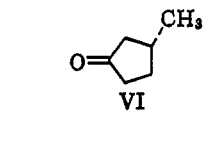
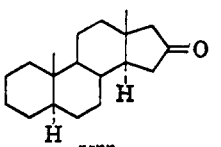
striking and unexpected is the situation illustrated in Fig. 3 with 5α-androstan-11-one (VIII), where the low temperature C.D. measurement did not only uncover the anticipated vibrational structure, but also resulted in an inversion of the C.D. curve from weakly positive (room temperature, in accord with earlier[13] O.R.D. results) to negative (liquid nitrogen). We interpret these results in terms of the existence of another, hitherto unsuspected, conformer of ring A at room temperature, the tendency toward departure from the standard all-chair form presumably being due to the interference between the equatorial C-1 hydrogen and the 11-keto function.

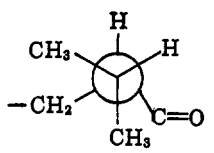
Finally, we cite in Fig. 4 an application of low temperature C.D. measurements to studies of free-rotational isomerism. While the rotational strengths of cholestan-3-one or its 2α-methyl homolog do not differ at −192° and 25°, a considerable increase is observed (Fig. 4) at lower temperature in 2α-isopropylcholestan-3-one (IX).[14] Of the three most obvious rotamers around the C-2 bond, according to the octant rule[10] IXB would be expected to have the least effect (as compared to cholestan-3-one), while IXC would yield a more negative and IXA a more positive rotatory contribution. Evidently at lower temperatures, conformer IXA is the preferred one, which seems reasonable since it does not exhibit the eclipsing between one of the methyl groups and the carbonyl function present in IXB and IXC.



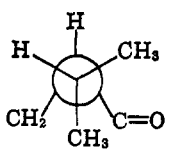
VI



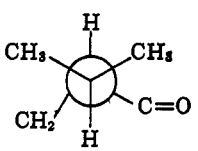
VII



IXa



IXb



IXc

In addition to the many qualitative applications in stereochemistry which are opened up by such low temperature measurements, we are also investigating some of the more interesting quantitative aspects. Thus, C.D. studies at several temperatures over the −192° to room temperature range offer a means of determining the energy differences between two conformers or rotamers and details of such work conducted in collaboration with Prof. A. Moscowitz of the University of Minnesota will be described in a forthcoming paper.

(13) E. W. Foltz, A. E. Lippman and C. Djerassi, *J. Am. Chem. Soc.*, **77**, 4359 (1955); C. Djerassi and W. Klyne, *J. Chem. Soc.*, 2390 (1963).

(14) P. A. Hart, unpublished observation from this Laboratory.

(15) National Institutes of Health Postdoctoral Research Fellow, 1962–1963.

DEPARTMENT OF CHEMISTRY
STANFORD UNIVERSITY
STANFORD, CALIFORNIA

KEITH M. WELLMAN[15]
E. BUNNENBERG
CARL DJERASSI

RECEIVED MAY 6, 1963

Some Observations Relative to the Mechanism of Chymotrypsin

Sir:

The inactivation of α-chymotrypsin by diphenylcarbamyl chloride (I), a reagent of high specificity, has recently been described.[1] The following are some of the characteristics of this reaction: (1) Inactivation resulted from a reaction of equimolar stoichiometry. (2) Inactivation could be competitively inhibited by indole. (3) The inactivation of chymotrypsin was approximately one hundred times faster than that of trypsin. (4) Reactivation was rapid and complete in the presence of nucleophilic agents such as hydroxylamine and isonitrosoacetone but occurred at a negligible rate in their absence. (5) No reaction occurred between I and chymotrypsinogen or diethylphosphorylchymotrypsin.

These characteristics, as well as analogy with the reaction of carbamates with acetylcholinesterase,[2] are consistent with the conclusion that I is a new type of specific substrate of chymotrypsin capable of participating in the acylation step of the catalytic mechanism but leading to a relatively stable acyl enzyme intermediate.[3] I, therefore, should be a valuable tool for the study of the acylation step of the mechanism by which specific substrates are hydrolyzed by chymotrypsin. This communication reports the results of such a study.

