

ANTIGEN RECOGNITION AND THE IMMUNE RESPONSE  
HUMORAL AND CELLULAR IMMUNE RESPONSES TO SMALL MONO-  
AND BIFUNCTIONAL ANTIGEN MOLECULES\*

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Based on the original observation that L-tyrosine-azobenzene-*p*-arsonate (RAT)<sup>1</sup> induced specific delayed hypersensitivity in guinea pigs (1), we have shown that this small molecule could serve as a carrier for a macromolecular hapten (2). Conjugates of RAT and poly- $\gamma$ -D-glutamic acid (PGA) raised humoral antibody specific for the PGA determinant and cellular immunity directed against the RAT determinant in guinea pigs. In addition, prior immunization with RAT potentiated the anti-hapten response (2). These observations prompted the conclusion that RAT, although having a molecular weight of only 409, is a true immunogen

The RAT-PGA conjugates had, on the average, 9-12 RAT groups per molecule of PGA. In the present communication, we report the preparation and use of better defined, small bifunctional antigen molecules composed either of one RAT carrier moiety and one dinitrophenyl (DNP) haptenic group or of two RAT moieties, separated by spacers of varying size. These molecules permitted an exploration of the spatial relationships between carrier and hapten required for a humoral antibody response, as well as the question of "self-help." The latter refers to the ability of a carrier determinant to cooperate in the humoral response to a second identical determinant. In addition, the structural features of RAT which are essential for immunogenicity have been investigated.

*Materials and Methods*

The names and structures of the compounds used as antigens in this investigation are shown in Table I. A detailed description of their preparation and characterization will be published elsewhere. All abbreviations used in this manuscript are also defined in Table I.

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<sup>1</sup> Abbreviations used in this paper: See Table I.

*Monofunctional Antigen Molecules.*—L-Tyrosine-azobenzene-*p*-arsonate (RAT) was prepared by coupling diazotized arsanilic acid to *N*-*tert*-butyloxycarbonyl(*t*-BOC)-L-tyrosine. This procedure invariably produced mono- (orange) and bis- (purple) substituted phenol derivatives, which are conveniently separated by column chromatography on Sephadex G-15 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) in dilute solutions of ammonia. This purification technique was used for various other derivatives described below, obtained by diazonium coupling to phenolic compounds. After appropriate purification, the N-protecting group was removed with formic acid (3) and the product was lyophilized. The purity of this derivative, as well as all those described below, was investigated by thin-layer chromatography (TLC) in a variety of solvents and by high-voltage electrophoresis at several pH values. The most useful solvent for investigating the purity of the diazonium compounds was found to be chloroform:methanol:concentrated ammonia (3:4:2). Invariably, the purple bis-substituted phenolic derivatives remained close to the origin, while the orange mono-substituted derivatives migrated to varying degrees.

Using similar methods, the following derivatives of L-tyrosine-azobenzene were obtained: *p*-sulfonate (SAT); *p*-acetamide (AAT); *p*-sulfonamide (SNAT); *p*-nitro (NAT); and *p*-trimethylammonium chloride (TAT).

A corresponding series of bovine serum albumin (BSA) and human  $\gamma$ G globulin (HGG) conjugates of the above diazonium compounds were prepared as described previously (4, 5). The extent of substitution in each product was determined spectrophotometrically, using extinction coefficients obtained from the corresponding tyrosine derivatives. The average substitution varied from 4 to 12 chromophores per molecule of BSA or HGG.

A series of phenolic derivatives in which the aliphatic moiety of tyrosine was varied were coupled to diazotized arsanilic acid to provide the following conjugates of azobenzene arsonate: *p*-hydroxyphenyl propionic acid (DRAT), *p*-hydroxyphenyl acetic acid (ROTTE), *p*-hydroxybenzoic acid (BRAT), *p*-cresol (RAP), and tyramine (TRAT).

6-Amino-caproyl-L-tyrosine-azobenzene-*p*-arsonate (SAC-RAT) was obtained by reacting *N*-*t*-BOC-6-amino-caproic acid with L-tyrosine-methyl ester using *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC). The methyl ester was hydrolyzed to yield *N*-*t*-BOC-6-amino-caproyl-L-tyrosine, which was coupled with diazotized arsanilic acid. After appropriate purification, the N-protecting group was removed with formic acid.

The corresponding compound containing tyramine instead of tyrosine was obtained by preparing *N*-*t*-BOC-6-amino-caproyl-tyramine. Reaction with diazotized arsanilic acid and removal of the N-protecting group yielded 6-amino-caproyl-tyramine-azobenzene-*p*-arsonate (SAC-TRAT).

*Bifunctional Antigen Molecules.*—

*DNP-RAT:* Direct reaction of tyrosine with 2,4-dinitrofluorobenzene (DNFB) yielded bis-*N*,*O*-2,4-dinitrophenyl tyrosine which has been shown to give rise to anti-DNP antibody (6), undoubtedly because the O-DNP bond is labile to transconjugation with sulfhydryl groups (7), which are available in proteins in injected animals. Singly substituted *N*-DNP-L-tyrosine was obtained by reaction of DNFB with L-tyrosine-*O*-*t*-butyl ether-*t*-butyl ester. Subsequent removal of the protecting groups with formic acid yielded a ninhydrin negative, Pauly diazo-positive product, which on TLC plates migrated very closely to *N*-2,4,6-trinitrophenyl tyrosine and differed from bis-*N*,*O*-2,4-dinitrophenyl tyrosine and *O*-2,4-dinitrophenyl tyrosine. Diazonium coupling and appropriate purification to remove the purple bis-substituted compound yielded the desired DNP-RAT product. The same compound could be obtained by reacting RAT with DNFB and chromatographically separating the bis-dinitrophenylated by-product. The latter reaction sequence proved to be less satisfactory.

