

## COMPETITION OF HAPTENS\*

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Competition of antigens, a phenomenon of some significance in vaccination procedures, may also serve to bring about a better understanding of the mechanism of antibody formation. The subject has been extensively studied by a number of authors, especially by Adler, who has recently reviewed his own work as well as that of others (1, 2).

Competition is manifested in that injection of certain antigens will suppress the response of the animal to a second antigen administered concurrently or within a certain time interval. While it is generally true that the suppressing antigen is considerably more immunogenic than the suppressed antigen, Adler (2) has warned that this may not be universally true. The possibility that suppression may be due to removal of a metabolite necessary for antibody synthesis has concerned various authors. This problem has been investigated by Cremer (3) in tissue culture and by Adler (2) through the administration of small amounts of antigen. Both authors have come to the conclusion that exhaustion of a metabolite necessary for the production of antibody is most unlikely. On the other hand, the possibility of competition for antibody-producing cells has been suggested. This possibility, however, is not compatible with direct clonal selection by antigen since it would imply that cells possess the potential to produce antibodies to two unrelated antigens with high frequency; i.e., the suppressed antibody could potentially be produced by a large number of those cells which are producing the antibody to the suppressing antigen. Our work indicates that competition can take place on the cellular level, in that it appears to depend on the ability of two antigenic determinants to reach the same cell simultaneously.

The current investigation followed an observation made during the course of a study with bovine serum albumin (BSA) to which two different haptenic groups, 2,4-dinitrophenyl (DNP) and *p*-azophenyl arsonate (R), had been conjugated. The study had been undertaken for the purpose of demonstrating an anamnestic response to a hapten group present in the original antigen, but

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lacking in the antigen used for rechallenge (4). In the course of this work it was found that the presence of a large number of DNP groups on the molecule will suppress the antibody produced to the R hapten.

Competition of antigenic determinants was reported by investigators (5, 6), who have used identical carriers to which different haptens had been conjugated. They showed both enhancement and suppression of antibody production depending on the hapten conjugated to the carrier. The interpretation of that work is not clear and would depend on an extensive study of changes in the tertiary structure or enzymatic degradation products of the substituted proteins. Two groups of authors (7, 8) have reported work with doubly conjugated carrier proteins, but neither noticed any degree of suppression of either anti-hapten antibody.

#### *Materials and Methods*

Keyhole limpet hemocyanin (KLH) was prepared according to the method of Campbell et al. (9). Sheep red cell stroma was prepared according to the method described by Mayer (10). Crystallized (5 ×) bovine plasma albumin (BSA) was purchased from Armour Pharmaceutical Co., Kankakee, Illinois, arsanilic acid was obtained from Eastman Kodak Co., Rochester, New York, and 2,4-dinitrobenzenesulfonic acid sodium salt (DNBS) from Nutritional Biochemicals Corporation, Cleveland. The latter material was recrystallized from hot water. Freund's complete adjuvant was obtained from Difco Laboratories, Inc., Detroit.

