removal of formic acid, the residue was carefully fractionated; 61 g. of product, b.p. 88° (13 mm.), was collected, $n^{20}D$ 1.4780, λ_{max} 242 mμ, ϵ_{max} 1330; reported[2] 88–90° (16 mm.), $n^{17}D$ 1.4778, λ_{max} 243 mμ, ϵ_{max} 1400.

Anal. Calcd. for $C_{11}H_{20}O$: C, 79.47; H, 10.91. Found: C, 79.46; H, 10.93.

2,2,6-Trimethylcyclohexene-1-glycolic Acid.—Five grams of the above compound and 4 g. of seleinous acid in 20 ml. of dioxane were refluxed for 3 hours. The ether extract of the residue, after removal of dioxane, was treated with sodium carbonate and then with 10% sodium hydroxide. Acidification of the hydroxide extract yielded the acid which after recrystallization from alcohol–water melted at 173° (with gas evolution).

Anal. Calcd. for $C_{11}H_{18}O_3$: C, 66.63; H, 9.14; neut. equiv., 198. Found: C, 66.48; H, 9.22; neut. equiv., 194.

β-Cyclocitral.—One gram of the glycolic acid in 25 ml. of glacial acetic acid was oxidized at 60–65° with 2.2 g. of lead tetraacetate added in two portions during two hours. To the cooled mixture 7 ml. of 1 *N* sulfuric acid was added, lead sulfate removed, the filtrate diluted with 300 ml. of ether and neutralized with a paste of sodium bicarbonate. The neutral ether layer yielded on evaporation 500 mg. of practically pure β-cyclocitral, $n^{25}D$ 1.4950, λ_{max} 248 mμ, ϵ_{max} 7880.

The semicarbazone was prepared and recrystallized from ethyl acetate; m.p. 165°, λ_{max} 274 mμ, ϵ_{max} 20700.

Anal. Calcd. for $C_{11}H_{19}ON_3$: C, 63.10; H, 9.16; N, 20.10. Found: C, 63.25; H, 8.90; N, 20.21.

All the recorded physical properties of β-cyclocitral and its semicarbazone are in agreement with those in the literature.

DEPARTMENT OF CHEMISTRY
THE MOUNT SINAI HOSPITAL
NEW YORK, N. Y.

Interactions of Homologs of Carcinogenic Azo Dyes and Bovine Serum Albumin[1-3]

BY R. K. BURKHARD AND F. A. MOORE

RECEIVED APRIL 25, 1955

Many studies have been made on the interactions between proteins and azo dyes, but the majority of these studies have been concerned with interactions involving cationic or anionic dyes.[4] Very little has been reported regarding the interactions of uncharged azo dyes and proteins.[5] This latter type of interaction might be of interest since certain of these uncharged azo dyes are involved in tumor formation.[6]

It has been reported that slight structural modifications in azo dye ions can affect the ability of these ions to complex with proteins.[7-9] This suggests that slight structural changes in uncharged azo dyes might also result in a similar effect. If this were true then we might have another clue to the relationship between carcinogenicity and chemical structure.

Protein–dye interactions involving 4-dimethylaminoazobenzene, 3′-methyl-4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminoazobenzene might be of value since these three dyes possess widely differing values for their relative carcinogenicities.[6] Studies with these dyes, however, are complicated by the fact that they are extremely insoluble in the aqueous solutions usually used for protein–dye interaction studies. The aminoazobenzene homologs of these compounds, however, are more soluble in water and yet possess the same structural relationships found among the members of the trio of carcinogens. Accordingly, it was thought desirable to study the interactions of 4-aminoazobenzene, 3′-methyl-4-aminoazobenzene and 4′-methyl-4-aminoazobenzene with proteins.

Bovine serum albumin was selected as the protein for this study since much is known concerning the nature of the complexes involving this protein. Hence any results obtained with bovine serum albumin might be more easily interpreted than results obtained with some less well characterized protein. If studies with bovine serum albumin proved to be successful, then the more complex systems involved in tumor formation might also be successfully studied.

