COMMUNICATIONS TO THE EDITOR

THE REACTION OF *p*-NITROPHENYL ACETATE WITH CHYMOTRYPSIN[1]

Sir:

The reaction of NPA with chymotrypsin at *p*H 5.0 results in a stable monoacetyl derivative which is an intermediate in the catalytic hydrolysis of the ester at higher *p*H.[2] Phosphorylating agents (*e.g.*, DFP) have a similar effect and give rise to stable monophosphoryl enzyme derivatives, the hydroxyl group of a serine residue being the final point of attachment of the phosphoryl residue.[3,4,5]

The reaction of NPA with chymotrypsin is a two-phase process,[6] a rapid initial "burst" of *p*-nitrophenol being followed by a slow liberation until NPA is exhausted. At low temperature and *p*H, using rapid mixing techniques, the initial acetylation reaction in isolation has been followed in the Cary recording spectrophotometer and its kinetics have been found to correspond to those of a bimolecular reaction. At higher *p*H and temperature, the slow zero order reaction, which represents the turnover of acetylchymotrypsin (de-acetylation being rate-limiting), has been followed. The acetylation reaction, with a maximum at *p*H 8–9, occurs with a velocity approximately one hundred fold that of de-acetylation (maximal at *p*H 8.5–10). The energy of activation for the acetylation reaction at *p*H 6.0 was found to be 13,700 cal. per mole and for the de-acetylation at *p*H 7.5, 15,700 cal. per mole (as compared to 18,400 cal. per mole for the base catalyzed hydrolysis of NPA[6]).

The following series of experiments were undertaken to study the attachment of the acetyl group to the enzyme. A difference spectrum (600–230 mμ) between two portions of the same acetyl-chymotrypsin solution, one of which had been allowed to de-acetylate at *p*H 8.0, 25°, showed that the two proteins were spectrally identical. Since a histidine side chain has been implicated in the action of proteolytic enzymes[7,8] and since the properties of acetyl-imidazole are known to include a characteristic ultraviolet absorption with a peak at 245 mμ,[7] the changes at 245 mμ were carefully followed during the acetylation reaction. The quantity of enzyme was chosen to provide an easily measurable change at 245 mμ if acetylimidazole were formed, but no such change was observed.

In contrast to acetyl esters such as ethyl acetate, the acetyl group in monoacetyl chymotrypsin is reactive toward hydroxylamine at *p*H 5.5 with the formation of one equivalent of hydroxamic acid.[2] However, when acetyl-α-chymotrypsin was denatured (reversibly) in 8 *M* urea at *p*H 3.0 and then reacted with hydroxylamine at *p*H 5.5, no formation of hydroxamate could be detected. (NPA reacted normally with hydroxylamine in the presence of 8 *M* urea.) The acetyl group remained bound to the protein, however, and when the urea was diluted out (with 0.001 *N* HCl at 0°), regained its normal properties. However, if chymotrypsin is initially denatured in 8 *M* urea, acetylation by NPA does not occur. These observations focus attention upon the modifying influence of the environment in the native protein upon the reactivity of amino-acid side chains. When the "reactive" side chain in the native state is first acetylated and the acetyl enzyme is then denatured, the modifying influence of the protein configuration is again in evidence, the acetyl group being (reversibly) transformed from a state in the native protein in which it is reactive toward hydroxylamine to an unreactive state in the denatured protein. It is clear, therefore, that the properties of this "specially reactive" group in the protein cannot be interpreted in terms of the known properties of the amino-acid side chains in isolation, the reactivity of the group in question being functionally related to the specific structure of the native protein. This unusual reactivity has also been demonstrated by the observation that at *p*H 5, the same group in chymotrypsin is rapidly acylated by acetic, propionic or butyric anhydride. It is proposed that the successive acylation and deacylation of this uniquely reactive group constitutes an important feature of the mechanism of hydrolysis of NPA by chymotrypsin.

