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Immunology

# Molecular determinants of immunogenicity: The immunon model of immune response

(polyacrylamide/dinitrophenyl-/receptor linkage)

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ABSTRACT **The immunological response *in vivo* to a series of size-fractionated linear polymers of acrylamide substituted with hapten has been measured in mice. A sharp threshold was observed in immunogenic response elicited by various polymer preparations. All polymers with less than 12 to 16 appropriately spaced hapten groups per molecule were nonimmunogenic, while those polymers with greater than this number were fully immunogenic. The results lead to the conclusion that the immunological response at its most elementary level is quantized, i.e., a minimum specific number of antigen receptors (approximately 12 to 16) must be connected together as a spatially continuous cluster, an immunon, before an immunogenic signal is delivered to the responding cell.**

The mechanism by which first exposure to an antigen stimulates the immune response, and the subsequent production of circulating antibody molecules specifically reactive with that antigen, has been the subject of intensive research and speculation for decades. In efforts to elucidate this mechanism of immunogenicity, studies of the humoral primary immune response have been carried out, using as antigens macromolecular substances as diverse as proteins, peptides, polysaccharides, viruses, cell walls, and cell organelles. In general, the production of antibodies against macromolecular substances of these types has been found to be a complex process, incapable of being resolved into the molecular parameters involved.

A great deal has been added to our knowledge of the humoral immune response when small organic compounds of defined structure (haptens) were covalently bonded to macromolecular biological molecules (carriers) and these substances used as antigens. Antibodies with specificity directed against the haptenic structure have been obtained when haptens have been attached to biological macromolecules of the types mentioned above.

In all cases studied, the immunological response has proven to be highly complex and dependent on dose, degree of substitution with hapten, molecular size, state of aggregation, and the chemical nature of the carrier molecule. In addition to these molecular complexities, the immune response often depends on complicated cellular interactions. Models to explain immunogenicity have been put forth on the basis of the number, pattern, density, or crosslinking of antigenic determinants (1–4), complex cell to cell interactions (3, 5), or the relationship of the haptenic groups to the carrier molecule (6). However, since data from different experimental systems have not been correlated in any quantitative sense, these models have remained rather vague and qualitative, as well as somewhat contradictory (7).

Because of these complexities, it seemed attractive to us to attempt to synthesize and then to study the immunogenicity of a specific "ideal model" antigen. Although attempts to study antigenic properties in this way have been made previously (8–10), the minimum requirements for triggering the primary immune response have remained unclear, and it seemed that a systematic variation of molecular parameters might give clues as to the mechanisms involved.

In this study, we sought to vary several molecular properties of our ideal antigen, looking for those responsible for the triggering of a bone-marrow-derived lymphocyte (B-cell) to differentiate and to produce specific antibody in the primary immune response. We desired as our ideal antigen a molecule with the following properties: (*i*) It should consist of a nonimmunogenic carrier or "backbone" structure made of repeating subunits and with hapten groups projecting from it. (*ii*) The molecular weight, and therefore the length, of the carrier should be manipulable. (*iii*) It should be nondegradable by the host organism. (*iv*) The molecule should be linear, flexible, uncharged, and hydrophilic so that it might interact freely with cell surface receptors in whatever geometrical arrangement they might happen to be. (*v*) The immune response to this molecule should require minimal complex cellular interactions, i.e., should be relatively independent of the thymus-derived-lymphocyte (T-cell). In regard to the haptenic groups on this carrier, one should be able to control the number of haptenic groups per molecule and the average spacing between haptenic groups.

As described below, linear polyacrylamide substituted with dinitrophenyl (Dnp) hapten groups proved to have the properties of the "ideal-model" molecule we sought. In addition, it may be synthesized to any desired molecular weight (degree of polymerization) and substituted with hapten to any desired extent (Fig. 1). We therefore used this molecule as our model antigen. In this paper, we show that the systematic variation of some of the parameters mentioned above leads to a most interesting finding, with profound implications for the understanding of the basic molecular mechanism of immune response induction.