Experimental

Synthesis and Properties of Dyes.—4-Aminoazobenzene was prepared in two steps by the preparation and rearrangement of diazoaminobenzene. 3′-Methyl-4-aminoazobenzene and 4′-methyl-4-aminoazobenzene were prepared in a manner similar to that used by Witt[10] in that *p*-nitrosoacetanilide was condensed with the appropriate toluidine in glacial acetic acid to yield the acetyl derivatives of the desired dyes. These were then hydrolyzed to yield the final products. All of the dyes were recrystallized from alcohol–water mixtures to give compounds melting at 122.5–123°, 96–97° and 151–152°, respectively (lit. values 125–126°[11] and 126°[12,13]; 89–91°[13]; 144–146°[13] and 153–154°,[14] respectively.)

The ionization constants for these dyes were determined colorimetrically by use of the Beckman model DU spectrophotometer. The solubilities of these dyes were determined by placing small amounts of the dyes in the phosphate buffer used for interaction studies and these mixtures agitated at 25°. Periodically, samples of each solution were withdrawn, filtered and diluted to concentration ranges wherein Beer's law was known to obtain. The concentrations of these solutions were then determined by use of the Beckman model DU spectrophotometer and from these values and the corresponding dilution factors the solubilities were obtained.

Interactions.—The spectral shift and equilibrium dialysis techniques developed by Klotz and his co-workers were used for these studies.[15,16] All spectral and analytical data were obtained by use of a Beckman model DU spectrophotometer using either 1 or 5 cm. cells. The dialysis studies were performed at 25°. The spectral and analytical data were taken at room temperatures. The concentrations of

(1) Contribution No. 517, Department of Chemistry, Kansas Agricultural Experiment Station, Manhattan, Kansas.

(2) Supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

(3) Presented in part before the Biological Section of the 124th National Meeting of the American Chemical Society, Chicago, Illinois, September 6, 1953.

(4) I. M. Klotz, "Protein Interactions," in "The Proteins," H. Neurath and K. Bailey, Editors, Academic Press, Inc., New York, N. Y., 1953, Vol. I, part B, pp. 727–806.

(5) I. M. Klotz and J. Ayers, THIS JOURNAL, **74**, 6178 (1952).

(6) J. A. Miller and E. C. Miller, "The Carcinogenic Aminoazo Dyes," in "Advances in Cancer Research," Vol. I, J. P. Greenstein and A. Haddow, Editors, Academic Press, Inc., New York, N. Y., 1953, pp. 340–396.

(7) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, *J. Phys. Chem.*, **56**, 77 (1952).

(8) I. M. Klotz, R. K. Burkhard and J. M. Urquhart, THIS JOURNAL, **74**, 202 (1952).

(9) R. K. Burkhard, B. E. Burgert and J. S. Levitt, *ibid.*, **75**, 2977 (1953).

(10) F. H. Witt, *Ber.*, **45**, 2380 (1912).

(11) O. N. Witt and E. G. P. Thomas, *J. Chem. Soc.*, **43**, 114 (1883).

(12) O. Silberrad and G. R. Rotter, *ibid.*, **89**, 169 (1906).

(13) L. von Michel and H. Stauffer, *Helv. Chim. Acta*, **24**, 151 (1941).

(14) P. Jacobsen, *Ann.*, **427**, 153 (1922).

(15) I. M. Klotz, THIS JOURNAL, **68**, 2299 (1946).

(16) I. M. Klotz, F. M. Walker and R. B. Pivan, *ibid.*, **68**, 1486 (1946).

the dye solutions ranged from approximately 4.5 to 11 $\times$ 10^{-6} mole/l. for 4-aminoazobenzene and 3.5 to 10 $\times$ 10^{-6} mole/l. for the methylated dyes. All protein solutions were 0.2% by weight. Dye and protein solutions were prepared by dissolving these substances in a phosphate buffer (*p*H 6.8) which contained 0.055 mole of Na_2HPO_4 and 0.044 mole of KH_2PO_4 per liter of solution. The protein used was Crystallized Bovine Plasma Albumin (referred to as bovine serum albumin in this paper) as obtained from the Armour Laboratories. Samples of the protein were dried to constant weight to determine the moisture content.

