hydrolysis catalyzed by the proteolytic enzyme, α-chymotrypsin. Spectrophotometric techniques have been utilized in studies involving oxidation–reduction enzymes, the course of reaction being followed by changes in absorption of the *enzyme* resulting from an enzyme–substrate interaction.[2] The present work exploits changes in the absorption of the *substrate*.

On the basis of kinetic studies, two catalytic steps and an acyl–enzyme intermediate have been postulated for the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate.[3,4] Degradation and inhibition studies indicate that acetylation of α-chymotrypsin by *p*-nitrophenyl acetate, phosphorylation by diisopropyl phosphofluoridate[5] and hydrolysis of acetyl-L-tyrosine ethyl ester[6] occur at the same position in the enzyme. Acetylchymotrypsin has been isolated[7] and its deacylation shown to be inhibited by 8 *M* urea.[8]

In contrast to previous studies, we have utilized an ester whose acid and alcohol components could be detected spectrophotometrically. At *p*H 7, *o*-nitrophenyl cinnamate (λ_{max}, 287 mμ; ϵ_{max}, 2.38×10^4) gives on hydrolysis cinnamate ion (λ_{max}, 268 mμ; ϵ_{max}, 1.93×10^4) and *o*-nitrophenol (λ_{max}, 360 mμ; ϵ_{max}, 2.53×10^3) (λ_{max}, 279 mμ; ϵ_{max}, 5.61×10^3). Approximately equimolar amounts ($0.42 \times 10^{-4} M$) of ester and α-chymotrypsin reacted in phosphate buffers containing 10% acetonitrile at 25° at *p*H's 5.48 to 8.24. Thus, at *p*H 6.2, these spectrophotometric observations were made: (1) at 350 mμ, the liberation of *o*-nitrophenol is practically complete in one minute; and (2) at 250 mμ, the absorption decreases reaching a minimum in about one minute; the absorption then rises to a maximum value in about 40 minutes. The absorption at infinity is the sum of *o*-nitrophenol and cinnamic acid absorptions (Fig. 1). Since cinnamic acid is not appreciably formed by the time *o*-nitrophenol formation is complete, the decrease in absorption at 250 mμ must correspond to the formation of a cinnamoyl-chymotrypsin intermediate.

The spectrum of cinnamoyl-chymotrypsin was obtained at lower *p*H's (5.48 and 4.5) to avoid complications arising from the formation of cinnamic acid. Cinnamoylchymotrypsin has a single peak at 293 mμ (ϵ_{max}, 1.6×10^4) which corresponds more closely to that of cinnamoyl esters (λ_{max}, ~285 mμ) than to cinnamoylimidazole (λ_{max}, 307 mμ). Cinnamoylchymotrypsin was isolated by Balls'[7] method and gave a spectrum similar to the above; its rate of deacylation at *p*H's 7.0 and 8.2 corresponded to reactions run directly at those *p*H's. The rates of acylation and deacylation of cinnamoyl-chymotrypsin depend on groups whose pK_a's are about 6.6 and 7.4, respectively, results similar to those obtained previously.[4]

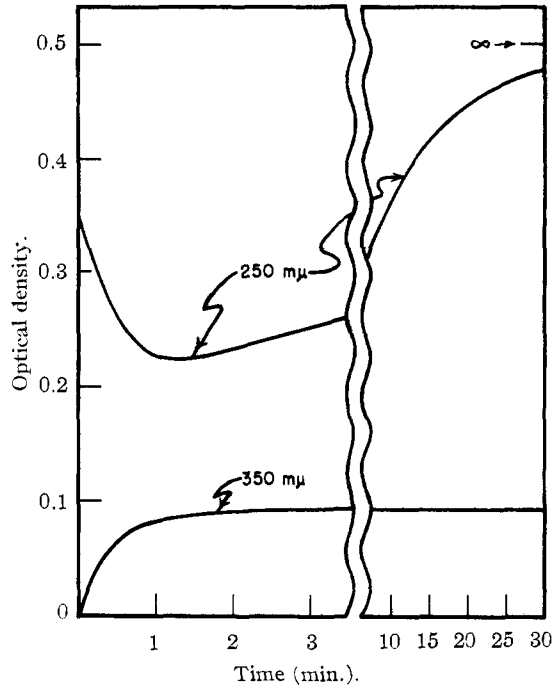

Fig. 1.—The α-chymotrypsin-catalyzed hydrolysis of *o*-nitrophenyl cinnamate at *p*H 6.2 at 25° in phosphate buffers containing 10% acetonitrile. Measurements were taken on a Beckman DK-2 recording spectrophotometer: $E = S \cong 0.42 \times 10^{-4} M$.

