For **malachite green** (Fig. 11) no influence of varied concentration and only an extremely small, perhaps doubtful one, in the presence of agar, has been observed.

In **phenosafranin** (Fig. 12) no influence of concentration in aqueous solution can be seen. In agar the curve is slightly depressed but not displaced. The behavior of the "normal" color in nucleic acid is the same as for strongly metachromatic dyes.

Figure 13 shows the ultraviolet band of methylene blue is independent of concentration and of agar, within the limits of error.

Figure 14 shows that also in thionine the ultraviolet band depends very little on the concentration, if at all, and even on extreme variation of concentration neither the peak of absorption is displaced nor any secondary band is established.

Summary

Many basic dyestuffs are adsorbed by stainable substrates in two different shades of color, designated as the normal and the metachromatic color. As a model for a substrate stainable in the normal color, a 3% solution of nucleic acid at *p*H 4.6, is chosen; as a model for a substrate stainable in the metachromatic color a solution or gel of agar at *p*H 4.6 is chosen. These model substrates allow of spectrophotometric measurement of the absorption curve. It is shown that all dyestuffs capable of metachromasy disobey Beer's law in aqueous solution, due to the fact that with increasing concentration dimeric molecular aggregates of the dye molecules are formed. The absorption maximum of the dimer lies at shorter wave lengths than that of the monomer as it exists in extremely dilute solution. In the presence of agar still higher molecular aggregates are formed and it is these aggregates which are adsorbed by agar. The absorption bands of these high polymers are still further displaced toward shorter wave lengths and are more diffuse. In contrast, in the presence of nucleic acid the absorption spectrum of all basic dyes is independent of the concentration of the dye and is similar to, although not identical with, that of the dye in extremely dilute solution. No polymerization takes place. Each cation of the dye is combined with one acidic side chain of the nucleic acid to form a stoichiometrically well-defined salt-like compound. Very little is known about the correlation of the chemical structure of a dye with its faculty of polymerization. On the other hand, this faculty of polymerization is always correlated with the metachromatic effect.

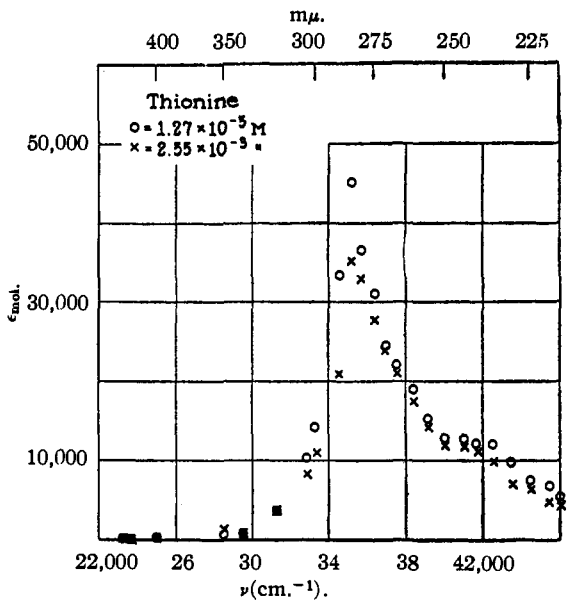

Fig. 14.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY NO. 1003]

The Serological Properties of Simple Substances. X. A Hapten Inhibition Experiment Substantiating the Intrinsic Molecular Asymmetry of Antibodies

BY DAVID PRESSMAN, JOHN H. BRYDEN, AND LINUS PAULING

It was shown by Landsteiner and van der Scheer[1] that by inoculating animals with suitable antigens antisera can be produced which can distinguish between optical isomers. Their first experiments were carried out with antisera prepared with use of immunizing antigens made by coupling proteins with diazotized *d*- and *l*-*p*-aminobenzoylphenylaminoacetic acid; each of the two antisera precipitated preferentially the test azoprotein containing the corresponding haptenic group, and the precipitation was inhibited preferentially by the corresponding hapten. Similar results were also obtained with azoproteins containing azo derivatives of the stereoisomeric tartranilic acids as haptenic groups.

