[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Competitive Interaction of Organic Anions with Bovine Serum Albumin[1]

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The accumulating evidence furnished by studies on the binding properties of serum albumins points to the conclusion that the intrinsic association constants of the binding sites, for any particular albumin, are not identical but may be spread over a wide range of values. Such variations suggest that there exist considerable structural differences among the several combining regions of the protein molecule. We have previously shown,[3,4] for example, that the binding by bovine albumin of two structurally dissimilar organic anions, a long-chain alkyl sulfate and a carboxylic azo dye, required in each instance the assumption of heterogeneity for its quantitative interpretation.

The heterogeneity of the binding sites of albumin raises an interesting problem which is particularly significant for the hypothesis of configurational adaptability which we have advanced to account for the distinctive binding properties of serum albumins.[4] This is the problem of the relative heterogeneity of the binding sites with respect to the various complexing anions. Do the sites which bind one anion most strongly also bind other anions most effectively? That the binding sites are not completely specific in their selection is clear from such studies as those of Klotz and co-workers[5,6] who showed by spectral means that various organic acids could displace methyl orange and azosulfathiazole from bovine albumin. These studies were not designed, however, to deal with the problem of relative heterogeneity.

In the attempt to elucidate this problem we have investigated the competitive binding by bovine serum albumin of sodium dodecyl sulfate and the azo dye, *p*-(2-hydroxy-5-methylphenylazo)-benzoic acid. These anions were selected because they had previously been studied[3,4] and because the analysis of each anion in the presence of the other is feasible.

Experimental

Materials.—The bovine serum albumin used in the study was crystallized protein obtained from Armour and Company. Corrections for moisture and ash were made as previously described.[4] The sodium dodecyl sulfate was a specially purified sample generously supplied by the Fine Chemicals Division of E. I. du Pont de Nemours and Company. It was the same sample employed in our earlier investigation[3] where its analysis was reported. The dye, *p*-(2-hydroxy-5-methylphenylazo)-benzoic acid, prepared by coupling diazotized *p*-aminobenzoic acid to *p*-cresol, was the same material described before.[4]

Dialysis Method.—The binding of the dye was determined by equilibrium dialysis with 15-ml. volumes inside and outside, in the manner previously indicated.[4] All the experiments were conducted at 25.0 ± 0.1° in 0.05 *M* phosphate buffer, *p*H 7.0 with an initial protein concentration, inside the bag, of 3.00×10^{-5} *M*. This is based on a molecular weight of 69,000 for bovine serum albumin.[7]

The effect of bound dodecyl sulfate on the binding of the dye was investigated by conducting a series of experiments in which various predetermined amounts of the detergent were included in the albumin solutions. These quantities were selected so that the average number of detergent molecules bound per protein molecule would be integral values. The range of these values was from 0 to 8, inclusive. For the values 1 to 6, a 1% excess over the amount of detergent which was to be bound was used and for the two higher values the necessary total concentrations were estimated from the binding curve of dodecyl sulfate.[8] In each case controls were included in which dye was omitted. It was also established that the dialysis times used, ten to twelve hours, were sufficient to permit the detergent distribution to reach equilibrium. Duplicate tubes were always used in the dialysis experiments.

Analytical Methods.—Dye concentrations were determined by spectrophotometric measurement in the manner previously described.[4] The analysis for dodecyl sulfate was carried out by a sensitive colorimetric method recently developed.[8] This method permits detection of the detergent at a minimum concentration of about 1×10^{-7} *M*. In the presence of the dye a small correction must be made due to its interfering spectral absorption.

Results

The experimental results are given in Table I. These include a correction for the casing adsorption of the dye; no correction is necessary for the detergent since it is not adsorbed. Table I also shows the values of r and r/c where r is the average number of moles of dye bound per mole of protein at the free dye equilibrium concentration c. These quantities have been plotted in Fig. 1 for the moles of detergent bound per mole of protein ranging from 0 to 8. The curve for detergent absent is taken from earlier data.[4] Curves have been drawn through the points according to the best visual fit and extrapolated linearly to the r/c axis. In each experiment analyses for dodecyl sulfate were carried out with the outside solutions of the control, in which no dye was used, and the samples containing the largest amount of dye. These determinations demonstrated that the integral values for the detergent bound were correct in all cases to within about 1%. Also, they showed that not more than 1% of the bound detergent was displaced by the bound dye.

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(2) Investigation conducted during tenure of a Fellowship in Cancer Research of the American Cancer Society, recommended by the Committee on Growth of the National Research Council; present address: The Neurological Institute, New York 32, N. Y.

