not a normal metabolic product but appears as a detoxification product following a high intake of nicotinic acid (not amide).

It appears that at normal levels of intake both nicotinic acid and nicotinamide are excreted by the rat chiefly as N^1-methylnicotinamide and/or a similar compound active toward *T. cremoris* (see ref. 17) as originally stated by Huff, *et al.*,[20] and by Rosen, *et al.*,[21] while when large doses of nicotinic acid or nicotinamide are given, much higher amounts of the other metabolites appear, some of which should be regarded as "detoxification products."

When *T. cremoris* bioautographs were run on the strips from these rat urines, zones of growth occurred at the nicotinic acid and nicotinamide positions and at the N^1-methylnicotinamide position. This again indicates that the spot identified as N^1-methylnicotinamide is a mixture of at least two compounds, one of which is active for *T. cremoris* (N^1-methylnicotinamide is not active for *T. cremoris;* see reference 17).

A study of the identity of the unknown spot 8 is at present under way. From Fig. 4 it is seen that this spot is due to a compound which does not react with cyanogen bromide.

There is considerable variation in the amount of radioactivity occurring in the feces; however, the values are comparatively small, indicating that only a small portion of the intraperitoneally injected vitamin was secreted into the intestine and excreted in the feces (see Table I).

The presence of C^{14} in the expired CO_2 demonstrates that decarboxylation of both nicotinic acid and its amide does occur in the body. It also appears that the excretion of $C^{14}O_2$ is higher in rats receiving C^{14}-nicotinamide than in those receiving C^{14}-nicotinic acid, indicating that a greater degree of decarboxylation has taken place with nicotinamide than with nicotinic acid. This observation is in accord with the data of Roth, *et al.*, using mice.[14]

URBANA, ILLINOIS

(20) J. W. Huff and W. A. Perlzweig, *Science*, **97**, 538 (1943).

(21) F. Rosen, J. W. Huff and W. A. Perlzweig, *J. Biol. Chem.*, **163**, 343 (1946).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, KANSAS STATE COLLEGE]

An Anomaly Among Interactions Involving Certain Azo Dye Anions and Bovine Serum Albumin[1,2]

BY R. K. BURKHARD, B. E. BURGERT AND J. S. LEVITT

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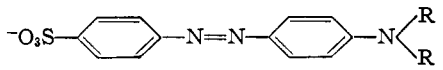
The 4′-aminoazobenzene-4-sulfonate anion is bound very slightly to bovine serum albumin at 25° and *p*H 7.3. If alkyl groups are placed on the amino nitrogen atom of this parent anion, or if the amino group of this parent anion is removed completely then the extent of interaction with bovine serum albumin increases. The interaction involving the 4′-dibutylaminoazobenzene-4-sulfonate anion (butyl orange) and bovine serum albumin exhibits an anomalous temperature dependence. This phenomenon is believed to be due to a temperature dependent association and dissociation of the butyl orange anions.

Recent investigations involving the interactions of various N,N-dialkylaminoazobenzene sulfonate anions with bovine and human serum albumins



have revealed that these two proteins evidently form similar complexes with certain azo dye anions when in solutions of *p*H 6.8, but form radically different complexes when in solutions of *p*H 9.2.[3,4] In the case of the complexes involving bovine serum albumin evidently the negatively charged sulfonate group of the azo dye is attracted to a positively charged ammonium group of the protein at both *p*H values. However, with human serum albumin this type of binding occurs only at the lower *p*H value, and when the *p*H is raised to 9.2 a second type of binding site becomes available in which the basic amino nitrogen atom of the azo dye can also be involved in the interaction by virtue of its ability to participate in hydrogen bonding to a phenolic tyrosine residue of the protein molecule.

One can thus easily account for the marked drop in the extent of interaction of various N,N-dialkylaminoazobenzene sulfonate anions with human serum albumin at *p*H 9.2 for as the size of the alkyl group is increased the reaction site involving the basic amino nitrogen atom of the dye is blocked. In the case of the interactions involving bovine serum albumin, however, this effect may not occur if only the charged sulfonate group of the dye is involved in complex formation.

It would appear then that additional support for the contention that the complexes involving bovine serum albumin and azo dyes related to methyl orange utilize only one reaction site of the dye, namely, the charged sulfonate group, could be gained by studying the interactions involving homologs of methyl orange and related compounds with bovine serum albumin in more detail. Accordingly, a study was undertaken to determine the influence which N-alkyl groups in N-alkylated aminoazobenzenesulfonate anions might have on complex formation with bovine serum albumin.

