HAPTEN INHIBITION OF PRECIPITATION OF ANTISERA HOMOLOGOUS TO THE o-, m-, AND p-AZOBENZOIC ACID GROUPS

Thesis by

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INTRODUCTION

Landsteiner and his coworkers investigated the effect of position of substituents in the benzene ring of haptens and haptenic groups on the precipitation reactions of antisera and azoproteins and the inhibition of precipitation by haptens during their extensive studies of the properties of antisera prepared by injecting animals with artificial conjugated antigens containing groups of known chemical structure.[1] They found that combination of antiserum and antigen or hapten is usually decreased by the presence of substituent groups in the precipitating antigen or hapten in positions other than those occupied in the immunizing antigen. Similar results have been obtained in work by Pauling, Pressman and collaborators with the antisera homologous to the p-azophenylarsonic acid,[2,3,4] p-(p-azophenylazo)phenylarsonic acid,[3,4] p-azobenzoic acid,[5] and the o-, m-, and p-azophenylarsonic acid groups.[6] In order to learn more about the intermolecular forces operating in these serological systems and to learn more concerning the steric configuration of the antibody-hapten complex, this work was undertaken with the quantitative hapten inhibition studies of antisera homologous to the o-, m- and p-azobenzoic acids using haptens containing both the carboxyl and azo groupings. Previously hapten inhibition work was done with simple substances usually containing only one homologous charged grouping. This group is presumably held in the antibody-hapten complex by a charge on the antibody of the opposite sign, by hydrogen bonds and by van der Waals forces due to the intimacy and close proximity of this grouping to the antibody formed by an homologous immunizing antigen.[7] The remaining portions of the homologous

hapten are held in the antibody-hapten complex by hydrogen bonds and by van der Waals forces of attraction. The presence of a substituent, not homologous, causes steric hindrance effects. Previously various substituent groups were placed on the arsonic acid or the benzoic acid haptens and the steric effect of substitution on the combination of the antibody and haptens was discussed from the standpoint of radial dilatation required for such a hapten to fit into the antibody molecule.[6] These results were ambiguous as to showing exactly how the hapten might fit into the antibody in the cases where there were two orientations which the hapten could assume in combining with the antibody-(e.g. ortho substituted haptens could fit into meta antiserum in two ways, meta substituted haptens could fit into ortho antiserum in two ways, etc.).

To eliminate ambiguity as to how a hapten with a substituent group in a non-homologous position oriented itself in the antibody, the o-, m-, and p-(p-hydroxyphenylazo)chlorobenzoic acids were prepared and used as haptens in inhibiting the specific precipitation of antiserum specific to the o-, m-, and p-azobenzoic acid groups - hereafter called anti-Xo,-Xm, and Xp sera respectively. These acids have two groups(carboxyl and azo) homologous to the immunizing antigen which determine their orientation when combining with antibody prepared from antigen having the azo and carboxyl in the same positions. Hence these two groupings firmly attracted to the antibody by strong forces of a more specific nature anchor the hapten in a definite configuration in the complex precluding the possibility of the hapten orienting itself in more than one way with the antibody

molecule. With the hapten oriented in the antibody in a known way, the placement of a chloro group on the benzene ring in some known position and the use of the subsequent hapten in the inhibition studies would give a measure of the steric effect of the substituent on the combination of antibody and hapten. All the monochloromonoazobenzoic acids except 3-chloro-4-azobenzoic acid were prepared and their relative combining powers with the antibodies were determined. The results of the investigation are given in this thesis.

EXPERIMENTAL

SIMPLE HAPTENS: The benzoic acid used as hapten was a Merck product of acidic equivalent weight of 123.1 and a melting point(hereafter designated by M.P.) of 119.5-120°C. The o- and m-(p-hydroxyphenylazo)benzoic acids were prepared by Dr. A. Recsei and the p-(p-hydroxyphenylazo)benzoic acid was prepared by Mr. John Bryden by the coupling of diazotized o-, m-, and p-aminobenzoic acids with phenol. The preparation of the chloro substituted haptens is discussed later in this thesis.

PROTEIN ANTIGENS: The immunizing antigens used for inoculations were prepared by diazotizing 2 grams of o-, m- and p-aminobenzoic acid and coupling with 200 ml. of beef serum at pH about 9. The product was purified by the method of Landsteiner and van der Scheer.[8]

The azoprotein test antigens were prepared by diazotizing 0.2 grams of o-, m- and p-aminobenzoic acid and coupling the product to 3 grams of ovalbumin at pH about 9. The azoproteins were dialyzed against tap water overnight, precipitated twice at pH 3.5 and finally dissolved at pH 7 in saline.

ANTISERA: Antisera were prepared by injecting rabbits with beef serum coupled to the o-, m- and p-aminobenzoic acids. The method was similar to that described previously for the preparation of anti-phenylarsonic acid sera.[9] The sera were pooled according to titer. A single pool of each antiserum was used for these experiments.

ANTIGEN-ANTIBODY REACTION OPTIMUM: For these inhibition experiments it was desired to find the concentrations of the antiserum and antigen that would give the optimum(that is the greatest) amount of precipitate when they were mixed. Therefore preliminary tests were set up for the precipitation of anti-Xo, -Xm and -Xp sera with Xo-, Xm- and Xp-ovalbumin antigen solution. To 1 ml. of antiserum and 1 ml. of borate buffer(giving supernates of pH 8.0) was added 1 ml. of antigen solution(in saline) of different concentrations. The mixture stood two hours at 37°C and over two nights in the refrigerator. Cross reactions were: The anti-Xo serum gave appreciable precipitate with Xm-ovalbumin, but none with the Xp-ovalbumin. The anti-Xm serum gave very slight precipitates with Xo-ovalbumin and none with the Xp-ovalbumin. The anti-Xp-serum gave appreciable precipitate with the Xm-ovalbumin and none with the Xo-ovalbumin. In table I below the optimums observed for each antiserum and antigen are listed.

TABLE I

Antigen	Antiserum	Amount of antigen added in µg						
		4.7	9.4	18.8	37.5	75	150	300
		Amount of precipitate in µg[a]						
Xo-ovalbumin	anti-Xo	11	40	119	199	315	216	76
Xm-ovalbumin	anti-Xm	43	102	176	199	165	87	41
Xp-ovalbumin	anti-Xp	64	117	245	314	363	363	152

a. Values are averages of triplicate analyses with a mean deviation of ± 4% and are corrected for blanks of serum and buffer - 12, 12 and 10 µg for the Xo-, Xm- and Xp- systems respectively.

REACTION OF ANTISERUM WITH ANTIGEN AND HAPTEN: The hapten, antigen and antiserum were mixed in the proper concentrations(using 1 ml. of each) and after shaking were allowed to stand at 37°C in a water bath for two hours and then at 5°C over two nights in a refrigerator.

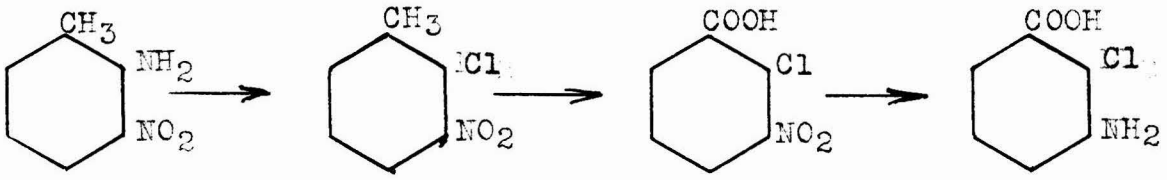
The precipitates formed were centrifuged and washed three times with 10 ml. portions of saline(0.9% sodium chloride) solution and were then analyzed by the standard method.[10]

PROTEIN ANALYSIS: From a burette 2.5 ml. of 1.00 N sodium hydroxide solution were added to the precipitate after the wash solution had been decanted off. The precipitate dissolved and the solution was transferred to a 15 ml. graduated centrifuge tube using water to wash. The total volume now was about 6 ml. The tubes were heated for 8 minutes in a boiling water bath and then cooled for 5 minutes in a cold water bath. The volume was then made up to 7.5 ml. using distilled water.

To each tube was added rapidly 2.5 ml. of the Folin reagent(prepared as described by Folin and Ciocalteu[11] and diluted with two volumes of water) making the total volume 10 ml. The tubes were shaken immediately using rubber stoppers and after allowing to stand just 10 minutes(in order to secure the maximum blue color caused by the Folin reagent and the protein antibody), the color intensity which is a measure of the amount of antibody present was read with a Klett-Summerson photoelectric colorimeter using a red filter transmitting 640-700 mμ. The amount of protein in μg(micrograms) was obtained by converting the Klett reading by a linear relation.

PREPARATION OF SUBSTANCES: The haptens that were used in these experiments were prepared by the coupling of phenol with the diazotized chloroaminobenzoic acids. There are ten monochloro-monoaminobenzoic acids all of which were prepared except the 3-chloro-4-aminobenzoic acid. Their preparations are described below. All observed melting points are corrected for exposed stem.

