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Antigenic Determinants in Lysine-Rich Histones†

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ABSTRACT: Antibodies to purified, unfractionated, calf thymus F1 histone were induced by immunizing rabbits with histone–RNA complexes. The reactions of these antibodies with chemically modified calf thymus F1 histone and with fragments of the F1 histone have been tested in order to approach the definition of serologically active regions of the molecule. Two fragments were obtained by treating the F1 histone with *N*-bromosuccinimide. The larger carboxyl-terminal portion (N1) reacted to give 40% complement fixation with a serum dilution (1:500) that gave over 85% complement fixation with intact F1 histone. The smaller amino-terminal portion (N2) failed to form a complement-binding complex with even higher serum concentrations (1:100). However, a 5-fold molar excess of the N2 fragment over intact F1 did completely inhibit the complement fixation of the F1 histone with a 1:1000 dilution of anti-F1 serum. The cleaved fragments have different antigenic determinants for the anti-F1 serum and do not recombine in dilute solution to re-form the native protein structure, since the complement fixation reaction of a mixture of peptides N1 and N2 was equal to that of N1 alone. Dinitrophenylated derivatives of N1 and F1 failed to fix complement upon reaction with anti-F1. The inhibitory capacity of N2, however, was not altered by dinitrophenylation. The results suggest that lysine residues in N1 but not in N2 are involved in immunogenic determinants. Compared to native F1 histone, F1 which was nitrated at the single tyrosine residue gave a weaker complement fixation reaction thereby pinpointing the tyrosine residue or its environment as an antigenic determinant. The tyrosine-containing peptide failed to inhibit the complement fixation activity of the F1–anti-f1 mixture at peptide concentrations which do not interfere with the complement system. However, an unfractionated tryptic digest of F1 did completely and specifically inhibit the system. When animals were immunized with RNA complexed with either N1 or N2, the animals immunized with N1 did not produce detectable antibodies, while those immunized with N2 did produce antibodies which reacted strongly with intact F1 and weakly with isolated N1 or N2 fragments. Both fragments N2 and N1 as well as poly(L-lysine) inhibited the reaction between anti-N2 and intact F1 suggesting that antibodies against lysine residues in N2 react with lysine containing determinants in N1 and F1. Indeed, when the reaction of this serum with dinitrophenylated derivatives of F1 and N1 was tested, it was found that the complement fixation reactivity of these derivatives was markedly reduced. On the other hand the N_2ph–N2 derivative fixed complement to somewhat a higher degree than N2. The latter results support the findings that N2 contained determinants other than lysine residues and suggest that lysine residues in fact inhibited effective complement-fixing aggregation of this fragment with antibody.

Complexes of histone and RNA, when injected into rabbits, elicit specific anti-histone antibodies (Stollar and Ward, 1970). Anti-F1 histone antibodies obtained in this way are not only specific for the F1 histone class in general, but in addition they can distinguish between subfractions of this class and between F1 molecules derived from various sources (Bustin and Stollar, 1972). Thus, the species and organ specificity of the F1 histone class, which has been detected by chromatographic (Bustin and Cole, 1968; Kinkade, 1969) and electrophoretic (Paynim *et al.*, 1971) techniques, is also immunologically demonstrable.

Structural studies on the F1 histone revealed that the molecule could be visualized as being composed of three regions, each of which displays characteristic amino acid composition and overall cationic charge (Bustin and Cole, 1970). Kinkade and Cole (1966b) mentioned the possibility that the structural differences between the various lysine-rich histones could be restricted to a particular portion of the primary structure of the molecule. Tryptic fingerprints of the two peptides isolated after *N*-bromosuccinimide cleavage of the molecule suggested that most of the differences between the various F1 molecules lie in the amino-terminal portion of the molecule (Bustin and Cole, 1969). Indeed, detailed sequence analysis (Rall and Cole, 1971) detected a variable region within the first 40 residues of the F1 molecule.

The presence of regions with distinct amino acid composition and overall cationic charge raises the possibility that these differences could be expressed conformationally and that the various parts of the histone molecule differ in their ability to interact with DNA (Boublick *et al.*, 1970; Bustin and Cole, 1970; Fasman *et al.*, 1971). Immunochemical methods may be useful in studying these conformational changes and interactions with DNA, especially if we can identify the immunologically reactive regions of the histone molecule. This article reports studies designed to gain further insight into the antigenic properties of the F1 histones.