*DNP-SAC-RAT and symmetrical RAT antigens:* Synthesis of *N*-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-azobenzene-*p*-arsonate (DNP-SAC-RAT) was achieved by EDC coupling of DNP-SAC and L-tyrosine-methyl ester. After hydrolysis of the methyl ester and

TABLE I  
Nomenclature and Structures of Mono- and Bifunctional Antigens

Monofunctional Antigens		Bifunctional Antigens	
Structure	Name	Structure	Abbreviation
Derivatives of L-tyrosine azobenzene			
	R		
	-AsO <sub>3</sub> H <sub>2</sub>	-p-arsonate	RAT
	-COOH	-p-carboxylate	CAT
	-SO <sub>3</sub> H	-p-sulfonate	SAT
	-NH-CO-CH <sub>3</sub>	-p-acetamide	AAT
	-SO <sub>2</sub> NH <sub>2</sub>	-p-sulfonamide	SNAT
	-NO <sub>2</sub>	-p-nitro	NAT
	-N(CH <sub>3</sub> ) <sub>3</sub> Cl	-p-trimethylammonium chloride	TAT
Bifunctional Antigens			
		p-hydroxyphenyl-propionic acid azobenzene-p'-arsonate	DRAT
		p-hydroxyphenyl-acetic acid azobenzene-p'-arsonate	ROTTE

$\begin{array}{c} \text{COOH} \\   \\ \text{C}_6\text{H}_3(\text{OH})-\text{N}=\text{N}-\text{C}_6\text{H}_4-\text{AsO}_3\text{H}_2 \end{array}$	p-hydroxy-benzoic acid azobenzene-p'-arsonate	BRAT
$\begin{array}{c} \text{CH}_3 \\   \\ \text{C}_6\text{H}_3(\text{OH})-\text{N}=\text{N}-\text{C}_6\text{H}_4-\text{AsO}_3\text{H}_2 \end{array}$	p-cresol-azobenzene-p'-arsonate	RAP
$\begin{array}{c} \text{H}_2\text{N}-(\text{CH}_2)_5-\text{CO}-\text{NH}-\text{CH}-\text{COOH} \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_3(\text{OH})-\text{N}=\text{N}-\text{C}_6\text{H}_4-\text{AsO}_3\text{H}_2 \end{array}$	6-amino-caproyl-L-tyrosine azobenzene-p-arsonate	SAC-RAT
$\begin{array}{c} \text{H}_2\text{N}-\text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_3(\text{OH})-\text{N}=\text{N}-\text{C}_6\text{H}_4-\text{AsO}_3\text{H}_2 \end{array}$	tyramine azobenzene-p-arsonate	TRAT
$\begin{array}{c} \text{H}_2\text{N}-(\text{CH}_2)_5-\text{CO}-\text{NH}-\text{CH}_2 \\   \\ \text{CH}_2 \\   \\ \text{C}_6\text{H}_3(\text{OH})-\text{N}=\text{N}-\text{C}_6\text{H}_4-\text{AsO}_3\text{H}_2 \end{array}$	6-amino-caproyl-tyramine azobenzene-p-arsonate	SAC-TRAT

TABLE I (Cont'd)

Bifunctional Antigens		
Structure	Name	Abbreviation
	N-2,4-dinitrophenyl-L-tyrosine azobenzene-p-arsonate	DNP-RAT
	N-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine azobenzene-p-arsonate	DNP-SAC <sub>3</sub> -RAT
	N-2,4-dinitrophenyl-(6-amino-caproyl) <sub>3</sub> -L-tyrosine azobenzene-p-arsonate	DNP-SAC <sub>3</sub> -RAT
	N-acetyl-(6-amino-caproyl) <sub>3</sub> -L-tyrosine azobenzene-p-arsonate	Ac-SAC <sub>3</sub> -RAT