In order to gain an insight into the number and types of functional groups participating in the acylation mechanism, the effect of pH upon the inactivation process was investigated. The rate of inactivation was determined by measurement of residual enzyme activity as a function of time.[4] Complete inactivation could be obtained throughout the pH range 5.0 to 9.5 with a rate maximum at about pH 7.5 (see Fig. 1). K_i ($= K_m$) remained constant ($0.6 \pm 0.1 \times 10^{-4}$ M) up to pH 8.2.

The stoichiometry of the inactivation process calls for the release of one mole of H^+ for the formation of each mole of diphenylcarbamyl (DPC) chymotrypsin. Titration studies, however, led to the findings shown in Fig. 2.[5] Close to a theoretical quantity of H^+ could be detected only at pH 5–5.5. Thereafter the amount of H^+ was consistently below the expected value.[6] As can be seen in Fig. 2, different conditions of ionic strength and temperature resulted in a shift but no marked change in the shape of the curve.

The low values between pH 5.5 and 7.5 agree with the findings of Guttreund and Sturtevant[7] and are consistent with their suggestion that acylation of the enzyme resulted in a change in the apparent pK_a of a functional

(1) B. F. Erlanger and W. Cohen, *J. Am. Chem. Soc.*, **85**, 348 (1963).

(2) *Cf.* I. B. Wilson, M. A. Harrison and S. Ginsburg, *J. Biol. Chem.*, **236**, 1498 (1961).

(3) Despite the fact that it causes inactivation, I is a substrate of chymotrypsin because, like conventional substrates, its hydrolysis is mediated by the enzyme *via* a multi-step mechanism involving acylation of chymotrypsin followed by deacylation [*cf.* B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **85**, 356 (1963)]. The deacylation step, however, proceeds very slowly. It should be considered to be a *specific* substrate because its rate of reaction with chymotrypsin is almost two orders of magnitude faster than its rate of reaction with trypsin and, as reported in this paper, it has a rather respectable K_m (or K_i depending upon one's point of view).

(4) The methods used were essentially the same as described in ref. 1.

(5) The preparation of α-chymotrypsin (Worthington 3× crystallized, lot no. CDI 6032) was found to be 88% pure (mol. wt. 25,000) by titration with I as well as by a method that utilized the specific chromogenic inactivator 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (B. F. Erlanger and F. Edel, in preparation). The reaction was carried out under nitrogen in a Radiometer pH-Stat using 0.0087 N NaOH standardized against a National Bureau of Standards preparation of benzoic acid. The diphenylcarbamylchloride used was from Distillation Products, Inc., and recrystallized three times from ethanol to constant m.p. 85° [H. Erdmann and P. Huth, *J. prakt. Chem.* (II), **56**, 7 (1897) report 85°]. It was stable in acetone and isopropyl alcohol and could be recovered pure (by melting point) after distillation of the solvent. Early experiments utilized dimethyl sulfoxide as a solvent for I. Use of this solvent was discontinued when it was found that considerable decomposition of I took place in 30 min. at room temperature. After each run, the apparatus and standard base were checked by the titration of a convenient quantity of standardized hydrochloric acid.

(6) That the low values were not due to denaturation of the enzyme was established by simultaneous assay with the chromogenic inactivator described in ref. 5.

(7) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U.S.*, **42**, 719 (1956).

