BINDING OF HOMOLOGS OF CERTAIN CARCINOGENIC AZO DYES WITH PROTEINS

by

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INTRODUCTION

Since 1906 when Fisher (5) first found that epithelial tumors were produced on the ears of rabbits when innoculated with scarlet red, scientists have been studying the relationship of azo dyes to cancer.

Yoshida, in 1934, was reportedly the first to successfully induce a tumor using o-aminoazotoluene at a site considerably removed from the site of application. However, the lack of complete experimental information and suspected diet deficiencies decreased the usefulness of many of these observations.

Kensler, et al. (10) first postulated that it is not the azo dye that initiates the carcinogenic process, but rather an enzyme poison, such as p-aminophenol or p-phenylenediamine formed in the metabolism of the azo compounds. Miller and Baumann (15) of the University of Wisconsin were lead to dispute this idea on the basis of their work and contend that the intact azo molecule is the true carcinogen. Their work involved 4-dimethylaminoazobenzene and its methyl homologs. Structural differences were related to carcinogenic activity with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) being greater than 4-dimethylaminoazobenzene (DAB), which is greater than 2'-methyl-4-dimethylaminoazobenzene (2'-Me-DAB), which is greater than 4'-methyl-4-dimethylaminoazobenzene (4'-Me-DAB). The Millers (16) proposed that the dyes initiate the carcinogenic process through the formation of linkages to the proteins contending that this binding of the dyes takes place at the $-N(CH_3)_2$ or possibly the ring to which this group is attached. Such processes should involve some type of complex formation between these dyes and protein molecules. Present knowledge of such complexes and their formations is very limited and thus this study was undertaken to

possibly help elucidate these phenomena. Relationships between the binding of the azo dyes and the structural qualities of the protein itself might aid in determining the manner in which the complex forms. Part of the work undertaken was to determine how important certain specific functional groups on the protein are in the binding of the dye and to determine whether there are specific sites on the protein for such interactions.

Previous experiments (Klotz and Ayers, 11) in which lysine, tyrosine, and carboxyl residues of bovine serum albumin have been modified indicates that binding of aminoazobenzene involves a hydrogen bond between a side chain of the protein and a electron pair of the uncharged molecule. From this one might expect the binding to involve a positive site on the protein, such as that of the ϵ -ammonium group of lysine. This was upheld as a decrease in binding was realized with acetylated bovine serum albumin compared to the unaltered protein. Residues, such as histidine, and arginine, might similarly enter into the reaction. These nitrogen containing side chains were not the only type involved in the interactions. The -OH group on the tyrosine residue should also have a hydrogen capable of hydrogen bonding with the dye. Experiments showed that the tyrosine residues too must be involved because of nearly a 50 per cent drop in binding with the iodinated protein. Since the change in net charge is less with the iodinated protein that with the acetylated, it would seem the decrease in binding with the iodoalbumin specifically must involve the tyrosine side chain also.

In considering the nature of these binding sites on the protein, each of the classes of small organic molecules or ions must be dealt with individually and the physical and chemical characteristics of each binding media must be considered separately. Nearly all research workers in the

field report that the number of sites on a given protein molecule, for example, serum albumin, is dependent upon the particular material being bound. The maximum number of methyl orange ions bound to a single serum albumin molecule has been found to be approximately 22 (Klotz et al., 14), while others such as the neutral molecule of p-aminoazobenzene, show about one to be bound per protein molecule.

Besides this type of bonding, surface attraction due to van der Waals forces may be important. This appears especially important when albumin-anion attractions are contrasted to interactions of albumins and neutral molecules, for in the latter case, a marked temperature dependence is found with a binding decrease upon raising the temperature (Klotz and Ayers, 11).

Here also was noted a substantial increase in binding as the pH was raised. Neutral molecules seem most likely to be more effectively bound as the protein acquires a more negative charge due to a raise in pH which may act to unfold the protein, opening new sites for binding and somehow provide greater surface area. Slight unfolding due to a raise in temperature has also been postulated (Burkhard, et al., 3) which might account for a small quantity of increased binding.

It is the purpose of this study to compare the bindings of three azo dyes: p-aminoazobenzene, 3'-methyl-4-aminoazobenzene, 4'-methyl-4-aminoazobenzene with both normal and altered bovine serum albumins and the soluble proteins in rat liver homogenate to determine their binding relationships and the dependence of these interactions upon various physical and chemical factors. These particular homologs were chosen for this study rather than the actual carcinogens used by Miller and Miller (16) because of the insolubility of the dimethylaminoazobenzene homologs. The anionic dye methyl orange, was used as a reference. Bovine serum albumin was chosen as a suitable protein

with which to work because of its availability, purity and the fact that it has been quite well studied and characterized (Brand, 1). Since the formation of tumors somehow involved the binding of azo dyes to natural proteins, rat liver homogenates provided a source of protein material known to be susceptible to cancer development. Work on this phase would act to substantiate previous studies carried out in this laboratory (Grossman, 7) utilising more carefully controlled methods of extraction and determination of protein material present in each sample.

PREPARATION OF BUFFERS

The pH 6.8 phosphate buffer was prepared by dissolving 7.87 grams of disodium hydrogen phosphate and 6.0 grams of potassium dihydrogen phosphate in distilled water and diluting to one liter.

The pH 9.1 glycinate buffer was prepared by adding 30.028 grams of glycine to enough NaOH to make one liter of a 0.100 N NaOH solution.

HOMOGENATE

The rat liver homogenates were previously prepared by the method of Grossman (7). This homogenate was then extracted with 6.8 phosphate buffer at 4°C. Approximately a 10 per cent homogenate solution was used for this extraction. Each individual sample was analysed separately by micro-Kjedahl technique to determine the nitrogen present.

BINDING TECHNIQUES

Equilibrium dialysis techniques as developed by Klotz and co-workers (14) were used. The following is an example of a typical binding run. The dye was prepared in a buffer solution previously described, and in some cases the solution was heated slightly on a steam cone to aid the dye in dissolving more quickly. From the extinction coefficients of the dyes (Burkhard and Moore, 4) the desired range of concentration was obtained using the Beers-Lambert law which applies in this range of concentration (Burkhard, 2),

$$OD = \log \frac{I_o}{I} = \epsilon C l$$

OD = optical density
I_o = intensity of light entering solution
I = intensity of transmitted light
C = concentration of dye (Moles per liter)
l = length of cell (1 cm. in all cases of this study)
ϵ = molar extinction coefficient

and by utilizing the relationship:

$$OD_1 V_1 = OD_2 V_2$$

A range of 0.1 to 1.0 x 10^{-5} mols. per liter was used. Several dilutions were made within this range. Eight ml. of each sample were placed in two tubes. Into one of these tubes the bag containing the protein material was placed, while the other acted as a control, having only the bag containing the buffer added to it. At least four tubes with buffer both in the bag and in the tube were used with each run to determine any amount that might have been bound by the bags themselves.

