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[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY RESEARCH AND EXPERIMENTAL BIOLOGY, ROSWELL PARK MEMORIAL INSTITUTE]

Rotatory Dispersion of a Serum Albumin–Azo Dye Complex

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Rotatory dispersion measurements are reported for human serum albumin in interaction with the anionic azo dye, *p*-(*p*-dimethylaminobenzeneazo)-benzoylaminoacetic acid at *p*H 7.4. The dispersion curves indicate structural changes in the albumin molecule as a result of dye binding. The sources and elimination of spurious rotations encountered in the presence of colored substances are discussed.

In a recent communication by Markus and Karush[1] dealing with the rotatory behavior of serum albumin in combination with a variety of azo dyes measured at 589 mμ in a visual polarimeter rotatory dispersion determinations were also reported for two of the dyes used (Fig. 6 in ref. 1). These dispersion measurements were made with a Rudolph dispersion polarimeter. The specific levorotation of serum albumin in a 0.001 *M* solution of the first dye, *p*-(*p*-dimethylaminobenzeneazo)-benzoylaminoacetic acid was found to decrease sharply around 630 mμ and to reach a specific rotation of −9° at 560 mμ. The same dye at half this concentration showed the same trend. We have recently repeated these measurements using a Keston photoelectric polarimeter attached to the Beckman DU spectrophotometer. At the beginning of these experiments we obtained curves very similar to those cited and in addition found that the dye alone, though lacking an asymmetric center showed a very similar change in dispersion below 570 mμ (Fig. 1, curves e and f). It was subsequently found that this behavior is due to an artifact and is caused by stray light partially polarized by reflection in the Keston attachment. (For an analysis of this artifact see below.) When the stray light was eliminated the dye alone showed zero optical rotation and the sudden change in apparent rotation between 500 and 600 mμ was no longer observed in the dispersion curve of the dye–protein complex. It must be emphasized, however, that the curves obtained under these conditions, using three dye concentrations at constant HSA concentration (Fig. 1) still showed very strong deviations from the dispersion of HSA in the absence of dye. The specific rotations measured at 589 mμ agreed well with the values obtained in the earlier work using a Stanley–Bellingham visual polarimeter with a double field where this type of artifact cannot be present.

The dispersion curves reported now are in agreement with the conclusions drawn in the previous paper on the basis of measurements at 589 mμ and are consistent with the view expressed there that the changes in optical rotation of serum albumin upon interaction with azo dyes represent changes in the configuration of the protein molecule.

The anomalies described are caused by stray light partially polarized by reflection and occur only in the wave length region where the substance under investigation shows high optical density and under the following conditions: (1) the incidence upon the detector of a portion of the output of the monochromator which has not, or has incompletely traversed the solution; (2) high wave length inhomogeneity of the radiation emergent from the monochromator (mostly at the very edges of the beam). Under these conditions, the undesired radiation will become increasingly

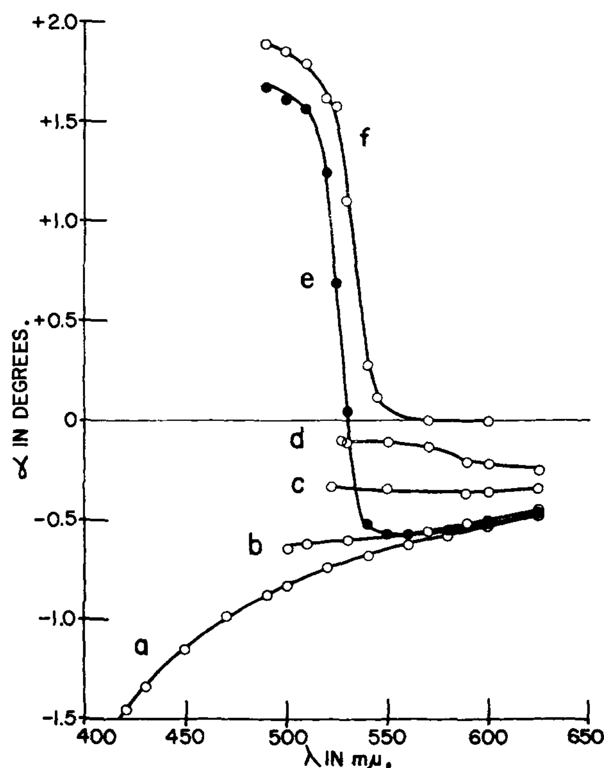

Fig. 1.—Rotatory dispersion of HSA–dye complexes: a, HSA alone; b, HSA in 1×10^{-4} *M* dye; c, HSA in 5×10^{-4} *M* dye; d, HSA in 1×10^{-3} *M* dye; e, HSA in 1×10^{-4} *M* dye showing the artifact; f, 1×10^{-4} *M* dye showing the artifact. HSA, 0.89%; NaCl 0.15 *M*; phosphate buffer, 0.02 *M*, *p*H 7.4.

important relative to the principal component as the latter is attenuated by absorption. The spurious rotation can be eliminated by using a cell blackened on the outer surfaces with a large black paper shield around the end of the cell facing

(1) G. Markus and F. Karush, THIS JOURNAL, **80**, 89 (1958).

the phototube and by reducing the aperture anterior to the analyzing polaroid. It is probable that in the dispersion curves obtained earlier with the Rudolph instrument the anomaly may also have been caused by stray light, partially polarized by reflection.

