[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY RESEARCH, ROSWELL PARK MEMORIAL INSTITUTE]

# The Annular Nitrogen of Pyridine as a Determinant of Immunologic Specificity

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The combination of rabbit antibodies homologous to the 3-azopyridine and azobenzene haptenic groups with various substances of related configuration was investigated. Closeness of fit of each antibody about the ring structure of the hapten was determined. The anti-3-azopyridine antibody showed distinct specificity for a ring nitrogen atom; the contribution of this atom to the negative free energy of interaction was 2.4 kcal./mole greater than that of a C–H group in the same position. Conversely, the presence of the nitrogen greatly hindered combination of haptens with the anti-azobenzene antibody. Each of various substituents in the *ortho* or *para* positions of pyridine interfered with combination with the anti-3-azopyridine antibody, indicating a tight fit of the antibody about these positions of the ring. Substituents were readily accommodated in the azo-specific 3-position as shown by a general increase in combining constant; the forces responsible for the interaction of these uncharged groups with antibody are discussed. The fit of anti-3-azopyridine antibody about the aromatic ring was further explored by measuring the combining constants of ring-substituted derivatives of N-phenylsuccinamate. An apparently anomalous finding is that substituents on the benzene ring *para* to the azo group are readily accommodated by the anti-3-azopyridine antibody, whereas substituents in the corresponding position on pyridine are not. Two possible models to account for the results are proposed; in one, which appears more probable, the antibody is assumed to form against pyridine with its ring nitrogen atom in the hydrated state.

Information concerning the closeness of fit and forces responsible for antibody–hapten interactions can be obtained by quantitative measurement of the ability of various structurally related haptens to combine with antibody prepared against a specific chemical group. This paper presents the results of such a study of antibody homologous to the 3-azopyridine haptenic group. In the course of the work the combination of certain haptens with antibody formed against the azobenzene grouping was also investigated. A qualitative study of the interaction of a few homologous haptens with anti-3-azopyridine antibody has been reported by Berger and Erlenmeyer.[1]

The anti-pyridine system was of particular interest for several reasons. First, there is little quantitative information available at present on the reaction of heterocyclic haptens with antibody. Secondly, there have been few attempts to analyze the forces responsible for antibody–hapten interaction in the case of uncharged haptenic groups. A third question of interest was that of the possible importance of the hydration of the annular nitrogen atom of pyridine as a determinant in specificity. In several antibody systems, it has been found that compounds having ring nitrogen systems acted as though the nitrogen were hydrated.[2–5] Thus, the *ortho*, *meta* and *para*-pyridine carboxylates in their combinations with antisera specific to the *ortho*, *meta* and *para*-azobenzoate groups acted as though there were a large substituent, presumably water of hydration, attached to the nitrogen. Similar evidence has been reported in the case of antibody to the 4-azophthalate ion in its reaction with pyridine dicarboxylate and pyrazine dicarboxylate.

These observations were in accord with the known fact that the annular nitrogen atom is strongly hydrated in aqueous solution, as shown by the miscibility of pyridine with water and its large heat of hydration (12 kcal./mole).[6] The possibility therefore exists that antibody may form against pyridine in its hydrated state; *i.e.*, that the water of hydration may act as a determinant of specificity. One objective of the present work was to investigate this possibility.

(1) E. Berger and H. Erlenmeyer, *Klin. Wochschr.*, **14**, 536 (1935).
(2) D. Pressman and L. Pauling, THIS JOURNAL, **71**, 2893 (1949).
(3) D. Pressman and M. Siegel, *ibid.*, **75**, 686 (1953).
(4) D. Pressman and M. Siegel, *ibid.*, **79**, 994 (1957).
(5) A. Nisonoff and D. Pressman, *ibid.*, **79**, 1616 (1957).
(6) G. Briegleb, *Z. Elektrochem.*, **53**, 350 (1949).

## Experimental

**Haptens.**—N-Phenylsuccinamic acid and its ring-substituted derivatives were prepared: (a) by the reaction of aniline, or the appropriate aniline derivative, with slightly less than an equimolar quantity of succinic anhydride. The reaction was carried out in chloroform solution at the boiling point, usually for an hour or less. In most instances the product precipitated out in a short time. Otherwise, to induce precipitation, some of the solvent was removed by distillation. The product was purified by recrystallization from chloroform or from a water–alcohol mixture. (b) the *ortho* and *para*-nitro derivatives of N-phenylsuccinamic acid were prepared by fusing a mixture of succinic anhydride, a large molar excess of *o*- or *p*-nitroaniline and a weight of decalin approximately equivalent to that of the aniline derivative; the fused mixture was kept at 170–180° for about an hour. The mixture was then cooled and dissolved in ethanol. The product precipitated upon addition of water and was recrystallized from an alcohol–water mixture.

N-2-Pyridylsuccinamic acid and N-3-pyridylsuccinamic acid were synthesized in chloroform solution as described above. N-4-Pyridylsuccinamic acid was prepared similarly except that pyridine was used as solvent. Each of the derivatives of succinamic acid described above was recrystallized until its equivalent weight agreed, within 2%, with the theoretical value.

The preparation of N-phenylmaleamic and N-phenylfumaramic acid has been described.[7]

Pyridine and its derivatives, other than the pyridylsuccinamates, were commercial products. Those derivatives which are solid at room temperature were recrystallized to the correct melting point. Liquids were purified by distillation, in most instances under vacuum and in the presence of an atmosphere of nitrogen. A sharp middle fraction was collected. The boiling point was checked at atmospheric pressure when practical or else at reduced pressure, and in each case agreed closely with values given in the literature.

**Protein Antigens.**—The antigen for injection was prepared by coupling at 5° 90 mg. of diazotized 3-aminopyridine with 6 g. of lyophilized whole beef serum in 60 ml. of water. The azoprotein was purified by dialysis for 3 days against saline–borate solution, $\mu = 0.16$, *p*H 8. The solution was then adjusted with saline–borate to a protein concentration of 10 mg./ml. A phenol solution was added to a final concentration of 0.1%. The test antigen was prepared by diazotizing and similarly coupling 20 mg. of 3-aminopyridine with 0.5 g. of crystallized ovalbumin (commercial product). This azoprotein was dialyzed for 4 days against several changes of saline–borate solution. Dialysis was carried out at 2–5°.

