Production of Antibodies with Specificity toward Adenosine, Uridine, and Ribose 5′-Phosphates*

Helen Van Vunakis, Edna Seaman, Peter Setlow, and Lawrence Levine

ABSTRACT: During the photooxidation of guanine-containing nucleotides and dinucleotides, the guanine moiety is destroyed to yield a reactive photointermediate which condenses with the amino groups of a copolymer composed of glutamyl and lysyl residues. Immunization of rabbits with such conjugates complexed to methylated bovine serum albumin results in the production of antibodies directed toward the haptens as well as toward the random copolymer. The specificities of the antisera to conjugates obtained with guanosine 5′-phosphate (GMP-5′), GpA, and GpU photoproducts were examined by complement fixation. The antibodies directed toward the mononucleotide conjugate show specificity toward the ribose 5′-phosphate moieties of the nucleotides. In sera of rabbits immunized with photooxidized GpA and GpU conjugates, the antibodies are directed toward the 5′-nucleotides, adenosine 5′-phosphate (AMP-5′) and uridine 5′-phosphate (UMP-5′), respectively. With the latter antisera, structural changes in the base, the sugar, or the position of the phosphate group alter the serologic activity of the nucleotide.

The anti-[G]pA conjugate immune system, in which AMP-5′ is the antigenic determinant, is inhibited by coenzymes and coenzyme analogs in a predictable manner, *i.e.*, diphosphopyridine nucleotide (DPN), adenosine diphosphate ribose (ADPR), and flavin–adenine dinucleotide (FAD) which contain AMP-5′ as an integral part of their structure are good inhibitors, while nicotinamide mononucleotide (NMN), triphosphopyridine nucleotide (TPN), and riboflavin 5′-phosphate (FMN) are poor inhibitors. With the poly Glu,Lys-[G]MP-5′ immune system in which ribose 5′-phosphate is the antigenic determinant, the order of effective inhibition is NMN > DPN > ADPR > FAD > TPN and FMN.

Based on the classic studies of Landsteiner (1936) in which antibodies specific to a variety of small molecules were obtained upon immunization with conjugates of such small molecules with macromolecules, antibodies specific for nucleic acids have been obtained by immunizing rabbits with conjugates composed of bases, nucleosides, or nucleotides linked to 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). In the majority of these syntheses, the nucleic acid component is usually linked *via* the sugar or phosphate residue to the carrier, and with most immune systems studied the antibodies produced using such immunogens exhibit specificity toward the purine or pyrimidine base. In sera of rabbits immunized with nucleoside 5′-carboxylic acids attached to multichain poly-DL-alanine (Sela *et al.*, 1964; Ungar-Waron *et al.*, 1967), antibodies showed specificity toward the entire nucleoside and in one of the five nucleoside immune systems tested, the antibodies differentiated the riboside from the deoxyriboside.

In the syntheses described here, guanine-containing mono- and dinucleotides are coupled to the amino groups of polyamino acids rich in lysine residues through a photointermediate formed during the photooxidation of the guanine moiety (Van Vunakis *et al.*, 1966). Antisera to such antigens contain antibodies directed toward the phosphate and sugar moieties when the mononucleotide is used as the hapten, and the phosphate, sugar, and base of the 5′-nucleotide when certain dinucleotides are used as haptens. The preparation of such antigens and the specificities of the resulting antibodies toward nucleotide derivatives, various coenzymes, and coenzyme analogs are the subject of this communication.

