[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Reactions of Antiserum Homologous to the p-Azomaleanilate and p-Azofumaranilate Ion Groups[1]

BY MALCOLM SIEGEL AND DAVID PRESSMAN

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Antibodies against maleanilate and fumaranilate ions were prepared and their combination with simple haptens was studied. Although succinanilate ion is known to exist in a *cis* configuration specific precipitation of anti-maleanilate antibody by hapten homologous antigen was only slightly inhibited by succinanilate ion, indicating a poor fit of succinanilate ion in the hapten specific region of the antibody formed complementary to the maleanilate ion. This suggests that the fit of the antibody about the maleanilate ion is so close that there is a steric effect of the larger methylene groups of the succinanilate ion. Antibodies against the *trans* fumaranilate ion were readily formed and their specific precipitation was inhibited by succinanilate and other ions known to exist in a *cis* form in aqueous solution (*i.e.*, benzoylpropionate, phenylhydantoate, etc.). Apparently those ions which had previously been shown to exist in a *cis* form can also exist in a *trans* form, and thus combine with antibodies against fumaranilate ion.

It has been shown that antibodies specific to the succinanilate ion (anti-S_p-antibodies) appear to be formed complementary to the succinanilate group when the latter exists in a coiled *cis* configuration. The coiled configuration was first suggested by Landsteiner and van der Scheer[2] from their observation that maleate ion inhibited the specific precipitation of anti-S_p antibodies by the hapten homologous antigen while fumarate ion did not. Later, in a quantitative study, Pressman, Bryden and Pauling[3] showed that maleanilate ion (which is more closely analogous to succinanilate ion than is the maleate ion) combines relatively strongly with anti-S_p-antibodies while fumaranilate ion does not. From this the conclusion was drawn that succinanilate ion prefers the *cis* configuration, or that antibodies are more readily formed against the *cis* configuration.

More recently we have also shown that antibodies prepared against the β-benzoylpropionate ion[4] are also formed against that substance while it exists in a *cis* configuration. This was again demonstrated by the greater interaction of the antibodies with maleanilate ion than with fumaranilate.

In the study we are reporting here, antisera homologous to the p-azofumaranilate ion (anti-F_p sera) and antisera homologous to the p-azomaleanilate ion (anti-M_p sera) were prepared in order to show that it is possible to prepare antibodies which will reflect an extended *trans* configuration as well as a coiled *cis* configuration. A study was also made of the properties of these antibodies with respect to the structural features required in the hapten for appreciable combination with the antibody.

Experimental Methods

Materials.—Most of the simple substances used in this work have been described previously,[3,5] or were commercial preparations crystallized to the correct melting point and acidic equivalent weight. The acids prepared for this investigation are described below. p-Acetaminomaleanilic acid was prepared by adding a solution of 0.20 mole of p-aminoacetanilide in 400 ml. of warm dioxane to a warm solution of 0.20 mole of maleic anhydride in 100 ml. of dioxane. A yellow precipitate separated from the mother liquor (91% yield), which on recrystallization from methyl cellosolve melted at 218–218.5°. p-Aminomaleanilic acid was prepared (10% yield) by heating 8 g. of the acetyl derivative in 64 ml. of 1 *N* potassium hydroxide at 90° for 3 hours. The solution was cooled and adjusted to pH 3 with 10% HCl. A yellow precipitate appeared which was washed and suspended for 5 minutes in 50 ml. of 1% H_2SO_4, then quickly filtered. The filtrate was adjusted to pH 3. Pale yellow crystals separated in clusters (m.p. 133–134° dec.). p-Nitromaleanilic acid was prepared in 55% yield by heating a mixture of 0.2 mole of maleic anhydride in 100 ml. of ethyl ether and 0.2 mole of p-nitroaniline in 100 ml. of dioxane. The solid which slowly separated after one hour was filtered, dissolved in aqueous alkali, and precipitated with HCl; on recrystallization from alcohol, m.p. 198.5–199°.

Anal. Calcd. for $C_{10}H_8O_5N_2$: C, 50.9; H, 3.4; acidic equiv. wt., 236.2. Found: C, 50.5; H, 3.4; equiv. wt., 236.

p-Nitrofumaranilic acid was prepared in 30% yield by the dropwise addition of 0.1 mole of p-nitroaniline in 500 ml. of warm chloroform to 0.2 mole of fumaryl chloride in 50 ml. of chloroform. After mixing for 10 minutes the reaction mixture was extracted with aqueous alkali and the aqueous layer adjusted to pH <2. The precipitated acid was suspended in 400 ml. of boiling water and filtered hot. The product was recrystallized from ethanol; m.p. 266.5–267°.

