ies. The EHT programs are available for use on IBM 7094 and IBM 360/50 systems. The modified CNDO programs have been used on IBM 360/50 (convergence criterion 0.0001 au in electronic energy) and CDC 3600 and 6500 (0.000001 au) systems. The electron density plotter functions on IBM 360/30 equipment. The program for calculating total interaction energy (HYTRAN) for the hydride ion was written for the CDC 6500 machine.

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Species Difference in the Competitive Binding of 2-(4′-Hydroxybenzeneazo)benzoic Acid (HBABA) and α-(4-Chlorophenoxy)-α-methylpropionic Acid (CPMPA) to Serum Albumin. A Possible Model System for Studying Allosteric Transitions[1a]

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The comparative competitive binding of 2-(4′-hydroxybenzeneazo)benzoic acid (HBABA) and α-(4-chlorophenoxy)-α-methylpropionic acid (CPMPA) with a number of serum albumin preparations obtained from different species was studied. Data derived from equilibrium dialysis, spectrophotometric, and extrinsic rotatory dispersion (ERD) measurements reveal a unique behavior for rat serum albumin (RSA). With serum albumins obtained from all other species studied CPMPA competitively inhibited the HBABA–protein interaction at all concentration levels. With RSA low concentrations of CPMPA actually enhanced the HBABA–protein interaction. These results suggest that CPMPA is able to cause a small molecular perturbation in the RSA molecule which liberates additional sites on the protein for HBABA binding. Rat albumin, in combination with certain drugs, seems to be a model for studying allosteric transitions.

In blood plasma the albumin is a very important constituent because of its relative abundance and homogeneity and also because of its special osmotic and transport properties.[2] In the past, serum albumin was often regarded as a single homogeneous protein with similar physicochemical properties in all species from which the crystalline protein had been isolated. However, exceptions have often been noted and it is now well documented in the literature that species differences do exist in amino acid content[3] and sequence[4] and in the binding of small molecules and ions.[5]

Witiak and Whitehouse[6] have shown that rat serum albumin (RSA) behaves abnormally when compared with plasma albumin fraction V preparations from other species in its interaction with 2-(4′-hydroxybenzeneazo)benzoic acid (HBABA) in the presence of drugs such as thyroxine, indoleacetates, and chlorophenoxyacetic acids and that the rat may be a singularly nonrepresentative species as far as drug binding to its albumin is concerned. This is well known with respect to thyroxine binding. Through results obtained utilizing equilibrium dialysis, spectrophotometry, and optical rotatory dispersion an explanation for this unique effect with RSA is suggested in this study.

HBABA, in 0.1 *M* sodium phosphate buffer, pH 7.4, exhibits a λ_{max} at 350 mμ (ε 19,125), but when combined with serum albumin in phosphate buffer a new λ_{max} of considerably lower intensity is observed at 475–480 mμ.[5a] The intensity of this peak depends on both the concentration of albumin and the amount of HBABA bound. The position of the λ_{max} 475–480 mμ does not change over a range of animal serum albumins, but the actual absorbance at 475 mμ is species dependent.[5a] Assigning a value of 1.0 to the absorbance with bovine serum albumin (BSA), the relative absorbance for other albumins are guinea pig, 1.35; mouse, 1.10; rat, 0.80; human, 0.75; dog, 0.70; sheep, 0.60; rabbit, 0.40; pig, 0.30; horse, 0.10.[6] When various phenoxyacetic acids and indomethacin are added to a phosphate buffer containing RSA and HBABA an increase in absorbance is observed at 475 mμ while phenylbutazone and nicotinic acid quenched the absorbance at all concentration levels. With albumins obtained from all other species[7] that Witiak and Whitehouse[6] investigated, the phenoxyacetic acids decreased the absorbance at 475 mμ.

Optical rotatory dispersion can be utilized in determining the interaction of small molecules with serum albumin. The ORD spectrum has been thoroughly investigated for bovine and human serum albumin[8] and many other polypeptides and proteins. Rotatory parameters derived from such curves have been attributed

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(2) F. W. Putnam, *Proteins*, **3**, 153 (1965).

(3) (a) G. R. Tristram and R. H. Smith, *Advan. Protein Chem.*, **18**, 227 (1963); (b) S. E. Allerton, D. Elwyn, J. T. Edsall, and P.-F. Spahr, *J. Biol. Chem.*, **237**, 85 (1962); (c) B. Jirgensons, "Natural Organic Macromolecules," Oxford University Press (Pergamon), London, 1962, p 207.

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to the effective right-handed α-helical content of the peptide backbone.[9,10] Such measurements, referred to as intrinsic rotatory dispersions,[11] are extremely important when studying the conformational structure of the peptide,[9] but provide no evidence concerning differences in amino acid sequence and in the peripheral arrangement of functional groups about the polypeptide backbone which are responsible for the binding of small molecules and ions. In order to probe into the species differences in arrangement of binding sites in serum albumin, extrinsic rotatory dispersions[9] of albumins complexed with HBABA were investigated. Extrinsic Cotton effects refer to the rotatory dispersion of the peptide in combination with small molecules and ions, *i.e.*, dyes, inhibitors, substrate analogs, metal ions, etc. The Cotton effects have been called extrinsic to distinguish them from the intrinsic ORD.

HBABA was chosen for these studies because (1) it has a well-defined affinity for serum albumin,[12] (2) the reaction is reversible and competitive inhibition of the absorbance at 475–480 mμ (and therefore the HBABA–protein reaction) has been studied with many acidic drugs,[13] and (3) because of the unique increase in absorbance observed at 475 mμ when various phenoxyacetic acids and indomethacin are added to a phosphate buffer solution of RSA and HBABA.