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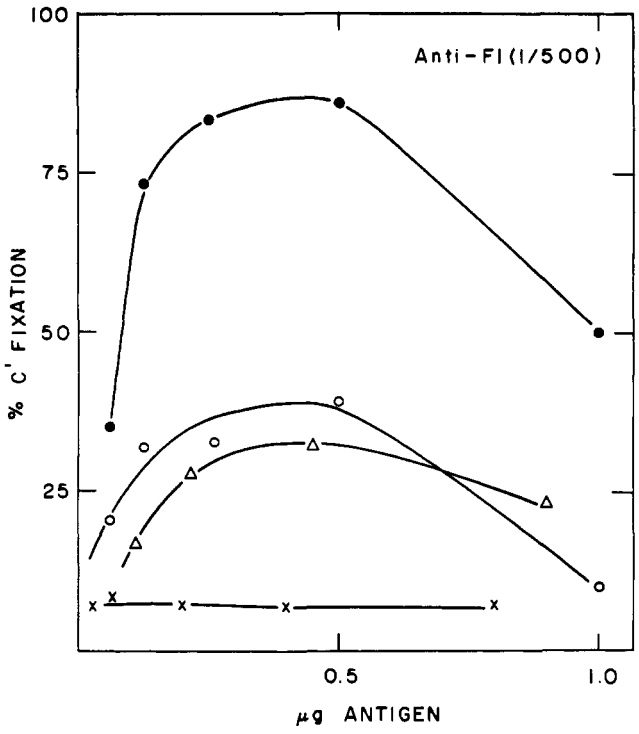

FIGURE 1: Complement fixation of F1 and peptides derived from F1 by cleavage with *N*-bromosuccinimide. Antisera to F1 diluted 500-fold: (●) F1, (○) N1, (×) N2, (△) N1 + N2.

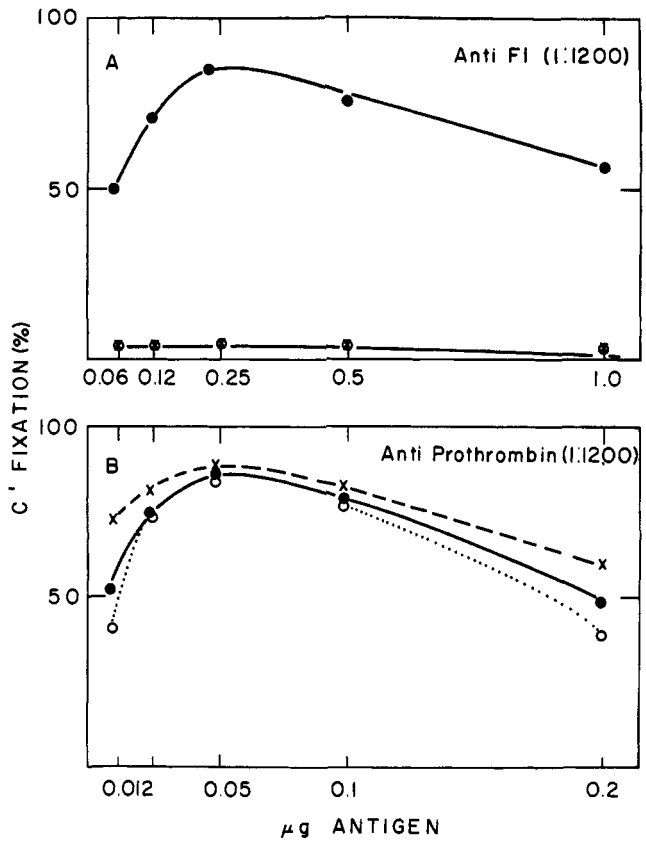

FIGURE 2: Inhibition of complement fixation by fragment N2 and by trypsin-digested-F1. Prior to the addition of antigen the antisera was incubated for 1 hr at 25° with 0.2 ml of the inhibitor: (○) N2, 8 μg/ml; (×) digest, 500 μg/ml; (●) homologous antigen.