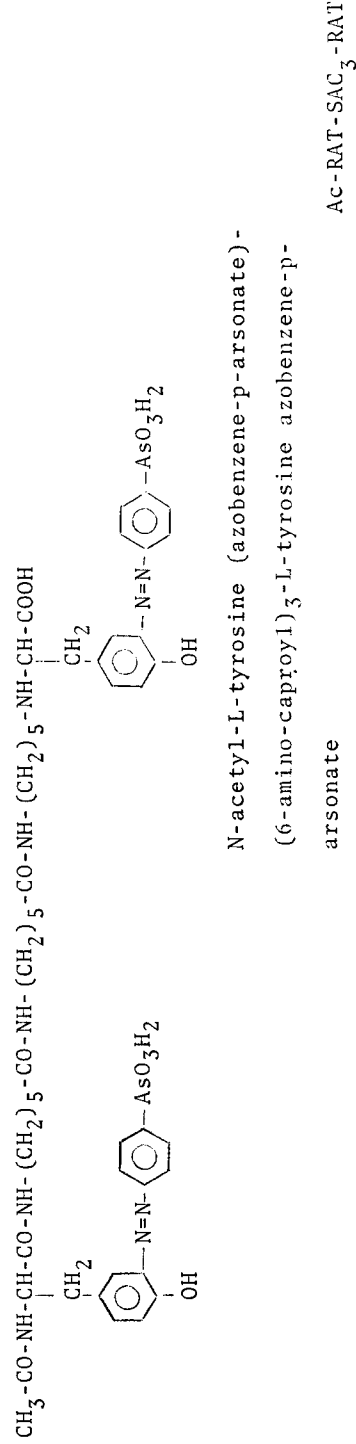
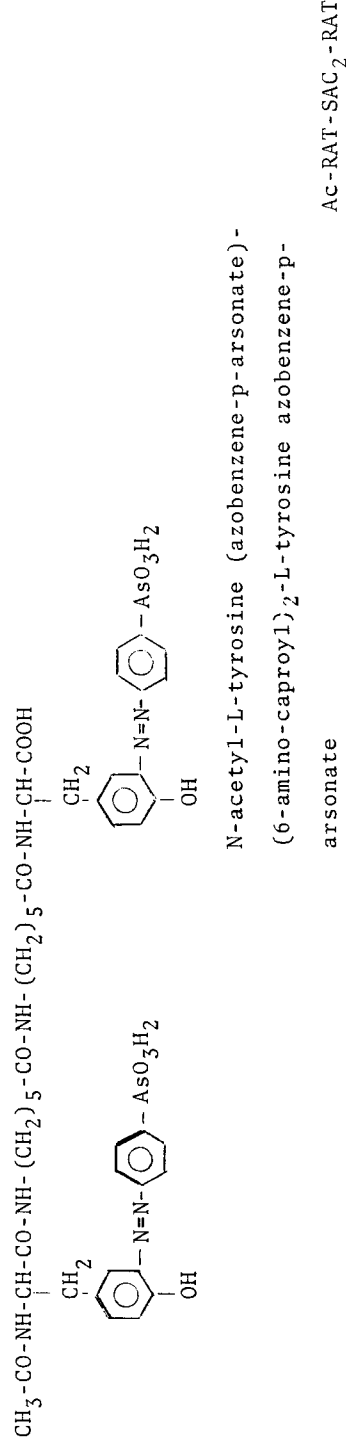
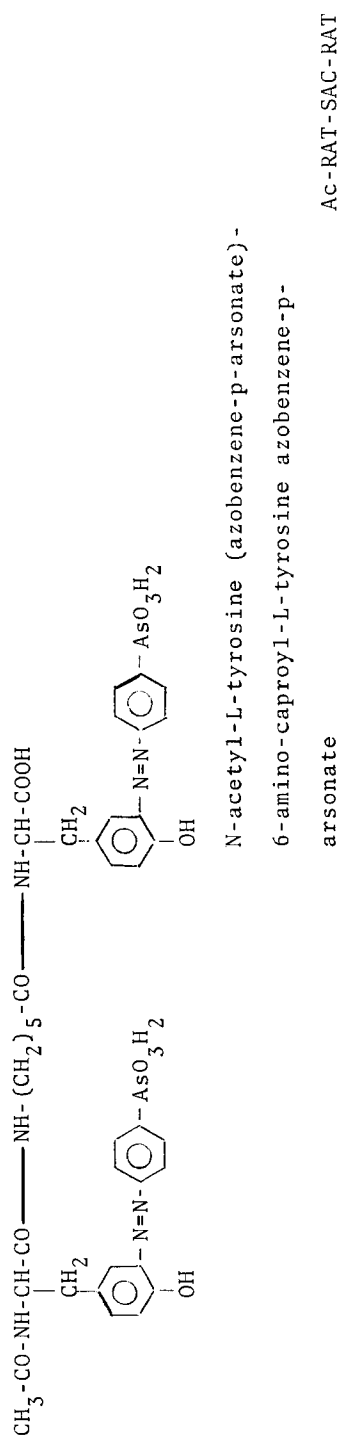


TABLE I (Cont'd)

Bifunctional Antigens		
Structure	Name	Abbreviation
<p>The structure shows a cyclo-L-tyrosine molecule where the para position of the phenyl ring is substituted with an azobenzene group (-N=N-C<sub>6</sub>H<sub>4</sub>-AsO<sub>3</sub>H<sub>2</sub>).</p>	cyclo-L-tyrosine (azobenzene-p-arsonate)	
	L-tyrosine azobenzene-p-arsonate	
	or	cyclo-L-RAT-L-RAT
	cyclo-L-tyrosine (azobenzene-p-arsonate)	
	D-tyrosine azobenzene-p-arsonate	
<p>The structure shows a D-tyrosine molecule where the para position of the phenyl ring is substituted with an azobenzene group (-N=N-C<sub>6</sub>H<sub>4</sub>-AsO<sub>3</sub>H<sub>2</sub>).</p>		cyclo-L-RAT-D-RAT

\* Other abbreviations used in this paper: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DNFB, 2,4-dinitrofluorobenzene; DNP, dinitrophenyl; EDC, N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide; HGG, human  $\gamma$ G globulin; OVA, ovalbumin; PGA, poly- $\gamma$ -D-glutamic acid; t-BOC, *tert*-butoxycarbonyl; TLC, thin-layer chromatography.

diazonium coupling, the product was purified as described above. Solid phase peptide synthesis (8) was used, with some modification, to prepare the following compounds: acetyl-(SAC)<sub>3</sub>-L-tyrosine, DNP-(SAC)<sub>3</sub>-L-tyrosine, acetyl-L-tyrosyl-SAC-L-tyrosine, acetyl-L-tyrosyl-(SAC)<sub>2</sub>-L-tyrosine, and acetyl-L-tyrosyl-(SAC)<sub>3</sub>-L-tyrosine. After extensive purification and diazonium coupling, the following products were obtained: acetyl-(SAC)<sub>3</sub>-RAT, DNP-(SAC)<sub>3</sub>-RAT, acetyl-RAT-SAC-RAT, acetyl-RAT-(SAC)<sub>2</sub>-RAT, and acetyl-RAT-(SAC)<sub>3</sub>-RAT.

The SAC chain is extremely flexible. In order to obtain symmetrical bifunctional RAT molecules with a rigid spacer for better controlled separation of the two determinants, two cyclic RAT compounds, cyclo-L-RAT-L-RAT, in which the side chains of tyrosine are *cis* with respect to the diketopiperazine ring, and the trans molecule, cyclo-L-RAT-D-RAT, were prepared. They were obtained by cyclization of the corresponding tyr-tyr-methyl ester formates to diketopiperazines (9), followed by diazonium coupling.

All the symmetrical, bifunctional RAT compounds showed the expected molar extinction coefficients in 0.1 N sodium hydroxide (4, 5), indicating two azobenzene-arsenate chromophores per molecule. In addition to spectrophotometric analysis, which can distinguish between mono- and bis-substituted phenolic derivatives as well as determine the number of chromophores per molecule, several compounds were quantitatively analyzed for arsenic. Elementary analyses were performed by Elek Microanalytical Laboratories, Torrance, Calif. The results were as follows:

- (a) *t*-BOC-SAC-RAT (C<sub>26</sub> H<sub>36</sub> O<sub>9</sub> N<sub>4</sub> As): expected 12.01%; found 12.59%.
- (b) *t*-BOC-RAT (C<sub>20</sub> H<sub>23</sub> O<sub>8</sub> N<sub>3</sub> As): expected 14.73%; found 14.63%.
- (c) DNP-RAT (C<sub>21</sub> H<sub>18</sub> O<sub>10</sub> N<sub>5</sub> As): expected 13.02%; found 11.75%.
- (d) DNP-SAC-RAT (C<sub>27</sub> H<sub>29</sub> O<sub>11</sub> N<sub>6</sub> As): expected 10.88%; found 10.94%.

