we assume that there are molecules in the glass having either cisoid or transoid configurations.[4] Because there should be a large amount of steric strain in the *cis* configuration we were a little surprised by the evidence that both configurations were appreciably populated at 77°K. Since the setting temperature of MCIP is significantly higher than 77°K., it seemed possible that non-equilibrium configurations might have been "frozen in." It has been reported[5] that a glass formed from isopentane (6 parts) and 3-methylpentane (1 part) has a setting temperature very close to 77°K. Therefore, we prepared a solution of β-naphthil in this glass with the thought that mobility would be maintained at lower temperatures and something closer to configurational equilibrium would be established in this system. As illustrated by Fig. 3, the phosphorescence spectrum of β-naphthil in this system has only two bands at 18,830 and 17,360 cm^{-1}. Probably only *trans* molecules are present in this glass. The experiment in the low setting glass also indicates that the "second" emission from β-naphthil in MCIP is not due to an impurity.

In light of the interpretation offered, we must presume that the emission from EPA solution arises essentially exclusively from *cis* triplets. While this leads to the conclusion that the ratio of *cis* to *trans* ground state configurations is greater in that solvent than in the hydrocarbons, it does not imply that the ratio is necessarily greater than unity. Since the lifetime of the *trans* triplet is obviously much shorter than that of the *cis* isomer, the decay process may have changed the *trans*/*cis* ratio by a large amount before emission is measured in any of our experiments.

Recently, evidence has been presented[6] which supports the existence in methanol–methylcyclohexane solutions of two retinene triplets. It was suggested that the "second" triplet is a retinene–methanol complex. In view of our observations with β-naphthil, we would like to propose as an alternative explanation the existence of two stereoisomeric retinene triplets.

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(7) National Science Foundation Predoctoral Fellow, 1961 to present.

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SUBSTRATE CONTROL OF CONFORMATION CHARACTERISTICS IN CHYMOTRYPSIN[1]

Sir:

In a stimulating series of studies Vaslow and Doherty[2] found that although the equilibrium constant for the binding of virtual substrates to α-chymotrypsin (CT) was nearly independent of pH, the standard enthalpy and entropy underwent large variation. The occurrence of a significant variation in entropy change compensated by enthalpy changes suggested to them

(1) The contents of this note were presented at the 46th Federation meeting, R. Lumry and H. Parker, *Fed. Proc.*, **21**, 246 (1962), and at the 142nd National Meeting, American Chemical Society, Atlantic City, New Jersey, September, 1962, Abstract 48-C.

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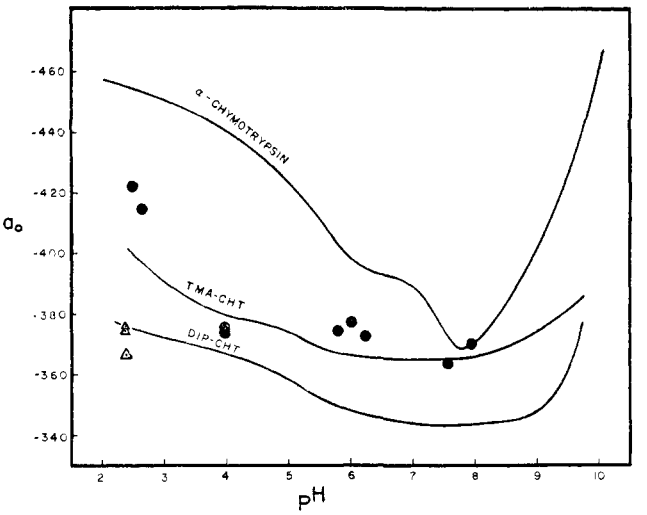

Fig. 1.—The value of the parameter a_0 for α-chymotrypsin and some derivatives. The solid circles are from α-chymotrypsin in the presence of 0.02 *M* hydrocinnamic acid; the open triangles from α-chymotrypsin in the presence of 0.007 *M* acetyl-L-tyrosine ethyl ester.

