3.2	3.0
Ala	5.9	6.0	7.2	7.0
Val	4.3[b]	5.0	3.9[b]	4.0
Met	0.1	0.0	0.1	0.0
Ile	1.0[b]	1.0	3.8[b]	4.0
Leu	1.0	1.0	5.0	5.0
Tyr	2.8[a]	3.0	2.0	2.0
Phe	0.0	0.0	1.9	2.0
His	1.0	1.0	2.0	2.0
Lys	13.8	15.0	5.0	5.0
Arg	3.0	3.0	4.6	5.0

[a] Extrapolated to zero time from 24-, 48-, and 72-h hydrolyses. [b] Calculated from 72-h hydrolysis.

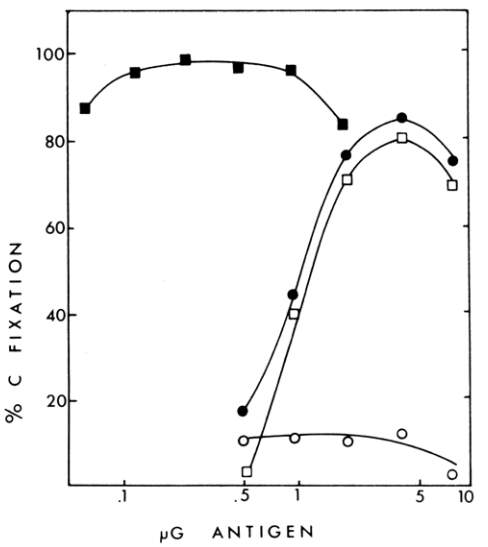

FIGURE 2: Microcomplement fixation reactions of anti-H2b serum, at a final dilution of 1:3000, with H2b (■), 1–58 (○), 63–125 (●), and a mixture of 1–58 and 63–125 (□) of calf thymus.

93% of the 1–58, and 90% of the 63–125 when they were centrifuged in the complement fixation buffer at 20 °C.

Specificity and Subpopulations of Anti-H2b Antiserum. All five rabbits immunized with H2b–RNA complexes produced specific anti-H2b antibody detectable in complement fixation assays. The sera reacted with intact H2b and with both cyanogen bromide fragments. At a given serum concentration, H2b reacted more strongly than either fragment and 63–125 reacted more strongly than 1–58 (Figure 2). In comparison with the amount of H2b used, 10–15-fold higher concentrations of the fragments were required for the reaction (Figure 2). The complement fixation curve obtained with a mixture of the two fragments did not resemble that of intact H2b and was nearly identical with that of 63–125 alone (Figure 2). These reactions were common to several anti-H2b sera.

Serum from the third bleeding of one animal was used for a more detailed analysis of specificity. The serum was tested at several dilutions in complement fixation reactions with H2b, 1–58, and 63–125. Serum titers were determined as the reciprocal of the dilution that resulted in a curve with 50% maximal complement fixation. The titers were 5400 for H2b,

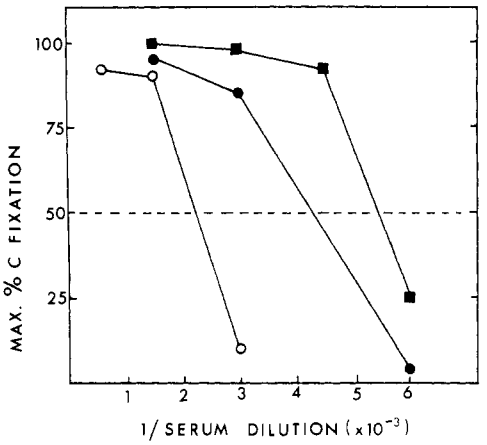

FIGURE 3: Determination of serum titers of anti-H2b antiserum with H2b (■), 1–58 (○), and 63–125 (●). Peak complement fixation levels were obtained from complement fixation curves with optimal concentrations for each antigen.