*R<sub>72</sub>KLH and R<sub>72</sub>DNP<sub>19</sub>KLH.*—Arsanilic acid (1.5 g) was dissolved in 100 ml of 0.1 N HCl by warming slightly in a water bath. The material was then placed in an ice bath and observed during cooling. When the first crystals settled out (at approximately 8 to 10° C) 0.5 g of sodium nitrite in 4 ml of water were quickly added. After ½ hr the solution of diazonium salt was added at 10- to 20-min intervals in 10 ml aliquots to 4 g of KLH in 50 ml of solution containing 1 g of tris-(hydroxymethyl)-aminomethane (Tris). Following each addition the pH was adjusted to 9 with 0.5 N NaOH. After addition of the complete volume of diazonium salt the solution was permitted to stand overnight and the protein was then precipitated by acidification with acetic acid. The precipitate was redissolved in 100 ml of solution containing 2 g of Tris, to which small amounts of 1 N NaOH were added in order to obtain complete solution. This solution was passed through Sephadex G-25 to remove the bulk of uncoupled material and side product, then through Sephadex G-50 to assure complete removal of uncoupled arsonate derivatives and possible low molecular weight protein degradation products. All passages on Sephadex were carried out in Tris-saline (normal saline containing 10% 0.1 N tris buffer, pH 8). No second band was obtained on Sephadex G-50. The final volume of the solution was 275 ml. To 125 ml of this solution, 50 ml of 0.2 N sodium carbonate containing 10 g of DNBS were added. The flask was rotated for 36 hr on an inclined drum at a speed of approximately 10 rpm. The conjugate was then precipitated with acetic acid, redissolved in Na<sub>2</sub>CO<sub>3</sub>, adjusted with NaOH to pH 10, and passed through Sephadex G-50. The arsenic content of this and subsequent preparations was determined by Elek Microanalytical Laboratories, Torrance, California. The DNP content was determined by subtracting the absorption of the R-KLH from that of R-DNP-KLH at 360 mμ using the specific absorption constant given by Eisen et al. (11), for calculation. This procedure appeared permissible since the absorption spectrum of a mixture of R-KLH and DNP-KLH and that of the doubly conjugated material were superimposable at wavelengths above 300 mμ. R<sub>72</sub>DNP<sub>19</sub>KLH showed two major peaks in the ultracentrifuge, calculated at 10S and 13S (extrapolated to infinite dilution). The smaller peak constituted somewhat more than 50% of the material. A minimum

molecular weight of 220,000 was calculated for this antigen, based on diffusion constant values for other hemocyanins (12). On this basis the smallest molecule present would contain 19 DNP groups and 72 R groups.

*R<sub>24</sub>BSA and R<sub>24</sub>DNP<sub>25</sub>BSA.*—To 1.1 g of arsenilic acid in 50 ml of 0.1 N HCl, 350 mg of NaNO<sub>2</sub> were added. Three 10 ml portions of this solution were added to 5 g of BSA in 250 ml of 0.1 N Na<sub>2</sub>CO<sub>3</sub> at 5-min intervals. The material was precipitated by acidification, redissolved, and passed through Sephadex as above. 95 ml of solution was recovered. To 55 ml of this solution, 45 ml of water and 100 ml of 0.2 N Na<sub>2</sub>CO<sub>3</sub> containing 10 g DNBS were added. The solution was stirred at medium speed on a magnetic stirrer for 12 hr. It was then precipitated by rapid addition of 1 N acetic acid to the ice cold solution, washed, and passed through Sephadex G-25 three times. Following the third passage, no further change in the 280/360 absorption ratio was seen.

*R<sub>24</sub>DNP<sub>8</sub>BSA.*—R<sub>24</sub>BSA was prepared as above. To 270 mg of this material in 10 ml of 0.1 N Na<sub>2</sub>CO<sub>3</sub> was added 1 g DNBS in 10 ml Na<sub>2</sub>CO<sub>3</sub>. The solution was stirred rapidly for 90 min, precipitated, and passed repeatedly through Sephadex until a constant 280/360 absorption ratio was obtained on two consecutive passages.

*DNP<sub>39</sub>KLH.*—To 2 g of KLH and 100 ml of saline were added 5 g of DNBS and 100 ml of 0.2 N Na<sub>2</sub>CO<sub>3</sub>. The solution was stirred overnight on a magnetic stirrer at medium speed and treated as above.

*DNP<sub>28</sub>BSA.*—To 3 g of BSA dissolved in 100 ml of 0.1 N Na<sub>2</sub>CO<sub>3</sub> were added 9 g of DNBS in 200 ml of 0.1 N Na<sub>2</sub>CO<sub>3</sub>. The solution was stirred overnight on a magnetic stirrer at medium speed and treated as above.

*R Stroma.*—To 440 ml of saline containing 1.5 mg of stroma nitrogen per ml, 220 ml of 0.2 N Na<sub>2</sub>CO<sub>3</sub> were added. Arsanilic acid (1.1 g) in 100 ml of 0.1 N HCl was diazotized as above. Three 10 ml portions of diazonium salt were added at 5-min intervals with stirring. The material was spun down and washed four times in saline to remove excess diazonium salt.

*R-DNP Stroma.*—To 300 ml of a suspension of R stroma containing 1 mg stroma nitrogen per ml were added 300 ml of 0.2 N Na<sub>2</sub>CO<sub>3</sub> containing 6 g of DNBS. The solution was stirred overnight on a magnetic stirrer at medium speed and washed four times until no further colored material was removed.