The immunizing antigen used for the preparation of anti-azobenzene antibodies was prepared by diazotizing 30 mg.

(7) D. Pressman, J. H. Bryden and L. Pauling, THIS JOURNAL, **70**, 1352 (1948).

TABLE I

COMBINATION OF PYRIDINE DERIVATIVES AND OTHER CYCLIC NITROGEN COMPOUNDS WITH ANTI-3AP ANTIBODY

| Hapten (pyridine derivative) | $K_0'$ | $\Delta F^\circ_{rel.}$ (cal./mole) | $\sigma$ | Hapten concn., molar × $10^5$: 0.16 | 0.65 | 2.61 | 41.7 | 167 | 667 | 2667 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Amount of precipitate[a] | | | | | | |
| Unsubstituted | 1.00 | 0 | 2.0 | | | | 75 | 47 | 17 | 4 |
| 2-Amino | 0.17 | 980 | 3.0 | | | | | 75 | 54 | 31 |
| 3-Amino | 0.75 | 160 | 2.0 | | | | 82 | 49 | 21 | |
| 4-Amino | 0.026 | 2020 | 4.0 | | | | | 89 | 73 | 59 |
| 2-Methyl | 0.63 | 260 | 2.5 | | | | | 58 | 27 | 9 |
| 3-Methyl | 3.3 | −660 | 1.5 | | | | 48 | 12 | 6 | |
| 4-Methyl | 0.41 | 490 | 2.5 | | | | | 57 | 39 | 12 |
| 2-Cyano | 0.14 | 1090 | 3.0 | | | | | 78 | 59 | 35 |
| 3-Cyano | 1.1 | −50 | 3.0 | | | | 66 | 47 | 24 | |
| 4-Cyano | 0.14 | 1090 | 3.0 | | | | | 77 | 55 | 39 |
| 2-Hydroxymethyl | 0.008 | 2760 | 4.0 | | | | | 95 | 82 | 73 |
| 3-Hydroxymethyl | 1.1 | −50 | 2.0 | | | | 71 | 49 | 16 | |
| 4-Hydroxymethyl | 0.072 | 1460 | 2.5 | | | | | 84 | 68 | 46 |
| 2-Chloro | 0.36 | 560 | 2.5 | | | | | 63 | 40 | |
| 2-Bromo | 0.38 | 530 | 2.5 | | | | | 66 | 40 | |
| 3-Bromo | 2.4 | −480 | 2.0 | | | | 56 | 27 | 4 | |
| 3-Acetyl | 3.5 | −690 | 1.5 | | | | 45 | 22 | 4 | |
| 3-Carboxy | 0.12 | 1170 | 2.0 | | | | | 88 | 62 | 31 |
| 2,4,6-Trimethyl | 0.14 | 1090 | 1.0 | | | | | 89 | 63 | 16 |
| 3-(4′-Hydroxyphenylazo) | 37 | −2000 | 2.0 | 94 | 78 | 63 | | | | |
| Imidazole | 0.027 | 2000 | 3.0 | | | | | 90 | 80 | 65 |
| Pyrrolidine | 0.013 | 2400 | 1.5 | | | | | 101 | 105 | 85 |
| Quinoline | 2.1 | −410 | 1.5 | | | | 61 | 22 | 7 | |

[a] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten; 205 μg. Experimental procedure described under "Methods."

of aniline and coupling to 100 ml. of whole beef serum. The corresponding azoprotein test antigen was made by coupling 30 mg. of diazotized aniline to 0.5 g. of ovalbumin dissolved in 10 ml. of a saline–borate mixture. In each case, the coupling reaction was carried out at 0° and *p*H 9–9.5. The azoproteins were purified by dialysis as described above.

**Preparation of Antisera.**—The method for obtaining and pooling antisera has been described previously.[8]

**Reactions of Antiserum with Antigen and Hapten.**—Solutions of haptens were prepared by dissolving in sodium-hydroxide solution and back-titrating with HCl to *p*H 7.5–8.5; hapten solutions with buffering capacity in this region were adjusted to *p*H 8.0. Solutions were 0.16 *M* in sodium ion. Dilutions of the hapten solutions were made with 0.16 *M* sodium chloride; dilutions of the antigen were made with borate buffer of *p*H 8.0 and ionic strength 0.16.

A preliminary series of experiments was run to determine the amount of antigen required to give optimum precipitation. This quantity was used in subsequent experiments in the presence of hapten. To test haptens as inhibitors of the precipitin reaction, 1.0 ml. each of solutions of antigen, hapten and antibody were added to a test-tube in that order; the mixture was permitted to stand for one hour at 37°, then for 5 days at 3–5°. Haptens were usually tested at three concentrations. Precipitates were centrifuged and washed three times with five 10-ml. portions of 0.16 *M* sodium chloride solution. The amount of protein in the precipitate was estimated by a modification of the procedure of Folin and Ciocalteu.[9] Control experiments were carried out in duplicate in which antigen and saline, or antibody and saline were incubated together. The mean value of the small blanks obtained was subtracted from the results of the analyses. Four to eight replicate experiments in which antigen and antibody, but not hapten, were present, were performed in each series to serve as a basis for calculating the degree of inhibition. Otherwise each experiment was carried out in duplicate. The arithmetic mean of all values of mean deviation is 3.0%.

In order to separate a γ-globulin fraction, the serum was fractionated 3 times with sodium sulfate by the method of Kekwick.[10] The effectiveness of this method has previously been checked by electrophoresis of samples of serum before and after fractionation. Only one major peak, corresponding to γ-globulin, was found to remain in the fractionated serum; no serum albumin was detectable.