Anal. Calcd. for $C_{10}H_8O_5N_2$: C, 50.9; H, 3.4; acidic equiv. wt., 236.2. Found: C, 50.9; H, 3.7; equiv. wt., 234.

p-Acetaminofumaranilic acid was prepared in 65% yield by the dropwise addition of 0.34 mole of p-aminoacetanilide in 2 l. of warm acetone to 0.66 mole of fumaryl chloride. A precipitate appeared immediately which was partially solubilized by the addition of 1 l. of water and sufficient sodium hydroxide solution to bring the pH to 8. The gummy residue was separated by centrifugation, acetone was removed from the supernate under reduced pressure, and the resulting alkaline aqueous solution acidified. The precipitate was recrystallized from acetic acid; m.p. 318–320°.

Anal. Calcd. for $C_{12}H_{12}O_4N_2$: C, 58.0; H, 4.8; acidic equiv. wt., 248.2. Found: C, 58.0; H, 5.1; equiv. wt., 250.

p-Aminofumaranilic acid was prepared by refluxing for three hours a solution of 0.09 mole of p-acetaminofumaranilic acid in 185 ml. of 1 *M* potassium hydroxide. The solution was cooled and adjusted to pH 3. The brown precipitate was redissolved in 0.2 *M* hydrochloric acid and reprecipitated as tan crystals by again adjusting to pH 3; m.p. 216–219° dec.

Protein Antigens.—The antigens for injection were prepared by coupling overnight at pH 9 and 5°, 0.001 mole of diazotized p-aminomaleanilic or p-aminofumaranilic acid with 50 ml. of regenerated lyophilized whole beef serum. The azoprotein was purified by dialysis for four days against saline–borate solution.

The test antigens were prepared by diazotizing and similarly coupling 25 mg. of p-aminomaleanilic or p-aminofumaranilic acid with 250 mg. of crystallized ovalbumin (com-

(1) This research was jointly supported by the Office of Naval Research and the U. S. Atomic Energy Commission.

(2) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **59**, 751 (1934).

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

(4) D. Pressman and M. Siegel, *ibid.*, **75**, 1376 (1953).

(5) D. Pressman, J. H. Bryden and L. Pauling, *ibid.*, **67**, 1219 (1945).

TABLE I

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-M_p SERUM WITH M_p-OVALBUMIN

Anti-M_p serum, 1.00 ml.; M_p-ovalbumin in borate buffer, 1.00 ml. (550 μg. protein); hapten in saline, 1.00 ml.; one hour at 37° and three days at 5°

Hapten concn., $M \times 10^5$	1.3	2.6	5.2	10.4	20.8	41.8	83.3	333			
Hapten	Amount of precipitate[a]								σ	K_0'[b]	ΔF_{rel}, cal.
Maleanilate		710		480		100			2.5	1.00	0
Fumaranilate								850			
p-Nitromaleanilate	600		410		90				2	9.7	−1300
Succinanilate					740		590	440	4	0.016	2300
p-Nitrosuccinanilate					660		440	160	3	.068	1500
Phenylhydantoate								590		.004	3000
β-Benzoylpropionate					900		820	690	2.5	.0032	3200

Hapten	333
Maleate	910
Succinate	960
Fumarate	970
Citraconate	890
Mesaconate	950
Valerate	900
Levulinate	900
Benzyl hydrogen succinate	790
Malonanilate	910
Gluteranilate	820
Adipanilate	890
d-Tartranilate	970
N-Methylsuccinanilate	850
Hydrocinnamate	830
γ-Phenylbutyrate	820
δ-Phenylvalerate	820
γ-Benzoylbutyrate	780
γ-Anilinobutyrate	950

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten; 174 μg. These values are averages of triplicate analyses with mean deviation of 2%. [b] The value of K_0' is corrected for albumin binding.

mercial product). These azoproteins were dialyzed for two days against saline–borate solution, three times precipitated at *p*H 3.6 from 50 ml. of solution, washed with 60% cold acetone, and finally dissolved in 50 ml. of saline at *p*H 8.