## MATERIALS AND METHODS

**Mice.** BALB/C mice were obtained from several alternative commercial suppliers. Nude homozygous and heterozygous mice were obtained from a local colony.

**Preparation of Polymers.** Linear polyacrylamide was made from the monomer in aqueous solution (11), giving preparations with average molecular weights ranging from $2 \times 10^4$ to $2 \times 10^5$, as determined by equilibrium sedimentation. Very high-molecular-weight linear polyacrylamide was obtained as Gelamide 250 from American Cyanamide, with average molecular weight $5 \times 10^6$. Polyacrylamide was substituted with ethylene diamine in a manner analogous to that previously used for polyacrylamide beads (12). Dnp derivatives were obtained by shaking the ethylene diamine substituted derivatives with excess fluorodinitrobenzene followed by extensive dialysis. The degree of substitution was determined from measurement of dry weight and optical absorbance at 360 nm. Preparations were labeled with $^{125}I$ by very short treatment with aqueous

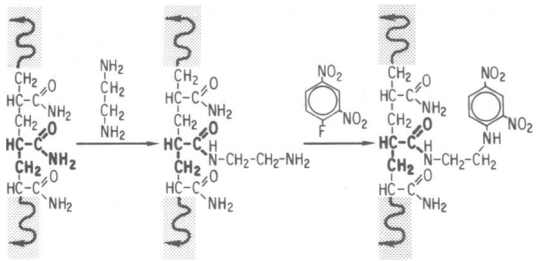

FIG. 1. The attachment of Dnp hapten to linear polyacrylamide. A section of three monomer subunits is shown, a single subunit being demarcated by a heavy line. The extended remainder of the polymer chain is indicated by shaded boxes.

hydrazine (12), followed by reaction with *p*-hydroxyphenyl proprionyl hydroxysuccinimide (13) and $^{125}I$ substitution using chloramine T (14). $^{125}I$ substitution levels of approximately one per 2500 monomer units were obtained, corresponding to less than one $^{125}I$ per molecule labeled.

Dnp-substituted polymers were fractionated by gel filtration through 1 m long columns of Bio-Gel A–0.5 M agarose beads. These original fractions were further fractionated three more times to obtain relatively homogeneous preparations, as determined by sedimentation equilibrium measurement in the analytical ultracentrifuge. Analysis of the molecular weight heterogeneity given by this method of preparation was done using interference optics and calculating the results with the aid of a computer program developed by Roark and Yphantis (15). The results showed that the ratio of weight average molecular weight to number average molecular weight and the ratio of Z average molecular weight to weight average molecular weight both were between 1.05 and 1.1, indicating a good degree of molecular weight homogeneity.

**Antibody Response.** Polymer solutions (0.25 ml) in isotonic saline were injected intraperitoneally into mice 6 to 10 weeks old. Blood was collected from the tail and serum stored frozen at −30° until analysis. Hemagglutination was performed using as indicators human cells substituted with trinitrobenzene sulfonate (16) and subsequently fixed with glutaraldehyde. Plaque determination was performed using sheep or donkey erythrocytes substituted with trinitrobenzene sulfonate (17) or with Dnp-substituted polyacrylamide coupled with carbodiimide. Spleen cells were counted in a hemocytometer. Concentration in serum of IgM antibody against Dnp was determined by a solid phase binding assay using as indicator $^{125}I$-labeled rabbit antibody against mouse IgM antibody (unpublished work).

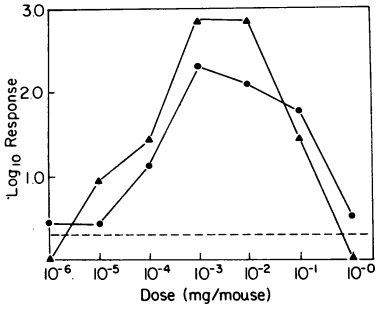


FIG. 2. The dose response curve of Dnp-polyacrylamide substituted to the level of 1 Dnp per 48 monomer units (molecular weight $5 \times 10^6$), measured 6 days after intraperitoneal polymer injection. (●) Plaque-forming cells per $10^6$ spleen cells; (▲) hemagglutination of human Tnp-erythrocytes. Plaque-forming cells of normal mice (- - - -).