## Results

Data which show the effect of various pyridine derivatives on the precipitation of anti-3-azopyridine (anti-3AP) antibody with the ovalbumin-3-azopyridine test antigen (3AP-oval) are given in Tables I, II, III and V. Data on the combination of derivatives of N-phenylsuccinamate with the same antibody are recorded in Table IV. Results describing the combination of various derivatives of N-phenylsuccinamate with the anti-azobenzene antibody are shown in Table VI. In each case, the data were interpreted with the aid of a theory based on the assumption that the heterogeneity of combining energies of the various antibody molecules with hapten can be described by a gauss distribution function.[11] The parameters obtained on application of the theory to data on inhibition of precipitation are: $K_0'$, which is an average equilibrium constant for the combination of hapten with antibody, $\Delta F^\circ_{rel.}$ $(-RT \ln K_0')$ and $\sigma$, an index of heterogeneity, which appears as an exponent in the distribution function. In the anti-3AP system, the $K_0'$ values given are relative to that of pyridine which is arbitrarily assigned a $K_0'$ value of 1.00; each $\Delta F^\circ_{rel.}$ value therefore represents a difference between the free energy of combination of the hapten with antibody, and that of pyridine with antibody. In the anti-azobenzene system, the $K_0'$ values given are relative to that of N-phenylsuccinamate, which is assigned the value $K_0' = 1.00$. The degree of heterogeneity of combining constants correspond-

(8) L. Pauling, D. Pressman, D. H. Campbell, C. Ikeda and M. Ikawa, THIS JOURNAL, **64**, 2994 (1942).

(9) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **51**, 357 (1943).

(10) R. A. Kekwick, *Biochem. J.*, **34**, 1248 (1940).

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

TABLE II

COMBINATION OF N-PYRIDYLSUCCINAMATES WITH ANTI-3AP ANTIBODY

| Hapten | $K_0'$ | $\Delta F^\circ_{rel.}$ (cal./mole) | $K_2$[a] | $\sigma$ | Hapten concn., molar × $10^5$ 41.7 | 167 | 667 | 2667 |
|---|---|---|---|---|---|---|---|---|
| | | | | | Amount of precipitate[b] | | | |
| Pyridine | 1.00 | 0 | 31 | 2.0 | 75 | 47 | 17 | 4 |
| N-Phenylsuccinamate | 0.032 | 1900 | 1.00 | 1.5 | | | 88 | 64 |
| N-2-Pyridylsuccinamate | 0.065 | 1510 | 2.0 | 1.5 | | 94 | 83 | 44 |
| N-3-Pyridylsuccinamate | 2.5 | −510 | 77 | 2.5 | 55 | 30 | 8 | |
| N-4-Pyridylsuccinamate | 0.10 | 1270 | 3.2 | 4.0 | 81 | 72 | 61 | |

[a] $K_2$ is the ratio of $K_0'$ of a hapten to $K_0'$ of N-phenylsuccinamate. [b] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten; 205 μg. Experimental procedure described under "Methods."

TABLE III

EFFECT OF SOLUBILIZING GROUP ON COMBINATION OF HAPTENS WITH ANTI-3AP ANTIBODY

| Hapten | $K_0'$ | $\Delta F^\circ_{rel.}$ (cal./mole) | $K_2$[a] | $\sigma$ | Hapten concn., molar × $10^5$ 41.7 | 167 | 667 | 2667 |
|---|---|---|---|---|---|---|---|---|
| | | | | | Amount of precipitate[b] | | | |
| Pyridine | 1.00 | 0 | 31 | 2.0 | 75 | 47 | 17 | 4 |
| N-Phenylsuccinamate | 0.032 | 1900 | 1.00 | 1.5 | | | 88 | 64 |
| N-Phenylfumaramate | .046 | 1700 | 1.4 | 1.0 | | 100 | 93 | 54 |
| N-Phenylmaleamate | .044 | 1730 | 1.4 | 1.5 | | 101 | 84 | 57 |

[a] $K_2$ is the ratio of $K_0'$ of a hapten to $K_0'$ of N-phenylsuccinamate. [b] The amount of precipitate is reported as per cent. of the amount found in the absence of hapten; 205 μg.

TABLE IV

COMBINATION OF DERIVATIVES OF N-PHENYLSUCCINAMATE WITH ANTI-3AP ANTIBODY

| Hapten | $K'_0$ | $\Delta F^\circ_{rel.}$ (cal./mole) | $K_2$[a] | $\sigma$ | Hapten concn., molar × $10^5$ 41.7 | 167 | 667 | 2667 |
|---|---|---|---|---|---|---|---|---|
| | | | | | Amount of precipitate[b] | | | |
| Pyridine | 1.00 | 0 | 31 | 2.0 | 75 | 47 | 17 | 4 |
| N-Phenylsuccinamate | 0.032 | 1900 | 1.00 | 1.5 | | | 88 | 64 |
| N-2-Methylphenylsuccinamate | .007 | 2750 | 0.20 | 2.5 | | 108 | 98 | 84 |
| N-3-Methylphenylsuccinamate | .045 | 1720 | 1.4 | 1.5 | | 104 | 85 | 56 |
| N-4-Methylphenylsuccinamate | .044 | 1730 | 1.4 | 2.5 | | 90 | 77 | 56 |
| N-2-Chlorophenylsuccinamate | .029 | 1960 | 0.88 | 1.5 | | 102 | 97 | 69 |
| N-3-Chlorophenylsuccinamate | .056 | 1590 | 1.7 | 2.0 | | 93 | 74 | 49 |
| N-4-Chlorophenylsuccinamate | .23 | 810 | 7.0 | 2.5 | | 71 | 50 | 17 |
| N-2-Bromophenylsuccinamate | .030 | 1940 | 0.91 | 2.5 | | 92 | 99 | 59 |
| N-3-Bromophenylsuccinamate | .075 | 1430 | 2.3 | 1.5 | | 84 | 83 | 37 |
| N-4-Bromophenylsuccinamate | .26 | 740 | 8.0 | 1.5 | | 60 | 52 | 7 |
| N-2-Iodophenylsuccinamate | .026 | 2020 | 0.80 | 1.5 | | 96 | 95 | 72 |
| N-3-Iodophenylsuccinamate | .46 | 430 | 14.0 | 2.5 | | 62 | 66 | 8 |
| N-4-Iodophenylsuccinamate | .72 | 180 | 22.0 | 2.5 | | 49 | 37 | 2 |
| N-2-Nitrophenylsuccinamate | .043 | 1740 | 1.3 | 2.5 | | 90 | 80 | |
| N-3-Nitrophenylsuccinamate | .12 | 1170 | 3.6 | 1.5 | | 79 | 67 | 31 |
| N-4-Nitrophenylsuccinamate | .33 | 610 | 10.1 | 1.5 | | 64 | 44 | 7 |
| N-3-Acetylphenylsuccinamate[c] | .42 | 480 | 13.0 | 3.0 | 74 | 59 | 29 | 6 |
| N-4-Acetylphenylsuccinamate[c] | 1.8 | −320 | 58.0 | 3.0 | 50 | 28 | 7 | 4 |
| N-2,4-Dichlorophenylsuccinamate | 0.045 | 1710 | 1.4 | 2.5 | | 91 | 79 | 51 |
| N-2,5-Dichlorophenylsuccinamate | 0.090 | 1330 | 2.8 | 1.5 | | 94 | 70 | 35 |