(3) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

(4) F. Karush, *ibid.*, **72**, 2705 (1950).

(5) I. M. Klotz, *ibid.*, **68**, 2299 (1946).

(6) I. M. Klotz, H. Triwush and F. M. Walker, *ibid.*, **70**, 2935 (1948).

(7) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *ibid.*, **69**, 1753 (1947).

(8) F. Karush and M. Sonenberg, *Anal. Chem.*, **22**, 175 (1950).

TABLE I

COMPETITIVE BINDING OF DODECYL SULFATE AND DYE BY BOVINE SERUM ALBUMIN AT 25° IN 0.05 *M* PHOSPHATE BUFFER, *p*H 7.0; INITIAL PROTEIN CONCN. 3.00×10^{-5} *M*

Initial dye concn. m./l. $\times 10^5$	Concn. of free dye(c) m./l. $\times 10^5$	r	$r/c \times 10^{-4}$
r for detergent = 1; initial detergent concn. 3.03×10^{-5} *M*			
17.80	3.10	3.66	11.8
11.80	1.73	2.67	15.4
7.88	1.00	1.89	18.9
5.94	0.72	1.45	20.15
3.95	0.45	0.984	21.9
17.73	3.01	3.70	12.30
11.80	1.71	2.68	15.7
7.86	1.01	1.88	18.7
5.90	0.71	1.445	20.4
3.96	0.44	0.997	22.6
r for detergent = 2; initial detergent concn. 6.06×10^{-5} *M*			
17.80	3.48	3.38	9.72
11.80	1.94	2.51	12.94
7.88	1.13	1.79	15.82
5.94	0.78	1.41	18.1
3.95	0.48	0.964	20.1
11.76	1.96	2.48	12.66
7.85	1.13	1.78	15.7
5.90	0.77	1.40	18.2
3.95	0.48	0.966	20.1
r for detergent = 3; initial detergent concn. 9.09×10^{-5} *M*			
17.82	3.93	3.06	7.80
11.78	2.24	2.29	10.22
7.90	1.32	1.67	12.6
5.95	0.91	1.313	14.4
3.96	0.57	0.901	15.8
r for detergent = 4; initial detergent concn. 12.12×10^{-5} *M*			
17.82	4.42	2.70	6.11
11.78	2.64	1.99	7.54
7.90	1.57	1.48	9.43
5.95	1.12	1.160	10.35
3.96	0.71	0.800	11.3
r for detergent = 5; initial detergent concn. 15.15×10^{-5} *M*			
14.75	4.03	1.97	4.89
9.85	2.51	1.44	5.74
6.90	1.64	1.097	6.70
4.92	1.13	0.810	7.16
2.98	0.64	0.524	8.2
r for detergent = 6; initial detergent concn. 18.18×10^{-5} *M*			
14.75	4.51	1.62	3.59
9.85	2.90	1.16	4.00
6.90	1.95	0.870	4.46
4.92	1.36	.644	4.74
2.98	0.79	.413	5.23
r for detergent = 7; initial detergent concn. 22.2×10^{-5} *M*			
9.83	3.20	0.935	2.92
6.89	2.20	.684	3.11
4.92	1.54	.511	3.32
2.96	0.94	.298	3.17
1.98	0.63	.198	3.14
r for detergent = 8; initial detergent concn. 26.0×10^{-5} *M*			
9.83	3.43	0.766	2.24
6.89	2.37	.560	2.36
4.92	1.70	.396	2.33
2.96	1.04	.224	2.16
1.98	0.67	.169	2.52

Discussion

Theoretical Considerations.—Before we attempt an interpretation of the data in terms of relative heterogeneity we wish to consider theoretically the problem of competitive binding and derive some useful relations. We shall designate the component whose binding is being measured (the dye) by A and the competitive inhibitor (the detergent) by B. We assume that