Materials and Methods

The coplymer of glutamic acid and lysine (poly $Glu_{60}Lys_{40}$, lot C-32; mol wt 115,000) was purchased from Pilot Chemicals. The guanine nucleotides and dinucleotides were obtained from Calbiochem. Methylene blue (zinc free), from Matheson Coleman and Bell, was used as the photosensitizing dye. The source of the coenzymes and their analogs was P-L Biochemicals for DPN^+,[1] TPN^+, NMN^+, and ADPR and Nutritional

* Publication No. 552 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received December 11, 1967.* Supported in part by research grants from the National Institutes of Health (AI-01940 and AI-02792) and the American Cancer Society, Inc. (E 222-G). H. V. V. is a recipient of a Public Health Service career award (5-K6-AI-2372), L. L. is an American Cancer Society Professor of Biochemistry (Award No. PRP-21), and P. S. is a predoctoral fellow of the Revson Foundation.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966) are: [G], the unknown photointermediate formed during the photooxidation of guanine which condenses with the ε-amino groups of lysine; C′, complement.

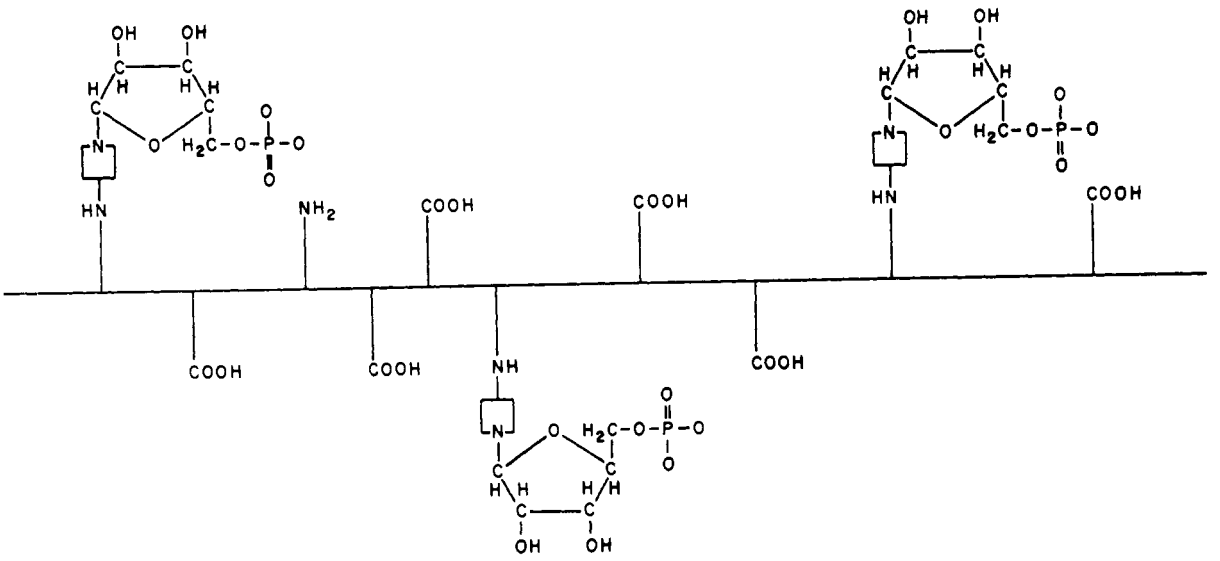

FIGURE 1: Schematic representation of the product resulting when GMP-5′ is photooxidized in the presence of poly Glu,Lys.

