The pK_a values thus determined show remarkable strengths for the cyanocarbon acids. Pentacyanopropene and the first ionization of hexacyanoisobutylene are comparable with the stronger mineral acids. Hydrochloric acid, for example, is thought to have a pK_a in the neighborhood of -7.[7] A high degree of resonance stabilization in the anions (which is not possible in protonated form) appears to be responsible for the high acidity.[1]

Smoothed values of the acidity function H_- calculated according to

$$H_- = pKa + \log C_A^-/C_{HA}$$

are listed in Table II along with values of H_0 for comparison.[2] The H^- function presented here is based entirely on *p*-(tricyanovinyl)-phenyldicyanomethane, methyl dicyanoacetate and bis-(tricyanovinyl)-amine.

H_- behaves in a manner surprisingly similar to H_0 although there are quantitative differences. At lower concentrations (<5 *M*) these appear to be of the kind expected on the basis of the differences in charge types. At higher concentrations H_- roughly parallels H_0. The similarity of these two acidity functions is further evidence that these indicator acidity functions depend primarily on the properties of the hydrogen ion in solution relatively unencumbered by activity coefficient effects of the indicators themselves.[7]

(7) R. P. Bell, "The Proton in Chemistry," Chaps. VI, VII, Cornell University Press, Ithaca, N. Y., 1959.

CONTRIBUTION NO. 714
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RECEIVED AUGUST 29, 1961

DEMONSTRATION OF THE "CAGE" EFFECT

Sir:

In 1934, Franck and Rabinowitch[1] pointed out that collision times of reactive molecules should be much longer in solution than in the gas phase. Further, in solution, after the colliding molecules have diffused apart by a distance small compared to the mean distance between reactive molecules, there remains a finite probability of re-encounter before diffusion to the mean distance. Effectively the solvent holds the reactive molecules together, forming a "cage." A more sophisticated treatment using the theory of random walks has been given by Noyes.[2]

Numerous observations have been explained by invoking the cage effect with varying degrees of certainty. Probably the best is a recent study by Herk, Feld, and Szwarc,[3] in which they reaffirm Szwarc's previous conclusion that in the photolysis of azomethane in liquid isoöctane, ethane is formed chiefly by recombination of methyl radicals inside the solvent cage. This conclusion was inferred from three observations: that the CH_4/N_2 ratio was independent of azomethane concentration, that ethane formation could be inhibited effectively by radical scavengers in the gas phase photolysis, but not in the liquid, and that the ratio of $CH_4 + 2\ C_2H_6/N_2$ was nearly 2.

We believe that since the cage effect is an important concept in solution kinetics, it is of value to confirm its existence directly and unequivocally. We wish in this paper to report such a confirmation.[4]

We have photolyzed mixtures of azomethane and d_6-azomethane in the gas phase and in isoöctane solution using a procedure essentially similar to that of Herk, Feld, and Szwarc.[3] Azomethane was prepared by oxidation of *sym*-dimethylhydrazine with mercuric oxide, while d_6-azomethane was purchased from Merck of Canada. Both materials were proven to be chemically pure by gas chromatography. The isotopic purity of the d_6-azomethane was checked by mass spectra. 3% $CD_3N_2CD_2H$ and no other impurities were found. The isoöctane was spectroscopic grade, further purified by passing through silica gel. Its purity and the cleanliness of our handling procedure were checked by ultraviolet spectra.

The reaction products were analyzed by mass spectroscopy. The cracking patterns of N_2, CD_3NNCD_3, CH_3NNCH_3, and C_2H_6 were determined for our mass spectrometer, while the cracking patterns of C_2D_6, C_2D_5H, and CD_3CH_3 were kindly provided by Mr. J. Bell of Harvard University.[5] C_2D_6, C_2D_5H, CD_3CH_3, and C_2H_6 were determined from the mass 36, 35, 33, and 27 peaks, respectively. From these peak heights, the remaining peaks in the 36 to 24 range could be predicted within 4%.

In the gas phase photolysis of azomethane one would expect ethane to be formed by random recombination of methyl radical. For such a random process, the ethane is formed in proportions such that $(CH_3CD_3)^2/(C_2H_6)(C_2D_6) = 4$. The observed ratio of parent peak heights is 4.1 ± 0.8.