In the physiologic pH range, the extinction coefficients of the bifunctional RAT compounds were low compared to RAT. At pH 7.4 in saline, RAT-SAC-RAT, cyclo-L-RAT-L-RAT, and cyclo-L-RAT-D-RAT exhibited 32, 34, and 23% hypochromism, respectively. Molecular models of these compounds indicated sufficient flexibility to permit the basic centers (-N=N- groups) to align intramolecularly with the acidic centers (arsenate groups) in a "deck of cards" geometry (10). This intramolecular stacking may serve to reduce the effective bifunctional character of these molecules.

*Animals.*—Randomly bred albino guinea pigs weighing about 600 g each were used in all experiments.

*Immunization.*—Antigens were dissolved in 0.15 M sodium chloride, the pH was adjusted to 7.0, and the aqueous solutions were emulsified with equal volumes of complete Freund's adjuvant (CFA; Difco Labs., Inc., Detroit, Mich.). Immunization protocols varied with different experiments and will be described with the results.

*Delayed Hypersensitivity.*—Skin tests of immunized animals were performed on a shaved area of the flank by intradermal injection of 50–100 μg of antigen in 0.1 ml of saline, pH 7.2. Skin sites were examined at 2–4 hr for signs of Arthus reactions and at 24 and 48 hr for delayed reactions. Reactions were considered positive if they consisted of an area of induration and erythema 5 mm in diameter or larger.

*Quantitative Precipitin Determinations.*—The BSA and HGG conjugates served as test antigens for detection of specific antibody. Sera were assayed using a micromodification of the Folin-Ciocalteu method that is useful for determining quantities of protein in the range of 1–10 μg, as previously described (11).

*Lymphocyte Stimulation.*—Lymph node or spleen cells were cultured and their response to antigen was assessed by incorporation of methyl-thymidine-<sup>14</sup>C (New England Nuclear Corp., Boston, Mass.) into DNA, as described previously (2).

## RESULTS

*Response to Bifunctional Antigens Containing DNP and RAT Determinants.*—All guinea pigs immunized with antigens containing DNP and RAT responded



with delayed hypersensitivity to RAT and circulating antibody to DNP (Table II). None of the animals developed cellular immunity to DNP, assessed by skin testing with (DNP)<sub>24</sub>-BSA. The humoral response to the RAT determinant was negligible, compared with the anti-DNP response, based on precipitation with (RAT)<sub>11</sub>-BSA. The efficacy of spacers between the two determinants in facilitating the anti-hapten response was explored by comparing DNP-RAT, DNP-SAC-RAT, and DNP-(SAC)<sub>3</sub>-RAT. Since each SAC chain has an extended length of about 8 Å, the maximum separation of the RAT and DNP determinants in DNP-SAC-RAT and DNP-(SAC)<sub>3</sub>-RAT was 8 and 24 Å, respectively. The anti-DNP responses to these two compounds were identical, within experimental error. However, although DNP-RAT evoked an anti-DNP response, it was significantly weaker than the responses to the DNP-SAC-RAT compounds.

It was considered possible that the anti-DNP response to DNP-RAT might have resulted from transconjugation of the DNP group to other carrier molecules, such as autologous proteins. DNP-RAT should be less stable than the DNP-SAC-RAT compounds because of the approximate position of the DNP and COOH groups on the side chain of tyrosine (TYR), both of which are strongly electronegative. In order to test this possibility, animals were immunized with DNP-TYR, which also contains the two electronegative groups on the side chain, and DNP-(SAC)<sub>3</sub>-TYR, both of which lack carrier activity and fail to induce delayed hypersensitivity. Neither gave rise to anti-DNP antibody (Table II), rendering improbable the transconjugation hypothesis. In addition, acetyl (Ac)-(SAC)<sub>3</sub>-RAT provoked delayed hypersensitivity to RAT but no anti-DNP response, as expected (Table II).

*Potentiation of Anti-DNP Response by Prior Immunization with RAT.*—For these experiments, two groups of four guinea pigs were primed with 0.5 mg of (DNP)<sub>20</sub>-ovalbumin(OVA) administered intraperitoneally in saline on two successive days. 1 wk later, one group was injected with 0.3 mg of RAT in CFA distributed between two footpads, while the other group received only CFA administered similarly. After an additional period of 3 wk, both groups received 0.5 mg of DNP-(SAC)<sub>3</sub>-RAT in CFA distributed between the other two footpads, and 0.5 mg of the same antigen intradermally in saline. All animals were bled 1 wk later and at weekly intervals thereafter. A comparison of the anti-DNP levels of the two groups is shown in Fig. 1. 1 wk after administration of DNP-(SAC)<sub>3</sub>-RAT the control group had no detectable circulating anti-DNP antibody whereas the group which had been preimmunized with RAT averaged 35 µg/ml; at 2 wk the values were 30 and 230 µg/ml, respectively. The difference at each time interval between the two groups was significant at  $P < 0.01$ . Thus, prior immunization with RAT significantly potentiated the anti-DNP response after immunization with DNP-(SAC)<sub>3</sub>-RAT.