Experimental Section

All albumin preparations (fraction V) were obtained from Pentex, Inc., Kankakee, Ill., and used without further modification; fraction V preparations for bovine, guinea pig, rat, human, dog, sheep, rabbit, pig, and horse were studied. All of these preparations were described as >95% pure by electrophoresis at pH 7.0. The rat serum albumin was purchased at many different times throughout this study and a sample was also obtained from Mann Laboratories, New York, N. Y. All rat serum albumin preparations afforded the same results. Crystallized samples of human, bovine, and rabbit serum albumin were also purchased from Pentex, Inc. Crystallized samples are described as 100% pure by electrophoresis at pH 5.1 and gave the same results as their corresponding fraction V preparations. A defatted sample of RSA was prepared according to Goodman.[14] This sample gave the same results as the original fraction V preparation. Purified HBABA, mp 205–206°, was purchased from Sigma Chemical Co., St. Louis, Mo., Dajac Laboratories, Philadelphia, Pa., and The British Drug Houses, Poole, Dorset, England, and was used without further purification. All HBABA samples produced the same general effects with RSA. α-(4-Chlorophenoxy)-α-methylpropionic acid (CPMPA) was synthesized from *p*-chlorophenol.[15]

Equilibrium Dialysis Experiments.—The procedure used to study the interaction of HBABA with serum albumin in the presence and absence of CPMPA is similar to the method reported by Klotz and coworkers.[16] Cellophane bags were made from 1-cm diameter dialysis tubing (Union Carbide Corp., Chicago, Ill.) and were soaked in distilled H_2O for 1 day prior to use. The bags were filled with exactly 4.0 ml of 1.0×10^{-4} *M* (assuming a molecular weight of 67,000 for all albumins) serum albumin in 0.1 *M* phosphate buffer, pH 7.4. The filled bag was immersed in a test tube of convenient diameter containing 4.0 ml of a solution of HBABA or HBABA plus CPMPA in the same buffer. Initial concentrations of HBABA outside the bag ranged from 1.2×10^{-5} to 2.0×10^{-3} *M*. CPMPA concentrations of 5.0×10^{-4} and 4.0×10^{-3} *M* were employed. The test tubes were covered and kept at a temperature of 4° for a period of not less than 72 hr, an interval sufficient to attain equilibrium.[16] All compounds were stable under these conditions.[6] The tubes were shaken periodically, the dialysis bags were removed, and the outside solutions were analyzed for HBABA content at 350 mμ (ϵ 19,125) using a Beckman Model DU spectrophotometer. CPMPA shows no absorption of visible light at 350 mμ.

Initially, several controls were also run in which only phosphate buffer and no albumin was placed inside the bag; other conditions remained the same. It was found that within an accuracy of about 5% all of the HBABA could be accounted for. Since the experimental error is larger than this value, controls were not run in subsequent experiments. Knowing the amount of HBABA remaining outside the bag after equilibrium, the amount of dye bound to the albumin could be calculated.

The Donnan effect on the ratio of monovalent ions inside and outside the bag has been calculated by McLean and Hastings[17] where the solution contained 1.2% total protein. In this calculation the concentration outside the bag would be 0.5% higher than inside. Since the concentration of albumin used in this study was even less (0.7%), any Donnan effect is negligible. The binding of HBABA to albumin was not corrected for protein volume because using the specific volume of 0.75 for serum proteins given by Svedberg and Sjogren[18] would mean that, at a concentration of 0.7% serum albumin, a maximum error of only 0.5% should result. The binding of HBABA in these studies is far greater than would be accounted for by protein volume alone.

Spectrophotometric Methods.—In addition to the equilibrium dialysis measurements binding of HBABA and CPMPA was studied spectrophotometrically in the region between 330 and 600 mμ using BSA and RSA for comparative purposes. Essentially following the method of Klotz, *et al.*,[19] the ϵ of HBABA bound to BSA and RSA was determined by holding the HBABA concentration (2.4×10^{-5} *M*) constant and varying the concentration of albumins up to 5.2×10^{-4} *M*. Then, the albumin concentration (either 4.5 or 5.0×10^{-5} *M*) and HBABA concentration (either 3.0 or 4.8×10^{-5} *M*) was held constant and the CPMPA concentration was varied from 1.5×10^{-5} to 18.0×10^{-3} *M*. The absorbance was determined at ambient temperature in a 1-cm cell as a function of wavelength using the Cary Model 15 spectrophotometer.

ORD Measurements.—The molar rotation as a function of wavelength for various albumins in the presence or absence of HBABA or CPMPA or both was calculated from ORD spectra in the region between 350 and 650 mμ using the Durrum-Jasco ORD-CD/UV5 spectropolarimeter with a cell of 1-cm light path. All measurements were made at ambient temperatures (29°) with concentrations of 5.0×10^{-5} *M* albumin, 5.0×10^{-5} *M* HBABA, and 5.0×10^{-4} *M* CPMPA in 0.1 *M* sodium phosphate buffer, pH 7.4.

Results

Equilibrium Dialysis.—The interactions observed between HBABA and bovine, human, rat, rabbit, and guinea pig serum albumins are typified by Figures 1 and 2 for bovine and rat serum albumins, respectively. The relationships between the moles of HBABA bound per mole of albumin and free HBABA concentration in the presence and absence of CPMPA are plotted. Through use of a Scatchard plot,[20,21] applied to these

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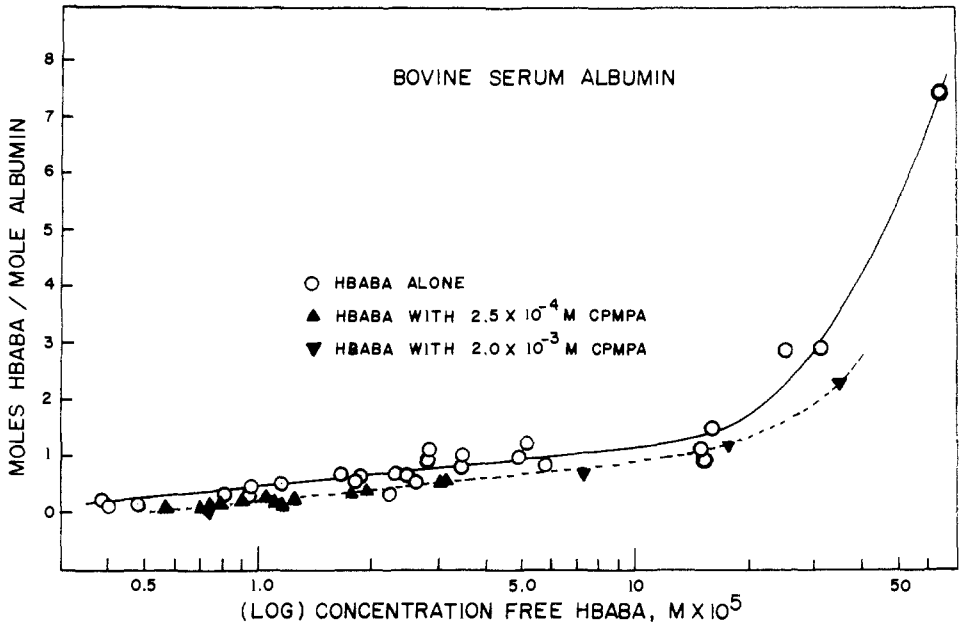

Figure 1.—The relation between the moles of HBABA bound per mole of BSA and free HBABA using equilibrium dialysis in the presence or absence of CPMPA.

