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The Nitro Group as a Determinant of Immunologic Specificity[1]

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The combination of rabbit antibodies homologous to the 3-azonitrobenzene (3-NB), 4-azonitrobenzene (4-NB) and 1-azo-3,5-dinitrobenzene (3,5-NB) haptenic groups, with small molecules related in configuration to each homologous hapten, was investigated by measurement of the inhibition of precipitation by various haptens. The contribution to the free energy of combination of a nitro group in the homologous position on the hapten was found to be −5.3 or −4.0 kcal./mole in the anti-3-NB or anti-4-NB systems, respectively. These values represent a large fraction of the total energy of interaction in antibody–hapten systems which have been investigated and show that a small uncharged group may contribute nearly all of the energy required for an antigen–antibody reaction. The data also show that an uncharged group may contribute as strongly to antigenicity as a charged group. The magnitude of the energy of interaction suggests that displacement of water from a non-polar portion of the antibody is largely responsible for the effect of the nitro group. The possibility of direct hydrogen bonding to the antibody appears unlikely since an iodo group present in the nitro-specific position interacts strongly with the anti-3-NB or anti-4-NB antibodies. In the anti-3,5-NB system, the loss of one nitro group decreases the free energy released on interaction by about 2.8 kcal./mole. The sum of the energies of interaction of both nitro groups (−4.5 kcal./mole) is comparable to that of a single nitro group interacting with antibody homologous to a mono-nitro derivative. This supports other evidence indicating the existence of a mechanism limiting the total free energy released in an antigen–antibody reaction.

The fact that antibodies can be prepared against a wide variety of chemical groupings (haptens) provides a means of investigating protein–small molecule interactions which are specific in nature but which involve small molecules that can be selected by the investigator and are not restricted to those normally present in the animal. In the present investigation we have studied the interaction, with various molecules related in configuration to the homologous hapten, of rabbit antibody prepared against three different haptens containing nitro groups—azo-3-nitrobenzene, azo-4-nitrobenzene and azo-3,5-dinitrobenzene—each linked to the immunizing protein through the azo group.

The strength of combination of the three antibodies with a variety of compounds related to the homologous haptens was measured to secure information as to the nature of the forces involved in the interaction of the uncharged nitro group with antibody, and the closeness of fit of antibody around the hapten. Another purpose was to determine the magnitude of the contribution of a single nitro group to the free energy of combination with antibody, for comparison with the known contributions of small charged groups, and to ascertain whether the position of the nitro group in the hapten influenced this quantity. Since the magnitude of the contribution of a nitro group is large, it was possible to obtain significant answers. The present investigation adds to the limited quantitative information in the literature concerning the antigenicity of small uncharged groups.

Since there is evidence that the energy of interaction of hapten with antibody is limited,[2] probably as a consequence of the mechanism of formation of antibodies,[3] it was of interest to determine whether antibody would form against a dinitro compound in a manner such that each nitro group contributed as much as does a single nitro group in its interaction with antibody homologous to a mono-nitro compound; or whether the sum of the contributions of the two nitro groups more closely approximated this value.

Finally, the possibility that antibody is linked to the nitro group through a hydrogen bond was tested by measuring the effect of substituting for the nitro group a non-polar group approximating the nitro in size but incapable of forming hydrogen bonds.

Experimental Methods

Preparation of Haptens.—Synthesis of 3,5-dinitroaniline was carried out by the azide method.[4] Ten g. of 3,5-dinitrobenzoyl chloride was treated with 3 g. of sodium azide in 30 ml. of glacial acetic acid. The product was precipitated by addition of water. Small amounts of the solid were added with caution to concentrated sulfuric acid to convert the azide to 3,5-dinitroaniline. The product was identified by its melting point after crystallization from water and was subsequently converted into N-3,5-dinitrophenylsuccinamic acid by fusion with an equimolar quantity of succinic anhydride.

The compounds N,N′-*m*- and N,N′-*p*-phenylenedisuccinamic acid were prepared from the corresponding phenylenediamines by refluxing with an excess of succinic anhydride in alcohol (*meta*-derivative) or chloroform (*para*-derivative).