*Immunogenicity of Analogs of RAT Modified at the Arsonate Position.*—A series of monofunctional analogs of RAT were synthesized and guinea pigs were immunized with each member of the series by a single injection of 250 µg in

TABLE II  
Humoral and Cellular Responses of Guinea Pigs to Nonsymmetrical Bifunctional Antigen Molecules

Test antigens	Immunizing agent*																				
	DNP-(SAC) <sub>3</sub> -RAT			DNP-SAC-RAT¶			DNP-RAT**			DNP-(SAC) <sub>3</sub> -TYR			DNP-TYR‡‡			Ac-(SAC) <sub>3</sub> -RAT					
	Ppt. Ab (μg/ml)	Mean ± SE	Range	Aver- age D.S.R.†	Ppt. Ab (μg/ml)	Mean ± SE	Range	Aver- age D.S.R.	Ppt. Ab (μg/ml)	Mean ± SE	Range	Aver- age D.S.R.	Ppt. Ab (μg/ml)	Mean ± SE	Range	Aver- age D.S.R.	Ppt. Ab (μg/ml)	Mean ± SE	Range	Aver- age D.S.R.	
(DNP) <sub>24</sub> -BSA (RAT) <sub>11</sub> -BSA§§	113-204	164 ± 12	3-9	0§	54-230	141 ± 31	<2	0§	13-102	52 ± 17	2-8	3	2-6	3 ± 0.8	0-6	2	0	0-2	<2	0	0
DNP-(SAC) <sub>3</sub> -RAT				16				14				6					0				17
DNP-(SAC) <sub>3</sub> -TYR				6				6				4					0				7

\* Five to six animals were immunized with each antigen.  
 † Delayed skin reaction.  
 ‡ Arthus at 2-4 hr which disappeared at 24-48 hr.  
 § Immunization Protocols:  
 || 1.0 mg distributed equally between the four footpads. Bled at 10 and 20 days. Skin tested at 21 days.  
 ¶ 0.75 mg distributed equally between the four footpads. Bled at 10 and 20 days. Skin tested at 21 days.  
 \*\* 0.70 mg distributed equally between the four footpads. Bled at 10 and 20 days. Skin tested at 21 days.  
 †† 0.50 mg distributed equally between the four footpads. Bled at 10 and 20 days. Skin tested at 21 days.  
 §§ Strictly speaking, this designation is incorrect since the tyrosine moiety implied in RAT, CAT, etc., is not external but rather an integral part of the protein molecule. This designation was preserved for the sake of simplicity.

CFA distributed between the four footpads. The animals were skin tested 15 days later and the results are given in Table III. Besides RAT, only the *p*-sulfonate (SAT) and *p*-trimethylammonium (TAT) compounds induced significant de-

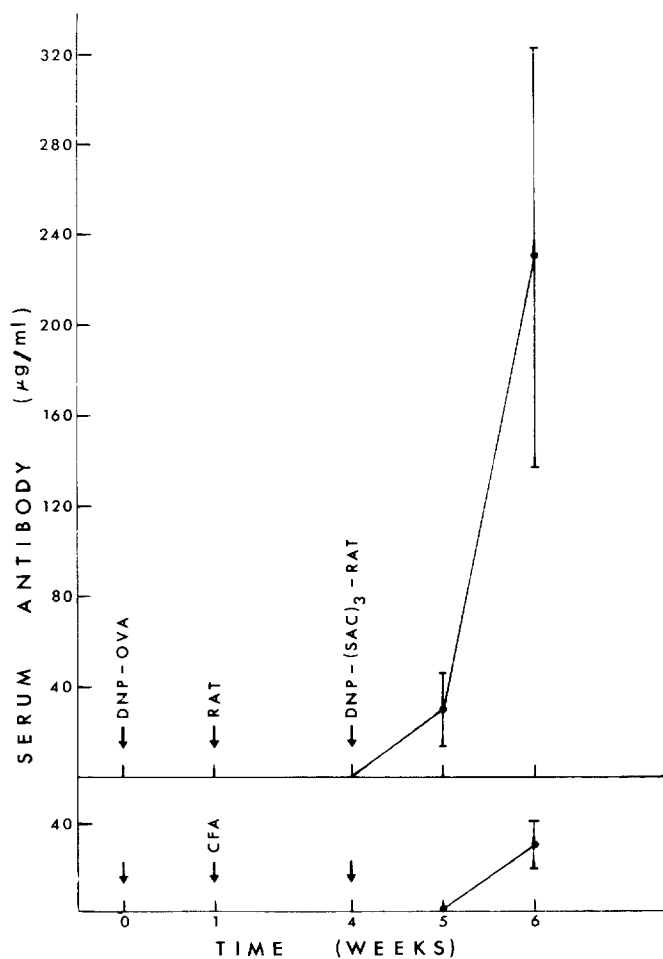


FIG. 1. Humoral antibody response to DNP by guinea pigs immunized sequentially with (DNP)<sub>20</sub>-OVA, RAT, and DNP-(SAC)<sub>3</sub>-RAT (*upper panel*), or with (DNP)<sub>20</sub>-OVA, CFA, and DNP-(SAC)<sub>3</sub>-RAT (*lower panel*). There were four animals in each group. Mean values and standard errors are shown. Differences between the two groups were significant at  $P < 0.01$ .

layed hypersensitivity using this regimen. Of the inactive compounds, only CAT carried a charged substituent (carboxylate) at the arsonate position. Thus, the arsonate group is not essential for immunogenicity of molecules with a tyrosine-azobenzene core, but a charged substituent, either anionic or cationic, appears to be required.

*Immunogenicity of Analogs of RAT Modified at the Tyrosine Position.*—Groups of animals were injected with 250  $\mu$ g of RAT or equivalent molar quantities of azobenzene-arsenate conjugated to various modified tyrosines (Table I). The antigens were incorporated in CFA and distributed between the four foot-

TABLE III  
*Induction of Delayed Hypersensitivity in Guinea Pigs\* by Analogs of RAT Modified at the Arsonate Position*

Test antigens	Immunizing agent‡							CFA
	RAT	SAT	CAT	SNAT	AAT	NAT	TAT	
(RAT) <sub>11</sub> -BSA	18§	6	0	0	0	0	0	0
(SAT) <sub>20</sub> -BSA	3	16	3	3	2	2	N.D.	3
(CAT) <sub>3</sub> -BSA	3	5	3	3	2	2	N.D.	4
(SNAT) <sub>5</sub> -BSA	0	2	0	0	0	0	N.D.	0
(AAT) <sub>4</sub> -BSA	0	0	0	0	0	0	N.D.	0
(NAT) <sub>12</sub> -BSA	0	0	0	0	0	0	N.D.	0
(TAT) <sub>5</sub> -BSA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	19	0

\* Two animals in each group. No reactions were seen 2–4 hr after injection of the test antigens.