[a] $K_2$ is the ratio of $K_0'$ of a hapten to $K_0'$ of N-phenylsuccinamate. [b] Amount of precipitate is reported as per cent. of amount found in the absence of hapten; 205 μg. [c] Data obtained in a separate run in which the amount of precipitate found was 257 μg. in the absence of hapten; 128, 184 and 220 μg. were found in the presence of 2667, 667 and 167 × $10^{-5}$ *M* N-phenylsuccinamate.

TABLE V

COMBINATION OF BROMOPYRIDINES WITH ANTI-3AP ANTIBODY

| Hapten | $K_0'$ | $\Delta F^\circ_{rel.}$ (cal./mole) | $\sigma$ | Hapten concn., molar × $10^5$ 41.7 | 167 | 667 | 2667 |
|---|---|---|---|---|---|---|---|
| | | | | Amount of precipitate[a] | | | |
| Pyridine | 1.00 | 0 | 2.0 | 75 | 47 | 17 | 4 |
| 3-Bromopyridine | 2.4 | −480 | 2.0 | 56 | 27 | 4 | |
| 3,5-Dibromopyridine | 0.065 | 1510 | 2.0 | | 92 | 75 | |

[a] Amount of precipitate is reported as per cent. of amount found in the absence of hapten; 205 μg.

ing to various σ-values has been described previously.[11] A σ-value of zero indicates complete uniformity of combining constants; increasing σ-values correspond to greater degrees of heterogeneity of antibody with respect to the hapten.

## Discussion

**Combination of Pyridine Derivatives with Anti-3AP Antibody.**—Equilibrium constants ($K_0'$) for the combination of a number of pyridine derivatives with the anti-3AP antibody are shown in the second column of Tables I and II. The corresponding standard free energy changes, relative to that of unsubstituted pyridine, are listed in the third column. Pyridines with substituents in the 2-, 3- and 4-positions were used.

It is evident that substitution in the 2-position or 4-position decreases the combining constant of pyridine in the case of each group tested (amino, methyl, cyano, hydroxymethyl or succinamate in the 2- or 4-positions; chloro or bromo in the 2-position).

Substitution of various groups in the 3-position results, in most instances, in an increase in the combining constant. Each substituent which was tested in all three positions exhibits its highest value of $K_0'$ when present in the 3-position.

These results indicate, first, that the ring nitrogen atom is an important determinant of specificity, and is not antigenically equivalent to the carbon–hydrogen group of benzene, despite the similarity in size of the two groups and the nearly identical structures of pyridine and benzene. If the C–H group and the N atom were equivalent, the position of a substituent relative to that of the nitrogen atom would not be significant; and large differences obviously exist. The combining constant of 3-hydroxymethylpyridine, for example, is over 100 times greater than that of the corresponding 2-substituted derivative.

Additional evidence establishing the annular nitrogen atom as an important determinant of specificity is provided by the data in Table II, which gives the equilibrium constants for the combination with anti-3AP antibody of N-phenylsuccinamate and the three isomeric N-pyridylsuccinamates. Each of the N-pyridylsuccinamates has a higher combining constant than N-phenylsuccinamate. The 3-pyridyl derivative, which most closely resembles the homologous 3-azopyridine group, has a value of $K_0'$ which is 77 times as high as that of unsubstituted N-phenylsuccinamate, indicating that the nitrogen atom contributes $-2.4$ kcal./mole to the free energy of interaction.

A second conclusion, which may be drawn from the low values of $K_0'$ for the 2- and 4-substituted derivatives of pyridine as compared with pyridine, is that there is a close fit of the antibody about both the 2- and 4-positions of the pyridine ring, and a much looser fit in the area corresponding to the 3-position. The fact that an amino group is not accommodated in either the 2- or 4-positions indicates that the spacing is less than 2 Å. in either of these positions. The ability of the antibody site to accommodate rather large substituents in the 3-position is readily attributable to the fact that this position corresponds to that occupied by the azo group in the immunizing antigen; the antibody can accommodate other substituents in the space directed against the azo group (Fig. 1, diagram A).

It is probable that two factors are largely responsible for the increase in $K_0'$ associated with the substitution of various groups for the hydrogen atom in the 3-position of pyridine. One is the greater polarizability of the larger groups as compared with the hydrogen atom, which may permit greater attraction through increased London dispersion forces. The second factor[12] is increased displacement of water from a non-polar portion of the surface of the antibody by the larger substituent, with a correspondingly greater release of interfacial energy.

Of the haptens tested, 3-(4′-hydroxyphenylazo)-pyridine has the highest combining constant ($K_0' = 37$). The hydroxyphenylazo group thus contributes $-2.0$ kcal. to the free energy of interaction. This indicates that some of the specificity of the antibody was directed toward the azo group of the immunizing antigen and also, in all probability, toward the benzene ring of tyrosine groups in the protein to which many of the azo-haptenic groups must have been coupled.

