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The Significance of the Protein Carrier in the Stimulation of DNA Synthesis by Hapten–Protein Conjugates in the Secondary Response

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Summary. Rabbits were immunized with 2,4-dinitrophenyl (DNP)–protein conjugates. Spleen cell suspensions were prepared and incubated in the presence of various DNP–protein conjugates, the proteins alone, and DNP–lysine. The antigen dependent stimulation of DNA synthesis was used as a measure of the antigenic 'activity' of the DNP preparations. It was found that the cells were strongly stimulated by the DNP–protein conjugates used for immunization, and weakly stimulated by the protein alone. Highly substituted DNP–protein conjugates were markedly more effective than lightly substituted conjugates. DNP-conjugates with proteins other than the one used during immunization were inactive. DNP–lysine alone was inactive but inhibited stimulation by the DNP–protein conjugate used for immunization. The significance of these findings is discussed.

INTRODUCTION

Many workers have shown that animals immunized with hapten–protein conjugates form antibodies both to the hapten moiety and to the 'carrier' protein (Landsteiner, 1945). Under appropriate conditions (e.g. Farah, Kern and Eisen, 1960), the formation of antibodies specific for the hapten predominate. Precipitation studies with the hapten coupled to other carriers (Landsteiner, 1945) and hapten inhibition studies (Karush, 1957; Kabat, 1956; and others) have led to the conclusion that such anti-hapten antibodies are specific for an antigenic site little bigger than the hapten itself. Others, however, have suggested that the size may be considerably greater (Porter, 1960; Press and Porter, 1962).

It is well known, however, that it is not possible to immunize animals with free hapten, and the protein carrier would therefore appear to play an essential role in immunization if not in reaction with the formed antibody.

The previous paper described an *in vitro* system for the study of the mechanism of antigenic stimulation. The addition of specific antigen to spleen cells from a previously immunized animal was shown to stimulate cell division and the incorporation of thymidine into DNA.† The degree of stimulation has been taken as a measure of antigen activity in the secondary response.

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† The abbreviations used in this work are as follows: DNP-, dinitrophenyl-; BGG, bovine γ-globulin; BSA, bovine serum albumin; HGG, human γ-globulin; HSA, human serum albumin; Ea, egg albumin; DNA, deoxyribonucleic acid.

In this paper, use has been made of hapten–protein conjugates as antigens to investigate the significance of the 'carrier' part of the molecule in this stimulation.

Animals were immunized with hapten–protein conjugates using dinitrophenyl (DNP) derivatives of heterologous proteins. Spleen cells from such animals were tested for their ability to respond *in vitro* to the immunizing complex, the carrier or hapten alone, and the hapten on other carriers.

MATERIALS AND METHODS

The materials and methods used were those described in the preceding paper. The following additional materials and methods were used:

Materials

2,4-Dinitrobenzenesulphonic acid was obtained from Eastman Kodak Co., Rochester, New York. The sodium salt was twice recrystallized from water before use. Epsilon–DNP–L-lysine HCl was obtained from Gallard Schlesinger Chemical Mfg. Corporation, Garden City, New York.

Methods

The DNP–protein conjugates were prepared by a modification of the technique described by Eisen, Kern, Newton and Helmreich (1959). Excess sodium dinitrobenzene sulphonate was removed by dialysis (preparations freed of the sulphonate by passage through ion-exchange resins proved toxic to spleen cells). The average number of DNP groups per molecule of protein carrier was calculated from the absorption readings at 360 and 290 mμ in alkaline solution (Eisen, Carsten and Belman, 1954). In these calculations, the molecular weight of the globulins was taken to be 160,000, of the serum albumins 68,000 and of egg albumin 40,000.

Conjugates with two to fifty-five DNP groups were prepared by varying the concentrations of the reactants. No information was obtained on the polydispersity of the preparations.