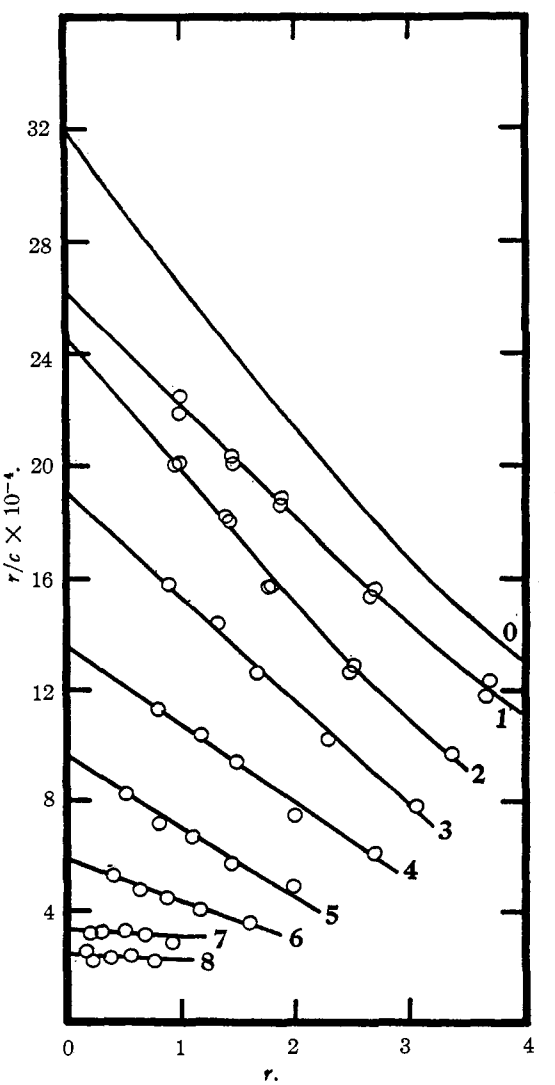

Fig. 1.—The effect of bound dodecyl sulfate on the binding by bovine serum albumin of an anionic azo dye at 25° in 0.05 *M* phosphate buffer, *p*H 7.0. The average number of detergent anions bound per molecule of protein is indicated at the right of the curves.

there are n moles of binding sites per mole of protein characterized by n values of the intrinsic association constant K, all of which may be different. We neglect any electrostatic interaction of the bound ions and assume the absence of any other interaction. If α_i is the fraction of the ith sites occupied at a particular concentration by the appropriately indicated component, then it follows readily from the law of mass action that

$$\alpha_i^A = \frac{c_A K_i^A}{1 + c_A K_i^A + c_B K_i^B} \tag{1}$$

and

$$r_A = \sum_i \alpha_i^A = \sum_i \frac{c_A K_i^A}{1 + c_A K_i^A + c_B K_i^B} \tag{2}$$

This can be put in the form

$$\frac{r_A}{c_A} = \sum_i \frac{\alpha_i^A}{c_A} = \sum_i \frac{K_i^A - \alpha_i^A K_i^A}{1 + c_B K_i^B} \tag{3}$$

In the absence of inhibitor

$$\frac{r_A}{c_A} = \sum_i (1 - \alpha_i^A) K_i^A \tag{4}$$

and

$$\lim_{c_A \to 0} \frac{r_A}{c_A} \equiv \left(\frac{r_A}{c_A}\right)_0 = \sum_i K_i^A \tag{5}$$

If inhibitor is present, then

$$\lim_{c_A \to 0} \frac{r_A}{c_A} \equiv \left(\frac{r_A}{c_A}\right)_{B,0} = \sum_i \frac{K_i^A}{1 + c_B K_i^B} \tag{6}$$

Under these circumstances, *i. e.*, when $c_A = 0$

$$1 + c_B K_i^B = 1/(1 - \alpha_i^B) \tag{7}$$

which on substitution in (6) yields

$$\left(\frac{r_A}{c_A}\right)_{B,0} = \sum_i (1 - \alpha_i^B) K_i^A \tag{8}$$

This expression on replacing α_i^B with α_i^A gives the equation for self-competition and becomes equivalent to (4). In this case the meaning of r_A/c_A is easily seen if one imagines the experiment in which is added to the protein solution, containing a particular concentration of free dye, an additional known amount of chemically identical dye, but experimentally distinguishable by virtue of the inclusion of a radioactive isotope. The value of r_A/c_A given by (8) would be the limiting value of r/c for the radioactive dye as its concentration approached zero.