‡ See Table I for structures corresponding to abbreviations.

§ Average delayed skin reaction in millimeters.

TABLE IV  
*Induction of Delayed Hypersensitivity in Guinea Pigs by Analogs of RAT Modified at the Tyrosine Position*

Test antigens	Immunizing agent*						CFA
	RAT	TRAT	DRAT	ROTTE	BRAT	RAP	
(RAT) <sub>11</sub> -BSA	17‡(3)§	15(4)	12(7)	13(3)	6(3)	0(3)	0(3)
(DRAT) <sub>4</sub> -BSA	15(3)	20(4)	15(7)	15(3)	2(3)	0(3)	0(3)
(TRAT) <sub>3</sub> -BSA	15(3)	17(4)	10(7)	15(3)	2(3)	0(3)	0(3)
BSA	0(3)	0(4)	0(7)	0(3)	0(3)	0(3)	0(3)

\* See Table I for structures corresponding to abbreviations.

‡ Averaged delayed skin reaction in millimeters.

§ Parentheses refer to number of animals tested.

pads. Skin tests for delayed hypersensitivity were performed 20 days after immunization. The results are shown in Table IV.

Removal of either the amino group (DRAT) or the carboxyl group (TRAT) of tyrosine did not appreciably diminish immunogenicity, but the compound which lacked both charged groups (RAP) did not induce a response. Shortening the side chain of DRAT by one carbon atom (ROTTE) did not affect immunogenicity, but removal of both methylene groups (BRAT) resulted in a less potent inducer of delayed hypersensitivity.

*Response to Symmetrical Bifunctional Antigens Containing Two RAT Determinants.*—The ability of RAT to serve as a carrier for an identical RAT determinant was evaluated by immunizing groups of animals with a series of bifunctional RAT molecules: RAT-SAC-RAT, RAT-(SAC)<sub>2</sub>-RAT, RAT-(SAC)<sub>3</sub>-RAT, and cyclo-L-RAT-D-RAT. In the cyclic compound, the spacer is more rigid and the arsonate groups are projected on opposite sides of the plane of the ring, providing somewhat better controlled separation of the determinants. Groups of four to eight animals were injected with 0.25, 1.0, or 2.5 mg of one of the compounds, administered as before in CFA distributed between the four footpads. The animals were bled 10 and 20 days later, skin tested 20 days after immunization, and the lymph node cells of some were assayed in vitro for stimulation of DNA synthesis by antigen. Most of the animals were bled weekly for 3 wk and none developed more than 2  $\mu$ g of precipitating antibody to RAT,

TABLE V  
*Humoral and Cellular Responses of Guinea Pigs to RAT-(SAC)<sub>3</sub>-RAT\**

Test antigens	Average delayed skin reaction	Average lymphocyte stimulation†	Anti-RAT Ab (mean)
	<i>mm</i>		$\mu$ g/ml
(RAT) <sub>11</sub> -BSA	18 (8)§	2.4 (2)	<2.0
RAT-(SAC) <sub>3</sub> -RAT	9 (8)	1.6 (2)	
RAT	6 (8)	3.0 (2)	
BSA	0 (8)	0.9 (2)	

\* Animals immunized with 1.0 mg of RAT-(SAC)<sub>3</sub>-RAT in CFA.

† Ratio of thymidine-<sup>14</sup>C incorporation in cultures with antigen present relative to cultures without antigen from the same animal.

§ Parentheses indicate number of animals tested.

although all developed cellular immunity. The group of animals immunized with 1.0 mg of RAT-(SAC)<sub>3</sub>-RAT was studied in greatest detail and the data from these animals are shown in Table V. All developed strong delayed hypersensitivity without significant humoral immunity. The lymph node cells from two of the guinea pigs were assayed for thymidine incorporation and were stimulated more than twofold by (RAT)<sub>11</sub>-BSA and RAT.

All animals which were immunized with 0.25 mg of RAT and RAT-(SAC)<sub>1-3</sub>-RAT were given a booster injection of 1.0 mg of their respective antigens 6 wk after the primary injection and bled 10 days later. As before, the antibody level averaged less than 2  $\mu$ g/ml. Thus, these symmetrical bifunctional antigens were much weaker inducers of humoral immunity than the asymmetric DNP-RAT compounds.

Since the bifunctional RAT compounds were unable to induce a significant primary humoral anti-RAT response, their capacity to promote a secondary response in appropriately prepared subjects was investigated. Eight guinea pigs were immunized with 10  $\mu$ g of (RAT)<sub>11</sub>-BSA in CFA and all developed anti-

arsonate antibody, determined by precipitation with  $(\text{RAT})_{13}\text{-HGG}$  (Fig. 2). 30 days later, four animals received 250  $\mu\text{g}$  of RAT in CFA while the other four were injected with the same quantity of  $\text{RAT-(SAC)}_3\text{-RAT}$  in CFA. The levels of anti-arsonate antibody 10 and 20 days later were either unchanged or lower, but all animals developed delayed hypersensitivity to RAT. The animals were rested until day 90, when the levels of anti-arsonate antibody were very low. Challenge at this time with 250  $\mu\text{g}$  of RAT or 200  $\mu\text{g}$  of  $\text{RAT-(SAC)}_3\text{-RAT}$  produced no increase in these levels (Fig. 2). Consequently, neither RAT nor RAT-