*DNP Stroma.*—To 240 ml of saline containing 1.5 mg of stroma nitrogen per ml were added 240 ml of 0.2 N Na<sub>2</sub>CO<sub>3</sub> containing 3.5 g DNBS. The solution was stirred overnight on a magnetic stirrer and the conjugate washed repeatedly in saline until no further colored material was removed. The R and DNP contents of these materials, estimated as above, were respectively: R stroma: 0.88  $\mu$ mole of R/mg stroma N. DNP stroma: 0.82  $\mu$ mole of DNP/mg stroma N. R-DNP stroma: 0.73  $\mu$ mole DNP/mg stroma N, 0.88  $\mu$ mole R/mg stroma N.

*Immunization of Animals.*—New Zealand white rabbits weighing from 5 to 8 lb. were used. Groups were randomized at the beginning of each experiment. Injections of antigens in Freund's adjuvant were administered intramuscularly and subcutaneously in at least three locations. The schedules and amounts injected are indicated in the corresponding experiments. Quantitative precipitin tests were carried out by the method of Campbell et al. (9 a), using DNP-ovalbumin and R-ovalbumin as precipitating antigens.

## RESULTS

The experiments were designed to determine if two antigenic determinants on the same immunizing molecule could significantly interfere with each other in immunogenicity. To accomplish this, we compared the response of animals injected with doubly conjugated antigens to that of animals injected with a mixture of singly conjugated antigens. Clearly, if one wishes to inject similar

amounts of hapten, it is possible to choose for the mixture of singly conjugated haptens either antigens containing a number of hapten groups per molecule similar to that possessed by the doubly conjugated antigen or molecules containing twice that number. In the former case one would have to introduce double the amount of total protein in order to achieve the administration of the same amount of hapten. In the experiments reported here both procedures have been used. It is our general impression from previous experience with this system that with courses of immunization such as those used here and with amounts of antigen in the range of those used, there is little or no difference in antibody levels produced due to variations of dosage over a rather wide range. This is especially true when complete adjuvant is used (13). We have chosen to compare only a mixture of singly conjugated antigens with the doubly conjugated material and have desisted from comparing the response to such mixtures with that induced by the components injected separately into animals. Previous experience indicates that the differences shown would probably require a very large number of experimental animals to obtain significant data.

*Response to R-DNP-KLH.—*

Two groups of six animals were used for this experiment. The animals received respectively 5 mg of  $R_{72}DNP_{19}KLH$  or 7.5 mg of a mixture containing 5 mg of  $R_{72}KLH$  and 2.5 mg of  $DNP_{39}KLH$  per injection. Injections were administered according to the following schedule: daily on days 1 to 5; 6 to 11, 15 to 18, and 22 to 25. In addition, the group receiving doubly conjugated antigen was injected on days 29, 31, 33, 35, 37, and 39. Animals were bled on days 14, 21, 28, and 42.

Results are shown in Fig. 1. The minimal requirement for positive ring test, established by using dilutions of high titer antisera diluted in normal rabbit serum, was 70  $\mu g$  of antibody. It is quite possible that somewhat larger amounts of early antibody are required to show positive ring tests.

The scattergram shows that the anti-R response of animals injected with doubly conjugated KLH appeared 2 wk later than the response in the group injected with the mixture of singly conjugated antigens. Furthermore, only 2 of 5 surviving animals showed antibody. The titers were low, and they decreased despite further courses of immunization for the next 2 wk. Anti-DNP titers, on the other hand, though somewhat lower initially, showed no significant difference. We have no ready explanation for the apparent decrease in the anti-DNP titers starting at wk 2 in the group injected with mixed, singly conjugated KLH.

Since, as will be discussed, it is conceivable that suppression may be due to some change in the physical-chemical properties of the protein due to substitution, we found it necessary to use a variety of systems to demonstrate competition.

