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

Interactions of Homologs of Carcinogenic Azo Dyes and Proteins[1-3]

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The interactions of 4-aminoazobenzene, 3'-methyl-4-aminoazobenzene, 4'-methyl-4-aminoazobenzene with bovine serum albumin and the proteins contained in rat liver extracts have been studied by the equilibrium dialysis technique at 0° and *p*H 6.8. The interaction between methyl orange and bovine serum albumin was repeated as a reference interaction. The data suggest the following order among the quantities of these dyes bound to bovine serum albumin: 3'-methyl-4-aminoazobenzene (greatest), 4'-methyl-4-aminoazobenzene, 4-aminoazobenzene, methyl orange (least). However, only in certain cases were the differences between these quantities found to be statistically significant at the 5% level. The data also suggest that the interaction of 4'-methyl-4-aminoazobenzene with bovine serum albumin is less temperature dependent than the interactions involving the other two uncharged dyes and this protein. The methylated aminoazobenzenes bind to the proteins in the rat liver extracts in larger quantities than aminoazobenzene and these quantities are about one third to one half as large as those observed with bovine serum albumin when both sets of data are expressed in terms of moles of dye bound per milligram of nitrogen.

It has been suggested that in the interaction of an uncharged azo dye such as 4-aminoazobenzene with bovine serum albumin cationic sites from the lysine residues and phenolic hydroxyl groups from the tyrosine residues in the protein engage in hydrogen bonding to the amino group of the dye.[4] Subsequent to this report it was found that the differences observed in the interactions of 4-aminoazobenzene, 3'-methyl-4-aminoazobenzene and 4'-methyl-4-aminoazobenzene with bovine serum albumin at 25° could not be accounted for solely by differences in basicities of the three dyes and it was suggested that factors other than basicity could be of major importance in interactions of this type.[5] In order to gain further insight into the nature of these protein–dye interactions it was thought desirable to study their temperature dependences for which the temperature of 0° was selected for additional studies. Along with these studies it was also thought desirable to repeat earlier studies involving the interactions between the proteins in rat liver extracts and azo dyes at 0°[6] and thus establish a firmer foundation for further studies involving the interactions of rat liver proteins and dyes. This paper reports the results obtained from these two lines of investigation.

Experimental

Sources of Dyes and Proteins.—The dyes used in these studies were prepared as previously outlined.[5] The serum protein was Crystallized Bovine Plasma Albumin (referred to as bovine serum albumin in this paper) as obtained from the Armour Laboratories. The albumin was dried over P_2O_5 *in vacuo* prior to its use. The rat liver extracts were prepared by the extraction of a dried blood-free rat liver homogenate with phosphate buffer near 0°. The livers used in this preparation were obtained by the *in situ* perfusion of these organs with cold 0.89% saline during anesthesia followed by extirpation and quick freezing. These livers were then pooled, homogenized near 0° in a Waring Blendor, dialyzed near 0° against distilled water until the resistance of the dialyzate matched that of the distilled water and the resultant material lyophilized. This dried homogenate was then examined for the presence of carbohydrates, blood and lipids. The di-*o*-anisidine test was used to test for carbohydrates[7]; the benzidine test (after a short period of boiling) was used to test for blood[8]; exhaustive extraction with chloroform by use of a Soxhlet extractor followed by gravimetric determination of the chloroform soluble material was used to test for lipids. These tests indicated that carbohydrates, blood and lipids were not present in detectable quantities in this rat liver homogenate. The quantity of nitrogen in each extract prepared from this homogenate was determined by the micro-Kjeldahl method of Stover and Sandin[9] using the mixed indicator of Sobel, *et al.*[10] Crushed ice-water baths were used to maintain the desired temperature during the preparation of the homogenate and extracts.