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(1) *p*-Nitrophenyl acetate will be abbreviated to NPA, and diisopropyl phosphofluoridate to DFP. This work was performed under Contract No. Nonr-477-04 between the University of Washington and the Office of Naval Research, Department of the Navy, and was supported also by funds made available by the people of the State of Washington, Initiative 171. Our thanks are due to Miss Dorothy Kauffman for technical assistance. Details of this work will be given in a paper now in preparation.

(2) A. K. Balls and F. L. Aldrich, *Proc. Nat. Acad. Sci.*, **41**, 190 (1955); A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(3) N. K. Schaffer, S. C. May and W. H. Summerson, *ibid.*, **206**, 201 (1954).

(4) R. A. Oosterbaan, P. Kunst, and J. A. Cohen, *Biochim. Biophys. Acta*, **16**, 299 (1955).

(5) G. H. Dixon, S. Go and H. Neurath, *ibid.*, **19**, 193 (1956).

(6) B. S. Hartley and P. Kilby, *Biochem. J.*, **50**, 672 (1952).

(7) E. Stadtmann, "Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., 1954, p. 581.

(8) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).

POLYPEPTIDES. X. CONFIGURATIONAL AND STEREOCHEMICAL EFFECTS IN THE AMINE-INITIATED POLYMERIZATION OF N-CARBOXY-ANHYDRIDES

Sir:

The usual formulation of the amine-initiated polymerization of N-carboxy-α-amino-acid anhydrides predicts a narrow molecular weight distribution provided the propagation rate is fast compared to initiation.[1] Recent work on the poly-

(1) (a) S. G. Waley and J. Watson, *Proc. Roy. Soc.* (*London*), **A199**, 499 (1949); (b) R. R. Becker and M. A. Stahmann, THIS JOURNAL, **74**, 38 (1952); (c) D. G. H. Ballard and C. H. Bamford, "Symposium on Peptide Chemistry," Special Pub. No. 2 Chemical Soc. (London), pp. 25–48 (1955).

merization of γ-benzyl-L-glutamate anhydride has shown that the molecular weight distribution is quite broad (beyond that attributable to a termination process[2]) and in fact two molecular species have been characterized.[3,4] We wish to report the discovery of two successive stages of chain growth in amine-initiated polymerizations which accounts for this polydispersity, and on the effect of optical isomers on the polymerization.

The rate of polymerization of γ-benzyl-N-carboxy-L-glutamate anhydride at 4% concentration in dioxane was determined at 25° by measuring the rate of evolution of carbon dioxide. The results for three different anhydride-initiator ratios (A/I) are shown in Fig. 1. The intercepts at zero

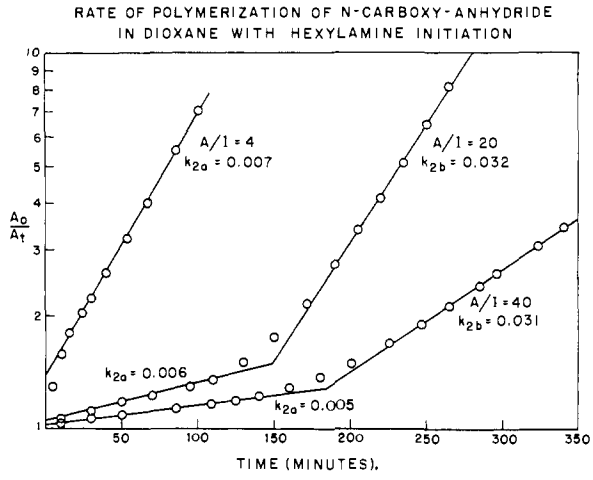

Fig. 1.—Log of ratio of original anhydride concentration to that at time *t versus* time for three different anhydride-initiator ratios.