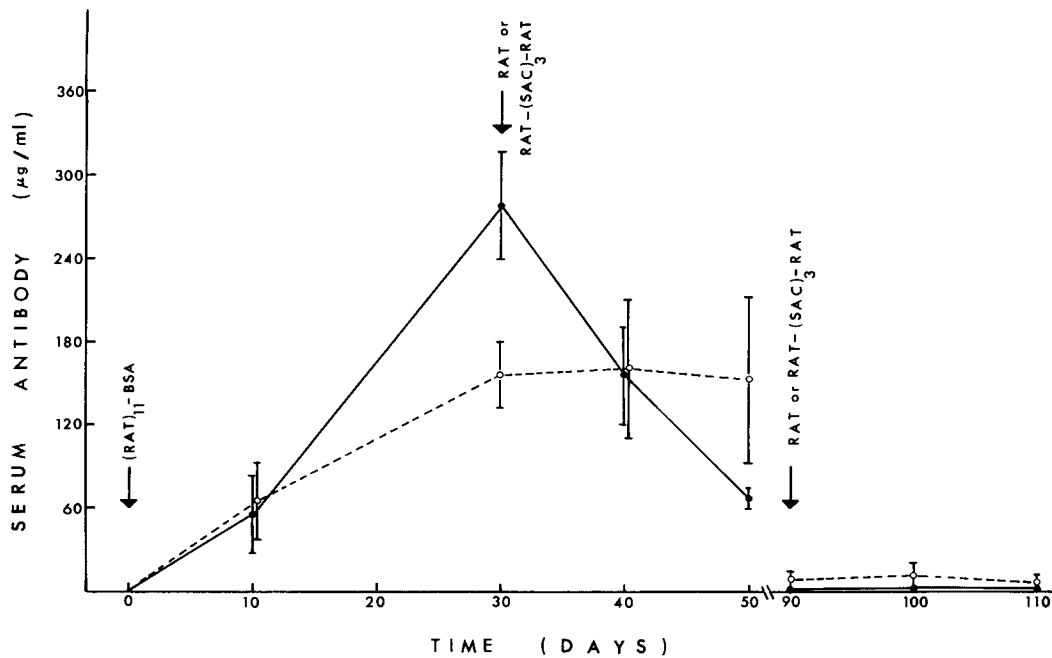


FIG. 2. Humoral anti-arsonate antibody response by guinea pigs immunized sequentially with  $(\text{RAT})_{11}\text{-BSA}$  and RAT (●) or with  $(\text{RAT})_{11}\text{-BSA}$  and  $\text{RAT-(SAC)}_3\text{-RAT}$  (○). There were four animals in each group. Mean values and standard errors are shown.

$(\text{SAC})_3\text{-RAT}$  was able to induce a secondary humoral response in animals which had both cellular and humoral immunity to RAT.

#### DISCUSSION

Based on our current understanding of the immune response, the minimum requirement for a molecule to function as a carrier for a hapten would be its "recognition" by at least one clone of thymic (T) lymphocytes. Large proteins function best as carriers, presumably because they present a diversity of determinants and so have a high probability of interacting with a number of T cells of different specificity. Small, uni-determinant molecules as a rule are nonimmu-

nogenic and serve only as haptens. In conjunction with an immunogenic carrier they evoke humoral antibody, but cellular immunity is usually confined to the carrier. RAT appears to be an exception in that it can, by itself, evoke cellular immune responses such as delayed hypersensitivity (1, 2) and a proliferative response by sensitized lymphoid cells in culture (2).

On the basis of the cellular immune response to RAT, it seemed logical that it could serve as a carrier for a hapten. This, indeed, proved to be the case when RAT was conjugated to a nonimmunogenic macromolecular polypeptide (2) as well as to DNP (this investigation). In both instances, the humoral response was predominantly directed against the haptenic determinant, while cellular immunity was restricted to RAT. This functional discrimination between the determinants of these synthetic conjugates is highly compatible with a two cell mechanism of induction of humoral immunity and has a parallel in the immune response to the natural polypeptide glucagon (12). In the latter investigation, it was found that humoral antibody had a predominant specificity for the amino-terminal heptadecapeptide, whereas only the carboxy-terminal undecapeptide was capable of stimulating sensitized cells in culture. Thus, there is evidence for a functional dichotomy of determinants in both natural and synthetic antigens.

This does not mean that carrier determinants cannot also function as haptens. Proteins substituted with azobenzene-*p*-arsonate groups can provoke anti-arsonate antibody. However, under circumstances where RAT is joined only to a hapten, it appears to function simply as a carrier. Analogously, when glucagon was conjugated to a protein carrier, the carboxy-terminal region behaved like a haptenic determinant (13).

As discussed previously (2), it cannot be rigorously excluded that the activity of RAT is due to an indirect mechanism, such as its adherence to a carrier *in vivo* and in cell culture. Arguing against such a mechanism are the observations that polymers of *D*-tyrosine-azobenzene-*p'*-arsonate are inactive (1) and that humoral antibody is not elicited by monofunctional arsanilate compounds (1, 2), but is by DNP-RAT bifunctional molecules. In addition, prior immunization with RAT potentiated the anti-hapten response (2; this investigation), as has carrier immunization in conventional hapten-protein systems (14, 15). Indeed, all observations made with RAT have parallels in systems in which macromolecules served as carriers.

A recent report on the immunogenicity of several dinitrophenylated amino acids (6) is in our judgement irrelevant to the findings presented here. The preparations in question always contained O-DNP or N(imidazolyl)-DNP substitutions which are known to transconjugate in the presence of sulfhydryl groups (7). We took great pains to ensure the absence of these labile derivatives from preparations used for immunization, and our preparations of DNP-TYR and DNP-SAC-TYR were inactive (Table II).

In the work referred to (6), DNP-SAC also induced the formation of anti-DNP antibody. In our preparation of this compound by the reaction of DNFB

with 6-amino-caproic acid, a by-product was observed by TLC. It was not possible to remove it by eight recrystallizations, but purification was achieved by chromatography on very large columns of silica gel. It is not known if this impurity could have been responsible for the putative immunogenicity of DNP-SAC (6), but in our hands DNP-SAC compounds which did not contain azobenzene-*o*-arsonate were inactive (Table II).