Interactions.—The equilibrium dialysis technique as developed by Klotz and his co-workers was used for these studies.[11] The fact that the optical densities of solutions containing dyes and rat liver extracts did not change over a 24-hour period was considered as evidence that the dyes were not undergoing metabolic changes during this period and that differences in optical densities observed during equilibrium dialysis studies were due to protein–dye interactions. All spectral data were obtained by use of a Beckman model DU spectrophotometer. A crushed ice-water bath was used to maintain the temperature for the equilibrium dialysis experiments. The dye solutions used varied in concentration from approximately 3×10^{-6} to 10×10^{-6} moles per liter. The protein solutions involving serum albumin were 0.2% by weight. The rat liver extracts contained approximately 0.1 mg. of nitrogen per ml. The exact nitrogen content of each extract was determined and was taken into consideration when computations were made. All interactions and extractions made use of 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.

Treatment of Data.[12]—The data obtained from studies involving bovine serum albumin 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[13]

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

The correlation coefficients for these lines were then calcu-

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(3) A portion of the data reported in this paper has been abstracted from a thesis to be presented by Roger Bauer, in partial fulfillment of the requirements for the degree of Master of Science at Kansas State College.

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

(5) R. K. Burkhard and F. A. Moore, *ibid.*, **77**, 6057 (1955).

(6) Robert M. Grossman, M.S. thesis, Kansas State College, 1954.

(7) F. Feigl, "Qualitative Analysis by Spot Tests," Elsevier Pub. Co., Inc., New York, N. Y., 1946, 3rd edition, pp. 410–411.

(8) P. B. Hawk, B. L. Oser and W. H. Summerson, "Practical Physiological Chemistry," The Blakiston Co., Philadelphia, Pa., 1947, 12th edition, pp. 433 and 437.

(9) N. M. Stover and R. B. Sandin, *Ind. Eng. Chem., Anal. Ed.*, **3**, 240 (1931).

(10) A. E. Sobel, H. Yuska and J. Cohen, *J. Biol. Chem.*, **118**, 443 (1937).

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

(12) The terminology and methods used in the statistical analysis of the data can be found in G. W. Snedecor, "Statistical Methods," Iowa State College Press, Ames, Iowa, 1956, 5th edition.

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

lated to establish the validity of such treatment. The data were also converted into values of r/A and the method of least squares again used to calculate the intercepts and slopes for the equations having the form[14]

$$r/A = nk - kr$$

Correlation coefficients were then calculated for the lines obtained from these plots but it was found in all cases that much higher correlation coefficients were obtained by the first method of analysis. The reason for this was probably due to the fact that when the Klotz type of plot was used each set of experimental points was almost equally spaced along the abscissa but when the Scatchard type of plot was used each set of experimental points formed a cluster. Since statistical analysis of the data ultimately was used it was felt that the precision of any calculated value could still be represented adequately even though the Klotz type of plot was used. Thus all values reported in this paper regarding bovine serum albumin were obtained by the method introduced by Klotz.[13] Estimated values for $1/r$ and the corresponding 95% confidence intervals were then calculated at selected values for $1/A$. The t-test was then used to determine the significance of the differences between these values and between the slopes of the least squares lines. The 5% probability level was used as the criterion for significance in all such comparisons. Thermodynamic quantities were then calculated from the slopes of the least squares lines obtained at 0° and 25°.[5] In the case of the free energy data calculations also were made from the 95% confidence limits to indicate the precision of each calculation but this treatment was not extended to the enthalpy and entropy calculations.

TABLE I

VALUES FOR $1/n$, $1/nk$ AND CORRELATION COEFFICIENTS ASSOCIATED WITH LEAST SQUARES LINES (0°) AND THE THERMODYNAMIC FUNCTIONS FOR THE BINDING OF THE FIRST DYE MOLECULE OR ION TO BOVINE SERUM ALBUMIN

Dye[a]	No. of detn.	$1/n$	$1/nk \times 10^3$	Cor. coeff.	$-\Delta F_1°$ (0°) (kcal./mole) From slope	$-\Delta F_1°$ (0°) (kcal./mole) From 95% limits	$-\Delta F_1°$ (25°)[b] (kcal./mole) From slope	$-\Delta F_1°$ (25°)[b] (kcal./mole) From 95% limits	$-\Delta H_1°$ (kcal./mole)	$\Delta S_1°$ (cal./mole/deg.)
AB	10	0.050	14.9	0.99	6.03	5.97–6.10	6.30[c]	6.19–6.44	3.3[c]	11[c]
3′-Me-AB	11	.432	8.87	.99	6.31	6.30–6.32	6.47	6.38–6.57	4.6	6
4′-Me-AB	9	− .0856	13.8	.85	6.07	5.87–6.55	6.44	6.34–6.56	2.0	15
MO	10	− .183	17.3	.94	5.95[d]	5.80–6.16	..	.....	..	..

