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Characterization of New Antigenic Determinants Introduced into Homologous Serum Albumin by Dinitrophenylation and Sulphanylation

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Summary. Studies on the immune response against hapten-autologous protein carriers in mice, guinea-pigs and rabbits have shown that the new antigenic determinants introduced in the carrier molecule by the hapten coupling reaction play an important role in the induction of both immunity and tolerance to these conjugates. The present experiments were designed to elucidate the specificity of the new antigenic determinants induced (1) by different haptens through the same coupling procedure and (2) by the same hapten coupled by different procedures. The results showed that both the nature of the hapten and the coupling procedure played a role in the serological specificity of the new antigenic determinants.

INTRODUCTION

The introduction of haptens into protein molecules creates new structures in the proteins (NADs). These structures seem to play an important role in the induction of anti-hapten antibody synthesis against hapten-homologous protein conjugates (St. Rose and Cinader, 1967; Paul, 1970; Rubin, 1972a, b, c, 1973; Rubin, Schirrmacher and Wigzell, 1972a, 1973; Rubin and Wigzell, 1973; Walters, Moorhead and Claman, 1972) and in the induction of anti-hapten antibody synthesis against hapten-heterologous protein conjugates in animals tolerant to the heterologous protein carrier (Boyden and Sorkin, 1962; Nachtigal and Feldman, 1964; Cinader, St. Rose and Yoshimura, 1967; Paul, Siskind and Benacerraf, 1967).

Studies in mice, guinea-pigs and rabbits have shown that upon immunization with hapten-homologous albumin conjugates specific antibodies are produced against (1) the haptenic groups and (2) the NADs. The latter kind of antibodies had no detectable affinity for either the haptenic group or the native protein molecule (St. Rose and Cinader, 1967; Paul, Siskind and Benacerraf, 1967; Rubin, 1972a, 1973). Two different haptens have been used: sulphanilic acid (sulph) and dinitrophenyl (DNP). The main characteristics of these two haptens are: (1) sulph is strongly hydrophilic and it is coupled to the carrier protein by diazo-linkage to tyrosine, histidine and lysine residues (Tabachnick and

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Sobotka, 1959), and (2) DNP is hydrophobic and it is coupled to the carrier protein by amino-linkage to lysine residues (Eisen, Carsten and Belman, 1954). In accordance with this, it was found that anti-sulph and anti-DNP antibodies on one hand and anti-NAD^{sulph} and anti-NAD^{DNP} antibodies on the other hand did not cross-react (Rubin, 1972a, b, 1973).

The present experiments were carried out in order to answer the following question: Is the specificity of the NADs determined by (1) the nature of the hapten, (2) by the hapten coupling reaction, or (3) by both? We studied the immune response to and the cross-reactivity between the following conjugates in the rabbit: $Sulph_9RSA$ (sulph coupled to rabbit serum albumin (RSA) by diazo-linkage), (2) $DNPA_{11}RSA$ (DNP coupled to RSA by diazo-linkage), and (3) DNP_7RSA (DNP coupled to RSA by amino-linkage). By choosing these conjugates we have the following combinations; (a) Sulph-RSA→ DNPA-RSA = different haptens but coupled by diazo-linkage, and (b) DNPA-RSA→ DNP-RSA = the same hapten but coupled by different procedures (diazo- or amino-linkage).

EXPERIMENTAL METHODS

Animals

White Danish country rabbits from a closed colony were obtained from Statens Seruminstitut. They were females and 2–3 months of age at the beginning of the experiment. Blood samples were obtained by ear-bleeding or heart-puncture. Antisera were heat-inactivated for 30 minutes at 56° and stored at −24°.

Immunogens

Rabbit serum albumin (RSA), human serum albumin (HSA) and ovalbumin (OA) were obtained from Koch-Light Laboratories (Colnbrook, Bucks, England). Sulphanilic acid (Merck AG, Darmstadt, Germany) was coupled to RSA by diazo-linkage* (Tabachnick and Sobotka, 1959). 2,4-dinitrophenyl-sulphonic acid (Eastman Kodak CO, Rochester, U.S.A.) was coupled to RSA and OA, and 2,4-dinitroaniline (Fluka AG, Buchs, Switzerland) was coupled to HSA and RSA by diazo-linkage as described by Eisen *et al.* (1954).

