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# Antibodies to Nucleic Acids. Immunochemical Studies on Dinucleoside Phosphate–Protein Conjugates*

Susan P. Wallace,† Bernard F. Erlanger, and Sam M. Beiser

ABSTRACT: Antibodies specific for purine dinucleoside phosphate were elicited by immunization with dinucleoside phosphate–bovine serum albumin complexes. The reactions of these antibodies were studied by microquantitative precipitin, agar gel diffusion, and when possible, complement fixation. The anti-dinucleoside phosphate sera recognize the purine–purine sequence. However, a greater portion of this specificity appears to be directed toward the coupled base than toward the terminal one, as indicated by cross-reaction with mononucleoside antigens corresponding to the purines in the homologous dinucleoside phosphate antigen. Similarly, anti-mononucleosides cross react with dinucleoside phosphate antigens whose coupled purine corresponds to the homologous antigen. The phosphodiester linkage also appears to be involved in the specificity of the anti-dinucleoside phosphate sera. This was indicated by the finding that some of the anti-dinucleoside phosphate sera reacted more strongly with other dinucleoside phosphate antigens containing the same coupled purine moiety than they did with the corresponding purine riboside antigen.

Antibodies reactive with nuclei acids can be elicited in various ways (Plescia and Braun, 1967). Among these has been immunization which conjugates of bases, nucleosides, and nucleotides with proteins or polyamino acids (Butler *et al.*, 1962; Tanenbaum and Beiser, 1963; Erlanger and Beiser, 1964; Sela *et al.*, 1964; Halloran and Parker, 1966; Ungar-Waron *et al.*, 1967). Conjugates have also been prepared using dinucleotides containing one photooxidized guanine residue complexed to a polyamine backbone (Van Vunakis *et al.*, 1968). In the former instances, the specificity is directed primarily toward the purine or pyrimidine base attached to the carrier molecule and in the latter case, the specificity is directed toward the non-photooxidized 5′-nucleotide. E. Nahon, B. F. Erlanger, S. M. Beiser (in preparation) have prepared several purine–pyrimidine and pyrimidine–purine conjugates using the method of Erlanger and Beiser (1964) in which the hapten is linked to the BSA[1] carrier. Antibodies induced by these dinucleotide conjugates are

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[1] Abbreviations used are: BSA, bovine serum albumin; GMP, guanosine 5′-monophosphate; ApG, adenylyl-3′,5′-guanosine; GpA, guanylyl-3′,5′-adenosine; ApA, adenylyl-3′,5′-adenosine.

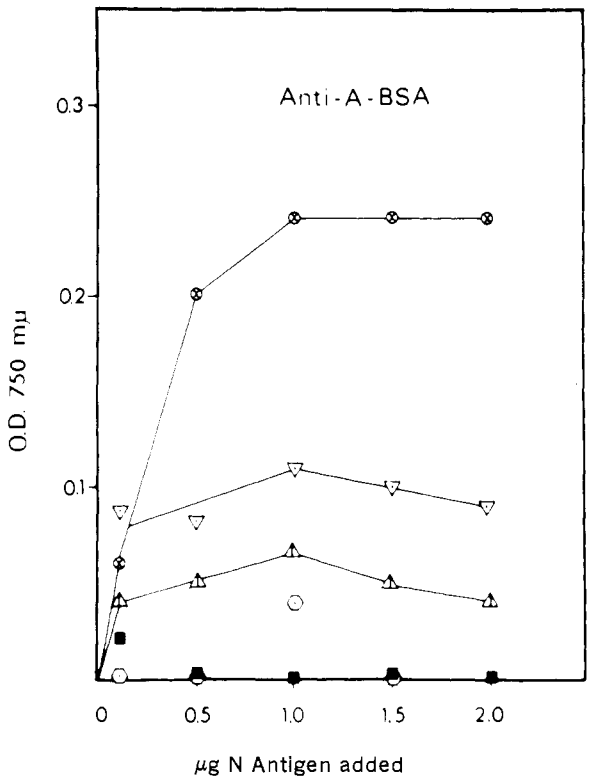


FIGURE 1: Precipitation of anti-A-BSA with mononucleoside and dinucleoside phosphate antigens. (⊗—⊗) A-BSA, (▽—▽) GpA-BSA, (△—△) ApA-BSA, (■—■) ApG-BSA, and (⊙—⊙) G-BSA.