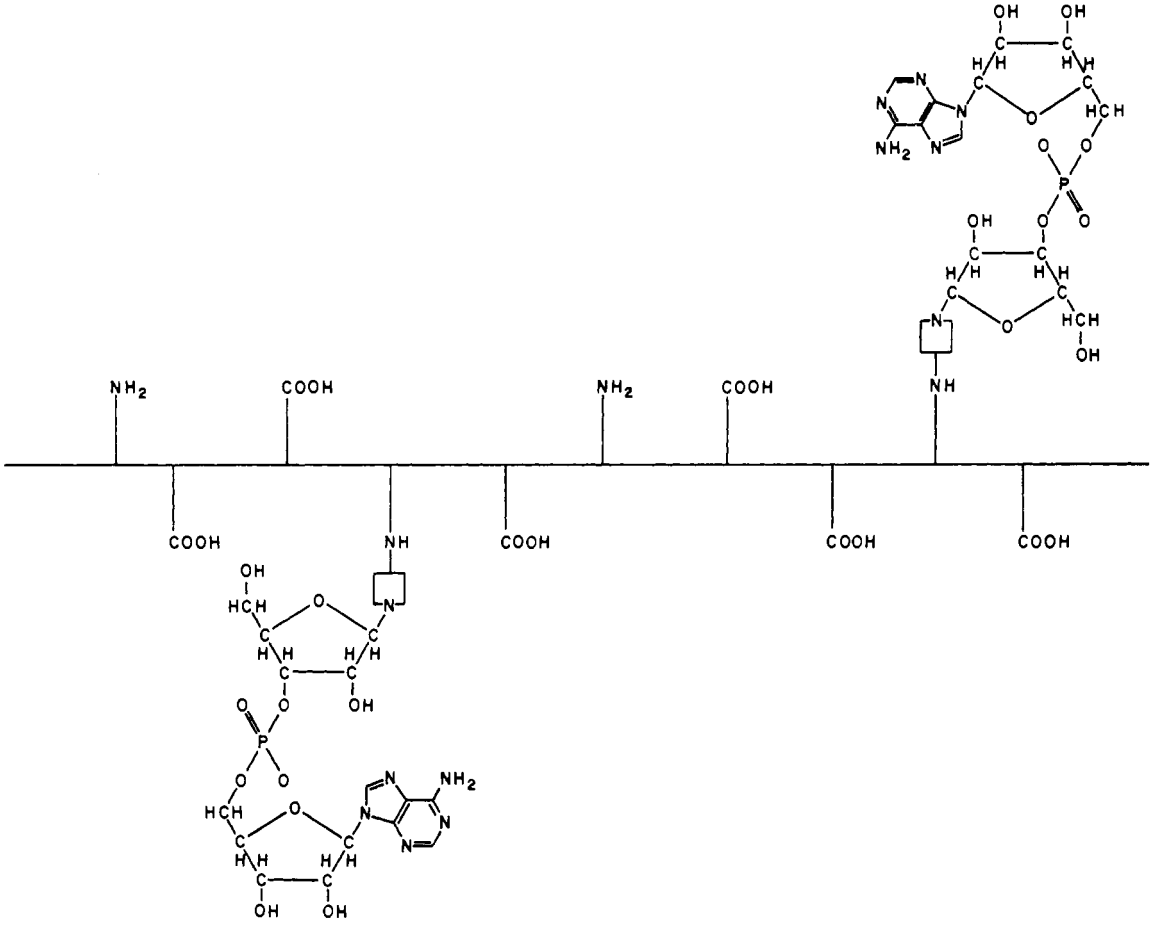

FIGURE 2: Schematic representation of the product resulting when GpA is photooxidized in the presence of poly Glu,Lys.

Biochemicals for FAD and FMN.

Antigenic Conjugates. The guanine derivative (5 mg/ml) was photooxidized in the presence of 5 mg/ml of the poly Glu,Lys (mol wt 115,000) at pH 8.5 and 10° using 20 μg/ml of methylene blue as the photosensitizing dye. O_2 was bubbled through the reaction mixture and the pH was maintained by addition of 0.2 M NaOH. The photooxidation apparatus used was similar to the one described by Simon and Van Vunakis (1962). Aliquots were removed periodically for spectral analysis and the reaction was terminated when there was no longer any change in the optical density at 250 mμ. After exhaustive dialysis to remove unbound photoproducts, aliquots of the reaction mixture were assayed

TABLE I: Extent of Incorporation of Photoproduct into the Copolymer.