The number of haptens introduced per protein molecule was determined spectrophotometrically (for the sulph hapten the formula given by Tabachnick and Sobotka (1959) was used, for the DNP hapten $\varepsilon = 17{,}400$ and for the DNPA hapten $\varepsilon = 7{,}980$ (Eisen *et al.*, 1954) were used.

The conjugates used were composed as follows: $sulph_9RSA$, DNP_7OA, DNP_7RSA, $DNPA_8HSA$ and $DNPA_{11}RSA$. DNP-lysine (DNP-lys) was obtained from Mann Research Laboratories (New York, U.S.A.). Sulph-tyrosine (sulph-tyr) and DNPA-tyrosine (DNPA-tyr) were prepared by coupling the haptens to *N*-chloroacetyl-tyrosine (Koch-Light) by diazotization (Schirrmacher, 1972).

* The actual coupling reaction for the Sulph hapten is thought to be: SO_3H-⬡-N_2^+ + tyrosine-protein → SO_3H-M-N = *N*-tyrosine-protein (or histidine and lysine instead of tyrosine).
For the DNPA hapten:
$(NO_2)_2$-⬡-N_2^+ + tyrosine-protein→ $(NO_2)_2$-⬡-N = *N*-tyrosine-protein (or histidine and lysine instead of tyrosine).
For the DNP hapten:
$(NO_2)_2$⬡-SO_3H + NH_3-lysine-protein→ $(NO_2)_2$-⬡-NH-lysine-protein.
Thus, the sulph and DNPA coupling to protein will be called diazo-linkage, and the DNP coupling to protein will be called amino-linkage (the DNP coupling creates a secondary 'amine').

Immunization

Primary immunization was carried out with the conjugates emulsified in Freund's complete adjuvant (FCA). Each rabbit received 1 ml of emulsion as five subcutaneous (s.c.) injections (0·2 ml) on the back. Protein concentration in the emulsion was 1 mg/ml. Secondary immunizations were performed with the conjugates dissolved in phosphate buffered saline (PBS). Each rabbit received 1 mg of conjugate, injected intravenously (i.v.).

Serology

Anti-hapten and anti-carrier antibodies were detected by passive haemagglutination using the microtitre equipment (Flow Laboratories, Glasgow, Scotland). Titration medium was 1 per cent normal rabbit serum (NRS) in PBS, i.e. 10^{-5} M RSA. Anti-sulph specific haemagglutination was carried out using sulphanylated sheep erythrocytes (sulph-SRBC) (Ingraham, 1952). Anti-DNP specific haemagglutination was performed using DNP_7OA coupled to SRBC (coupling reagent = glutaraldehyde (Rubin, 1972a, 1973). The specificity of the anti-sulph and the anti-DNP haemagglutination titres was verified by complete inhibition with 10^{-3}–10^{-5} M of free hapten (sulph-tyr or DNP-lys). Anti-DNP antibodies from anti-$DNPA_{11}RSA$ antisera were tested against both DNP_7OA-SRBC and $DNPA_8HSA$-SRBC. The specificity of these reactions will be dealt with in the Results section.

Anti-NAD antibodies were tested against the homologous hapten-RSA conjugate coupled to SRBC (glutaraldehyde), the anti-hapten antibodies being inhibited with 10^{-3} M of the relevant free hapten during the test. Thus, $sulph_9RSA$-SRBC, $DNPA_{11}RSA$-SRBC or DNP_7RSA-SRBC was used to detect anti-NAD^{sulph} anti-NAD^{DNPA}-, or anti-NAD^{DNP} antibodies, respectively.