These various derivatives of N-phenylsuccinamic acid were prepared by refluxing the appropriate aniline derivative with an equimolar quantity of a 10% molar excess of succinic anhydride in the solvent specified: N-2-carboxyphenylsuccinamic acid (chloroform); N-3-nitro-5-carboxyphenylsuccinamic acid and N-3-carboxyphenylsuccinamic acid (acetone–chloroform mixture); N-3,5-dicarboxyphenylsuccinamic acid (pyridine). The preparations of the other derivatives of N-phenylsuccinamic acid used have been described.[5]

Other haptens were purchased from commercial sources. All haptens were recrystallized from ethanol, water or an ethanol–water mixture until the acid equivalent differed by less than 2% from the theoretical value. Melting points were in good agreement with values reported by others, for those compounds for which such data could be found in the literature.

Preparation of Antigens.—The antigens used for immunizing rabbits were prepared by diazotizing 150 mg. of the appropriate nitroaniline (3-nitroaniline, 4-nitroaniline or 3,5-dinitroaniline) and coupling at *p*H 9–9.5 and 0° to 5 g. of bovine γ-globulin (Armour Fraction II); 1 *N* NaOH was used to maintain the desired *p*H during coupling.

(1) Presented at the 42nd meeting of the Federation of American Societies for Experimental Biology, Philadelphia, April, 1958.

(2) For the several systems in which the standard free energies of hapten–antibody interactions have been determined, the values vary between approximately −5 and −8 kcal./mole. Also, the free energies of combination of *p*-iodobenzoate with anti-*p*-azobenzoate antibody from a number of individual, immunized rabbits and from several serum pools varied between the limits −5.6 to −6.2 kcal./mole (A. Nisonoff and D. Pressman, *J. Immunol.*, **80**, 417 (1958).

(3) L. Pauling, THIS JOURNAL, **62**, 2643 (1940).

(4) J. J. Blanksma and G. Verberg, *Rec. trav. chim.*, **53**, 988 (1934).

(5) A. Nisonoff and D. Pressman, THIS JOURNAL, **79**, 5565 (1957).

TABLE I

COMBINATION OF HAPTENS WITH ANTI-3-NB ANTIBODY

Hapten	K_0' × 10^8	$\Delta F°$ rel. (kcal./mole)	σ	Hapten concn., molar × 10^6: 0.63	2.5	10	40	160	640	2560
				Amount of precipitate[a]						
N-3-Nitrophenylsuccinamate	1000	0	2.5	58	24	11				
N-Phenylsuccinamate	<0.3 (0.07)[c]	>4.5 (5.3)[b]	4.0					86	81	71
N-2-Nitrophenylsuccinamate	15	2.3	2.5				55	26	14	
N-4-Nitrophenylsuccinamate	400[c]	0.51	(2.5)			18	0	0		
N-Phenylfumaramate	0.2	4.7	4.0					83	75	59
m-Phenylenedisuccinamate	<0.3	>4.5	..					83	75	83
p-Phenylenedisuccinamate	<0.3	>4.5	..					79	68	88
N-2-Iodophenylsuccinamate	0.7	4.0	2.5					84	55	42
N-3-Iodophenylsuccinamate	40	1.8	4.0			58	38	34		
N-4-Iodophenylsuccinamate	20	2.2	2.5				47	26	6	
N-2-Chlorophenylsuccinamate	0.3	4.5	1.0					87	84	63
N-3-Chlorophenylsuccinamate	2	3.4	4.0				71	54	45	
N-4-Chlorophenylsuccinamate	5	3.0	4.0			87	59	56		
N-2-Carboxyphenylsuccinamate	<0.3	>4.5	4.0					91	77	66
N-3-Carboxyphenylsuccinamate	<0.3	>4.5	..				88	81	91	
N-4-Carboxyphenylsuccinamate	<0.3	>4.5	..				81	75	97	
Benzoate	<0.3	>4.5	..					98	95	89
m-Nitrobenzoate	9	2.6	3.0			78	61	46		
m-Phthalate	<0.3	>4.5	0.5					96	92	72
Trimesate	<0.3	>4.5	..					104	89	
N-3,5-Dinitrophenylsuccinamate	>100	<1.3	..			20	32	0		
N-3-Nitro-5-carboxyphenylsuccinamate	1	3.6	1.5					55	67	13
N-3,5-Dicarboxyphenylsuccinamate	<1	>3.8	..				84	72	78	

[a] Amount of precipitate is reported as per cent. of the amount found in the absence of hapten; 54.8 μg. Experiments carried out in duplicate with mean deviation 1.9 μg. [b] Value estimated by extrapolation using σ = 4.0. [c] Value estimated by extrapolation using σ = 2.5.

These antigens were purified by dialysis in the cold against three 10-liter portions of saline–borate buffer, *p*H 8, $\Gamma/2$ = 16.