Materials and Methods

Materials. Tetranitromethane was obtained from Fluka, yeast RNA from Sigma, guanidinium chloride (Ultra Pure) from Mann, Amberlite IRC-50 from Bio-Rad, and *N*-bromosuccinimide from BDH. Anti-prothrombin serum and prothrombin were gifts of Dr. S. Shapiro.

Protein Samples. Lysine-rich histones were extracted directly from calf thymus with 5% trichloroacetic acid (De Nooij and Westenbrink, 1962; Kinkade and Cole, 1966a). The lyophilized fraction was further purified by stepwise elution with 10.5% guanidinium chloride from Amberlite IRC-50 columns (Kinkade and Cole, 1966a; Bustin, 1972). The purity of the histones was tested by amino acid analysis (Moore and Stein, 1963), electrophoresis in polyacrylamide gels (Panyim and Chalkley, 1969), and gel filtration on Sephadex G-100 columns eluted with 0.02 N HCl (Bustin, 1968).

Nitration Procedure. Nitration was performed as recommended by Sokolowsky *et al.* (1966). Purified F1, at a concentration of 2 mg/ml in 0.01 M Tris buffer (pH 8.0), was treated with an 8-fold molar excess of tetranitromethane (as a 0.1 M stock solution) as described previously (Bustin, 1971). Amino acid analysis of the nitrated F1 preparation revealed the presence of 0.82 residue of nitrotyrosine and less than 0.04 residue of tyrosine per histone molecule.

Cleavage of F1 Histone by N-Bromosuccinimide. Purified calf thymus F1 was treated with *N*-bromosuccinimide as described previously (Bustin and Cole, 1969). The cleavage products (N1 peptide containing the carboxyl-terminal portion of the molecule, and N2 peptide containing the amino-terminal portion of the molecule) were separated by gel filtration on Sephadex G-100 (Bustin and Cole, 1969). The purity of the peptides was confirmed by amino acid analysis.

Dinitrophenylation. Purified F1, N1, and N2 at concentration of 0.1, 0.4, and 0.3 μmole per ml in solution of 0.5 M $NaHCO_3$ in 50% dioxane were treated respectively with a 180-, 100-, and 60-fold excess of fluorodinitrobenzene (added as 10% ethanolic solution). At various intervals the reactions were stopped by acidification with 6 N HCl. The reaction mixtures were passed through Sephadex G-10 columns eluted with 0.05 M sodium phosphate (pH 6.8). Part of the dinitrophenylation material and dinitrophenol bound tightly to the resin. Only fractions that passed freely through the G-10 columns were used. Concentrations of F1, N1, and N2 in the dinitrophenylated samples were determined by amino acid analysis (Bustin and Cole, 1969). The extent of dinitrophenylation was determined using a molar extinction coefficient of 17,400 for N_2ph-lysine. The following derivatives were obtained (values in parentheses denote extent of dinitrophenylation): $(N_2ph)_8$-F1 (12%); $(N_2ph)_{16}$-F1 (24%); $(N_2ph)_7$-N1 (13%); $(N_2ph)_{15}$-N1 (28%); $(N_2ph)_{3.8}$-N2 (32%).

Tryptic Digestion. For tryptic digestion, the histone was dissolved in 0.2 M NH_4HCO_3 (pH 8.2) (Kinkade and Cole, 1966b) and subjected to enzymatic hydrolysis for 10 hr at 37° at a substrate to enzyme ratio of 75:1 (w/w). The tyrosine-containing peptide 3 was obtained as described previously (Bustin, 1972).

Immunological Procedures. Rabbits were immunized with complexes made of yeast RNA and purified F1 histone or peptides N1 or N2 (Bustin and Stollar, 1972). Insoluble complexes of F1 or N1 were formed by mixing with equal amounts of RNA in 0.14 M NaCl–0.01 sodium phosphate (pH 6.8). Mixing of N2 with RNA failed to form insoluble complexes. In this case the final ratio of N2 to RNA was 2:1 (w/w).