These experimental results show the significance of spatial configuration in serological reactions. They do not, however, depend in any way on the fact that antibodies themselves have inherent optical activity. Because of the optical activity (*l*-configuration) of the amino acid residues of proteins, the possibility exists that an antiserum made by use of an antigen prepared from an optically inactive haptenic substance may combine preferentially with one of a pair of optically isomeric substances. We have prepared such an antiserum (anti-S_p serum), by injecting rabbits with an azoprotein made from the inactive

(1) K. Landsteiner and J. van der Scheer, *J. Exptl. Med.*, **48**, 315 (1928); **50**, 407 (1929).

substance *p*-aminosuccinanilic acid, and have found by hapten-inhibition experiments that the antibodies in the serum combine more strongly with *l*-N-(α-methylbenzyl)-succinamic acid than with the *d* isomer.

Experimental Methods

Protein Antigens.—The immunizing antigen was prepared by diazotizing 0.006 mole of *p*-aminosuccinanilic acid and coupling with 100 ml. of sheep serum at *p*H 9. The antigen was purified according to the directions of Landsteiner and van der Scheer.[1]

The test antigen (S_p-ovalbumin) was prepared by diazotizing 0.050 g. of *p*-aminosuccinanilic acid and coupling the product with 0.50 g. of ovalbumin at about *p*H 9. The azoprotein was dialyzed overnight against tap water, precipitated twice at *p*H 3.5 from 50 ml. of solution, and finally dissolved in saline solution at *p*H 7.

Antisera.—The method of preparing and pooling antisera was similar to that described previously for the preparation of anti-R_p sera.[2]

Reaction of Antiserum with Antigen and Hapten.—The reactants were mixed and permitted to stand for one hour at room temperature and over two nights at 5°. The precipitates were centrifuged and washed three times with 10-ml. portions of 0.9% sodium chloride solution and were analyzed by our standard method.[3]

Preparation of Substances

Succinanilic acid was prepared by the method of Auwers[4] by slowly adding 0.16 mole of aniline to a boiling solution of 0.15 mole of succinic anhydride in 150 ml. of chloroform. The reaction took place immediately with precipitation of succinanilic acid. The product was recrystallized from water, with a yield of 68%, m. p., 147.2–147.7°; reported 148.5°.[4] *Acidic equivalent weight:* calcd. for $C_{10}H_{11}O_3N$, 193.1; found, 191.4, 191.7.

***d*- and *l*-N-(α-methylbenzyl) succinamic acids** were prepared similarly using approximately 0.08 mole of *d*- or *l*-α-methylbenzylamine with an equivalent amount of succinic anhydride. It was necessary to extract the product from the chloroform solution with sodium hydroxide. Practically the theoretical quantity was used. The substituted succinamic acid was precipitated with hydrochloric acid and recrystallized from water. Yields were about 65%; m. p.: *d*-, 100.9–101.1°; *l*-, 100.9–101.2°. $[\alpha]^{25}D$ of sodium salt at *p*H 9 in water: *d*-, +96.7° (α, +2.14°, 2 dm., 11.09 g./liter); *l*-, −91.4° (α, −2.01°, 2 dm., 11.01 g./liter). $[\alpha]^{25}D$ of free acid in water: *d*-, +129.2° (α, +2.59°, 2 dm., 10.01 g./liter); *l*-, −127.0° (α, −2.54°, 2 dm., 10.00 g./liter). *Acidic equivalent weight:* calcd. for $C_{12}H_{15}O_3N$, 221.1; found, *d*-, 220.9, 221.5; *l*-, 221.6, 221.9.

The *d*- and *l*-α-methylbenzylamines were prepared from redistilled *dl*-α-methylbenzylamine (b. p. 186.0–186.6° uncor.) by the method of Lovén.[5] The *dl*-amine (0.434 mole) was added to 0.436 mole of *l*-malic acid in 235 ml. of water. *d*-α-Methylbenzylammonium *l*-malate crystallized out overnight with a crude yield of 94%. The mother liquor was made basic with sodium hydroxide and the amine extracted with ether. The amine was recovered by evaporation of the ether and was added to 0.22 mole of *d*-tartaric acid in 130 ml. of water. *l*-α-Methylbenzylammonium *d*-tartrate crystallized overnight with a crude yield of 61%. The crude salts were crystallized twice from 100 ml. of water. The *d*- and *l*-amines were recovered by liberation of the free amines by sodium hydroxide solution, extraction with ether, and subsequent fractional distillation. Yield was *d*-, 44%; *l*-, 38%; b. p.: *d*-, 185.5–186.0° uncor.; *l*-, 186.0–186.5° uncor.; D.: *d*-, $0.9475^{26.5}_4$, *l*-, $0.9466^{26.5}_4$; $[\alpha]^{26.5}D$: *d*-, +39.2° (α, +37.2°, 1 dm.); *l*-, −39.4° (α, −37.3°, 1 dm.); reported[6] $[\alpha]^{15}D$ *d*-, +40.16°.