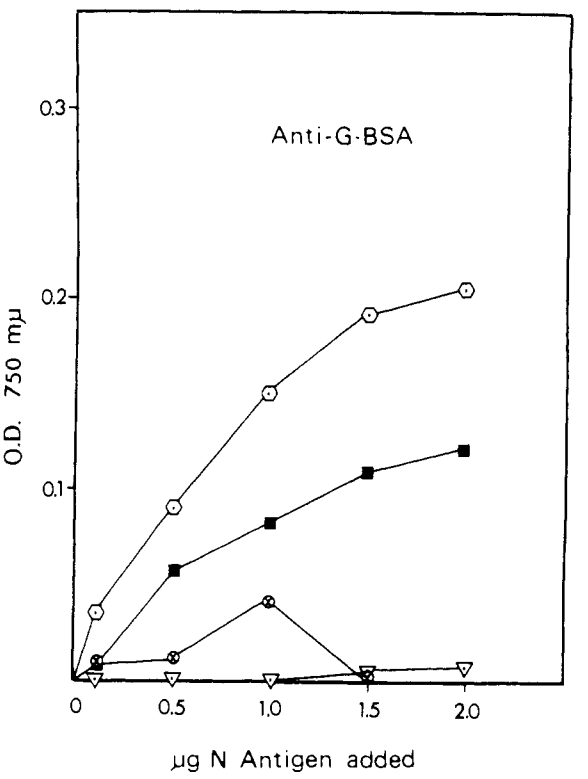


FIGURE 2: Precipitation of anti-G-BSA with mononucleoside and dinucleoside phosphate antigens. (⊙—⊙) G-BSA, (■—■) ApG-BSA, (⊗—⊗) A-BSA, and (▽—▽) GpA-BSA.

specific for the dinucleotide, and mainly for the base which is covalently linked to the BSA molecule.

The present studies describe the specificity of several antisera to purine–purine dinucleoside phosphates. Dinucleotides with purine linkages were used to minimize the possibility of cleavage of the external nucleoside moiety from the immunogen by circulating RNase in the rabbit serum. As a further precaution, the rabbits were preimmunized with RNase. Sela *et al.* (1964) reported that RNase in rabbit serum was inhibited by anti-RNase. For example, polyuridylic acid and RNA reacted with rabbit antiuridine antiserum in the presence of anti-RNase, but not when the anti-enzyme was omitted.

The specificities of the anti-dipurine antisera are similar to those seen with anti-purine–pyrimidine antisera (Beiser and Erlanger, 1966; E. Nahon, B. F. Erlanger, and S. M. Beiser, in preparation) insofar as all sera tested react most strongly with the homologous antigen, and cross react strongly with mononucleoside conjugates containing the internal nucleoside of the particular anti-dinucleoside phosphate investigated.

## Materials and Methods

*Antigens.* The dinucleoside phosphate haptens were conjugated to BSA by a modification of the method of Erlanger and Beiser (1964). Before the BSA was used for conjugation, it was treated with bentonite to remove any RNase present. Bentonite (20 mg) was added to 100 mg of BSA (Armour fraction V) in 8 ml of water. After stirring for 20 min at room temperature, the bentonite was removed by centrifugation. The procedure is then the same as that described for the conjugation of mononucleosides, until the addition of sodium borohydride. After this reduction, the conjugate was left in the cold for only 3 hr instead of 18 hr. No formic acid was added, and the dinucleoside phosphate conjugates were dialyzed overnight against running tap water and lyophilized Dinucleoside phosphates were obtained from Sigma Pharmaceuticals. All the conjugates prepared in this manner had absorption maxima identical with that of the corresponding dinucleoside phosphate hapten, and contained seven or eight groups for each BSA molecule. A-BSA and G-BSA, prepared as described previously (Erlanger and Beiser, 1964), were also studied. Concentrations of antigens were determined by Kjeldahl analysis (Kabat and Mayer, 1961).