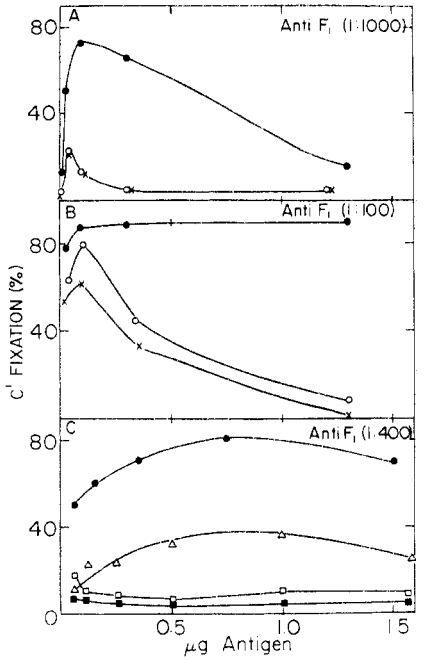

FIGURE 3: Complement fixation of anti-F1 sera with dinitrophenylated derivatives of F1 and N1: (●) F1, (○) $(N_2ph)_8$-F1, (×) $(N_2ph)_{16}$-F1, (△) N1, (□) $(N_2ph)_7$-N1, (■) $(N_2ph)_{15}$-N1.

The solutions were emulsified with 1.5 volumes of complete Freund's adjuvant. Each rabbit was injected at multiple intradermal sites with 1.5 ml containing either 550 μg of F1, 550 μg of N1, or 1200 μg of N2. Booster injections identical with immunizing injections were given 14 days later. Animals were bled 7, 14, and 30 days after the boost injections. Quantitative micro-complement fixation was performed according to the method of Wasserman and Levine (1960) in a total volume of 1.4 ml/reaction mixture, with a buffer of 0.14 M NaCl–0.01 M Tris (pH 7.4), 0.5 mM Mg^{2+}, 0.15 mM Ca^{2+}, and 0.1% bovine albumin.

Results

Distinct Determinants in the Amino-Terminal and Carboxyl-Terminal Portions of the F1 Molecule. By cleaving the F1 histone molecule with *N*-bromosuccinimide (Bustin and Cole, 1969) it is possible to separate the amino-terminal third of the molecule from the carboxyl-terminal portion. The two portions display characteristic amino acid compositions and cationic charge. To determine whether they are distinct immunologically, their ability to react with antisera to the whole F1 was tested. As shown in Figure 1, intact F1 histone at a 1:500 dilution of homologous serum reacted to give more than 85% complement fixation. The carboxyl-terminal fragment alone (N1) reacted to give 40% complement fixation with this dilution of the serum, and 85% complement fixation with a 1:300 dilution of serum. The N2 fragment gave less than 20% complement fixation even with a 1:100 dilution. When a mixture of N1 and N2 was added to the anti-F1 serum, the amount of complement fixation was very close to that observed with the N1 alone. These findings indicate that mixing of the two peptides does not result in a reconstitution of native histone antigenic structure. Furthermore, since the N2 did not inhibit the reaction of N1, they indicate that if antigenic sites are present in N2, they are distinguishable from those present in N1. That antigenic

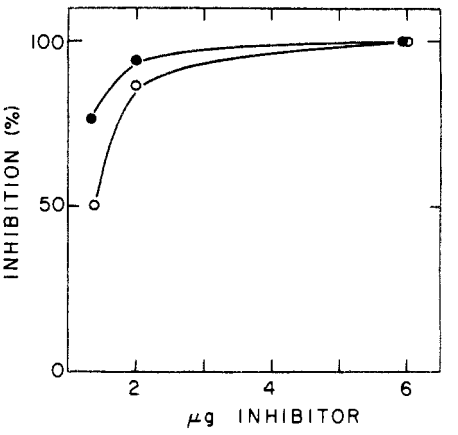

FIGURE 4: Inhibition of complement fixation in F1–anti-F1 system by N2 and $(N_2ph)_{3.8}$-N2, (○) N2; (●) $(N_2ph)_{3.8}$-N2.

regions are indeed present in the amino-terminal N2 third of the molecule is shown by the fact that a 5-fold molar excess of the N2 (compared to F1) completely inhibited the complement fixation reaction between F1 and anti-F1 (Figure 2A). A 2-fold molar excess of the N2 inhibited the reaction by more than 50%, and the inhibition involved a drop in the complement fixation curve without a shift to higher antigen concentrations, so that it was probably not due to an interaction between the fragments themselves and a simple removal of a fraction of the effective antigen molecules from the mixture. That the inhibition is specific is indicated by the fact that N2 had no effect on the complement fixation reaction of an unrelated immune system consisting of prothrombin and anti-prothrombin antiserum (Figure 2B).