The only compound showing a distinctly unfavorable effect of substitution in the 3-position is the 3-carboxypyridine (nicotinate), which has a $K_0'$ value of 0.12. This suggests the presence of a negatively charged group in or near the antibody site, but more evidence on this point is required.

**Use of Derivatives of N-Phenylsuccinamate for Investigating the Fit of Anti-3AP Antibody about the Pyridine Ring of the Homologous Hapten.**—The results discussed so far indicate that a great deal of the specificity of anti-3AP antibody is directed against the ring nitrogen atom, and that a C–H group is not an effective substitute for the nitrogen atom. The nitrogen atom in pyridine differs markedly from a C–H group in benzene with respect to ability to form secondary bonds, because it possesses an unshared pair of electrons. The observed specificity for nitrogen may arise from hydrogen bond formation between the antibody and the nitrogen atom, or the nitrogen atom may retain its water of hydration (attached through its unshared electron pair) during the process of antibody formation. In the latter case, the water of hydration would be an important structural feature, and the antibody site would possess a large concavity in juxtaposition to the nitrogen atom. It was considered desirable to test this possibility.

As indicated earlier in the discussion, closeness of fit of anti-3AP antibody about the pyridine ring was partially investigated by determining the effect of ring substituents on the combination of pyridine with antibody. These compounds, however, did not give information concerning the fit about the nitrogen atom, which is related to the question of hydration of this atom. N-Substituted pyridine derivatives would not be useful in this consideration because such substitution results in the introduction of a positive charge on the nitrogen atom, which may in itself greatly alter the ability of the hapten to combine with antibody.

To avoid this difficulty, a series of ring-substituted derivatives of benzene, rather than pyridine, were tested for ability to combine with anti-3AP antibody. Actually, the parent compound employed was N-phenylsuccinamate, and the haptens tested were ring-substituted derivatives of this compound. The succinamate group served the necessary purpose of making these haptens water-soluble under the experimental conditions, *i.e.*, *p*H 8.

However, in order to interpret the data, it was necessary to know the orientation of succinamate derivatives with respect to the antibody site when combination takes place; or, more specifically, to

(12) H. Eyring in "Symposium on the Mechanism of Enzyme action," pp. 10 and 16, W. D. McElroy and B. Glass, ed., The Johns Hopkins Press, Baltimore, Md., 1954.

determine which position on the benzene ring of N-phenylsuccinamate, when combined with antibody, corresponds to that of the nitrogen atom in the pyridine ring of the immunizing antigen. This information would provide a frame of reference for interpreting data obtained with N-phenylsuccinamates, substituted in various positions, in terms of corresponding positions on the pyridine ring of the haptenic group of the immunizing antigen.

As discussed earlier, the results in Table II indicate that the succinamate group can only be accommodated by the antibody site in the position *meta* to the nitrogen atom of pyridine; the *ortho* and *para* N-pyridylsuccinamates combine very weakly. The $K_0'$ values, relative to pyridine, of the 2-, 3- and 4-N-pyridylsuccinamates are 0.065, 2.5 and 0.10. These data conform with the fact that the antibody was formed to accommodate an azo group in the 3-position and can therefore accommodate other substituents. It was therefore possible to investigate the fit of antibody about the various positions of the pyridine ring, by measuring the ability of variously substituted N-phenylsuccinamates to combine with the antibody and interpreting the data on the assumption that the succinamate group occupies the azo-homologous position, or conversely, that the 3-position of N-phenylsuccinamate corresponds to that which would normally be occupied by the annular nitrogen atom of pyridine. Results of such experiments will be described in the next section.

It is apparent from the data in Table III that the combining constant undergoes little change if the succinamate group in N-phenylsuccinamate is replaced by a fumaramate or maleamate group. The combining constants of each of the latter two haptens, relative to that of N-phenylsuccinamate, is 1.4, indicating that the space in the azo-specific portion of the antibody site is large enough so that the exact configuration of the solubilizing group substituted in this position is not very significant. These results also show that substitution of a succinamate group in the azo-specific position does not interfere sterically with the fit of the pyridine ring in the antibody site.

**Closeness of Fit of Anti-3AP Antibody about the Ring Nitrogen Atom of Pyridine.**—The data in Table IV show the effect of substitution of various groups in the benzene ring of N-phenylsuccinamate on the equilibrium constant for the combination of hapten with anti-3AP antibody. The ratio of the combining constant of each hapten to that of unsubstituted N-phenylsuccinamate ($K_2$) is given in the fourth column.

It is evident that substituents can readily be accommodated in the 3-position of N-phenylsuccinamate, since an increase in $K_0$ results from substitution of each group tested (methyl, chloro, bromo, iodo, nitro or acetyl) for the hydrogen atom in this position.

Similarly, there appears to be a loose fit of anti-3AP antibody about the 4-position of N-phenylsuccinamate; each of the 4-substituted derivatives tested has a higher value of $K_0'$ than the parent compound.

Substituent groups tested in both the 3- and 4-positions show a somewhat greater enhancing effect on $K_0'$ when present in the 4-position. For example, the $K_0'$ values, relative to N-phenylsuccinamate, of the 3-acetyl and 4-acetyl derivatives are 13 and 58, respectively.

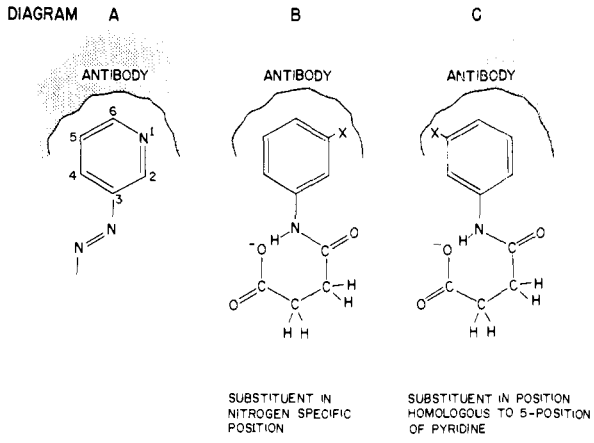


Fig. 1.—Diagrams B and C illustrate two possible modes of combination of a *meta*-substituted N-phenylsuccinamate with anti-3AP antibody. Orientation of the homologous 3-azopyridine in the same site is shown in Diagram A.