Table II: Recovery of Specific Antibody from Anti-H2b Serum by Affinity Chromatography

	affinity reagent		
	H2b-Sepharose	(1–58)-Sepharose	(63–125)-Sepharose
protein applied (mg)[a]	7.1	14.3	14.3
protein eluted with BBS[b] wash (μg)	20	0	0
protein eluted with ammonia, pH 11 (μg)	298	60	518
antibody recovd (μg/mg of IgG applied)	42	4.2	36

[a] IgG was purified from anti-H2b antiserum by DEAE-cellulose chromatography. The concentration was 4.8 mg/mL in the solution applied to the affinity reagent. The affinity reagent with bound antibody was washed with 0.1 M NaCl-0.01 M phosphate, pH 7, before the BBS and ammonia elution steps. [b] BBS (1 M NaCl and 0.1 M borate, pH 8.5) and 0.1% Tween-20.

2300 for 1–58, and 4300 for 63–125 (Figure 3). At a 1:1500 dilution, which gave over 90% complement fixation with any of these three antigens, there was no significant complement fixation (0–12%) with H1, H2a, H3, or H4.

More precise quantitation of the amount of antibody reacting with each antigen was obtained by affinity chromatography on antigen–Sepharose columns. The IgG was first isolated from serum by DEAE-cellulose chromatography to minimize nonspecific interactions of serum proteins with the immobilized histones (Stollar & Rezuke, 1978). As has been noted with other antihistone sera (Bustin et al., 1977; Stollar & Rezuke, 1978), there were two peaks of antihistone IgG. Both fractions showed the same reactions with H2b and were pooled.

IgG was applied separately to H2b–Sepharose, (1–58)–Sepharose, and (63–125)–Sepharose. Columns were washed with PBS and 1 M NaCl–borate–Tween 20 solution and PBS again before elution of specifically bound antibody with 0.5 M NaCl–0.5 M NH_4OH, pH 11. The results revealed a marked difference in the amount of antibody directed toward the two halves of the molecule. The (63–125)–Sepharose yielded 0.9 as much antibody as did H2b–Sepharose, whereas only 0.1 as much antibody was obtained from the (1–58)–Sepharose (Table II). In control experiments, normal rabbit IgG was eluted completely with the first two solutions and no protein was recovered after a subsequent wash with the saline–ammonia.

Table III: Complement Fixation Reactions of Affinity-Purified Antibody Populations

anti-H2b purified on	antibody dilution	maximal % complement fixation with H2b	1–58	63–125
H2b-Sepharose	1:30	98	86	93
	1:60	98	15	76
	1:120	92	0	32
	1:240	27	0	0
(1–58)-Sepharose	1:6	97	96	10
	1:15	93	88	0
	1:30	34	5	0
(63–125)-Sepharose	1:30	98	0	89
	1:60	98	0	48
	1:120	97	0	18
	1:180	10		

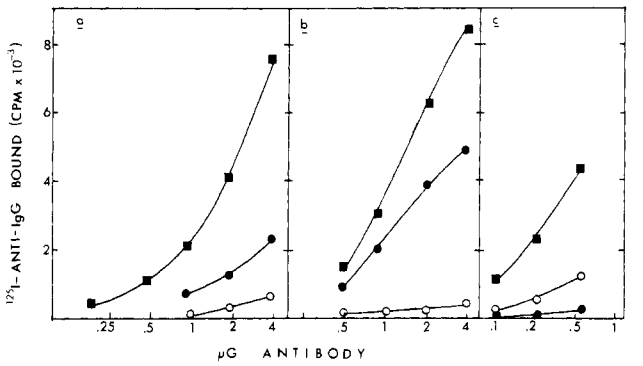

FIGURE 4: Solid-phase radioimmunoassay for anti-H2b antibodies purified on H2b–Sepharose (a), (63–125)–Sepharose (b), or (1–58)–Sepharose (c), tested with polystyrene tubes coated with H2b (■), 1–58 (○), or 63–125 (●). Maximal binding by normal IgG (from 0.25–10 μg/tube) was 200 cpm of ^{125}I-labeled anti-IgG.

The yields of antibody specific for each fragment were unchanged when the sera were passed sequentially through the fragment–Sepharose columns. That is, when the flow-through material from (1–58)–Sepharose was applied to (63–125)–Sepharose, there was still a yield of 42 μg/mg of IgG from the latter. Similarly, when the flow-through from (63–125)–Sepharose was applied to (1–58)–Sepharose, the yield from the latter was 4 μg/mg of IgG. The flow-through from either column alone still reacted with intact H2b, but IgG that passed through both fragment–Sepharose columns in sequence no longer did so.