Reaction of Dinitrophenylated F1 and the N1 and N2 Fragments with Anti-F1 Serum. Samples of F1 histone were dinitrophenylated to the extent of 8 and 16 mol of N_2phOH per mol of protein. The complement fixing reactivity of both of these derivatives with serum diluted 1:1000 was markedly reduced (Figure 3A). Even with a 1:100 serum dilution a reduced reactivity of the derivatives was evident (Figure 3B). Similarly the reactivity of dinitrophenylated N1 with a 1:400 serum dilution was completely destroyed. The N2 fragment and its derivative with 3.8 mol of N_2ph/mol of peptide were tested for the ability to inhibit the F1–anti-F1–reaction. In this case dinitrophenylation did not markedly alter the reactivity of the peptide (Figure 4). These data suggest that for antiserum to intact F1, some lysine residues in the N1 but not in the N2 fragment are involved in antigenic determinants. The role of lysine was further demonstrated by the finding that 0.2 μg of poly(L-lysine) caused complete inhibition of the F1–anti-F1 reaction. Poly(L-lysine) by itself does not fix complement when reacted with anti-F1. It is possible that in the case of intact F1 part of the loss of complement-fixing reactivity is due to dinitrophenylation of the single tyrosine residue.

Tyrosine Region as an Antigenic Determinant. Reaction of F1 with tetranitromethane modifies the single tyrosine residue and converts it to nitrotyrosine. The reaction is conducted under mild conditions (Sokolowsky *et al.*, 1966), thereby minimizing side reactions. Indeed, amino acid analysis of the nitrated F1 showed that the tyrosine was the only residue affected by nitration (Bustin, 1971). Therefore the results presented in Figure 5 pinpoint the tyrosine residue or its immediate vicinity as an antigenic determinant since, in com-

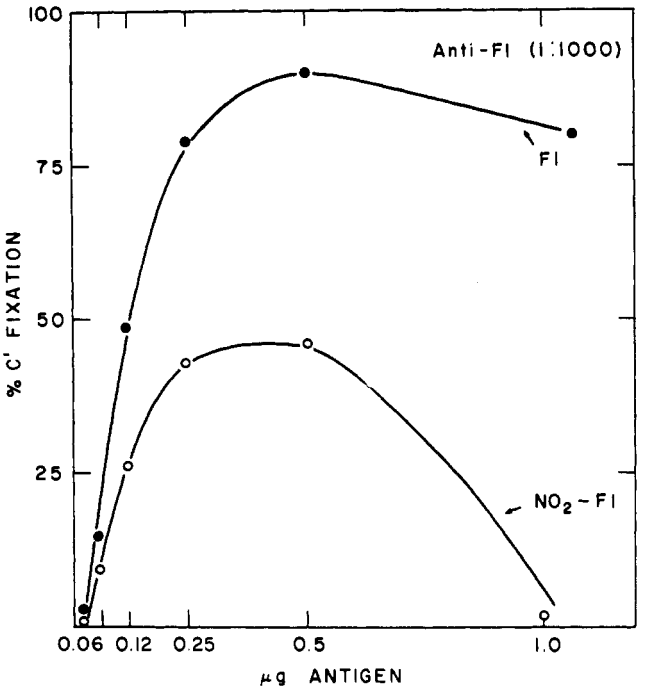

FIGURE 5: Effect of nitration on the complement fixation of F1 with anti-F1.