A sample of protein material was dried over night in vacuo over P_2O_5 in a drying pistol. A 0.2 per cent solution of the protein was prepared with the appropiate buffer. Two ml. of this protein-buffer solution was pipetted into Visking Nojax casing which had previously been boiled in distilled

water for three half hour periods. The bag was securely knotted at both ends after expelling all the air possible and placed in the dye solution. A size 40 white thread previously attached to it provided a means of removing the bag after equilibrium was reached. The tubes were then placed in either a crushed ice bath or a 25°C constant temperature bath and agitated for 24 hours by means of a Burrell wrist action shaker. After equilibrium was reached, the bags were removed from the tubes which were then allowed to come to room temperature. The optical densities of the solutions remaining in both the protein and buffer containing tubes were then read on a Beckman model DU spectrophotometer using 1 cm. silica absorption cells. A wave length of 375 mμ. was used for all but the methyl orange which required a wave length of 465 mμ. The concentrations of all the tubes were calculated and the amount bound determined by subtracting the amount of dye in the protein containing tube from that in the buffer blank. The amount bound either per mole of protein or per mg. nitrogen was then calculated. This could be plotted against the log of the free dye concentration, A.

MICRO-KJEDAHL

The nitrogen determinations were made on each sample of the rat liver extracts by micro-Kjedahl techniques similar to that of Sobel, et al (17). One ml. of the protein extract was digested until fuming with one ml. of concentrated H_2SO_4 using a potassium sulfate-copper sulfate catalyst. Thirty per cent H_2O_2 was added drop-wise to nearly decolorize the solution, leaving it a pale green color. The sample was again heated to fuming to remove any excess H_2O_2.

The sample was transferred to a distillation apparatus and diluted with 5-10 ml. H_2O and 7 ml. 30 per cent NaOH. This was steam distilled for four minutes into a 4 per cent boric acid solution to which had been added a methylene blue-methyl red indicator. This solution was titrated back to a pale green color again with 0.00978 N HCl. The nitrogen was then calculated from the formula:

$$\text{Mg. N} = \text{ml. HCl} \times \text{N of HCl} \times .014$$

Protein in binding studies was expressed in Mg. N per bag.

PREPARATION OF ACETYLATED BSA

Acetylated bovine serum albumin was prepared by a method similar to that of Frankeal-Conrat, et al. (6). A sample of normal BSA was suspended as a 5 per cent solution of one half saturated sodium acetate. After the solution was allowed to cool to 0°C, 2.4 ml. of acetic anhydride were added per gram of protein the solution was then allowed to remain standing in the cracked ice bath for one hour. The sample was then placed in dialysis casings and dialysised against distilled water at 0°C in a constant flowing dialysis chamber. This dialysis was carried out until the resistance measurements showed that the water leaving the dialysis chamber had the same resistance as that of the distilled water entering the chamber. These resistance measurements were carried out on a model RC 16 conductivity bridge, as supplied by Industrial Instruments Inc. This dialysis was usually complete in approximately 48 hours. The sample was then lyophylized and stored in a refrigerator. A yield of approximately 80 per cent was obtained.

FORMOL TITRATION

The number of ϵ -amino groups of lysine remaining after acetylation was determined by a potentiometric titration method as outlined by Kekwick and Cannan (9). This consisted of making a 0.020 gr. sample of acetylated bovine serum albumin up to a 1 per cent solution with distilled water. This was then adjusted to a pH of 9.0 by adding 0.0109 N NaOH. Eight ml. of formalin, previously adjusted to a pH of 9.0, were added. The sample was then readjusted to a pH of 9.0 with 0.0109 N NaOH. The number of unacetylated ϵ -amino groups on the lysine residue was then calculated from the last amount of NaOH used. The number of equivalents of NaOH used should be equal to the number of ϵ -amino groups available. Subtracting this value from the known number of ϵ -amino groups originally on the bovine serum albumin (Brand, 1), the percentage of acetylation could be calculated. The pH measurements were carried out using a Beckman Model G pH meter equipped with external electrodes.

PREPARATION OF IODINATED BSA

Iodination of bovine serum albumin was carried out following the procedure of Hughes and Straessle (8) which will iodinate the greatest number of tyrosine groups to diiodotyrosine with minimum denaturation or oxidation of the protein. A 5 per cent solution of BSA in a precooled borate buffer solution of pH 9.1 was prepared. Iodine in the form of 0.1 N iodine-0.2 M iodide solution was added while the mixture remained at 0°C. After a period of four or five hours at 0°C the solution lost its iodine color. The mixture was then placed in a dialysis casing and dialysed in a constant flowing dialysis chamber for 72 hours to remove any unreacted iodine or iodide. The

mixture was then lyophylised and stored in a refrigerator.

DETERMINATION OF DIIODOTYROSINE

Determination of the number of iodine atoms substituted into the tyrosine residue as diiodotyrosine was made by calculations from the spectral shift. In a solution of pH 10.75 diiodotyrosine of serum albumin has exhibited a shift in absorption maximum for the normal at 279 mμ. to one at 312 mμ. (Fig. 1). The height of this maximum is dependent upon the number of iodine atoms per mole in the form of diiodotyrosine.

RESULTS AND DISCUSSION

The binding studies of the rat liver homogenate suggest that the 3'-methyl-4-aminoazobenzene is bound slightly more than the 4'-methyl-4-aminoazobenzene and both of these compounds are bound in greater quantities than p-aminoazobenzene when expressed as mean per cent of dye bound per mg. of nitrogen in the rat liver homogenate.

TABLE 1. Mean per cent dye bound per mg. nitrogen

Dye	Per cent bound / mg. N	Standard deviation
p-aminoazobenzene	18.4	6.1
3'-methyl-4-aminoazobenzene	28.8	4.9
4'-methyl-4-aminoazobenzene	25.7	8.6

In Table 1, the results are shown with their corresponding sample standard deviations (s). Figure 2 graphically illustrates this binding. These values compare, within sample standard deviations with earlier work

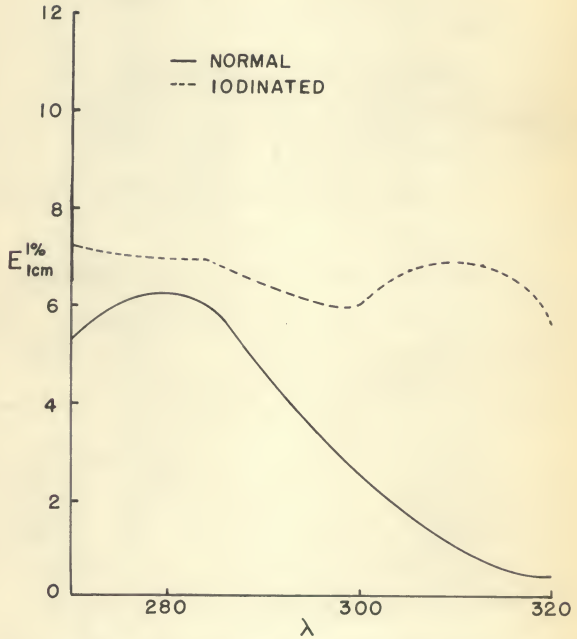

Fig. 1. Spectra of normal and iodinated bovine serum albumin at pH 10.75.