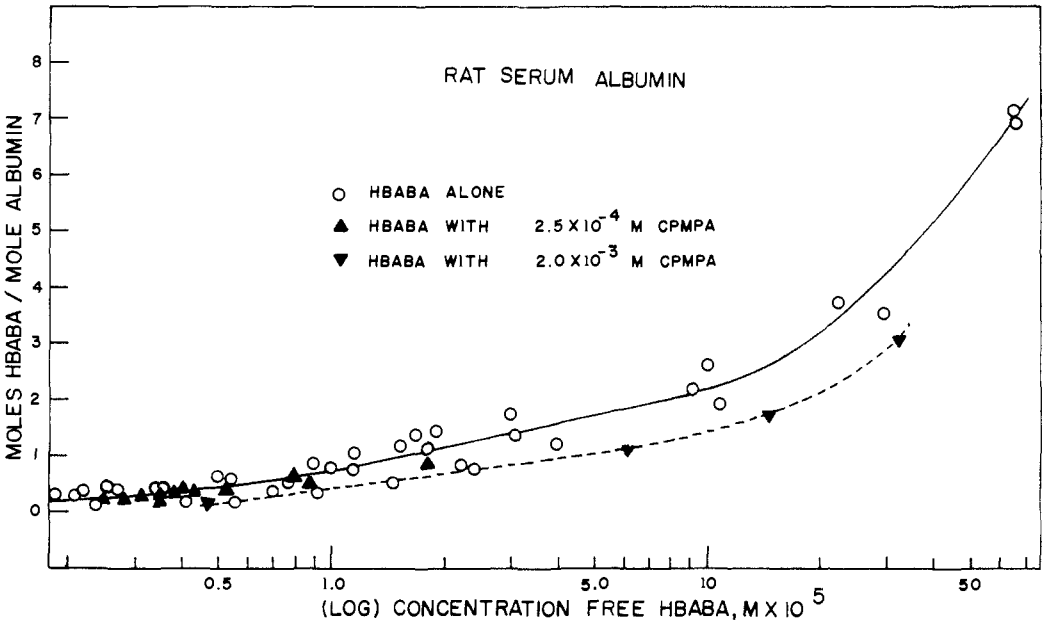

Figure 2.—The relation between the moles of HBABA bound per mole of RSA and free HBABA using equilibrium dialysis in the presence or absence of CPMPA.

data for all five albumins, two different sites (*i.e.*, sites exhibiting different equilibrium constants) were observed for the binding of HBABA to bovine, human, rat, rabbit, and guinea pig serum albumin. This is in agreement with the work of Baxter[5a] who studied the interaction of HBABA with serum albumins obtained from a number of different species, but apparently contradicts the finding of Moriguchi and coworkers[13] who only observed one site using BSA. To conveniently estimate species differences in the first (*i.e.*, stronger) binding constant and the corresponding maximum number of moles of HBABA molecules bound to this site, a Langmuir treatment[21,22] of the same data was employed at free HBABA concentrations less than about 4.0×10^{-4} *M* (where the large initial negative slope on the Scatchard plot was observed).

These results (Table I) show that HBABA interacts with bovine and human serum albumins in a similar manner. The equilibrium constants and the maximum number of moles of HBABA which may be bound to the stronger binding site are the same within experimental error. On the other hand, rat, rabbit, and guinea pig serum albumins exhibit similar equilibrium constants, but the constants are significantly larger than those observed for bovine and human serum albumin. In fact, when compared to RSA, BSA shows about one-sixth the free energy of binding by HBABA.

When the equilibrium constants were determined in the presence of a constant concentration of CPMPA (2.5×10^{-4} *M*) no significant change in equilibrium

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TABLE I

A COMPARISON OF REPRESENTATIVE STRONGER BINDING CONSTANTS AND THE CORRESPONDING MAXIMUM MOLES BOUND OF HBABA TO VARIOUS SERUM ALBUMINS IN THE PRESENCE AND ABSENCE OF CPMPA AS CALCULATED STATISTICALLY FROM THE REGRESSION OF $1/\bar{\nu}$ ON $1/A$ WHERE $\bar{\nu}$ AND A ARE OBTAINED FROM EQUILIBRIUM DIALYSIS EXPERIMENTS[a]

System	Regression slope ± std error[b] × 10^5	Representative binding constant[c] ± max error[d] × 10^{-4}	Max no. of moles of HBABA bound/mole of albumin ± max error[d]
Bovine albumin			
No CPMPA	2.6 ± 0.2	1.6 ± 0.1	2.4 ± 0.7
With CPMPA	3.8 ± 1.6	2.2 ± 1.1	1.2 ± 0.7
Human albumin			
No CPMPA	1.8 ± 0.3	2.4 ± 0.5	2.2 ± 0.8
Rat albumin			
No CPMPA	0.65 ± 0.12	9.8 ± 1.1	1.6 ± 0.6
With CPMPA	0.91 ± 0.33	7.8 ± 3.1	1.4 ± 0.7
Rabbit albumin			
No CPMPA	0.49 ± 0.24	17 ± 11	1.2 ± 1.0
Guinea pig albumin			
No CPMPA	0.60 ± 0.24	14 ± 7	1.2 ± 0.7

[a] $\bar{\nu}$ = number of moles of HBABA bound/mole of albumin and A = molar concentration of free HBABA. The regression analysis seems more appropriate to the Langmuir treatment of binding data since regression assumes that error is due to only the error in the dependent variable. [b] Calculated at a probability level of 0.05. [c] The binding constant tabulated here reflects the tendency of HBABA to bind to the stronger of two sites in serum albumin. The real binding constant would probably be slightly different from this number, but the relative strengths observed between the various albumins as reflected in the values given in this table should parallel the relative binding strengths between albumins using real binding constants. Primarily because of the small volumes (4.0 ml) used in these experiments (necessitated by the expense of some of the albumins) the resultant error does not permit a precise treatment of data according to Scatchard to get real binding constants. [d] The error tabulated here was determined by calculating the representative binding constant or maximum number of moles of HBABA/mole of albumin at maximum and minimum values of the regression slope. The average error between these two calculated values is given here. In the systems bovine and rat serum albumin (both in the presence of CPMPA) the larger value of the regression slope led to a value for the maximum number of moles of HBABA bound/mole of albumin that were small negative numbers. Hence, in these two systems, the maximum number of moles of HBABA bound was calculated from the minimum value for the regression slope, and the difference between this calculated value and the average number (1.21 or 1.41) is reported as the error.

constant for RSA or BSA could be detected. However, an apparent decrease in the maximum moles of HBABA which could be bound to the stronger binding site was observed for BSA. For RSA no such decrease was observed at this concentration of CPMPA.