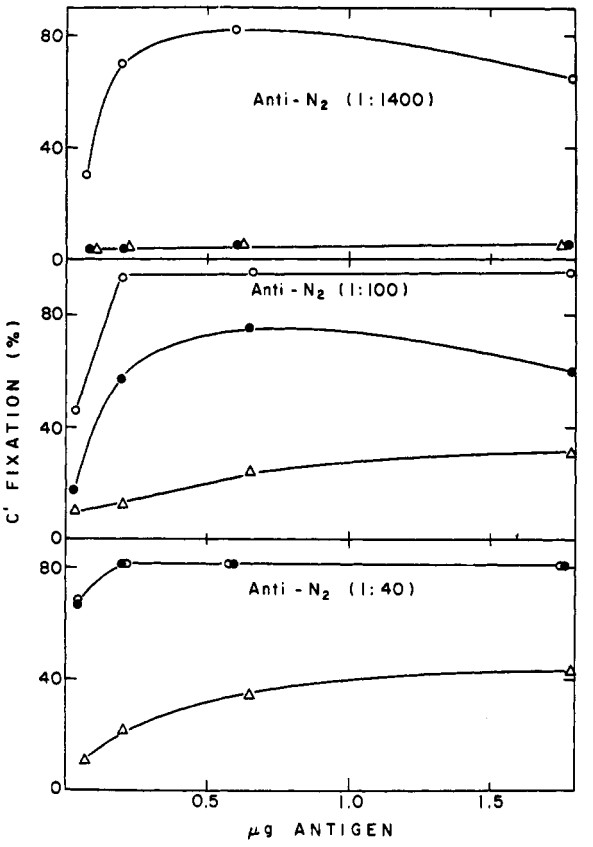

FIGURE 6: Complement fixation of anti-N2 sera with F1, N1, and N2: (○) F1, (●) N1, (△) N2.

parison with unmodified F1, the nitrated F1 reacted less effectively with anti-F1 antiserum.

The tyrosine-containing tryptic peptide can be readily isolated from a tryptic digest of F1 (Bustin, 1972). A 20-fold molar excess of this free peptide did not inhibit the F1–anti-F1 reaction. Higher concentrations could not be tested because they interfered with the complement system itself.

Intact Determinants in a Tryptic Digest of F1. While the isolated tyrosine-containing peptide was not inhibitory, the ability of a tryptic digest of F1 to inhibit the complement fixation reaction of F1 and anti-F1 was tested. This was done to see whether other fragments containing determinant structures might be identifiable. As shown in Figure 2A the presence of a 100-fold molar excess of trypsin-digested F1 completely inhibited the complement fixation reaction in the F1–anti-F1 system, while it had no effect on the prothrombin–anti-prothrombin system (Figure 2B). A 20-fold molar excess of the tryptic digest lowered the amount of complement fixation in the F1–anti-F1 system from 86 to 43%. Thus, determinants other than the tyrosine region, perhaps lysine-containing peptides, may be responsible for the inhibitory activity of the tryptic digest.

Immunogenicity of the N1 and N2 Fragments. When the ability of the separate fragments to induce specific antibodies was tested, a more complex picture was observed. The two fragments behaved differently during preparation of the immunizing complexes with RNA. While N1 formed a visible precipitate with RNA in the same way as did intact F1, the N2 fragment failed to form such a precipitate, even over a wide range of N2:RNA concentration ratios. Sera from animals immunized with the N1–RNA complexes failed to react with N1 or F1 alone or with N1–RNA complexes. Sera induced by the N2–RNA mixture did react, at a 1:40 dilution, to give 40% complement fixation with the N2 fragment (see Figure 6). Surprisingly, N1 reacted more strongly (70% complement fixation at 1:100 dilution) and intact F1 reacted to give 80% complement fixation with a 1:1400 serum dilution (Figure 6). The latter reaction with F1 was inhibited to the extent of 50% by 0.90 μg of N1 and by 0.22 μg of N2 (Figure 7). Apparently, the smaller N2 fragment induced sera which, unlike antiserum to intact F1, reacted with determinants common to both fragments and more effectively expressed in N1. This finding could be explained if lysine residues in isolated N2 participate in a determinant structure so that N1 and F1 were more effective by virtue of their greater polyvalency resulting from a higher lysine content. The role of lysine residues was again suggested by the finding that 0.4 μg of poly(L-lysine) completely inhibited the F1–anti-N2 reaction. Furthermore, dinitrophenylation of the lysine residues in N1 and F1 reduced their reactivity with anti-N2 sera (Figure 8). While the basis for reactivity of N1 and F1 with this serum may therefore have been their content of lysine-containing determinants, the N2 may also have had other reactive structures. Indeed dinitrophenylated N2 reacted more strongly than untreated N2 (Figure 8C), so that other determinants were more effectively expressed when some lysine residues were blocked.