Fig. 2. Mean per cent of dye bound per mg. of Nitrogen.

carried out in this laboratory (Grossman, 7). These results were expressed in this manner since a rigorous analysis of this data did not seem to be warranted and it was found that the per cent of total dye bound was independent of the dye concentration and thus permitted averaging of the data. (Table 2).

TABLE 2. Correlation coefficients for total dye vs. per cent bound

Dye	Correlation coefficient	Degrees of freedom
p-aminoazobenzene	0.10	18
3'-methyl-4-aminoazobenzene	-0.28	14
4'-methyl-4-aminoazobenzene	0.31	17

It is not exactly known how these protein dye complexes form, but it is thought to take place by forces such as hydrogen bonding and/or van der Waals forces. It would be of some interest to determine how important these two factors are in interactions of this type. It has been shown (Burkhard and Moore, 4) that a relationship between solubilities and the extent of dye protein in complex formation exists with bovine serum albumin. This also was found to be true in this study and suggests again that factors other than basicity are of major importance in these interactions. To further examine this hypothesis it was thought desirable to chemically modify a protein such as BSA and study the effect of such modifications on the interactions with the azo dyes.

The interactions of acetylated serum albumin with methyl orange were first examined and showed (Fig. 3) a marked decrease in binding as has been reported in literature (Klotz, et al., 12). The acetylation of the protein masks the ϵ-ammonium groups which could lend themselves to the binding with the anionic methyl orange. The acetylated BSA used in this experiment had over 90 per cent of these groups removed.

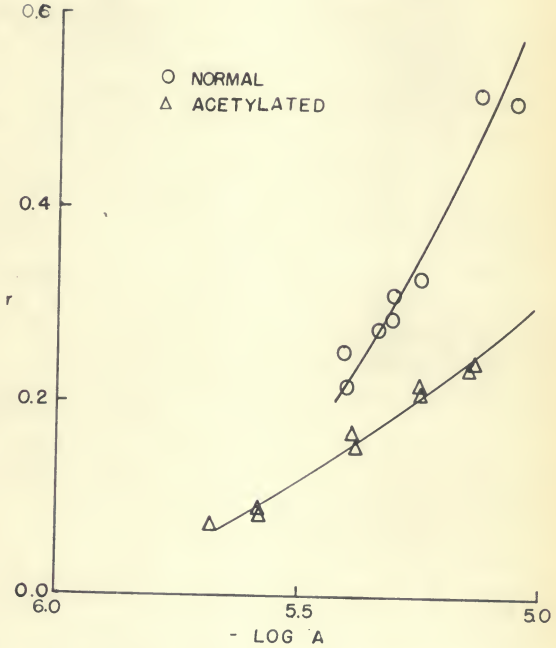

Fig. 3. Binding of methyl orange with normal and acetylated bovine serum albumin at 0°C and pH 6.8.

The neutral molecules of the p-aminoazobenzene, however, showed a slight increase in binding and both the 3'-methyl-4-aminoazobenzene and the 4'-methyl-4-aminoazobenzene showed an even greater increase in binding after the acetylation of the protein (Figs. 4, 5 and 6). Since a greater part of the interaction of the protein and the neutral molecules may be due to a surface attraction, any change of enlarging the net surface area could then cause an increase in the complex formation. If by acetylation of the ϵ-ammonium group and the removal of its positive charge a change in the net charge of the protein molecule were effected, some unraveling or unfolding of the protein could occur and allow an increase in binding.

The iodination of 60 per cent of the tyrosine groups to diiodotyrosine resulted in increased binding (Figs. 7-10). Of the neutral dyes, p-aminoazobenzene showed the most marked rise in the binding ability as the protein was iodinated (Fig. 8). 3'-Methyl- and 4'-methylaminoazobenzene (Figs. 9 and 10) show nearly equal increases in binding ability. Methyl orange seemed to show the smallest increase in binding upon iodination of the protein. These results suggest that the tyrosine residues are not specifically involved in the binding of these dyes to the protein. The enhanced binding might again be accounted for by unfolding or opening of the complex protein molecule upon iodination.

The binding studies of acetylated BSA at pH 6.8 in a phosphate buffer and a temperature of 0°C were extended to studies at 25°C and utilization of a pH 9.1 glycine buffer. By raising the temperature and the pH of the binding media the study was expected to show any dependence of the binding on these factors which might indicate that either surface attraction or ionic attraction played the major role in the complex formation.

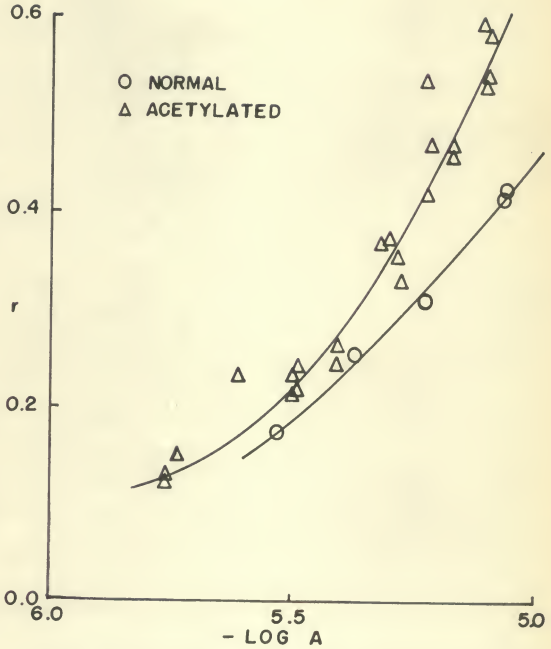

Fig. 4. Binding of p-aminoazobenzene with normal and acetylated bovine serum albumin at 0°C and pH 6.8.