The differences in the rotations of the salts (5%) and acids (2%) are undoubtedly due to the presence of the optical isomer. The presence of a non-optically active substance is doubtful since the method of preparation of *dl*-α-methylbenzylamine from formamide and acetophenone makes very improbable the presence of any other amine and the result of the equivalent weight determinations shows that succinic acid is not present.

***p*-Nitrosuccinanilic acid** was prepared by Dr. W. B. Renfrow by mixing hot solutions of 0.2 mole of succinic anhydride and 0.2 mole of *p*-nitroaniline in 50-ml. portions of dioxane and heating in a boiling water-bath for five minutes. The *p*-nitrosuccinanilic acid crystallized on cooling; m. p. 194–195°, reported 194–195°.[6]

***p*-Aminosuccinanilic acid** was prepared by Dr. W. B. Renfrow by reducing 0.08 mole of *p*-nitrosuccinanilic acid in 175 ml. of 90% methanol with hydrogen at 3 atm. over a catalyst of palladium on calcium carbonate. The aminosuccinanilic acid precipitated on cooling. It was dissolved in the theoretical amount of sodium hydroxide solution, filtered, and reprecipitated with the theoretical amount of hydrochloric acid; m. p. 184–185°, reported 183–184°.[6]

Discussion

The results of the hapten inhibition experiments are given in Table I and Fig. 1. The values 0.8 to 3.5 of the heterogeneity index σ obtained on application of the theory of heterogeneous antisera[7] to the data are similar in magnitude and in trend to those found for other systems. The values of the hapten inhibition constant K_0' (aside from the difference for the *d* and *l* haptens) are reasonable; succinanilic acid, which is very closely related in structure to the haptenic group of the immunizing antigen (the N-(*p*-azophenyl)-succinamic acid group), combines five to ten times more strongly with the antibody than the optically active haptens, and about fifty times more strongly than the simple hapten succinic acid.

Data were obtained for three different amounts of the precipitating antigen, S_p-ovalbumin, extending into the regions of antibody excess and antigen excess. It is interesting that in these experiments a given amount of hapten was found to be more effective in inhibiting precipitation the greater the amount of antigen; this corresponds to the simple theory of homogeneous antibody,[8] but is contrary to earlier work with another type of antiserum.[7]

The striking result of the experiments is the difference in inhibiting power shown by the *d* and *l* isomers of N-(α-methylbenzyl)-succinamic acid, amounting in the region of antibody excess to a factor in K_0' greater than 2, and to a difference of about 500 cal. per mole in the standard free energy of combination of hapten and antibody. This difference is presumably to be attributed to the presence of optically active amino acid residues in the antibody γ-globulin, causing the stable con-

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

(3) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **15**, 357 (1943).

(4) K. Auwers, *Ann.*, **309**, 326 (1899).

(5) J. M. Lovén, *J. prakt. Chem.*, **72**, 310 (1905).

(6) K. Landsteiner and J. van der Scheer *J. Exptl. Med.*, **56**, 399 (1932).

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

(8) L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, *ibid* **64**, 3003 (1942).

TABLE I

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

Antigen solution in borate buffer at *p*H 8, 1 ml.; antiserum, 1 ml.; hapten solution in saline, 1 ml.; *p*H of supernates 8.2.[a]

Amount of antigen, μg	Hapten[b]	K_0'	σ	Moles of hapten added, × 10^6 15.6	31.3	62.5	125	250	500	1000
				Amount of precipitate[c]						
35	*d*-I	0.070	3.5				845	720	657	577
	l-I	.167	3.5				724	594	540	435
110	*d*-I	.092	3.5		909	864	777	717	642	485
	l-I	.165	2.5		927	864	(791)	685	553	397
	II	1.00	1.5	883	785	652	439			
	III								864	799
440	*d*-I	0.17	3.0		903	(834)	(749)	656	552	394
	l-I	.19	2.0		887	(871)	786	702	543	363
	II	1.00	0.8	966	(877)	741	503			
	III								927	882