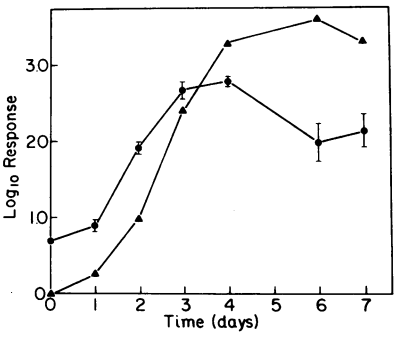


FIG. 3. The time response curve to $10^{-3}$ mg of Dnp-polyacrylamide substituted to the level of 1 Dnp per 48 monomer units (molecular weight $5 \times 10^6$). (●) Plaque-forming cells per $10^6$ spleen cells; (▲) hemagglutination of human Tnp-erythrocytes.

## RESULTS AND DISCUSSION

The immunological response at 6 days to various doses of Dnp-substituted polyacrylamide of high molecular weight ($5 \times 10^6$) is shown in Fig. 2. The response at times from 1 to 7 days to an optimal dose is shown in Fig. 3. Very similar results were obtained using different degrees of substitution of the polyacrylamide with Dnp, varying from 1 Dnp per 30 acrylamide monomer units to 1 per 100. Similar results were also obtained using inbred mice other than BALB/C, including SWR/J, A/J, C57Bl/6J, SJL/J, and BDP/J. These strains were selected and tested in order to uncover possible influences on the immune response of differing Ir genes (18), but no such influences were found.

The immune response to Dnp-substituted linear polyacrylamide shown in Figs. 2 and 3 is similar to that of many thymus-independent polymers, both simple and hapten-substituted (19). In particular, the dose response curve peaking at about $10^{-3}$ mg per mouse and falling off steeply at high dose is reminiscent of the behavior of pneumococcal polysaccharides as antigens (20). The similarity to thymus-independent polymeric antigens is further supported by the following observations: (*i*) a very fast rise of direct plaque-forming units in the spleen (Fig. 3), together with undetectable indirect plaque stimulation; (*ii*) the abolishment of hemagglutination titer by 0.1 M mercaptoethanol, indicating the major antibody product to Dnp-polyacrylamide is an IgM protein; (*iii*) a high level of response of homozygous athymic mice (*nu/nu*) relative to their heterozygous thymus functional siblings (*nu/+*); and (*iv*) the

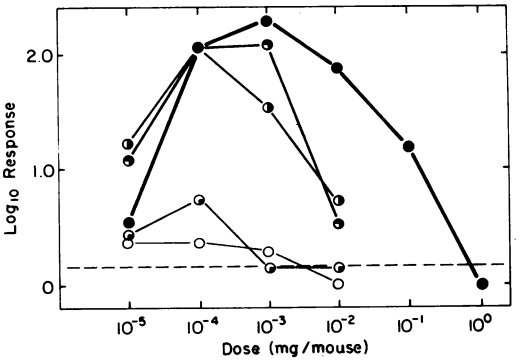


FIG. 4. The response at 4 days (plaque-forming cells per $10^6$ spleen cells) to different molecular weight polymers substituted to the level of approximately 1 Dnp group per 40 acrylamide monomer units. Molecular weight: (○) $0.4 \times 10^5$; (◔) $0.8 \times 10^5$; (◑) $1.4 \times 10^5$; (◕) $1.8 \times 10^5$; (●) $5 \times 10^6$. The dashed line represents the response of normal mice, 1.5 plaque-forming cells per $10^6$ spleen cells.