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(2) Presented in part at the Meeting in Miniature sponsored by the Kansas City Section of the American Chemical Society, Kansas City, Missouri, November 18, 1952.

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

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

Experimental

The extent of interaction was determined by the equilibrium dialysis technique as developed by Klotz and his coworkers at Northwestern University.[5] The studies were performed at two temperatures (0 and 25°) and at two *p*H values (6.8 and 7.3). The determinations at 0° were realized by using a cracked ice-water bath. The determinations at 25° were realized by using a thermostatically controlled constant temperature bath. Phosphate buffers were used to maintain the *p*H of the solutions. The 6.8 buffer contained 0.056 mole of Na_2HPO_4 and 0.044 mole of KH_2PO_4 per liter of solution. The 7.3 buffer contained 0.160 mole of Na_2HPO_4 and 0.050 mole of KH_2PO_4 per liter of solution. All analytical determinations were performed on a Beckman model DU spectrophotometer. The protein concentration used for these studies was 0.2%. The protein was obtained from the Armour Company.

The ability of butyl orange anions to form micelles in aqueous solution was studied by two independent methods—spectrophotometry and conductometry. All spectral studies were performed at *p*H 6.8 and at room temperature by the use of a Beckman model DU spectrophotometer. The analysis of a wide range of concentrations was realized by the use of both one-centimeter cells and nine-millimeter inserts. Spectral data were taken only at the wave length of maximum absorption. Conductance measurements were performed at 0 and 25° by use of an Industrial Instruments model RC-16 conductance bridge. The temperatures were realized by the methods used for the equilibrium dialysis studies. The cell constant of the conductance cell was 0.246. The specific conductance of the water was 0.850×10^{-6} ohm^{-1} cm.$^{-1}$ at 0° and 1.54×10^{-6} ohm^{-1} cm.$^{-1}$ at 25°. These values were taken into consideration when the data were examined.

Azobenzene-4-sulfonic acid trihydrate was prepared according to the procedure outlined by Stein and Moore.[6] 4'-Aminoazobenzene-4-sulfonic acid was prepared by converting the sodium salt of this compound as obtained from the Eastman Kodak Company into the free sulfonic acid. 4'-Methylaminoazobenzene-4-sulfonic acid was prepared by coupling diazotized sulfanilic acid with methylaniline. The sodium salt of this compound was collected and then converted into the free sulfonic acid. The purity of each of these sulfonic acids was determined by titration with sodium hydroxide. Sodium 4'-dimethylaminoazobenzene-4-sulfonate (methyl orange) was obtained from commercial sources and was recrystallized before use. 4'-Dibutylaminoazobenzene-4-sulfonic acid (butyl orange) was obtained from Dr. I. M. Klotz of Northwestern University.

Results and Discussion

Equilibrium dialysis studies involving the interactions of bovine serum albumin and the following dye anions at 25° and *p*H 7.3 were first undertaken: azobenzene-4 sulfonate, 4'-aminoazobenzene-4-sulfonate, 4'-methylaminoazobenzene-4-sulfonate, 4'-dimethylaminoazobenzene-4-sulfonate (methyl orange) and 4'-dibutylaminoazobenzene-4-sulfonate (butyl orange). These studies were undertaken to determine whether the presence and size of N-alkyl groups on an aminoazobenzenesulfonate anion have any influence on the ability of the anion to complex with this protein. The azobenzene-4-sulfonate anion was added to determine the effect of replacing the amino group of the aminoazobenzenesulfonate anion by a hydrogen atom. It was found (Fig. 1) that at 25° and *p*H 7.3 the 4'-aminoazobenzene-4-sulfonate anion is bound to bovine serum albumin in very small amounts and that as alkyl groups are placed on the amino nitrogen atom of this parent anion the extent of interaction increases. Also the removal of the amino group of the 4'-aminoazobenzene-4-sulfonate anion increases the extent of interaction. These findings are in agreement with those of other workers who have found that the introduction of polar groups such as hydroxyl or amino groups into an anion decreases the ability of the anion to form complexes with bovine serum albumin.[7]

Thus one can account for these findings on the basis that the polar amino nitrogen atom of the 4'-aminoazobenzene-4-sulfonate anion is responsible for the slight interaction observed. As this amino nitrogen atom is masked or removed the extent of interaction increases. If this nitrogen atom is masked by large butyl groups as in the case of butyl orange then not only would the masking effect tend to increase binding but also the increase in molecular weight would cause an increase in binding due to increased van der Waals forces. In the case of the azobenzene-4-sulfonate anion where the polar nitrogen atom is removed entirely the extent of interaction increases but not as much as in the case of butyl orange because the contribution due to van der Waals forces is somewhat lower than that of butyl orange. Thus one might conclude that N-alkyl groups in certain azo dyes related to methyl orange have an indirect role in binding by making the dye anion more compatible to the bovine serum albumin.