The conjugates were dialysed against distilled water, adjusted to pH 8–9 by the addition of Na_2CO_3. Under these conditions, the preparations remained in solution. The preparations were spun at 30,000 ***g*** for 1 hour and the concentration and number of DNP groups were determined immediately before use. The preparations were then diluted as required in complete medium. In some cases this lowering of pH and increase in salt concentration caused the DNP–globulin preparations to become turbid. Such preparations were not used except where indicated below.

Immunization

The rabbits were immunized by the standard immunization schedule previously described. DNP–BGG preparations used for immunization contained either thirty-six or fifty DNP groups per molecule of BGG and the DNP–BSA preparation twenty-eight groups per molecule BSA. (It so happened that the rabbit immunized with DNP–BSA had been previously immunized with HSA.)

RESULTS

IMMUNIZATION

The sera of rabbits immunized with DNP–BGG contained antibody which would precipitate with DNP–BGG. Little or no antibody precipitated with BGG. The rabbits

had thus responded preponderantly to the hapten and not to the carrier. The rabbit immunized to DNP–BSA had been previously immunized to HSA immediately before the start of the DNP course. Sera from this rabbit contained antibody to HSA at the start of DNP–BSA immunization. At the end of immunization to DNP–BSA, the serum contained, in addition, anti-DNP–BSA and some antibody to BSA. This animal was thus immunized to the hapten DNP and to the protein HSA. It was slightly immunized to the carrier BSA. This information is summarized in Table 1.

In vitro CHALLENGE

1. *Hapten on Carrier Used for Immunization*

Spleen cells from the DNP–BGG immunized rabbits responded well to DNP–BGG and slightly to BGG (Fig. 1). It can be seen that approximately one hundred times more BGG was required to produce the same stimulation as DNP–BGG.

Similarly, cells from the DNP–BSA immunized rabbit responded well to DNP–BSA but poorly to BSA.

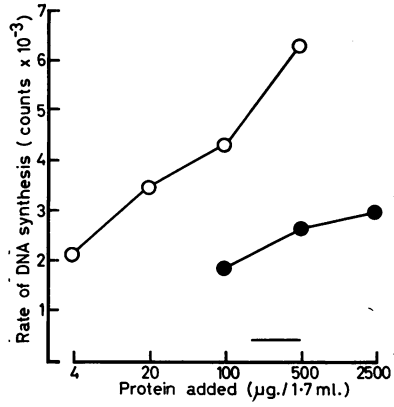

FIG. 1

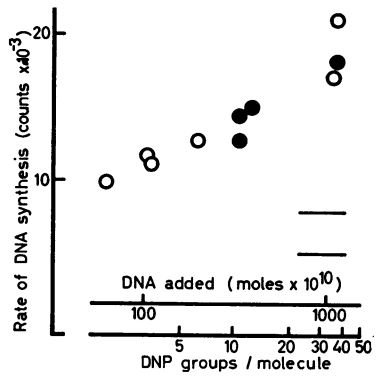

FIG. 2

FIG. 1. Stimulation of DNA synthesis in spleen cells from a rabbit (No. NB 1) immunized with DNP–BGG. In this and subsequent figures, approximately 1×10^7 cells were incubated in 1·7 ml. of medium containing the additions indicated. 'DNA synthesis' represents the total counts incorporated from 0·2 µc. of [^{14}C]thymidine in a 24 hour period starting 24 hours after the addition of antigen. The bar to the right of the figure represents the DNA synthesis in the absence of any addition. ○, DNP–BGG; ●, BGG.

FIG. 2. The effect of the number of DNP groups per molecule on the stimulation of DNA synthesis by spleen cells from a rabbit (No. 214) immunized with DNP–BGG. DNP–BGG preparations carrying varying numbers of DNP groups were added as 500 µg. of BGG. ○, clear DNP–BGG preparation; ●, turbid DNP–BGG preparation. The bars on the right of the figure represent (in descending order) DNA synthesis in the presence of 500 µg. of BGG; no addition. (See also legend to Fig. 1.)