The technique of labeling the active sites of enzymes by highly absorbing groups should help elucidate the mechanisms of enzymatic catalysis.

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(3) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **50**, 672 (1952); **56**, 288 (1954).

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(5) R. A. Oosterbaan and M. E. van Adrichem, *Biochim et Biophys. Acta*, **27**, 423 (1958).

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(8) G. H. Dixon and H. Neurath, THIS JOURNAL, **79**, 4558 (1957).

THE SYNTHESIS OF BIS-TRIPHENYLCYCLOPROPENYL, AN UNDISSOCIATED DIMER OF THE TRIPHENYLCYCLOPROPENYL RADICAL, AND ITS ISOMERIZATION TO HEXAPHENYLBENZENE

Sir:

In view of the striking contrast in the cyclopropenyl series between derivatives of the stable cation[1] and those of the unstable anion[2] it seemed desirable to obtain evidence on the stability of a cyclopropenyl radical. Accordingly we have reacted *sym*-triphenylcyclopropenyl bromide[1] (I) with zinc dust[3] in benzene and obtained (66%) the dimer (II), m.p. 225–226° (with rapid resolidification to an isomer, m.p. 430°). Found: C, 94.40; H, 5.57. The ultraviolet spectrum, λ_{max} 306 mμ (log ε 4.5) with shoulders at 290 and 320 mμ, is similar to that of other diphenylcyclopro-

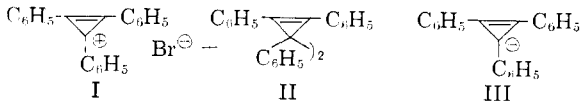
(1) R. Breslow and C. Yuan, THIS JOURNAL, **80**, 5991 (1958).

(2) R. Breslow and M. Battiste, *Chem. & Ind.*, 1143 (1958).

(3) The tropylium ion has been dimerized with zinc by W. Doering and L. H. Knox, THIS JOURNAL, **79**, 352 (1957).

pene systems[1] but of lower intensity. With bromine II is reconverted to 1.6 moles of I, as expected from ionic bromination. The n.m.r. spectrum,[4] with suitable controls, demonstrates the absence of any aliphatic hydrogen; with the bromination results this proves the position of coupling.

Although II is the dimer of the *sym*-triphenylcyclopropenyl radical (III), a solution of II gives no evidence of radicals in the electron spin resonance spectrometer,[4] and the compound is recovered unchanged from several days exposure, in solution, to oxygen or iodine or from two hours reflux with potassium permanganate in pyridine in the pres-



I II III

ence of oxygen (except for 20% conversion to the high melting isomer under these conditions). The dimer is thus remarkably stable with respect to the radical, although III is formally very similar to I, the cation. We take this as further strong evidence for a special stabilization in cyclopropenyl cations which is not shared by either the radical or the anion.

When II is held at its melting point for a few seconds it is quantitatively transformed to an isomer, m.p. 430–432°, which also is formed when II is refluxed in xylene for a few hours or irradiated in solution with an ultraviolet lamp at room temperature. Although we early rejected the possibility that this was hexaphenylbenzene, because of what we now recognize to have been an experimental error, and entertained the idea that this isomer was hexaphenylprismane, careful re-examination establishes that this compound is in fact identical with authentic hexaphenylbenzene.[5] Several possible mechanisms can be formulated for this interesting rearrangement.

(4) We wish to thank Mr. John Martin for the n.m.r., and Mr. Herbert Strauss for the e.s.r., determinations.