When considering the effect of CPMPA on the actual number of moles of HBABA bound to bovine and rat serum albumin (rather than focusing on the maximum number that can be bound) it appears that CPMPA decreases the amount of HBABA bound at both concentrations of 2.0×10^{-3} and 2.5×10^{-4} M for BSA, but that CPMPA decreases HBABA bound to RSA only at its higher concentration (Figures 1 and 2, respectively). To study this relationship further a considerably more sensitive spectrophotometric analysis was used.

Spectrophotometric Analysis.—Comparative spectrophotometric analysis for BSA and RSA complexed with HBABA was studied with and without added CPMPA in the region 330–600 mμ. The spectrum for BSA–HBABA is shown in Figure 3. Addition of CPMPA causes a decrease in the absorbance at 477 mμ with a proportional increase in the absorbance at 345 mμ. Since the absorption at shorter wavelength is directly proportional to the amount of bound HBABA, the same number of moles of HBABA bound per mole of protein may be calculated through use of either eq 1 or 2.[19]

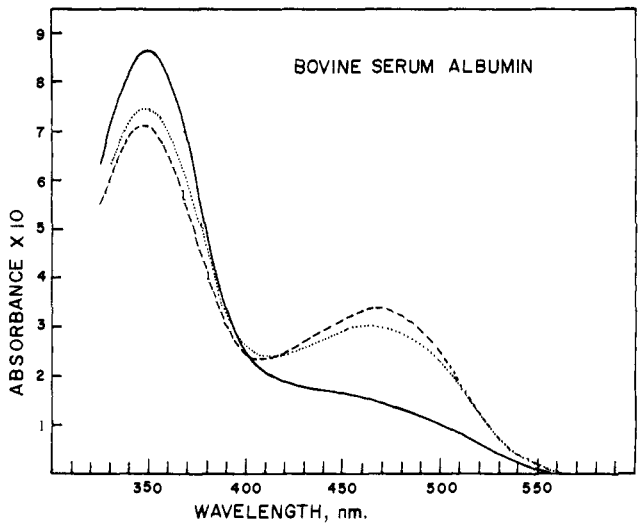

Figure 3.—Absorbance as a function of wavelengths for HBABA (4.8×10^{-5} M) and BSA (4.5×10^{-5} M) in the presence of 1.5×10^{-5} M (- - -), 1.8×10^{-4} M (· · ·), and 1.8×10^{-3} M (—) CPMPA in 0.1 M phosphate buffer, pH 7.4.

$$\phi_F^{345} = \frac{\epsilon_{app}^{345} - \epsilon_2^{345}}{\epsilon_1^{345} - \epsilon_2^{345}} \quad (1)$$

$$\phi_B^{477} = \frac{\epsilon_{app}^{477} - \epsilon_1^{477}}{\epsilon_2^{477} - \epsilon_1^{477}} \quad (2)$$

In these equations ϕ_F^{345} = fraction of HBABA free, ϵ_{app}^{345} = the observed absorbance divided by the total concentration of HBABA added, ϵ_1^{345} = extinction coefficient at 345 mμ for HBABA in 0.1 M sodium phosphate buffer, pH 7.4, and ϵ^{2344} = extinction coefficient for HBABA bound to protein at 345 mμ. The latter constant was obtained from a study of the apparent extinction coefficient as a function of increasing albumin to HBABA concentration ratio (Figure 4). Analogously, ϕ_B^{477} = fraction of HBABA bound and ϵ_{app}^{477}, ϵ_1^{477}, and ϵ_2^{477} are similar to the definitions for ϵ^{345} except that the absorabnce was determined at 477 mμ. In this case, however, ϵ_2^{477} was determined from the maximum increase in absorbance due to HBABA bound (Figure 4). The results are shown in Table II and substantiate the predictability of reversible competition of CPMPA for HBABA bound to the stronger site on BSA.

In contrast to the excellent mathematical correlation obtained for BSA and the competitive binding of CPMPA and HBABA, an anomalous effect was observed in a similar study with RSA. This is illustrated with Figures 5 and 6. At lower CPMPA concentrations (0.15×10^{-4} and 1.8×10^{-4} M) the 477-mμ peak increased and the λ_{max} at the shorter wavelength decreased in absorbance with increasing CPMPA concentration (Figure 5). Paralleling the latter decrease in

TABLE II

SPECTROPHOTOMETRIC DETERMINATION OF THE BINDING OF HBABA TO BOVINE SERUM ALBUMIN IN THE PRESENCE OF VARYING CONCENTRATIONS OF CPMPA

CPMPA concn, $M \times 10^4$	Moles of HBABA/mole of albumin At 345 mμ[a]	At 477 mμ[b]
0.15[c]	0.60	0.58
1.8[c]	0.51	0.51
6.0[d]	0.22	0.29
12.0[d]	0.18	0.18
18.0[c]	0.17	0.15
18.0[d]	0.13	0.15

[a] Calculated from 1 − fraction free = $1 - (\epsilon_{app} - \epsilon_2)/(\epsilon_1 - \epsilon_2)$ where ϵ_{app} = absorbance at 345 mμ/total HBABA concentration, ϵ_2 = absorptivity of bound HBABA at 350 mμ = 11,510, ϵ_1 = absorptivity of HBABA in absence of albumin at 350 mμ = 19,125. [b] Calculated from fraction bound = $(\epsilon_{app} - \epsilon_1)/(\epsilon_2 - \epsilon_1)$ where ϵ_{app} = absorbance at 477 mμ/total HBABA concentration, ϵ_2 = absorptivity of bound HBABA at 477 mμ = 11,447, ϵ_1 = absorptivity of HBABA at 477 mμ in the absence of albumin = 1312. [c] Initial HBABA concentration = 4.8×10^{-5} *M*, initial albumin concentration = 4.5×10^{-5} *M*. [d] Initial HBABA concentration = 3.0×10^{-5} *M*, initial albumin concentration = 5.0×10^{-5} *M*.