a modification in protein structure on substrate binding, with a maximal effect at about pH 7.5, the lowest pH of maximum catalytic activity. Schellman and Vaslow[3] could find little or no substrate dependence of optical rotation though the experiments were not carried out under optimum conditions. Simpson, Jacobs and Lumry[4] repeated the experiments of Vaslow and Doherty using trypsin, an enzyme known to be very similar to CT in its catalytic properties. Dialysis-equilibrium studies showed single-site binding with pH and temperature dependencies essentially identical with those reported for CT. Addition of virtual substrates at pH 8 produced no change in viscosity, specific rotation or tryptophan spectrum for trypsin solutions. Subsequently Brandts[5] observed that the characteristic small substrate of CT, acetyl-L-tyrosine ethyl ester (ATEE) at pH 2 greatly reduced the entropy and enthalpy of the reversible denaturation of chymotrypsinogen. This observation coupled with the report by Rupley, Dreyer and Neurath[6] of a pH-dependent change in specific rotation of CT lead us to suspect that conformation changes due to bound substrates might occur at low pH values rather than at high. Such was found to be the case in our early studies of the specific acylating reagents: diisopropylfluorophosphate (product DIPCT) and *p*-nitrophenyltrimethylacetate (product TMACT).[1] There were significant changes in protein physical parameters at low pH and also at high (a_0 in Fig. 1 for example) some of which were independently observed by Hess and co-workers.[7] We have now found that real and virtual substrates produce quite as large changes in optical rotation as do the specific acylating reagents. The a_0 parameter computed from rotatory-dispersion data using the Moffitt equation[8] is presented as a function of pH at 23° in Fig. 1. Hydrocinnamate ion (HC) is a good competitive inhibitor though a poor virtual substrate. The Moffitt b_0 parameter varies from form to form with pH

(3) Personal communication from F. Vaslow.

(4) J. Simpson and R. Lumry, 139th National Meeting, American Chemical Society, St. Louis, Missouri, March, 1961, Abstract 19-O.

(5) J. Brandts, Dissertation, "The Reversible Thermal Denaturation of Chymotrypsinogen," University of Minnesota, 1961.

(6) J. Rupley, W. Dreyer and H. Neurath, *Biochim. et Biophys. Acta*, **18**, 162 (1955); H. Neurath, J. Rupley and W. Dreyer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).

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but at pH values below 7 its change is too small to be reported without full statistical analysis.

Optical rotation differences between CT and modified forms are greatest at low and at high pH values. Since b_0 differences become large at pH values above 8, it is probable that two different domains of the protein dominate our observations—one at low pH, the other at high. Above pH 8 reliable data for HC binding have not been obtained since HC greatly increases the autolysis rate of CT. In the pH region below 7.5 a_0 differences appear to consist of a large pH-independent change supplemented by a smaller pH-dependent variation which reflects the pH dependence of a_0 in unmodified CT. As measured by a_0 the normal conformation for catalysis appears to be that at pH 7.5 and the larger change mentioned above is either a consequence of acylation and substrate binding or a necessary preliminary process.

Acetyl-D-tyrosine ethyl ester (A-D-TEE) produces no change in rotation at pH 2.4. With ATEE rotation change shows a first-order dependence on substrate concentration with an equilibrium constant at pH 2.5 of approximately 2×10^{-3} M. Dialysis-equilibrium studies[2] show only one binding site. Hence rotation changes are to be attributed to the binding of one molecule of ATEE and not to any indirect effect of ATEE on solvent properties. Rotation changes due to HC at pH 4 are also first-order in HC concentration with an equilibrium constant of 6×10^{-3} M in good agreement with constants from fluorescence studies,[9,10] from virtual substrate binding studies[2] and from some studies of HC as a competitive inhibitor of CT catalysis.[11,12] Hence, again we can attribute the effect to binding of substrate. In current interpretations, changes in a_0 without corresponding b_0 changes are due to changes in freedom of vicinal groups or environment composition at asymmetric carbons. Hence a_0 variations are to be attributed to conformation changes, though these may involve only alterations in structural rigidity and conformational relaxation times. There is no proof in this work that substrates influence conformational properties through binding to groups of the catalytic site. However, though a second binding site may be involved, there is thus far no evidence which makes this a probable situation. Substrate binding at low pH can be divided into two parts: (1) a change in conformational properties and (2) direct interaction of substrate and protein at the binding site. At pH 7.5 the change in conformation indicated by a_0 is a minimum and the direct interaction appears to dominate the thermodynamics. However, fluorescence studies to be reported suggest that this analysis is too simple.