However in the isoöctane solution photolysis, the mass 33 peak is less than 2% of the 36 peak. This peak can be accounted for satisfactorily by the contributions of isotopic carbon and CD_5H. The authors estimate that if any CD_3CH_3 is formed, it must be less than 0.3% of the total ethane.

(1) Franck and Rabinowitch, *Trans. Faraday Soc.*, **30**, 120, 9 (1934).

(2) R. M. Noyes, *J. Chem. Phys.*, **18**, 999 (1950), and the following papers.

(3) Herk, Feld, and Szwarc, *J. Am. Chem. Soc.*, **83**, 2998 (1961).

(4) We have recently become aware of an unpublished study by Ausloos and co-workers at the National Bureau of Standards, whose results somewhat parallel our results.

(5) J. Bell, private communication.

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RECEIVED SEPTEMBER 11, 1961

ROTATORY-DISPERSION CHANGES DURING THE THERMAL DENATURATION OF CHYMOTRYPSINOGEN AND CHYMOTRYPSIN

Sir:

The effects of denaturing reagents such as urea in producing drastic unfolding of proteins have become so well known as to direct our attention away from the search for other interpretations of major denaturation reactions. In particular, the large entropy changes associated with reversible thermal denaturations may not be the result of

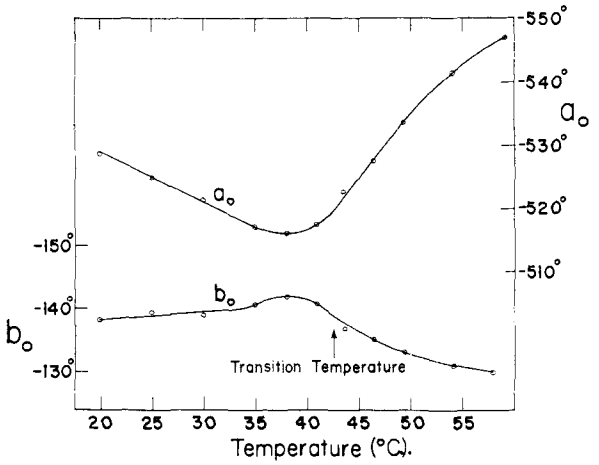

Fig. 1.—Changes in rotatory dispersion constants of chymotrypsinogen during thermal denaturation at pH 2.0. The system was completely reversible from 58° back to 20°.

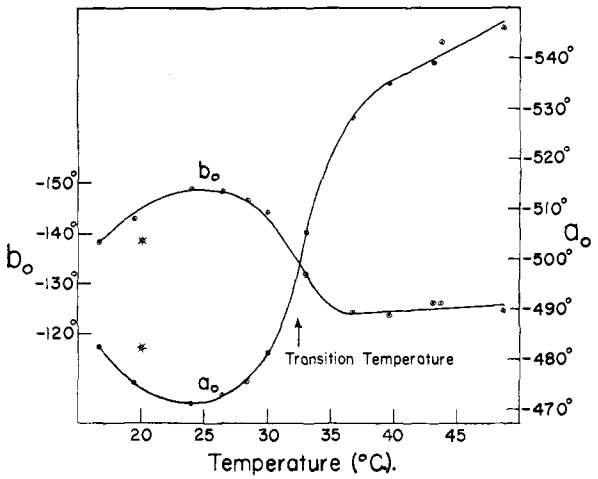

Fig. 2.—Changes in rotatory dispersion constants of chymotrypsin during thermal denaturation at pH 2.0. Starred points show reversal back to 20° from 49°.

drastic transconformation processes involving the polypeptide backbone and may reveal information about quite different aspects of protein conformation and interaction with solvent media.

Schellman[1] has noted that the specific rotation of chymotrypsinogen did not change significantly through the temperature range of reversible denaturation at pH 2 even though the standard entropy change for the process was found to be 316 e.u.[2] which, if attributable to helical unfolding, would correspond to at least ten per cent. of the residues changing from helix to coil. The lack of change in rotation means that there is either no change in backbone conformation, or optical rotation is not to be interpreted as current opinion suggests. We have measured the rotatory dispersion of chymotrypsinogen in order to confirm and extend Schellman's findings. The rotatory dispersion of chymotrypsin also was studied since its denaturation appears more typical of other proteins in that the changes in rotation are fairly large.[1] The data suggest, however, that the chymotrypsin denaturation is to a first approximation the sum of the effects present in chymotrypsinogen plus effects from the uncoiling of a small segment of α-helix.