2-Chloro-3-aminobenzoic acid



The starting material obtained commercially was 2-amino-3-nitrotoluene. For the diazotization 80 grams(0.51 mols) of it were suspended in 77 ml. of conc. hydrochloric acid and 157 ml. of water.[12] An additional 137 ml. of conc. hydrochloric acid were added. The mixture was cooled to 2°C in an ice bath and 32 grams(0.51 mols) of sodium nitrite dissolved in 40 ml. of water were added dropwise with vigorous stirring over a period of several hours. After 4 hours at 2°C with vigorous stirring and with a slight excess of nitrite present(tested for by the starch-iodide test), the material was filtered to remove any unreacted 2-amino-3-nitrotoluene. The filtrate was carefully added to a solution of cuprous chloride in water and conc. hydrochloric acid. (The cuprous chloride solution was made by dissolving 160 grams(0.63 mols) of crystallized copper sulfate and 54 grams(0.93 mols) of sodium chloride in 575 ml. of water. The cuprous chloride was precipitated when 26 grams

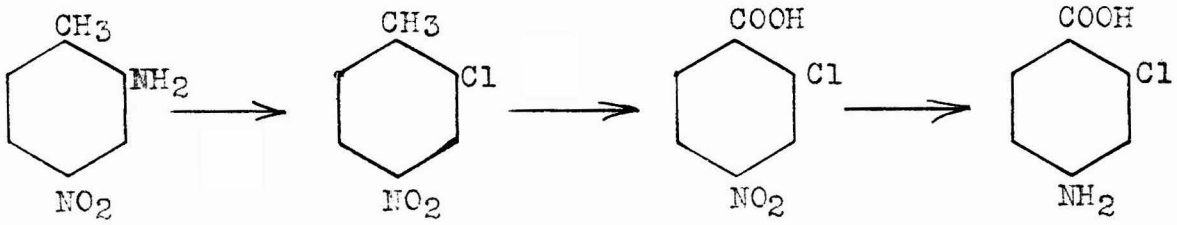
(0.22 mols) of sodium bisulfite dissolved in a little water were added. The cuprous chloride was centrifuged, washed and dissolved to form $CuCl_2^-$ in 260 ml. of water and 200 ml. of conc. hydrochloric acid.[12]) Nitrogen was evolved and a heavy oil was formed when the diazonium salt decomposed in the $CuCl_2^-$ solution. The oil was steam distilled to give pure 2-chloro-3-nitrotoluene. A yield of 70 grams(85%) was obtained. M.P.= 21.5°C; reported = 21.5°C[13]

The 2-chloro-3-nitrotoluene was oxidized to 2-chloro-3-nitrobenzoic acid by refluxing with permanganate.[14] A mixture of 25 grams(0.146 mols) of 2-chloro-3-nitrotoluene and 54 grams (0.34 mols) of potassium permanganate in 1 liter of water was refluxed for 5 hours on an oil bath with vigorous stirring till all the permanganate was converted into manganese dioxide. From the original 25 grams, 9 grams of 2-chloro-3-nitrotoluene were recovered by steam distillation. The manganese dioxide formed was filtered off and washed with 500 ml. of hot water. The hot combined filtrates were acidified with 6N hydrochloric acid to pH 2. The 2-chloro-3-nitrobenzoic acid crystallized out upon the cooling of the solution. The acid was recrystallized from water. A yield of 10 grams(55% on the basis of the 2-chloro-3-nitrotoluene not recovered) was obtained. M.P. = 183°C;reported = 185°C [15] acidic equivalent weight ≠ calculated for $C_7H_4O_4NCl$ = 201.5; found = 205.8 and 206.2.

The 2-chloro-3-nitrobenzoic acid was reduced to 2-chloro-3-aminobenzoic acid by a low pressure catalytic hydrogenation using platinum oxide as the catalyst. In 100 ml. of 96% ethanol

26.7 grams(0.133 mols) of the acid were dissolved and 0.1 gram of the catalyst was added. A drop of 33.8 pounds pressure(0.423 mols) of hydrogen compared to the calculated 0.399 mols was observed. The alcohol solution was carefully evaporated to a smaller volume and needles of 2-chloro-3-aminobenzoic acid crystallized out of the cooled solution. The needles were recrystallized from water to give 13 grams(60% yield).M.P.= 156-158°C; reported = 158°C [16] Acidic equivalent weight calculated for $C_7H_6O_2NCl$= 171.5; found = 170.2 and 172.5.

2-Chloro-4-aminobenzoic acid



The starting material obtained commercially was 2-amino-4-nitrotoluene of which 80 grams(0.53 mols) were suspended in 70 ml. of conc. hydrochloric acid and 145 ml. of water.[12] An additional 145 ml. of conc. hydrochloric acid were added with vigorous stirring. The mixture was cooled to 2°C and 33.6(0.53 mols) of sodium nitrite dissolved in a little water were added dropwise over a period of several hours. The material was kept cold throughout and was filtered. The filtrate was added cautiously to a solution of cuprous chloride in water and conc. hydrochloric acid. The cuprous chloride was prepared as described previously starting with 168 grams(0.591 mols) of crystallized copper sulfate and 57 grams(0.97 mols) of sodium chloride. With the copious evolution of nitrogen, a reddish oil was formed from which 80 grams(85% yield) of semi-pure 2-chloro-4-nitro-

toluene were steam distilled. M.P. = 57.6°C; reported = 65°C.[17]

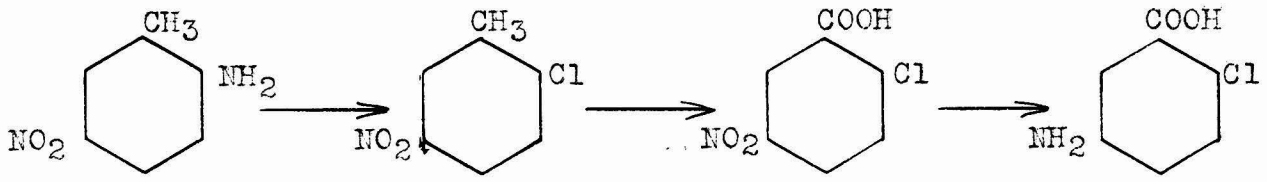
The 2-chloro-4-nitrotoluene was oxidized to 2-chloro-4-nitrobenzoic acid by refluxing with permanganate.[14] The mixture of 25 grams(0.146 mols) of 2-chloro-4-nitrotoluene, 2 grams of potassium hydroxide and 54 grams(0.34 mols) of potassium permanganate in 1 liter of water was refluxed on an oil bath with vigorous stirring for 2 1/2 hours till all the permanganate was converted into manganese dioxide. From the 25 grams of starting material, 13 grams were recovered by steam distillation. The manganese dioxide formed was filtered and washed with 500 ml. of hot water. The combined filtrates were acidified to pH 2 by the addition of 6N hydrochloric acid and were evaporated to a small volume from which the 2-chloro-4-nitrobenzoic acid crystallized out. The yield was 5.5 grams(40% on the basis of the 2-chloro-4-nitrotoluene not recovered). M.P. = 140.9°C; reported = 140-142°C. [18] Acidic equivalent weight calculated for $C_7H_4O_4NCl$ = 201.5; found = 204.8 and 205.7.

The yield was not spectacular so some variations in procedure were tried. The oxidation using potassium permanganate was tried using 4 liters of water instead of 1 liter of water as the medium in the hope of getting more of the"toluene" in contact with the permanganate solution decreasing the time of oxidation. The yield was 7.5 grams compared to the 5.5 grams secured previously. However the increase in yield did not warrant the greater inconvenience in handling of large volumes. The use of carbon dioxide as a buffer against the hydroxide formed by adding small pieces of dry ice to the refluxing mixture was unsuccessful due to the too rapid evolution of carbon

dioxide in the flask. The use of magnesium sulfate as a buffer (0.146 mols for the above procedure) gave unsatisfactory results (75% of the starting material was recovered and a yield of 4 grams of the desired acid was obtained). Another modification involved the oxidation of the compound with permanganate exactly as described in the first procedure with double quantities for all reactants, but at the conclusion of the oxidation half as much more permanganate was added without separating the product, starting material or the manganese dioxide and the oxidation was continued. The yield was 10 grams. The yields were not spectacularly changed by the different variations in procedure.

The 2-chloro-4-nitrobenzoic acid was reduced to the 2-chloro-4-aminobenzoic acid by a low pressure catalytic hydrogenation using 0.1 grams of platinum oxide as catalyst. In 100 ml. of 95% ethanol were dissolved 22.7 grams (0.113 mols) of the acid. A pressure drop of 26.8 pounds (0.335 mols) of hydrogen compared to the 0.339 mols calculated was observed. The alcohol solution was evaporated to dryness on a water bath and the residue was recrystallized from water and dilute alcohol. A little material insoluble in water and difficultly soluble in alcohol was also formed. By a neutralization equivalent of 530.3 it was believed to be a trimer of the desired chloroaminobenzoic acid. A yield of 10 grams (53%) of the 2-chloro-4-aminobenzoic acid was obtained. M.P. = 212-213°C; reported = 213°C [19] Acidic equivalent weight calculated for $C_7H_6O_2NCl$ = 171.5; found = 171.1 and 173.2.