*Antisera.* All antisera were prepared as described by Butler *et al.* (1962) except that the rabbits were immunized with RNase prior to immunization with the dinucleoside phosphate conjugates. The same procedure was used for preimmunization with RNase as was used for immunization with the conjugates; 0.1 ml of 1 mg/1 ml of antigen in complete Freund's adjuvants into each footpad three times at weekly intervals. All sera that were tested by quantitative precipitin or agar gel diffusion were first absorbed with BSA.

*Immunochemical.* Quantitative precipitin tests were done by the micromethod described in Kabat and Mayer (1961) which employs the Folin–Ciocalteu color reaction for the analysis of specific precipitates. Complement fixation was done by the method of Wasserman and Levine (1961) with the substitution of Tris-chloride buffer for Veronal (Butler *et al.*, 1965). Micro agar gel diffusion experiments were performed as described by Lacour *et al.* (1962). Gel hapten inhibition tests were carried out by diffusing the inhibiting hapten 24 hr before the antigens and antibodies were added.

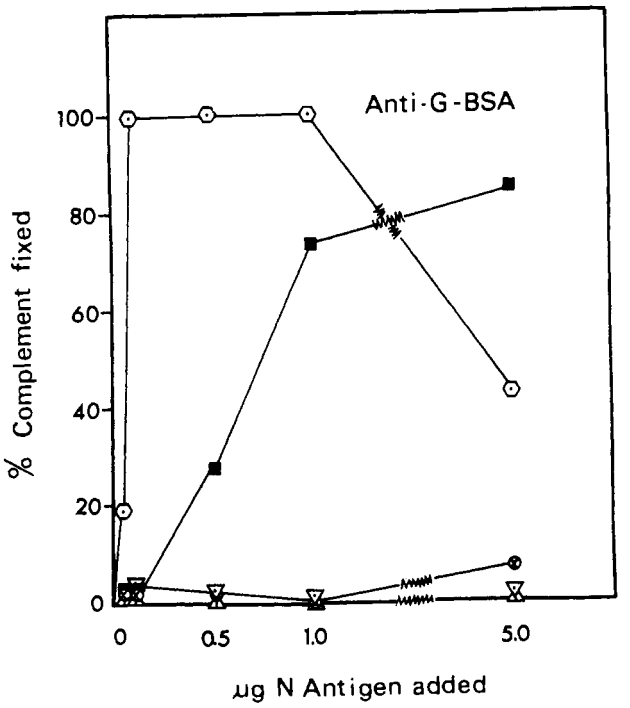


FIGURE 3: Complement fixation of anti-G-BSA with mononucleoside and dinucleoside phosphate antigens. (⊙—⊙) G-BSA, (■—■) ApG-BSA, (▽—▽) GpA-BSA, (⊗—⊗) A-BSA, and (△—△) ApA-BSA.

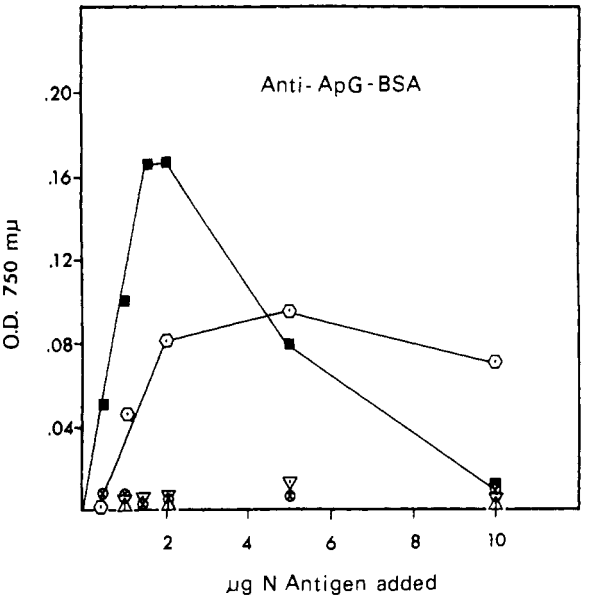


FIGURE 4: Precipitation of anti-ApG-BSA with mononucleoside and dinucleoside phosphate antigens. (■—■) ApG-BSA, (⊙—⊙) G-BSA, (▽—▽) GpA-BSA, (⊗—⊗) A-BSA, and (△—△) ApA-BSA.