The data obtained at 25° and *p*H 7.3 were quite unexpected for it had been shown previously that at 0° and *p*H 6.8 very little difference existed between the extents of interaction observed with methyl orange and butyl orange with bovine serum albumin.[8] Thus it was desired to recheck these interactions at 0°. Determinations were made thus at 0°, *p*H 6.8 and *p*H 7.3 which showed that at this lower temperature the extent of interaction between butyl orange and bovine serum albumin is markedly lower than that observed at 25° (Fig. 2). It was quite evident that this interaction is very much dependent upon the temperature of the system. If this be true then the butyl orange–bovine serum albumin system shows a peculiar temperature dependence for previous work by Klotz and his coworker has shown that in many cases protein-dye interactions are relatively independent of temperature.[9]

One can speculate as to the cause for this peculiar temperature dependence. One suggestion is that at 25° the bovine serum albumin molecule undergoes a slight unfolding which might make new reaction sites available for the butyl orange anion that could not be enjoyed by other anions. Since the butyl orange anion has the amino nitrogen atom masked by alkyl groups it would appear that if a new type of reaction site were available that favors butyl orange then it would essentially involve the long hydrocarbon chains attached to the azo dye anion. If, for example, at 25° the serum albumin molecule unfolded so as to release hydrophobic residues that could attract the hydrocarbon-like groups of an azo dye then certainly an anion such as butyl orange would bind more to the protein at the higher temperature than an anion that had less hydrocar-

(5) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(6) W. H. Stein and S. Moore, "Biochemical Preparations," Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1949, p. 15.

(7) J. M. Luck, *Disc. Faraday Soc.*, **6**, 44 (1949).

(8) R. K. Burkhard, Ph.D. Thesis, Northwestern University, 1950.

(9) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 847 (1949).

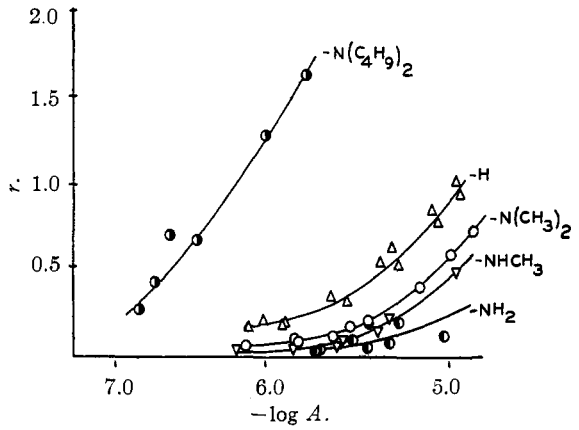

Fig. 1.—The interactions of various azo dye anions. ($X-C_6H_4-N{=}N-C_6H_4-SO_3^-$) and bovine serum albumin at 25° and *p*H 7.3. *r* is the ratio of the moles of dye anion bound per mole of protein; *A* is the molar concentration of the free dye anion.

bon characteristics. Accordingly, it was thought that the temperature dependence of an azo dye anion which has no amino nitrogen atom should be studied. Such an azo dye would perhaps be more hydrocarbon-like in nature than methyl orange and yet not have the bulky alkyl groups that butyl orange has. Temperature studies with the azobenzene-4-sulfonate anion and bovine serum albumin were undertaken and it was found that this anion shows the typical temperature dependence observed with other systems (Fig. 3). Thus it appears that