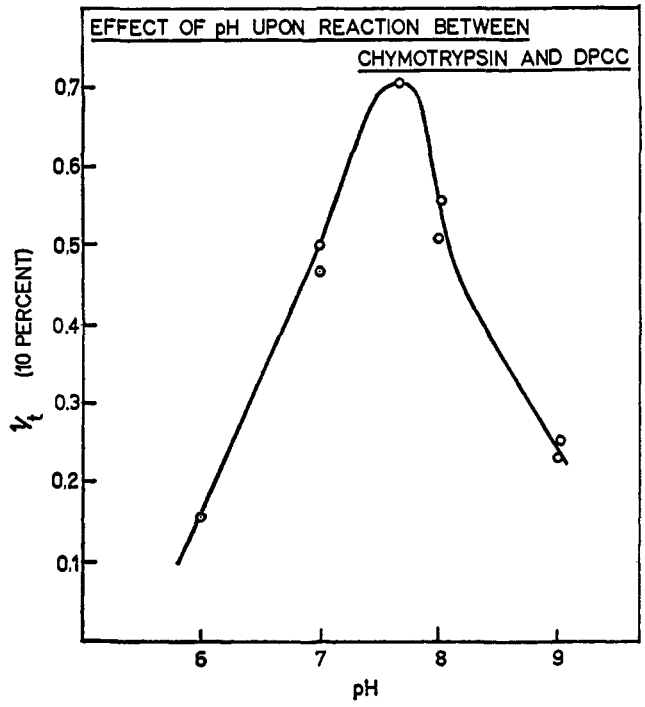


Fig. 1.—Effect of pH upon reaction of I with chymotrypsin. The reaction mixtures contained 1×10^{-6} *M* chymotrypsin, 2×10^{-6} *M* diphenylcarbamyl chloride in 0.06 *M* acetate–maleate–borate buffer containing 0.036 *M* $CaCl_2$. Ordinate is in min.$^{-1}$ for 10% decrease in activity.

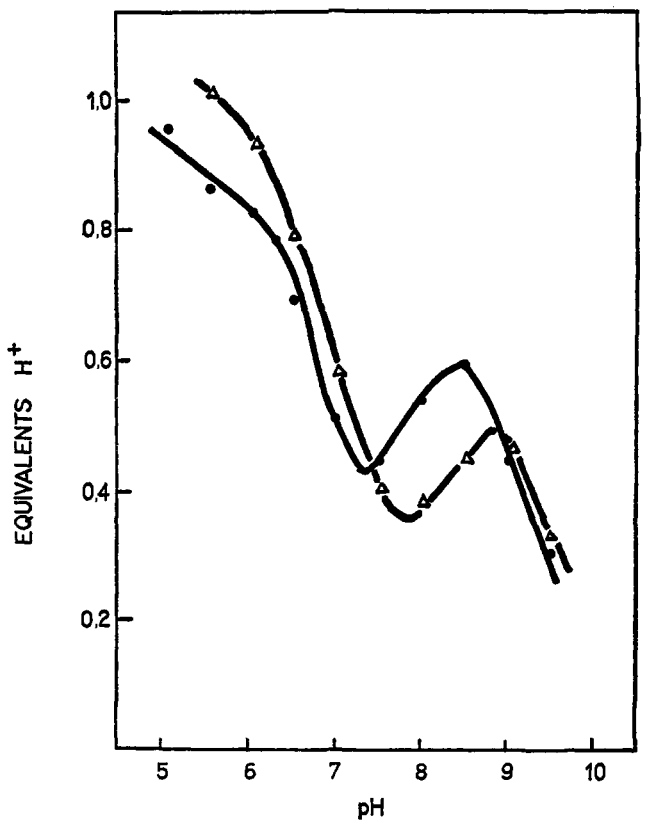


Fig. 2.—Measurement of liberation of hydrogen ions: ●—●, 1.22×10^{-6} mole of α-chymotrypsin in 9.8 ml. of 0.02 *M* $CaCl_2$, 0.02 *M* NaCl, 3% acetone; added 3.7×10^{-6} mole of I in 0.2 ml. of acetone at 25°; △—△, 2.4×10^{-6} mole of α-chymotrypsin in 9.8 ml. of 0.02 *M* $CaCl_2$, 0.20 *M* KCl, 3.06% 2-propanol; added 3.6×10^{-6} mole of I in 0.2 ml. of 2-propanol at 15°.

group, in our case from approximately 6.6 to 7.4, with a resulting uptake of H^+ by the enzyme.[8,9] The failure to reach the theoretical H^+ value at conditions of higher pH, however, cannot be reconciled with any previous findings. In order to investigate this phenomenon the following experiment was carried out.