*Response to R-DNP-BSA.—*

Animals were assigned to one of three groups according to the immunizing antigen. One group received 2 mg of  $R_{24}DNP_{25}BSA$ , another group received 2 mg of  $R_{24}DNP_8BSA$ , and a third group received a mixture of 2 mg of  $R_{24}BSA + DNP_{28}BSA$ . Each antigen was injected initially as 2 ml in complete Freund's adjuvant. Second and third injections were made into each group of animals, using  $1\frac{1}{2}$  times the initial injection dose. The group immunized with  $R_{24}DNP_8BSA$  received no further injection whereas the other two groups received intravenous injections of 0.5 or 1 mg of the respective antigen in saline, administered weekly for an additional 3 wk. All groups were bled weekly.

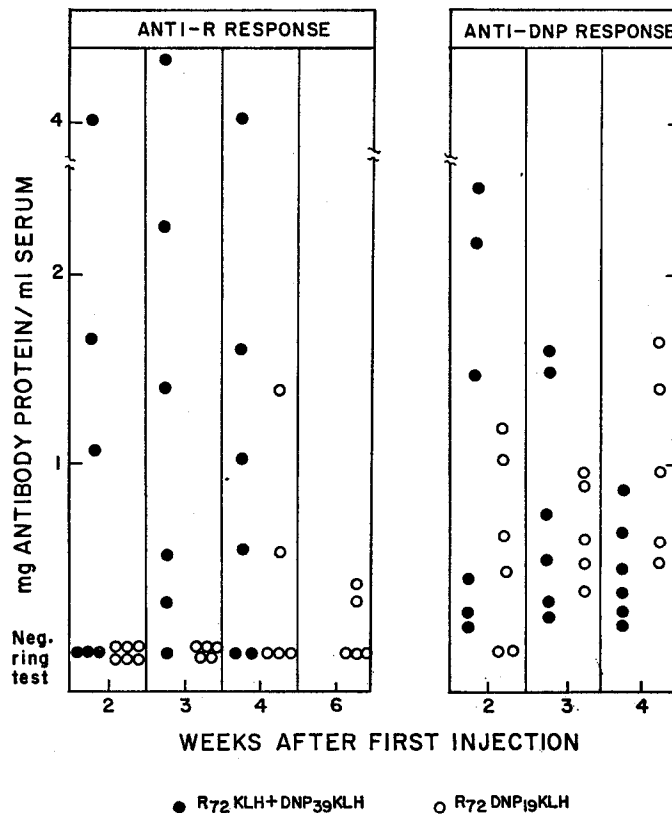


FIG. 1. Antibody response to hapten-KLH

Table I shows the antibody content of these sera. It will be noted that animals challenged with  $R_{24}DNP_{25}BSA$  produced no anti-R antibody at any time. It was due to this result that we resorted to additional intravenous injections, which frequently raise the antibody titers of adjuvant-injected animals. The sera of this group were tested also by passive cutaneous anaphylaxis (14) and

TABLE I  
*Antibody Response to Hapten-BSA in Complete Freund's Adjuvant*

Antibody specificity	Time after 1st injection	Antigens		
		R <sub>24</sub> BSA + DNP <sub>28</sub> BSA	R <sub>24</sub> DNP <sub>28</sub> BSA	R <sub>24</sub> DNP <sub>8</sub> BSA
Anti-BSA	<i>wk</i>			
	4	5/5 (850)	4/6 (70)	
	6	5/5 (1500)	4/6 (85)	
Anti-DNP	7	5/5 (1330)	4/6 (85)	
	4	3/5 (150)	5/6 (270)	4/4
	6	5/5 (860)	5/6 (670)	
Anti-R	7	5/5 (400)	5/6 (575)	4/4 (1980)
	4	4/5 (120)	0/6	4/4
	6	5/5 (500)	0/6	
	7	5/5 (400)	0/6	4/4 (920)

Fractions represent the proportion of challenged animals showing positive ring tests. Numbers in parentheses are the average antibody titers in  $\mu\text{g/ml}$  serum. The antibody content of sera showing negative ring tests was considered to be 0 for purposes of calculation of averages.

only one out of six sera showed a reaction to a 1:25 serum dilution but not to a 1:250 dilution. It would appear that at this coupling ratio, anti-R response has been suppressed completely. On the other hand, the material which was more lightly substituted with DNP, i.e. R<sub>24</sub>DNP<sub>8</sub>BSA, showed both anti-R and anti-DNP titers which were considerably higher than those of the group receiving mixed, singly coupled antigens. The result will be discussed below. We wish