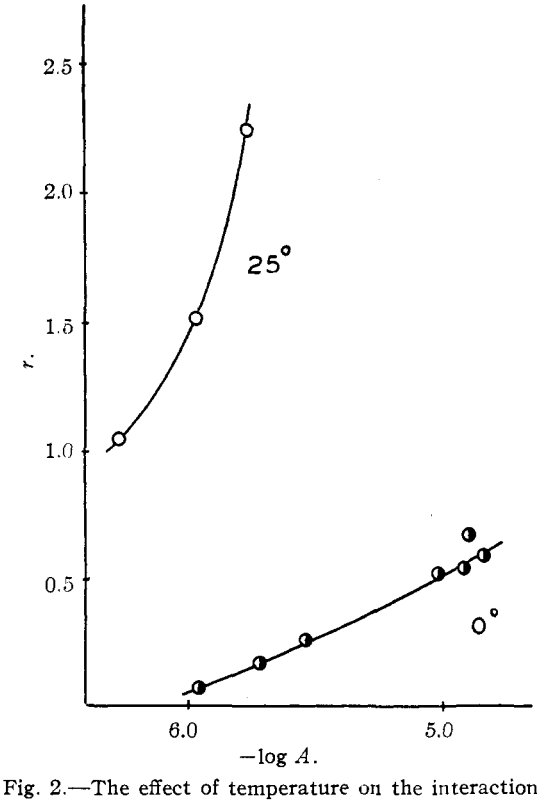

Fig. 2.—The effect of temperature on the interaction involving butyl orange and bovine serum albumin at *p*H 6.8. *r* is the ratio of the moles of dye anion bound per mole of protein; *A* is the molar concentration of the free dye anion.

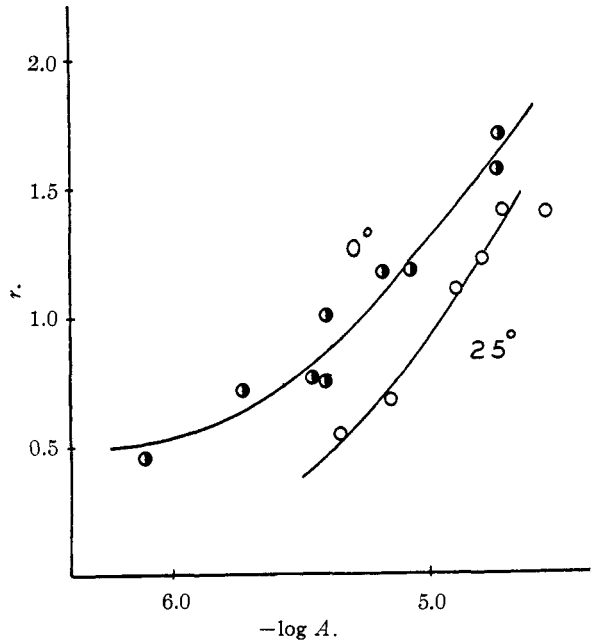

Fig. 3.—The effect of temperature on the interaction involving the azobenzene-4-sulfonate anion and bovine serum albumin at *p*H 6.8. *r* is the ratio of the moles of dye anion bound per mole of protein; *A* is the molar concentration of the free dye anion.

the temperature dependence observed with butyl orange is probably not directly due to the lack of, or the steric hindrance of, a polar amino group, both of which tend to increase the hydrocarbon-like characteristics of the azo dye anion. This could then be extended to imply that the temperature dependence observed with butyl orange is probably not due to an uncoiling of the protein to yield more favorable reaction sites but is due to some other factor.

Another suggested explanation for the observed temperature effect is that butyl orange anions might form micelles under conditions of the experiment while other azo dye anions might not. It appears very likely that the ability of a single anion to react with a protein will differ from that of an aggregate of these anions. If the extent of aggregation is greatly affected by temperature then the over-all observed ability of the anion in question to combine with proteins will probably also be greatly affected by temperature. Thus a study of the state of azo dye anions in aqueous solutions was undertaken to determine whether micelle formation could account for the unusual temperature effect observed.

It was found that at *p*H 6.8 and at room temperature solutions of methyl orange obey Beer's law over a wide concentration range, but that solutions of butyl orange obey Beer's law only at concentrations less than 1×10^{-4} mole per liter (Fig. 4). Above this concentration butyl orange shows a marked drop in the molar extinction coefficient indicating that at these higher concentrations micelle formation could be occurring. A solution of butyl orange of concentration 1×10^{-4} mole per liter has an optical density greater than three if one-centimeter cells are used and thus this deviation from Beer's law would likely go undetected in routine analysis.