Test antigens were prepared by similarly diazotizing and coupling the same aniline derivatives to ovalbumin; the proportions used were 30 mg. each of *m*- and *p*-nitroaniline or 40 mg. of 3,5-dinitroaniline per gram of ovalbumin. Each antigen was dialyzed against three 10-liter portions of cold saline–borate buffer, *p*H 8, $\Gamma/2$ = 0.16, and further purified by acidifying with HCl to *p*H 4–4.5 and adding quickly to four times its own volume of acetone at −20°. After centrifuging and washing with water, the precipitate was dissolved immediately by addition of 0.01 *N* NaOH to a final *p*H of 8.0. This resulted in essentially complete solution of the antigen. A small amount of turbidity was eliminated by centrifuging in the cold at 25,000 × *g*. Sodium chloride was added to adjust the ionic strength to 0.16.

Preparation of Antisera.—Rabbits were immunized by inoculating three times weekly for 2 to 4 months with 1–2 ml. of a 1% solution of antigen. Sera from a number of rabbits, bled by heart puncture, were pooled. A globulin fraction of each pool of antiserum was prepared by two precipitations with sodium sulfate, added to a concentration of 18% in the first precipitation and 14% in the second. The precipitate was then dissolved in borate buffer and dialyzed at 3–5° against 10 liters of saline–borate solution, *p*H 8.0, $\Gamma/2$ = 0.16.

Reactions of Antibodies with Antigens and Haptens.—Solutions of haptens were prepared by dissolving in sodium hydroxide solution and back-titrating to *p*H 8.0 with hydrochloric acid. The ionic strength of each solution was adjusted to 0.16. Hapten solutions were then diluted to desired concentrations with 0.16 *M* sodium chloride.

Dilutions of the test antigens were made with saline–borate buffer, *p*H 8.0, $\Gamma/2$ = 0.16. The amount of test antigen required for optimum precipitation of the homologous antibody solution was determined. Then 0.20-ml. portions of test antigen (at optimum concentration), hapten and antibody solution were added to a test-tube in that order and mixed. Mixtures were permitted to stand for 1 hr. at room temperature, then for 5 days at 3–5°. The precipitates were centrifuged, washed twice with saline–borate buffer and twice with saline (0.6 to 0.8-ml. portions). Each precipitate was dissolved in 0.02 *N* sodium hydroxide and diluted to 0.40(0) ml. in a calibrated quartz microcuvette. The optical density was recorded at 280 mμ and in the visible region at a wave length corresponding to the absorption maximum of the test antigen, which was yellow or orange. The contribution of the antigen to the reading at 280 mμ was calculated from the known ratio of its absorbance at 280 mμ to that at the wave length used in the visible region. (A small secondary correction was first made for the absorption of the antibody at 450 mμ.) The net contribution of the antibody to the reading at 280 mμ was calculated and the protein concentration was obtained from this value.[6]

Control experiments were carried out in duplicate in which very high concentrations of good inhibitors were used; the mean value of the small blanks obtained was subtracted from the results of the analyses. The results of five or six replicate experiments in which no hapten was present were used as the basis for calculating the degree of inhibition by haptens in each system.

Precipitates usually dissolved within a few minutes after adding sodium hydroxide and the optical densities were read within 1–2 hr. after the addition. In a number of instances, optical density readings were repeated after several hours contact with 0.02 *N* sodium hydroxide and were found to be unchanged. Experiments were carried out in duplicate with an average mean deviation of 2.2 μg. Owing to the small amounts of precipitate measured (55–71 μg. in the absence of hapten) and the gradual rate of change in amount of precipitate with concentration of hapten, 2.2 μg. corresponds to an error in *K* of a factor of about 1.5 to 1.8. The corresponding error in $\Delta F°$ is approximately 0.2 kcal./mole which is small compared with the quantities under discussion (contribution to $\Delta F°$ of one or two nitro groups, contribution of an iodo group, effect of substitution of carboxylate for nitro, etc.).

Results

The effect of haptens on the combination of test antigen with homologous antibody is shown by the data in Tables I, II and III, corresponding to antibodies prepared against the azo-3-nitrobenzene, azo-4-nitrobenzene and azo-3,5-dinitrobenzene groups, respectively. For convenience, these anti-

(6) D. Gitlin, *J. Immunol.*, **62**, 437 (1949).