TABLE II  
*Antibody Response to Hapten-Coupled Sheep Red Cell Stroma*

Antibody specificity	Time after 1st injection	Antigens	
		DNP <sub>0.73</sub> R <sub>0.88</sub> stroma*	DNP <sub>0.82</sub> stroma + R <sub>0.88</sub> stroma*
Anti-DNP	<i>days</i>		
	20	4/5 (660)	4/4 (700)
Anti-R	28	5/5 (900)	4/4 (850)
	20	2/5 (160)	4/4 (1030)
	28	3/5 (260)	4/4 (1400)

\* Hapten subscripts denote  $\mu\text{mole/mg}$  stroma N. See footnote, Table I.

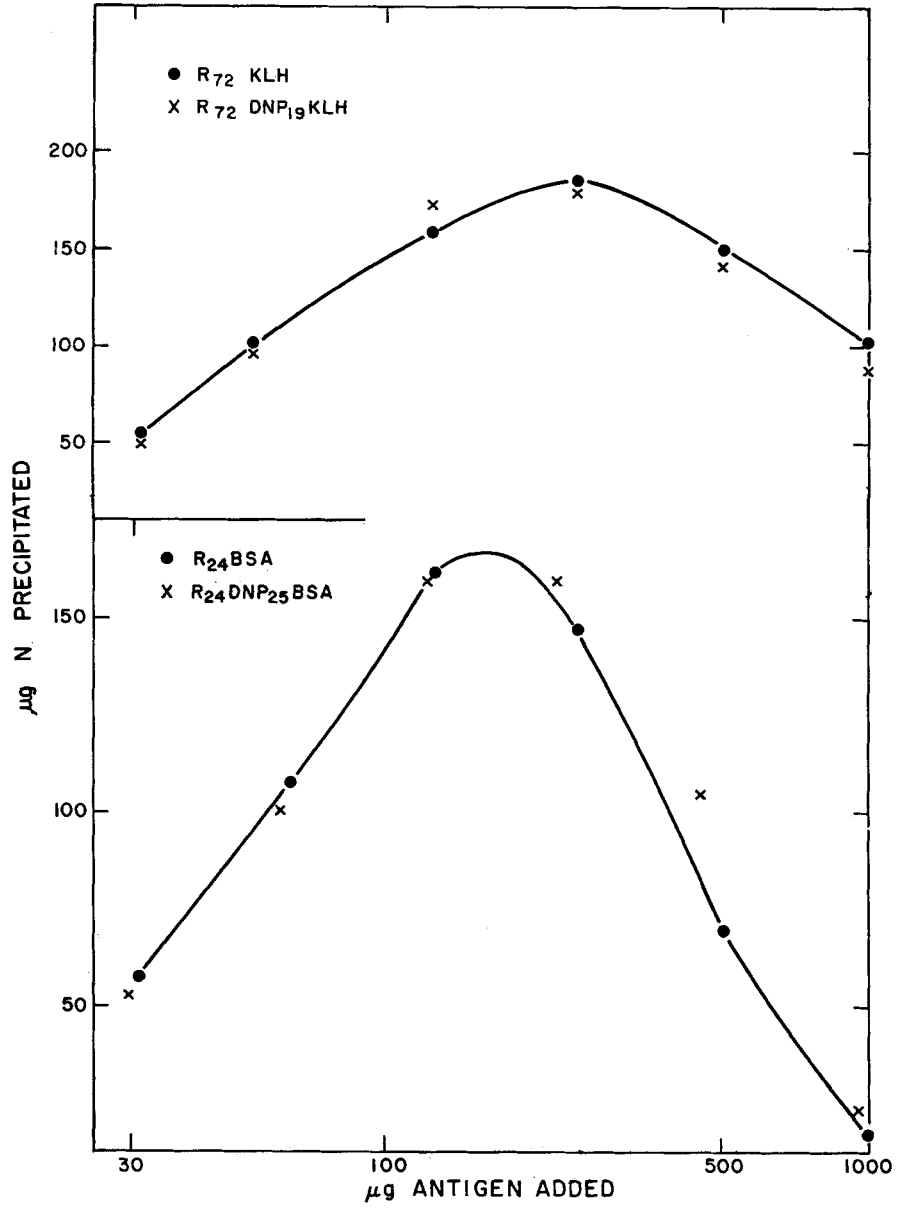


FIG. 2. Precipitation by anti-R-stroma serum

to point out here, however, that the hapten/protein weight ratios for  $R_{24}DNP_8BSA$  are quite close to those for  $R_{72}DNP_{19}KLH$ , a material which showed significant suppression of anti-R response following intravenous injection.