Chymotrypsin (1.22 μmoles in 10 ml. of a solution containing 0.02 *M* $CaCl_2$, 0.1 *M* KCl, 3% acetone, at 25°) was inactivated by an equimolar quantity of I in 0.2 ml. of acetone, the reaction being followed in a Radiometer pH-Stat at pH 7.0. After the inactivation was complete (H^+ = 49.2% of theory), a titration curve was determined up to pH 10.6 using 0.00986 *M* NaOH containing 0.1 *M* KCl. An identical control experiment, lacking I only, was carried out. The titration data showed one less titratable group in DPC–chymotrypsin, the difference appearing between pH 7.3 and 9.7. A similar result was obtained at 15° using 2.40 μmoles of chymotrypsin and an equimolar quantity of I.

The above results suggest the following conclusions concerning the acylation of chymotrypsin by a specific substrate:

(1) The presence of a pH optimum implies the participation of a minimum of two functional groups in the acylation mechanism. One of them is a base with a pK_a of 6.6; the other a conjugate acid of a base with a pK_a that can be calculated from the data in Fig. 1 to be 8.7. (Also, see below.) Additional evidence for the presence of a second, conformation-sensitive group at the active center of chymotrypsin was given previously.[10]

(2) As suggested by Gutfreund and Sturtevant,[7] the acylation of chymotrypsin might affect the pK_a of a functional group presumably involved in the catalytic mechanism, shifting it from approximately 6.6 to 7.4 under our experimental conditions. On the other hand, the data are consistent also with acylation of the alkoxide form of an abnormally acidic serine side chain (pK_a *ca.* 7).

(3) The absence of one titratable group in DPC–chymotrypsin despite its apparently higher basicity (as reflected by the uptake of an acid shown in the curve of Fig. 1 between pH 7.5 and 9.5) can be explained in one of two ways. Either acylation takes place on the functional group having the pK_a of 8.7, accompanied by the appearance of a "new" group with a pK_a above 10.6, or, as a result of the reaction of I with chymotrypsin, the pK_a of this group shifts from 8.7 to a value above 10.6. The possibility that arginine could participate in the latter type of phenomenon has been discussed previously.[11]

The suggestions given are, of course, tentative and will be tested experimentally. We can conclude, how-

(8) According to M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961), the apparent shift is due to differences in the sensitivities of the acylation and deacylation reactions to electrostatic effects. In any case, at the salt concentrations used in these studies, uptake of H^+ would occur.

(9) While this paper was in preparation, D. E. Fahrney and A. M. Gold, *J. Am. Chem. Soc.*, **85**, 349 (1963), reported on a study of the reaction of phenylmethanesulfonyl fluoride with chymotrypsin. A number of their findings are apparently at variance with the results reported here. For example, they reported a release of 1.00 ± 0.02 equivalent of H^+ throughout the pH range 7 to 8. On the basis of these results, the point was made that no evidence existed for a pK_a shift near neutrality and, therefore, that a serine–histidine interaction was unlikely. Since their reaction conditions (*i.e.*, 0.1, 0.3 and 1.0 *M* KCl, 1% isopropyl alcohol) were somewhat different from ours, we repeated the DPC–chymotrypsin reaction under conditions which simulated theirs (0.3 *M* KCl, 1% isopropyl alcohol, 3 μmoles of I, 6 μmoles of chymotrypsin, 15°) at pH 7.0. A release of 0.68 equivalent of H^+ occurred. Apparently the reaction with chymotrypsin is influenced by the nature of the acylating group, *i.e.*, whether it is a sulfonyl or a carboxyl group. R. Kitz and I. B. Wilson, *J. Biol. Chem.*, **237**, 3245 (1962), have reported that methanesulfonylated acetylcholinesterases have anomalous characteristics also in that they cannot be reactivated by hydroxylamine or pyridine-2-aldoxime methiodide.