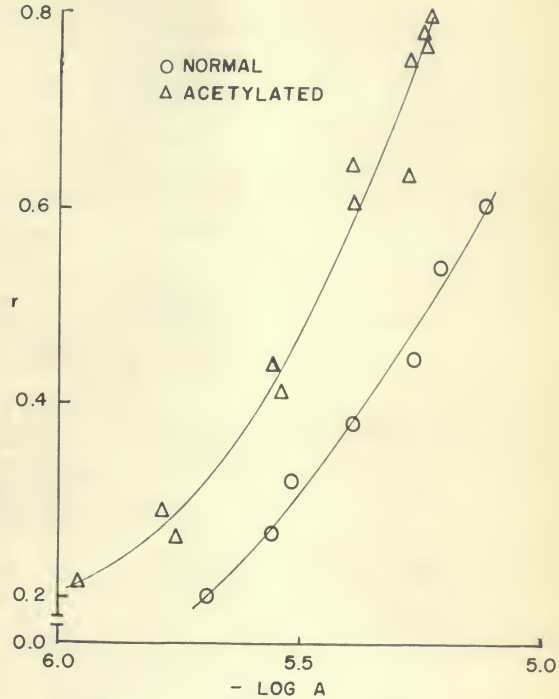

Fig. 5. Binding of 3'-methyl-4-aminoazobenzene with normal and acetylated bovine serum albumin at 0°C and pH 6.8.

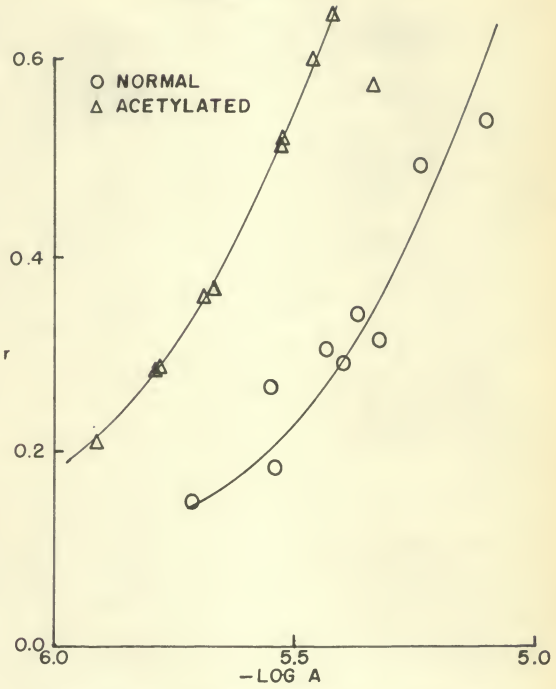

Fig. 6. Binding of 4'-methyl-4-aminoazobenzene with normal and acetylated bovine serum albumin at 0°C and pH 6.8.

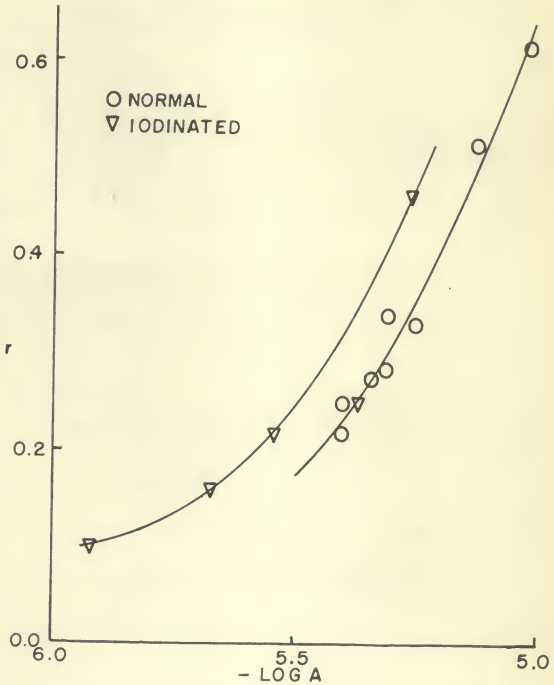

Fig. 7. Binding of methyl orange with normal and iodinated bovine serum albumin at 0°C and pH 6.8.

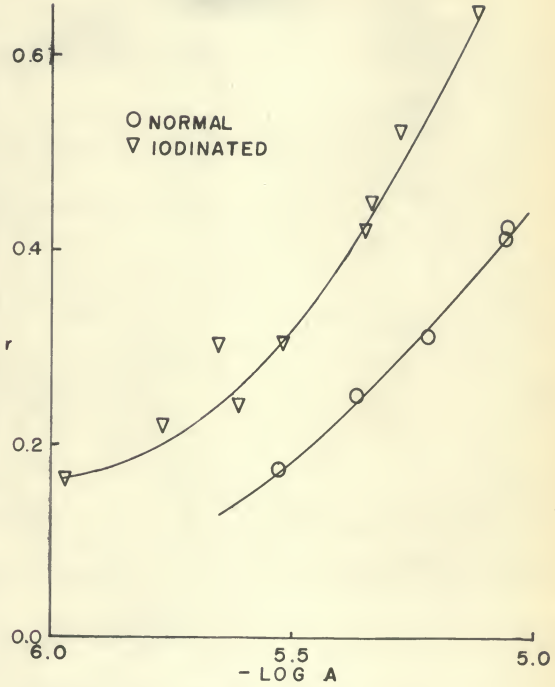

Fig. 8. Binding of p-aminoazobenzene with normal and iodinated bovine serum albumin at 0°C and pH 6.8.

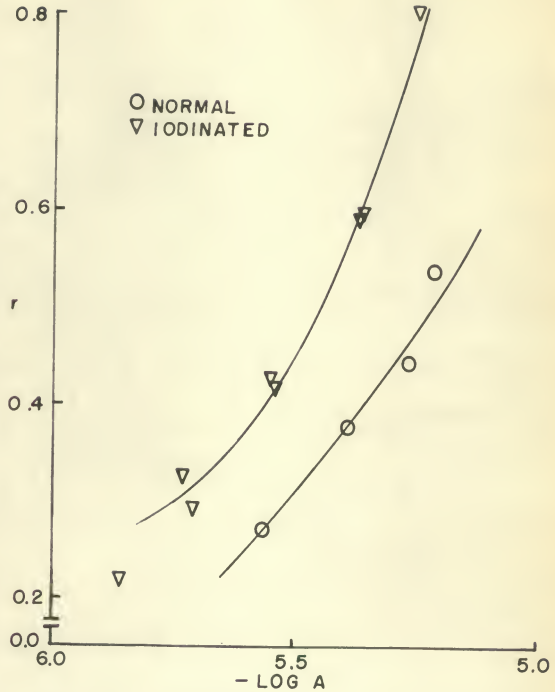

Fig. 9. Binding of 3'-methyl-4-aminoazobenzene with normal and iodinated bovine serum albumin at 0°C and pH 6.8.