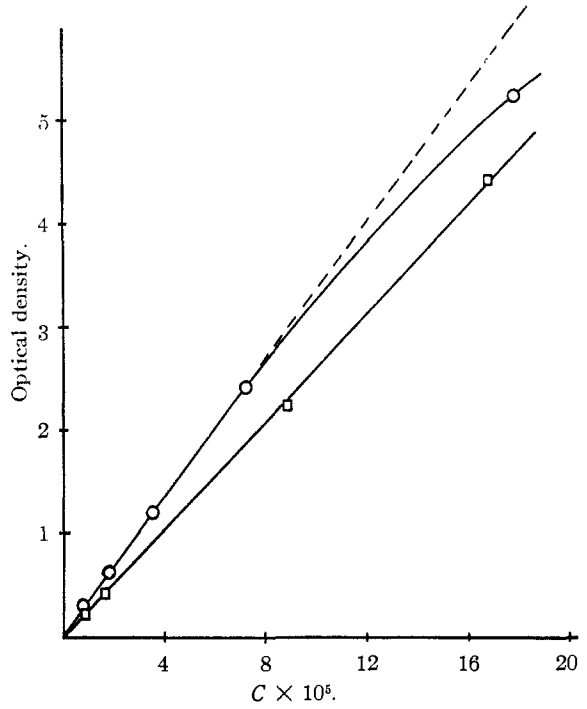

Fig. 4.—The relationship between optical density of and the molar concentration (C) of methyl orange and butyl orange solutions at room temperature and pH 6.8: □, represents methyl orange; O, represents butyl orange. The dashed line represents the extrapolation that would be expected if Beer's law had been obeyed.

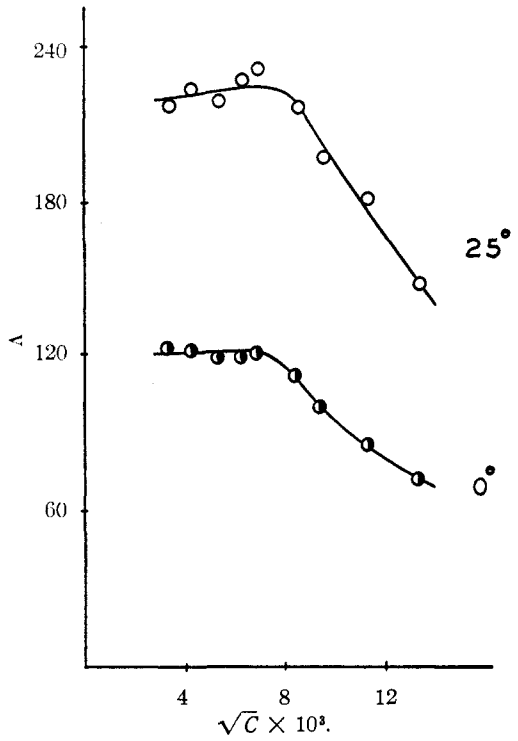

Fig. 5.—The relationship between equivalent conductance (Λ) and the molar concentration (C) of butyl orange solutions at 0 and 25°.

Conductance measurements at both of the temperatures, 0 or 25°, showed a marked decrease in equivalent conductance of butyl orange solutions of concentrations higher than 5×10^{-5} mole per liter (Fig. 5). Conductance measurements of methyl orange solutions in approximately the same concentration range were performed and they showed no such drop in equivalent conductance and agreed essentially with the results obtained by Robinson and Garrett who likewise concluded that methyl orange does not form micelles in this concentration range.[10] Thus it appears that butyl orange anions can undergo micelle formation in solutions that approximate those used in equilibrium dialysis studies and that the temperature effect observed with butyl orange and bovine serum albumin is probably due to a temperature dependent association and dissociation of the butyl orange anions.

These findings suggest a word of caution in regard to protein–dye interactions. Many dye analyses are done on a spectrophotometer in a concentration range suited to maximum precision. However, as pointed out above the mere fact that micelle formation cannot be shown under the conditions of routine analysis does not rule out the possibility of micelle formation at concentrations slightly higher than those which can be conveniently determined spectrophotometrically. It is of utmost importance, therefore, to determine whether an anion which is used in protein binding studies has a tendency to form micelles under the conditions used for these binding studies. If micelle formation is apt to occur in a system then one may be misled as to the true significance of the results obtained with such a system.

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(10) C. Robinson and H. E. Garrett, *Trans. Faraday Soc.*, **35**, 771 (1939).