Rotatory-dispersion data were obtained using a Rudolph spectropolarimeter sensitive to 0.001° of rotation and accurate in wave length to 1 Å. Rotations were measured at these lines of the mercury emission spectrum: 365, 405, 436, 546, and 578 millimicrons. The protein samples were obtained from the Worthington Biochemicals Corporation. Only chymotrypsinogen was examined carefully for impurities and was found to contain less than 1% foreign protein contamination.

We have used the Moffitt equation (Eq. 1) for the analysis of our data.

$$\frac{3}{N^2+2}\frac{M_0}{100}[\alpha] = \frac{a_0\lambda^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \quad \text{(Eq. 1)}$$

The data are well represented with λ_0 chosen as 209 millimicrons. The results of our studies are shown in Figs. 1 and 2. The experiments were carried out with no neutral salt added, pH being adjusted by addition of HCl. Under these conditions our agreement with Schellman on the $[\alpha]_D$ and λ_c values for the native forms of the two proteins is good. In particular, for chymotrypsin, the transition temperature and the total changes in $[\alpha]_D$ agree within the total of errors.

In the case of chymotrypsinogen, we found very small changes in specific rotations and dispersion constants though the temperature dependencies of the dispersion constants, usually thought to measure rigidity of conformation, change drastically. The situation for chymotrypsin is somewhat different and may be analyzed at least tentatively using the popular method based on the studies on poly-amino acid helix-coil transitions.[3] On this basis, and using our λ_0, we expect a change in b_0 from 0 to $-650°$ as coil goes to helix. b_0 is thus to be considered a measure of the helix content of a protein and leads to estimates of the helix content of chymotrypsin as 23% in the native protein and 19% in the denatured form or a loss of helix folding in nine residues. In the case of chymotrypsinogen, the very small changes in b_0 indicate no unfolding during denaturation. Neurath and Dixon[4] have postulated that chymotrypsin differs from chymotrypsinogen in having a small additional segment of helix. Imahori[5] has since established this state of affairs for the two proteins at pH 7.7. Our results support this interpretation.

The measured changes in the entropy and enthalpy of denaturation for the two proteins are given in Table I. Using Schellman's estimates[6] for the entropy and enthalpy changes per residue in helix formation and our estimate of the number of residues unfolded in the chymotrypsin denatura-

(1) J. A. Schellman, *Compt. rend. Lab. Carlsberg, Ser. Chim.*, **30**, 450-461 (1958).

(2) M. A. Eisenberg and G. W. Schwert, *J. Gen. Physiology*, **34**, 583-606 (1951).

(3) K. Imahori, *Biochim. Biophys. Acta*, **37**, 336-341 (1958).

(4) H. Neurath and G. H. Dixon, *Federation Proc.*, **16**, 791 (1957).

(5) K. Imahori, A. Yoshida and H. Hashizume, *Biochim. Biophys. Acta*, **45**, 380-381 (1960).

(6) W. F. Harrington and J. A. Schellman, *Compt. rend. Lab. Carlsberg, Ser. Chim.*, **30**, 21 (1955).

tion we would expect the difference in ΔS to be 45 e.u. and in ΔH to be 18 kcal. These are in good agreement with the observed differences (Table I) when one considers that the estimates of both the number of residues involved and the changes per residue must necessarily be crude since the end effects will be large when dealing with such short segments of helix. We also have oversimplified the problem by neglecting side chain interactions. However, the denaturation region lies at lower temperatures for chymotrypsin than for chymotrypsinogen, indicating that more profound changes in the stability of the structure occur during activation of the zymogen than are revealed by the production of a new helix segment. Nevertheless, at temperatures above the denaturation region, the two proteins are very nearly identical in a_0 and b_0 values, and are thus probably in very similar conformational states. b_0 is about $-130°$ for both denatured proteins at 50° and in both cases there is only a small temperature dependence. a_0 for denatured chymotrypsinogen is $-538°$ at 50°, while a_0 for denatured chymotrypsin is $-548°$ at the same temperature. a_0 also shows nearly the same temperature dependence for the two denatured proteins.