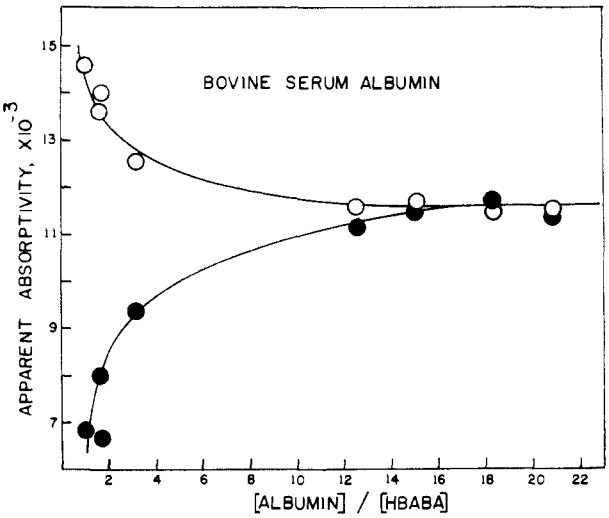

Figure 4.—The apparent absorptivity for HBABA as a function of BSA to HBABA ratio at wavelengths of 345 (○) and 477 mμ (●).

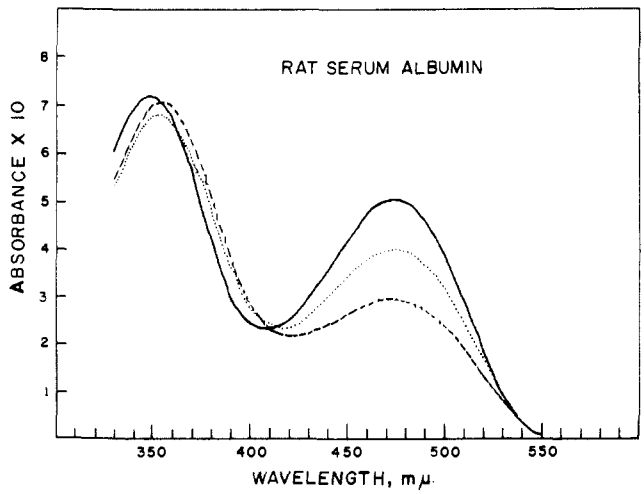

Figure 5.—Absorbance as a function of wavelength for HBABA (4.8×10^{-5} *M*) and RSA (4.5×10^{-5} *M*) in the presence of 1.5×10^{-5} *M* (- - -), 1.8×10^{-4} *M* (· · ·), and 1.8×10^{-3} *M* (—) CPMPA in 0.1 *M* phosphate buffer, pH 7.4.

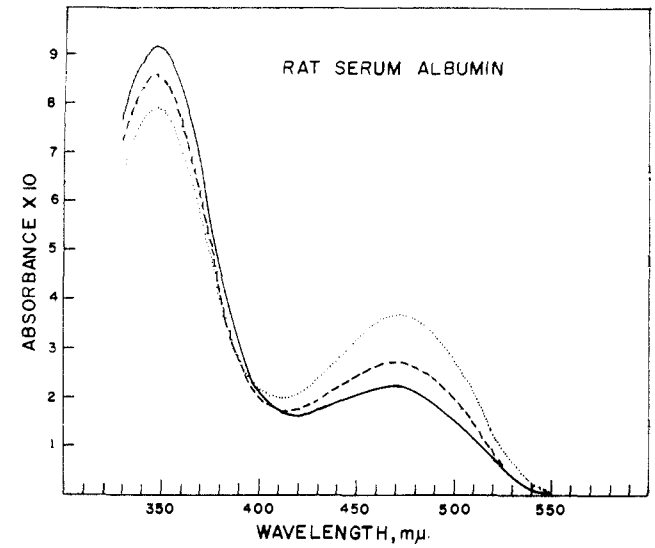

Figure 6.—Absorbance as a function of wavelength for HBABA (4.8×10^{-5} *M*) and RSA (4.5×10^{-5} *M*) in the presence of 6.0×10^{-3} *M* (· · ·), 1.26×10^{-2} *M* (- - -), and 1.8×10^{-2} *M* (—) CPMPA in 0.1 *M* phosphate buffer, pH 7.4.

absorbance a hypsochromic shift from 355 to 348 mμ was observed. Such a shift indicates a decrease in anionic resonance contribution; conversely, the 355-mμ peak seems to be a reflection of an interaction of RSA with the *p*-phenolic OH group of HBABA in which the OH proton is partly attracted to a binding site on RSA. The result is increased resonance contribution of the anionic form. This is analogous to the explanation for the bathochromic shift observed when increasing the pH of a solution of HBABA. In the absence of RSA, CPMPA has no effect on the HBABA absorption spectrum. At higher CPMPA concentrations (1.8×10^{-3} to 1.8×10^{-2} *M*) the 477-mμ peak decreased in absorbance and the λ_{max} at 350 mμ increased as in the case with BSA (Figure 6). However, even at higher CPMPA concentrations, no mathematical correlations corresponding to eq 1 and 2 were applicable with RSA where values of ϵ_2^{350} and ϵ_2^{477} were determined to be 14,200 and 7020, respectively.

These differences in data between BSA and RSA may be further analyzed with the use of Figure 7. With in-

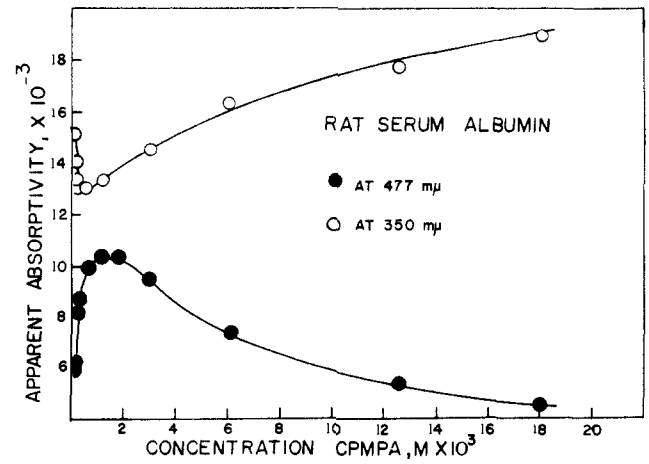

Figure 7.—Apparent absorptivity for HBABA as a function of increasing CPMPA concentration at 350 and 477 mμ.

creasing concentration of CPMPA there is an initial decrease in ϵ_{app}^{350} and then an increase which asymtotically approaches ϵ_1^{350} (19,125). Alternatively, increasing concentrations of CPMPA causes an initial increase in ϵ_{app}^{477} followed by a decrease, which, at a concentration of CPMPA where ϵ_1^{350} is approached, ϵ_{app}^{477} = 4500. Actually, ϵ_1^{477} = 1300; this suggests that HBABA is interacting with RSA even at high CPMPA concentrations.