6-Chloro-3-aminobenzoic acid



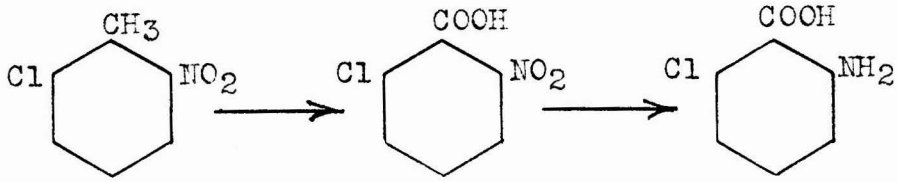
The starting material was 2-amino-5-nitrotoluene obtained commercially of which 100 grams(0.66 mols) were suspended in 82 ml. of conc. hydrochloric acid and 170 ml. of water.[12] An additional 170 ml. of conc. hydrochloric acid were added with vigorous stirring. The mixture was cooled to 2°C and 45 grams(0.66 mols) of sodium nitrite dissolved in 115 ml. of water were added dropwise over a period of several hours. The cold solution was then filtered to remove unreacted starting material and the filtrate was carefully added to a solution of cuprous chloride in conc. hydrochloric acid and water with the evolution of nitrogen and the formation of a heavy oil. The cuprous chloride solution prepared as described previously starting with 200 grams(0.79 mols) of crystallized copper sulfate and 67 grams(1.15 mols) of sodium chloride. The oil was steam distilled to give 93 grams(93% yield) of semi-pure 2-chloro-5-nitrotoluene. M.P. = 40°C; reported = 44°C. [20]

The 2-chloro-5-nitrotoluene was oxidized to 2-chloro-5-nitrobenzoic acid by refluxing 100 grams in 25 gram portions with permanganate.[14] The mixture of 25 grams(0.146 mols) of 2-chloro-5-nitrotoluene, 20 grams(0.36 mols) of potassium hydroxide and 54 grams(0.34 mols) of potassium permanganate in 1 liter of water was refluxed for several hours till all the permanganate was converted to manganese dioxide. By steam

distillation 15 grams of the starting material were recovered. The manganese dioxide formed was filtered and washed; the combined filtrates were acidified to pH 2 and the 2-chloro-5-nitrobenzoic acid crystallized out upon cooling of the solution. An overall yield of 19 grams(43%) of the acid was obtained. Two samples with different equivalent weights were secured. M.P.= 162-164°C(no. 1); 163-165°C(no. 2); reported = 165°C [21] Acidic equivalent weight calculated for $C_7H_4O_4NCl$ = 201.5; found=191.8(no. 1) and 206.9 and 204.9(no. 2).

The 2-chloro-5-nitrobenzoic acid was reduced by low pressure catalytic hydrogenation to 6-chloro-3-aminobenzoic acid using 0.1 gram of platinum oxide as catalyst. In 100 ml. of 96% ethanol were dissolved 12 grams(0.0595 mols) of the 2-chloro-5-nitrobenzoic acid(largely that of the lower M.P. and lower equivalent weight). The alcohol solution was heated prior to the absorption of the hydrogen to facilitate the reaction. An absorption of 16.5 pounds (pressure drop) (0.206 mols) of hydrogen compared to the 0.179 mols calculated was observed. The alcohol solution was partially evaporated and allowed to cool. From this solution a precipitate separated believed at first to be the desired product, but it gave a M.P. of above 250°C and an equivalent weight of 110. The filtrate was then carefully evaporated almost to dryness to give a residue which was recrystallized from a minimum of water to give 2 grams(20% yield) of 6-chloro-3-aminobenzoic acid. M.P. = 186-187°C; reported = 185°C [22] Acidic equivalent weight calculated for $C_7H_6O_2NCl$ = 171.5; found = 167.8 and 178.0. (M.P. reported = 188-188.5°C [23] also).

6-Chloro-2-aminobenzoic acid



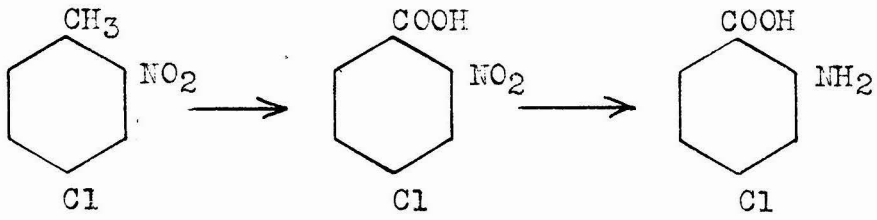
The 6-chloro-2-nitrotoluene obtained commercially was oxidized to 6-chloro-2-nitrobenzoic acid by refluxing with permanganate.[14] A mixture of 25 grams(0.146 mols) of 6-chloro-2-nitrotoluene and 54 grams(0.34 mols) of potassium permanganate was refluxed on an oil bath with vigorous stirring for several hours till all the permanganate was converted to manganese dioxide. By steam distillation 15 grams of the starting material were recovered. The manganese dioxide was filtered and washed, and the combined filtrates were acidified with 6N hydrochloric acid to pH 2. Upon cooling of the solution 5 grams(42% yield) of 6-chloro-2-nitrobenzoic acid were obtained. M.P.= 161.6-163.6°C.

A variation in procedure was tried. In one flask was placed in addition 25 grams(0.45 mols) of potassium hydroxide to facilitate the reaction. The time needed for the oxidation with the hydroxide was half that without it(4 hours were cut to 2 hours). The yield was improved from 46%(for the flask without the hydroxide) to 58%(for the flask with it). The acid obtained was treated with norite and gave a M.P. of 161-162°C; reported = 161°C [24] Acidic equivalent weight calculated for $C_7H_4O_4NCl$ = 201.5; found = 205.2 and 206.2.

The 6-chloro-2-nitrobenzoic acid was reduced by catalytic low pressure hydrogenation to 6-chloro-2-aminobenzoic acid

using 0.12 gram of platinum oxide as catalyst. In 90 ml. of 96% ethanol were dissolved 28 grams(0.14 mols) of 6-chloro-2-nitrobenzoic acid. The solution was heated prior to the absorption of the hydrogen to facilitate the reduction. An absorption of 34.5 pounds (pressure drop) (0.43 mols) of hydrogen compared to the 0.42 mols calculated was observed. The alcohol solution was evaporated almost to dryness on a water bath and the residue(M.P. = 135°C) was recrystallized from benzene to give 7 grams(29%) yield of 6-chloro-2-aminobenzoic acid. M.P. = 146-147°C; reported = 146-147°C [25] Acidic equivalent weight calculated for $C_7H_6O_2NCl$ = 171.5; found = 168.1 and 168.4.

4-Chloro-2-aminobenzoic acid

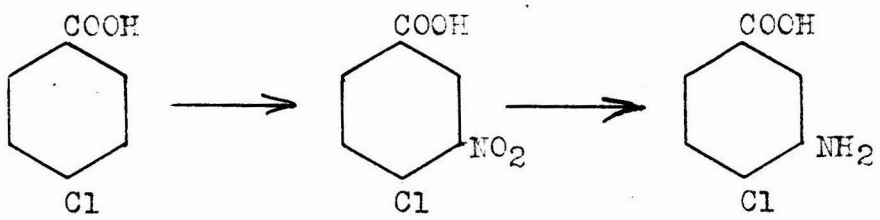


The 4-chloro-2-nitrotoluene obtained commercially was oxidized to 4-chloro-2-nitrobenzoic acid by refluxing with permanganate.[14] A mixture of 25 grams(0.146 mols) of 4-chloro-2-nitrotoluene and 54 grams(0.34 mols) of potassium permanganate in 1 liter of water was refluxed with vigorous stirring on an oil bath till all the permanganate was converted to manganese dioxide. By steam distillation 14 grams of the starting material were recovered. The manganese dioxide was filtered and washed; the combined filtrates were acidified to pH 2 with 6N hydrochloric acid. Upon cooling 6 grams(46% yield) of 4-chloro-2-nitrobenzoic acid crystallized out. M.P. = 141-143°C; reported = 140-143°C [26]

The procedure above was carried out using 50 grams of 4-chloro-2-nitrotoluene, 108 grams of potassium permanganate and 5 grams of potassium hydroxide. The yield was only 27% with 24 grams of the starting material being recovered. Acidic equivalent weight calculated for $C_7H_4O_4NCl$= 201.5; found = 203.1 and 203.9.

The 4-chloro-2-nitrobenzoic acid was reduced to 4-chloro-2-aminobenzoic acid by low pressure catalytic hydrogenation using 0.1 gram of platinum oxide as catalyst. In 100 ml. of 96% ethanol were dissolved 26 grams(0.129 mols) of 4-chloro-2-nitrobenzoic acid. The solution was heated prior to the absorption of the hydrogen to facilitate the reaction. An absorption of 28.8 pounds (pressure drop) (0.361 mols) of hydrogen compared to the 0.387 mols calculated was observed. A precipitate separated out on cooling which was recrystallized from dilute alcohol to give 12 grams(55% yield) of 4-chloro-2-aminobenzoic acid. M.P. = 234.5°C; reported = 235-236°C [27] Acidic equivalent weight calculated for $C_7H_6O_2NCl$ = 171.5; found 169.9 and 170.1.