2. *The Number of DNP Groups per Carrier Molecule*

A series of DNP–BGG preparations carrying from five to thirty-five DNP groups per molecule were tested for stimulatory capacity (Fig. 2). All the preparations were added at the same concentration of carrier (0·5 mg. of BGG per 1·7 ml.) and the rate of DNA synthesis is plotted against the number of DNP groups per molecule (a second scale, DNP added in moles $\times 10^{10}$ is given for the purpose of comparison with the data in later

TABLE 1

ROUGH PRECIPITIN DATA ON SERA FROM BLEEDINGS 5 DAYS AFTER LAST INJECTION

Rabbit No.	*Immunized with*	*Precipitation observed after addition* of*																	
		DNP–BGG			*BGG*	*DNP–BSA*			*BSA*		*DNP–HSA*			*DNP–Ea*			*HSA*		
		28†			—	19			—		29			5			—		
		52‡			54	49			49		56			97			66		
		0·1§	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·2	0·1	0·1
207	DNP–BGG	+	tr	–	–	+	–		Not done		+	–		+	–		Not done		
208	DNP–BGG	+	–		–	+	–		Not done		+	–		+	–		Not done		
213	DNP–BGG	+	–		–	+	–		Not done		+	–		tr	–		Not done		
214	DNP–BGG	+	tr		–	–			Not done		–			–			Not done		
215	DNP–BGG	+	+	+	–	+	+	–	Not done		+	+	–	+	+	+	Not done		
216	DNP–BGG	+	+	–	–	+	–		Not done		+	–		+	+	–	Not done		
218	DNP–BGG	+	+	±	–	+	tr	–	Not done		+	–		+	+	–	Not done		
222	HSA, then DNP–BSA	+	+	±	Not done	+	+	–	+	–	+	+	–	+	tr	–	+	+	+

* 0·1 or 0·2 ml. of the antigen solution indicated was added to 0·5 ml. of the antiserum. The presence or absence of precipitation was recorded after 30 minutes incubation at 37° followed by 1 hour at 4°. The precipitate was discarded and a further 0·1 ml. of the antigen solution added to the supernatant. Precipitation was recorded as before. In some cases a third 0·1 ml. of antigen solution was added. The columns record the presence or absence of precipitation after each addition of antigen.

† Number of DNP groups per molecule.

‡ Concentration (μg. N per ml.).

§ Amount added (ml.).

figures). It can be seen that the more DNP groups per molecule, the greater is the stimulation. Some of the DNP–BGG preparations become turbid when diluted in medium. These preparations are plotted as solid points, the rest as open circles.

Similar findings were obtained for a series of DNP–BSA preparations with a DNP–BSA immunized animal (Fig. 3).

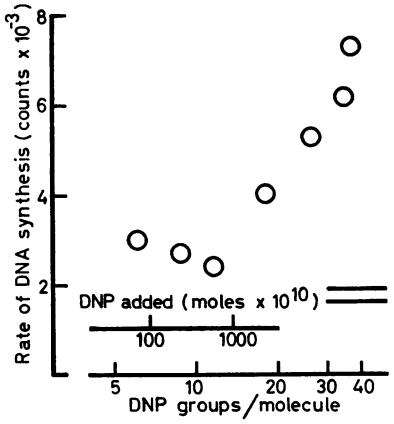

FIG. 3

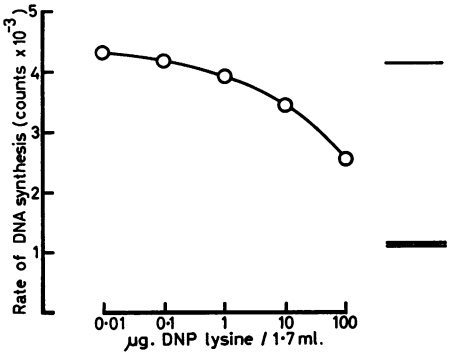

FIG. 4

FIG. 3. The effect of the number of DNP groups per molecule on the stimulation of DNA synthesis by spleen cells from rabbit No. 222, immunized with DNP–BSA. DNP–BSA preparations carrying varying numbers of DNP groups were added as 600 µg. of BSA. The bars on the right of the figure represent (in descending order) DNA synthesis in the presence of 600 µg. of BSA; no addition. (See also legend to Fig. 1.)