It is convenient to define two experimentally determinable quantities $\Delta(A)$ and $\Delta(B)$ which give parallel measures of self-competition and non-self-competition, respectively.

$$\Delta(A) = \left(\frac{r_A}{c_A}\right)_0 - \frac{r_A}{c_A} = \sum_i \alpha_i^A K_i^A \quad \left(\sum_i \alpha_i^A = r_A\right) \tag{9}$$

$$\Delta(B) = \left(\frac{r_A}{c_A}\right)_0 - \left(\frac{r_A}{c_A}\right)_{B,0} = \sum \alpha_i^B K_i^A \quad \left(\sum_i \alpha_i^B = r_B\right) \tag{10}$$

We now inquire as to the relation between $\Delta(A)$ and $\Delta(B)$ when there is identical heterogeneity in the binding of A and B. We define identical heterogeneity to mean that K_i^A/K_i^B is the same for all i, which ratio is indicated by d. Since $\Delta(A)$ is determined in the absence of inhibitor and $\Delta(B)$ is found under the condition $c_A \to 0$, then α_i^A of (9) and α_i^B of (10) are given by

$$\alpha_i^A = \frac{K_i^A c_A}{1 + c_A K_i^A}, \quad \alpha_i^B = \frac{K_i^B c_B}{1 + c_B K_i^B} \tag{11}$$

Under the condition that $r_A = r_B = r$, it follows that

$$\left.\begin{aligned} r_A &= \sum_i \alpha_i^A = \sum_i (1 - \alpha_i^A) c_A K_i^A = r \\ r_B &= \sum_i \alpha_i^B = \sum_i (1 - \alpha_i^B) c_B K_i^B = r \end{aligned}\right\} \tag{12}$$

which on substituting dK_i^B for K_i^A gives

$$\left.\begin{aligned} \sum_i (1 - \alpha_i^A) c_A d K_i^B &= r \\ \sum_i (1 - \alpha_i^B) c_B K_i^B &= r \end{aligned}\right\} \tag{13}$$

Since the K^B's are independent then the corresponding coefficients must be equal

$$(1 - \alpha_i^A) = \frac{c_B}{c_A d} (1 - \alpha_i^B) \tag{14}$$

By virtue of the fact that we have assumed $\sum_i \alpha_i^A = \sum_i \alpha_i^B$, it follows that $c_B/c_A d = 1$ and $\alpha_i^A = \alpha_i^B$. In view of equations (9) and (10) we have therefore the experimental criterion for identical heterogeneity that

$$\Delta(A) = \Delta(B) \text{ (for all } r_A = r_B) \tag{15}$$

In the case that the binding of either constituent is homogeneous simple relations are obtained. If the binding of A is homogeneous, which means that the K_i^A's are identical, it follows from (10) that

$$\Delta(B) = K^A \sum_i \alpha_i^B = K^A r_B \tag{16}$$

For the homogeneous binding of B, $\alpha_i^B = r_B/n$. On substitution in (10), this gives

$$\Delta(B) = \frac{r_B}{n} \sum_i K_i^A \tag{17}$$

Thus, in both instances, it would be observed that successive unit increases in r_B would be accompanied by equal successive increments of $\Delta(B)$. It is worth noting that if $\Delta(B)$ does not change in this way with r_B, it can be inferred that the binding of both constituents is heterogeneous.

Relative Heterogeneity.—In order to evaluate our data in terms of the criteria developed above, we have listed in Table II the competition differentials $\Delta(A)$ and $\Delta(B)$ and their increments. For the case of self-competition we have used the dye binding data previously obtained.[4] The quantities $(r_A/c_A)_{B,0}$ are, of course, found by the extrapolation of the dye binding curves obtained

with the several values of r_B. For low values of r_B significant error could be introduced if the linear extrapolation were not valid. For $r_B = 1$, in particular, there is considerable uncertainty about the extrapolation and we shall therefore, in our main discussion, not include reference to the corresponding value of $\Delta(B)$.

TABLE II

THE COMPETITIVE EFFECT OF DODECYL SULFATE ON THE BINDING OF A DYE AT 25° IN 0.05 *M* PHOSPHATE BUFFER, *p*H 7.0. A COMPARISON OF SELF-COMPETITION AND DETERGENT COMPETITION IN TERMS OF THE COMPETITION DIFFERENTIALS, $\Delta(A)$ AND $\Delta(B)$, AND THEIR INCREMENTS