The use of small, structurally defined bifunctional antigens permitted study of the spatial displacement of the two determinants needed to elicit an antibody response. A single SAC spacer with a maximum extended length of 8 Å permitted as strong an anti-DNP response as a spacer three times that size (Table II). These spacers were flexible chains (rigid rods would have been preferable) so the effective spacing at the critical time of the response remains hypothetical. Joining the two determinants without a spacer resulted in a weaker but still pronounced anti-DNP response. It is difficult to visualize how two cells could simultaneously interact with so small a molecule, if direct contact between T and B lymphocytes is required. Alternatively, a soluble mediator released by the T cell which activates the B lymphocyte remains a possibility (16).

The compounds in which DNP and RAT are separated by spacers should be very stable, particularly in the physiologic pH range. However, DNP-RAT would be expected to be less stable because the side chain of tyrosine in this conjugate possesses two electronegative moieties, the carboxyl and DNP groups. Therefore, it was considered possible that the anti-DNP response to DNP-RAT might have resulted from transconjugation of the DNP group to other carriers, such as autologous proteins. However, the inactivity of DNP-tyrosine in stimulating an anti-DNP response (Table II) appears to effectively vitiate this possibility.

Since it is unusual for a molecule as small as RAT to be immunogenic, a systematic exploration of the structural features which contribute to its immunogenicity was undertaken. A series of analogs with substitutions for the arsonate group, some members of which carried negatively charged substituents while others were neutral or positively charged, was tested. Only the sulfonate (SAT) and trimethylammonium (TAT) compounds induced delayed hypersensitivity with the single immunizing regimen employed. These findings suggest that a charged substituent is needed at the arsonate position, but is not necessarily sufficient since CAT was inactive, and that either a cationic or anionic group may fill the role. It is worth noting that there was little, if any, cross-reactivity in delayed hypersensitivity between RAT, SAT, and TAT (Table III), so different clones of antigen-reactive cells seem to be involved in the responses to these immunogens.

Tests with the series of analogs modified at the tyrosine position showed, again, the importance of polar groups for immunogenicity. Removal of either the carboxyl or amino group from the side chain (TRAT and DRAT, respectively) did not abrogate the induction of delayed hypersensitivity, but RAP, which carried neither charge, was inactive (Table IV). Since BRAT, the *p*-



hydroxy-benzoate compound was palpably less active than DRAT or ROTTE, the size of this side chain apparently plays a part and it will be necessary to test compounds with longer uncharged hydrophobic and hydrophilic side chains than RAP before the necessity of a charged group at this position can be firmly established.

It is noteworthy that there are several discrepancies between these results and those of Leskowitz and his coworkers, who found that RAP induced delayed hypersensitivity in guinea pigs (1) whereas SAT did not (17). One possibly significant difference in protocol was their use of more mycobacteria in the preparations used for immunization than is included in commercial Freund's adjuvant. This could conceivably account for the inactivity of RAP in our hands but obviously would not explain the inactivity of SAT in theirs.

Monofunctional RAT appears to induce a pure cellular immune response (1, 2). There is now a reasonable basis for the view that at least two antigenic determinants are required for a humoral antibody response (14). The question of self-help, whether an immunogenic molecule with two identical determinants can provoke a humoral response, remains to be definitely answered. Immunization with symmetrical bifunctional RAT-RAT compounds induced cellular immunity without detectable humoral antibody, in marked contrast to the behavior of asymmetric bifunctional DNP-RAT antigens. It might be argued that DNP is a more potent hapten than arsonate, but one would still expect RAT-RAT to influence the level of anti-arsonate antibody in animals pretreated with (RAT)<sub>II</sub>-BSA and RAT in order to prepare them for a secondary anti-hapten response with a second carrier. Bifunctional RAT antigens had no effect on the level of anti-arsonate antibody in such animals (Fig. 2).

Several conceivable explanations, which are not equally plausible, to account for the inability of RAT-RAT compounds to engender a humoral response might bear consideration. One is that the bifunctional compounds used in this study are sufficiently flexible to permit the two determinants to be bound by receptors on the same cell surface, thus failing to satisfy the requirement for cell cooperation. Another, related to the first, is that flexibility results in intramolecular stacking of the RAT determinants, compromising their bifunctional character. The hypochromism observed with these compounds, including the cyclic dityrosine molecules which were expressly prepared for greater rigidity, suggests that this occurs to a marked degree (discussed in Materials and Methods). An approach to this problem is the preparation of bifunctional molecules with more rigid spacers, which is currently in progress. A third possibility is that there is a biological block to self-help.

#### SUMMARY

L-Tyrosine azobenzene-*p*-arsonate (RAT) induced cellular immunity without antibody production in guinea pigs. Bifunctional antigens were prepared consisting of one RAT carrier moiety linked either directly to a dinitrophenyl

(DNP) haptenic determinant or through one or more 6-amino-caproyl (SAC) spacers. Each SAC unit has an extended span of 8 Å. Guinea pigs immunized with these conjugates developed cellular immunity directed against the RAT determinant and antibody specific for the DNP determinant. The anti-DNP response was the same with one or three SAC spacers, but was significantly weaker when the two determinants were joined without a spacer. Animals immunized with either DNP-SAC-TYR or DNP-TYR developed neither cellular nor humoral immunity. Prior immunization with RAT potentiated the secondary anti-hapten response to DNP-SAC-RAT.

Modification of RAT at either the arsonate or tyrosine positions showed that other charged groups (sulfonate and trimethylammonium) could substitute for arsonate without loss of immunogenicity. Removal of either the amino or carboxyl group from the side chain of tyrosine did not abolish immunogenicity, but immunogenicity was lost upon removal of both.

Immunization with symmetrical bifunctional RAT-(SAC)<sub>n</sub>-RAT and cyclo-(L-RAT-D-RAT) antigens led to cellular immunity but no anti-arsonate antibody, suggesting a barrier to "self-help." These compounds were also ineffective in inducing a secondary anti-arsonate response in animals primed with arsonate-BSA conjugates and RAT.

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