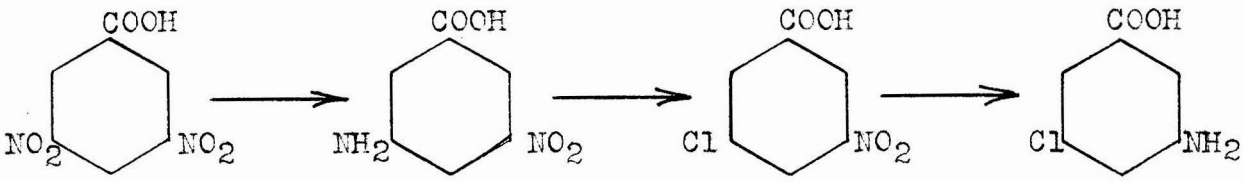
4-Chloro-3-aminobenzoic acid



The 4-chloro-3-aminobenzoic acid was synthesized by Mr. Dan Rice by the nitration of p-chlorobenzoic acid with fuming nitric acid.[28] The 4-chloro-3-nitrobenzoic acid was recrystallized from aqueous alcohol. It was then reduced by catalytic

low pressure hydrogenation to 4-chloro-3-aminobenzoic acid using platinum oxide as catalyst. Acidic equivalent weight calculated for $C_7H_6O_2NCl$(the latter compound) = 171.5; found = 173.2 and 175.0.

5-Chloro-3-aminobenzoic acid



The starting material obtained commercially was 3,5-dinitrobenzoic acid of which 100 grams(0.47 mols) were dissolved in an excess of ammonia(giving a brick-red colored solution) and boiled.[29] To the boiling solution was added via a capillary tube hydrogen sulfide for 20 minutes. The solution was heated to expel hydrogen sulfide and ammonia and then evaporated almost to dryness on a hot plate and completely to dryness on a water bath. The residue was taken up in water and the sulfur removed by filtration. The filtrate was then acidified to pH 3 with the 5-amino-3-nitrobenzoic acid crystallizing out on cooling. Evaporation of the filtrate gave more of the product. The acid was recrystallized from hot water. A yield of 60 grams(66%) was obtained. If hydrogen sulfide is passed in for over 20 minutes(or less for less material), appreciable quantities of undesirable sulfur and other products accumulate which complicate the isolation of the desired acid.M.P. = 205-207°C; reported = 208°C [29]

The 5-amino-3-nitrobenzoic acid was diazotised by dis-

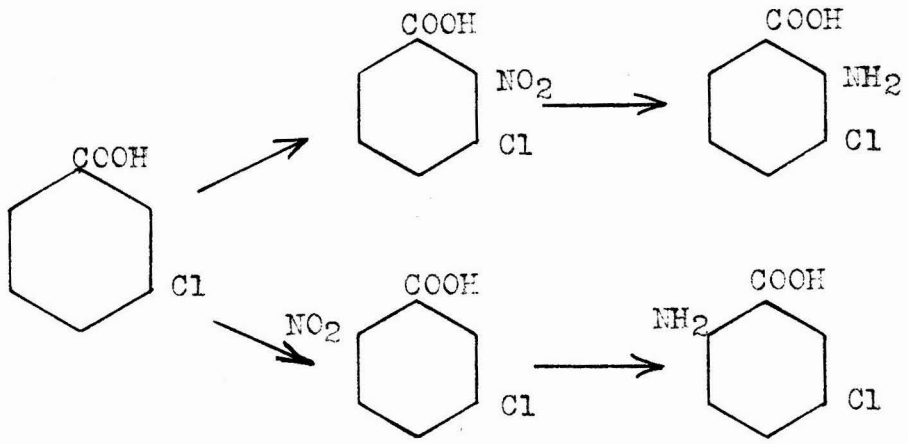
solving 60 grams(0.33 mols) of it in 170 ml. of 2N sodium hydroxide(0.33 mols) for the inverted diazotization method.[12] This solution was added along with 27 grams(0.39 mols) of sodium nitrite to 125 ml. of 12N hydrochloric acid slowly keeping the temperature below 10°C. The material was kept cold and was vigorously stirred for 2 hours. Excess nitrite was present at the end of the 2 hours(tested for by the starch-iodide test). The material was filtered to remove unreacted starting material and the filtrate was carefully added to a solution of cuprous chloride in 125 ml. of conc. hydrochloric acid and 170 ml. of water. The cuprous chloride was made as previously described starting with 105.3 grams (0.42 mols) of crystallized copper sulfate and 34.5 grams (0.60 mols) of sodium chloride. Copious quantities of nitrogen were evolved and a reddish material formed. The material was heated to decompose any remaining diazonium salt("R" acid was used to test for the presence of diazonium salt). The precipitate was filtered and had a M.P. of 140-141°C. It was recrystallized from water. A yield of 15 grams(23%) was obtained. M.P.= 145.9-146.9°C; reported = 147°C [30] Acidic equivalent weight calculated for $C_7H_4O_4NCl$ = 201.5; found = 201.1 and 203.4.

The diazotization of the 5-amino-3-nitrobenzoic acid did not proceed too readily. The isolation of the resulting chloro substituted acid was rendered more difficult as it was not volatile in steam. The procedure of diazotizing the 5-amino-3-nitrobenzoic acid by forming the diazonium salt and then boiling in conc. hydrochloric acid to secure the 5-chloro-3-nitrobenzoic acid was unsuccessful.[30]

Oils and resins were obtained preventing the isolation of the desired compound.

The 5-chloro-3-nitrobenzoic acid was reduced by a low pressure catalytic hydrogenation using 0.1 gram of platinum oxide as catalyst. In 100 ml. of 96% ethanol were dissolved 7.5 grams(0.037 mols) of 5-chloro-3-nitrobenzoic acid. The solution was heated prior to the absorption of the hydrogen to facilitate the reaction. An absorption of 7.5 pounds (pressure drop) (0.094 mols) of hydrogen compared to the 0.111 mols calculated was observed. The alcohol solution was partially evaporated and a precipitate was formed upon the addition of a little water. A yield of 5 grams(78%) of the 5-chloro-3-aminobenzoic acid was obtained. The M.P. of the acid obtained was over a wide range-- darkening at 215°C and decomposing at 230°C. reported M.P. = 216°C [30] Acidic equivalent weight calculated for $C_7H_6O_2NCl$ = 171.5; found = 172.8.

3-Chloro-2-aminobenzoic acid and
5-Chloro-2-aminobenzoic acid



The starting material obtained commercially was m-chlorobenzoic acid of which 20 grams(0.127 mols) were dissolved in 100 ml. of fuming nitric acid(sp.gr. = 1.49-1.50).[31] This was

just sufficient to dissolve the m-chlorobenzoic acid. The solution was heated for 10 minutes and was then diluted with 2 liters of water. The solution(with a little sandy precipitate in it) was evaporated to dryness to give a residue of M.P. = 128-130°C(which indicated a mixture of the 3-chloro-2-nitro and 5-chloro-2-nitrobenzoic acids from known melting points). The residue was taken up in a small amount of water to form an aqueous solution and an oil. The oil was allowed to solidify under the aqueous phase - which was then decanted off. Crystals that crystallized out from the aqueous phase on cooling had a M.P. of 132.7-134.7°C(still a mixture).

The solidified oil was extracted two times with 100 ml. portions of hot water. An insoluble portion of M.P. = 232.5-234°C was obtained. This portion was chiefly the 3-chloro-2-nitrobenzoic acid.

Nitrations were carried out at 70°C and at -20°C, but the proportions of the two isomers obtained were not noticeably altered.

At 70°C 60 grams(0.381 mols) of m-chlorobenzoic acid were nitrated by dissolving in 300 ml. of fuming nitric acid. After 10 minutes the solution was poured into 2 liters of water. A precipitate(found later to be chiefly the 3-chloro-2-nitrobenzoic acid) settled out and was filtered off. The filtrate was evaporated to dryness and the residue was extracted with successive portions of hot water ~~w~~ till all the 5-chloro-2-nitrobenzoic acid dissolved. Upon cooling the acid crystallized out with a M.P. of 130°C. The acid was then recrystallized

from hot benzene. The yield was 50 grams. M.P. = 136-138°C; reported = 137-138°C [31] Acidic equivalent weight calculated for $C_7H_4O_4NCl$ = 201.5; found = 205.1 and 206.1.

The impure 3-chloro-2-nitrobenzoic acids secured above were treated with hot benzene to remove the 5-chloro-2-nitrobenzoic acid. The 3-chloro-2-nitrobenzoic acid remaining behind was recrystallized from a minimum of hot water and alcohol. The yield was 8 grams. M.P. = 236.3-238.3°C; reported = 235°C [31] Acidic equivalent weight calculated for $C_7H_4O_4NCl$ = 201.5; found = 202.8 and 203.3.

The overall yield of the two isomers was 47% with the ratio of the 5-chloro to the 3-chloro as about 8 to 1.