Discussion

To study antigenic determinants of the F1 molecule, we have employed two approaches which have been applied to other protein antigens. These involve (1) the isolation of fragments which still react directly or which inhibit the reaction between the native antigen and its antiserum and (2) the comparison of serological reactivity of a specifically modified antigen with that of the native molecule (see Arnon, 1971, for numerous examples).

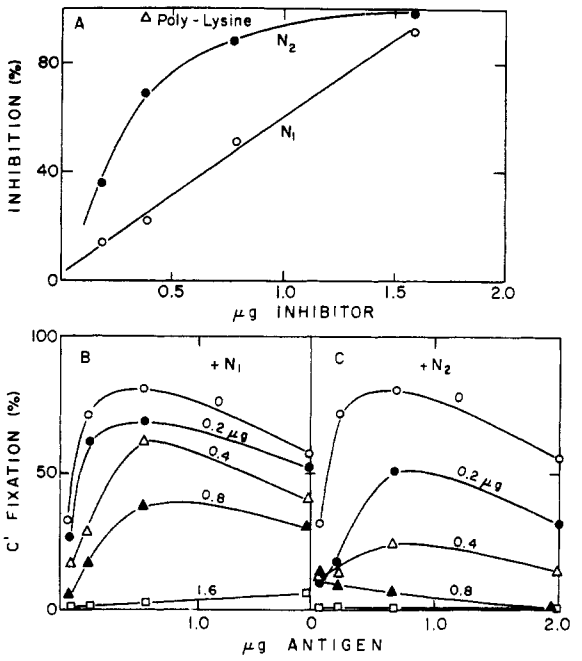

FIGURE 7: Inhibition of complement fixation in F1–anti-N2 system by N1, N2, and poly(L-lysine): (A) dependence of per cent inhibition on the amount of N1 and N2; (B) complement fixation curve in presence of various amounts of fragment N1; (C) complement fixation curve in presence of various amounts of N2. In all experiments anti-N2 was diluted 1400-fold. Prior to addition of antigen the sera were incubated for 1 hr at 25° with 0.2 ml containing the denoted amount of inhibitor.

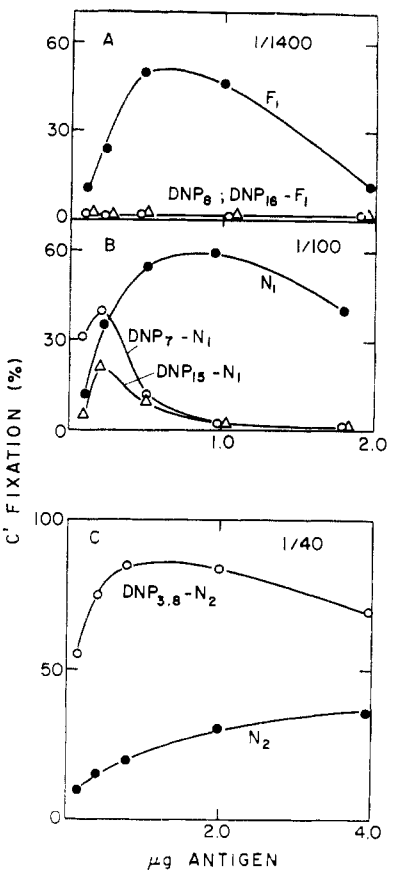

FIGURE 8: Complement fixation by anti-N2 sera reacted with F1, N1, and N2 and with their dinitrophenylated derivatives.

The results presented in this study reveal that distinct antigenic sites are located in both the amino-terminal third of the molecule and in the remaining carboxyl-terminal part. The amino-terminal portion (N2) failed to bind complement upon its interaction with anti-whole F1, but did specifically inhibit the F1–anti-F1 reaction, while the larger carboxyl-terminal portion (N1) was still directly reactive in complement fixation, though less so than the intact F1. The different behavior of the two fragments may reflect a difference in the number of antigenic sites in the two regions, as a polyvalent antigen is required for formation of a complement-binding aggregate with antibody. The finding that under the conditions used the amino-terminal portion (N2) did not inhibit the reaction of the carboxyl-terminal portion (N1) with anti-F1 indicates that the two fragments have different antigenic sites.