For halogens substituted in the 3- or 4-positions, the combining constants are in the order Cl < Br < I which is also the order of increasing size and polarizability of the atom. Increase of combining affinity with increasing size of the halogen, when substituted in positions where the antibody can accommodate a substituent, has been observed in other antibody systems. It can be attributed to the greater polarizability of the larger halogen atoms and consequent increase in van der Waals attractive forces; or to the fact that a larger atom is capable of displacing more water from a non-polar portion of the antibody surface, with a consequently greater release of interfacial energy.

For groups containing more than one atom, the steric configuration of the group may be of importance in its interaction with antibody, so that the direct relationship of size to combining constant might not necessarily be expected to hold. However, the correlation with size is quite good for those groups other than the halogens which were tested. The acetyl group, the largest of those investigated, shows by far the greatest increase in $K_0'$ when substituted in the 4-position relative to the succinamate group, and a larger enhancing effect in the 3-position than that of any other group except iodo. The nitro group, which is about as large as bromo, increases the combining constants in the 3- and 4-positions to a slightly greater extent than does the bromo substituent. The contribution of the methyl group to the $K_0'$ value is somewhat lower than would be predicted on the basis of its size (about equal to bromo). This may be ascribed to the fact that it is less polarizable than the bromine atom and hence capable of a smaller degree of van der Waals interaction with the antibody.

The fact that there is a somewhat greater enhancing effect of substitution in the 4-position than in the 3-position can be explained on the assumption that there is a space large enough to accommodate substituents in either position, but that the space is

TABLE VI

COMBINATION OF HAPTENS WITH ANTI-AZOBENZENE ANTIBODY

| Hapten | $K'_0$ | $\Delta F^\circ_{rel}$, (cal./mole) | $\sigma$ | Hapten concn., molar × $10^5$: 2.6 | 10.4 | 41.7 | 167 | 667 | 2667 |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Amount of precipitate[a] | | | | | |
| N-Phenylsuccinamate | 1.00 | 0 | 4.0 | 75 | 58 | 47 | | | |
| N-2-Chlorophenylsuccinamate | 0.099 | 1280 | 4.0 | | 86 | 77 | 53 | | |
| N-3-Chlorophenylsuccinamate | 0.96 | 20 | 4.0 | 74 | 61 | 49 | | | |
| N-4-Chlorophenylsuccinamate | 2.6 | −530 | 3.5 | 68 | 50 | 29 | | | |
| N-2-Iodophenylsuccinamate | 0.055 | 1610 | 4.0 | | 84 | 78 | 64 | | |
| N-3-Iodophenylsuccinamate | 1.5 | −220 | 4.0 | 70 | 55 | 40 | | | |
| N-4-Iodophenyluccinamate | 5.8 | −970 | 3.0 | 58 | 39 | 12 | | | |
| N-2-Pyridylsuccinamate | 0.22 | 840 | 4.0 | | 76 | 64 | 46 | | |
| N-3-Pyridylsuccinamate | 0.018 | 2220 | 4.0 | | | 86 | 77 | 59 | |
| N-4-Pyridylsuccinamate | 0.019 | 2190 | 1.5 | | | | 87 | 78 | 34 |

[a] Amount of precipitate is reported as per cent. of amount found in the absence of hapten; 166 $\mu$g.

so large and so oriented that a substituent in the 3-position can be accommodated, but does not approach the walls of the antibody closely enough to interact very effectively with the antibody; a somewhat closer fit about a substituent in the 4-position would account for the higher $K'_0$ values for substituents in this position.

Substitution of a chloro, bromo, iodo or nitro group for the hydrogen atom in the 2-position of N-phenylsuccinamate has little effect on $K'_0$ (Table IV). The values of $K'_0$, relative to that of unsubstituted N-phenylsuccinamate vary between 0.80 and 1.3. However, the relative combining constant of the 2-methyl derivative is only 0.2. A simple interpretation of the results of substitution in the 2-position is that there is a small amount of steric interference with combination, which is compensated for by van der Waals attractive forces in the case of the halogens and nitro group but not by the less polarizable methyl group.

The data discussed so far indicate that there is a fairly close fit of antibody around the 2-position, a rather loose fit about the 3- and 4-positions of N-phenylsuccinamate (loose enough to accommodate an acetyl group), and that the fit is somewhat closer about a substituent in the 4-position than in the 3-position.

We are particularly interested in the space about the 3-position—the position occupied by the annular nitrogen atom of the immunizing haptenic group. The 3-substituted N-phenylsuccinamates can conceivably fit the anti-3AP antibody in two ways; with the substituent occupying the nitrogen-specific 1-position or the 5-position (illustrated in diagrams B and C of Fig. 1). That the former is the case is shown by results obtained with 3-bromopyridine and 3,5-dibromopyridine (Table V). The $K_0$ value for 3-bromopyridine is 2.4, while that for 3,5-dibromopyridine is only 0.065. The ability of antibody to accommodate a single *meta*-substituent readily can be attributed to a loose fit in the azo-specific 3-position. A loose fit in the azo-specific region also has been observed in other anti-hapten antibody systems. The interference with combination by a second *meta*-substituent must therefore be attributed to a tight fit about the 5-position (Fig. 1, diagram A). The *meta*-substituents in N-phenylsuccinamate must then be accommodated in the 1-position; *i.e.*, there is a loose fit of antibody about the annular nitrogen atom. This observation is consistent with the possibility that the nitrogen atom of the pyridine ring is in the hydrated state when the antibody is formed against it.