Preparation of Antisera.—The method for obtaining and pooling antisera has been described previously.[6]

Reaction of Antiserum with Antigen and Hapten.—Equal volumes of antiserum, antigen and hapten solution were mixed and allowed to stand about one hour at 37° and three to four days at 3–5°. The amount of antigen used was that which gave maximum precipitation in the absence of hapten. Hapten solutions were prepared with 0.9% sodium chloride solution, and the antigen solution with borate buffer, *p*H 8.0.[7] The precipitates were centrifuged, washed three times with 8-ml. portions of 0.9% sodium chloride solution and analyzed by a modification of the Folin–Ciocalteu method.[8]

Binding to Normal Serum Proteins.—10.0-ml. portions of a threefold borate buffer dilution of normal rabbit serum (19.2 mg. protein/ml. by Nessler analysis) were placed in dialysis bags and immersed in vials containing 10.0-ml. portions of a borate solution of hapten. The vials were rocked for 4 days to reach equilibrium. The concentration of the hapten both inside and outside the dialysis bag was determined by measurements of optical density. The percentage of the hapten inside the dialysis bag bound to the protein was calculated either from the difference in optical density of the hapten inside and outside the bag, or from the change in optical density of the outer phase.

Results and Discussion

The Formation of Anti-M_p and Anti-F_p Antibodies.—Both the anti-M_p sera and the anti-F_p sera gave specific precipitates with the homologous antigens, M_p-ovalbumin and F_p-ovalbumin, respectively. As in other antigen–antibody systems, the largest amount of precipitate was obtained with an optimum amount of antigen and less was obtained, at constant amounts of antibody, with either more or less of the antigen. Since antibodies can be formed against the *trans* structure (fumaranilate) as well as against the *cis* (maleanilate), the *cis* specificity of anti-succinanilate and anti-benzoylpropionate antibodies cannot be due to an inability of antibodies to be formed around the extended form. However, our experiments do not rule out the possibility that in the case of succinanilate and benzoylpropionate ions antibodies form preferentially around the *cis* configuration.

The Extent of Combination of Haptens with Antibody.—The extent of combination of haptens with antibody was determined by the ability of the hapten to inhibit the precipitation of the antibody with antigen. The amount of antigen used was that which gave the optimum amount of precipitate. The *p*H was kept at 8, since at this *p*H the carboxylic acids would be essentially completely dissociated and since several other systems have been investigated under the same conditions.

Data on the combination of hapten with antibody are given in Tables I and II. Values of the relative hapten inhibition constant K'_0, and the heterogeneity index σ, obtained on application of the theory of heterogeneous antisera,[9] and a value for the relative free energy change ΔF_{rel} accompanying the combination of hapten with antibody are also listed.[10] Since these experiments were carried out with whole sera, correction was made in the concentration of hapten depending on the extent of binding of the hapten to the serum proteins present.[11] The correction was made by using the concentration of free hapten present at the fiducial point[9] in place of the total hapten concentration. Most of the haptens used were at concentrations so high as to make binding to serum albumin a negligible factor (see ref. 11). In the case of the few haptens for which serum binding might be appreciable, binding was measured at two concentrations of hapten, one above and one below the fiducial concentration of the hapten in inhibiting the precipitation of anti-maleanilic or anti-fumaranilic

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

(7) D. Pressman, D. H. Brown and L. Pauling, *ibid.*, **64**, 3015 (1942).

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

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

(10) ΔF_{rel} is the difference between the free energy of combination with antibody of a particular hapten and the free energy of combination of the homologous furmaranilate or maleanilate hapten.

(11) D. Pressman and M. Siegel, THIS JOURNAL, **75**, 686 (1953).

TABLE II

EFFECT OF HAPTENS ON THE PRECIPITATION OF ANTI-FUMARANILATE SERUM WITH F_p-OVALBUMIN

Anti-F_p serum, 0.50 ml.; F_p-ovalbumin in borate buffer, 0.50 ml. (277 μg. protein); hapten in saline, 0.50 ml.; one hour at 37° and four days at 5°

Hapten concn., $M \times 10^5$	5.2	20.8	83.3	167	333	1330			
Hapten			Amount of precipitate[a]				σ	K_0'[c]	ΔF_{rel}, cal.
Fumaranilate[b]	930	820	350				2	1.00	0
Maleanilate					900				
p-Nitrofumaranilate	610	410	160				3	16	−1600
p-Acetaminofumaranilate	630	390	110				2.5	6.0	−1000
Succinanilate[b]			750		410	0	2	0.14	1100
p-Nitrosuccinanilate			380	170	110		3	1.3	−100
N-Methylsuccinanilate[b]					890	730	2.5	0.007	2700
Phenylhydantoate[b]					700	390	2	.030	1900
β-Benzoylpropionate[b]					840	550	2	.016	2300
γ-Benzoylbutyrate[b]					750	620	3	.012	2400
γ-Phenylbutyrate[b]					890	790	3	.0035	3200
δ-Phenylvalerate[b]					950	690	2	.008	2700
			333						
Malonanilate			920						
Glutaranilate			850						
Adipanilate			850						
d-Tartranilate			970						
Benzyl hydrogen succinate			1020						
Maleate			970						
Fumarate			930						
Succinate			990						
Levulinate			920						
Valerate			990						
Citraconate			970						
Mesaconate			1040						