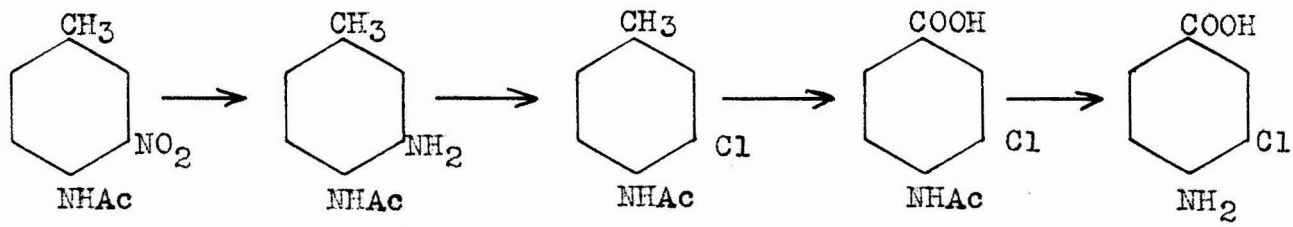
The 5-chloro-2-nitrobenzoic acid was reduced by low pressure catalytic hydrogenation to 5-chloro-2-aminobenzoic acid using 0.14 gram of platinum oxide as catalyst. In 120 ml. of 96% ethanol were dissolved 40 grams(0.199 mols) of the 5-chloro-2-aminobenzoic acid. The solution was heated prior to the absorption of the hydrogen to facilitate the reaction. An absorption of 49 pounds (pressure drop) (0.612 mols) of hydrogen compared to the 0.597 mols calculated was observed. The 5-chloro-2-aminobenzoic acid separated out on cooling of the solution. The acid was recrystallized from alcohol. The alcohol solution gave a striking violet fluorescence to the glass walls of the flask in the sunlight as reported by Eller and Klemm.[32] A yield of 21 grams (62%) was obtained. M.P. = 204-205°C(softening at 180-190°C); reported = 204°C [33] Acidic equivalent weight calculated for $C_7H_6O_2NCl$ = 171.5; found = 165.3 and 165.5.

The 3-chloro-2-nitrobenzoic acid was reduced by low pressure catalytic hydrogenation to 3-chloro-2-aminobenzoic acid using 0.1 gram of platinum oxide as catalyst. In 50 ml. of 96% ethanol were dissolved 6 grams(0.0298 mols) of the 3-chloro-2-nitrobenzoic acid. The solution was heated prior to the absorption of the hydrogen to facilitate the reaction. An absorption (pressure drop) of 7 pounds(0.0874 mols) of hydrogen compared to the 0.0894 mols calculated was observed. The alcohol solution was partially evaporated and the 3-chloro-2-aminobenzoic acid crystallized out on cooling. The acid gave a remarkable violet fluorescence to the glass wall of the flask. A yield of 4 grams (78%) was obtained. M.P. = 189-190.5°C A mixture of this compound and the 5-chloro-2-nitrobenzoic acid had a mixed melting point of 155-160°C. Acidic equivalent weight calculated for $C_7H_6O_2NCl$= 171.5; found = 171.6

Hübner and Weiss[34] prepared a mixture of the two isomers above. A M.P. of 136°C was observed for the nitro acid mixture and a M.P. of 148°C for the amino acid mixture. They did not consider their compounds to be mixtures. Later however, Hübner[31] and Ulrich separated the two nitro, but not the two amino, isomers.

Attempted preparations of 3-Chloro-4-aminobenzoic acid

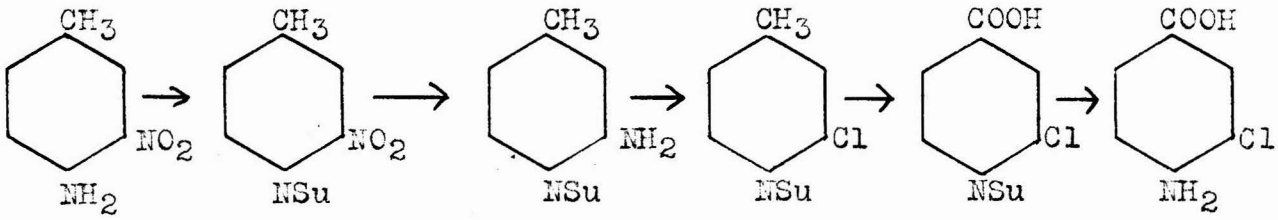
method I (Ac = $-COCH_3$)



The reduction of 4-acetylamino-3-nitrotoluene obtained commercially to 4-acetylamino-3-aminotoluene in 77% yield by

low pressure catalytic hydrogenation went easily. M.P. = 130-131°C; reported = 130-131°C [35] The diazotization of the 4-acetylamino-3-aminotoluene to form 4-acetylamino-3-chlorotoluene was unsuccessful as the diazonium salt formed coupled internally to form 1-acetyl-5-methyl-bentriazole of M.P. = 130.2°C; reported = 130.5°C [35] This method therefore had to be abandoned. (Su = $-COCH_2CH_2CO-$ as ring)

method II

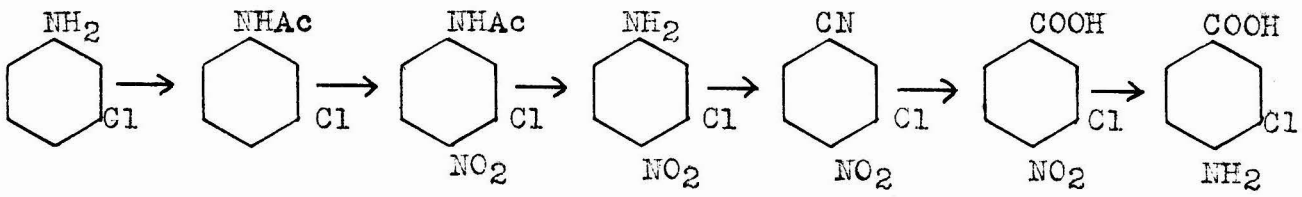


The succination of the 4-amino-3-nitrotoluene obtained commercially by fusing equal mols with succinic acid went smoothly. The molten mass was cooled and then taken up in 96% ethanol. Yellow crystals of N-(4-methyl-2-nitrophenyl)-succinimide separated out on cooling. M.P. = 136.8-139.8°C.

This material was reduced by catalytic hydrogenation to N-(4-methyl-2-aminophenyl)-succinimide of M.P. = 164-166°C easily. Treatment with mild sodium hydroxide in its purification clove the succinimide ring to give presumedly N-(4-methyl-2-aminophenyl)-succinamic acid as the compound obtained exhibited acid characteristics. Therefore this method, too, was dropped.

method III

(see next page for formulae)



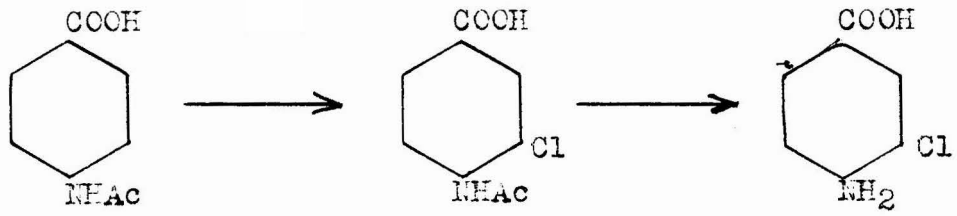
The m-chloroaniline obtained commercially was easily acetylated by refluxing with glacial acetic acid and acetic anhydride to form m-chloroacetanilide. M.P. = 75-77°C; reported = 72.5°C [36]

The nitration of the m-chloroacetanilide was carried out in glacial acetic and sulfuric acids with fuming nitric acid at temperatures below 15°C. The mixture stood over night and was then poured onto cracked ice and filtered. The mixture of the 4-nitro-3-chloroacetanilide and the 6-nitro-3-chloroacetanilide was hydrolyzed by boiling 1/2 hour with conc. hydrochloric acid. The reddish-yellow solution was neutralized in an ice bath with ammonia with the precipitate formed taken up in a minimum of 96% ethanol. The 4-nitro-3-chloroaniline crystallized out first leaving the other undesired isomer behind: (the two isomers may also be separated by steam distilling off the 6-nitro-3-chloroaniline). M.P. of the 4-nitro-3-chloroaniline = 153.2-154.7°C; reported = 156-157°C [37]

The diazotization of this 4-nitro-3-chloroaniline and the addition of the diazonium salt formed to cuprous cyanide solution resulted in the formation of a resinous mass from which the 4-nitro-3-chlorobenzoic acid was not obtained after the hydrolysis of the intermediary nitrile with sulfuric acid.[38] More exact work with much larger quantities of the 4-nitro-3-

chloroaniline might result in the securing of the desired product.