Table 1. Characteristics of preparations of fractionated Dnp-polyacrylamide

| Preparation | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Immunogenic ? | No | No | Yes | Yes | No | Yes |
| Molecular weight, $\times 10^{-5}$ | 0.5 | 0.8 | 1.4 | 1.8 | 1.3 | 3.3 |
| Acrylamide monomer subunits/molecule | 670 | 1050 | 1850 | 2350 | 1830 | 4650 |
| Extended length of polymer chain, Å | 1700 | 2600 | 4600 | 6000 | 4600 | 11,600 |
| Acrylamide monomer subunits/Dnp | 48 | 42 | 38 | 36 | 230 | 270 |
| Average distance between Dnp groups, Å | 120 | 105 | 95 | 90 | 575 | 675 |
| Total Dnp groups/molecule | 14 | 25 | 48 | 66 | 8 | 17 |
| "Effective" Dnp groups/molecule | 5–7 | 8–12 | 16–24 | 22–33 | 7–8 | 16–17 |

long-lasting antigenic effect of a single dose of $10^{-3}$ mg of Dnp-polyacrylamide, which produced high titer antiserum for periods of up to 100 days, as measured by hemagglutination.

The unique aspect of this study is the use as antigen, of sharply fractionated molecular sizes of haptenated polymer, with varying numbers and spacings of the Dnp hapten per molecule, as shown in Table 1. From Fig. 4 it may be seen that Dnp-substituted polyacrylamide preparations of molecular weight $1.4 \times 10^5$ (Table 1, C) and $1.8 \times 10^5$ (Table 1, D) are essentially as immunogenic by plaque assay as Dnp-polyacrylamide of molecular weight $5 \times 10^6$, although the fall off in response at high dose is somewhat faster. On the other hand, Dnp-substituted polymer of molecular weight $0.5 \times 10^5$ (Table 1, A) is completely nonimmunogenic and that of molecular weight $0.8 \times 10^5$ (Table 1, B) is essentially nonimmunogenic.

Similar results obtained by measuring serum antibody concentration are shown in Fig. 5, where the contrast between the immunogenic $1.4 \times 10^5$ molecular weight polymer (Table 1, C) and the almost completely nonimmunogenic $0.8 \times 10^5$ molecular weight polymer (Table 1, B) is particularly striking. Moreover, not only are the $0.8 \times 10^5$ (Table 1, B) and $0.5 \times 10^5$ molecular weight (Table 1, A) polymers nonimmunogenic, but they markedly inhibit the immunogenic effect of high-molecular-weight Dnp-polyacrylamide, as shown in Table 2.

Such results might possibly be the result of differential retention within the body of the polymers of molecular sizes above and below the apparent response threshold. That such is not the case is shown in Fig. 6, where the time-dependent loss from the body of $^{125}$I-labeled polymer fractions of molecular weight $0.8 \times 10^5$ (Table 1, B) and $1.4 \times 10^5$ (Table 1, C) is shown to be very similar. Despite the faster initial loss of the lower-molecular-weight polymer, the amount within the body of the nonimmunogenic polymer is never less than 70% of that of the immunogenic polymer, so that the lack of immunogenic response at all doses cannot be attributed to simple concentration differences. Furthermore, no large differences in distribution of polymers B and C were found in individual organs, either at 2 days or at 19 days after injection. Each polymer was found predominately in the liver (45%), the intestines (22%), and the skin (12%).

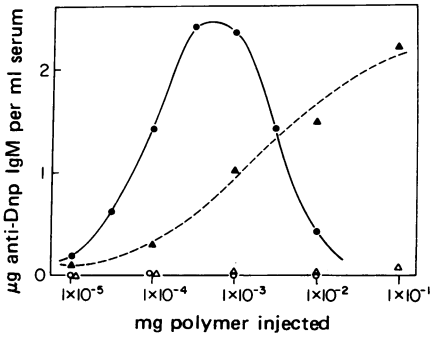


FIG. 5. The dose response curve to Dnp-polyacrylamide at 6 days, measured as concentration in serum of IgM antibody against Dnp. Each point represents the averaged response of three mice. The symbols represent the response to different preparations of fractionated polymer: (○) preparation B, (●) preparation C, (△) preparation E, (▲) preparation F, which are described in Table 1.