[a] Optimum precipitation 850 μg. at 220 μg. of antigen. [b] I = N-(α-Methylbenzyl)-succinamic acid; II = succinanilic acid; III = succinic acid. [c] The amounts of precipitate are in parts per mille of the amounts in the absence of hapten: 680, 660, and 239 μg. for 440, 110 and 35 μg. of antigen, respectively. Blanks of serum and buffer 8, 8, and 16 μg., respectively. Values are averages of triplicate analyses, with mean deviation ±2%, except for duplicate analyses in parentheses.

figurations complementary to the symmetric haptenic group of the immunizing antigen to be asymmetric. An alternative but less likely explanation could be based on the presence of optically active amino acid residues in the protein part of the immunizing antigen; this explanation is rendered improbable by the fact that the part of the haptenic group in the immunizing antigen which corresponds to the asymmetric carbon atom of the *d* and *l* haptens is separated from the asymmetric carbon atom of the amino acid residue by a considerable distance (the length of a benzene ring, azo group, and the amino acid side chain).

Our present knowledge of the structure of antibodies is not sufficiently detailed to permit an interpretation to be made of the observation that it is the *l* isomer which combines the more strongly with the antibody.

A reasonable explanation can be given of the observed dependence of the optical isomer effect on the amount of precipitating antigen and on the amount of hapten. The antiserum is heterogeneous—it contains antibodies which in configuration approximate only roughly to the haptenic group of the immunizing azoprotein and the precipitating azoprotein, and hence exert only weak attraction for these groups, and antibodies which fit very closely to the haptenic group, and form strong bonds with it. It is to be expected that weak antibodies, fitting the haptenic group only loosely, could be built in a great many different ways, of which some would combine more strongly with the *d* isomer of a pair of asymmetric haptens than with the *l* isomer, and others would combine more strongly with the *l* isomer than with the *d* isomer, and that these preferential effects would largely cancel each other. A very good antibody molecule, however, would have to assume a very well-defined configuration, bringing it into the closest approximation to the haptenic group and locating and orienting its charged groups and hydrogen-bond-forming groups in the most satisfactory way for attracting and holding the haptenic group; the nature of this most complementary configuration would be determined by the fact that the building stones are *l*-amino acid residues, and the configuration would accordingly be expected to correspond more closely to one than to the other of an enantiomorphic pair of asymmetric molecules.

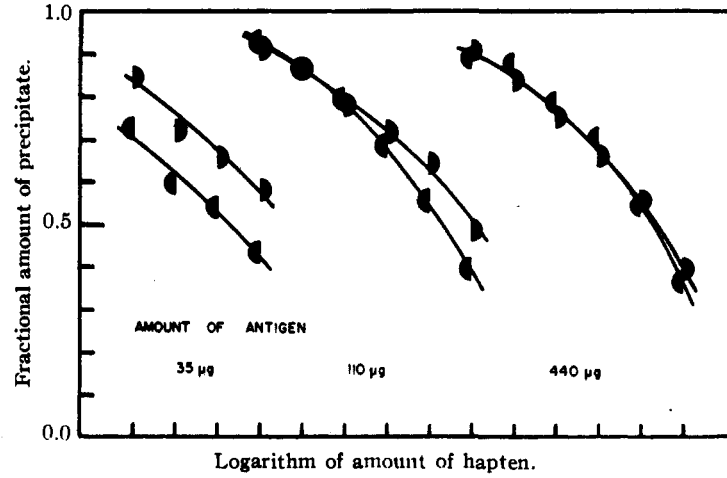

Fig. 1. Effect of *d*- and *l*-N-(α-methylbenzyl)-succinamic acid in inhibiting the precipitation of anti-S_p serum and S_p-ovalbumin; points for the *d* and *l* haptens, respectively, are represented by the right and left halves of circles.

When a small amount of antigen is added to a

portion of antiserum it is the antibody molecules with the greatest attraction for the antigen which form the precipitate with it. The argument given above leads to the expectation that the optical isomer effect should be larger in this case than when a large amount of precipitating antigen is used. This expectation is confirmed by the experimental results.