## Results

*Reaction of Anti-mononucleoside Antibodies with Dinucleotide Antigens.* The reactions of anti-A and anti-G with the various mononucleoside and dinucleoside phosphate conjugated antigens were studied by precipitation, complement fixation, and agar diffusion. Figure 1 shows the precipitation of anti-A with homologous antigen and with ApG-BSA, ApA-BSA, and GpA-BSA. Anti-A reacts best with A-BSA, and then with GpA-BSA and ApA-BSA, which have the adenosine moiety attached directly to the BSA molecule. Similarly, the precipitation curves depicted in Figure 2 show that ApG-BSA, in which the guanosine is attached to the BSA, reacts better with anti-G than does GpA-BSA. Anti-A, by complement fixation, appeared to react almost equally with ApG-BSA, GpA-BSA, and ApA-BSA. On the other hand, as seen in Figure 3, anti-G reacts preferentially with G-BSA and ApG-BSA. The results obtained by using gel diffusion are recorded in Table I. These data reflect clearly the reactivity of anti-mononucleoside antisera with dinucleoside phosphate–BSA conjugates which contain the respective mononucleoside directly coupled to the protein. No reaction with the pyrimidine mononucleoside containing antigens was noted. In hapten inhibition, as seen in Table II, each component of a dinucleoside phosphate could inhibit the corresponding antibody.

*Reactions of Anti-ApG.* Anti-ApG antiserum, absorbed with G-BSA to eliminate some cross-reactivity with this conjugate, reacts primarily with ApG-BSA and G-BSA (Figure 4). Complement fixation tests, using unabsorbed antisera, demonstrated that A-BSA also reacts with this antiserum, but bound only about 50% of the amount of complement fixed in the reaction with G-BSA or ApG-BSA. No fixation was observed with ApA-BSA, GpA-BSA, or BSA.

*Reactions of Anti-GpA Sera.* The precipitation of anti-GpA with homologous and heterologous antigens is shown in Figure 5. The greatest precipitation is observed with GpA-BSA followed by A-BSA then ApG-BSA. There is also some precipitation with ApA-BSA and G-BSA. Table III shows the results of agar gel diffusion of three different anti-GpA sera. All three sera show the strongest bands with GpA-BSA followed by ApA-BSA and ApG-BSA. The reaction of anti-GpA-BSA with homologous antigen is inhibited by GpA and ApA, but is only slightly inhibited by ApG. Com-

TABLE I: Reactions of Anti-A and Anti-G with Conjugated Antigens.

| | A-BSA | G-BSA | ApA-BSA | GpA-BSA | ApG-BSA | BSA |
|---|---|---|---|---|---|---|
| Anti-A | 4+ | – | + | 2+ | – | – |
| Anti-G | – | 2+ | – | – | + | – |

TABLE II: Hapten Inhibition of Reactions of Anti-A and Anti-G with Homologous Antigens.[a]

| | A | G | Gmp | ApA | GpA | ApG |
|---|---|---|---|---|---|---|
| Anti-A + A-BSA | + | – | – | 3+ | + | + |
| Anti-G + G-BSA | – | 4+ | 3+ | – | + | 2+ |

[a] Hapten concentration is 1 μmole/ml; 4+ no band; 3+ barely visible band; 2+ faint band; 1+ visible band, but definitely lighter than control.