**Closeness of Fit of Anti-azobenzene Antibody about the Homologous Hapten.**—It was considered desirable to investigate, as a control, the closeness of fit of antibody formed against the azobenzene haptenic group, which of course is not hydrated and is identical with 3-azopyridine except for the replacement of the ring nitrogen atom by a C–H group. As in the case of anti-3AP antibody, the closeness of fit of anti-azobenzene antibody about the benzene ring was investigated by testing the ability of various derivatives of N-phenylsuccinamate to combine with the antibody. Here again, the succinamate group served the dual purpose of solubilizing the haptens and orienting them with respect to the antibody site. The large succinamate group is assumed to occupy the azo-specific portion of the site.[13]

The data on combination of haptens with this antibody are shown in Table VI. Values of $K'_0$ are relative to that of unsubstituted N-phenylsuccinamate which is arbitrarily assigned the value, $K'_0 = 1.00$. The results indicate that a chloro or iodo group substituted in the 3- or 4-position of N-phenylsuccinamate does not hinder combination with the antibody. Indeed, substitution of a chloro, or especially an iodo group, in the 4-position results in an increase in $K'_0$. There is little effect on $K'_0$ attending the substitution of a chlorine or iodine atom for the hydrogen atom in the 3-position.

While there are important differences between the anti-3AP and anti-azobenzene systems, which will be considered below, there is similarity in that neither of the antibodies fits tightly about the unsubstituted N-phenylsuccinamate group; in both systems, substitution of rather large groups for the hydrogen atom in the 3- or 4-positions can be made without a reduction in combining constant. Thus the existence of an area of loose fit around the annular nitrogen atom of pyridine does not in itself constitute evidence that the antibody is formed against the hydrated form of azopyridine, since a

(13) The effect of substitution of a succinamate group in an azo-specific site has been determined in three systems. In each case, there is an enhancement of $K'_0$ upon substitution, indicating accommodation without steric interference. Such data for the 3-azopyridine system were already discussed. For the anti-3-azo-5-nitrobenzoate and anti-*p*-azobenzoate antibodies, substitution results in increases in $K'_0$ by factors of 9 and 23, respectively (unpublished data).

similar type of accommodation is observed for antibody against the closely related, non-hydrated azobenzene group. Nevertheless, there are marked differences between the two systems which give further evidence of the importance of the ring nitrogen atom as a determinant in specificity, and provide the basis for a further discussion of the possibility of hydration. These are discussed in the next section.[14]

**Combination of N-Pyridylsuccinamates with Anti-azobenzene Antibody.**—Equilibrium constants for the combination of the N-2-, N-3- and N-4-pyridylsuccinamates with anti-azobenzene antibody are listed in Table VI. Each value of $K'_0$ is considerably lower than that of N-phenylsuccinamate. Thus, the presence of a ring nitrogen in the hapten greatly hinders combination with the anti-azobenzene antibody. This is in sharp contrast with the anti-3AP system, in which each of the N-pyridylsuccinamates combined more effectively than N-phenylsuccinamate. The N-3-pyridylsuccinamate, which most closely resembles the homologous 3-azopyridine, had a combining constant 77 times as large as that of N-phenylsuccinamate in the anti-azopyridine system.

These results clearly indicate that the combining site of anti-azobenzene antibody differs markedly from the combining site of anti-3AP antibody, and that a large degree of specificity of the anti-3AP antibody is directed toward the ring nitrogen atom. The latter conclusion is in agreement with the data discussed earlier which showed that the nitrogen atom of various pyridine derivatives had to be present in the position corresponding to that of the nitrogen atom of the hapten on the immunizing antigen in order for effective combination with the antibody to take place.

**Comparison of the Combination of Derivatives of Pyridine and of N-Phenylsuccinamate with Anti-3AP Antibody.**—To return to a discussion of the anti-3AP system, we note that there is an apparent inconsistency between the combining constants with anti-3AP antibody of the N-phenylsuccinamates and the pyridine derivatives (Tables I and IV). The results obtained with the methyl, chloro, bromo, iodo, nitro and acetyl-N-phenylsuccinamates demonstrate quite conclusively that there is a loose fit of antibody around the 6-position of the 3-azopyridine group (Fig. 1, diagram A); substitution of these larger groups for the hydrogen atom in the corresponding position of N-phenylsuccinamate resulted in the formation of stronger antibody–hapten bonds. On the other hand, substitution of various groups in the *ortho* position of pyridine markedly reduces the combining constant (Table I), indicating a close fit about both the 2- and 6-positions of pyridine.

Two alternative hypotheses to account for these results are proposed. The apparent inconsistency can be accounted for on the assumption that the antibody is specific for pyridine with its nitrogen atom in the hydrated state, and that the water of hydration forms part of the linkage between the hapten and the antibody. This would account for the observed loose fit between the antibody and the benzene ring of N-phenylsuccinamate. It would also explain the fact that substitution of various groups in the same position in pyridine results in a large decrease in combining constant, since *ortho* substituents would alter the structure of the water of hydration surrounding the nitrogen atom. This would disturb the normal orientation of the water with respect to the antibody and reduce the combining constant, if the water of hydration participates in the interaction. If, for example, the water acts by forming a hydrogen bond with an atom in the antibody site, it would be particularly sensitive to steric effects because of the large variation of the strength of a hydrogen bond with the distance between the interacting atoms.

An alternative explanation, which does not involve hydration, does require that the site be flexible. Thus, for example, the antibody could interact directly with the pyridine nitrogen atom through a hydrogen bond. Substituents *ortho* to the nitrogen atom could interfere with the formation of such a bond and thus lower the combining constant, as observed. Substituents of N-phenylsuccinamate in the nitrogen-specific region (position 1 in Fig. 1, diagram A) might, however, be accommodated if the antibody site were flexible and could assume various orientations.

However, we know of no other good evidence for a flexible antibody site, and there are a great many instances of steric interference with combination by substituted small atoms which indicate rigidity of the antibody site. The explanation of the anomalous effects of *ortho* substitution based on hydration appears more plausible. It also is consistent with the large energy of hydration of pyridine. In order for the antibody to form against the unhydrated form of pyridine acting as template, enough energy would have to be supplied to remove this water from the pyridine during the process of formation of the antibody molecule.