ORD Studies.—Although there are small differences in molecular weight[3a] for serum albumins (fraction V) derived from different species, within the accuracy of our instrument, we observed the same negative anomalous optical rotatory dispersion (ORD) for all albumins at a concentration of 0.004% with the same molar rotation as those reported for bovine and human serum albumin. All albumins showed the same intensity trough at 233 mμ. Addition of 1.33×10^{-4} *M* HBABA and/or 1.33×10^{-4} *M* CPMPA yielded no detectable change in the molar rotation at any wavelength.

At higher protein concentration (5.0×10^{-5} *M*) only the region of the negative Cotton effect between 350 and 650 mμ may be studied. For bovine, human, and sheep serum albumin, addition of HBABA (5.0×10^{-5} *M*) gave no change in the molar rotation in this region of the ORD spectrum (Figure 8). For dog, horse, and pig

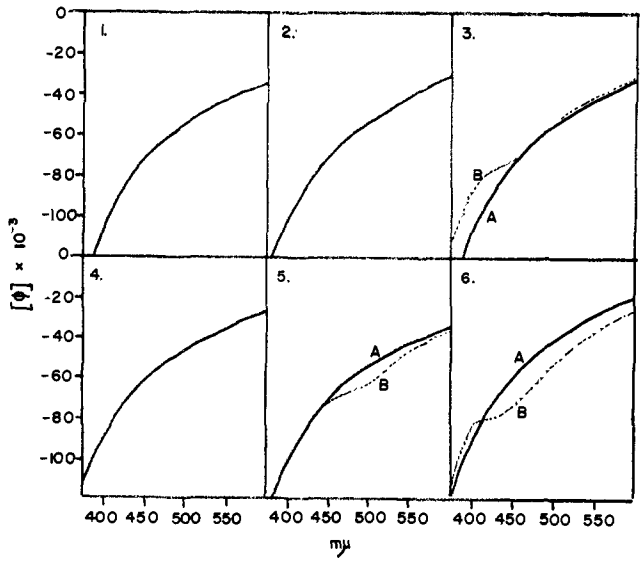

Figure 8.—Molar rotation as a function of wavelength at albumin concentrations of 5.0×10^{-5} *M* with or without 5.0×10^{-5} *M* HBABA in 0.1 *M* phosphate buffer, pH 7.4. 1, BSA with or without HBABA; 2, human SA with or without HBABA; 3, dog SA, (A) without HBABA, (B) with HBABA; 4, sheep SA with or without HBABA; 5, horse SA, (A) without HBABA, (b) with HBABA; 6, pig SA, (A) without HBABA, (B) with HBABA.

serum albumins small differences (curves labeled B in Figure 8) could be detected at these higher concentrations. However, with rat (Figure 9), rabbit (Figure 10), and guinea pig (Figure 11) serum albumins, a somewhat stronger induced anomalous optical rotatory dispersion (curves labeled C in the respective figures) was observed. This effect seems to parallel the equilibrium constant and therefore the free energy of binding; *i.e.*, bovine and human serum albumins with small equi-

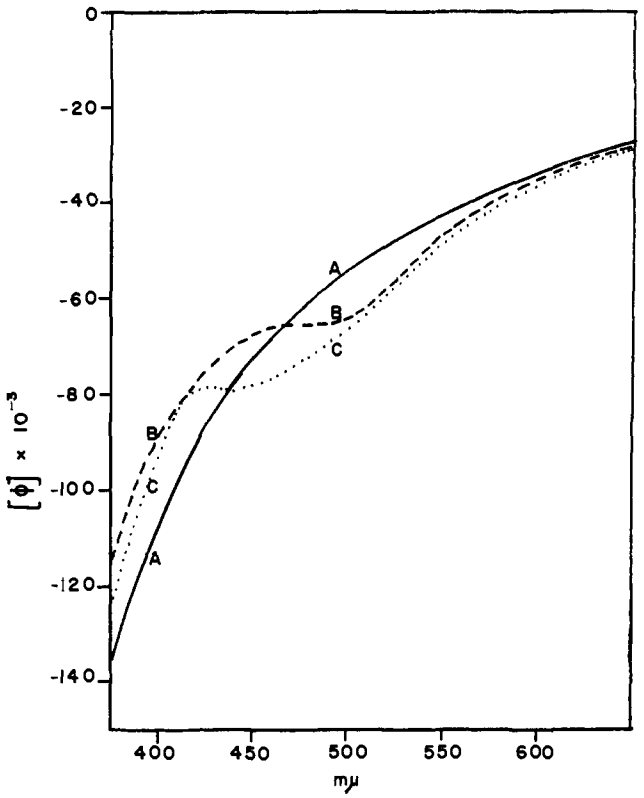

Figure 9.—Molar rotation as a function of wavelength at RSA concentrations of 5.0×10^{-5} *M*: A, without HBABA, but with or without CPMPA added; B, with 5.0×10^{-5} *M* HBABA and 5.0×10^{-4} *M* CPMPA added; C, with 5.0×10^{-5} *M* HBABA added, but no CPMPA.

librium constants show no change in their ORD spectra upon addition of 5.0×10^{-5} *M* HBABA, while rat, rabbit, and guinea pig serum albumins with larger equilibrium constants afford similar ERD curves.

With rabbit serum albumin and to a lesser extent with guinea pig serum albumin, addition of 5.0×10^{-4} *M* CPMPA blocks the ERD effect (curves B in Figures 10 and 11, respectively). This competitive block parallels the decrease in absorbance at 477 mμ observed spectrophotometrically when CPMPA is added to a solution of these albumins and HBABA. However, when CPMPA is added to a solution of RSA and HBABA the induced anomalous ORD is not blocked, but rather shifts 26 mμ to a higher wavelength (curve B in Figure 9). In the absence of HBABA, CPMPA has no effect on the ORD curve for RSA at this concentration.