Further support for this notion can be found in the dinitrophenylation studies. Dinitrophenylation of about 13% of the lysines in either F1 or N1 suffices to completely abolish its reactivity with anti-F1 sera, while a derivative of N2 in which more than 30% of the lysine residues were dinitrophenylated can still fully inhibit the complement fixation in the F1–anti-F1 system (Figure 4). It can be concluded therefore that with anti-F1 sera lysine residues in N1 but not in N2 are involved in antigenic determinants. Because F1 molecules in solution are known to be practically devoid of ordered structure, it is not likely that the changes observed upon dinitrophenylation reflect conformational changes in the antigen.

An additional amino acid residue which seems to be involved in forming an antigenic determinant in the F1 molecule is the single tyrosine in position 72 (Rall and Cole, 1972). The reaction of tetranitromethane with the tyrosine residue in F1 (Bustin, 1971) is similar to that reported for other exposed tyrosine residues in proteins (Sokolowsky *et al.*, 1966). Thus, the finding that the interaction of the nitrated F1 with anti-F1 serum fixes less complement than does the interaction between native F1 and anti-F1 pinpoints the tyrosine residue, or its immediate vicinity, as an antigenic determinant. The tyrosine-containing peptide was isolated in an attempt to confirm this, but at concentrations which did not interfere with the complement system, it did not cause specific inhibition of the F1–anti-F1 reaction. Inhibition of protein–anti-protein reactions by peptide fragments has also been unsuccessful in other cases in which a specific determinant site has been identified by other means, as in the cytochrome *c*–anticytochrome *c* system (Reichlin *et al.*, 1970). On the other hand, some protein determinants can be identified by inhibition techniques (Arnon, 1971), and it is possible that other of the F1 histone determinants may be approached by this technique, since a tryptic digest of F1 did give full inhibition of the F1–anti-F1 complement fixation reaction.

As mentioned previously, on immunization with the whole F1 molecule the N1 portion appeared to contain more antigenic sites than did the N2 fragment. Yet complexes of just the N1 portion with RNA did not stimulate detectable antibody formation. A more complex picture was observed when the sera elicited by mixtures of N2 and RNA were tested. In this case, anti-N2 reacted weakly with the immunogen N2, more strongly with peptide N1 and even stronger with the intact F1 molecule (see Figure 6). The reaction with F1 could be explained if the F1 molecules form aggregates in solution so that the N2 fragment, which by itself has too low

a valence to form a complement-binding lattice with antibody, was effectively converted into a polyvalent form in the aggregate, without the N1 directly contributing antigenic sites. Such an influence of polyvalency in aggregates of antigen occur in other immune systems, such as cytochrome *c* (Reichlin *et al.*, 1970) for example. Alternatively if a determinant structure (or part of it) in N2 were fortuitously repeated in the N1 region, this might make the whole F1 molecule polyvalent and therefore effective in complement fixation. Indeed the N1 fragment contains determinants that cross-react with anti-N2 sera. Lysine residues seem to be involved in this determinant since polylysine, which by itself does not react with anti-N2, abolishes the reaction between anti-N2 and both F1 and N1. Moreover dinitrophenylation of part of the lysines in either N1 or F1 virtually abolishes their ability to fix complement upon interaction with anti-N2 sera. It can be concluded from these studies that lysine residues in isolated N2 fragment are involved in immunogenic determinants. However other determinants are also present in this fragment, since dinitrophenylated N2 reacted with anti-N2. The presence of such determinants was already detected in studies with sera against intact F1. Actually from Figure 8C it appears that dinitrophenylated N2 reacted somewhat stronger than untreated N2 (the reaction was still weak since it required a 1:40 sera dilution for 80% complement fixation). At face value however these results imply that blocking of some lysine residues leads to a situation where other determinants are more effectively expressed. It is pertinent in this respect to note that in the complexes used for immunization the lysine residues may interact with the phosphate groups in the RNA so that other regions of the fragment are immunodominant.

The behavior of the separated fragments as immunogens differs from their behavior as part of one molecule. Interestingly, the effects of the separated fragments in modifying the circular dichroism spectra of DNA were also not identical with the effects they produced as parts of the intact molecule (Fasman *et al.*, 1971).

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