	Self competition			Detergent competition		
r	r_A/c_A $\times 10^{-4}$	$\Delta(A)$ $\times 10^{-4}$	$\Delta(A)_r - \Delta(A)_{r-1}$ $\times 10^{-4}$	$(r_A/c_A)_{B,0}$ $\times 10^{-4}$	$\Delta(B)$ $\times 10^{-4}$	$\Delta(B)_r - \Delta(B)_{r-1}$ $\times 10^{-4}$
0	32.0	0	...	32.0	0	...
1	26.8	5.2	5.2	26.2	5.8	5.8
2	21.6	10.4	5.2	24.6	7.6	1.8
3	16.9	15.1	4.7	19.0	13.0	5.4
4	13.2	18.8	3.7	13.5	18.5	4.5
5	10.3	21.7	2.9	9.5	22.5	4.0
6	8.1	23.9	2.2	5.8	26.2	3.7
7	6.5	25.5	1.6	3.3	28.7	2.5
8	5.4	26.6	1.1	2.4	29.6	0.9
9	4.6	27.4	0.8			
10	4.0	28.0	.6			
11	3.5	28.5	.5			
12	3.1	28.9	.4			
13	2.7	29.3	.4			
14	2.4	29.6	.3			

From Table II several interesting inferences can be made about the relative heterogeneity in the binding of the dye and detergent. In the first place, the inequality in the increments of $\Delta(B)$ verifies the conclusion previously reached that the binding of both dye and detergent is heterogeneous. Secondly, the lack of agreement of $\Delta(A)$ and $\Delta(B)$ demonstrates that this heterogeneity is not identical for these two anions. Thirdly, it will be noted that for low values of r, $\Delta(A)$ is larger than the corresponding values of $\Delta(B)$. For $r = 6$ and larger, however, $\Delta(B)$ exceeds $\Delta(A)$. This means, in general terms, that for small r self-competition is more effective than detergent competition. At high r values, the reverse is true.

For a more detailed interpretation of this reversal let us recall that the dye binding sites can be divided into two groups: Group 1 contains between four and five sites and is characterized by a high binding constant and Group 2 with about seventeen sites and a relatively low binding constant.[4] We also note that the $\Delta(B)$ increments exceed those of $\Delta(A)$ for r ranging from 3 to 7. The picture emerges, then, that there is at least one site, and perhaps two, which binds the detergent strongly. This site (or sites) is not a member of Group 1, but may belong to Group 2. Aside from this site, the Group 1 sites are not only most effective for the dye but also bind the dodecyl sulfate most strongly. In fact, the superiority of these sites for complexing with detergent relative to any other sites (including Group 2) which also bind detergent is greater than their superiority relative to the Group 2 sites in the binding of the dye. This permits the explanation of the fact that the effect on dye binding of only seven bound detergent anions is equivalent to the effect of more than 11 bound dye anions. To put it another way, it accounts for the fact, already pointed out, that the $\Delta(B)$ increments exceed the $\Delta(A)$ increments in the r range of 3 to 7.

These results, on the whole, serve to support the idea that the binding properties of serum albumins are associated with the configurational adaptability of a number of regions of the protein.[4] The fact that the same sites (Group 1) bind most strongly these two structurally different anions, suggests that these sites have a high degree of structural adaptability, *i. e.*, they can assume structures which are complementary to a wide range of configurations. Thus, it would follow that it is this property which confers upon the albumins the distinctive ability to form complexes with a wide variety of anions. From this point of view, the Group 2 sites could be interpreted as being more restricted in their range of adaptability with, consequently, smaller binding constants and therefore more selective in the anionic structures with which they associate.

Since the binding of dye and detergent anions probably involves a displacement of buffer anions,[9] it is unclear what net contribution, if any, is made to the competition differentials by the electrostatic effect of these bound anions. In any case, our conclusions would not be affected by the inclusion of an electrostatic correction. Thus, for example, the maximum effect on the dye binding of 7 bound detergent anions was calculated on the assumption that these decrease the net charge on the protein molecule by 7. The calculation was carried out in a manner similar to that employed by Scatchard and Black.[10] If the reduction in the number of available sites is taken into account, a value of 14.1×10^4 is obtained for $(r_A/c_A)_{B,0}$ compared to the experimental value of 3.3×10^4. This indicates that there is heterogeneity in the dye binding sites and that the detergent binds preferentially to the Group I sites. Otherwise the experimental value would not be less than 14.1×10^4.

With the knowledge now available of the relative heterogeneity of the binding sites of bovine serum albumin it is possible to account for some interesting but unexplained observations of Klotz, Triwush and Walker.[5] They attempted to determine the binding constant of bovine albumin for sodium dodecyl sulfate by its competitive displacement of methyl orange. This was measured by the spectral shift associated with the binding of the dye. On the assumption that the binding,

(9) L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

(10) G. Scatchard and E. S. Black, THIS JOURNAL, **53**, 88 (1949).

both of the dye and detergent, is homogeneous it was calculated from the experimental results that more detergent was bound than was added. The explanation is, undoubtedly, that there is heterogeneity in the binding of both anions and that the same sites are most effective in both instances. Under these circumstances the detergent is a more effective competitor than the homogeneity assumption would predict. In fact, if, as is probably the case, it is the Group 1 sites which are involved, the experimental observations are readily accounted for.