Haemagglutination inhibition was performed as follows. After titration of the individual antisera, different concentrations of the inhibitors (25 μl of 2×10^{-x} M) were added to all plates and indicator cells were added directly. The haemagglutination patterns were scored after 1 hour at 37°, and the titre read as the last well giving unequivocal haemagglutination. Negative haemagglutination of undiluted antisera was designated $\log_2 = 0$. Antisera were not absorbed with SRBC as our rabbits showed very little natural anti-SRBC activity ($\log_2$ titre = 0·0–1·0). The concentration of inhibitor giving 75 per cent inhibition of the haemagglutination titre was taken as an indication of the avidity of the inhibited antibodies. This concentration of inhibitor was converted to $-\log_{10}$ units.

Statistics

Statistical significance of the haemagglutination results was evaluated by the Student's *t*-test. A difference of two $\log_2$ units or more was found significant ($P<0{\cdot}05$).

RESULTS

1. GENERAL DESIGN AND REMARKS

Forty-five rabbits were divided into three groups of fifteen designated groups I-III. Group I was immunized with 1 mg of $sulph_9RSA$, group II with 1 mg of $DNPA_{11}RSA$ and group III with 1 mg of DNP_7RSA. All rabbits were bled at weekly intervals and antisera tested for anti-hapten and anti-NAD specific haemagglutinating antibodies. Significant amounts of anti-sulph and anti-DNP antibodies were detected from the third

week, anti-DNPA antibodies were detected from the fifth week. Conjugate specific anti-NAD antibodies were detected simultaneously with the anti-hapten antibodies and they were usually 2–4 $\log_2$ titres higher than the anti-hapten antibody titres.

Sixty days following immunization the three groups of rabbits were subdivided each into three groups of five rabbits (groups I^a, I^b, I^c and so forth). Groups I^a, II^a and III^a were restimulated with 1 mg of $sulph_9RSA$, groups I^b, II^b and III^b with 1 mg of $DNPA_{11}$ RSA and groups I^c, II^c and III^c with 1 mg of DNP_7RSA, respectively. They were bled 4, 7 and 14 days later. Only results from the day of restimulation and 7 days after restimulation will be presented. The purpose of this design was to test the *in vivo* cross-reactivity of the NADs, i.e. the cross-reactivity of the NADs on both the helper cell level and the antibody-forming precursor cell level. If the NADs introduced into RSA by DNP and DNPA were identical or cross-reacted one would expect secondary anti-DNP and anti-NAD responses in (1) groups II^b and II^c, and (2) in groups III^b and III^c. If the NADs introduced by diazo-linkage were identical or cross-reactive one might expect (1) secondary anti-NAD responses in groups I^b and II^a, and (2) enhanced anti-hapten responses in groups I^b and II^a due to 'carrier' pre-immunization (Rajewsky, Schirrmacher, Nase and Jerne, 1969; Katz, Paul, Goidl, and Benacerraf, 1970). The findings will be correlated with the serological data on the cross-reactivity between the different NADs in $sulph_9RSA$, $DNPA_{11}RSA$ and DNP_7RSA.

2. CROSS-REACTIVITY BETWEEN THE NADS IN $SULPH_7RSA$, $DNPA_{11}RSA$ AND DNP_7RSA ON THE LEVEL OF *in vivo* SECONDARY STIMULATION

Table 1 gives the results of the secondary anti-hapten responses. It can be seen that in all cases only homologous stimulation is observed, i.e. groups I^a, II^b and III^c (underlined in the Table). This finding was observed whether tested 4, 7 or 14 days after restimulation.