The activity and specificity of the ammonia-eluted antibodies were tested in complement fixation assays. Antibodies eluted from H2b–Sepharose, like the starting IgG, reacted more strongly with H2b than with 63–125 or 1–58. Antibodies recovered from either of the fragment–Sepharose columns reacted with the corresponding fragment and with intact H2b (Table III). The anti-(1–58) population was equally reactive with 1–58 and H2b. The anti-(63–125) population reacted more strongly with H2b than with 63–125 (Table III). There was no cross-reaction between the two fragments.

Since complement fixation requires multivalent interaction, the possibility of cross-reactions between fragments was tested further in a radioimmunoassay. In this assay, IgG and purified anti-H2b reacted very much better with intact H2b than with either fragment (Figure 4a). Purified antifragment antibodies reacted with intact H2b and with the corresponding fragment, but in both cases the reactions with H2b were stronger (parts

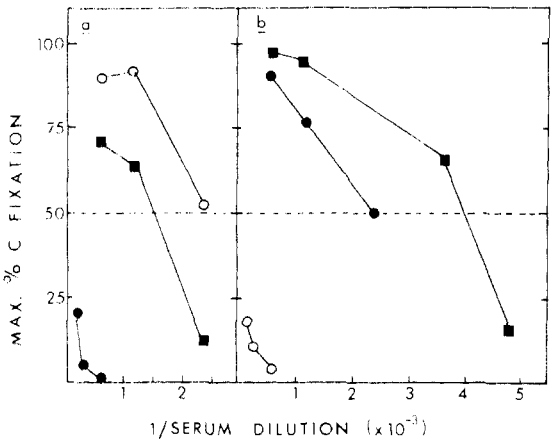

FIGURE 5: Determination of complement fixation titers of anti-(1–58) antiserum (a) and anti-(63–125) antiserum (b), tested with H2b (■), 1–58 (○), or 63–125 (●).

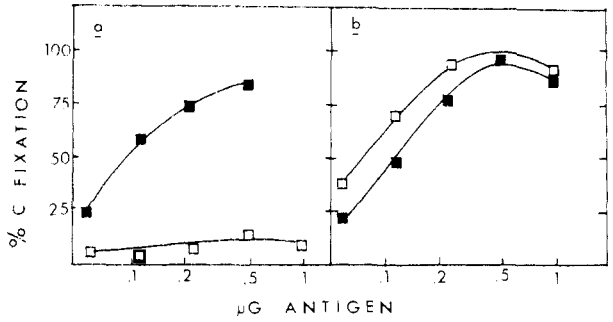

FIGURE 6: Complement fixation reactions of *Drosophila* H2b (□) and calf H2b (■) with (a) anti-(1–58) serum (1:300) and (b) anti-(63–125) serum (1:3600).

b and c of Figure 4). Again, there was no cross-reaction between the fragments.

Specificities of Antisera Induced by H2b Fragments. Two rabbits were immunized with (1–58)–RNA and two with (63–125)–RNA complexes. All animals produced antifragment antibodies. Early sera from animals immunized with 1–58 reacted best with isolated 1–58 but also gave complement fixation reactions with intact H2b. The cross-reaction with H2b diminished as immunization progressed, so that late sera reacted only with the 1–58. All of the anti-(63–125) sera, early and late, reacted with intact H2b; indeed, the complement fixation titer with H2b was higher than that with the immunogen 63–125 itself. There was little or no cross-reaction between the fragments (Figure 5).