*Response to R-DNP-Sheep Red Cell Stroma.*—This experiment was carried out in order to add to our experience that of a particulate material containing a

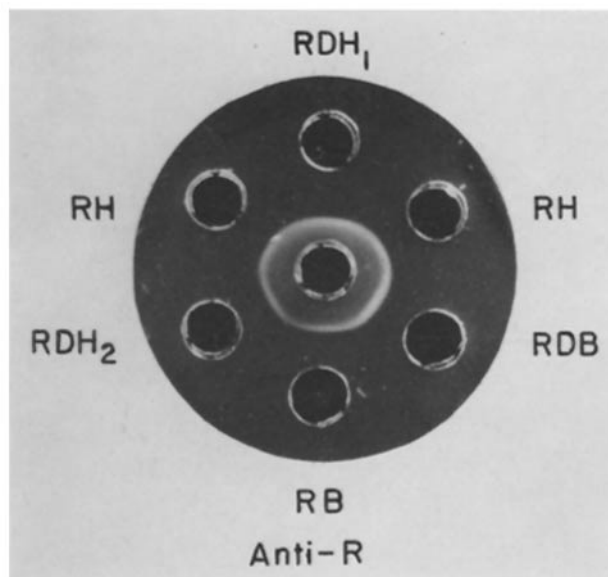


FIG. 3.  $RDH_1 = R_{440}DNP_{39}KLH$   
 $RDH_2 = R_{72}DNP_{19}KLH$   
 $RH = R_{72}KLH$   
 $RDB = R_{24}DNP_{25}BSA$   
 $RB = R_{24}BSA$

All antigen concentrations: 1 mg/ml. Center well contains anti-R-stroma.

number of different proteins in a single particle. The possibility suggested itself that one or several of the protein components of stroma might contain DNP/R ratios favorable for unsuppressed production of anti-R antibody. The system would then offer the possibility to determine if suppression occurred at the stage of phagocytosis or at a later stage in antibody production (see Discussion).

Animals received intravenous injections on 5 consecutive days containing 1 mg of R-DNP-stroma or 1 mg each of the R-stroma and the DNP-stroma preparations respectively. Animals were rested for 1 day and then received double the previous dose daily on days 7 to 11, 13 to 17, and 21 to 25. They were bled on day 20 and exsanguinated on day 28.

Table II shows that while anti-DNP production was remarkably similar in



the two groups, anti-R response was significantly suppressed in the animals injected with the doubly conjugated material.

*Availability of Antigenic Determinants.*—To demonstrate that the suppressed determinant was still available on the surface of the molecule, quantitative precipitin tests were carried out with the R-coupled material prior to and following coupling with DNP, using antiserum produced by a rabbit injected with R stroma. Fig. 2 shows the precipitin curves for KLH and BSA derivatives. It

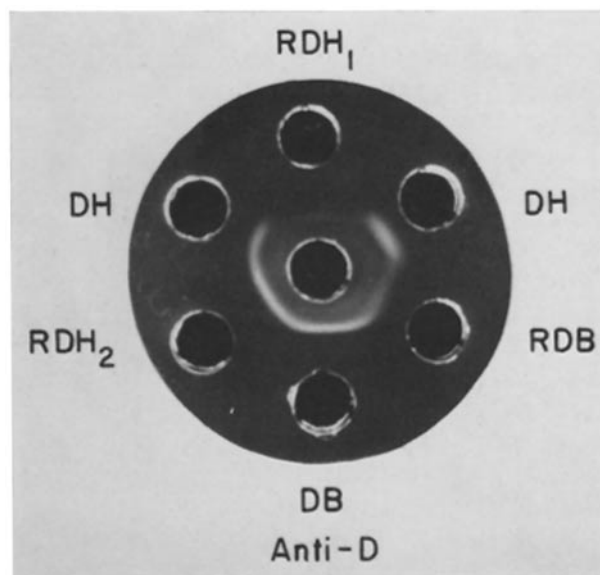


FIG. 4. DH = DNP<sub>39</sub>-KLH  
DB = DNP<sub>28</sub>-BSA  
(For others see Fig. 3) Center well contains anti-DNP-stroma.

will be seen that the presence or absence of DNP groups on the precipitating antigens does not significantly affect the shape of these curves.