TABLE II

COMBINATION OF HAPTENS WITH ANTI-4-NB ANTIBODY

Hapten	K_0' × 10^3	$\Delta F°$ rel. (kcal./mole)	σ	Hapten concn., molar × 10^5: 0.16	0.63	2.5	10	40	160	640	2560
				Amount of precipitate[a]							
N-4-Nitrophenylsuccinamate	1000	0	3.0	68	56	36	16				
N-Phenylsuccinamate	0.7	4.0	4.0						68	64	41
N-2-Nitrophenylsuccinamate	2	3.5	2.5						71	49	16
N-3-Nitrophenylsuccinamate	70	1.5	2.5	87	92	76	58				
N-Phenylfumaramate	1	3.8	2.5						72	61	29
m-Phenylenedisuccinamate	0.3	4.5	0.5						90	88	59
p-Phenylenedisuccinamate	<0.3	>4.5	..						105	97	85
N-2-Iodophenylsuccinamate	<1	>3.8	..					106	101	83	
N-3-Iodophenylsuccinamate	3	3.2	2.0					86	62	33	
N-4-Iodophenylsuccinamate	50	1.6	2.5		93	76	67				
N-2-Chlorophenylsuccinamate	0.5	4.2	3.0						79	67	43
N-3-Chlorophenylsuccinamate	3	3.2	3.0					68	64	33	
N-4-Chlorophenylsuccinamate	35	1.9	3.5		86	82	64				
N-2-Carboxyphenylsuccinamate	0.2	4.7	2.0						87	81	54
N-3-Carboxyphenylsuccinamate	<0.3	>4.5	1.5						82	99	63
N-4-Carboxyphenylsuccinamate	0.2	4.7	1.5						94	110	57
Benzoate	<0.3	>4.5	2.5						94	90	69
p-Nitrobenzoate	30	2.0	4.0		96	76	66	46			
m-Nitrobenzoate	4	3.0	4.0				72	78	52		
p-Phthalate	<0.3	>4.5	..						109	95	80
N-3,5-Dinitrophenylsuccinamate	60	1.6	4.0	95	77	76	57				
N-3-Nitro-5-carboxyphenylsuccinamate	2	3.5	0.5						73	59	0
N-3,5-Dicarboxyphenylsuccinamate	<0.3	>4.5	..						104	102	

[a] Amount of precipitate is reported as per cent. of the amount found in the absence of hapten, 71.4 μg. Experiments in duplicate with mean deviation 2.7 μg.

bodies will be referred to as anti-3-NB, anti-4-NB and anti-3,5-NB.

Results were interpreted by application of a theory of heterogeneity of combining sites of antibody,[7] based on the assumption of a Gaussian distribution of combining energies, to give values of K_0' and σ. K_0' is the average equilibrium constant for the combination of the hapten with antibody, relative to that of a reference hapten arbitrarily assigned the value $K_0' = 1.00$. The reference haptens in the anti-3-NB, anti-4-NB and anti-3,5-NB systems are N-3-nitrophenylsuccinamate, N-4-nitrophenylsuccinamate and N-3,5-dinitrophenylsuccinamate, respectively. The value of σ, which is an exponent in the distribution function, is zero for a homogeneous group of combining sites and increases with increasing heterogeneity.

Discussion

Use of Derivatives of N-Phenylsuccinamate as Haptens.—Since the nitrobenzenes and certain other compounds of interest related to these haptens are not water soluble, it was necessary for inhibition studies to use derivatives containing a solubilizing group; N-phenylsuccinamates with various substituents in the benzene ring were prepared for this purpose. In some cases, derivatives of benzoate were also used. Most interpretations of data, such as the effect of removal of a nitro group or replacement of nitro by iodo or chloro, were made on the basis of differences between haptens containing the solubilizing group in the same relative position.

The K_0' values for the combination of the 2-, 3- and 4-N-nitrophenylsuccinamates with each of the three antibodies indicate that the large succinamate group is best accommodated by each antibody in the azo-specific position of the combining site. For example, in the anti-4-NB system, N-4-nitrophenylsuccinamate, in which the phenylsuccinamate group necessarily occupies the azo-specific position, has the highest K_0' value of the three isomeric nitro derivatives. In the anti-3-NB system, the 3-nitro derivative has the highest value of K_0'. In this case, there are two positions *meta* to the nitro group which might conceivably be occupied by the phenylsuccinamate group. However, experience with antibodies against a number of other phenylazo derivatives has shown that, in each case, the azo-specific position best accommodates large groups; and the fact that the 3-nitro derivative is the most effective here may be interpreted on the same basis. However, the interpretation of our results in terms of the contribution of the nitro group does not depend on this factor. In the anti-3,5-NB system, the 3-nitro derivative has the highest K_0' value of the mononitro-N-phenylsuccinamates, and the N-3,5-dinitrophenylsuccinamate is much more effective than any other hapten tested, including 3,5-dinitrobenzoate. Here again, the data are consistent with the postulate that the succinamate group can best be accommodated in the azo-specific position. Additional evidence on the same point is the fact that the homologous nitro- or dinitrophenylsuccinamates combine very strongly with the antibody, as shown by their effectiveness in inhibiting precipitation at very low concentrations ($\sim 10^{-5}$ *M*).