(10) W. Cohen and B. F. Erlanger, *Biochim. Biophys. Acta*, **52**, 604 (1961).

(11) B. F. Erlanger, *Proc. Natl. Acad. Sci. U. S.*, **46**, 1430 (1960).

ever, that the acylation step in the mechanism of chymotrypsin is accompanied by a number of rather remarkable changes in properties of the side chain functional groups; deacylation must restore them to their original state. It is not unreasonable to attribute these changes to conformational alterations occurring at least in the region of the active center of the enzyme. We can conclude also that at least one of the functional groups involved in the acylation step of the catalytic mechanism is in an "abnormal" state due to the influence of neighboring groups. One wonders, therefore, about the applicability to the elucidation of enzyme mechanisms of information derived from studies on simple model compounds. It is becoming apparent that enzyme mechanisms exploit the capacity of proteins, as macromolecules, to assume special conformations, not only as a means of inducing specificity of binding, but also to enhance or suppress the activities of side-chain functional groups participating in the catalytic mechanism itself.[11a]

(11a) NOTE ADDED IN PROOF.—Titration to pH 3.5 was recently carried out using 0.011 *N* HCl containing 0.1 *M* KCl subsequent to the inactivation of 2.4 μmoles of chymotrypsin by 2.4 μmoles of I in 10 ml. of a solution containing 0.02 *M* $CaCl_2$, 0.1 *M* KCl and 3% isopropyl alcohol at pH 7.0, 15°, and the results compared with a control chymotrypsin solution lacking I. Between pH 6.4 and 4.8, chymotrypsin consumed approximately one more mole of acid than did DPC-chymotrypsin. However, within the pH range 4.8 to 3.5, DPC-chymotrypsin required approximately one additional mole of acid. These results suggest a shift in the p*K* of a carboxyl group from *ca.* 5.5 to *ca.* 3.7 as a result of the acylation reaction, possibly because of the disruption of a hydrogen bond.

(12) The support of the Office of Naval Research (Nonr-266 (73) and the National Institute of Health AI-01672-06 is gratefully acknowledged.

DEPARTMENT OF MICROBIOLOGY BERNARD F. ERLANGER[12]
COLLEGE OF PHYSICIANS AND SURGEONS HARRIET CASTLEMAN
COLUMBIA UNIVERSITY A. G. COOPER
NEW YORK 32, N. Y.

RECEIVED APRIL 12, 1963

A New Organometallic Semiconductor

Sir:

Although a variety of conjugated organic molecules are known to act as semiconductors, the carrier mobilities in them usually are very low. This is due to the difficulty electrons experience in jumping from one molecule to another, and so the carrier mobility in compounds of this kind increases with increasing molecular size.[1]

On this basis one would expect coördination polymers to show interesting electrical properties, if prepared from a suitable transition metal and a double aromatic ligand capable of binding two metal atoms at different points. For easy conduction throughout the polymer, each metal atom must provide a conducting path for π-electrons from one adjacent ligand to the other; this will be the case if the ligands are coplanar, the metal forming $d\pi:p\pi$ bonds to both ligands by the same d-orbital. This in turn requires that the ligands be of chelate type, and that the metal forms either square planar complexes (*e.g.*, Cu^{II}, Ni^{II}), or octahedral complexes in which two opposite sites are occupied by ligands of some other kind. The former alternative seems much the more attractive, the more so since such complexes are commonly linked by metal–metal bonds perpendicular to the plane (*cf.* nickel dimethylglyoxime); bonds of this kind would tend to increase the mobility of electrons between adjacent molecules.

(1) For reviews, see D. D. Eley and M. R. Willis, and H. Akamatu and H. Inokuchi, in H. Kallmann and M. Silver, Ed., "Symposium on Electrical Conductivity in Organic Solids," Interscience Publishers, New York, N. Y., 1961, pp. 257, 277; H. A. Pohl, p. 134; H. A. Pohl, J. A. Bornmann, and W. Itoh, in J. J. Brophy and J. W. Buttrey, Ed., "Organic Semiconductors," Macmillan Co., New York, N. Y., 1962, pp. 134–142.