Substituted Polymer	Photoproduct : Lysine[a]
Poly Glu,Lys-[G]MP-5′	1:10
Poly Glu,Lys-d[G]MP-5′	1:11
Poly Glu,Lys-[G]pA	1:13
Poly Glu,Lys-[G]pU	1:11
Poly Glu,Lys-Ap[G]	1:18
Poly Glu,Lys-Cp[G]	1:12
Poly Glu,Lys-[G]pC	1:14

[a] In micromoles.

for phosphate content (Chen *et al.*, 1956). The ratio of phosphates to lysine residues (Table I) indicates the extent of covalent linkage of the guanine derivative to the polymer. The macromolecular antigens formed by such syntheses are shown schematically in Figure 1 for poly Glu,Lys-[G]MP-5′ and in Figure 2 for poly Glu,Lys-[G]pA.

Antisera. Rabbits were immunized with a complex of the above synthetic antigens (containing 0.1 μmole of photoproduct) and methylated bovine serum albumin (Plescia *et al.*, 1964) in complete Freund's adjuvant *via* the toepads at weekly intervals, and were bled 1 week after the fourth injection. At monthly intervals thereafter, booster injections were given intramuscularly. The C′ fixation and the C′ fixation inhibition methods of Wasserman and Levine (1960) were used in the serologic analyses. Immune precipitation experiments have not been done. Thus, the antibody nitrogen content of the antisera has not been determined.

Results

The C′ fixation curves obtained with two antisera to poly Glu,Lys-[G]MP-5′ with the antigens, poly Glu,-Lys-[G]MP-5′ and poly Glu,Lys-d[G]MP-5′, and the carrier, poly Glu,Lys, are shown in Figure 3. It can be seen that at the dilutions of antisera used in these experiments, the antibodies show specificity toward the [G]MP-5′ residues. The two antisera differ in their degree of specificity, antiserum A being able to distinguish ribose from deoxyribose to a greater extent than antiserum B. Antiserum B contains antibodies directed toward the carrier poly Glu,Lys whereas at the antiserum dilution used antiserum A does not. It should be noted that antibodies to poly Glu,Lys are also present in antiserum A and can be demonstrated if the antiserum is used at a higher concentration.

The specificity of antiserum A toward the base, the sugar, and phosphate of various nucleotides was tested using a more discriminating assay, inhibition of the homologous immune systems by small molecules of known structure (Table II). In the poly Glu,Lys-[G]MP-5′–anti poly Glu,Lys-[G]MP-5′ system, all of the ribo-

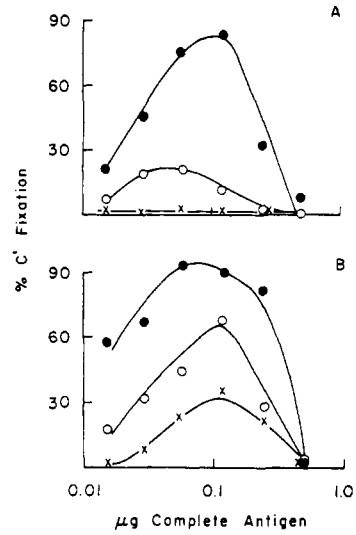

FIGURE 3: Complement-fixation reaction of two anti-poly Glu,Lys-[G]MP-5′ sera with poly Glu,Lys (×), poly Glu,Lys-d[G]MP-5′ (○), and poly Glu,Lys-[G]MP-5′ (●).

nucleoside 5′-phosphates tested were about equally effective as inhibitors. In addition, GMP photooxidized in ε-aminocaproic acid to form an adduct which would resemble the ε-NH_2-Lys-[G]MP-5′ linkage in the immunogen was no more effective as an inhibitor than the untreated nucleotides or GMP photooxidized in the presence of other molecules such as lysine or Tris. The purine or pyrimidine structures do not have to be present in order to effectively inhibit the immune reaction. In fact, complete absence of the base as in ribose 5′-phosphate yields an inhibitor only two to three times less effective than the nucleotides themselves. The antibody-combining site therefore does not encompass the base although a C–N bond at the ribose 1 position appears to be required for maximal inhibition.