time correspond to the reaction of equimolar amounts of anhydride and initiator showing that the initiation is fast relative to propagation. At the low A/I of 4 a single propagation rate (k_{2a} = 0.007 liter/mole/sec.) is observed. At an A/I value of 20, essentially the same propagation rate is observed but when about one-third of the anhydride has reacted the propagation shifts to a faster rate (k_{2b} = 0.032). It is clear that the growing chains which first attain the condition necessary for the second rate constant will by more rapid growth consume most of the remaining anhydride and thereby produce the broadening of the molecular weight distribution observed.[5] The midpoint of the transition from the slow to the fast propagation rate occurs when a degree of polymerization of about 8 has been reached. Infrared spectral and rotatory dispersion studies[4,6] show that in this polypeptide–solvent system the helical configuration becomes stable at about this degree of polymerization.[3,7] This coincidence strongly suggests that the higher propagation rate is associated with the helical configuration.

The polymerization of racemic mixtures followed the form shown in Fig. 1, but the rates were diminished: k_{2a} = 0.004 and k_{2b} = 0.015. To explore this effect, L polymer was used to initiate the polymerization of D anhydride, whereupon it was found that an induction period preceded attainment of first order growth. This is indicative of a strong preference of the growing chain for its own optical isomer. However, a study of the optical rotation during this reaction suggests a different explanation wherein this retardation arises from a gradual transition from one type of helix to another. Results for the polymerization of identical amounts of L, DL, and D anhydrides initiated by equal portions of the same L polymer are shown in Fig. 2. The rise in rotation for the L anhydride is

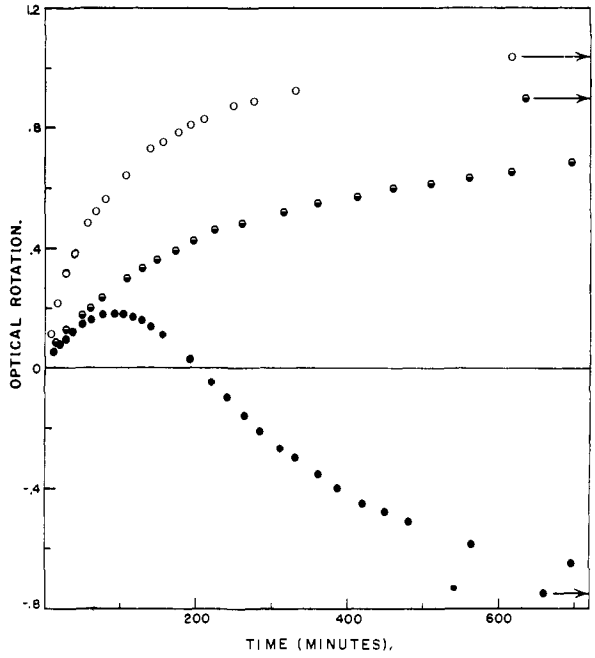

Fig. 2.—The change in optical rotation of L(○), DL(◒), and D(●), carboxy-anhydrides of γ-benzyl glutamate during polymerization initiated by aliquots of an L-polymer; Initial anhydride concentration in dioxane, 2.00%; polarimeter tube length = 20 cm.

due to the conversion of the anhydride ($[\alpha]_D$ −16° to L polypeptide $[\alpha]_D$ +10°). The surprising change in rotation due to the polymerization of the racemic (DL) anhydride cannot be the result of preferential incorporation since the rotation does not return to its original value upon completion of the reaction. The rise is actually due to the continuation of the helix of the initiating polymer.[8] The maximum observed in the case of D anhydride has the following explanation: the first D residues to add continue in the configuration of the initiating helix, contributing a strong positive rotation.[8]

(2) M. Sela and A. Berger, THIS JOURNAL, **75**, 6350 (1953); **77**, 1893 (1955).

(3) A. E. Woodward and P. Doty, to be published.

(4) E. R. Blout and A. Asadourian, THIS JOURNAL, **78**, 955 (1956); E. R. Blout and R. H. Karlson, *ibid.*, **78**, 951 (1956).