TABLE 1

ANTI-HAPTEN ANTIBODY SYNTHESIS AGAINST HAPTEN-AUTOLOGOUS PROTEIN CONJUGATES

Group No.	Primary† antigen	Secondary‡ antigen	$\log_2$ anti-hapten haemagglutination titre*	
			Days after restimulation‡	
			0	7
I^a	$Sulph_9RSA$	$Sulph_9RSA$	5·7 ± 2·2	7·9 ± 1·4
I^b	$Sulph_9RSA$	$DNPA_{11}RSA$	5·5 ± 1·9	5·8 ± 0·4
I^c	$Sulph_9RSA$	DNP_7RSA	4·1 ± 1·6	4·5 ± 2·0
II^a	$DNPA_{11}RSA$	$Sulph_9RSA$	1·3 ± 1·2	2·1 ± 1·4
II^b	$DNPA_{11}RSA$	$DNPA_{11}RSA$	1·8 ± 1·9	7·1 ± 1·4
II^c	$DNPA_{11}RSA$	DNP_7RSA	0·5 ± 0·7	1·8 ± 2·2
III^a	DNP_7RSA	$Sulph_9RSA$	7·5 ± 1·0	6·5 ± 1·9
III^b	DNP_7RSA	$DNPA_{11}RSA$	5·1 ± 2·8	5·2 ± 2·4
III^c	DNP_7RSA	DNP_7RSA	6·9 ± 0·7	9·3 ± 0·3

* Group No. I, anti-hapten titre measured against sulph-SRBC. Group No. II, anti-hapten titre measured against $DNPA_8HSA$-SRBC. Group No. III, anti-hapten titre measured against DNP_7OA-SRBC.

† 1 mg of primary antigen given s.c. in FCA.

‡ 1 mg of secondary antigen given i.v. in PBS.

§ Restimulation on day 60 following primary immunization.

Table 2 presents the secondary anti-NAD responses obtained. Only homologous secondary anti-NAD responses were achieved , i.e. groups I[a], II[b] and III[c] (underlined in the Table). Similar results were obtained with antisera from day 4 or day 14.

TABLE 2

ANTI-NAD ANTIBODY SYNTHESIS AGAINST HAPTEN-AUTOLOGOUS PROTEIN CONJUGATES

Group No.	Primary† antigen	Secondary‡ antigen	Indicator§ SRBC	Log$_2$ anti-hapten haemagglutination titre* Days after restimulation¶ 0	7
I[a]		Sulph$_9$RSA	Sulph$_9$RSA--		12·0±1·9
I[b]	Sulph$_9$RSA	DNPA$_{11}$RSA	Sulph$_9$RSA-	7·8±1·8	7·7±1·5
I[c]		DNP$_7$RSA	Sulph$_9$RSA-		7·1±2·7
II[a]		Sulph$_7$RSA	DNPA$_{11}$RSA-		4·4±1·9
II[b]	DNPA$_{11}$RSA	DNPA$_{11}$RSA	DNPA$_{11}$RSA	3·8±2·2	9·5±1·3
II[c]		DNP$_7$RSA	DNPA$_{11}$RSA-		4·0±1·3
III[a]		Sulph$_9$RSA	DNP$_7$RSA-	9·3±0·3	8·2±1·1
III[b]	DNP$_7$RSA	DNPA$_{11}$RSA	DNP$_7$RSA-		9·7±1·3
III[c]		DNP$_7$RSA	DNP$_7$RSA-		12·8±0·8

* Anti-NAD titre measured against the indicator cells shown in (§) in presence of 10^{-3} M of the relevant hapten.
† 1 mg of primary antigen given s.c. in FCA.
‡ 1 mg of secondary antigen given i.v. in PBS.
§ Indicator cells used to detect conjugate-specific anti-NAD antibodies (see Materials and Methods).
¶ Restimulation on day 60 following primary immunization.

The antisera from groups I[a] and I[b] and groups II[a] and II[b] were then tested for anti-DNPA and anti-sulph antibodies. As can be seen from Table 3, no enhanced anti-hapten antibody responses were detected either in group I[b] or in group II[a]. The same results were obtained when testing the antisera from day 14. In summary, there seems to be no detectable *in vivo* cross-reactivity between the NADs introduced into RSA by (1) diazotization with sulph or DNPA or (2) by the same hapten coupled by two different coupling procedures.