The polymer preparations A, B, C, and D discussed above have very similar degrees of substitution with hapten, all containing approximately 1 Dnp group per 40 acrylamide monomer units with an average distance between Dnp groups of approximately 100 Å (Table 1). Two other polymer preparations were made with substantially larger spacing between Dnp groups. These two preparations, E and F (Table 1), contain approximately 1 Dnp group per 250 acrylamide monomer units, with an average spacing between Dnp groups of approximately 600 Å. The smaller of the two (E) is nonimmunogenic, while the larger (F) is essentially as immunogenic as polymer C but requires larger doses (Fig. 5).

In order to test for the influence of unsubstituted polyacrylamide ("carrier") on the immune response to Dnp-polyacrylamide, the response to an immunogenic dose of $1 \times 10^{-3}$ mg of Dnp-polyacrylamide, molecular weight $1.4 \times 10^5$ (C), was measured both with and without the simultaneous injection of 1 mg of unsubstituted polyacrylamide, molecular weight $2 \times 10^5$. The presence of a 1000-fold excess of unsubstituted polymer had no measureable effect on antibody produced against Dnp hapten, the response being $0.96 \pm 0.06$ of that of the $1 \times 10^{-3}$ mg of Dnp polymer alone.

At the concentrations used in these experiments, all attempts

Table 2. Suppression of response by nonimmunogenic Dnp-polyacrylamide

| Molecular weight of Dnp-polyacrylamide | Dose, mg | Fractional suppression when given: Simultaneously | Fractional suppression when given: 5 Days previously |
|---|---|---|---|
| $0.8 \times 10^5$ | $10^{-3}$ | 0.53, 0.91 | 0.82, 0.84 |
| | $10^{-2}$ | 0.83, 0.93 | 0.93, 0.95 |
| $0.4 \times 10^5$ | $10^{-3}$ | 0.07, 0.29 | 0.80, 0.83 |
| | $10^{-2}$ | 0.70, 0.78 | 0.85, 0.88 |

Direct plaque response, expressed as plaques per $10^6$ spleen cells, was measured 4 days after injection of $10^{-3}$ mg. of Dnp-polyacrylamide, molecular weight $5 \times 10^6$. This high-molecular-weight polymer was injected either alone, or in addition to an equal weight ($10^{-3}$ mg) or 10-fold weight excess ($10^{-2}$ mg) of low-molecular-weight polymer given either simultaneously or 5 days previously. The numbers represent triplicate analysis of direct plaque on individual mice.

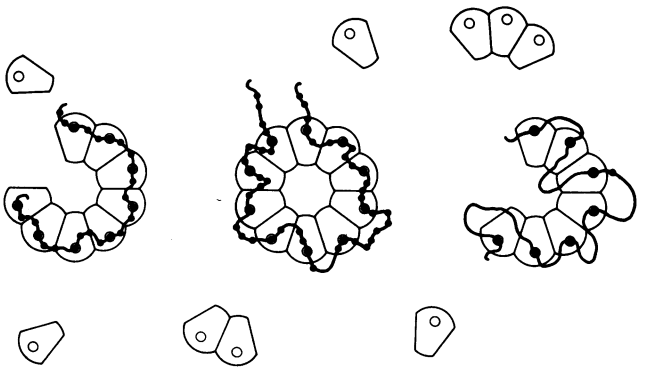

FIG. 7. A diagrammatic representation of the immunon concept. The wedge-shaped objects represent mobile receptor protein molecules containing single binding sites (circles). The heavy dark lines represent polyacrylamide molecules with substituted Dnp groups shown as solid circles. The lengths of the polyacrylamide molecules and the number of Dnp groups are those for preparation B (left), preparation C (center), and preparation E (right), as described in Table 1. Only preparation C is capable of forming a complete immunon because it has an adequate number of "effective" Dnp groups capable of simultaneously linking the receptor sites of a total immunon structure (center). The other preparations, for different reasons, cannot stabilize a complete immunon structure. Receptor proteins in various states of spontaneous aggregation are illustrated, but highly aggregated subunits cannot form without stabilizing crosslinkage.