(5) Two successive propagation rates occur in other solvents, but in dimethylformamide only linear, first order kinetic plots are observed (k_2 = 0.055). Polymers thus formed have much narrower molecular weight distributions.

(6) P. Doty and J. T. Yang, THIS JOURNAL, **78**, 498 (1956).

(7) J. C. Mitchell, unpublished work.

(8) J. T. Yang, E. R. Blout and P. Doty (to be published) have shown that the helix core characteristic of L-residues contributes about +50° to the specific rotation of poly-γ-benzyl-L-glutamate in dioxane.

However, when several residues have added, this configuration becomes unstable and following a transitional period the D residues take up their own stable configuration, the mirror image of the L peptide helix.[9]

(9) This work was supported by the Office of Naval Research (N5ori-07654). The anhydrides were kindly furnished by Dr. E. R. Blout and R. H. Karlson.

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N-MONOALKYLATION AND ARYL BROMINATION OF CERTAIN AMINES WITH ETHYL BROMIDE IN DIMETHYL SULFOXIDE[1]

Sir:

In a study of alkylation of certain weak aromatic amines by alkyl phosphates and phosphonates,[2] we found that alkyl bromides in trialkyl phosphates gave good yields, for example, of N-monoalkylated 2-aminofluorenone.[3] We have therefore tried a limited number of other solvents, with other reaction conditions unchanged, finding none as good as the phosphates (or phosphonates), until use of dimethyl sulfoxide (generously donated by the Stepan Chemical Co., Chicago) resulted in a novel reaction which we wish to report briefly.

From 2-aminofluorenone and ethyl bromide in dimethyl sulfoxide, kept under reflux at a bath temperature of 150° for 1.5 hours, stirred into cold water and purified, there was obtained a product in crude yields of 50–60%, which we have identified as 2-N-ethylamino-3-bromofluorenone (I), m.p.[4] (of analytical sample) 164.5–165.5°. *Anal.* Calcd. for $C_{15}H_{12}BrNO$: C, 59.62; H, 4.00; Br, 26.45; N, 4.64. Found: C, 59.65; H, 3.98; Br, 26.63; N, 4.91. About 8–15% of 2-amino-3-bromofluorenone (II) was also isolated, m.p. 215.5–216°. *Anal.* Calcd. for $C_{13}H_8BrNO$; N, 5.11. Found: N, 5.04.

A similar reaction with *p*-nitroaniline gave 2-bromo-4-nitro-N-ethylaniline, m.p. 66.5–68° (reported[5] m.p. 65–66°), and 2-bromo-4-nitroaniline, m.p. 103.5–104.5° (m.p.[6] 104.5°). *Anal.* Calcd. for $C_6H_5BrN_2O_2$: N, 12.91. Found: N, 12.92.

Finding no report of direct bromination of 2-aminofluorenone, we attempted this reaction at 20° in acetic acid, obtaining 80–85% of a crude product (III), m.p. (after two crystallizations from benzene) 215.5–216°; the mixture m.p. with II was not depressed. *Anal.* Calcd. for $C_{13}H_8BrNO$: N, 5.11. Found: N, 5.09. Monoethylation[2] of III gave I (m.p. and mixture m.p.). Diazotization of III and treatment with hypophosphorous acid[7] (1°) for 22 hours gave 3-bromofluorenone (IV), m.p. 165.5–166° (reported m.p. 162°,[8a] 165.5°[8c]). *Anal.* Calcd. for $C_{13}H_7BrO$: C, 60.26; H, 2.72; Br, 30.84. Found: C, 60.34; H, 2.91; Br, 30.90. Reduction of the latter compound with sodium borohydride[9] gave 3-bromofluorenol, m.p. 169.5–170.5° (reported[8b] m.p. 142–145°). *Anal.* Calcd. for $C_{13}H_9BrO$: C, 59.79; H, 3.47; Br, 30.61. Found: C, 60.00; H, 3.71; Br, 30.73. This upon further reduction with phosphorus and iodine[8b] yielded 3-bromofluorene (V), m.p. 89–90° (reported[8b] m.p. 90–91°).