[a] Abbreviations: AB, (4-aminoazobenzene); 3′-Me-AB, (3′-methyl-4-aminoazobenzene); 4′-Me-AB, (4′-methyl-4-aminoazobenzene; MO, (methyl orange). [b] The 25° values were obtained from data of Burkhard and Moore.[5] [c] Literature values for these thermodynamic functions are −6150 cal./mole for the free energy change at *p*H 6.9, −3700 cal./mole and 11 cal./mole/deg. for the enthalpy and entropy changes, respectively, at *p*H 9.2.[4] [d] Literature value for this free energy change is −6049 cal./mole.[17]

The data obtained from studies involving the rat liver extracts were converted into units of the percentage of total dye bound per milligram of nitrogen in the extract. These rather unusual units were used in reporting these data since it was felt that a preparation such as this type of rat liver extract was too heterogeneous to allow a rigorous analysis of the data. Correlation coefficients relating these percentages to the total dye concentrations were calculated and it was found that over the concentration range studied these percentages were independent of the total dye concentration. Thus these percentages were merely averaged and the arithmetic means and standard deviations reported. The F-test was used to determine whether significant differences existed among these means. The t-test was then used to locate wherein these differences lay. The 5% probability level was again used as the criterion for significance in these comparisons. For the purpose of comparing these data to the data associated with bovine serum albumin certain of both sets of data were selected and converted into units of moles of dye bound per milligram of nitrogen. To achieve this conversion the value of 16.07[15] was used for the percentage of nitrogen in bovine serum albumin.

Results and Discussion

The values for $1/n$, $1/nk$ and the correlation coefficients associated with each least squares line involving bovine serum albumin interactions are listed in Table I. Estimated values for $1/r$ and the corresponding 95% confidence intervals are listed in Table II. From these data it was concluded that in the majority of the cases an excellent linear relationship existed between $1/r$ and $1/A$. Even though the correlation coefficient associated with 4′-methyl-4-aminoazobenzene was small it still indicated a linear relationship did exist between $1/r$ and $1/A$. Statistical analysis of the data revealed that over the common free dye concentration range 3′-methyl-4-aminoazobenzene was bound in significantly larger quantities than 4-aminoazobenzene, however, in the case of 3′-methyl-4-aminoazobenzene and its 4′-isomer only at concentrations less than 6×10^{-6} mole per liter were significantly larger quantities of the 3′-substituted dye bound. It was also found that over the common range examined no significant differences were found in the amounts of binding of 4′-methyl-4-aminoazobenzene and 4-aminoazobenzene. These results again suggest[5] that factors other than basicity are of major importance in determining the abilities of these uncharged dyes to complex to bovine serum albumin.

It has been reported that 4-aminoazobenzene binds to bovine serum albumin in somewhat smaller quantities than methyl orange at *p*H 6.9 and 25°.[4] Burgert also has studied these interactions at 25° but at *p*H 7.3.[16] He obtained the same results that Klotz and Ayers did in that at a free dye concentration of 1×10^{-5} mole per liter, for example, the amount of methyl orange bound to the protein was 0.40 mole per mole of protein and with 4-aminoazobenzene the amount was 0.35. Thus while there appears to be greater binding of methyl orange at 25° it does not appear to be true at 0° and *p*H 6.8. The data in Table II even suggest that methyl orange is bound in smaller quantities than 4-aminoazobenzene but statistical analysis of these data revealed that the differences between these amounts were not significant. Comparison of methyl orange to 3′-methyl-4-aminoazobenzene, however, revealed that over the entire common range the unchanged dye was bound in larger quantities, but in the case of 4′-methyl-4-aminoazobenzene *versus* methyl orange the uncharged dye was bound in significantly larger quantities only at concentrations lower than 6×10^{-6} mole per liter.