To test the possibility that the succinamate group might also be accommodated in the nitro-

(7) L. Pauling, D. Pressman and A. L. Grossberg, THIS JOURNAL, **66**, 784 (1944).

TABLE III

COMBINATION OF HAPTENS WITH ANTI-3,5-NB ANTIBODY

Hapten	K_0' × 10^3	$\Delta F°$ rel. (kcal./mole)	σ	Hapten concn., molar × 10^5: 0.16	0.63	2.5	10	40	160	640	2560	10240
				Amount of precipitate[b]								
N-3,5-Dinitrophenylsuccinamate[a]	1000	0	3.5	82	56	40	24					
N-3-Nitrophenylsuccinamate[a]	<10	>2.5	4.0			81	83	81	69			
N-Phenylsuccinamate[a]	<0.5	>4.2	..						107	107	88	
N-2-Nitrophenylsuccinamate	3	2.0	2.5						71	56	20	
N-4-Nitrophenylsuccinamate	9	2.6	4.0			88	69	69	50			
N-Phenylfumaramate	<0.5	>4.2	..					111	109	107	125	
m-Phenylenedisuccinamate	0.3	4.5	2.5						91	85	59	
p-Phenylenedisuccinamate	<0.5	>4.2	2.5						94	92	74	
N-2-Iodophenylsuccinamate	1	3.7	3.0						77	65	35	
N-3-Iodophenylsuccinamate	2	3.4	3.5				102	90	68	58		
N-4-Iodophenylsuccinamate	2	3.4	5.0				75	77	76	53		
N-2-Chlorophenylsuccinamate	20	2.1	5.0						36	26	21	
N-3-Chlorophenylsuccinamate	3	3.2	2.0						68	51	18	
N-4-Chlorophenylsuccinamate	4	3.0	2.5						61	43	0	
N-2-Carboxyphenylsuccinamate	0.6	4.1	2.5						74	84	49	
N-3-Carboxyphenylsuccinamate	0.4	4.3	4.0						84	68	57	
N-4-Carboxyphenylsuccinamate	0.2	4.7	4.0						75	83	62	
Benzoate	<0.5	>4.2	3.5						93	85	72	
m-Nitrobenzoate	1.0	3.8	0.5						106	105	33	
3,5-Dinitrobenzoate	30	1.9	4.0	107	95	80	72					
5-Nitro-*m*-phthalate	0.5	4.2	3.5					104	82	73		
m-Phthalate	<0.5	>4.2	2.5						89	97	77	
Trimesate	<0.5	>4.2	..						89	96		
N-3-Nitro-5-carboxyphenylsuccinamate	3	3.2	1.5						81	51	0	
N-3,5-Dicarboxyphenylsuccinamate	<0.5	>4.2	4.0						87	73		
N-3,5-Dinitrophenylsuccinamate[c]	1000	0	2.5	61	51	24	6					
N-3-Nitrophenylsuccinamate[c]	6	2.8	3.5				77	61	49	20		
N-Phenylsuccinamate[c]	0.3	4.5	1.5							79	48	0

[a] In the first series of experiments, the concentrations of N-3-nitrophenylsuccinamate were not sufficiently high to give adequate inhibition. Therefore, a second series of experiments was run with this hapten and, for comparison, with N-3,5-dinitrophenylsuccinamate and N-phenylsuccinamate. Results are given at the bottom of the table. [b] Amount of precipitate is reported as per cent. of the amount found in the absence of hapten; 58.5 μg. All experiments carried out in duplicate with mean deviation 2.1 μg. [c] Data obtained in a separate run in which the amount of precipitate found in the absence of hapten was 52.3 μg.

specific position, the compounds N,N-*m*-phenylenedisuccinamate and N,N′-*p*-phenylenedisuccinamate were tested for their ability to combine with each of the three antibodies. In each case (Tables I, II, III), the disuccinamates were very poor inhibitors, indicating that the large negatively charged succinamate group cannot be accommodated in the nitro-specific position or in the adjacent position.