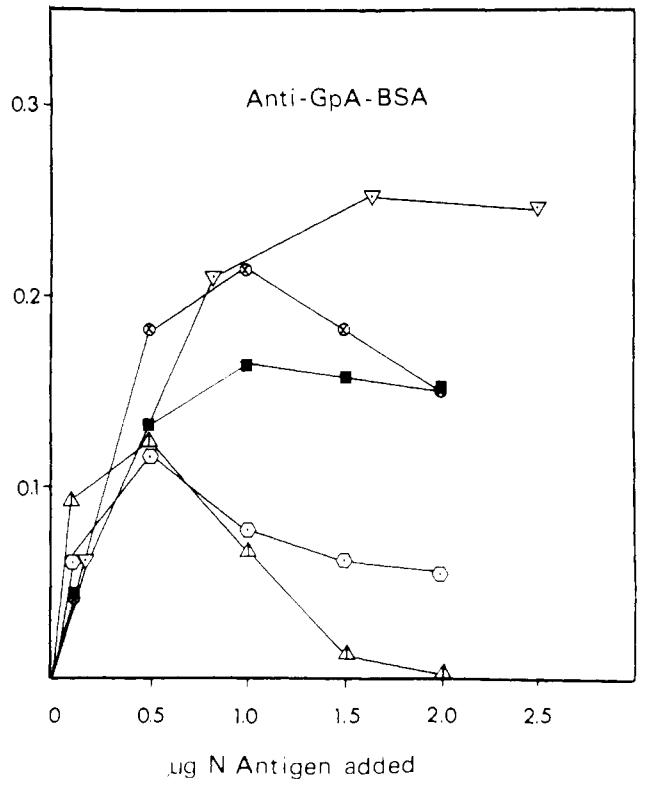


FIGURE 5: Precipitation of anti-GpA-BSA with mononucleoside and dinucleoside phosphate antigens. (▽—▽) GpA-BSA, (⊗—⊗) A-BSA, (■—■) ApG-BSA, (△—△) ApA-BSA, and (⊙—⊙) G-BSA.

plement fixation could not be done with these sera because they were anti-complementary.

*Reactions of Anti-ApA.* The results of agar gel diffusion with three anti-ApA sera are shown in Table IV. All three sera precipitate best with ApA-BSA and GpA-BSA and, in addition, sera 1 and 3 precipitate A-BSA. The reaction of anti-ApA with ApA-BSA in gel is inhibited by ApA, ApG, AMP, and GpA, but not by A, GMP, or G. No complement fixation was done because all sera were anticomplementary.

## Discussion

The anti-dinucleoside phosphate antibodies studied in these experiments, as expected, all react most strongly with their homologous antigens. These antibodies also cross react with mononucleoside antigens which have a purine moiety corresponding to the conjugated purine–ribose in the homologous dinucleoside phosphate antigen, and with other conjugates with the same purine coupled directly to the protein. When the anti-dinucleotide antibodies were tested by gel diffusion, no cross-reaction was observed with mononucleoside antigens having a purine moiety corresponding to the terminal purine in the homologous dinucleoside phosphate antigen. However, by complement fixation, a reaction of A-BSA with anti-ApG could be detected. Reichlin *et al.* (1964) also found complement fixation to be a more sensitive method in their studies on abnormal hemoglobins. However, the specificity of anti-dinucleoside phosphate sera for the coupled moiety is apparent.

Similarly, gel diffusion shows that the anti-mononucleoside antibodies react most strongly with homologous antigens, and cross react with dinucleotide antigens containing the same nucleoside directly coupled to BSA. Here again, complement fixation tests appear to be more sensitive in that anti-A reacted equally well with ApG-BSA, GpA-BSA, and ApA-BSA. Anti-G by complement fixation, gave the same pattern as was seen in gel diffusion, and reacted only with ApG-BSA of the dinucleoside phosphate conjugates tested. Thus the specificity of the anti-mononucleoside sera encompass more than just the base, and probably includes at least part of the conjugated ribose.

The anti-dinucleotide sera recognize the purine sequence as indicated by their reactions with the homologous antigens. Further, the specificity is directed more toward the coupled base, including the conjugated ribose, rather than toward the terminal moiety. This can be deduced from the fact that anti-ApG reacted well with G-BSA, but not with GpA-BSA or with A-BSA. Anti-GpA reacted both with ApA-BSA and with ApG-BSA, but A-BSA reacted better with this antiserum than did G-BSA.

The phosphate moiety may also be involved in specificity. This is particularly evident from the data recorded in Table IV. Anti-ApA did not react with G-BSA, but reacted better with GpA-BSA than with A-BSA. In fact, one serum did not react with either A-BSA or G-BSA, but reacted with GpA-BSA. The phosphate moiety has also been shown to play a role in the specificity of anti-adenosine monophosphate sera (Klein *et al.*, 1966). Recently, Van Vunakis *et al.* (1968) have shown that antibodies directed toward photooxidized mononucleotide antigens and photooxidized dinucleotide antigens show specificity for the ribose 5′ moiety. In addition, the second purine (G) may alter, somewhat, the configuration of the first purine (A), so that the A in GpA-BSA more closely resembles ApA-BSA than does the A in A-BSA.