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CHEMICAL HYDROXYLATION OF 12a-DEOXYTETRACYCLINE

Sir:

Initial attempts to prepare tetracycline by perbenzoic acid oxidation of 12a-deoxytetracycline were unsuccessful.[1] There was some evidence from ultraviolet data that 12a-hydroxylation occurred but, if so, 12a-epitetracycline must have been formed. Similar results have been reported with perbenzoic acid oxidation of 12a-deoxydedimethylaminotetracycline.[2] Muxfeldt and Kreutzer[3] have recently reported, however, on the preparation of 12a- and 12a-epi-hydroxylated derivatives of 12a-deoxydedimethylamino-anhydrotetracycline-10-monomethyl ether by this method. The encouraging results obtained from the microbiological process[4] prompted attempts to improve the yield of tetracycline via this method. It was thought that by altering the fermentation conditions (*e.g.*, type of medium and time of harvest) an enzyme specific for 12a-hydroxylation might be produced preferentially. While this objective proved unsuccessful, one medium was found capable of 12a-hydroxylation prior to inoculation. The chemical agent responsible for this reaction was found to be sodium nitrite, present as an impurity in the sodium nitrate used in the medium.

Study of the reaction revealed that the optimum *p*H, as in the case of the microbiological process, was from 4.4–4.6. This common optimal *p*H range may reflect the importance of a specific tautomeric form of 12a-deoxytetracycline for optimum reaction rates. The rate of disappearance of 12a-deoxytetracycline, as measured by spectrophotometric assay in 0.1 *M* sodium borate, increased with increasing concentrations of sodium nitrite employed, but at molar ratios of sodium nitrite to 12a-deoxytetracycline of 1 or higher, the yield of tetracycline was reduced. The action of sodium nitrite appears to be in part direct and in part catalytic. Under anaerobic conditions no tetracycline was formed but the 12a-deoxytetracycline and sodium nitrite were consumed in equimolar quantities. Two gases were evolved; carbon dioxide and in much lesser quantity a neutral gas of unknown identity. Aerobically, oxygen was consumed and tetracycline, as indicated by paper chromatography, was produced in .25–35% yield. The catalytic nature of sodium nitrite in the aerobic reaction is suggested by the fact that at low sodium nitrite to 12a-deoxytetracycline ratios (0.05–0.01) considerably more 12a-deoxytetracycline than sodium nitrite was consumed.

For proof of tetracycline formation, 150 ml. of 10 mg./ml. aqueous solution of 12a-deoxytetracycline (*p*H to 1.5 with HCl) was added to 1200 ml. of *p*H 4.4 McIlvaine buffer[5] plus 150 ml. of sodium nitrite solution at 0.808 mg./ml. After two hours of shaking on a reciprocating shaker, spectrophotometric assay indicated 97% consumption of 12a-deoxytetracycline and microbiological assay suggested a 29% yield of tetracycline. The isolation procedure was similar to that previously reported.[4] Three ultraviolet absorbing fractions were obtained by column chromatography. Crystalline tetracycline, as characterized by infrared, ultraviolet, paper chromatography and bioassay was isolated from the most polar fraction (fraction 3). Fraction 1 displayed non-distinctive ultraviolet absorption spectra. However fraction 2 exhibited spectra in 0.1*N* HCl and 0.1*M* sodium borate very similar to those of fraction 2 material obtained from the microbiological reaction.[4] The similarity was further extended by failure to observe anhydro formation upon refluxing with methanolic HCl. It is believed that an 11a-

(1) Unpublished results of Dr. A. Green of these laboratories.

(2) Discussed by C. R. Stephens at the Gordon Research Conference, Medicinal Chemistry, August, 1957.

(3) H. Muxfeldt and A. Kreutzer, *Naturwissenschaften*, **46**, 204 1959).

(4) Holmlund, *et al.*, THIS JOURNAL, **81**, 4750 (1959).

(5) "Handbook of Chemistry and Physics," Chemical Rubber Publishing Co., Cleveland, Ohio, 24th Edition, 1940–1941, p. 1374.