Contribution of a Nitro Group to the Free Energy of Interaction with Anti-3-NB or Anti-4-NB Antibodies.—Comparing N-phenylsuccinamate with N-3-nitrophenylsuccinamate in the anti-3-NB system (Table I), we find that substitution of a nitro group for a hydrogen atom in the 3-position has a large effect on the combining constant. The ratio of the K_0' values, >3000/1, corresponds to a difference in free energy of combination of more than 4.5 kcal./mole. The free energy difference estimated by extrapolation of the data for N-phenylsuccinamate to 50% inhibition of precipitation is 5.3 kcal./mole. Since the total standard free energy of combination of homologous haptens with antibody, in systems for which such values are available, varies between limits of about −5 to −8 kcal./mole,[8−14] it is evident that the loss of a nitro group has a drastic effect on the ability of hapten to combine with the anti-3-NB antibody. The large contribution of the nitro group is confirmed by a comparison of the combining energies of benzoate and *m*-nitrobenzoate. (The difference, >1.9 kcal./mole, cannot be estimated with greater precision because of the very low combining affinity of benzoate.)

A somewhat smaller, but still large, contribution of the homologous nitro group is noted in the anti-4-NB system (Table II), in which the difference in the free energies of combination of N-4-nitrophenylsuccinamate and N-phenylsuccinamate is 4.0 kcal./mole. The fact that the contribution of the nitro is large is confirmed by a comparison of benzoate with *p*-nitrobenzoate (the difference is >2.5 kcal./mole).

It is difficult to attribute effects of this magnitude to London dispersion forces acting between the nitro group and the antibody, because of the small size of the group.[15,16] Also, the K_0' values for the iodophenylsuccinamates, which will be discussed below, indicate that the interaction is

(8) H. N. Eisen and F. Karush, THIS JOURNAL, **71**, 363 (1949).
(9) M. E. Carsten and H. N. Eisen, *ibid.*, **77**, 1273 (1955).
(10) S. I. Epstein, P. Doty and W. C. Boyd, *ibid.*, **78**, 3306 (1956).
(11) F. Karush, *ibid.*, **78**, 5519 (1956).
(12) A. Nisonoff and D. Pressman, *Federation Proc.*, **16**, 976 (1957).
(13) F. Karush, THIS JOURNAL, **79**, 3380 (1957).
(14) A. Nisonoff and D. Pressman, *J. Immunol.*, **80**, 417 (1958).
(15) L. Pauling and D. Pressman, THIS JOURNAL, **67**, 1003 (1945).
(16) D. F. Waugh, *Advances in Protein Chem.*, **9**, 325 (1954).

probably not due to direct hydrogen bonding of the nitro group to antibody. The observed contribution to the combining energy may, however, be accounted for by the usual mechanism of interaction of non-polar surfaces in an aqueous medium; the source of energy is largely the formation of new hydrogen bonds between water molecules which accompanies a reduction in oil–water interface, and the quantitative expression of the energy released is the interfacial tension. Assuming a value for the latter of 50 ergs/cm.^{2}, one finds that -5.3 kcal./mole corresponds to a reduction in surface of about 80 sq. Å. per molecule, which is the surface of a hemisphere of radius 3.6 Å.; this is of the right order of magnitude for a nitro group reacting with a non-polar surface if a "surrounding" type of fit of the antibody is assumed. It is of course unrealistic to discuss the interactions of a small group of atoms in terms of the macro value of the interfacial tension and the calculation was made only to compare orders of magnitude. An accurate quantitative approach would require a knowledge of the arrangement of the water molecules in the combining region of the antibody, in the presence or absence of the nitro group. This would permit an estimate of the average number of new hydrogen bonds formed as a result of the presence of the group. In the absence of this type of information, it appears reasonable to assume that a nitro group may displace an average of one or two water molecules from a non-polar surface of the antibody, with the formation of one or more additional hydrogen bonds.[17]

The large contribution of the nitro group to the free energy of interaction shows that a small uncharged group can contribute most of the energy required for antigen–antibody interaction. It is also of interest that the energy is comparable in magnitude to that observed in the combination of antibodies with haptens containing charged groups. For example, the free energy of interaction of antibody with the nitro group in the anti-3-NB or anti-4-NB systems (4–5 kcal./mole) is as large as that of anti-*p*-azobenzoate antibody with the carboxylate ion of the homologous hapten; the total free energy of combination of the latter antibody with benzoate is -6 kcal./mole,[14] of which the benzene ring contributes an appreciable part.