FIG. 4. The inhibition of stimulation with ε-DNP–L-lysine. The open circles indicate the DNA synthesis observed in the presence of 100 µg. of DNP–BGG plus DNP–lysine in amounts indicated on the horizontal scale. The bars at the right of the figure represent (in descending order) the DNA synthesis in the presence of 100 µg. of DNP–BGG; 100 µg. of BGG; no addition. Rabbit No. 207 immunized with DNP–BGG. (See also legend to Fig. 1.)

TABLE 2

THE EFFECT OF ε-DNP–LYSINE ON THE RATE OF DNA SYNTHESIS

Rabbit	*Immunized to*	*Addition (mg./1·7 ml.)*		*DNA synthesis (total counts/min.)*
R176	DNP–BGG	None		910
		DNP–BGG	1·0	2502
		DNP–lysine	0·1	814
		DNP–lysine	0·01	920
R207	DNP–BGG	None		1150
		DNP–BGG	0·5	4996
		BGG	0·5	2365
		BGG +DNP–lysine	0·5 + 0·1	2200
RNB5	DNP–BGG	None		676
		DNP–BGG	0·1	3145
		DNP–lysine	0·1	580
		DNP–lysine	0·01	620

DNA synthesis was measured by the uptake of [^{14}C]thymidine over the period 24–48 hours after the start of incubation.

3. *Challenge with Hapten Alone*

Epsilon–DNP–L-lysine did not stimulate when added alone (Table 2). It did not appear to be toxic in the concentration used, as it did not affect the response to BGG in DNP–BGG stimulated animals (Table 2). It did, however, inhibit the stimulation due to DNP–BGG (Fig. 4). The molar ratio of DNP as DNP–lysine, to DNP as DNP–BGG for '50 per cent inhibition', was of the order of 20 to 1 in this and a similar experiment.

4. *Challenge with Hapten Coupled to Proteins Other than One Used During Immunization*

When preparations of DNP coupled to carriers other than the one used for immunization were added *in vitro*, there was little or no response (Figs. 5–8), regardless of the number of DNP groups per molecule. (For the purpose of comparison, the antigen additions are plotted as DNP added in moles $\times 10^{10}$.)

Some further features are illustrated in these figures. Different preparations of DNP–BGG varied markedly in their stimulatory activity relative to their DNP content (Figs. 5 and 6). In general, the heavily substituted preparations were more effective than the lightly substituted ones.

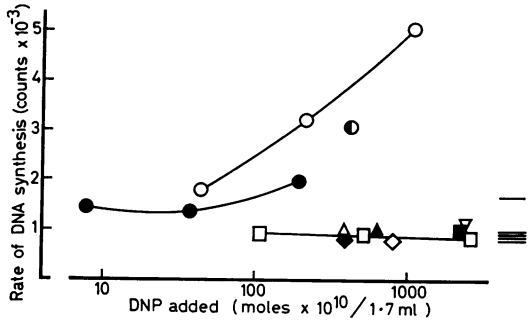

FIG. 5. The stimulation of DNA synthesis by DNP–protein conjugates. Rabbit No. 216 immunized with DNP–BGG.

Symbol	*Preparation*	*DNP groups per molecule*	*Conjugate added as µg. of carrier protein*
○	DNP–BGG	31	500, 100, 20
◐	DNP–BGG	24	500
●	DNP–BGG	9	500, 100, 20
▲	DNP–Ea	7	350
△	DNP–Ea	5	350
□	DNP–HSA	30	600, 120, 24
■	DNP–HSA	26	600
◇	DNP–HSA	9	600
◆	DNP–HSA	5	600
▽	DNP–BSA	26	600

The bars at the right of the figure represent (in descending order) the DNA synthesis observed in the presence of 500 µg. of BGG; no addition; 600 µg. of BSA; 350 µg. of Ea; 600 µg. of HSA. (See also legend to Fig. 1.)