Experimental

p-(*p*-Dimethylaminobenzeneazo)-benzoylaminoacetic acid was synthesized according to Karush.[2]

Human serum albumin was obtained from Cutter Laboratories.

(2) F. Karush, *J. Phys. Chem.*, **56**, 70 (1952).

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[CONTRIBUTION NO. 1504 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

The Mechanism of Chymotrypsin-catalyzed Reactions. III

BY TREVOR SPENCER AND JULIAN M. STURTEVANT

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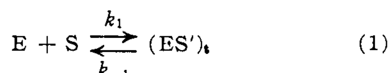
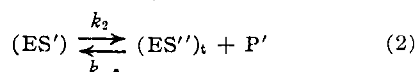
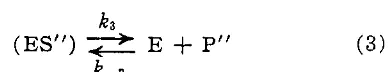
Kinetic experiments, employing fast reaction techniques, on the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate are reported. These experiments demonstrate that the acetylchymotrypsin isolated by Balls and Wood is a true intermediate in the reaction and give further indication that the position of acylation is at a serine hydroxyl rather than at a histidine imidazolyl group. The rate of the final hydrolysis of the acetyl enzyme is controlled by a group at the catalytic site having an apparent pK of 7.4. It is shown on the basis of experiments involving competition between *p*-nitrophenyl acetate and a specific substrate, acetyl-L-tyrosine ethyl ester, that hydrolysis of the latter probably involves the same three-step mechanism as the hydrolysis of *p*-nitrophenyl acetate. This conclusion emphasizes that the specificity of the enzyme can be even more important in later stages of the catalysis than it is in the initial formation of a Michaelis–Menten complex. A new stopped-flow fast reaction apparatus is briefly described.

Introduction

It has been shown in previous publications[1,2] that the kinetics of the chymotrypsin (CT)-catalyzed hydrolysis of *p*-nitrophenyl acetate (NPA) is consistent with a mechanism involving three distinct steps







In the first step there is a very rapid formation of a Michaelis–Menten complex, ES′, which in the second step is decomposed to give the product P′ (*p*-nitrophenol (NP) in the case of NPA) and an acylated derivative, ES″, of the enzyme. Finally, the acyl enzyme is hydrolyzed to regenerate the enzyme and form the product P″ (acetate in the case of NPA). The rates of the second and third steps are dependent on the state of ionization of a group in the catalytic site, only the unprotonated form being active. In equations 1 to 3, $(ES')_t$ and $(ES'')_t$ represent the sum of both the ionized and unionized forms of the intermediates, the distribution between these forms following, at least approximately, the equation for a single group ionization

$$K_i' = a_H \frac{[ES']}{[ES'H^+]} \qquad K_i'' = a_H \frac{[ES'']}{[ES''H^+]} \quad (4)$$

where the quantities in square brackets represent the molar concentrations of the corresponding species and a_H is the hydrogen ion activity (in this paper operationally defined as the quantity measured by a glass electrode).

(1) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(2) H. Gutfreund and J. M. Sturtevant, *Proc. Nat. Acad. Sci.*, **42**, 719 (1956).

The differential equations of this kinetic scheme are readily solved by the method of Laplace transforms, if attention is confined to the early stages of the reaction so that the substrate concentration can be assumed to remain essentially constant at its initial value, $[S]_0$, and the reversal of the second and third steps can be neglected. It can be shown that the concentration of the product P′ is given by the expression

$$[P'] = k_1k_2'[E]_0[S]_0 \left[\frac{nm - k_3'(n+m)}{n^2m^2} + \frac{k_3'}{nm}t + \frac{k_3' - m}{m^2(n-m)}e^{-mt} - \frac{k_3' - n}{n^2(n-m)}e^{-nt}\right] \quad (5)$$

where t is the time, $k_2' = k_2K_i'/(K_i' + a_H)$ and $k_3' = k_3K_i''/(K_i'' + a_H)$ are apparent first-order rate constants, and m, n are given by

$$2m = k_1A' + k_3' - \sqrt{(k_1A' + k_3')^2 - 4k_1(k_2'[S]_0 + k_3'A')} \quad (6)$$

$$m + n = k_1A' + k_3' \quad (7)$$

$$A' = [S]_0 + K_m' \quad (8)$$

$$K_m' = \frac{k_{-1} + k_2'}{k_1} \quad (9)$$

The second exponential in equation 5 reflects the build up of the steady-state concentration of the Michaelis–Menten complex ES′, while the first exponential describes the formation of the steady-state concentration of the acyl enzyme, ES″. In the present case, available techniques are inadequate to detect the phase of the reaction involving the second exponential so that we may conclude that $n >> m$. This implies that $k_1A' >> k_2'$ and $k_1A' >> k_3'$. It then follows with adequate accuracy that

$$m = \frac{k_2'[S]_0 + k_3'A'}{A'};\ n = k_1A' \quad (10)$$

Equation 5 becomes, after decay of the second exponential