[a] The amount of precipitate is reported in parts per mille of the amount present in the absence of hapten; 135 μg. These values are averages of triplicate analyses with mean deviation of 3%. [b] Inhibitions with these haptens were measured one week later on the same pool of antiserum. The amount of precipitate in the absence of hapten was 110 μg. [c] The value of K_0' is corrected for albumin binding.

antibody. The binding at the fiducial concentration was determined by linear interpolation between the above two points. Binding values appear in Table III.

TABLE III

BINDING OF HAPTENS AT FIDUCIAL CONCENTRATION TO RABBIT SERUM[a]

Hapten	Equil. concn. in protein phase, molar $\times 10^4$	Bound, %	Fiducial concn., molar $\times 10^4$	Calcd. % bound at fiducial concn.
Maleanilate	0.56	75	0.9	73
	1.1	72		
Succinanilate	7.6	29	18	21
	19	20	24	16
p-Nitrosuccinanilate	2.9	63	4.9	53
	7	43	5.4	50
p-Nitromaleanilate	0.25	90	0.26	90
	.58	86		
Fumaranilate	3.9	59	6	50
	6.6	48		
p-Nitrofumaranilate	0.57	87	1.1	82
	1.9	75		
p-Acetaminofumaranilate	0.48	63	1.1	54
	1.9	41		

[a] Threefold borate dilution of normal serum.

The Maleanilate System.—The anti-M_p antibodies fit very closely about the *cis* maleanilate ion, as would be expected. They did not combine with the *trans* fumaranilate ion under the conditions of the experiment, nor did they combine well with the succinanilate ion (as is shown by the increase of ΔF_{rel} of 2300 calories). Succinanilate is known to exist in a *cis* configuration. This lack of combination of the anti-M_p antibody with succinanilate ion suggests that the antibody fits so closely around the flat maleanilate group that it cannot accommodate the thicker methylene groups of the succinanilate ion. However, of all the heterologous substances tested, *i.e.*, those not directly derived from maleanilate ion, succinanilate combined to the greatest extent. Phenylhydantoate and benzoyl propionate, somewhat further removed from the maleanilate structure than is succinanilate, combine less, with ΔF_{rel} values of 3000 and 3200 cal., respectively. In phenylhydantoate a CH_2 of succinanilate is replaced by an NH group; in benzoylpropionate the NH group is omitted. All of the other structural changes other than substitution on the benzene ring resulted in changes in ΔF_{rel} of more than 3200 cal., the largest ΔF_{rel} which was measured with accuracy. The anilino-grouping is very important since replacing it in maleanilate by a negative oxygen to give maleate ion essentially eliminates all combination under the experimental conditions.

Substitution of a nitro group in the *para* position on the ring increases the combination of both maleanilate and succinanilate. The ΔF_{rel} values decrease 1300 and 800 cal., respectively. This increase is apparently due to the greater van der Waals interaction of the nitro group with the antibody region complementary to the azo group of the immunizing antigen.

The Fumaranilate System.—In this system also, any change in hapten structure other than a substitution in the benzene ring greatly decreased the combining power. As was to be expected, antibodies to this *trans* compound did not react with maleanilate ion. However, they did react to an appreciable extent with succinanilate ion. The ΔF_{rel} value was 1100 cal. The succinanilate ion can apparently exist in a *trans* configuration in aqueous solution, for it would have to exist in such a form to be able to combine with anti-fumaranilate antibody. It seems especially true in view of the fact that the combination cannot be attributed

to the side chain alone since fumarate ion which is very close in structure to the fumaranilate ion does not combine with anti-F_p antibody. The antibodies against the fumaranilate group must not be as closely fitting as those against maleanilate since they can accommodate the larger methylene groups of the succinanilate ion. Changes other than just saturation of the double bond to form succinanilate ion decrease the combining power still further. Other substances such as phenylhydantoate and benzoylpropionate which have been shown to exist in the coiled configuration[4] are also able to exist in an extended configuration since they combine with the anti-fumaranilate antibodies. The replacement of a CH_2 group of succinanilate by NH (in phenylhydantoate) decreases combination by increasing ΔF_{rel} by 800 cal. Placing a methyl group on the anilino NH (N-methylsuccinanilate) increases ΔF_{rel} by another 800 cal. Replacing the anilino group to give fumarate ion decreases the strength of combination by at least 3200 cal. The importance of the γ-carbonyl group is also shown by the fact that γ-phenylbutyrate ion (ΔF_{rel} 3200 cal.) combines to a lower extent than does the β-benzoylpropionate ion (ΔF_{rel} 2300 cal.).