In view of the heterogeneity in the binding of anions, the interpretation of the binding of a substance by its displacing effect is subject to considerable limitation. Depending on the relative heterogeneity, the binding of the competitor may be underestimated or even overlooked entirely. When compared to data obtained by other methods, *e. g.*, electrophoretic mobility, considerable disagreement in the results may appear.

Acknowledgment.—I am indebted to Dr. C. P. Rhoads for making available to me, during the conduct of this investigation, the excellent laboratory facilities of the Sloan–Kettering Institute for Cancer Research. This work was supported by the Office of Naval Research.

Summary

The effect of bound sodium dodecyl sulfate on the binding by bovine serum albumin of an anionic azo dye has been investigated at 25° in 0.05 *M* phosphate buffer, *p*H 7.0. The amount of bound detergent was varied from 1 mole per mole of protein to 8 moles per mole protein. The data are analyzed in terms of a comparison of self-competition *versus* detergent competition. For this purpose there are introduced the quantities Δ(A) and Δ(B) which are named competition differentials and whose values can be derived from the experimental data. It is shown that the criterion for identical heterogeneity is that Δ(A) = Δ(B) for $r_A = r_B$. A comparison of Δ(A) and Δ(B) over the whole range of r_A and r_B studied leads to the conclusion that those sites (Group 1) which bind the dye most strongly also, for the most part, bind the detergent most effectively. In terms of configurational adaptability the Group 1 sites are interpreted as being able to assume structures complementary to a wide range of configurations whereas the other sites are more restricted in this respect. The latter bind less strongly and are more selective.

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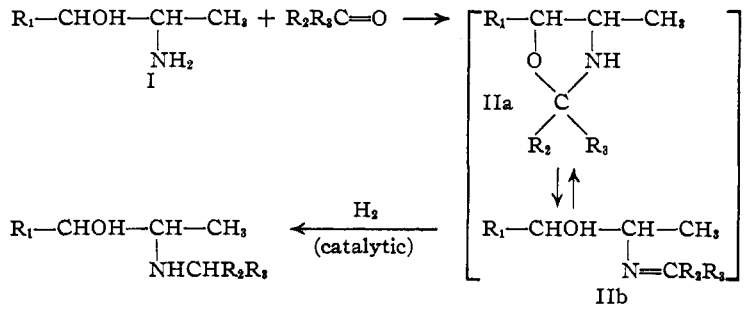
[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, MEDICAL RESEARCH DIVISION, SHARP AND DOHME, INC.]

The Reductive Alkylation of Arylalkanolamines

BY EDWARD L. ENGELHARDT, FRANK S. CROSSLEY AND JAMES M. SPRAGUE

Cope and Hancock[1] have described a convenient method for the preparation of N-alkyl derivatives of alkanolamines by the catalytic reduction of mixtures of the alkanolamine and an aldehyde or ketone. As a part of a pharmacological study of N-alkyl derivatives of phenethanolamine we have extended the method of Cope and Hancock to the alkylation of 1-phenyl-2-amino-1-propanol and certain nuclear substituted derivatives.

With aliphatic aminoalcohols, Cope and Hancock have shown that the structure of the anhydro compounds (II) that are thought to be intermediates in the reaction, depends upon steric factors in the carbonyl compound. Aldehydes and unhindered ketones give oxazolidines (IIa) while highly branched ketones give alkylidene-aminoalcohols (IIb). The alkylaminoalkanol results either from reduction of the alkylidene intermediate (IIa) or from hydrogenolysis of the oxazolidine (IIb). Although in the present work the nature of the anhydro compounds formed from norephedrine (I, R_1 = phenyl) has not been studied, the steric effect of the phenyl group might be expected to favor the alkylidene-aminoalcohol (IIb).



The only failures encountered in the norephedrine series (Table I) occurred when acetaldehyde or acetophenone was employed as the carbonyl component. The former was polymerized, the latter apparently failed to condense with the aminoalcohol. 3,4-Dihydroxynorephedrine gave good yields of the expected products when ketones were employed as the carbonyl

(1) Cope and Hancock, THIS JOURNAL, **64**, 1503 (1942); **66**, 1453 (1944); Hancock and Cope, *ibid.*, **66**, 1738 (1944).