In agreement with the results obtained by direct fixation (Figure 3), the deoxyribonucleotide (dCMP-5′) is less effective than the ribonucleotide (CMP-5′) as an inhibitor. There is no inhibition by the ribonucleosides (the derivatives of the four bases commonly found in RNA were tried), indicating that the phosphate group is an essential part of the antigenic determinant.

The inhibitory effectiveness of molecules which have the phosphate group esterified to the various hydroxyl positions of the sugars was next tested. Whereas 40 mμmoles of ribose 5′-phosphate gave 50% inhibition of the poly Glu,Lys-[G]MP-5′ immune system, ribose 1′-phosphate gave no inhibition even when 470 mμmoles of this compound was used. No inhibition at 100-mμmole levels was obtained with nucleotides bearing the phosphate groups at the 2′- or 3′-hydroxyls, *i.e.*, AMP-2′ and AMP-3′. The antibody can also distinguish between a mono- and diesterified phosphate. Cyclic AMP-3′,5′ and the dinucleotides GpU and UpG which contain the required ribose 5-phosphate moiety in a diesterified form fail to inhibit the reaction at least at the 100-mμmole level. The decreasing order of effectiveness of the mono-, di-, and triphosphates may not be due to the proximity of monoesterified phosphate, but rather to discrimination against all molecules

TABLE II: Inhibition of the Poly Glu,Lys-[G]MP-5′ Immune System.[a]

Inhibitor	mμmoles Required for 50% Inhibn	Inhibitor	mμmoles Required for 50% Inhibn	Inhibitor	mμmoles Required for 50% Inhibn
GMP-5′	13	AMP-3′	>100[b]	Ribose 5′-phosphate	40
		AMP-2′	>100[b]		
ε-NH_2-caproic-[G]-MP-5′	6	Cyclic AMP-3′,5′	>100[b]	Ribose 1′-phosphate	>470[b]
Lysine-[G]MP-5′	6	CMP-5′	16	Uridine	>400[b]
Tris-[G]MP-5′	11	CDP	>100[c]	Guanosine	>400[b]
AMP-5′	8	CTP	>100[b]	Cytidine	>400[b]
UMP-5′	15	dCMP-5′	90	Adenosine	>400[b]

[a] Immune system: poly Glu,Lys-[G]MP-5′, 0.1 μg; antiserum diluted 1:1200. [b] 10% inhibition or less at these levels. [c] 22% inhibition with 100 mμmoles.

with substituents other than a monoesterified phosphate on the 5′-hydroxyl, with discrimination becoming more severe the bulkier the substituent group.

Although antibodies have been produced to several guanine-containing dinucleotides, only the antibodies produced against poly Glu,Lys-[G]pA and poly Glu,-Lys-[G]pU will be considered here. Antibodies in the poly Glu,Lys-[G]pA serum appear to be directed toward the adenosine 5′-phosphate moiety of the [G]pA. As measured by C′ fixation this serum reacts only with the homologous antigen and, at the dilution of antiserum used, does not react with the poly Glu,Lys or with the antigens made by photooxidizing ApG, CpG, GpC, or GpU in the presence of the carrier. The homologous immune system is inhibited most effectively by GpA and AMP-5′. GMP-5′ is inhibitory at a concentration 20 times higher than AMP-5′, while the pyrimidine ribonucleotides are ineffective at the highest concentrations used (Table III). Adenosine is inhibitory at higher concentrations than AMP-5′. The 5′-phosphate moiety appears to play a lesser role here than it did in the mononucleotide immune systems. AMP-2′, AMP-3′, and cyclic AMP-3′,5′ are poor inhibitors of this system. A possible reason for the poor inhibition of the AMP-2′ and AMP-3′ is discrimination against the negative charges or bulky groups in this position. Alternatively, perhaps the presence of the phosphate group on carbons 2 or 3 may change the confirmation of the nucleotide sufficiently to render it unrecognizable by the antibody. The sugar of the nucleotide is also involved in the specificity, since dAMP-5′ is ineffective as an inhibitor even when used at tenfold greater concentration than AMP-5′. With the dinucleotide inhibitors, GpA is the most effective. Dinucleotides in which the 5′-nucleotide is a guanine, *i.e.*, ApG, CpG, and UpG, are also inhibitory, but at five- to tenfold higher concentrations.