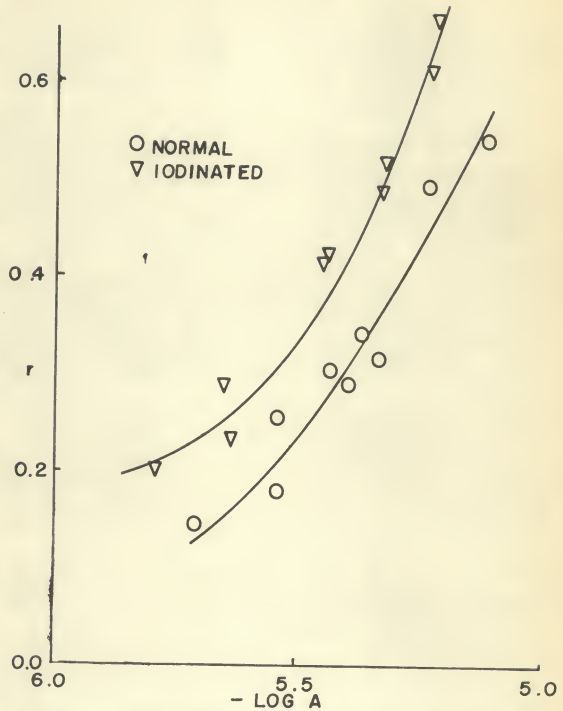

Fig. 10. Binding of 4'-methyl-4-aminoazobenzene with normal and iodinated bovine serum albumin at 0°C and pH 6.8.

Klotz and Urquhart (13) studied the competitive effects of various buffers on the protein dye complex formation and found the glycinate buffer (pH 9.1) had the least inhibitory effect while phosphate ranked second as a non-competitor out of 13 buffers studied. Using a glycinate buffer, the pH could be raised without acting as a stronger competitor than the phosphate of the pH 6.8 buffer.

The results here show, that while at 0°C and a pH of 6.8, acetylation causes a very slight increase in binding ability under certain other conditions a decrease in binding appears. (Compare Fig. 4 to Fig. 11, 12, and 13). This would suggest that at 25°C and either pH 6.8 or 9.1 the ϵ-ammonium groups of lysine are involved in the binding of p-aminoazobenzene, while at 0°C only at pH 9.1 are these groups involved. These results agree with those of Klotz and Ayers (11) and suggest that under certain conditions the ϵ-ammonium groups of lysine can be of importance in binding of uncharged dyes while under other conditions (such as pH 6.8 at 0°C) they may not be of importance.

From this study it seems that at the low temperature and pH employed the chemical bonding of specific groups on the protein plays a lesser role in the complex formation with neutral organic dyes than does the physical aspect of surface attraction. If either of these conditions are allowed to change a shift in the relationship of major importance of the factors effecting binding may occur.

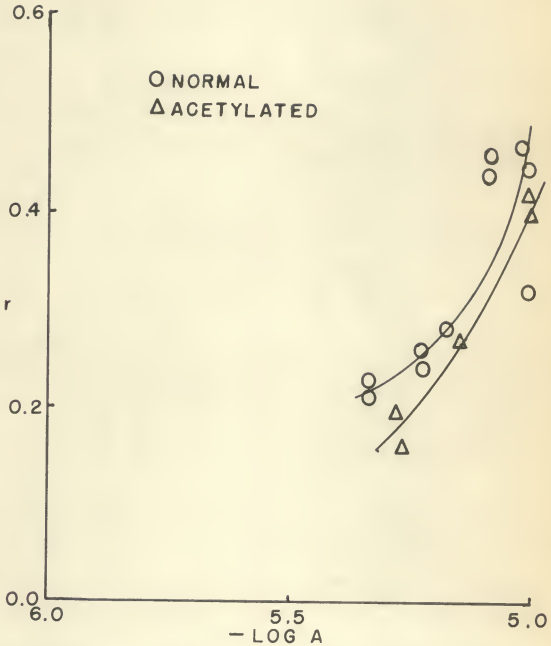

Fig. 11. Binding of p-aminoazobenzene with normal and acetylated bovine serum albumin at 25°C and pH 6.8.

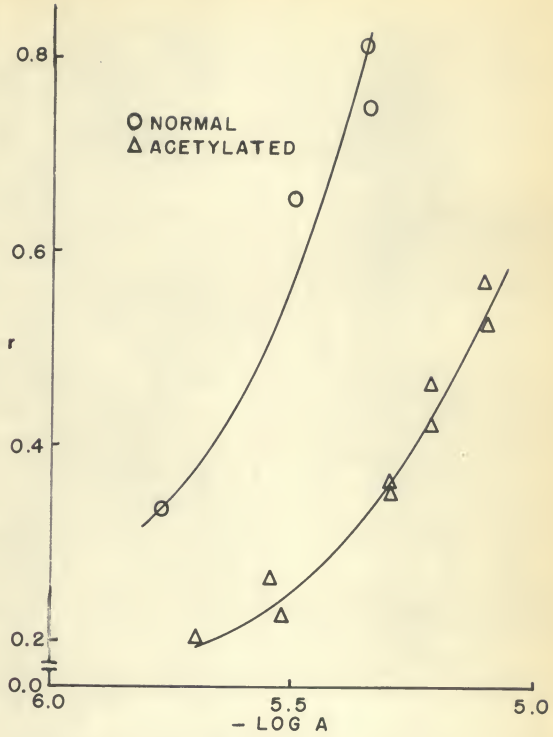

Fig. 12. Binding of p-aminoazobenzene with normal and acetylated bovine serum albumin at 0°C and pH 9.1.

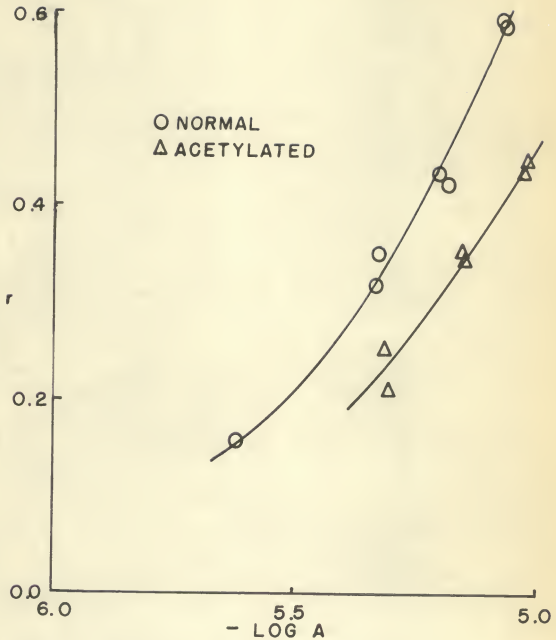

Fig. 13. Binding of p-aminoazobenzene with normal and acetylated bovine serum albumin at 25°C and pH 9.1.

SUMMARY

The binding of uncharged azo dyes with rat liver homogenate was studied and it suggested that 3'-methyl-4-aminoazobenzene was bound only slightly more than was 4'-methyl-4-aminoazobenzene and that both of these were bound to a greater extent than was p-aminoazobenzene. This substantiated earlier work that rat liver homogenates do bind with neutral azo dyes and bind them in different quantities.