TABLE 3

ANTI-HAPTEN ANTIBODY SYNTHESIS AGAINST HAPTEN-AUTOLOGOUS PROTEIN CONJUGATES

Group No.	Primary† antigen	Secondary‡ antigen	Log$_2$ anti-hapten haemagglutination titre* Anti-DNPA titre		Anti-sulph titre	
			Days after restimulation§ 0	7	0	7
I[a]	Sulph$_9$RSA	Sulph$_9$RSA	0·4±0·8	0·0	5·7±2·2	7·9±1·4
I[b]	Sulph$_9$RSA	DNPA$_{11}$RSA	0·1±0·2	0·4±0·5	4·1±1·6	4·5±2·0
II[a]	DNPA$_{11}$RSA	Sulph$_9$RSA	1·3±1·2	2·1±1·4	0·0	0·0
II[b]	DNPA$_{11}$RSA	DNPA$_{11}$RSA	1·8±1·9	7·1±1·4	0·6±0·5	0·0

* Anti-DNPA titre measured against DNPA$_8$HSA-SRBC. Anti-sulph titre measured against sulph-SRBC.
† 1 mg of primary antigen given s.c. in FCA.
‡ 1 mg of secondary antigen given i.v. in PBS.
§ Restimulation on day 60 following primary immunization.

3. SEROLOGICAL STUDY ON THE CROSS-REACTIVITY BETWEEN NADs IN $SULPH_9RSA$, $DNPA_{11}RSA$ AND DNP_7RSA

We have shown previously that there was no detectable cross-reactivity (1) between anti-sulph and anti-DNP antibodies and (2) between anti-NAD^{sulph} and anti-NAD^{DNP} antibodies (Rubin, 1972a, b, 1973). When coupling the DNP hapten to RSA by diazotization, the DNP group is linked to tyrosine, histidine and lysine residues, as is the case with the sulph hapten (Eisen *et al.*, 1954; Tabachnick and Sobotka, 1959). No cross-reactivity between anti-sulph and anti-DNPA antibodies could be detected suggesting that the amino-acid residues are a less important part in the haptenic determinant (Kabat, 1966). This finding holds true whether anti-DNPA antibodies are tested against DNP_7OA-SRBC (Table 4) or $DNPA_8HSA$-SRBC (unpublished data). Anti-DNP titres of anti-$DNPA_{11}$ RSA antisera against DNP_7OA-SRBC could be inhibited by both DNPA-tyr and DNP-lys (Table 4). Differences in the inhibitory effectiveness might be due to the purity of the

TABLE 4

SPECIFICITY OF ANTI-DNP ANTIBODIES INDUCED BY DNP_7RSA AND $DNPA_{11}RSA$

Antiserum†	Inhibitor	Log$_2$ anti-DNP haemagglutination titre* — Molar concentration of inhibitor							
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	PBS
Anti-DNP_7RSA	DNP-lys	1·0	1·0	2·0	5·0	7·0	8·0	8·0	8·0
	DNPA-tye	3·0	5·0	6·5	7·0	8·0	8·0	8·0	8·0
	Sulph-tyr	8·0	7·5	8·0	—	—	—	—	8·0
Anti-$DNPA_{11}RSA$	DNP-lys	0·5	1·5	3·5	5·5	8·0	8·0	7·5	7·5
	DNPA-tyr	1·0	3·0	6·0	8·5	8·0	9·0	9·0	8·5
	Sulph-tyr	8·5	9·0	9·0	—	—	—	—	8·5

* Anti-DNP titre measured against DNP_7OA-SRBC.
† Secondary antisera from group III[c] and group II[b], respectively.

DNPA-tyr and the DNP-lys haptens. Again similar findings were obtained when using $DNPA_8HSA$-SRBC as indicator cells in the haemagglutination assay (unpublished data). However, anti-DNP titres of anti-DNP_7RSA antisera were always inhibited better with DNP-lys than with DNPA-tyr, suggesting that anti-DNP-lys-RSA, antibodies have a higher affinity for DNP-lys than for DNPA-tyr. Therefore, anti-NAD antibodies in anti-$DNPA_{11}RSA$ and anti-DNP_7RSA antisera were tested in the presence of 10^{-3} M DNPA-tyr or DNP-lys, respectively.