Very few coördination polymers of this type have been prepared and little has been done on their electrical properties; an exception is the work of Kanda and Kawaguchi,[2] who studied the copper derivatives of 1,6-dihydroxyphenazine, 2,5-dihydroxy-*p*-benzoquinone and rubeanic acid. It is also known that copper polyphthalocyanin is a semiconductor, but this is not a true coördination polymer; in it the monomer units are linked by carbon–carbon bonds rather than by metal coördinate links. Most of the true coördination polymers that have been prepared have been made either from "insulating" double ligands (where there can be no through-conjugation between the metal atoms) or from metals of unsuitable types (*e.g.*, ones forming tetrahedral complexes). The object of these investigations has been to prepare thermally stable polymers rather than semiconductors.

We became interested in this problem while studying electron transfer processes in aromatic compounds, our idea being that polymers of this kind might act as catalysts for electron transfer processes. We now wish to report the synthesis of a new organometallic semiconductor of this kind, the cupric derivative of the dioxime of 1,5-diacetyl-2,6-dihydroxynaphthalene. The dioxime, prepared in the usual way from 1,5-diacetyl-2,6-dihydroxynaphthalene, had m.p. 247–248° (*Anal.* Calcd. for $C_{14}H_{14}N_2O_4$: N, 10.2. Found: N, 10.0). It reacted with cupric acetate in the presence of acetic acid to form a dark brown solid, m.p. >300°, insoluble in all the usual solvents (*Anal.* Calcd. for $(C_{14}H_{12}N_2O_4Cu)_n$: C, 50.1; H, 3.58; N, 8.35; Cu, 18.9. Found: C, 50.1; H, 4.15; N, 8.15; Cu, 17.4). The copper content indicates a degree of polymerization of about ten. The conductivity of the polymer was measured in compressed disks (prepared at about 9×10^4 p.s.i.) at room temperature. Three samples of the polymer, prepared in different experiments, had resistivities of 8.6×10^7, 7.4×10^7 and 8.0×10^7 ohm-cm. We have not yet been able to measure the resistivity as a function of temperature, but an indication of the energy gap seems to be given by the spectrum of the compound in potassium bromide or iodide disks. In each case absorption began at 2.2 μ and increased steadily with decreasing wave length. An absorption edge at 2.2 μ would correspond to an energy gap of 0.56 e.v.; this would give a value of $\rho_0 = 1.8 \times 10^3$ ohm-cm., comparable with the values reported by Akamatu and Inokuchi[1] for very large aromatic systems. We are extending our studies to other polymers of this type.

Acknowledgment.—This work was supported by a grant (AF 62-104) from the Air Force Office of Scientific Research.

(2) S. Kanda and S. Kawaguchi, *J. Chem. Phys.*, **34**, 1070 (1961).

GEORGE HERBERT JONES LABORATORY MICHAEL J. S. DEWAR
UNIVERSITY OF CHICAGO AMBALAL M. TALATI
CHICAGO 37, ILLINOIS

RECEIVED MARCH 20, 1963

Cyclic Phosphate Esters from the Hydrolysis of Cyclic Oxyphosphoranes. Evidence for Pentavalent Phosphorus in the Oxyphosphoranes[1]

Sir:

We have proposed[2] a cyclic *oxyphosphorane* structure (I) with pentavalent phosphorus, for the crystalline *2:1 adduct* derived from biacetyl and trimethyl phosphite. This structural hypothesis (I) was based on

(1) Work supported by the Cancer Institute of the National Institutes of Health (CY-4769); the National Science Foundation (G19509) and the Petroleum Research Fund of the American Chemical Society (286-A).

(2) F. Ramirez, N. Ramanathan and N. B. Desai, *J. Am. Chem. Soc.*, **84**, 1317 (1962).