If we assume that the responding cell contains a large number of individual hapten receptors and that their clustering into a sufficient number of immunons per cell triggers it to immunological activity, then the general shape of the dose response curve (Fig. 2) may be explained. At low doses of immunogen, the number of cells with the necessary number of assembled immunons increases with increasing dose, giving increasing response up to a maximum. Beyond that optimum dose, the number of polymer molecules reaching a cell becomes so large that they compete with each other in the gathering together of enough receptors to form an immunon, and the number of completed immunons per cell then falls with increasing dose. Furthermore, the competitive effect of lower molecular weight, subthreshold sized polymers (Table 2) becomes understandable, since they bind receptors in clusters that are too small to act as immunons, simultaneously reducing the number of free receptors available to larger immunogenic molecules, so that these latter molecules can no longer form the necessary number of immunons to trigger the cell to antibody production (Fig. 7).

Nothing has been said in this discussion about the location of immunon formation. It is reasonable to think of cell surface receptors as being clustered to critical size for immunological triggering, but nothing in the data above rules out immunon formation occurring within cells or even at interfaces between cells. In this study, the linkage device that binds the receptors into an immunon is a covalent polyacrylamide chain. However, antibody molecules or molecules on the surface of a "helper" cell may aggregate a small antigen and thus act as comparable linkage devices. We imply only that somewhere, and somehow, critical clustering of the necessary minimum number of receptors must occur to trigger the primary immune response.

We believe that much confusion about the immunogenic properties of T-cell-independent antigens is present in the published literature because of the prevalent use of immunogens that are, in reality, mixtures of molecules of widely differing molecular weights. If some molecular weight components are immunogenic and others are too small to be immunogenic, the response to the mixture will depend on the relative amounts of each present (for example, see data in Table 2). Since molecular weight homogeneity of immunogens previously studied has not in general been achieved or even sought after in immunological studies, critical interpretation of most published results in the terms discussed in this paper is not possible.

In summary, we conclude that the immunological response at its most elementary molecular level is quantized. A minimum number of antigenic receptors, probably of the order of 10 to 20, must be connected together as a spatially continuous cluster, an immunon, before an immunogenic signal is delivered to the responding cell. Immunological response then occurs when a sufficient number of such signals per cell has been delivered.

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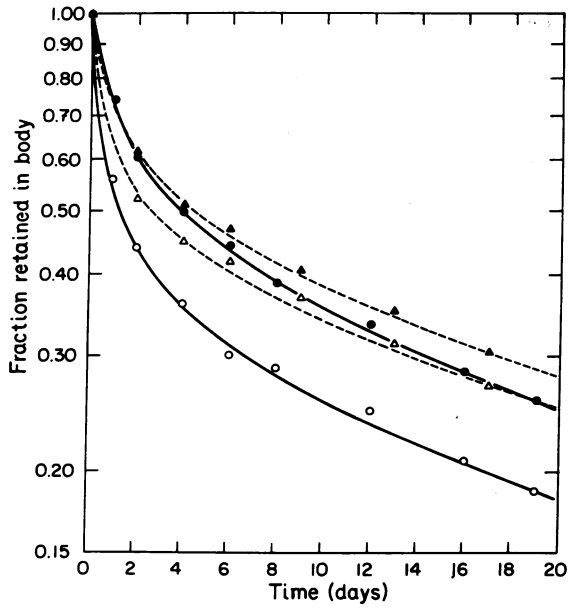


FIG. 6. The time-dependent whole body loss of $^{125}$I-labeled polyacrylamide of two different molecular weights. Open symbols represent $0.8 \times 10^5$ and filled symbols $1.4 \times 10^5$ molecular weight; triangles represent $2 \times 10^{-4}$ mg dose and circles $2 \times 10^{-3}$ mg dose.

to demonstrate either mitogenic effects or polyclonal stimulatory effects were unsuccessful.

In this study two different degrees of substitution of the polyacrylamide chain with Dnp groups are examined, the higher level of approximately 1 Dnp per 40 monomer units being represented in preparations A, B, C, and D of Table 1, while the lower level of approximately 1 Dnp per 250 monomer units is shown by preparations E and F of Table 1. Preparations A, B, and E are not immunogenic, while preparations C, D, and F are fully immunogenic (Figs. 4 and 5). Although preparations A and B are not immunogenic, they are capable of affecting the immune system, since their presence is strongly suppressive of the immune response caused by the larger molecules (Table 2).