Anti-NAD^{sulph} antibodies showed almost no serological cross-reactivity with anti-NAD^{DNPA} or anti-NAD^{DNP} antibodies (Table 5). On the other hand, anti-NAD^{DNPA} antibodies showed some avidity for the NADs in $sulph_9RSA$ (Table 5). Anti-NAD^{DNP} antibodies displayed no detectable avidity for the NADs in $sulph_9RSA$ and only some for the NADs in $DNPA_{11}RSA$. A summary of the study of the serological cross-reactivity between the NADs in $sulph_9RSA$, $DNPA_{11}RSA$ and DNP_7RSA is presented in Fig. 1. These results indicate that the coupling of sulph and DNPA to RSA by diazotization creates new antigenic determinants which are highly dependent on the nature of the hapten (especially in the sulph situation).

The cross-reactivity observed between the NADs in $sulph_9RSA$ and $DNPA_{11}RSA$ might be due to effects of the diazotization reaction on the RSA molecule. We have found previously (Rubin, 1972a) that anti-NAD^{sulph} antibodies reacted with RSA coupled to

TABLE 5

SPECIFICITY OF ANTI-NAD ANTIBODIES INDUCED BY $SULPH_9RSA$, $DNPA_{11}RSA$ AND DNP_7RSA

Antiserum†	Inhibitor	Log2 anti-NAD haemagglutination titre*							
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	PBS
Anti-$Sulph_9RSA$	$Sulph_9RSA$	0·0	0·0	0·0	3·0	5·5	7·0	8·0	9·0
	$DNPAR_{11}SA$	6·5	8·5	9·0	9·0	9·0	9·0	9·0	9·0
	DNP_7RSA	7·0	9·0	9·0	9·0	9·0	9·0	9·0	9·0
Anti-$DNPA_{11}RSA$	$Sulph_9RSA$	1·0	1·0	3·0	5·0	6·0	7·0	7·0	7·0
	$DNPA_{11}RSA$	0·0	0·0	1·0	3·0	4·0	6·0	7·0	7·0
	DNP_7RSA	4·5	6·0	7·0	7·0	7·0	7·0	7·0	7·0
Anti-DNP_7RSA	$Sulph_9RSA$	9·0	11·0	11·0	11·0	11·0	11·0	11·0	11·0
	$DNPA_{11}RSA$	2·0	5·0	9·0	11·0	11·0	11·0	11·0	11·0
	DNP_7RSA	0·0	0·0	0·0	1·0	3·0	8·0	10·0	11·0

* Anti-NAD titre of anti-$sulph_9RSA$ antiserum measured against $sulph_9RSA$-SRBC in presence of 10^{-3} M Sulph-tyr. Anti-NAD titre of anti-$DNPA_{11}RSA$ antiserum measured against $DNPA_{11}RSA$-SRBC in presence of 10^{-3} M DNPA-tyr.
SRBC in presence of 10^{-3} M DNPA-tyr. Anti-NAD titre against anti-DNP_7RSA antiserum measured against DNP_7RSA-SRBC in presence of 10^{-3} M DNP-lys.
† Three individual secondary antisera from groups I[a], II[b] and III[c], respectively.

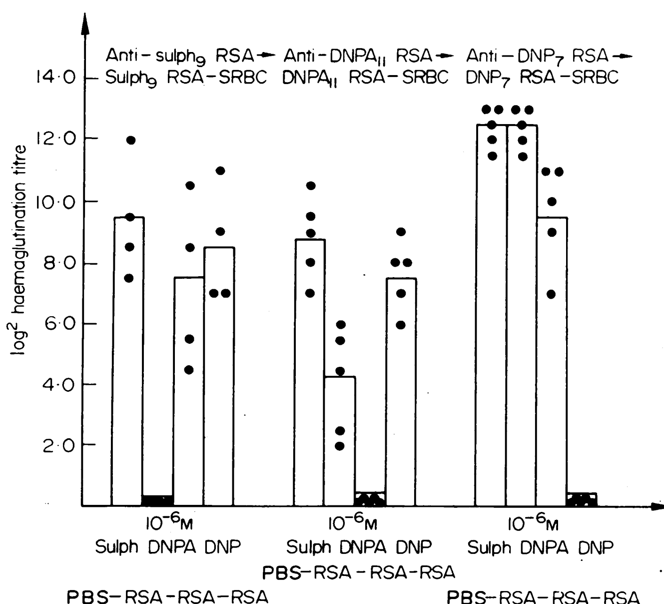

FIG. 1. Cross-reactivity between anti-NAD antibodies induced by $Sulph_9RSA$, $DNPA_{11}RSA$ and DNP_7RSA. Ordinate: Anti-NAD haemagglutination titres measured in presence of 10^{-3} M of the relevant free hapten. Abscisse: Molar concentration of the indicated inhibitors.