TABLE III: Inhibition of Poly Glu,Lys-[G]pA Immune System.[a]

Inhibitor	mμmoles Required for 50% Inhibn	Inhibitor	mμmoles Required for 50% Inhibn
GpA	18	AMP-2′	>300[c]
AMP-5′	24	AMP-3′	>300[c]
GMP-5′	580[b]	Cylic AMP-3′5′	>288[c]
UMP-5′	>225[c]	ApG	91[b]
CMP-5′	>250[c]	CpG	92[b]
Adenosine	140	UpG	267[b]
dAMP	>300[c]	GpU	>70[c]
ADP	22	GpC	>70[c]
ATP	20		

[a] Immune system: poly Glu,Lys-[G]pA, 0.5 μg; antiserum diluted 1:400. [b] Extrapolated value. [c] 10% inhibition or less at these levels.

The antisera directed against poly Glu,Lys-[G]pU also showed specificity toward the uridine 5′-monophosphate moiety of the dinucleotide. At dilutions of anti-poly Glu,Lys-[G]pU in which the homologous antigen gave 85% fixation, the antigen in which cytosine had replaced uracil, poly Glu,Lys-[G]pC, showed approximately a 20% cross-reaction while poly Glu,-Lys-[G]pA, poly Glu,Lys-Ap[G], poly Glu,Lys-Cp[G], and poly Glu,Lys did not react.

Again, hapten inhibition experiments were done to study the specificity of the [G]pU immune system (Table IV). GpU and UMP-5′ are potent inhibitors of the immune system and equally effective. The pyrimidine nucleotides, CMP-5′ and rTMP-5′, are 15 to 25 times less effective while the purine ribonucleotide monophosphates are not inhibitory. Significant alterations in the uracil moiety, *i.e.*, by subjecting UMP-5′ to ultraviolet radiation to form dihydrouridylic acid or by addition of a carboxyl group in the ring structure (orotidylic

TABLE IV: Inhibition of Poly Glu,Lys-[G]pU Immune System.[a]

Inhibitor	mμmoles Required for 50% Inhibn	Inhibitor	mμmoles Required for 50% Inhibn	Inhibitor	mμmoles Required for 50% Inhibn
Uridine	640	UMP-5′ (ultraviolet irradiated)	>100[b]	GpU	30
UMP-5′	24	CMP-5′	330	UpU	23
UDP	65	Orotidylic acid	>296[b]	ApG	>200[b]
UTP	90	rTMP-5′	560	GpA	>200[b]
UMP-3′(2′)	>310[b]	AMP-5′	>288[b]	GpC	>210[b]
dUMP-5′	>325[c]	GMP-5′	>276[b]	CpG	>210[b]
				UpG	700

[a] Immune system: poly Glu,Lys-[G]pU, 0.2 μg; antiserum diluted 1:600. [b] 10% inhibition or less at these levels. [c] 25% inhibition at this level.

acid), decrease the effectiveness of the inhibitors. Uridine is about 30 times less efficient than UMP-5′ in causing an equivalent amount of inhibition while dUMP-5′ is relatively inactive. These results demonstrate the requirement of a pyrimidine base closely resembling uracil and the necessity of the ribose structure in contrast to the deoxyribose for complementarity.

The requirement of the 5′-phosphate as opposed to other linkages is shown by the lack of inhibition by UMP-3′(2′) and the relatively effective inhibition of UDP and UTP. Of the dinucleotides, UpU was as effective an inhibitor as GpU, but GpC showed no inhibition. UpG in which the phosphate linkage to uridine is 3′ at the hydroxyl of the sugar is 20 to 30 times less inhibitory. ApG, CpG, and GpA nucleotides in which a purine has replaced a pyrimidine on the 5′-nucleotide end were predictably ineffective as inhibitors.