The measured immunogenicity of our "ideal" model T-cell-independent antigen is dependent in some way on the haptenic groups attached to the carrier, the number of which increases directly with increasing molecular weight at constant degree of substitution. The data above suggest that there exists a threshold in some molecular property, above which immunogenicity appears. What can this property be? Table 1 contains a number of molecular properties that can be examined sequentially to determine which, if any, might be correlated with the appearance of immunogenicity.

Properties directly related to the size of the molecule (molecular weight, number of acrylamide monomer units per molecule, and the extended length of the polymer chain) are reasonable candidates for the critical threshold parameter, but they are all eliminated by a comparison of preparations C and E, which have almost identical values for each of the above-mentioned properties, but still differ completely in immunogenicity. Acrylamide monomer units per Dnp, or the directly related spacing between Dnp groups [epitope density (10)], cannot be the determining factor since, although B and C (or E and F) have similar spacings, they differ completely in immunogenicity. Similarly, total number of Dnp groups on the molecule is not the crucial determinant, since A and F have similar numbers but differ widely in immunogenic properties.

At the bottom of Table 1 there remains one physical property of the molecule that is directly related to immunogenicity. This we term "effective" Dnp groups, a property that is determined by the total number *and* spacing of Dnp groups on the molecule, as will be elucidated further in the discussion below.

It can be seen from Table 1 that all polymer preparations that have more than 12 to 16 "effective" Dnp groups per molecule are fully immunogenic, while those with less than this threshold number are completely nonimmunogenic. Furthermore, molecules with less than the threshold number cannot cooperate with each other, *in vivo*, even at high doses, to do something that molecules having more than the threshold number can do even at low doses. What can this "something" be? And what are its implications for the induction of immunity?

We propose that the fundamental molecular event in the induction of the primary immune response is the linking together by a single antigen molecule of a critical number of separate hapten receptors into a molecularly connected entity. For the purposes of discussion, and in order to solidify the concept, we should like to call this minimum sized unit of linked receptors an "immunon", defined as the elementary unit of immune responsiveness.

The receptor molecules on the immunoresponsive cells that bind the Dnp hapten groups are likely to be protein molecules (21, 22), and as such are likely to have an average diameter of approximately 100 Å. In order to link together receptors, the Dnp groups on one extended polyacrylamide molecule must be spaced apart by at least the diameter of a receptor protein molecule; Dnp groups that are closer together cannot independently bind receptor molecules. Thus, if we define as "effective" Dnp groups those which are far enough apart to separately and independently bind to receptor groups, the total number of Dnp groups per molecule must be corrected for that fraction which are too close to each other and therefore not "effective".

Because of the method used to attach Dnp groups to the polymer (Fig. 1), substitution is random along the chain and the resulting distance between Dnp groups is itself random and may be described by an exponential distribution function (23). In such a distribution more groups are spaced at shorter distances than at longer, and only 37% of consecutive groups are spaced at distances equal to or greater than the average distance of separation.

Accordingly, for polymer preparations A, B, C, and D, the total number of Dnp groups must be corrected by a factor (approximately ½ to ⅓) to allow for those Dnp groups spaced too closely (i.e., closer than 100 Å) to independently bind receptors. This leaves the number of "effective" Dnp groups substantially smaller than the total number per molecule. On the other hand, for polymer preparation E and F, the average distance between Dnp groups is likely to be much larger than the minimum distance between receptor molecules, and the "effective" number of Dnp groups may be expected to be quite close to the total number per molecule.

Thus the two different degrees of Dnp substitution lead to the same conclusion, namely, that polymers that have fewer than 8 to 12 effective Dnp groups (A, B, and E) are totally nonimmunogenic, while those with more than 16 to 20 effective Dnp groups (C, D, and F) are fully immunogenic. Allowing for experimental error, we can therefore conclude that some number between 10 and 20 is the number of linked receptors that constitutes an immunon. A pictorial representation of the immunon concept is given in Fig. 7, where for purposes of illustration the immunon size is taken as 10 linked receptor molecules.