TABLE I

CHANGES IN THERMODYNAMIC FUNCTIONS DURING DENATURATION

	Chymotrypsin[1]	Chymotrypsinogen[2]	Difference
ΔS, e.u.	360	316	44
ΔH, kcal.	110	99.6	10.4

It is quite clear for both proteins that the major contributions to the entropy and enthalpy change in reversible denaturation are not associated with large changes in optical rotation. The best description of the denaturation reactions reconciling the thermodynamic and rotatory dispersion data which we have been able to find is this: The entropy change is the result of a coöperative, first-order-like melting of the tangle of side chain interactions, probably predominantly hydrophobic in nature, at the protein–solvent intefrace. The melted sections are similar to liquid condensed phases in surface film studies. Water is able to penetrate the forest of side chains after denaturation, but infrequently before. Although the side chains in the melted sections gain considerable freedom, there is little unfolding of the protein in the usual sense and the conformation of the backbone is essentially unaltered in the process so that a_0 and b_0 do not change. Other studies to be reported shortly support this interpretation.

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THE EFFECT OF LITHIUM BROMIDE ON THE STRUCTURAL TRANSITION OF RIBONUCLEASE IN SOLUTION

Sir:

Interest has evidenced itself recently on the effect of aqueous lithium bromide solutions on the thermodynamic stability of the ordered structures in the fibrous and globular proteins. Contrary conclusions have been reached by different investigators.[1-5] Harrington and Schellman[1] reported a decrease in the levorotation of various proteins dissolved in such solutions, with particula attention being given to the behavior of ribonuclease. Based upon the correlations obtained between chain conformation and optical rotation for the simple homopolypeptides by Doty and coworkers,[6,7] it was deduced that the helical structures were stabilized by the action of lithium bromide. Since it is well known that the activity of water is abnormally low in such solutions,[8] the stabilization inferred could be attributed conveniently to this cause. These deductions have, however, been seriously questioned recently as a result of more extensive solution measurements, which unfortunately were restricted to a single temperature.[2]

On the other hand, investigations of the dimensional changes exhibited by a variety of fibrous proteins, when immersed in a large excess of aqueous lithium bromide solutions, have led to the conclusion that at appropriate temperatures a transformation from the ordered to disordered state occurs.[3,4,5] The transformation in these cases is accompanied by an axial contraction. A significant disparity exists, therefore, between these latter results, typical of higher polymer concentrations, and the widely accepted interpretation placed on the optical rotation data obtained in dilute solution. The suggestion has been made that the nature of the lithium bromide interaction may be strongly dependent on the protein concentration, and a mechanistic model supporting this hypothesis has been offered.[9]

Since the structural transformation of ribonuclease that occurs in pure aqueous solutions has been studied extensively,[1,10,11,12] the apparent discrepancy cited above and the associated speculation should be resolvable by studying any variations that take place in the transformation temperature caused by the addition of lithium bromide

(1) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. chim.*, **30**, 167 (1957).
(2) C. C. Bigelow and I. I. Geschwind, *ibid.*, **32**, 89 (1961).
(3) L. Mandelkern, J. C. Halpin, A. F. Diorio, and A. S. Posner, *J. Am. Chem. Soc.*, in press.
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(5) L. Mandelkern, W. T. Meyer, and A. F. Diorio, *J. Phys. Chem.*, in press.
(6) J. T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).
(7) P. Doty, *Rev. of Mod. Phys.*, **31**, 107 (1959).
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(9) A. R. Haly and J. W. Snaith, *Biochim. et Biophys. Acta*, **44**, 180 (1960).
(10) J. G. Foss and J. A. Schellman, *J. Phys. Chem.*, **63**, 2007 (1959).
(11) J. Hermans, Jr., and H. A. Scheraga, *Biochim. et Biophys. Acta*, **36**, 534 (1959).
(12) J. Hermans and H. Scheraga, *J. Am. Chem. Soc.*, **83**, 3283 (1961).