Some of the coenzymes and coenzyme analogs which are important in biological systems have structures related to the nucleotides. Table V gives the concentrations of several coenzymes and coenzyme analogs which give 50% inhibition of the poly Glu,Lys-[G]MP-5′ and poly Glu,Lys-[G]pA immune systems. For comparative purposes, values are also presented for AMP-5′. In the poly Glu,Lys-[G]pA immune system, DPN^+, FAD, and ADPR which contain AMP-5′ as an integral part of their structure are equally as effective as AMP-5′. TPN^+, FMN, and NMN^+, however, which do not contain the AMP-5′ moiety are ineffective as inhibitors. The reaction of these compounds in the poly Glu,-Lys-[G]MP-5′ immune system is slightly more complex. TPN^+ and FMN are ineffective inhibitors, while ADPR and FAD show 50% inhibition at rather high concentrations compared to AMP-5′. DPN^+ is one-tenth as effective as AMP-5′, while NMN^+ is one-half as effective, but this latter difference may not be significant.

TABLE V: Inhibition of Poly Glu,Lys-[G]MP-5′ and [G]-pA Immune Systems.

	mμmoles Required for 50% Inhibition	
	Anti-[G]pA	Anti-[G]MP-5′
AMP-5′	24	7
DPN^+	22	70
ADPR	14	280
FAD	21	300
NMN^+	>572[a]	15
TPN^+	<240[a]	>240[b]
FMN	>456[a]	>456[a]

[a] 10% inhibition or less at these levels. [b] 19% inhibition at this level.

Discussion

During irradiation of guanine derivatives with visible light in the presence of a photosensitive dye, a photointermediate is formed which condenses with the free ε-amino groups in the copolymer poly Glu,Lys to yield the macromolecules which served as antigens in this study. The sugar, phosphate, and bases other than guanine are not photooxidized under our conditions. These moieties are not involved in forming bonds with the carrier and are thus exposed to the antibody synthesizing site. Without knowing the chemistry of the reaction in detail, it was predicted that the antibodies formed against these haptens would have a combining site which would encompass at least the phosphate and sugar moieties of the mononucleotide, and the base, sugar, and phosphate moieties in the dinucleotides. As shown, the antibodies formed against poly Glu,Lys-[G]MP-5′ are directed primarily toward the monoesterified ribose 5′-phosphate moiety of the nucleotide, the presence and

the position of the phosphate on the ribose being of prime importance. The antibodies formed against poly Glu,Lys-[G]pA or poly Glu,Lys-[G]pU are directed toward the purine or the pyrimidine nucleotide attached to the photooxidized guanosine residues.

Keeping the specificity requirements in mind, one can see why DPN^+, ADPR, and FAD are equally as effective as AMP-5′ in inhibiting the anti-poly Glu,Lys-[G]-pA immune system. These compounds contain AMP-5′ as an integral part of their structure and fulfill the specificity requirements of this system. The substituents on the 5′-phosphate differ for the three molecules, but the combining site of the antibody does not appear to extend beyond this phosphate as shown by the almost identical reactivity of GpA, AMP-5′, ADP, and ATP (Table III). One point which might seem surprising is the inability of the antisera to discriminate between DPN^+ or FAD to a greater extent. Both of these molecules exist in an "internal complex" (Weber, 1950, 1957) which might be expected to hinder the accessibility of the AMP-5′ portion of the molecule. It is also possible that the combination of the coenzyme with the antibody is sufficiently strong to disrupt any noncovalent linkages which exist. FMN, NMN^+, and TPN^+ are inert in this immune system. FMN and NMN^+ have no adenine moieties and TPN^+, although containing adenine and ribose, has an additional 2′-phosphate on the AMP-5′ portion of the molecule.