**Combining Constants of Other Haptens with Anti-3AP Antibody.**—Further evidence concerning the specificity of anti-3AP antibody is provided by the $K'_0$ values for imidazole and pyrrolidine (Table I). The five-member imidazole ring with its two

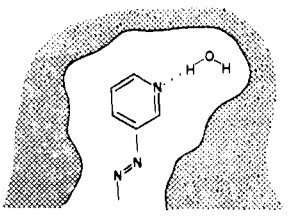

Fig. 2.—Sketch illustrating relative closeness of fit of anti-3AP antibody about the various positions of the 3-azopyridine group as derived from data presented here.

nitrogen atoms combines very weakly with the antibody ($K'_0$ = 0.027) as does the saturated five-member ring of pyrrolidine with its single nitrogen

(14) It is possible that the loose fit in the 3- and 4-positions in the anti-azobenzene system is due to steric interference by the succinamate group preventing close approach of the benzene ring to the antibody and that there is actually a tight fit around the 4-position of the homologous azobenzene hapten, in contrast to the loose fit observed in the pyridine system. However, the evidence discussed in footnote 13 argues such steric interference of the succinamate group.

atom ($K_0' = 0.013$). On the other hand, the combining constant of quinoline is somewhat higher than that of pyridine ($K_0' = 2.1$). This may be attributed to the large polarizability of quinoline and to the fact that a large part of the benzene ring of quinoline occupies the space *meta* to the annular nitrogen atom, corresponding to the location of the azo group of the immunizing antigen, and can therefore be accommodated by the antibody.

A sketch illustrating the relative closeness of fit of the anti-3AP antibody molecule about various parts of the 3-azopyridine haptenic group is shown in Fig. 2.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

# The Synthesis of Lysine-vasopressin[1]

BY VINCENT DU VIGNEAUD,[2] M. FREDERICK BARTLETT AND ALBERT JÖHL

RECEIVED JULY 17, 1957

The synthesis of an octapeptide amide with the structure proposed for lysine-vasopressin has been accomplished through the coupling of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (VIII) with S-benzyl-L-cysteinyl-L-prolyl-$N^{\epsilon}$-tosyl-L-lysylglycinamide in the presence of N,N'-dicyclohexylcarbodiimide to yield the protected nonapeptide amide, followed by reduction with sodium in liquid ammonia and oxidation. VIII was prepared by the coupling of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosine with L-phenylalanyl-L-glutaminyl-L-asparagine according to the mixed anhydride method. The synthetic octapeptide amide was purified by countercurrent distribution and electrophoresis. Comparison of the chemical, physical and biological behavior of the synthetic product with that of lysine-vasopressin isolated from hog posterior pituitary glands has led to the conclusion that the synthetic product is lysine-vasopressin.

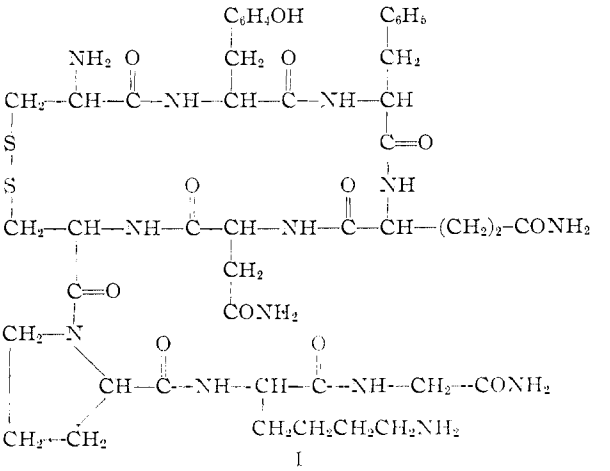
Popenoe, Lawler and du Vigneaud[3] reported the isolation, partial purification and amino acid content of lysine-vasopressin isolated from hog posterior pituitary glands. They found that hydrolysates of lysine-vasopressin contain phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, lysine and cystine in approximately equimolar quantities and ammonia in a molar ratio of 3 to any one amino acid. This differs from the amino acid content of arginine-vasopressin[4] in the replacement of arginine by lysine.

Degradative studies on arginine-vasopressin[5-7] led to the amino acid sequence

CyS-Tyr-Phe-Glu($NH_2$)-Asp($NH_2$)-CyS-Pro-Arg-Gly($NH_2$)

proposed by Popenoe, Lawler and du Vigneaud[3] and also by Acher and Chauvet.[8] The similarity in the results of enzymatic degradation of arginine-vasopressin and lysine-vasopressin and the similarity in the biological behavior of the two vasopressins led du Vigneaud, Lawler and Popenoe[5] to propose structure I for lysine-vasopressin.

The synthesis of an octapeptide having structure I was undertaken by du Vigneaud, Popenoe and Roeske[9] by a pathway which closely paralleled the synthesis of oxytocin.[10] This work led to biologically active material, but in contrast to the synthesis of oxytocin the yield of the active compound was insufficient to allow studies on purification.



I

Another approach to the synthesis of I was made in this Laboratory through the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (II)[11] with S-benzyl-L-cysteinyl-L-prolyl-$N^{\epsilon}$-tosyl-L-lysylglycinamide (III),[12] followed by removal of the protecting groups from the resulting nonapeptide derivative and oxidation to the disulfide octapeptide amide. A synthetic product which assayed approximately 100 pressor units per mg. after purification by electrophoresis and countercurrent distribution was finally obtained. In the course of this work it was observed that some decomposition occurred in the saponification of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine ethyl ester and a strong odor of

(1) A preliminary report of part of this work has appeared [M. F. Bartlett, A. Jöhl, R. Roeske, R. J. Stedman, F. H. C. Stewart, D. N. Ward and V. du Vigneaud, THIS JOURNAL, **78**, 2905 (1956)].

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(8) R. Acher and J. Chauvet, *ibid.*, **12**, 487 (1953).

(9) This approach was reported in a footnote to a communication by du Vigneaud, Lawler and Popenoe, see ref. 5.

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(12) R. Roeske, F. H. C. Stewart, R. J. Stedman and V. du Vigneaud, *ibid.*, **78**, 5883 (1956).