Discussion

The spectrophotometric studies made at constant HBABA and BSA concentrations with varying CPMPA indicate that with this albumin CPMPA competes with HBABA for an interaction site at all concentrations of CPMPA. From equilibrium dialysis studies the site involved at the concentrations of HBABA used is most likely the stronger site ($K \sim 2 \times 10^4$) on the albumin. When, however, RSA is used under similar experimental conditions, low CPMPA concentrations increase the absorbance at 477 mμ and decrease the absorbance at 350 mμ (indicating more HBABA bound). Furthermore, at high CPMPA concentrations (in the RSA system) the absorbance at 350 mμ approaches that of

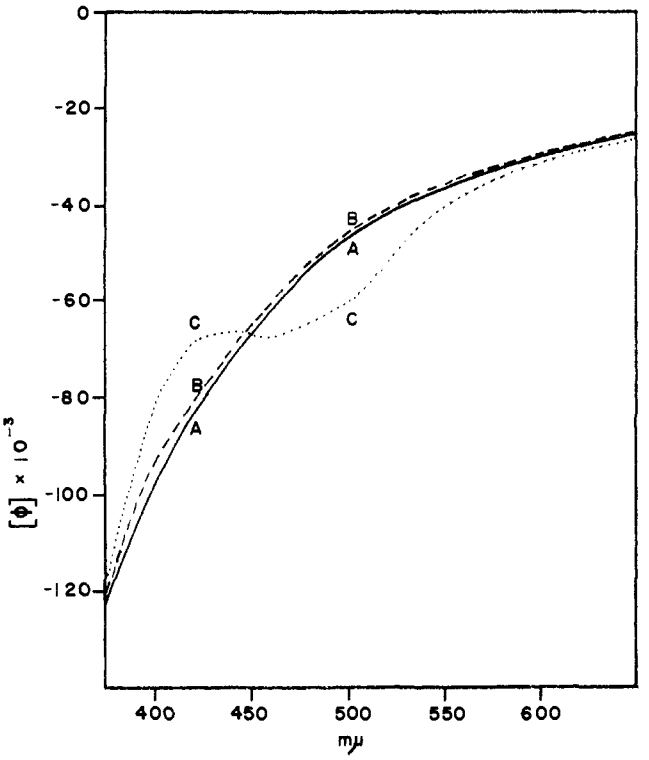

Figure 10.—Molar rotation as a function of wavelength at rabbit serum albumin concentrations of 5.0×10^{-5} *M*: A, without HBABA, but with or without CPMPA added; B, with 5.0×10^{-5} *M* HBABA and 5.0×10^{-4} *M* CPMPA added; C, with 5.0×10^{-5} *M* HBABA added, but no CPMPA.

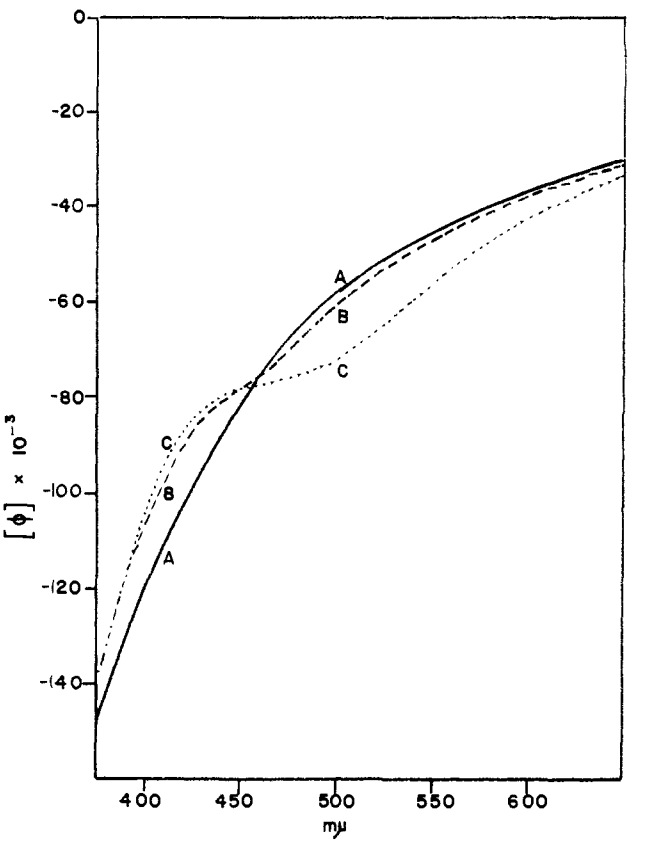

Figure 11.—Molar rotation as a function of wavelength at guinea pig serum albumin concentrations of 5.0×10^{-5} *M*: A, without HBABA, but with or without CPMPA added; B, with 5.0×10^{-5} *M* HBABA and 5.0×10^{-4} *M* CPMPA added; C, with 5.0×10^{-5} *M* HBABA added, but no CPMPA.

free HBABA while the absorbance at 477 mμ does not. It should be noted that neither CPMPA alone nor CPMPA in the presence of RSA absorbs at 477 mμ. These observations, indicating an increase in binding of HBABA upon addition of CPMPA, suggest that there are at least three different sites of interaction of HBABA on RSA: a strong and weak binding site (present in all species different albumins studied as indicated by equilibrium dialysis), and a third site liberated upon addition of CPMPA; the ϵ^{477} for the third site may also be greater than the ϵ^{477} for the stronger (first) site for RSA–HBABA. Further work is in progress in order to determine to what extent increased binding of HBABA contributes to ϵ^{477} in the presence of CPMPA. The increase in binding of HBABA could be the result of a small molecular perturbation on the RSA molecule caused by the binding of CPMPA to an allosteric site.[23,24] This results in the freeing of a new site to which HBABA may also bind. The RSA–HBABA system may be thought of as analogous to an enzyme system where the term allosteric site is used to differentiate between two different sites on an enzyme.[23a] In the RSA–HBABA system HBABA may be thought of as binding to an "active site" and is competitively inhibited by CPMPA. However, CPMPA concomitantly binds reversibly to an "allosteric site" which releases a new "active site" for competitive binding of HBABA and CPMPA.