Contribution of Each Nitro Group to the Energy of Combination with Anti-3,5-NB Antibody.—Comparing the free energies of combination with anti-3,5-NB antibody of N-3,5-dinitrophenylsuccinamate and N-3-nitrophenylsuccinamate (Table III, bottom), we find that the loss of one nitro group results in an increase in ΔF° of 2.8 kcal./mole. Loss of the second nitro group causes a further increase of 1.7 kcal./mole. The total contribution of both nitro groups is 4.5 kcal./mole.

The value 4.5 kcal./mole is comparable to that of a single nitro group in the homologous position interacting with antibody against a mononitro compound (-5.3 and -4.0 kcal./mole in the anti-3-NB and anti-4-NB systems respectively). This finding is consistent with Pauling's hypothesis[3] that there should be an upper limit to the free energy released in antigen–antibody reactions. He considered that an antibody, formed against an antigen acting as template, which combined too strongly with the antigen would not be released into the blood stream. In agreement with this suggestion, we recently have found that the free energy of combination with homologous hapten of purified antibody from five individual rabbits, immunized with the same antigen, varied over a small range of values (-5.6 to -6.2 kcal./mole).[14] Also, Karush has observed[13] that for two different anti-hapten antibodies, the ΔF° values for the combination with homologous hapten were similar despite large differences in ΔH°. And, as already mentioned, a survey of the values of ΔF° that have been reported for antibody–hapten interactions suggests a limit of about -8 kcal./mole. The present results provide additional support for Pauling's hypothesis. If each nitro group in the anti-3,5-NB system contributed as much to the free energy of interaction with antibody as a single nitro group interacting with antibody homologous to a mononitro derivative, the total energy of combination would have been -8 to -10 kcal./mole, exclusive of the energy attributable to the rest of the hapten molecule. The fact that a single nitro group interacts less strongly when there are two such groups present in the molecule suggests that the reason for the apparent upper limit is actually a mechanism operating in the rabbit, and not that the haptens which have been studied are too small to provide negative energies of combination with protein exceeding 8 kcal./mole.

Substitution of Carboxylate for the Homologous Nitro Group.—In each of the three systems investigated, substitution of carboxylate for a nitro group in the homologous position resulted in a very large decrease in K_0' (N-3-carboxyphenylsuccinamate, Table I; N-4-carboxyphenylsuccinamate, Table II; N-3,5-dicarboxyphenylsuccinamate, Table III). For each of these compounds, the K_0' value, relative to that of the corresponding nitro or dinitro derivative, is less than 10^{-3}. Since the nitro and carboxylate groups are almost identical in size and configuration, the large differences in K_0' can probably be attributed to the negative charge of the carboxylate. This again is consistent with the other evidence suggesting that the nitro group combines with a non-polar region of the antibody molecule. Owing to the very large energy of hydration of charged groups,[18] their combination with a non-polar surface would be energetically unfavorable.[19]

The fact that carboxylate cannot successfully be

(17) The low solubility of nitrobenzene in water suggests that strong hydrogen bonding does not occur between the nitro group and water. The breaking of such bonds on combination with antibody is probably not an important factor.

(18) W. M. Latimer, "Oxidation Potentials," 2nd Ed., Prentice-Hall, Inc., New York, N. Y., 1952.

(19) Epstein and Singer (THIS JOURNAL, **80**, 1274 (1958)) have suggested that the combination of the negatively charged carboxylate group with an uncharged surface can be brought about as a result of a favorable entropy change associated with release of bound water from the carboxylate. The heats of hydration of charged groups, however, appear to be much too large to be overcome by this relatively small change in entropy. If bound water is to be released, the large heat of hydration must be balanced by a favorable interaction, such as that between a positive and negative charge.

substituted for a nitro group is confirmed by the very low K_0' values of benzoate in the anti-3-NB and anti-4-NB systems, and of benzoate, *m*-phthalate, trimesate and N-3-nitro-5-carboxyphenylsuccinamate in the anti-3,5-NB system.