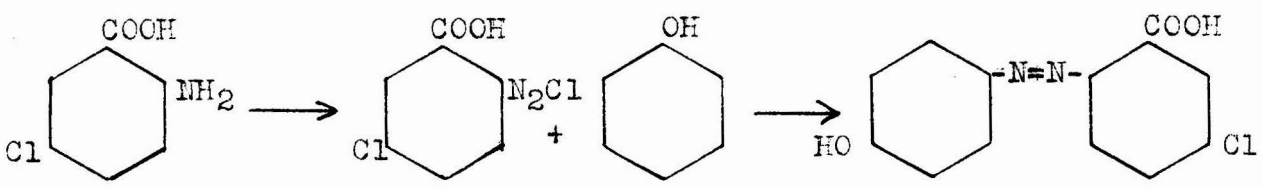
method IV



Partial success was attained by this method, but the 4-amino-3-chlorobenzoic acid was not isolated. The p-acetylaminobenzoic acid obtained commercially was dissolved in glacial acetic acid and a large excess of HTH(high test hypochlorite-calcium hypochlorite) was added slowly to the solution. The solution was allowed to stand overnight with vigorous stirring. Crystals of M.P. = 243.5-245°C crystallized out and gave a positive halogen test upon elementary analysis(no chloride ion as salt was present in the unknown as the solution gave no precipitate with silver nitrate). The equivalent weight of the compound was 186 indicating about 20% halogenation of the p-acetylaminobenzoic acid. The chlorination was repeated followed by hydrolysis with conc. hydrochloric acid. The amino acids present were not separated. Further work on this mixture may result in the isolation of the desired 4-amino-3-chlorobenzoic acid.

Azophenol chlorobenzoic acids

example:



The chloroaminobenzoic acids were diazotized and coupled to phenol to produce the azophenols. In the preparation of the azophenols the chloroaminobenzoic acids were dissolved in sodium hydroxide solution. To this solution were added hydrochloric acid and a little ice to form a finely divided precipitate and to keep the temperature low. Slowly sodium nitrite dissolved in a little water was added to diazotize the amino group. The excess nitrite at the end of the diazotization(tested for by the starch-iodide test) was eliminated by the addition of 10% sulfamic acid. The diazonium salt was filtered to remove unreacted chloroaminobenzoic acid.

A solution of ten times the theoretical amount of phenol and sufficient sodium carbonate dissolved in water was cooled with ice. The carbonate neutralized the hydrochloric acid present in the diazonium salt solution and brought the pH to 9 when the diazonium salt solution was added to the phenol-carbonate solution to form a reddish-orange dye. The reaction was carried out at pH 9 so as to keep the phenol dissolved as the phenoxide ion. After all the diazonium salt was added, the solution was acidified to pH 7 with 6N hydrochloric acid. The free phenol formed was extracted with ether. The aqueous phase was boiled to eliminate any residual ether and was then acidified to pH 2 with the precipitation of the colored dye. The dye was filtered off and recrystallized from aqueous alcohol. The quantities used for the different amino acids and the yields obtained are given in table II below.

Table II

Hapten Prepared	Hapten Number	Amino acid used	grams of acid used	ml. of 2N NaOH	ml. of 6N HCl	grams $NaNO_2$	grams phenol	grams Na_2CO_3	Yield
3-Cl-2-azo	3	3-Chloro-2-amino	3	15	15	1.2	16.5	33	3 gms- 62%
4-Cl-2-azo	4	4-Chloro-2-amino	4	20	20	1.62	22	44	3 gms-47%
5-Cl-2-azo	5	5-Chloro-2-amino	2	10	10	0.81	11	22	2 gms-62%
6-Cl-2-azo	6	6-Chloro-2-amino	3	15	15	1.2	16.5	33	4 gms-82%
2-Cl-3-azo	8	2-Chloro-3-amino	4	20	20	1.62	22	44	6 gms-93%
4-Cl-3-azo	9	4-Chloro-3-amino	4	20	20	1.62	22	44	3 gms-47%
5-Cl-3-azo[a]	10	5-Chloro-3-amino	2	10	10	0.81	11	22	0.5 gms-16%
6-Cl-3-azo	11	6-Chloro-3-amino	2	10	10	0.81	11	22	2 gms-62%
2-Cl-4-azo	13	2-Chloro-4-amino	3	15	15	1.2	16.5	33	3 gms-62%
a. inverted method was used with hapten 10									
5-Cl-3-azo	10	5-Chloro-3-amino	2	5	20	0.81	11	22	0.5 gms-16%

general footnotes

All went smoothly with the exception of 4 which was slower to diazotize and 10 which formed a gel upon the addition of the conc. hydrochloric acid(6N). Even with the inverted method the diazotization was quite poor for 10.

A diaz otization using butyl nitrite in 96% ethanol was tried on 10. In 20 ml. of 96% ethanol were dissolved 2 grams(0.0117 mols) of 5-chloro-3-aminobenzoic acid. The solution was cooled to 5oC after the addition of 3 grams of conc. sulfuric acid. To this solution was added dropwise 1.4 grams(0.014 mols) of butyl nitrite. The nitrite was taken up as shown by its disappearance when tested for by the starch-iodide test. With an excess of nitrite present at the conclusion of the diazotization, the solution was poured into 250 ml. of water and ice. The precipitate which formed was filtered off(the precipitate didm't appear to be diazonium salt by testing with "H" acid). Sulfamic acid was added to eliminate the excess nitrite and the diazonium salt solution was poured into the phenol-carbonate solution as above. The dye(0.5 grams= 16% yield) was treated with norite.

DISCUSSION

The hapten-inhibition data in Table III were interpreted according to the heterogenity theory[4] based on the assumption that the heterogenity of antisera can be described by an error-function distribution in free energy of interaction of antibody and hapten in competition with antigen. Application of this theory leads to the evaluation of two constants for each antibody-hapten-antigen system: one of these constants K_o' is an average hapten inhibition constant representing the average bond strength of antibody and hapten relative to that of antibody and antigen; the other constant σ (sigma) is the index of the effective heterogenity of the antiserum.

The reference point for K_o' has been ascribed as $K_o' = 1.00$ for benzoic acid as hapten. The values of K_o' range from 5.80 to less than 0.04 for the haptens studied.

The values of K_o' for the unsubstituted azohapten homologous to the immunizing antigen are the largest by far as is to be expected due to the very intimate manner in which the unsubstituted homologous hapten can fit into the antibody molecule. The addition of a chloro group into the benzene ring of the hapten brings about steric hindrance resulting in the decreasing of the stability of the antibody-hapten complex as evidenced by the lower values of K_o'.

The dependence of the inhibition constant with the anti-Xo serum on the position of the chloro group in the benzene ring for the 2-azo haptens is $6 > 3 = 5 > 4$. The value for benzoic acid is intermediary between the 6 and 3 values of K_o'. These values show that the placement of a chloro group in position 6 has the least effect on decreasing the stability of the antibody-

Table III

Effect of Haptens on the Precipitation of Anti-Xo, Anti-Xm, and Anti-Xp Serum with Xo-, Xm- and Xp-ovalbumin Respectively.

Antigen solution in borate buffer at pH 8.0, 1 ml.(90 ug of Xo- and Xp-ovalbumin, 33 ug of Xm-ovalbumin); antiserum, 1 ml; hapten solution in saline, 1 ml; pH of supernates = 8.0 - 8.1.

			Moles of Hapten added x 10^8								
			3.9	7.8	15.6	31.3	62.5	125	250	500	1000
			Anti-Xo serum and Xo-ovalbumin								
Hapten[a]	K'_o	σ[b]	Amount of precipitate[c]								
1	1.00	2.5			830		600		320		
2	5.2	2.5	790		560		260				
3	0.98	2			860		630		290		
4	0.46	2		970		900		650			
5	0.96	2			910		630		290		
6	1.75	2			(780)		490		180		
7	0.36	2					790		520		220
8	0.30	2.5					860		560		300
9	0.12	2.5					950		740		480
10	0.24	2					900		630		290
11	0.44	2				910		660		370	
12	0.03	(2)							920	870	760
13							970	940	860		

Table III(cont)

Hapten[a]	K'_o	σ [b]	3.9	7.8	15.6	31.3	62.5	125	250	500	1000
			Anti-Xm serum and Xm-ovalbumin								
1	1.00	3.5				670		480		280	
2	0.40	3					760		490		280
3	0.48	5					630		470		330
4	0.41	3			930		720		510		
5	0.14	3							710	520	460
6	0.13	3							720	580	460
7	2.9	2.5			660		400		50		
8	0.90	4				670		520		280	
9	0.63	3				770		570		290	
10	0.68	2.5				870		(540)		270	
11	0.46	2.5				950		660		350	
12	0.093	2.5							760	690	500
13	0.13	(2.5)					1030	(950)	(730)		
			Anti-Xp serum and Xp-ovalbumin								
1	1.00	1.5							770	630	410
2									920	830	700
3									810	840	860
4							1000	970	880		
5									1010	890	830
6									1040	(910)	850
7	1.79	1.5					950		660		250
8	1.18	2.5					870		680		400
9	1.52	2					1010		660		300
10	0.73	1.5					1060		910		500
11	0.58	(1.5)				970		900		750	
12	5.8	1.5			920		710		300		
13	2.1	2			960		830		580		

Footnotes for Table III

a. Haptens used were:

1 = Benzoic acid

2 = o-(p-Hydroxyphenylazo)benzoic acid

3 = 3-Chloro-2-(p-hydroxyphenylazo)benzoic acid

4 = 4-Chloro-2-(p-hydroxyphenylazo)benzoic acid

5 = 5-Chloro-2-(p-hydroxyphenylazo)benzoic acid

6 = 6-Chloro-2-(p-hydroxyphenylazo)benzoic acid

7 = m-(p-Hydroxyphenylazo)benzoic acid

8 = 2-Chloro-3-(p-hydroxyphenylazo)benzoic acid

9 = 4-Chloro-3-(p-hydroxyphenylazo)benzoic acid

10 = 5-Chloro-3-(p-hydroxyphenylazo)benzoic acid

11 = 6-Chloro-3-(p-hydroxyphenylazo)benzoic acid

12 = p-(p-Hydroxyphenylazo)benzoic acid

13 = 2-Chloro-4-(p-hydroxyphenylazo)benzoic acid

b. Values of o in parentheses are assumed.