The conformational changes may prove to be trivial or too sluggish to be related to fast catalytic processes.[10] On the other hand, the relationship between ΔH or ΔS for virtual substrate binding[2] and the pH dependence of a_0 for CT indicates an important relationship between the pH-dependent conformational processes associated with the formation of the specific nucleophilic site for catalysis and virtual substrate binding. There are relatively small but essential readjustments in the protein fabric which convert CT from a passive binding agent to a catalyst and these are directly or indirectly coupled to substrate binding. The effects of substrate binding are similar to and, where comparisons are possible, as large as those produced by specific acylation. The data suggest that if specific acylation occurs at the catalytic site, so does the binding of more normal substrates responsible for the changes in a_0. It is important to note, however, that there is no basis in this work upon which to postulate the occurrence of important conformation changes during normal catalysis. Of the substances tested only diisopropylfluorophosphate produces significant a_0 changes at pH 7.5 and there is no reason to suppose that the interaction of this substance with CT parallels the processes of normal catalysis. Current high-speed kinetic studies and static fluorescence studies may provide some basis for postulating conformational changes during normal catalysis and these will be reported. Several authors have proposed that primary bond rearrangement within the protein is essential to the formation of the specific nucleophilic site.[13] It is our thesis that an equally essential aspect of the development of this site is the distortion of primary bonds which provides the necessary local electronic properties for catalysis.[14] We shall defend this thesis and present details of our results in forthcoming papers.

Experimental.—Rotatory dispersion measurements were made with a Rudolph Model 200 spectropolarimeter modified to increase ease of reading and precision. Protein concentrations were usually 0.3%. The molecular weight of the protein was taken as 24,800. The routine standard deviation in a_0 is 3° but variations in protein concentration often increase this error to 5°. The λ_0 parameter was 2390 Å., the best-fit value found by Brandts.[5] Worthington CT and chymotrypsinogen were used throughout without further purification. TMACT, acetylCT and DIPCT were made by well-known procedures.[15]

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H ATOM ADDUCTS—NEW FREE RADICALS?

Sir:

The question as to whether molecules such as water or ammonia in the gas phase have an affinity for a hydrogen atom is of considerable interest since it implies the stability of free radicals of the type HA where A is a saturated proton acceptor molecule. An answer to this question may be obtained by considering the bond dissociation energies involved in a conventional hydrogen bonded system such as X–H. . .A. The bond dissociation energy in the isolated hydrogen donor molecule XH in the gas phase is given by

$$D(\mathrm{X-H}) = \Delta H_f(\mathrm{X}) + \Delta H_f(\mathrm{H}) - \Delta H_f(\mathrm{XH}) \quad (1)$$

where ΔH_f is the heat of formation in the gas phase.

In the complex

$$D(\mathrm{X{-}HA}) = \Delta H_f(\mathrm{X}) + \Delta H_f(\mathrm{HA}) - \Delta H_f(\mathrm{XHA}) \quad (2)$$

the enthalpy of hydrogen bond formation may be written as

$$-\Delta H = D(\mathrm{XH{-}A}) = \Delta H_f(\mathrm{A}) + \Delta H_f(\mathrm{XH}) - \Delta H_f(\mathrm{XHA}) \quad (3)$$

To construct a cycle we may write the bond dissociation energy of the HA radical as

$$D(\mathrm{H{-}A}) = \Delta H_f(\mathrm{H}) + \Delta H_f(\mathrm{A}) - \Delta H_f(\mathrm{HA}) \quad (4)$$

so that from these equations one readily obtains the