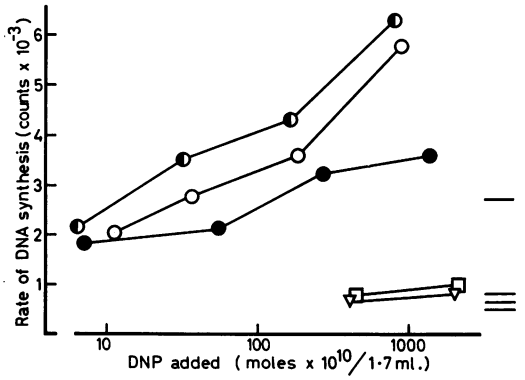

FIG. 6. The stimulation of DNA synthesis by DNP–protein conjugates. Rabbit No. NB 1 immunized with DNP–BGG.

Symbol	*Preparation*	*DNP groups per molecule*	*Conjugate added as μg. of carrier protein*
○	DNP–BGG	30	500, 100, 20, 4
◐	DNP–BGG	27	500, 100, 20, 4
●	DNP–BGG	9	2500, 500, 100, 20
□	DNP–HSA	24	600, 120
▽	DNP–BSA	23	600, 120

The bars at the right of the figure represent (in descending order) the DNA synthesis observed in the presence of 500 μg. of BGG; 600 μg. of BSA; 600 μg. of HSA; no addition. (See also legend to Fig. 1.)

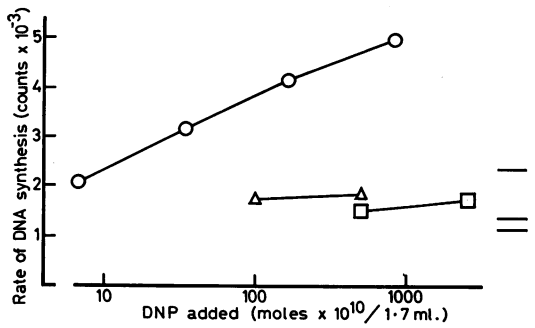

FIG. 7. The stimulation of DNA synthesis by DNP–protein conjugates. Rabbit No. 207 immunized with DNP–BGG.

Symbol	*Preparation*	*DNP groups per molecule*	*Conjugate added as μg. of carrier protein*
○	DNP–BGG	28	500, 100, 20, 4
△	DNP–HGG	17	500, 100
□	DNP–HSA	20	600, 120

The bars at the right of the figure represent (in descending order) the DNA synthesis in the presence of 500 μg. of BGG; 500 μg. of HGG; no addition. (See also legend to Fig. 1.)

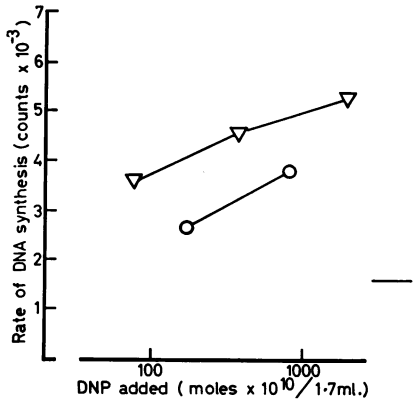

FIG. 8. The stimulation of DNA synthesis by DNP–protein conjugates. Rabbit No. 222 immunized with DNP–BSA.

Symbol	*Preparation*	*DNP groups per molecule*	*Conjugated added as µg. of carrier protein*
○	DNP–BGG	27	500, 100
▽	DNP–BSA	22	600, 120, 24

The bar at the right of the figure represents the DNA synthesis in the absence of any addition. The addition of 600 µg. of BSA (not shown) stimulated incorporation only slightly (1950 counts/min.). (See also legend to Fig. 1.)