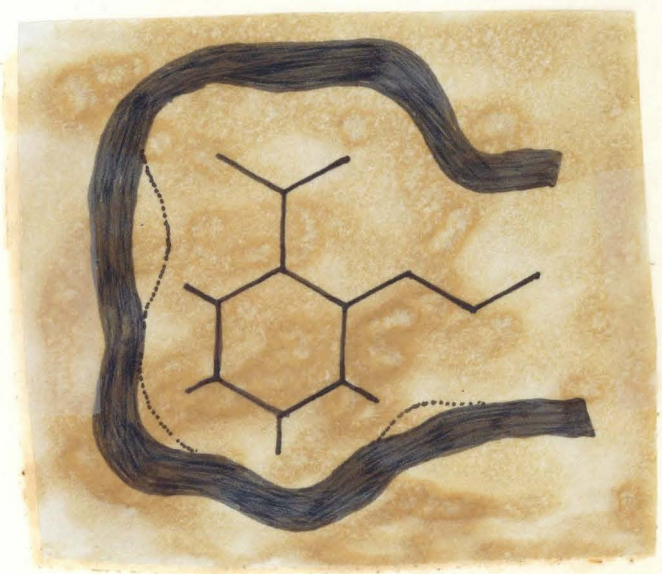
c. The amounts of precipitate are in parts per mille of the amounts in the absence of hapten, 437, 173, and 403 μg. for Xo-, Xm- and Xp- systems respectively. Blanks of serum and buffer 10, 6, and 9 μg. respectively were obtained. Values are averages of triplicate analyses with a mean deviation of ± 2.5%, except for duplicate analyses in the parentheses.

hapten complex. This clearly indicates that the antibody does not approach the hapten as closely at position 6 as at the other positions since this position is adjacent to the carboxyl group. The carboxyl group's large size does not permit the antibody to approach the benzene ring closely at the 6 position. Therefore the chloro group can fit somewhat into the small non-specific hole in the antibody at that position and thus is not as effective sterically.

The placement of the chloro group in either the 3 or 5 position causes slightly less inhibition than benzoic acid itself. The antibody appears to be formed more closely around the 3 and 5 positions(a little less so around the 3 position as it is adjacent to the 2-azo opening) than around position 6. The values of $K_o^!$ are a relative measure of the closeness of fit of the antibody to the hapten.

The placement of a chloro group in position 4 causes the greatest steric hindrance thus indicating the closest fit of the anti-Xo antibody for the 2-azo haptens at that position.

A model of the antibody-hapten complex showing the closeness of fit is postulated and ascribed below from the experimental data obtained.



(Dotted lines show normal van der Waals radii)

The dependence of the inhibition constant with the anti-Xm serum on the position of the chloro group in the benzene ring for the 3-azo haptens is 2 > 5 = 4 > 6. The benzoic acid causes greater inhibition than any of the 3-azo haptens except the non-substituted 3-azo hapten. The placement of the chloro group in position 2 between the azo and carboxyl groups causes the least lowering of the inhibition as there the chloro group is relatively small compared to the two large groups adjacent to it on both sides. The antibody itself does not fold down between the azo and carboxyl groups to approach the hapten at position 2. Therefore there is a non-specific hole in the antibody into which the chloro group in position 2 can fit without causing appreciable steric hindrance.

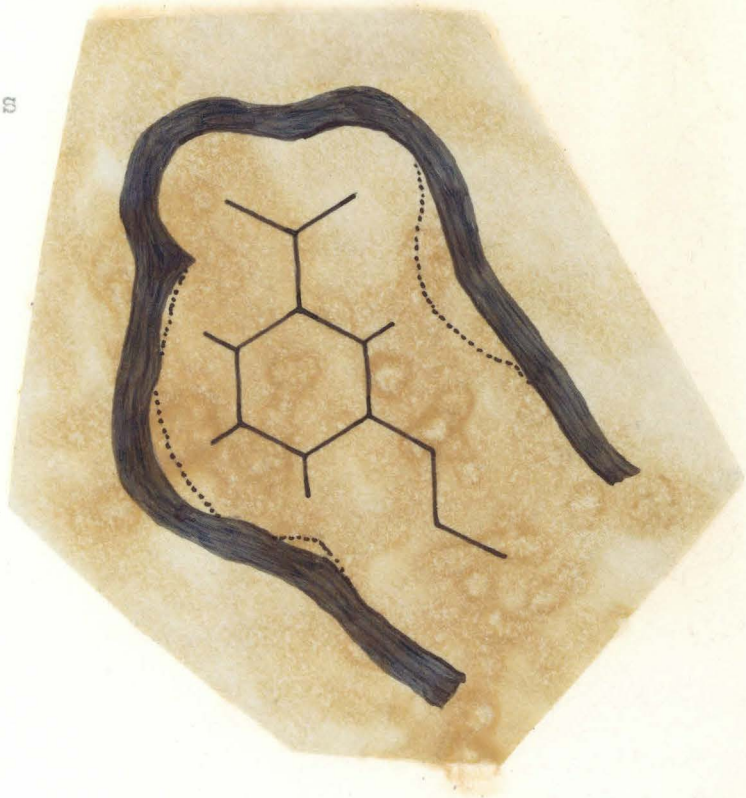
The substitution of a chloro group at position 6, however, causes great steric hindrance. This indicates that the antibody approaches the benzene ring at position 6 rather specifically inspite of the fact that it is adjacent to the large carboxyl group and thus differs from the anti-Xo antibody in that respect.

The placement of the chloro group in the 4 or 5 position causes less inhibition than in position 2 and more than in position 6. With a chlorine in position 5 the hapten molecule can orient itself by shifting both the carboxyl and azo groups. The carboxyl shifts toward the non-specific hole at position 2 and the azo groups slides further out of the 3-azo opening in the antibody. This allows the hapten with the chloro group at position 5 to assume a stable position(the whole hapten being shifted over a small distance). There is probably a small non-specific hole

at position 4 since it is adjacent to the large 3-azo group.

A model of the antibody-hapten complex showing the closeness of fit is postulated and ascribed from the experimental data obtained.

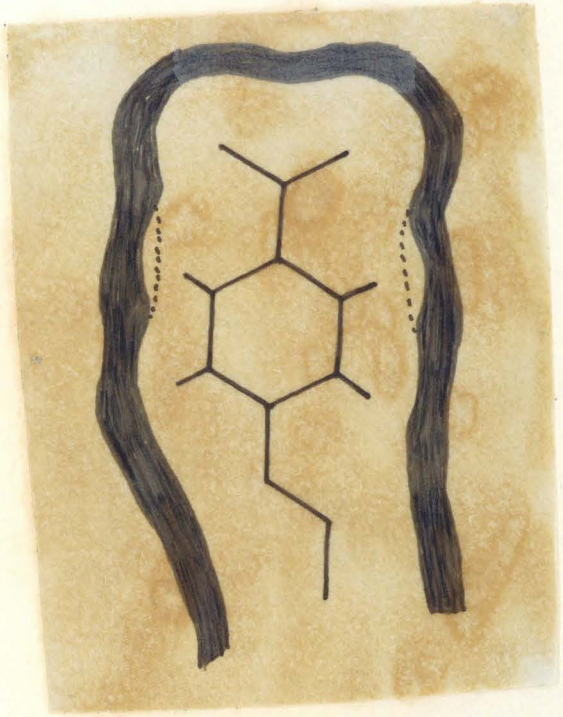
(Dotted lines show normal van der Waals radii)



The dependence ofthe inhibition constant with the anti-Xp serum on the position of the chloro group in the benzene ring for the 4-azo haptens is that the unsubstituted is greater than the 2-chloro. Both give greater inhibition than benzoic acid. Unfortunately the 3-chloro-4-azohapten was not prepared. The inhibition of the 2-chloro-4-azo hapten is one third that of the unsubstituted 4-azohapten. This shows a rather close fit of the hapten and antibody. Nevertheless the lack of steric hindrance on the part of benzoic acid was not sufficient to overcome its lack of the azo homologous group and so the 2-chloro-4-azo hapten inhibited twice as much as benzoic acid itself.

A model of the antibody-hapten complex showing the closeness of fit is postulated and ascribed below from the experimental data obtained.

(Dotted lines show normal van der Waals radii)



The values of $K_o^!$ for the chloro-3-azo and the chloro-4-azo haptens with the anti-Xo serum, for the chloro-2-azo and the chloro-4-azo haptens with the anti-Xm serum and for the chloro-2-azo and chloro-3-azo haptens with the anti-Xp serum are reasonable from the models of the antibodies described above.

Using the anti-Xo serum the unsubstituted m-azo hapten gave much greater inhibition than did the p-azo hapten as is to be expected for steric reasons.