The binding of the neutral dyes to BSA at 0°C and pH 6.8 after acetylation of the ϵ-ammonium group of lysine was greater than that observed with normal BSA. Under certain conditions the acetylation of the protein caused a decrease in the binding of the one neutral dye examined. Methyl orange, an anionic dye, acted as a reference and upon acetylation of the protein was found to be bound less. Iodination of the tyrosine residue to diiodotyrosine resulted in an enhanced binding for all of the azo dyes examined.

It appears from this that the binding of a charged dye may be affected by removal of possible ionic binding sites, but the uncharged azo dyes of this study are not so affected. It is suggested that the complexes involving uncharged dyes and proteins at 0°C are mostly due to surface attraction between the dye and protein molecules. Both the acetylation and iodination of the protein in some manner seem to cause an enhanced binding at 0°C. Such modifications of the protein could increase the surface area due to unfolding of the molecule by altering the charge on the molecule and thus account for the enhanced binding observed.

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APPENDIX

Normal rat liver homogenate and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

TUBE #	CONC. M/L(10^{-5})	Δ CONC. (10^{-5})	AMT. BOUND (10^{-7})	% BOUND	MG. N	% BOUND: MG. N	DEV.	DEV^2
1B	0.642							
1	0.597	0.045	0.045	7.02	0.339	20.6	2.2	4.84
2B	0.835							
2	0.783	0.052	0.052	6.22	0.339	18.4	0	------
3B	1.058							
3	0.987	0.071	0.071	6.72	0.339	19.8	1.4	1.96
4B	0.632							
4	0.612	0.020	0.020	3.16	0.316	10.0	8.4	70.56
5B	0.840							
5	0.788	0.052	0.052	6.20	0.316	19.6	1.2	1.44
6B	1.060							
6	0.976	0.084	0.084	7.93	0.316	25.1	6.7	44.89
7B	1.060							
7	0.973	0.087	0.087	8.21	0.316	26.0	7.6	57.76
8B	0.637							
8	0.607	0.030	0.030	4.71	0.316	14.9	3.5	12.25
9B	0.841							
9	0.826	0.015	0.015	1.79	0.316	5.65	12.8	163.84
10B	1.030							
10	0.967	0.063	0.063	6.11	0.316	19.3	.9	.81
11B	1.050							
11	0.997	0.053	0.053	5.05	0.316	16.0	2.4	5.76
12B	0.685							
12	0.597	0.088	0.088	12.80	0.462	27.8	9.4	88.36
13B	0.903							
13	0.792	0.111	0.111	12.30	0.462	26.6	8.2	67.24
14B	1.110							
14	0.995	0.115	0.115	10.4	0.462	22.4	4.0	16.00
15B	1.050							
15	1.014	0.036	0.036	3.43	0.462	7.42	11.0	121.00

Normal rat liver homogenate and p-aminoazobenzene (concl.)

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

TUBE: #	CONC. :M/L(10^{-5})	:Δ CONC.: (10^{-5})	ANT. BOUND (10^{-7})	:% BOUND	MG. N / DAG	:% BOUND : MG. N	DEV.	DEV.^{2}
16B	0.690							
16	0.646	0.054	0.054	7.84	0.462	16.9	1.5	2.25
17B	0.903							
17	0.812	0.091	0.091	10.10	0.462	21.8	3.4	11.56
18B	1.110							
18	1.014	0.096	0.096	8.65	0.462	18.7	.3	.09
19B	1.110							
19	1.040	0.070	0.070	6.31	0.462	13.6	4.8	23.04

Normal rat liver homogenate and 3'-methyl-4-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

TUBE: #	CONC.: M/L(10^{-5})	△ CONC.: (10^{-5})	AMT. BOUND: (10^{-7})	% BOUND	MG. N: EXP	% BOUND: MG. N	DEV.	DEV.^{2}
1B	0.593							
1	0.504	0.089	0.089	15.00	0.508	29.5	.7	.49
2B	0.739							
2	0.644	0.095	0.095	14.70	0.508	25.3	3.5	12.25
3B	0.982							
3	0.860	0.122	0.122	12.40	0.508	24.4	4.4	19.36
4B	0.595							
4	0.513	0.082	0.082	13.70	0.508	27.0	1.8	3.24
5B	0.588							
5	0.507	0.081	0.081	13.80	0.508	27.1	1.7	2.89
6B	0.748							
6	0.635	0.113	0.113	15.10	0.508	29.7	.9	.81
7B	0.762							
7	0.664	0.098	0.098	12.80	0.508	25.3	3.3	10.89
8B	0.978							
8	0.844	0.134	0.134	13.70	0.508	26.9	1.9	3.61
9B	0.998							
9	0.880	0.118	0.118	11.80	0.508	23.2	5.6	31.36
10B	0.347							
10	0.340	0.007	0.007	2.02	0.382	5.3	23.5	552.25
11B	0.602							
11	0.462	0.140	0.140	23.20	0.382	60.8	32.0	1024.25
12B	0.742							
12	0.657	0.085	0.085	11.40	0.382	29.8	1.0	1.00
13B	0.988							
13	0.851	0.137	0.137	13.90	0.382	36.4	7.6	57.76
14B	0.376							
14	0.334	0.042	0.042	11.20	0.378	29.6	.8	.64
15B	0.582							
15	0.501	0.081	0.081	13.90	0.378	36.8	8.0	64.00

Normal rat liver homogenate and 3'-methyl-4-aminoazobenzene (concl.)

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

TUBE #	CONC. M/L(10^{-5})	Δ CONC. (10^{-5})	AMT. BOUND (10^{-7})	% BOUND	DIV. N BAG	% BOUND NO. N	DEV.	DEV.^{2}
16B	0.752							
16	0.689	0.063	0.063	8.40	0.378	22.2	6.6	43.56
17B	0.988							
17	0.874	0.114	0.114	11.60	0.378	31.2	2.4	5.76

Normal rat liver homogenate and 4'-methyl-4-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

TUBE: #	CONC. :N/L(10^{-5})	Δ CONC.: (10^{-5})	ATT. BOUND : (10^{-7})	% BOUND	MG. N : BAG	% BOUND : MG. N	DEV.	DEV.^{2}
1B	0.545							
1	0.478	0.067	0.067	12.30	0.388	31.8	7.3	53.29
2B	0.690							
2	0.240	0.050	0.050	7.25	0.388	18.7	5.8	33.64
3B	0.959							
3	0.819	0.140	0.140	14.60	0.388	37.6	13.1	171.61
4B	0.897							
4	0.827	0.068	0.068	7.59	0.388	19.6	4.9	24.01
5B	0.545							
5	0.500	0.045	0.045	9.01	0.346	23.2	1.3	1.69
6B	0.753							
6	0.648	0.105	0.105	13.90	0.346	40.1	16.4	268.96
7B	0.906							
7	0.784	0.122	0.122	13.40	0.346	38.6	14.1	198.81
8B	0.897							
8	0.772	0.125	0.125	13.90	0.346	40.1	15.6	243.36
9B	0.500							
9	0.423	0.077	0.077	15.40	0.644	23.9	.6	.36
10B	0.698							
10	0.590	0.108	0.108	15.50	0.644	24.1	.4	.16
11B	0.894							
11	0.762	0.132	0.132	14.80	0.644	23.0	1.5	2.25
12B	0.894							
12	0.762	0.132	0.132	14.80	0.644	23.0	1.5	2.25
13B	0.735							
13	0.599	0.136	0.136	18.50	0.644	28.7	4.2	17.64
14B	0.500							
14	0.446	0.054	0.054	10.80	0.644	16.8	7.7	59.29
15B	0.492							
15	0.427	0.065	0.065	13.20	0.644	20.6	3.9	15.21

Normal rat liver homogenate and 4'-methyl-4-aminoazobenzene (concl.)