In addition the soluble derivatives were tested by double diffusion in agar for identity, using antihapten antibody prepared in rabbits by injection of hapten stroma conjugates. It may be seen (Figs. 3 and 4) that with the exception of RDH<sub>1</sub> which will be discussed later, all materials show reactions of complete identity, indicating that the nature of the antigenic determinant has not been modified.

#### DISCUSSION

Our findings indicate that the DNP group can suppress formation of antibody to arsanilate when both determinants are present on the same molecule.

To establish the significance of these findings, it was necessary to show that the result is not due to a change in physical-chemical properties of the macromolecule nor to the unavailability of the suppressed determinant. The first possibility can unfortunately not be totally eliminated, since a change in charge will occur in all molecules in which either basic amino groups are removed by DNP coupling or acid azophenyl arsonate groups are added. It is difficult, however, to visualize how such a change would specifically affect the antigenicity of one type of determinant. It would furthermore be quite surprising if such an effect were to manifest itself in the antibody responses to all of the antigens used in this experiment. That those antigenic determinants to which antibody response is suppressed are equally available to the antibody, after conjugation with the suppressing determinant, was shown by demonstrating a lack of change in the precipitin curve (Fig. 2). While this procedure shows accessibility of the suppressed hapten to the respective antibody, direct proof that the hapten is equally available to the antigen-recognizing or antigen-processing mechanism can as yet not be obtained. In this respect, the uncertainty is similar to that raised in the first problem discussed above.

We have also attempted to reverse the direction of inhibition by increasing the R/DNP ratio. This can be accomplished only by first conjugating carrier with DNP, since extensive conjugation with R may occupy too many of the  $\epsilon$ -amino groups available for conjugation with DNP (15). When carrying out the reaction in this order it is possible that the DNP group is modified by azo-coupling in the 6-position of the benzene ring. Animals were injected with an R<sub>440</sub>DNP<sub>39</sub>KLH derivative prepared in this manner. Intravenous injection did not produce any detectable antibody. Injection with adjuvant, however, produced up to 1 mg of anti-R antibody but no measurable (by precipitation) amounts of anti-DNP antibody, although three of six animals showed positive ring tests with DNP-ovalbumin. The anti-DNP precipitin curve changed considerably, and the behavior of this material, RDH<sub>1</sub>, in agar diffusion indicates partial identity (Fig. 4) regarding the DNP determinant.

In recent times the possibility of a two stage mechanism of antibody formation, involving two different cell types, has become widely accepted (16-18). Even without postulating the participation of two intrinsically immunologically competent cells in this process, it is clear that phagocytosis is intimately involved in the antibody-making mechanism, as has been discussed (18). Whether, following phagocytosis, information is passed on to the antibody-producing cell in the form of degraded antigen, RNA-coupled antigen (19, 20), or totally lacking in antigen (21) is still a matter of controversy. The question arises whether competition of haptens takes place at the stage of phagocytosis or at the stage of antibody production, and to what extent it is similar to the competition shown between macromolecular antigens.

The data indicate clearly that effective competition requires the presence of

both haptens on the same molecule implying that it is necessary for both determinants to reach the same cell. Since in the mixture of singly substituted antigens more than  $10^{15}$  molecules of each kind are administered in each injection, cells must also encounter both haptens with high frequency in this situation. The following are some of the mechanisms that might account for the difference in response:

Competition takes place at the level of antigen processing. (a) The processing cell seals itself off immediately after encountering an antigen and is impermeable to further antigen molecules. (b) The processing apparatus becomes determined very rapidly to produce a single type of information and is impervious to additional antigenic stimulation. (c) The information transcribing equipment of the cell is limited to recognizing single topographic features in a given segment of antigen. The difference in the character of the hapten, one charged and the other hydrophobic, may be fundamental in this selection.