Treatment of Data.—The data obtained were converted into values for $1/r$ (the reciprocal of the moles of dye bound per mole of protein) and $1/A$ (the reciprocal of the free dye concentration). The method of least squares was then used to calculate the intercepts and slopes for the equations having the form[17]

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nkA}$$

Estimated values for $1/r$ for given values of $1/A$ and the 95% confidence intervals for these estimated values were then calculated from these least squares equations. The *t*-test was then used to determine the statistical significances of the differences of the slopes and the calculated $1/r$ values obtained from data involving the two methylated dyes.

Results and Discussion

All three of the dyes exhibited changes in molar extinction coefficients and shifts in wave lengths of maximum absorption toward longer wave lengths when mixed with bovine serum albumin (Table I). The small change observed with 4-aminoazobenzene and bovine serum albumin suggested that this dye might be bound to the protein in the smallest quantity. This was confirmed by equilibrium dialysis studies. The fact that all of the spectral shifts were toward longer wave lengths suggested that these dyes might be bound to the protein in a common manner.

TABLE I

SPECTRAL PROPERTIES OF DYES AND THEIR PROTEIN COMPLEXES

System[a]	Wave length of max. absorption (Å.)	Molar extinction coefficient $\times 10^{-4}$
AB	3750	2.06
AB and BSA	3800	2.02
3′-Me-AB	3800	3.13
3′-Me-AB and BSA	3850	3.26
4′-Me-AB	3750	2.22
4′-Me-AB and BSA	3800	2.10

[a] Abbreviations: AB, (4-aminoazobenzene); 3′-Me-AB, (3′-methyl-4-aminoazobenzene); 4′-Me-AB, (4′-methyl-4-aminoazobenzene); BSA, (bovine serum albumin).

The values for $1/n$ and $1/nk$ as obtained from least squares calculations involving the equilibrium dialysis data are listed in Table II. Estimated values for $1/r$ and their 95% confidence intervals for selected values of $1/A$ are listed in Table III.

TABLE II

VALUES FOR $1/n$ AND $1/nk$ FROM LEAST SQUARES CALCULATIONS

System[a]	No. of determn.	$1/n$	$1/nk$
AB and BSA	19	0.523	24.0×10^{-6}
3′-Me-AB and BSA	12	− .126	18.1×10^{-6}
4′-Me-AB and BSA	18	.0439	19.1×10^{-6}

[a] Abbreviations: same as in Table I.

(17) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946).

TABLE III

ESTIMATED VALUES AND 95% CONFIDENCE INTERVALS FOR $1/r$ AT GIVEN VALUES FOR $1/A$

$1/A$ (1/mole)	System[a]	$1/r$	95% confidence interval
1.00×10^5	AB and BSA	2.92	2.58–3.26
	3′-Me-AB and BSA	1.68 *	1.30–2.06
	4′-Me-AB and BSA	1.87	1.49–2.25
1.67×10^5	AB and BSA	4.52	3.92–5.12
	3′-Me-AB and BSA	2.89 *	2.65–3.13
	4′-Me-AB and BSA	3.24	2.99–3.48
2.00×10^5	AB and BSA	5.31	5.04–5.55
	3′-Me-AB and BSA	3.49 *	3.28–3.70
	4′-Me-AB and BSA	3.87	3.63–4.11
2.50×10^5	3′-Me-AB and BSA	4.39	4.12–4.67
	4′-Me-AB and BSA	4.83 *	4.50–5.16
2.86×10^5	3′-Me-AB and BSA	5.05	4.74–5.35
	4′-Me-AB and BSA	5.51	5.09–5.94

[a] Abbreviations: same as in Table I. Values separated by * differ significantly at the 5% level. Values for AB are not listed for $1/A$ values of 2.50 and 2.86×10^5 due to the fact that the observations involving AB did not extend to these concentrations.