For further confirmation, acetylation of III (*i.e.*, II) followed by reduction with sodium borohydride[9] to the corresponding 9–01 and further reduction with phosphorus and iodine[8b] gave 3-bromo-2-acetamidofluorene, m.p. 208–209° (after melting, this substance solidified with pressure and remelted 210–211°). *Anal.* Calcd. for $C_{15}H_{12}BrNO$: C, 59.62; H, 4.00; Br, 26.45; N, 4.64. Found: C, 59.70; H, 3.86; Br, 26.50; N, 4.30. Bell and Mulholland[10] report isolation of "3 (or 1)-bromo-2-acetamidofluorene," m.p. 206–207°. In support of evidence in the preceding paragraph our substance cannot be the 1-bromo derivative since diazotization of III would have given 1-bromofluorenone which is reported to melt at 134–134.3°.[11] It is also highly unlikely that the 1-position would be attacked in this reaction to the exclusion of significant amounts of other isomers.

It would appear that dimethyl sulfoxide, offering a favorable environment for alkylation with ethyl bromide, reacts with eliminated hydrogen bromide, giving $(CH_3)_2SBr_2$.[12] The latter then effects ring bromination of the N-alkylated amine (or the free amine remaining) and is finally released as dimethyl sulfide, a supposition which is in agreement with the odor of the filtrate after aqueous treatment of the crude reaction product.

(1) This work was supported in part by a research grant (C-1744) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

(2) Mention of the effect of lithium bromide on alkyl phosphate alkylations of 2-aminofluorenone was included in T. L. Fletcher, M. E. Taylor and A. W. Dahl, *J. Org. Chem.*, **20**, 1021 (1955).

(3) Included in a further report which will be presented shortly by this Laboratory.

(4) All melting points are corrected, and were taken on a Fisher-Johns apparatus. We wish to thank Mr. Murray E. Taylor of this Laboratory for nitrogen microanalyses.

(5) M. S. Kharasch and I. M. Jacobson, THIS JOURNAL, **43**, 1894 (1921).

(6) B. H. Nicolet and W. L. Ray, *ibid.*, **49**, 1801 (1927).

(7) N. Kornblum, in "Organic Reactions," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 277.

(8) (a) H. F. Miller and G. B. Bachman, THIS JOURNAL, **57**, 2443 (1935); (b) H. F. Miller and G. B. Bachman, *ibid.*, **57**, 2447 (1935). The wide m.p. reported for 3-bromofluorenol may have resulted from impurity. Reported Br analysis 30.45 (no C or H). A small amount of IV remaining in the reduction product would change this analysis only slightly. Our melting points for IV and V agree with the literature; (c) P. J. Montagne and J. M. v. Charante, *Rec. trav. chim.*, **32**, 164 (1913).

(9) The reduction of approximately fifteen fluorenone derivatives in high yield has been carried out in this Laboratory and forms part of a paper in preparation.

(10) F. Bell and D. B. Mulholland, *J. Chem. Soc.*, 2020 (1949).

(11) E. H. Huntress, K. Pfister, 3rd, and K. H. T. Pfister, THIS JOURNAL, **64**, 2845 (1942).

(12) See, for example, R. Connor in "Organic Chemistry, An Advanced Treatise," ed. H. Gilman, Vol. I, 2nd ed., John Wiley and Sons, Inc., New York, N. Y., p. 872.

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21-FLUORO DERIVATIVES OF 9α-FLUORO- AND 1-DEHYDROCORTICOIDS

Sir:

The preparation of a series of 21-fluorinated steroids by the action of silver fluoride on 21-iodo-