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

TUBE #	CONC. M/L(10-5)	Δ CONC. (10-5)	AMT. BOUND (10-7)	% BOUND	MG. N EXC	% BOUND MG. N	DEV.	DEV.2
16B	0.681							
16	0.604	0.077	0.077	11.3	0.644	17.5	7.0	49.00
17B	0.878							
17	0.784	0.094	0.094	10.7	0.644	16.6	7.9	62.41
18B	0.856							
18	0.762	0.094	0.094	11.0	0.644	17.1	7.4	54.76

Acetylated Bovine Serum Albumin and Methyl Orange

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	.868				
1	.730	.138	.2380	.765	-5.137
2B	.860				
2	.726	.134	.2290	.726	-5.140
3B	.694				
3	.564	.130	.2240	.564	-5.249
4B	.690				
4	.567	.123	.2120	.567	-5.247
5B	.506				
5	.408	.098	.1690	.408	-5.390
6B	.506				
6	.416	.090	.1550	.416	-5.381
7B	.307				
7	.260	.047	.0812	.260	-5.585
8B	.310				
8	.260	.050	.0863	.260	-5.585
9B	.252				
9	.209	.043	.0742	.209	-5.680

Acetylated Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	.948				
1	.675	.273	.471	.675	-5.171
2B	.948				
2	.685	.263	.455	.685	-5.165
3B	.719				
3	.530	.189	.326	.530	-5.276
4B	.729				
4	.524	.205	.354	.524	-5.281
5B	.535				
5	.393	.142	.245	.393	-5.406
6B	.549				
6	.393	.156	.269	.393	-5.406
7B	.374				
7	.233	.141	.243	.233	-5.633
8B	.384				
8	.248	.136	.234	.248	-5.606
9B	.272				
9	.184	.088	.152	.184	-5.736

Acetylated Bovine Serum Albumin and 3'-methyl-4-aminoazobensene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	.890				
1	.523	.367	.633	.523	-5.282
2B	.965				
2	.527	.438	.755	.527	-5.279
3B	.755				
3	.403	.352	.608	.403	-5.395
4B	.775				
4	.398	.377	.650	.398	-5.401
5B	.533				
5	.278	.255	.440	.278	-5.556
6B	.526				
6	.287	.239	.412	.287	-5.543
7B	.324				
7	.173	.151	.261	.173	-5.762
8B	.330				
8	.163	.167	.288	.163	-5.788
9B	.233				
9	.108	.125	.216	.108	-5.967
10B	1.039				
10	.575	.464	.800	.575	-5.241
11B	1.028				
11	.565	.563	.798	.565	-5.248
12B	1.010				
12	.569	.441	.761	.569	-5.245
13B	1.015				
13	.562	.453	.783	.562	-5.251
14B	.910				
14	.439	.471	.813	.439	-5.358

Acetylated Bovine Serum Albumin and 4'-methyl-4-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	Amt. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	.907				
1	.559	.348	.601	.348	-5.459
2B	.937				
2	.555	.382	.659	.382	-5.418
3B	.726				
3	.424	.302	.521	.302	-5.520
4B	.735				
4	.432	.303	.523	.303	-5.519
5B	.519				
5	.306	.213	.368	.213	-5.672
6B	.514				
6	.306	.208	.359	.208	-5.782
7B	.370				
7	.203	.167	.288	.167	-5.778
8B	.365				
8	.198	.167	.388	.167	-5.778
9B	.243				
9	.122	.121	.209	.121	-5.918
10B	1.109				
10	.700	.409	.705	.700	-5.155
11B	1.045				
11	.681	.364	.628	.681	-5.167
12B	1.055				
12	.632	.423	.730	.623	-5.200
13B	1.110				
13	.645	.465	.802	.645	-5.191
14B	.843				
14	.496	.357	.616	.496	-5.305
15B	.800				
15	.469	.331	.571	.469	-5.329

Acetylated Bovine Serum Albumin and 4'-methyl-4-aminoazobenzene (concl..)

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
16B	.582				
16	.338	.244	.421	.338	-5.472

Iodinated Bovine Serum Albumin and methyl orange

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	.823				
1	.556	.367	.461	.556	-5.26
2B	.582				
2	.437	.145	.250	.437	-5.36
3B	.416				
3	.289	.127	.219	.289	-5.54
4B	.307				
4	.213	.094	.162	.213	-5.67
5B	.180				
5	.119	.061	.101	.119	-5.92

Iodinated Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	1.140				
1	.765	.375	.647	.765	-5.12
2B	.826				
2	.520	.306	.528	.520	-5.28
3B	.719				
3	.456	.263	.454	.456	-5.34
4B	.685				
4	.442	.243	.420	.442	-5.35
5B	.407				
5	.228	.179	.309	.228	-5.64
6B	.204				
6	.107	.097	.167	.107	-5.97
7B	.480				
7	.301	.179	.309	.301	-5.52
8B	.388				
8	.247	.141	.243	.247	-5.61
9B	.398				
9	.276	.122	.210	.276	-5.56
10B	.296				
10	.165	.131	.226	.165	-5.77

Iodinated Bovine Serum Albumin and 3'-methyl-4-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	1.033				
1	.566	.467	.806	.566	-5.25
2B	1.040				
2	.572	.468	.805	.572	-5.24
3B	.780				
3	.432	.348	.600	.432	-5.36
4B	.791				
4	.442	.349	.600	.442	-5.35
5B	.530				
5	.288	.242	.418	.288	-5.54
6B	.533				
6	.281	.252	.435	.281	-5.55
7B	.370				
7	.186	.184	.328	.186	-5.73
8B	.363				
8	.193	.170	.294	.193	-5.71
9B	.265				
9	.137	.128	.221	.137	-5.86

Iodinated Bovine Serum Albumin and 4'-methyl-4-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-3} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	.992				
1	.609	.383	.610	.609	-5.22
2B	1.000				
2	.613	.387	.668	.613	-5.21
3B	.757				
3	.474	.283	.488	.474	-5.32
4B	.775				
4	.474	.301	.520	.474	-5.32
5B	.613				
5	.365	.248	.428	.365	-5.44
6B	.595				
6	.351	.244	.422	.351	-5.45
7B	.396				
7	.225	.171	.295	.225	-5.65
8B	.370				
8	.234	.136	.234	.234	-5.63
9B	.280				
9	.162	.118	.204	.162	-5.79

Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	1.115				
1	.670	.245	.423	.670	-5.061
2B	1.105				
2	.865	.240	.414	.865	-5.063
3B	.778				
3	.598	.180	.310	.598	-5.224
4B	.573				
4	.426	.147	.254	.426	-5.371
5B	.398				
5	.296	.102	.176	.296	-5.529

Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0°C. pH 9.1

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	1.150				
1	.539	.611	1.055	.539	-5.269
2B	1.175				
2	.564	.611	1.055	.564	-5.249
3B	.894				
3	.461	.433	.746	.461	-5.337
4B	.695				
4	.315	.380	.655	.315	-5.502
5B	.364				
5	.170	.194	.335	.170	-5.770

Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 25°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	1.145				
1	.992	.153	.264	.992	-5.004
2B	1.160				
2	.977	.183	.316	.977	-5.011
3B	.812				
3	.651	.161	.278	.651	-5.187
4B	.825				
4	.662	.163	.281	.662	-5.180
5B	.593				
5	.461	.132	.228	.461	-5.337
6B	.583				
6	.461	.122	.211	.461	-5.337
7B	1.229				
7	.957	.272	.469	.957	-5.020
8B	1.219				
8	.961	.258	.445	.961	-5.018
9B	1.068				
9	.802	.266	.459	.802	-5.096
10B	1.048				
10	.796	.252	.435	.796	-5.100
11B	.738				
11	.588	.150	.259	.588	-5.231
12B	.728				
12	.588	.140	.242	.588	-5.231

Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 25°C. pH 9.1

5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-1})	r	A (10^{-5})	LOG A
1B	1.185				
1	.841	.344	.593	.841	-5.076
2B	1.190				
2	.850	.340	.587	.850	-5.071
3B	.898				
3	.655	.243	.419	.655	-5.184
4B	.884				
4	.632	.252	.435	.632	-5.200
5B	.651				
5	.466	.185	.319	.466	-5.332
6B	.670				
6	.466	.204	.352	.466	-5.332
7B	.330				
7	.238	.094	.159	.238	-5.624

Acetylated Bovine Serum Albumin and p-aminoazobenzene — Beckman Spectrophotometer Model DU

Temp. 0°C. pH 9.1 — 5.8×10^{-8} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	ANT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	1.110				
1	.806	.304	.524	.806	-5.094
2B	1.120				
2	.788	.332	.572	.788	-5.104
3B	.879				
3	.608	.270	.465	.608	-5.217
4B	.855				
4	.612	.243	.419	.612	-5.214
5B	.714				
5	.510	.204	.352	.510	-5.293
6B	.709				
6	.496	.213	.367	.496	-5.305
7B	.426				
7	.296	.130	.224	.296	-5.529
8B	.441				
8	.286	.155	.267	.286	-5.544
9B	.315				
9	.199	.116	.200	.199	-5.702

Acetylated Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 25°C. pH 9.1

5.8×10^{-6} Moles Protein / Bag

TUBE #	CONC. (10^{-5})	AMT. BOUND (10^{-7})	r	A (10^{-5})	LOG A
1B	1.210				
1	.948	.262	.452	.948	-5.024
2B	1.195				
2	.934	.251	.434	.934	-5.030
3B	.889				
3	.689	.200	.345	.689	-5.162
4B	.889				
4	.684	.205	.354	.684	-5.165
5B	.622				
5	.495	.127	.209	.495	-5.306
6B	.632				
6	.485	.147	.254	.485	-5.315

Acetylated Bovine Serum Albumin and p-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 25°C. pH 6.8

5.8×10^{-8} Moles Protein / Bag

TUBE #	$CONC_F$ (10^{-5})	AMT. BOUND (10^{-7})	r	A_F (10^{-5})	LOG A
1B	1.215				
1	.972	.243	.419	.972	-5.013
2B	1.223				
2	.995	.228	.394	.995	-5.003
3B	.865				
3	.709	.156	.269	.709	-5.150
4B	.855				
4	.695	.160	.276	.695	-5.158
5B	.632				
5	.539	.093	.160	.539	-5.269
6B	.640				
6	.525	.115	.198	.525	-5.280

BINDING OF HOMOLOGS OF CERTAIN CARCINOGENIC AZO DYES WITH PROTEINS

by

ROGER DUANE BAUER

B. S., Beloit College, 1953

AN ABSTRACT OF THESIS

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Ever since the earliest workers showed that azo dyes were involved in the formation of tumors, scientists have shown an increasing interest in the formation and mechanism of protein dye complexes. This particular study was undertaken to help elucidate, if possible, the method of this complex formation between both normal and altered proteins and uncharged azo dyes which are homologs of a known carcinogenic agent, 4-dimethylaminoazobenzene. The three uncharged dyes, p-aminoazobenzene, 3'-methyl-4-aminoazobenzene, and 4'-methyl-4-aminoazobenzene were chosen for this study rather than the 4-dimethylaminoazobenzene and its homologs because of their increased solubility in aqueous media. The anionic dye, methyl orange, was used as a reference. The method of equilibrium dialysis was used for all the bindings in this study.

Liver homogenate from normal rats provided one source of protein for the study as well as both normal and altered bovine serum albumins. It had previously been thought that serum albumin was rather unique in the formation of complexes with these neutral dyes, but this study shows an appreciable amount of binding between the liver homogenate proteins and these azo dyes.

The -amino groups of lysine in bovine serum albumin (BSA) were acetylated with acetic anhydride to block these normally charged amino groups. Over 90 per cent of the original amino groups were found to be acetylated by means of formal titration. Binding of anionic dyes to this group was found to be blocked by acetylation. The binding of uncharged azo dyes of this study was found to be enhanced under certain conditions, however, upon acetylation of the protein. This would suggest that under certain conditions the -amino groups of lysine are not of major importance in the interaction with uncharged dyes. The increase in binding could possibly be due to unfolding of the complex protein molecule upon acetylation.

The tyrosine groups of the normal BSA were iodinated to diiodotyrosine by use of iodine in iodide solution to the extent of blocking approximately 60 per cent of the originally available tyrosine residues resulting in enhanced binding of all the dyes. These results suggest that the tyrosine residues are not specifically involved in the binding of these dyes to the protein. This enhanced binding might again be accounted for by unfolding of the complex protein molecule upon iodination.

It appears from this that the binding of a charged dye may be affected by removal of possible ionic binding sites, but the uncharged azo dyes of this study are not so affected. It is suggested that the complexes involving charged dyes and proteins at 0°C are mostly due to surface attraction between the dye and protein molecules.