In one experiment (Fig. 7), the cells were challenged with DNP coupled to a protein carrier HGG, which might be expected to cross-react with the antibody to the carrier used for immunization (BGG). There was no obvious enhancement of activity.

In another experiment (Fig. 8), the rabbit was immunized with DNP–BSA. In this case it was the DNP–BSA rather than the DNP–BGG which was the more active on an *in vitro* challenge.

DISCUSSION

Evidence has been presented (Dutton and Eady, 1964) that the specific stimulation of DNA synthesis that follows the addition of antigen to spleen cells from a previously immunized animal can, in all probability, be taken as the *in vitro* counterpart of the cellular proliferation that follows secondary stimulation *in vivo*.

In this paper, therefore, it has been assumed that the stimulation of the incorporation of [^{14}C]thymidine into spleen cells is a measure of the activity of a given antigen preparation. With this system, the significance of the protein carrier in the response to DNP–protein complexes has been studied.

The results described above have been taken to establish, or confirm, the following:

1. Animals immunized with heavily conjugated DNP–protein complexes make anti-DNP antibody but little anti-protein antibody (Table 1) (Farah, Kern and Eisen, 1960).
2. The DNP–protein complex used for immunization is something like one hundred times more active than the carrier protein alone on *in vitro* challenge (Fig. 1).

3. The anti-DNP antibody raised against one DNP–protein complex will precipitate (at least partially) with DNP coupled to a different protein (Table 1). The carrier is thus not a crucial factor in the reaction of the antibody with a DNP group.

In contrast, the presence and the nature of the carrier in secondary stimulation is of considerable importance.

4. DNP coupled to proteins other than the one used in the immunizing complex is only weakly, or, in some experiments, completely inactive in the stimulation of DNA synthesis (Figs. 5–8). This is true regardless of the number of DNP groups per carrier molecule and in spite of the fact that these same preparations precipitated well with the antisera obtained from these same animals (Rabbit 207 in Fig. 7 and Table 1; Rabbit 216 in Fig. 5 and Table 1; and less clearly, Rabbit 222 in Fig. 8 and Table 1).

The stimulation by the immunizing DNP–protein complex must presumably represent the sum of two components, stimulation by the DNP determinants and stimulation by the carrier protein. How far, therefore, is the superior activity of the immunizing complex due to a contribution from the protein carrier? There is, unfortunately, no clear basis for determining what this contribution might be. The response to the carrier might be enhanced, suppressed or unaffected by the attachment of DNP groups. It was found that in animals immunized with human serum albumin, DNP–HSA had slightly less stimulatory activity than HSA. Therefore, it is probably safe to assume that the activity of the carrier in the DNP–protein complex is not greater than that which would be given by an equivalent quantity of the protein alone. When this correction was applied, i.e. when the stimulation given by a corresponding concentration of carrier was subtracted from the stimulation caused by the complex for the data in Figs. 5–8, the stimulation by the complex used for immunization was still clearly greater than that of DNP on any other carrier.

5. At a given concentration of protein carrier, conjugates with many DNP groups are much more effective than ones with few (Figs. 2 and 3). Furthermore, a given quantity of DNP is more effective if presented on a smaller number of highly substituted carrier molecules than on a larger number of lightly substituted molecules (Figs. 5 and 6).

6. DNP–lysine is inactive *in vitro* when added alone (Table 2). It does, however, reach a critical receptor site since it can block the stimulation by the DNP–protein complex (Fig. 4).

Three of these observations would seem to require some further consideration:

1. The finding that hapten coupled to carriers other than the one used for immunization is inactive in this system is analogous to the results reported by Salvin and Smith (1960). They showed that intracutaneous injection of DNP–Ea would sensitize guinea-pigs to a secondary response on challenge with DNP–Ea, whereas only a primary response could be obtained if DNP–BGG had been used for sensitization. Similar results were obtained using the corresponding picryl derivatives. In these experiments, very small quantities of conjugate were used for sensitization and it seemed possible that the results might only be obtained under these conditions. In the present study, however, the animals were hyperimmunized to a particular complex and still failed to respond to hapten on other carriers.