Comparison of these results with those obtained

(14) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
(15) E. Brand, *ibid.*, **47**, 187 (1946).
(16) B. E. Burgert, M.S. thesis, Kansas State College, 1951.
(17) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 847 (1949).

TABLE II

ESTIMATED VALUES AND 95% CONFIDENCE INTERVALS FOR $1/r$ AT GIVEN VALUES OF $1/A$ FOR BINDING OF DYES TO BOVINE SERUM ALBUMIN

$1/A$ (l./mole × 10^{-5})	Dye[a]	$1/r$	95% Confidence interval
1.67	AB	2.54	2.30–2.78
	3′-Me-AB	1.91	1.88–1.94
	4′-Me-AB	2.26	1.58–2.94
	MO	2.71	2.41–3.01
2.00	AB	3.03	2.80–3.26
	3′-Me-AB	2.20	2.17–2.23
	4′-Me-AB	2.70	2.20–3.20
	MO	3.28	3.00–3.56
2.50	AB	3.77	3.35–4.19
	3′-Me-AB	2.65	2.60–2.70
	4′-Me-AB	3.33	2.94–3.72
	MO	4.14	3.69–4.59
2.86[b]	3′-Me-AB	2.97	2.94–3.00
	4′-Me-AB	3.80	3.31–4.29

[a] Abbreviations: same as in Table I. [b] Values for AB and MO systems are not listed at 2.86×10^5 due to the fact that observations involving these dyes did not extend to this concentration.

at 25°[5] reveals an inverse relationship between temperature and extent of interaction for the uncharged dyes. However, this temperature dependence must not be identical in all cases since at 25° it was found that at most of the concentrations examined 3′-methyl and 4′-methyl-4-aminoazobenzene were not bound in significantly different quantities but both were bound in significantly larger amounts than 4-aminoazobenzene while at 0° 3′-methyl-4-aminoazobenzene was bound in significantly greater quantities at most concentrations examined than both 4′-methyl-4-aminoazobenzene and 4-aminoazobenzene and that these latter two dyes were not bound in significantly different quantities. If the natures of the interactions of 4-aminoazobenzene and 3′-methyl-4-aminoazobenzene with bovine serum albumin are such that the equilibrium constants associated with these two are more temperature dependent than that involving 4′-methyl-4-aminoazobenzene then one can qualitatively account for these observations. That this might be the case is suggested by the thermodynamic functions listed below.

TABLE III

AMOUNTS OF DYE BOUND TO PROTEINS IN RAT LIVER EXTRACT AND THEIR COMPARISONS TO AMOUNTS BOUND TO BOVINE SERUM ALBUMIN (0°, *p*H 6.8)

Dye[a]	No. of detn.	Mean % of total dye bound per mg. N in RLE[a]	Stand. dev.	Binding expressed as moles of dye bound per mg. N × 10^8 at total dye concn. of 5×10^{-6}, mole/l. RLE	BSA[a,b]
AB	19	18	6	1.1	2.98
3′-Me-AB	15	28	4	1.9	4.09
4′-Me-AB	18	26	8	1.8	3.35

[a] Abbreviations: same as Table I for the dyes; RLE (rat liver extract); BSA (bovine serum albumin). [b] These values were obtained from the data used to prepare Tables I and II.

In order to evaluate the thermodynamic functions for these interactions it was first thought desirable to calculate the correlation coefficients for the 25° data previously reported.[5] These coefficients were found to be 0.94, 0.96 and 0.94 for 4-aminoazobenzene, 3′-methyl-4-aminoazobenzene and 4′-methyl-4-aminoazobenzene, respectively. Thus from the previously and currently reported $1/nk$ values the thermodynamic functions associated with these interactions were calculated.

It was stated previously that there was not a significant difference between the slopes of the lines derived from data involving the methylated uncharged dyes at 25°.[5] Further calculations involving 4-aminoazobenzene revealed, however, that the slope of this line was significantly different from that involving 3′-methyl-4-aminoazobenzene (t = 2.27, D.F. = 27) but not different from the slope of the line involving 4′-methyl-4-aminoazobenzene (t = 1.64, D.F. = 33).[12] Calculations involving the slopes obtained at 0° revealed that these relationships held also at the lower temperature. The slope of the line involving methyl orange was found to differ significantly only from that observed with 3′-methyl-4-aminoazobenzene (t = 4.24, D.F. = 17).