Serological Comparisons of Histones of Varying Species Origin. H2b was obtained from several vertebrate and invertebrate organisms and, when necessary, purified by preparative electrophoresis. The H2b's were compared in quantitative complement fixation assays with several dilutions of anti-H2b, anti-(1–58), and anti-(63–125). Early bleedings of the anti-(1–58) were used so that cross-reactivity could be measured with whole H2b samples. As reported by Bustin et al. (1977), anti-H2b serum did not distinguish between calf and *Drosophila* H2b; the same was true for anticalf 63–125 (Figure 6b). In contrast, anticalf 1–58 showed little or no reaction with *Drosophila* H2b at a serum concentration that gave 80% complement fixation with calf H2b (Figure 6a). The anti-(1–58) serum also failed to react with either sea urchin or trout H2b at the same or higher concentration (Table IV). This serum reacted nearly identically with calf, chick, frog, and mouse H2b (Table IV). Anti-(63–125) antibodies did not distinguish among calf, chick, *Drosophila*, or trout H2b's but did detect a serological difference between calf and sea urchin proteins (Table IV).

Table IV: Complement Fixation Reactions of Anti-(1–58) and Anti-(63–125) Antisera with H2b from Various Species

	anticalf 1–58		anticalf 63–125	
H2b source	50% complement fixation titer	ID[a]	50% complement fixation titer	ID[a]
calf	960	0	3720	0
mouse	840	5.7	3300	5.3
chick	930	1.2	3600	1.7
frog	930	1.2	3300	5.3
trout	*b*	≥50	*c*	0
Strongylocentrotus	*b*	≥50	2200	22
Drosophila	*b*	≥50	3810	0

[a] ID = immunological distance = 100 × log [(titer for calf H2b)/(titer for heterologous H2b)]. [b] Less than 15% complement fixation with a 1:300 serum dilution. [c] Tested in a different experiment, in which the serum titer was 2400 for calf H2b and 2520 for trout H2b.

Discussion

Complement fixation assays have been used for comparative studies of many proteins. Prager & Wilson (1971) extended the quantitative interpretation of such comparisons by evaluating the correlation between immunological cross-reactivity and sequence homologies among lysozymes. They described an "index of dissimilarity" as the factor by which antiserum concentration must be raised in order for a heterologous antigen to give a complement fixation curve equal in height to that produced by homologous antigen. They then defined "immunological distance" as 100 times the log of the index of dissimilarity and found that the percent sequence difference (x) was related to the immunological distance (y) by the relationship $y = 5x$. This correlation would be optimal if all parts of the sequence contributed to antigenic reactivity and if antibodies in the analytical test serum were distributed equally to all antigenic determinants. It is likely, however, that in most proteins some particular amino acids contribute disproportionately to antigenic determinants and that the distribution of antibodies to different determinants is biased, as in cytochrome *c* and hemoglobin (Reichlin, 1975).

In many systems, the variable contributions of amino acids to antigenic sites and the variable distribution of antibodies to different antigenic sites may be statistically averaged, especially in large molecules with many determinants (Prager & Wilson, 1971). This averaging may be aided experimentally by the use of pooled sera and by reciprocal tests with antibodies induced by each of the proteins being compared (Prager & Wilson, 1971).

The distribution of anti-H2b antibodies resulting from immunization with H2b–RNA complexes was particularly biased, so that a large segment consisting of half of the molecule played little role in the reaction with anti-H2b sera. The unusual distribution of antibody specificity explained earlier findings, confirmed in our study, that *Drosophila* and calf H2b's were indistinguishable with anti-H2b sera (Bustin et al., 1977) even though there are major sequence differences between the two proteins (Elgin et al., 1979). In this case the problem was exaggerated; whereas most of the antibodies were directed to the 63–125 portion, nearly all the sequence differences were in the 1–34 region, numbered in relation to the calf sequence. When antibodies to 1–58 itself were prepared, they readily distinguished among proteins with differences in

this region, as seen in comparisons of *Drosophila*, trout, or sea urchin H2b with calf H2b. It was not possible to define an immunological distance for these comparisons. A 1:960 serum dilution gave 50% complement fixation with calf H2b, whereas a more than threefold higher serum concentration gave less than 15% complement fixation with these three histones; higher serum concentrations were anticomplementary. Trout H2b, closest of the three in sequence to calf H2b, differed from it by two deletions and six substitutions (a 14% sequence difference). Chick, frog, and mouse H2b's were similar to calf H2b in reactions with anti-(1–58) antibodies. With these histones, the largest immunological distance was 5.7 (for mouse H2b), which would predict only a 1% sequence difference.