Statistical analysis of these data revealed not only that 4-aminoazobenzene was bound to the protein in smaller quantities than the methylated dyes, but also that over the majority of the free dye concentration range studied there were not statistically significant differences at the 5% level between the amounts of 3′-methyl-4-aminoazobenzene and 4′-methyl-4-aminoazobenzene which were bound to the protein. It was previously reported that 3′-methyl-4-aminoazobenzene was bound to bovine serum albumin in slightly larger amounts than 4′-methyl-4-aminoazobenzene.[18] However, statistical analysis of the original and subsequent data has not entirely borne out this observation. This analysis showed that at free dye concentrations of 4 and 5 $\times$ 10^{-6} mole/l. there were significant differences at the 5% level between the bindings of the two methylated dyes with the 3′-methyl isomer being bound slightly more than the 4′-methyl-isomer (t = 2.18 and 2.31, respectively, D.F. = 26). However, at concentrations of 3.5 $\times$ 10^{-6} and from 6 to 10 $\times$ 10^{-6} mole/l. the observed differences were not found to be significant at the 5% level. This is probably due to the fact that the variance about a least squares line is at a minimum at the center of the line. The reciprocals of 4 and 5 $\times$ 10^{-6} mole/l. fall near this area when $1/r$ is plotted against $1/A$. Statistical analysis showed further that the slopes of the lines obtained from data involving the methylated dyes were not significantly different at the 5% level (t = 0.44, D.F. = 26).

One suggestion as to the cause for these differences in extents of interaction is the possible existence of a relationship between the basicities of the dyes and their abilities to complex with bovine se-

(18) R. K. Burkhard, Abstracts, 124th National Meeting of the American Chemical Society, Chicago, Illinois, 1953, p. 15C.

rum albumin. This would appear likely since it has been shown that the interaction of 4-aminoazobenzene and bovine serum albumin involves the amino group of the dye.[5] It was found, however, that 4'-methyl-4-aminoazobenzene was the strongest base of the three dyes examined and that 3'-methyl-4-aminoazobenzene and 4-aminoazobenzene had similar basicities (Table IV).

TABLE IV

pK_a's FOR DYES

Dye[a]	No. of detmn.	Mean pK_a	Standard error
AB	5	2.90	0.0132
3'-Me-AB	4	2.88	.0123
4'-Me-AB	4	3.04	.0230

[a] Abbreviations: same as in Table I. The lit. values for the pK_a of AB include 2.90 as calculated from the k_a of 1.25×10^{-3} reported by Farmer and Warth[19] and 2.80 as reported by Hammett and Paul.[20]

Thus if a relationship exists between basicity and the extent of interaction, one would expect 4-aminoazobenzene and 3'-methyl-4-aminoazobenzene to be bound to the protein in similar quantities. This was not observed and thus it was concluded that basicities could not be directly correlated to the binding observed in these cases.

Another suggested explanation for the binding observed might be the possible existence of a relationship between solubility and the extent of interaction. It was found that 3'-methyl-4-aminoazobenzene and 4'-methyl-4-aminoazobenzene were much less soluble than 4-aminoazobenzene when the phosphate buffer was used as a solvent (Table V). Thus if solubility were related to the extent of binding then one would expect that 3'-methyl-4-aminoazobenzene and 4'-methyl-4-aminoazobenzene both would be bound to bovine serum albumin in nearly equal quantities. This was observed and thus it was concluded that in this case solubility could be related to the extent of binding in an inverse manner.

TABLE V

SOLUBILITIES OF DYES IN PHOSPHATE BUFFER (pH 6.8, 25°)

Dye[a]	Time of agitation, hr.	Solubility (mole/l.)
AB	48	1.39×10^{-4}
	84	1.37×10^{-4}
3'-Me-AB	48	1.79×10^{-5}
	84	1.79×10^{-5}
4'-Me-AB	48	1.59×10^{-5}
	84	1.58×10^{-5}

[a] Abbreviations: same as Table I.