A similar carrier specificity has been demonstrated for delayed hypersensitivity reactions to a variety of haptens conjugated to carrier proteins (Benacerraf and Gell, 1959; Benacerraf and Levine, 1962; Gell and Silverstein, 1962). These authors conclude that the recognition of antigen in the cellular response involves a wider area of the antigen surface

than is involved in the reaction of antigen with antibody. They have further suggested that this may represent a significant difference between the cellular and humoral responses. However, it would seem more proper to compare the specificity of the delayed hypersensitivity response with that for the secondary stimulation of a humoral response. The present results would suggest that there is then no difference.

However, it still remains necessary to discuss the apparent discrepancy between the lack of carrier specificity for precipitation and the marked carrier specificity for stimulation.

This discrepancy may be less clear cut than has often been supposed. Various workers (Hooker and Boyd, 1933; Haurowitz and Schwerin, 1943; Francis, Mulligan and Wormall, 1955) have shown that the amino acid or acids adjacent to a small hapten group may be involved in the specificity of the antigenic site.

Eisen, Carsten and Belman (1954) showed that only 41 per cent of all the DNP binding capacity of a serum raised against DNP–BGG could be precipitated with DNP–Ea. Some of the anti DNP antibodies of the anti DNP–BGG sera were thus specific for DNP coupled to BGG.

It is most probable, therefore, that the surface of the carrier molecule in the immediate neighbourhood of the DNP group contributes to the specificity of the antigenic site and thus the DNP sites cannot be considered antigenically equivalent. By an extension of this reasoning, it is conceivable that a DNP conjugate of BGG may have no antigenic sites which are identical with any of those on DNP–HSA. The antibody formed to any one of these sites can be shown to bind DNP–lysine and have a limited affinity for some of the other DNP sites. Similar specificities may be postulated for the receptor sites responsible for recognition of the antigen in the secondary response. It is clear from the experiments here that combination of such a site with DNP–lysine is not sufficient to activate the response. If this explanation is to hold together it must be further supposed that combination with a 'cross-reacting' DNP site is also inadequate. There would seem to be no obvious reason why this should be so and the hypothesis is, therefore, not wholly satisfactory.

It should be noted, however, that the mechanism of the antigenic stimulation of DNA synthesis is not understood and it is not clear how far quantitative data from this system can be related to quantitative data from immunochemical measurements. In particular, there may be a threshold for the interaction of receptor site and antigen below which no response results. Complete lack of stimulation of DNA synthesis, therefore, may not necessarily mean that no interaction has occurred.

It is possible to devise more complex hypotheses based on two step processes such as suggested by Weigle (1962) to explain the breaking of neonatal tolerance to serum proteins by cross-reacting proteins.

'Recognition' of the carrier molecule based on previous contact could be conceived of as aiding the passage of the molecule through a first stage (phagocytosis, pinocytosis?) on its journey to a second site capable of responding to individual determinants. Further experiments will be necessary to test such a hypothesis.

2. The findings that more heavily conjugated molecules are more effective than lightly conjugated molecules could be explained on either of the hypotheses advanced above. In the experiments reported, the animals were immunized with highly conjugated preparations. If all the DNP sites are different, only highly conjugated proteins could contain all the antigenic determinants to which the animals were immune. On the other hand, it could be supposed that the more DNP groups a molecule possessed the more likely it would be to pass through the 'transport' step of a two stage process.

It is possible also that possession of a large number of DNP groups may grossly modify the physical and chemical properties of the molecule so as to facilitate its uptake and hence stimulatory activity.

3. DNP–lysine clearly binds a receptor site essential for the stimulation of DNA synthesis by the DNP complex used for immunization. In the absence of any knowledge of the mechanism of the response, it is difficult to advance any explanation why the binding of DNP–lysine does not itself lead to stimulation.

ACKNOWLEDGMENT

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