SRBC by means of bis-diazotized benzidine (BDB) (RSA diazotized to SRBC (Rubin 1972a)). Thus the anti-$sulph_9RSA$ and the anti-$DNPA_{11}RSA$ antisera in the present study were tested for reactivity to RSA-BDB-SRBC. All ten antisera (from groups I[a] and

II[b]) showed haemagglutination titres against RSA-BDB-SRBC ($\log_7$ titres = 5·0–7·0), whereas the titre of anti-DNP_7RSA antisera were negative. The anti-RSA-BDB-SCBC titres could be inhibited completely when using $sulph_9RSA$ or $DNPA_{11}ESA$ as inhibitors (10^{-4}–10^{-6} M). This means that the chemical build-up of the NADs introduced by sulph or DNPA is influenced also by an 'unspecific' component due to diazotization. As shown in Table 6, the NADs in $sulph_9RSA$ seem to contain more of the 'diazo' component than do the NADs in $DNPA_{11}RSA$, i.e. $sulph_9RSA$ was superior as inhibitor of the RSA-BDB-SRBC haemagglutination reaction, when tested against both anti-$sulph_9$-RSA and anti-$DNPA_{11}RSA$ antisera.

TABLE 6

ANTI-$SULPH_9RSA$- AND ANTI-$DNPA_{11}RSA$ ANTIBODIES WITH ACTIVITY FOR DIAZOTIZED RSA

Antiserum	Inhibitor	$-\text{Log}_{10}$ to the inhibitor concentration giving 75 per cent inhibition of RSA-BDB-SRBC titre.* Serum No.			
		1	2	3	4
Anti-$sulph_9RSA$	$Sulph_9RSA$	9·0	9·3	9·7	8·1
	$DNPA_{11}RSA$	6·0	7·0	6·2	6·9
Anti-$DNPA_{11}RSA$	$Sulph_9RSA$	7·0	8·0	8·0	7·8
	$DNPA_{11}RSA$	6·0	7·0	7·0	6·8

* See Experimental Methods.

DISCUSSION

Studies on the induction of antibody synthesis against haptens and new antigenic determinants introduced by the haptenic coupling have been performed using either hapten-homologous protein conjugates (St. Rose and Cinader, 1967; Paul, 1970; Rubin, 1972a, b, c, 1973; Rubin *et al.*, 1972a, 1973; Rubin and Wigzell, 1973) or hapten-heterologous protein conjugates in animals tolerant to the native carrier protein (Boyden and Sorkin, 1962; Nachtigal and Feldmann, 1964; Cinader *et al.*, 1967; Paul *et al.*, 1967). The induction of anti-hapten antibody synthesis in the latter animals is probably mediated by a mechanism similar to that demonstrated for hapten-autologous carrier conjugates, i.e. by cooperation between hapten-specific B lymphocytes and 'conjugate' or NAD-specific T lymphocytes (Rubin *et al.*, 1972a, b; Rubin and Wigzell, 1973). Several authors have described the production of 'anti-carrier' antibodies with preferential affinity for the hapten-carrier conjugate over the free carrier (= anti-NAD antibodies) in such systems. These antibodies have been detected using different techniques such as passive haemagglutination (Boyden and Sorkin, 1962; Cinader *et al.*, 1967; St. Rose and Cinader, 1967; Rubin, 1972a, d), gel precipitation (Nachtigal and Feldmann, 1964; Rubin, 1972a), quantitative precipitation (Eisen *et al.*, 1954; Paul *et al.*, 1967) and antigen-binding (Paul *et al.*, 1967).