Results derived from optical rotatory dispersion studies are in accord with the possibility of the existence of an "allosteric site." However, these studies do not suggest a large change in secondary structure. ERD and optical rotation changes at the sodium D line for albumins bound to azo dyes have been attributed to a distortion of secondary structure.[25] While major changes in the ORD spectrum at 233 mμ resulting from binding with extrinsic chromophores (dyes) is a reflection of a large structural change in the protein, optical rotation changes at only one wavelength (such as the sodium D line), as was observed for HBABA, need not necessarily reflect such a change in secondary structure. Stryer and Blout[26] measured a number of optical rotatory dispersions throughout the absorption bands of extrinsic chromophores bound to synthetic polypeptides and have suggested that the acquired optical rotatory power of the bound dye molecule in their system could be of only two types: (1) configurationally induced optical rotatory power arising from the interaction of the symmetric dye chromophore with the local asymmetric environment of the α-carbon atom of the polypeptide or (2) conformationally induced optical rotatory power resulting from the interaction among several symmetric dye chromophores which have been oriented in a specific fashion to one another by binding to the asymmetric polypeptide. Our results are in closer agreement with the Stryer–Blout proposal (either type

(23) (a) J. Monod, J.-P. Changeux, and F. Jacob, *J. Mol. Biol.*, **6**, 306 (1963); (b) J. Monod, J. Wyman, and J.-P. Changeux, *ibid.*, **12**, 88 (1965).

(24) (a) J. H. Wang and W. J. Black, *J. Biol. Chem.*, **243**, 4641 (1968); (b) D. E. Atkinson, J. A. Hathaway, and E. C. Smith, *ibid.*, **240**, 2682 (1965).

(25) (a) G. Markus and F. Karush, *J. Amer. Chem. Soc.*, **80**, 89 (1958); (b) M. H. Winkler and G. Markus, *ibid.*, **81**, 1873 (1959).

(26) (a) L. Stryer and E. R. Blout, *ibid.*, **83**, 411 (1961); E. R. Blout and L. Stryer, *Proc. Natl. Acad. Sci. U. S.*, **45**, 1591 (1959).

1 or 2, above) since (a) the induced anomalous ORD could only be detected in the region of maximum absorption for the HBABA–protein complex, (b) the ERD effect was small, and (c) no change was observed in the ORD spectrum at 233 mμ. These data, however, do not differentiate between configurationally or conformationally induced rotatory activity.

Irrespective of the mechanism of induced optical rotatory activity, these data complement the results obtained spectrophotometrically and by equilibrium dialysis and do suggest that if any structural change in the protein is involved, the change must be exceedingly small and/or occurring at the end of the polypeptide chain. Certainly, a large rearrangement of secondary structure is not involved. That a small change in structure is occurring with RSA is suggested by the displacement of the induced ORD to a longer wavelength (a 26-mμ shift) upon adding CPMPA. This shift in the induced ORD is not paralleled by a bathochromic shift of the λ_{max} at 477 mμ; *i.e.*, HBABA seems to be binding to a new site in the presence of CPMPA. Again, it is difficult to explain the unmasking of this new site without invoking the concept of allosteric transition. How general this phenomenon is and how important it may be in drug transport mechanisms remains to be seen.

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Molecular Orbital Calculations on Anticonvulsant Drugs

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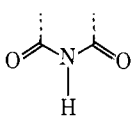
Molecular orbital calculations on a number of anticonvulsant drugs and related compounds have been completed by two methods, extended Hückel and complete neglect of differential overlap. Calculated dipole moments indicate that the latter method is more suitable for assessing net atomic charges. The calculated atomic charges at a "biologically active center" proposed by Perkow, together with those at atoms capable of forming hydrogen bonds, have been compared with observed anticonvulsant activity. The "biologically active center" does not appear to effect activity, while the hydrogen-bonding atoms, although common to all the drugs studied, are not proved responsible for variations in activity.

Widespread research on anticonvulsant drugs has led to numerous theories[1] which ascribe their CNS activity to a variety of simple physicochemical properties, but none appears to account satisfactorily for all the observed facts. A selection of anticonvulsant drugs which have proved useful clinically, together with some related compounds, is shown in Table I. These compounds all have a similar structure, and the presence of the grouping I appears to be a possible factor in their activity. Furthermore, it seems feasible that variation in the net atomic charges in this part of the molecule might change the CNS activity of the drugs by altering hydrogen-bonding behavior.



I

II

Another hypothesis has been put forward by Perkow,[2] who suggests that the net charge at a biologically active center (BAC), starred in II, is partly responsible for the type and degree of CNS activity. In this work both hypotheses have been tested by completing molecular orbital calculations on the compounds shown in Table I.

Methods

The molecular orbital calculations used were the extended Hückel theory (EHT) of Hoffmann[3] and the complete neglect of differential overlap calculation (CNDO/2) devised by Pople and Segal.[4] The original atomic parameters have been retained, except for some of the valence-state ionization potentials employed in the EHT calculations, which were averaged from atomic spectral data.[5] The values used were (in eV) H_{1s}, 13.6; C_{2s}, 20.8; C_{2p}, 11.3; N_{2s}, 26.5; N_{2p}, 13.6; O_{2s}, 33.0; O_{2p}, 16.2. The calculation of atomic charges by both methods, and of dipole moments by the CNDO/2 method, is described in the original papers. The EHT dipole moments, μ, were evaluated from expressions 1 and 2 where Q_A is the net charge on atom A, x_A is the

$$\mu_x = 4.80 \sum_{A}^{\text{atoms}} Q_A x_A - 7.337 \sum_{A}^{\text{atoms}} P(2s,2p_x)_A / Z_A \quad (1)$$

$$\mu^2 = \mu_x^2 + \mu_y^2 + \mu_z^2 \quad (2)$$

x coordinate, $P(2s,2p_x)_A$ is the bond order[4] between the 2s and $2p_x$ orbitals, and Z_A is the Slater exponent. The first term in eq 1 is the contribution from the net atomic charges, and the second is the atomic polarization contribution.[4]

Using Hoffmann's program[6] as a basis, a Fortran IV program[6] has been written which does EHT calculations for systems involving up to 96 atomic orbitals. The CNDO/2 calculations were done with a Fortran IV program[6] written by Segal, which handles a maximum of 72

(1) T. C. Butler, *Pharmacol. Rev.*, **2**, 121 (1950).
(2) W. Perkow, *Arzneimittel-Forsch.*, **10**, 284 (1960).
(3) R. Hoffmann, *J. Chem. Phys.*, **39**, 1397 (1963).
(4) (a) J. A. Pople and G. A. Segal, *ibid.*, **43**, S136 (1965); (b) J. A. Pople and G. A. Segal, *ibid.*, **44**, 3289 (1966).
(5) (a) H. A. Skinner and H. O. Pritchard, *Chem. Rev.*, **55**, 745 (1955); (b) G. Pilcher and H. A. Skinner, *J. Inorg. Nucl. Chem.*, **24**, 937 (1962).
(6) Available from Quantum Chemistry Program Exchange, University of Indiana, Bloomington, Ind.