The fact that solubility and extent of binding can be related to each other may not be the only way in which the observed data can be explained. The solubility of a molecule can be attributed to a number of factors among which is the weight of the molecule involved. The weight of the molecule may not only affect the solubility of the molecule but it might also affect the ability of the molecule to engage in protein binding through van der Waals forces. Since it was observed that the two methylated dyes were bound to the protein in equal (or nearly equal) and larger quantities than 4-aminoazobenzene, it was concluded that in this study the extents of interaction also could be related to the molecular weights of the dyes. Thus it appears that factors such as solubility, or molecular weight, or van der Waals forces are more important than basicity in determining the abilities of these three dyes to form complexes with bovine serum albumin.

Acknowledgment.—The authors are indebted to Dr. Holly C. Fryer, Department of Mathematics, Kansas State College, for advice regarding the statistical analysis of the observed data.

DEPARTMENT OF CHEMISTRY
KANSAS STATE COLLEGE
MANHATTAN, KANSAS

(19) R. C. Farmer and F. J. Warth, *J. Chem. Soc.*, **85**, 1713 (1904).
(20) L. P. Hammett and M. A. Paul, THIS JOURNAL, **56**, 827 (1934).

The Halogenation of Some 2- and 3-Amino Derivatives of Dibenzothiophene

BY HENRY GILMAN AND GENE R. WILDER

RECEIVED JULY 5, 1955

Earlier work on the halogenation[1] and nitration[2-5] of amino derivatives of dibenzothiophene indicate that isomers formed during electrophilic attack on the amino derivative are influenced both by the size of the group attached to the amine, *i.e.*, the carbonyl component, and the size of the electrophile.

The bromination of 2-acetamidodibenzothiophene[1,6] yields 2-acetamido-3-bromodibenzothiophene[1] as has been shown by deamination followed by oxidation to the known 3-bromodibenzothiophene-5-dioxide.[7] Also reported was the chlorination of 2-acetamidodibenzothiophene with sulfuryl chloride to give a chloro-2-acetamidodibenzothiophene which was believed by analogy to be the 3-isomer.[1] This material was actually 1-chloro-2-acetamidodibenzothiophene as was shown in the present work by deamination and comparison with an authentic specimen of 1-chlorodibenzothiophene.[4]

In the nitration of 3-acetamido- and 3-benzamidodibenzothiophene,[5] good yields of the 4-nitro isomer were obtained in both cases. Chlorination, however, affords mixed isomers in both cases, namely, the 2- and 4-chloro isomers. The 2-chloro isomer was identified by deamination and oxidation to the 2-chlorodibenzothiophene-5-dioxide which corresponds to the 2-chlorodibenzothiophene-5-dioxide reported by Courtot,[8] who obtained it by treating 2-nitrodibenzothiophene with thionyl chloride followed by oxidation.

Chlorination appears to follow nitration in the cases of the 2-acetamido- and 2-benzamidodibenzothiophene,[5] *i.e.*, chlorination of 2-benzamidodibenzothiophene goes in the 3-position as does bromination. The bromo-2-benzamidodibenzothiophene was identified by hydrolysis to the known 3-bromo-

(1) H. Gilman and S. Avakian, THIS JOURNAL, **68**, 1514 (1946).
(2) E. Sawicki, *J. Org. Chem.*, **18**, 1942 (1953).
(3) E. Sawicki, *ibid.*, **19**, 608 (1954).
(4) H. Gilman and G. Wilder, THIS JOURNAL, **76**, 2906 (1954).
(5) H. Gilman and G. Wilder, *ibid.*, **77**, 3920 (1955).
(6) H. Gilman and A. L. Jacoby, *J. Org. Chem.*, **3**, 108 (1938).
(7) H. Gilman, A. L. Jacoby and H. A. Pacevitz, *ibid.*, **3**, 120 (1938).
(8) M. Courtot, *Compt. rend.*, **198**, 2260 (1934).