The thermodynamic functions associated with the binding of each first dye molecule or ion are shown in Table I. It should be noted that the free energy changes include values calculated from the 95% confidence limits of the various slopes while the enthalpy and entropy changes do not. Comparison of these values with previously reported ones shows favorable agreement.[4,17] They, however, do not allow one to evaluate with certainty the importance of the various factors which might be involved in the binding of such dyes to bovine serum albumin. Further experimentation is now in progress in this Laboratory in an attempt to correlate the structure of uncharged dyes with their ability to form complexes with proteins.

The correlation coefficients for the dependence of the percentage of total dye bound per milligram of nitrogen on total dye concentration in the case of the rat liver extracts were found to be 0.09 (D.F. = 17) for 4-aminoazobenzene, −0.22 (D.F. = 15) for 3′-methyl-4-aminoazobenzene, and 0.30 (D.F. = 18) for 4′-methyl-4-aminoazobenzene.[12] From these it was concluded that the percentage of total dye bound was independent of the total dye present and thus these percentages could be averaged for each system and the mean percentages reported. These values and the standard deviations are listed in Table III.

Analysis of variance revealed that these means were not all the same (F = 10, $D.F._1$ = 2, $D.F._2$ = 49). Further analysis revealed that there was not a significant difference between the means for the methylated compounds but that there were significant differences between the means for the methylated compounds and aminoazobenzene (for example, t = 3.31, D.F. = 35 for 4′-methyl-4-aminoazobenzene *vs.* 4-aminoazobenzene).[12] These results agree with those obtained earlier by Grossman[6] and also suggest that factors other than basicity are of major importance in determining the extents of interaction of these three uncharged dyes with the proteins in these rat liver extracts.

The amounts of these uncharged dyes bound to both bovine serum albumin and the proteins in the

rat liver extracts at a free dye concentration of 5×10^{-6} mole per liter are also listed in Table III. It will be noted that at this particular free dye concentration and in terms of the units used the amount of any one dye bound to the proteins in the rat liver extracts is approximately one third to one half of the amount bound to bovine serum albumin. This observation may be of some significance since it has been found by other investigators that neutral dye molecules do not bind to bovine γ-globulin or pepsin.[4] The composition of these rat liver extracts is currently unknown, but an attempt is being made in this laboratory to isolate and characterize the protein or proteins involved in the formation of these complexes with uncharged dyes.

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[CONTRIBUTION FROM THE ORGANIC CHEMICALS DIVISION, ST. LOUIS RESEARCH DEPARTMENT, MONSANTO CHEMICAL CO.]

Studies in Steroid Total Synthesis. V. Abnormal Cleavage of Some Tricyclic Ketone Epoxides

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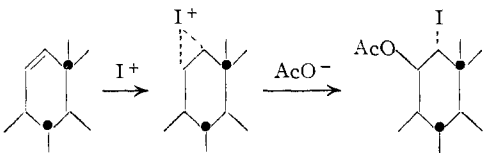
Reductive cleavage of the 5β,6β-epoxide function of the tricyclic ketone IIIa does not give the axial 5β-ol (IIc) predicted by conformational theory but gives the equatorial 6β-ol (IId). Both IIIa and the isomeric 5α,6α-epoxide IIIb are opened abnormally by methoxide and thiophenoxide anions giving products to which we have assigned structure VIIIa,b,c and IXa,b.

In the course of work designed to prepare ring B,C,D-tricyclic intermediates containing a potential C-11 oxygen, the chemistry of the isomeric 5,6-oxides derived from *dl-anti-trans*-1,6a,7,7a,10a,-11,11a,11b - octahydro - 4,6a,9,9 - tetramethylphenanthro[2,3][1,3]dioxol-3(2H)-one (Ic) was investigated. The course of the reactions in most cases was found to be extremely unpredictable. Abnormal cleavage by methoxide and thiophenoxide anions and the abnormal reductive cleavage of the β-oxide form the subject of the present paper.