In connection with the present experiments we asked the following question. Is the nature and the serological specificity of the new antigenic determinants introduced in homologous albumins by the hapten coupling reaction determined (1) by the chemical nature of the hapten and/or (2) by the coupling procedure? By studying the cross-

reactivity of antibodies produced against sulph-RSA (sulph dizao-linked to RSA) and DNP-RSA conjugates (DNP diazo-linked or amino-linked to RSA) we found that both the chemical nature of the hapten and the coupling procedure played a role in the serological specificity of the NADs.

The following results supported the role of the coupling reaction on the build-up of the NADs: (1) rabbits immunized with $DNPA_{11}RSA$ or DNP_7RSA produced anti-NAD antibodies which showed almost no serological cross-reactivity (Table 2), (2) rabbits immunized with $sulph_9RSA$ or $DNPA_{11}RSA$ produced antibodies which showed a relatively high titre against RSA-BDB-SRBC (= diazo-linkage influenced NADs).

On the other hand, the high degree of specificity of the anti-NAD antibodies produced against $sulph_9RSA$ (as measured with $sulph_9RSA$-SRBC, see Table 5) indicates that the sulph hapten plays a highly significant role in the serological specificity of the NADs in $sulph_9RSA$. As shown in Table 6, $sulph_9RSA$ was a more efficient inhibitor of the antibody titre against RSA-BDB-SRBC than was $DNPA_{11}RSA$. This may be due either (1) to a more profound influence of the diazo-linkage with the sulph hapten over that with the DNPA hapten, or (2) to the availability on the surface of the RSA molecule of the $NADs^{sulph}$ over the $NADs^{DNPA}$ (Hanna *et al.*, 1972). Thus the diazo-linkage of haptens to proteins seems to create at least two different kinds of NADs: diazo-linkage specific NADs and hapten-influenced NADs.

In the mouse we have presented direct evidence that induction of anti-hapten antibody synthesis against hapten-autologous protein conjugates is mediated via co-operation between anti-hapten reactive B lymphocytes and 'conjugate' specific T lymphocytes (Rubin *et al.*, 1972a, b; Rubin and Wigzell, 1973). Similar results were obtained by Walters *et al.* (1972). The present data indicate that anti-hapten specific B lymphocytes with similar specificity are induced by $DNPA_{11}RSA$ and DNP_7RSA (Table 4). Yet, no secondary anti-DNP responses were obtained either in rabbits primarily stimulated with $DNPA_{11}RSA$ and restimulated with DNP_7RSA or *vice versa* (Table 1). This means that the helper cells (T lymphocytes) induced by these conjugates do not cross-react, a finding which is in agreement with the specificity pattern of the delayed hypersensitivity reactions in guinea-pigs stimulated with similar hapten-carrier conjugates (Benacerraf and Levine, 1962; Gell and Silverstein, 1962; Hanna *et al.*, 1972).

The NADs introduced by sulph and DNPA into RSA showed serological cross-reactivity (Tables 5 and 6). However, no enhanced primary anti-sulph or anti-DNPA antibody responses were obtained as a consequence of pre-immunization with the cross-reactive NADs (Table 3). This might be due either (1) to the sensitivity of the *in vivo* pre-immunization test being too low to detect the cross-reactivity or (2) the cross-reactivity between the NADs in $sulph_9RSA$ and $DNPA_{11}RSA$ (in particular that influenced by the diazo-linkage) was not recognized at the T cell level.

The present studies give further evidence to the distinct serological specificity of antibodies against NADs and establish the cross-reactivity pattern of $sulph_9RSA$, $DNPA_{11}$-RSA and DNP_7RSA at the humoral antibody level and partly also at the level of *in vivo* secondary stimulation (probably T cell level). Further studies in the mouse on the specificity of co-operating T lymphocytes (Rubin and Wigzell, 1973) immune to a series of conjugates analogous to those in the present article (sulph-MSA = sulph-mouse serum albumin, DNPA-MSA and DNP-MSA) are in progress, using an adoptive cell transfer system (Rubin *et al.*, 1972a).

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