Competition takes place at the level of antibody production. Probably a very small proportion of the antigen is converted into antigenic information (22), and the probability of encounter with two different types of information-bearing units is relatively small.

At present, we cannot readily distinguish between these alternatives.

An attempt to determine whether simultaneous phagocytosis of antigens is insufficient to cause competition was made using hapten-coupled stromata. The composition of stroma is not yet accessible enough for the purpose of calculating hapten ratios. Electrophoretically eight proteins have been found in stroma, immunoelectrophoretically one can demonstrate an even larger number (23). Failure to demonstrate competition could then be imputed to single proteins substituted mainly with arsanilate, and would show that simultaneous arrival of the determinants in the phagocytic cell is not sufficient. Assuming that approximately 70% of stroma nitrogen is protein nitrogen, and an average amino acid composition of protein, it can be calculated that the compounds used contained approximately twelve R groups and 10 DNP groups per protein of mol wt 70,000. Thus, even small proteins would have significant probability of containing both haptenic groups and the experiment is not conclusive with regard to the site of competition. Experiments are in progress attempting to couple a mixture of singly conjugated proteins to stroma and to immunize animals with such antigens.

The mode of immunization seems to be important in the manifestation of hapten competition. Animals immunized with highly DNP-substituted R-DNP-BSA in adjuvant showed complete suppression of the anti-R response. Unfortunately, a similar highly conjugated KLH derivative could not be prepared. It is thus not clear whether it is the role of adjuvant which determines this complete blockage. Since inhibition of anti-R antibody production did not occur in animals injected with  $R_4DNP_8BSA$  in adjuvant, it would appear that

competition depends either on the hapten ratio per se—which would probably be of greatest importance at the level of the phagocytic cell—on the degree of proximity of the two determinants on the carrier molecule, or on the probability of encountering both determinants on the ultimate immunogenic<sup>4</sup> degradation product of the antigen. The R/DNP ratio of R<sub>72</sub>DNP<sub>10</sub>KLH, which showed suppression of anti-R on intravenous immunization, is not significantly different from that of R<sub>24</sub>DNP<sub>8</sub>BSA. A group of animals were immunized with the KLH derivatives in complete adjuvant. In this experiment very low levels of antibody were obtained but an equal number of animals in the group injected with mixed, singly substituted hemocyanins and that injected with doubly conjugated material showed ring tests to arsaniolate. Recently, several investigators have reported production of antibody to antigens of very low molecular weight using Freund's adjuvant (24, 25). It is therefore possible that adjuvant is important in the conversion of certain small degradation products into antigenic information. The stage of the process of antibody formation at which this influence is exerted requires further elucidation.

#### SUMMARY

Groups of rabbits were injected with either bovine serum albumin, sheep red cell stroma, or keyhole limpet hemocyanin to which 2,4-dinitrophenyl and/or *p*-azophenyl arsonate groups had been coupled. Groups of animals received either doubly coupled antigen or an equivalent mixture of singly coupled antigens. Materials were injected intravenously as a solution or subcutaneously and intramuscularly in complete Freund's adjuvant.

The presence of dinitrophenyl groups on the immunizing antigen could suppress, partially or completely, the antibody response to *p*-azophenyl arsonate when this hapten was located on the same molecule. Suppression was dependent on the ratio of haptenic groups on the molecule, appeared to be greatly affected by the method of immunization, and could be demonstrated in all three antigen systems. Partial suppression was manifested in decreased frequency and delayed appearance of the response as well as decreased maximal antibody titers. These findings appear irreconcilable with the possibility of direct clonal selection of antibody-producing cells by unprocessed antigen.

*Note Added in Proof.*—We have recently learned that poly-DL-phenylalanyl groups will suppress the formation of antibody to poly-DL-alanyl groups on the same carrier (M. Sela, personal communication). The system differs from that reported here in that a similar suppression is found in animals immunized with a mixture of singly substituted carriers (Schechter, I., *Biochim. et Biophysica Acta*, 1965, **104**, 303).

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