Moreover, in the region of the equivalence zone, with the precipitate containing poor antibody as well as good antibody, the part of the precipitate which is dissolved first on addition of hapten is that containing the poor antibody (with small combining power for the haptenic group of the immunizing antigen and precipitating antigen); the good antibody tends to remain in the undissolved precipitate. Accordingly, by the argument given above, the difference in effect of *d* and *l* haptens would be small at low hapten concentrations and larger at high hapten concentrations, at which a considerable fraction of the precipitate is dissolved. This effect, shown clearly by the middle pair of curves in Fig. 1, leads to a difference in apparent heterogeneity of the antiserum with respect to the isomers.

The small difference shown by the isomeric haptens in the region of slight antigen excess may be the result of the action of the excess of antigen in favoring the formation of soluble complexes involving good antibody molecules.

This investigation was carried out with the aid of a grant from The Rockefeller Foundation. We wish to thank Dr. W. B. Renfrow, Jr., and Mr. Dan Rice for assistance in analyses and the preparation of compounds.

Summary

An antiserum has been prepared by injecting rabbits with an azoprotein made by coupling sheep serum with diazotized *p*-aminosuccinanilic acid, the molecules of which have a plane of symmetry. It has been found that the *d* and *l* isomers of N-(α-methylbenzyl)-succinamic acid differ in their power to inhibit the precipitation of this antiserum by an azoprotein made by coupling ovalbumin with diazotized *p*-aminosuccinanilic acid, the *l* isomer having the greater inhibiting power. This behavior is presumably an effect of the presence of optically active amino acid residues in the antibody molecules.

PASADENA 4, CALIFORNIA RECEIVED APRIL 7, 1945

NOTES

A New Synthesis of 2-Diethylaminoethyl *p*-Aminothiolbenzoate

BY NOEL F. ALBERTSON AND R. O. CLINTON

2-Diethylaminoethyl *p*-aminothiolbenzoate (*Thiocaine*) was first prepared by Hansen and Fosdick,[1] and was shown to have anesthetic activity. Later work[2] indicated this activity to be from four to six times that of procaine hydrochloride, while the toxicity was not proportionately increased. The compound in the form of its hydrochloride was, however, relatively unstable in solution.[3] These facts suggested that a further investigation of its salts was desirable.

The simplest synthesis of this substance would involve reaction between 2-diethylaminoethanethiol and *p*-nitrobenzoyl chloride, followed by reduction of the thiol ester. An attempt to use this method was made by Lischer and Jordan,[4] but they were unable to prepare the thiol. A subsequent failure to prepare it also has been reported.[5] Both of these attempts were based upon the reaction of sodium hydrosulfide with 2-bromotriethylamine hydrobromide. In the present work we have been able to prepare the desired compound in about 40% yield by the reaction of alcoholic sodium hydrosulfide with 2-chlorotriethylamine. A more satisfactory procedure, however, involves the reaction of 2-chlorotriethylamine or 2-chlorotriethylamine hydrochloride with thiourea, followed by alkaline hydrolysis of the isothiouronium salt. The amine is less satisfactory than the hydrochloride, since the tertiary amino group is sufficiently basic to produce partial decomposition of the isothiouronium chloride with resultant loss of product.

Hansen and Fosdick[1] have reported a melting point of 52.0–52.5° for their base. In the present work material of this melting point was obtained in one experiment; however, all other preparations gave a base with melting point 75.0–75.5°. Seeding a melt of the low melting form with the high melting form produced a conversion to the latter. It is therefore evident that the compound exists in dimorphic forms.

A number of new salts of 2-diethylaminoethyl *p*-aminothiolbenzoate were prepared. Of these

(1) Hansen and Fosdick, THIS JOURNAL, **55**, 2872 (1933); U. S. Patent 2,090,756.

(2) Fosdick and Hansen, *J. Pharmacol.*, **50**, 323 (1934); Nolle, *Farm. i. Farmakol.* (U. S. S. R.), **1937**, No. 2, 1; (*Chem. Abstr.*, **34**, 3820 (1940)).

(3) Private communication from Dr. H. L. Hansen.

(4) Lischer and Jordan, THIS JOURNAL, **59**, 1623 (1937).

(5) Cook and Kreke, *ibid.*, **61**, 2971 (1939).