The importance of the correct benzene-carboxyl distance is reflected by the fact that phenylvalerate combines more strongly (by 500 cal.) than does phenylbutyrate, which is one carbon short of the distance between the groups in the homologous hapten. The effect of substitution in the benzene ring in the *para* position (the position homologous to that for attachment of the immunizing hapten) is to increase the combining power. The presence of a nitro group in the *p*-position increases the extent of combination by decreasing the relative free energy by 1600 cal. in the case of *p*-nitrofumaranilate ion, and 1200 cal. in the case of *p*-nitrosuccinanilate. The acetamino group has a similar effect (1000 cal.) in the fumaranilate system.

The results of these experiments show that succinanilate, N-methylsuccinanilate, benzoylpropionate, and phenylhydantoate ion can exist in aqueous solution in the *trans* configuration (or readily assume that configuration) as well as in the *cis* configuration which is responsible for their combination with antibodies to succinanilate ion.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Effect of Temperature on the Reversible *p*H-dependent Denaturation of Horse Ferrihemoglobin[1]

BY ETHEL M. ZAISER AND JACINTO STEINHARDT

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Velocity and equilibrium constants for the acid denaturation of horse ferrihemoglobin have been reported previously for *p*H 3.1 to 4.6 at 25°. It was shown that denaturation as characterized by loss of solubility at the isoelectric point can also be followed quantitatively by measuring the absorption of light of 4060 Å. wave length, and that denaturation is accompanied by the all-or-nothing appearance in each molecule of 36 acid-binding groups. In new experiments the effect of temperature on the kinetics and equilibria is examined in formate buffers over the range 15 to 35°. A formulation of the equilibrium constant based on a proposed cyclic model of the reaction leads to an explanation of the observed zero heat of reaction as an over-all heat of a reaction comprising dissociation as well as denaturation and regeneration steps. Thus, the observed ΔH (zero between 15 and 25°) gives no direct information as to the heat of transformation of native protein into denatured protein in the same ionic state. The observed rates of approach to equilibrium are analyzed for the contributions of the opposing denaturation and regeneration reactions. According to the model, different rate-determining acid dissociations contribute to the effect of temperature on denaturation and regeneration rate constants. In the case of denaturation, the earlier identification of the "trigger" groups as carboxyls (which have negligible heats of dissociation) makes it possible to attribute all of the small energy of activation, 16.2 kcal./mole at 15 to 25°, to the transformation of native into denatured protein in the same state of ionization. The apparent energy of activation for regeneration, however, contains heats of dissociation which are not yet evaluated. Some differences in the effect of *p*H at 35° as compared with 15 or 25° are examined in the light of the reaction model.

Introduction

Consideration of the kinetics and equilibria observed in the reversible denaturation of horse ferrihemoglobin with acid[2] has led to the view that the combination of hydrogen ion with a small number (2 to 3) of trigger groups suffices to initiate the appearance (as a unit) in each molecule of 36 acid-binding groups which were formerly unreactive. This change occurs simultaneously with a loss of solubility at the isoelectric *p*H and a decrease in spectroscopic absorption at the 4060 Å. band. The 36 extra acid-binding groups combine quantitatively with hydrogen ion at the *p*H at which they are unmasked[3]; thus it appears likely that they are the ϵ-amino groups of the 36 lysine residues, or possibly, in part, guanidino groups of arginine. In unbuffered solutions these 36 basic groups combine with hydrogen ion at the expense of carboxylate and imidazole groups which have already been titrated, and the net effect actually appears to be an increase in carboxylate and imidazole groups. All information available as to the identity of the 2 to 3 trigger groups whose titration determines the rate of denaturation is consistent with the possibility that they are carboxylate groups.

This paper is concerned with the effect of temperature on the kinetics and equilibria of the denaturation reaction and provides further information about the reaction. For convenience, the spectro-

(1) A brief account of this work was presented at the meeting of the American Chemical Society at Chicago, Ill., in September, 1953.

(2) J. Steinhardt and E. M. Zaiser, THIS JOURNAL, **75**, 1599 (1953).

(3) E. M. Zaiser and J. Steinhardt, *ibid.*, **76**, 1788 (1954).