Four of the coenzymes and coenzyme analogs exhibit a range of inhibitory effectiveness in the poly Glu,-Lys-[G]MP-5′ immune system. NMN^+, which contains a ribose 5′-phosphate with a C–N bond at the 1 position of the ribose, meets the structural requirements of the combining site of the antibody and is essentially as effective as AMP-5′ in this system. DPN^+ is about one-fifth as effective as NMN^+ even though it has two ribose 5′-phosphate moieties. However, the antiserum discriminates against molecules with substituents on the 5′-phosphate of the ribose, *e.g.*, ribonucleoside diphosphates are not nearly as effective as monophosphates (Table II). ADPR is an even poorer inhibitor than DPN^+, and the reason is undoubtedly the presence of a hydroxyl group at the C-1 position of the terminal ribose. The low reactivity of FAD can also be explained both by the presence of a substituent on the ribose 5′-phosphate moiety and the presence of a straight-chain deoxyribose instead of a ribose. FMN and TPN^+, however, are ineffective inhibitors in this system. FMN contains a straight-chain deoxyribose instead of ribose, and TPN^+ not only contains a ribose 2′-phosphate moiety, but also has substituents on the 5′-phosphates of both ribose molecules.

Although not described here, antibodies have also been produced using [G]MP-3′, d[G]MP-5′, d[G], r[G], Cp[G], and Ap[G] as haptens. In these antisera, the combining site appears to extend even to the photoproduct. Thus far, the limiting factor in obtaining antibodies to different terminal residues by this procedure appears to be the availability of the suitable guanine derivatives.

These antibodies are potentially useful reagents. Purified free of serum nucleases, they may be able to detect specific terminal groupings in nucleic acids. They may also serve as inhibitors of biological processes which require a given structure on the terminal ends of nucleic acids for activity, *e.g.*, the inhibition or termination of syntheses or the ability of tRNA to accept amino acids. Since some of the coenzymes can compete successfully for the combining sites of the antibody, the effect of the purified antibodies on coenzyme-dependent enzymic activities can also be tested. Recently, antibodies specific for the coenzymes folic acid (Jaton and Ungar-Waron, 1967; Ricker and Stollar, 1967) and pyridoxal (Ungar-Waron and Sela, 1966; Cordoba *et al.*, 1966) have been produced. Antibodies to pyridoxal did inhibit the pyridoxal-dependent enzyme, glutamic–oxalacetic transaminase (Ungar-Waron and Sela, 1966).

References

Butler, V. P., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, S., and Bendich, A. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1597.

Chen, Jr., P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1756.

Cordoba, F., Gonzalez, C., and Rivera, P. (1966), *Biochim. Biophys. Acta 127*, 151.

Erlanger, B. F., and Beiser, S. M. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 68.

Halloran, M. J., and Parker, C. W. (1966), *J. Immunol. 96*, 379.

Jaton, J., and Ungar-Waron, H. (1967), *Arch. Biochem. Biophys. 122*, 157.

Landsteiner, K. (1936), The Specificity of Serological Reactions, Cambridge, Mass., Harvard University.

Plescia, O. J., Braun, W., and Palczuk, N. C. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 279.

Ricker, R., and Stollar, B. D. (1967), *Biochemistry 6*, 2001.

Sela, M., Ungar-Waron, H., and Shechter, Y. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 285.

Simon, M. I., and Van Vunakis, H. (1962), *J. Mol. Biol. 4*, 488.

Tanenbaum, S. W., and Beiser, S. M. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 662.

Ungar-Waron, H., Hurwitz, E., Jaton, J. C., and Sela, M. (1967), *Biochim. Biophys. Acta 138*, 513.

Ungar-Waron, H., and Sela, M. (1966), *Biochim. Biophys. Acta 124*, 147.

Van Vunakis, H., Seaman, E., Kahan, L., Kappler, J. W., and Levine, L. (1966), *Biochemistry 5*, 3986.

Wasserman, E., and Levine, L. (1960), *J. Immunol. 87*, 290.

Weber, G. (1950), *Biochem. J. 47*, 114.

Weber, G. (1957), *Nature 180*, 1409.