With the 3-azo haptens the dependence of t he inhibition constant on the position of the chloro group in the benzene ring is 6 > unsubstituted > 2 > 5 > 4. The benzoic acid is twice as good an inhibitor as the 6-chloro.

There is some tendency for the 3-azo unsubstituted hapten to swing the azo group into the opening at the 2 position in the

anti-Xo antibody. In the anti-Xo serum there is a non-specific hole at position 6(as observed from the data above with the homologous haptens). The 6-chloro-3-azo hapten causes greater inhibition than the 3-azo hapten itself since the chloro group in position 6 stabilizes the antibody-hapten complex formed. The hapten must swing a little so as to let the 3-azo group go into the 2-azo hole of the antibody. As this occurs the non-specific hole at 6 is able to accomodate a chloro group without steric hindrance permitting van der Waals attraction due to the chlorine's juxtaposition along the antibody to become effective. The complex with the chloro group there assumes greater stability as the hapten cannot now swing back for the 6-chloro holds the hapten in the new position by steric considerations. and as the additional van der Waals forces increase the specificity of the antibody for the hapten.

The placement of a chloro group in the 2 position causes less inhibition than the unsubstituted 3-azo since the chloro group itself partially takes up the 2-azo opening of the antibody.

The effect of placing a chloro group at the 4 or 5 position causes large steric effects as expected. Less inhibition is observed with the chloro group in the 4 position since we have seen that the antibody fits most closely around the 4 position.

The unsubstituted 4-azo hapten causes very small inhibition but the placement of a chlorine even in the 2 position on the benzene ring reduces the stability of the antibody-hapten complex to a very small amount indeed.

With the anti-Xm serum, the unsubstituted o-azo hapten gave much greater inhibition than did the p-azo hapten as is to be expected from the non-specific hole in the 2 position.

With the 2-azo haptens the dependence of the inhibition constant on the position of the chloro group in the benzene ring is 3>4 = unsubstituted> 5 = 6. The benzoic acid is twice as good an inhibitor as the 3-chloro.

The chloro group in position 3 stabilizes the combination since there is a large hole at position 3 which can accomodate the chlorine and can bring into play its van der Waals forces of attraction due to the chlorine's juxtaposition along the antibody.

With the chloro group at position 4 there is still a loose fit of antibody around the hapten in this position as indicated with the 4-chloro-3-azo hapten allowing space for the chlorine to bring into play its van der Waals forces of attraction.

If the chloro group is placed at position 5 or 6, the combination is greatly decreased as expected from the closeness of fit of the antibody to the hapten in these positions.

Benzoic acid inhibits better than the 2-chloro-4-azo hapten which in turn is a better inhibitor than the unsubstituted 4-azo hapten. This is expected for there are steric reasons for the small inhibition of the unsubstituted 4-azo hapten. However as has been seen before, there is a loose fit of antibody in the 2 position which gives greater play to the van der Waals forces of attraction for a chlorine in the 2 position. These added forces stabilize the antibody-hapten complex formed

with the 2-chloro hapten.

With the anti-Xp serum the unsubstituted m-azo hapten gave much greater inhibition than did the unsubstituted o-azo hapten. This is expected from steric considerations.

With the anti-Xp serum values of K_0' less than about 0.5 could not be determined because of the high concentrations of hapten required. All of the chloro-2-azobenzoic acid haptens had values below 0.5 and below that for the unsubstituted 2-azo hapten.

With the 3-azo haptens the dependence of the inhibition constant on the position of the chloro group in the benzene ring is unsubstituted > 4 > 2 > 5 > 6. The benzoic acid has a K_0' of value between that of 2 and 5. The inhibition of the unsubstituted 3-azo and the 4-chloro-3-azo haptens were only a little less than that of the 2-chloro-4-azo hapten.

If a chloro group is at position 4 of the hapten, it shares the 4 opening in the antibody with the 3-azo group requiring great dilatation of the antibody causing less combination of antibody and hapten.

With a chloro group in position 2 there is some stabilizing influence of the chloro group on the orientation of the 3-azo group to the 4-azo position of the antibody. The effect is similar to that caused by the 6-chloro group on the 3-azo hapten in the anti-Xo serum. However with the chloro group in the 5 or 6 position the steric hindrance brought about by the large chloro group does not allow the 3-azo group to swing to the 4-azo position. As these haptens cannot form as stable antibody complexes, they cause much less inhibition.

SUMMARY

Experiments have been made on the precipitation of antisera homologous to the o-, m-, and p-azobenzoic acid groups, prepared by injecting rabbits with beef serum coupled with diazotized o-,m- and p-aminobenzoic acids, with azoovalbumins containing these homologous groups and on the effect of various monochloro substituted azobenzoic acid haptens in inhibiting the precipitation.

The monochloro substituted azobenzoic acid haptens were prepared from the corresponding chloroaminobenzoic acid. These chloroaminobenzoic acids were synthesized from commercially available starting materials. They were then diazotized and coupled to phenol to form the desired haptens used in these experiments.

The closeness with which the antibody fits its homologous azohapten was determined by determining the effect of chloro groups in every position in the benzene ring not occupied by an homologous group on the inhibition of the specific precipitation of the antisera. This was done for the antibodies of the anti-Xo, anti-Xm and anti-Xp sera using the appropriate haptens for each.

Cross reactions using the 3-azo and 4-azo(monochloro substituted and unsubstituted) haptens with the anti-Xo serum, the 2-azo and 4-azo(chloro substituted and unsubstituted) haptens with the anti-Xm serum, and the 2-azo and 3-azo(chloro substituted and unsubstituted) haptens with the anti-Xp serum were determined. The results obtained corresponded to what was expected as predicted from the data secured from inhibition studies for the closeness of fit of the antibody and its

homologous haptens.

Diagrams showing the closeness of fit of the various antibodies and their homologous haptens are given.

BIBLIOGRAPHY

1. K. Landsteiner - "The Specificity of Serological Reactions"- Charles C. Thomas- Springfield, Illinois 1936

2. D. Pressman, D.H. Brown, L. Pauling - J. Am. Chem. Soc.,64, 3015(1942)

3. D. Pressman, J.T. Maynard, A.L. Grossberg, L. Pauling - ibid, 65, 728(1943)

4. L. Pauling, D. Pressman, A.L. Grossberg - ibid, 66, 784(1944)

5. D. Pressman, S.M. Swingle, A.L. Grossberg, L. Pauling - ibid, 66, 1731(1944)

6. L. Pauling, D. Pressman - ibid, 67, 1003(1945)

7. L. Pauling, D.H. Campbell, D. Pressman - Physiol. Rev.,23, 203(1943)

8. K. Landsteiner, J. van der Scheer - J. Exptl. Med., 55, 781(1932)

9. L. Pauling, D. Pressman, D.H. Campbell, C. Ikeda, M. Ikawa - J. Am. Chem. Soc., 64, 2994(1942)

10. D. Pressman - Ind. Eng. Chem., Anal. Ed., 15, 357(1943)

11. O. Folin, V. Ciocaltéu - J. Biol. Chem., 73, 627(1927)

12. H. Gilman, A.H. Blatt - "Organic Syntheses" - John Wiley and Sons, New York, N.Y. - Collective Volume I - 1941 - p. 162

13. Holleman - Rec. trav.chim., 27, 456(1908)

14. A.H. Blatt - "Organic Syntheses" - John Wiley and Sons, New York, N.Y. - Collective Volume II - 1943 - p. 135

15. Holleman, de Bruyn - Rec. trav. chim., 20, 209(1901)

16. Holleman, Voerman - ibid, 21, 57(1902)

17. F. Ullmann, C. Wagner - Ann., 355, 360(1907)

18. J.B. Cohen, D. McCandlish - J. Chem. Soc., 87, 1271(1905)

19. F. Kunckell, A. Richartz - Ber., 40, 3395(1907)

20. H. Goldschmidt, M. Hönig - ibid, 19, 2443(1886)

21. H. Hübner - Ann., 222, 195(1884)

22. P. Griess - Ber., 19, 316(1886)

23. E.Bamberger, J. de Werra - ibid, 35, 3719(1902)

24. A.G. Green, T.A. Lawson - J. Chem. Soc.,59,1019(1891)

25. Cohn - Monatsh., 22, 488(1901)

26. J.B. Cohen, H.P. Armes - J. Chem. Soc., 89, 458(1906)

27. Cohn - Monatsh., 22, 485(1901)

28. D. Rice - private communication

29. H. Hübner - Ann., 222, 81(1884)

30. H. Hübner - ibid, 222, 89(1884)

31. H. Hübner, M. Ulrich - ibid, 222, 95(1884)

32. W. Eller, L. Klemm - Ber., 55, 221(1922)

33. Dorsch - J. prakt. Chem., (2), 33, 50(1886)

34. H. Hübner, G. Weiss - Ber., 6, 175(1873)

35. P. Bössneck - ibid, 19, 1757(1886)

36. F. Beilstein, A. Kurbatow - Ann., 182, 104(1876)

37. F. Beilstein, A. Kurbatow - ibid, 182, 107(1876)

38. Claus, Kurz - J. prakt. Chem., (2), 37, 200(1888)