Anti-dinucleoside phosphate sera may have specificities that do not occur in anti-mononucleoside sera. It was even possible to obtain an anti-ApA serum that did not react with A-BSA. It would seem probable, therefore, that antisera to tri-, tetra-, or pentanucleotide sequences might also have unique specificities, and might be used for structural studies of nucleic acids. Preliminary experiments indicate that these

TABLE III: Reactions of Anti-GpA with Conjugated Antigens.

| | GpA-BSA | ApA-BSA | ApG-BSA | A-BSA | G-BSA | C-BSA | BSA |
|---|---|---|---|---|---|---|---|
| Anti-$GpA_1$ | 2+ | + | + | ± | − | − | − |
| Anti-$GpA_2$ | 4+ | 2+ | 2+ | + | − | − | − |
| Anti-$GpA_3$ | 4+ | 2+ | 2+ | + | ± | − | − |

TABLE IV: Reactions of Anti-ApA with Conjugated Antigens.

| | ApA-BSA | GpA-BSA | ApG-BSA | A-BSA | G-BSA | BSA |
|---|---|---|---|---|---|---|
| Anti-$ApA_1$ | 2+ | 2+ | − | + | − | − |
| Anti-$ApA_2$ | 2+ | 2+ | − | − | − | − |
| Anti-$ApA_3$ | 2+ | 2+ | ± | + | − | − |

antibodies to dinucleoside phosphate react to a lesser extent with denatured DNA than do anti-mononucleosides. This decreased reaction could be caused by a difference in structure between the dinucleotide hapten and the dinucleotide linked in the denatured DNA molecule, or may reflect the more frequent occurrence of a mononucleoside than of a specific dinucleotide. We are attempting to determine if the anti-dinucleotide sera show more specificity in their reactions with DNAs of varying base composition than do anti-mononucleoside sera.

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# Fate of *Bacillus subtilis* Transforming Deoxyribonucleic Acid Incorporated into Transformable *Diplococcus pneumoniae**

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ABSTRACT: The fate of foreign DNA within a bacterial cell was examined by incorporation of biologically and isotopically labeled *Bacillus subtilis* 168$^+$ DNA (donor) into genetically transformable and biologically labeled *Diplococcus pneumoniae* R1 cells (recipient). DNA was subsequently extracted from recipient cells and studied physically and biologically. Portions of DNA extracted from recipient cells after exposure to donor DNA were denatured by heat or alkali, sonicated, sonicated and denatured, or untreated, and then subjected to CsCl equilibrium density gradient centrifugation. Gradient fractions were analyzed for physical (radioactivity and heavy density of donor DNA) and biological (transforming activity of recipient DNA) markers. The results indicated that elements of donor DNA too small to manifest the heavy density label were incorporated by covalent linkage into resident DNA. It was not possible to isolate from recipient cells as early as 1 min after exposure to native donor DNA heavy density native or denatured donor DNA, or DNA which contained the biological activity of donor DNA or was specifically hybridizable with donor DNA. Recipient cells were not transformed for the donor biological markers sulfanilamide and erythromycin resistance, nor were their growth rates altered over a 3-hr period. Presence in the transformation medium of deoxynucleosides and deoxynucleotides in about 160 times greater abundance than present in the deoxynucleotide components of donor DNA failed to decrease and usually increased incorporation of donor DNA into recipient DNA despite isotopic evidence for simultaneous incorporation of the deoxynucleosides and deoxynucleotides into recipient DNA. Inhibition of DNA synthesis by 50 $\mu$g/ml of fluorodeoxyuridine also failed to decrease incorporation of elements of donor DNA into resident DNA. These results suggest the possibility that small oligodeoxynucleotides derived from native DNA of a disparate species may be incorporated into the DNA of *Diplococcus pneumoniae* cells under transforming conditions.

Results of genetic recombination experiments by transformation with *Diplococcus pneumoniae*, *Bacillus subtilis*, and *Hemophilus influenzae* systems have been consistent with a model of covalent linkage of a single stranded segment of transforming DNA to recipient DNA presumably at the homologous region as dictated by base-pair recognition

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