Reactions of anti-(63–125) antibodies resembled those of anti-H2b and were consistent with the highly conserved structure of that region. *Drosophila* and trout H2b's were just as reactive as calf H2b with anticalf H2b. They differ from calf H2b in only one position in each case (residues 75 and 77, respectively) (Isenberg, 1979). The immunological distance for sea urchin H2b in relation to the calf protein was 22, predicting a 4 to 5% sequence difference. Sequence data for *Strongylocentrotus* were not available, but H2b of the sea urchin *Psammechinus* differs from calf H2b at six positions in this region. The differences are clustered (residues 70, 81, 82, 85, 123, and 124); the large region of 86–122 is identical in the two proteins.

With another anticalf H2b, Absolom & van Regenmortel (1978) localized part of the antigenic activity to a peptide fragment 36–50 of H2b. Roland & Pallotta (1978) found significant serological differences between calf and rye H2b, using serum raised against H2b from either source. The sera were induced by histone–RNA complexes. With anticalf H2b, the immunological distance between calf and rye H2b was 36–40, predicting a 7 to 8% sequence difference. A larger value was observed with antirye H2b antibodies in the reciprocal test. The localization of sequence differences has not been reported.

Studies of other antihistone antibodies have not identified a predictable pattern of distribution of antigenic determinants among charged and uncharged regions of the proteins. H1 fragments with both properties combined with anti-H1 antibodies (Bustin & Stollar, 1973b). Antisera induced by H3–RNA complexes also contained antibodies to both charged and uncharged regions (Sapeika et al., 1976). An example of narrowly focused specificity was observed with anti-H2a antisera, which recognized a difference between two variants that differ in only two sequence positions (Blankstein et al., 1977). A methionine at position 51 in one of the variants contributed to an antigenic determinant for homologous antibodies. When anti-H5 antibodies were induced without RNA carrier, the major part of the antibody specificity was directed to the globular central portion of the protein (Mura, 1979).

The studies with other antihistone systems used either measurements of cross-reactions, usually in complement fixation assays, or measurements of inhibition by histone fragments. Affinity chromatography provides a more definitive quantitation of the distribution of antibody specificity. There was only a twofold difference in the titer of the anti-H2b when it was tested with H2b and 1–58. This was difficult to evaluate, however, because of another difference in their reactions; a much higher concentration of the fragment was required. This may have reflected a lower affinity of the fragment for antibody or an equilibrium state in which only a small part of the fragment population is in the proper conformation for reaction with antibody (Furie et al., 1975). The radioimmunoassay, probably influenced by both the amount of antibody reacting and the quantity of antigen required, did show a large difference between H2b and the fragments but did not give quantitative measurements of anti-(1–58) and anti-(63–125).

The amount of antibody isolated from anti-H2b serum with 1–58 and 63–125 affinity reagents was equal to the amount obtained with H2b–Sepharose. In addition, IgG that was passed through both columns no longer gave a complement fixation reaction with intact H2b. These results indicated that there was little if any antibody unique to the intact structure that could not be accounted for by determinants in the isolated fragments. Each fragment was able to give a complement fixation reaction and was probably still multivalent. Within the 1–58 fragment, the 1–34 region played a major role in serological reactivity, either by providing one or more determinants or by interacting with the rest of the fragment. This was evident from the large differences in reactivity of H2b proteins varying in the 1–34 sequence but sharing nearly identical sequence in the rest of the fragment.

As the localization of antigenic sites of histones becomes more precise and antibodies directed to more localized regions of the histones are prepared, they become increasingly useful reagents, both for comparative studies and for exploring the nature of histone structures within chromatin. For example, chromatin was able to absorb anti-(1–58) antibodies under a variety of conditions in which little or no anti-(63–125) could be absorbed, possibly reflecting differences in accessibility of the two portions of the molecule in chromatin (Di Padua Mathieu, 1979).

References

Absolom, D., & van Regenmortel, M. H. V. (1978) *FEBS Lett. 85*, 61.

Alfageme, A., Zweidler, A., Mahowald, A., & Cohen, L. H. (1974) *J. Biol. Chem. 249*, 3729.

Blankstein, L. A., Stollar, B. D., Franklin, S. G., Zweidler, A., & Levy, S. B. (1977) *Biochemistry 16*, 4557.