The readily available tricyclic ketone Ic[1] served as the starting point for the preparation of both oxides. Reaction of Ic with approximately one mole of silver acetate and iodine in glacial acetic acid gave the 5β-acetoxy-6α-iodoketone IIa which upon treatment with aqueous base gave the 5β,-6β-epoxide IIIa, m.p. 200–201°.[2] The isomeric 5α,6α-epoxide IIIb, m.p. 214–217°, readily was obtained by treatment of Ic with perbenzoic or monoperphthalic acids. Configurational assignments were made assuming backside attack by peracids would give the α-epoxide, whereas silver acetate–iodine treatment would result in an initial α-oriented attack by I^+ followed by opening with acetate ion to form the 5β-acetoxy-6α-iodo configuration assigned. Base treatment would then yield the β-epoxide.



Catalytic reduction of the α-epoxide IIIb yielded the 6α-alcohol IIb. As expected for an axial hydroxyl located vinylogously beta to a carbonyl group, mild treatment with base caused elimination of water and formation of the dienone Ic. Several attempts to reduce the isomeric epoxide IIIa catalytically did not give clear results. Reduction with zinc and acetic acid proceeded smoothly to yield a new alcohol, m.p. 187–188°, λ_{max}^{alc} 250, ϵ 15,380. If the reduction had occurred in a normal manner, IIc would be the expected structure for the product. However, ready acetylation at room temperature with pyridine and acetic anhydride indicated the presence of an equatorial hydroxyl function.[3] Attempts to oxidize the alcohol with manganese dioxide[4a,b] or chromic anhydride in pyridine[5] yielded chiefly starting material, again indicative of a non-allylic equatorial hydroxy group.

The rearrangement of Δ^4-cholestenone-6-ol systems to the corresponding 3,6-diones under alkaline conditions has been well established.[6a,b,c] Moreover, it has been shown both in the Δ^4-3-one-6-ol[6b] system and the Δ^8-11-α-ol-7-one[7] system that it is significantly easier to effect rearrangement when the hydroxyl group is axial than when it is equatorial. The axial alcohol represented by

(1) The ketone Ib was first prepared by R. B. Woodward, F. Sondheimer, D. Taub, K. Heusler and W. M. McLamore (THIS JOURNAL, **74**, 4223 (1952)) as the minor β-*cis*-glycol resulting from osmium tetroxide oxidation of the unsaturated ketone Ia. Subsequently, Woodward and Brutcher (private communication) prepared the same glycol (m.p. 181–182°) by silver acetate–iodine oxidation of Ia.

(2) Compounds IIa and IIIa were first prepared at Harvard by F. V. Brutcher and R. B. Woodward and were disclosed to us by private communication.

(3) The isomeric alcohol IIb acetylated incompletely under the same conditions. This somewhat slower acetylation would be expected for an axial hydroxyl at C-6. The analogous C-12 α- and β-alcohols in the bile acid series show similar behavior [B. Koechlin and T. Reichstein, *Helv. Chim. Acta*, **25**, 918 (1942)].

(4) (a) *Cf.* S. Ball, T. W. Goodwin and R. A. Morton, *Biochem. J.*, **42**, 516 (1948); F. Sondheimer, C. Amendola and G. Rosenkranz, THIS JOURNAL, **75**, 5930, 5932 (1953). (b) Sondheimer, *et al.* [*J. Chem. Soc.*, 1226 (1954)], has used manganese dioxide for the oxidation of 6β-hydroxyprogesterone to the corresponding 6-ketoprogesterone, thus effecting successful oxidation of a system structurally similar to our formula IIc.

(5) *Cf.* G. I. Poos, G. E. Arth, R. E. Beyler and L. H. Sarett, THIS JOURNAL, **75**, 422 (1953).

(6) (a) B. Ellis and V. A. Petrow, *J. Chem. Soc.*, 1078 (1939); (b) P. T. Herzig and M. Ehrenstein, *J. Org. Chem.*, **16**, 1050 (1951), (c) see reference cited in footnote.[4b]

(7) J. Romo, G. Stork, G. Rosenkranz and C. Djerassi, THIS JOURNAL, **74**, 2918 (1952).