Effect of Substitution of Iodine for a Nitro Group in the Homologous Position.—In the anti-3-NB and anti-4-NB systems, the presence of an iodine atom in the nitro-specific position contributes markedly to the free energy of interaction with antibody. In the anti-3-NB system, the ΔF° value for N-3-iodophenylsuccinamate is 3.5 kcal./mole more negative than that of N-phenylsuccinamate; and in the anti-4-NB system, the value for N-4-iodophenylsuccinamate is 2.4 kcal./mole more negative than that of the unsubstituted compound. These energies represent 60 to 65% of the values associated with substitution of a nitro group in each of the corresponding homologous positions. The effectiveness of an iodo group in each case is consistent with the hypothesis that the interaction of the nitro group with antibody is largely the result of non-polar forces. Despite the fact that an iodo group does not ordinarily form hydrogen bonds, it still contributes a large fraction of the energy of interaction of the homologous nitro group with each antibody. The remaining difference can probably be ascribed to a difference in complementariness rather than to the nature of the force of attraction although hydrogen bonding cannot be ruled out completely. (The fact that a large change in ΔF° can be associated with a small change in the size of the substituent group is substantiated by the comparison of chloro and iodo substitution, discussed below.)

The possibility of ion–dipole interaction with a positive charge in the molecule similarly appears improbable. A positive charge, if present, would interact strongly with carboxylate; actually, substitution of carboxylate for nitro results in each case in a large decrease in K_0', despite the fact that carboxylate and nitro are almost identical in size and configuration.

Substitution of a chloro group for a hydrogen atom in the nitro-specific position in the anti-3-NB or anti-4-NB systems similarly results in improved combination with antibody. In the latter system, the chloro is nearly as effective as iodo; in the former, the chloro group is considerably less effective (the ratio of K_0' values is about 18/1). The higher K_0' values of the iodo compounds, as compared with chloro derivatives, may be attributed to the closer similarity in size of iodine to the homologous nitro group.

The effect of substitution of a halogen for nitro in the anti-3,5-NB system cannot readily be discussed because disubstituted halogen derivatives were not tested and because of the uncertainty in the K_0' value of N-3-nitrophenylsuccinamate in the first run (Table III).

Other Observations.—The anti-3-NB antibody was found to fit closely about the 3-nitrophenylsuccinamate ion, as shown by the fact that placing a second nitro group on the opposite side (in the 5-position) increased the free energy of combination by about 1.3 kcal./mole. If a carboxylate group is placed in the 5-position instead of the nitro group, there is a much greater increase (3.6 kcal./mole) which may be due either to the hydration of the carboxylate, which may increase its effective size; or to the presence of a negative charge in or near the combining site of the antibody, which would repel the carboxylate-substituted hapten.

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[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION[1]]

Cysteine Thioethers from Chloroethylenes[2]

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Both *cis*-dichloroethylene and vinylidene chloride reacted with disodium cysteinate in liquid ammonia to give the same dithioether I in good yield, but *trans*-dichloroethylene failed to react. Trichloroethylene reacted to give only a monothioether II, while tetrachloroethylene gave both a mono- (III) and a dithioether (IV). The hyperconjugation and conjugative effect of a vinyl group attached to a sulfur atom is evidenced by a bathochromic shift in the ultraviolet which is accentuated by the presence of chlorine atoms on the vinyl group. The trichloroethylene derivative II is unique in giving a typical mustard gas color test and in exhibiting a high order of toxicity which is not necessarily a function of chlorine beta to sulfur.

Introduction

The discovery that treatment of disodium L-cysteinate with trichloroethylene in liquid ammonia produced S-(dichlorovinyl)-L-cysteine which on oral administration to calves produced an aplastic anemia syndrome identical in all respects to that observed from trichloroethylene-extracted soybean oil meal[3] prompted a further study of the thioethers formed from chloroethylenes. This paper describes the products obtained by treating disodium cysteinate in liquid ammonia with *cis*-dichloroethylene, vinylidene chloride or tetrachloroethylene, along with further observations of the reaction with trichloroethylene and comparison of the properties of the products.

(1) One of the Divisions of the Agricultural Research Service, U. S. Department of Agriculture.

(2) Presented before the Division of Agricultural and Food Chemistry at the Symposium on Deleterious Compounds in Foods and Feeds, 134th Meeting, American Chemical Society, Chicago, Illinois, September 7–12, 1958.

(3) (a) L. L. McKinney, F. B. Weakley, A. C. Eldridge, R. E. Campbell, J. C. Cowan, J. C. Picken, Jr., and H. E. Biester, THIS JOURNAL, **79**, 3932 (1957); (b) L. L. McKinney, J. C. Picken, Jr., F. B. Weakley, A. C. Eldridge, R. E. Campbell, J. C. Cowan and H. E. Biester, *ibid.*, **81**, 909 (1959).