Blankstein, L. A., Stollar, B. D., & Levy, S. B. (1980) *Anal. Biochem.* (in press).

Bustin, M. (1978) *Cell Nucl. 4 (Part A)*, 195.

Bustin, M., & Stollar, B. D. (1973a) *J. Biol. Chem. 248*, 3506.

Bustin, M., & Stollar, B. D. (1973b) *Biochemistry 12*, 1124.

Bustin, M., Reeder, R. H., & McKnight, S. L. (1977) *J. Biol. Chem. 252*, 3099.

Di Padua Mathieu, D. (1979) Thesis, Tufts University, Boston, MA.

Elgin, S. C. R., Schilling, J., & Hood, L. E. (1979) *Biochemistry 18*, 5679.

Furie, B., Schechter, A. N., Sachs, D. H., & Anfinsen, C. B. (1975) *J. Mol. Biol. 92*, 497.

Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159.

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

Johns, E. W., Forrester, S., & Riches, P. L. (1972) *Arch. Biochem. Biophys. 152*, 287.

Mura, C. V. (1979) Thesis, Carleton University, Ottawa, Canada.

Porath, J., Aspberg, K., Drevin, H., & Axen, R. (1973) *J. Chromatogr. 86*, 53.

Prager, E. M., & Wilson, A. C. (1971) *J. Biol. Chem. 246*, 5978.

Reichlin, M. (1975) *Adv. Immunol. 20*, 71.

Roland, B., & Pallotta, D. (1978) *Can. J. Biochem. 56*, 1021.

Sapeika, G., Absolom, D., & Van Regenmortel, M. H. V. (1976) *Immunochemistry 13*, 499.

Simpson, R. T., & Bustin, M. (1976) *Biochemistry 15*, 4305.
Smith, J. A., Hurrell, J. G. R., & Leach, S. J. (1978) *Anal. Biochem. 87*, 299.
Stollar, B. D. (1978) *Methods Cell Biol. 18*, 105.
Stollar, B. D., & Ward, M. (1970) *J. Biol. Chem. 245*, 1261.
Stollar, B. D., & Rezuke, W. (1978) *Arch. Biochem. Biophys. 190*, 398.
Zweidler, A. (1978) *Methods Cell Biol. 17*, 223.

Adenosine Analogues as Substrates and Inhibitors of *S*-Adenosylhomocysteine Hydrolase in Intact Lymphocytes†

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ABSTRACT: A number of adenosine analogues have been examined for their ability to interact with *S*-adenosyl-L-homocysteine (SAH) hydrolase in intact mouse lymphocytes. In the presence of *erythro*-9-(2-hydroxy-3-nonyl)adenine, 3-deazaadenosine, 8-azaadenosine, formycin A, 2-aminoadenosine, 2-fluoroadenosine, N^6-methyladenosine, N^6-hydroxyadenosine, purine ribonucleoside and inosine were each metabolized to radioactive analogues of SAH when cells were labeled with either L-[2-^{3}H]methionine or L-[^{35}S]homocysteine. Tubercidin was shown to undergo metabolism both to *S*-[^{3}H]tubercidinyl-L-methionine and to *S*-[^{3}H]tubercidinyl-L-homocysteine in cells labeled with [2-^{3}H]methionine. 9-β-D-Arabinofuranosyladenine and 2′-deoxyadenosine caused marked elevations of [^{3}H]SAH in cells preloaded with [2-^{3}H]methionine but were not themselves metabolized detectably to SAH analogues. Adenine and 5′-deoxyadenosine also caused substantial elevations of [^{3}H]SAH under these same conditions. Some of the adenosine analogues shown to be metabolized to SAH analogues also caused an elevation of SAH in the lymphocytes. These results indicate the potential of adenosine analogues to interfere with cellular methylation reactions due either to their inhibition of SAH hydrolase or to their metabolism, via this enzyme, to SAH analogues.

Adenosine (Ado)[1] and many of its structural analogues are inhibitory to lymphocyte-mediated cytolysis (LMC) in vitro (Wolberg et al., 1975, 1978; Zimmerman et al., 1976). The LMC-inhibitory activity of the majority of these Ado analogues appears to be related to the ability of these agents to stimulate an elevation of adenosine 3′,5′-monophosphate (cAMP) within the cytolytic lymphocytes. However, a few compounds, such as 9-β-D-arabinofuranosyladenine (AraA), 2′-deoxyadenosine (2′-dAdo) and adenine, were found to be inhibitory to LMC in the absence of any appreciable effects on cAMP metabolism.

Several recent reports have drawn attention to the potential of Ado and its analogues to perturb cellular physiology due to their interaction with *S*-adenosyl-L-homocysteine (SAH) hydrolase (EC 3.3.1.1), the enzyme responsible for catabolism of SAH in eucaryotic cells (De La Haba & Cantoni, 1959; Walker & Duerre, 1975). The importance of this enzymatic reaction derives from the observations that SAH is a potent inhibitor of a number of *S*-adenosyl-L-methionine (SAM)-utilizing methyltransferases (Borchardt, 1977; Cantoni et al., 1979) and that cells appear unable to eliminate intact SAH across their plasma membrane (Walker & Duerre, 1975; Trewyn & Kerr, 1976). Since the equilibrium constant for SAH hydrolase favors greatly the synthesis of SAH over its hydrolysis (De La Haba & Cantoni, 1959), cells exposed simultaneously to Ado and L-homocysteine rapidly accumulate high levels of SAH (Kredich & Martin, 1977). Moreover, some Ado analogues, including 3-deazaadenosine (Chiang et al., 1977), AraA, 2′-dAdo, 5′-deoxyadenosine (5′-dAdo), and adenine (Hershfield & Kredich, 1978; Hershfield, 1979; Hershfield et al., 1979; Kredich et al., 1979; Palmers & Abeles, 1979; Ueland & Saebø, 1979), have been shown to be inhibitors of SAH hydrolase and, in the cases of 3-deazaadenosine and adenine, to cause an elevation of SAH within cells.

The recent finding that 3-deazaadenosine inhibits LMC and causes an elevation of lymphocyte SAH without any effect on cAMP levels indicates that SAH, like cAMP, is an intracellular modulator of this lymphocyte function (Zimmerman et al., 1978). It then became of interest to examine the interaction of other Ado analogues with SAH hydrolase as a possible basis for the inhibition of LMC by these agents. The present report describes the ability of a number of Ado analogues to serve as substrates or inhibitors of SAH hydrolase in mouse cytolytic lymphocytes.

Materials and Methods

L-[^{35}S]Homocysteine thiolactone (11 Ci/mol) and L-[2-^{3}H]methionine (2.9 Ci/mmol) were products of the Amersham Corp. *S*-[8-^{14}C]Adenosyl-L-homocysteine was kindly provided by Dr. E. M. Wise, Jr., and K. T. Calvert of these laboratories. *S*-3-Deazaadenosyl-L-homocysteine, *S*-8-azaadenosyl-L-homocysteine, *S*-[5′-(9-arabinofuranosyladenyl)]-L-homocysteine, *S*-tubercidinyl-L-homocysteine, and *S*-tubercidinyl-L-methionine were generous gifts from Dr. R. T. Borchardt of the University of Kansas (Borchardt et al., 1974, 1976a,b; Borchardt & Wu, 1975). SP-Sephadex C-25 was a product of Pharmacia Fine Chemicals. Boric acid gel was obtained from the Aldrich Chemical Co., Inc. The samples of adenine, 2′-dAdo, AraA, and 3′-deoxyadenosine (3′-dAdo) used in the present study were passed through small columns of boric acid

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[1] Abbreviations used: Ado, adenosine; AraA and adenine arabinoside, 9-β-D-arabinofuranosyladenine; cAMP, adenosine 3′,5′-monophosphate; 2′-dAdo, 2′-deoxyadenosine; 3′-dAdo, 3′-deoxyadenosine; 5′-dAdo, 5′-deoxyadenosine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; LC, high-performance liquid chromatography; LMC, lymphocyte-mediated cytolysis; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; SAraAH, *S*-(5′-arabinofuranosyladenyl)-L-homocysteine; STH, *S*-tubercidinyl-L-homocysteine; STM, *S*-tubercidinyl-L-methionine.

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