chloride in diglyme,[4] followed by chromic acid oxidation of the resulting 6-boron conanine derivative (II), gave 3β-dimethylaminoconanin-6-one (III), m.p. 198–203°,[5] $[\alpha]^{25}D$ +11.1°, λ^{KBr}_{max} 5.88 μ, C, 77.46; H, 10.59; N, 7.80, dinitrophenylhydrazone dec. about 250°. Reaction of the corresponding bismethiodide (IV) with potassium *t*-butoxide in boiling *t*-butyl alcohol afforded 18-dimethylamino-3,5-cyclopregn-20-en-6-one (V), m.p. 73–75° and 87–88°, $[\alpha]^{25}D$ +51.0°, λ^{KBr}_{max} 5.93 and 6.12 μ, C, 80.82; H, 10.30, in over 80% yield.

The metho-*p*-toluenesulfonate of V (VI) was hydroxylated with aqueous potassium chlorate and osmium tetroxide as a catalyst to yield a mixture of the corresponding 20α,21-diol (VIIa) and 20β,21-diol (VIIb), which was then refluxed with potassium *t*-butoxide in *t*-butyl alcohol, affording 21-hydroxy-18,20β-epoxy-3,5-cyclopregnan-6-one (VIII), m.p. 185–188°, $[\alpha]^{25}D$ +62.9°, λ^{KBr}_{max} 2.84 and 5.95 μ, C, 76.36; H, 9.13, as the major neutral product.

Treatment of VIII with *p*-toluenesulfonyl chloride in pyridine resulted in the corresponding tosylate IX, m.p. 142–143°, which on reaction with dimethylamine yielded the 21-dimethylamino derivative (X), m.p. 145–147°, C, 77.20; H, 10.05; N, 4.20. Reduction of X with lithium aluminum hydride led to a mixture of epimeric 6-hydroxy derivatives, m.p. 133–137°, that was isomerized with formic acid to the corresponding 21-dimethylamino-18,20β-epoxy-5-pregnen-3-ol (XI), m.p. 160–163°. Oppenauer oxidation of XI yielded 21-dimethylamino-18,20β-epoxy-4-pregnen-3-one (XII), m.p. 123–124.5°; λ^{KBr}_{max} 5.98, 6.20 μ and 240.5 mμ, ε 17,140. The corresponding N-oxide (XIII) smoothly eliminated dimethylhydroxylamine in refluxing *t*-butylbenzene, affording 18,20-epoxy-4,20-pregnadien-3-one (XIV), $\lambda^{CS_2}_{max}$ 5.95, 6.19 and 12.57 μ.

The enol ether XIV in presence of dilute acids, was hydrolyzed readily to 20-hydroxy-18,20-epoxy-4-pregnen-3-one (XV), m.p. 173–182°, λ^{KBr}_{max} 2.92, 5.96, 6.18 μ and 241 mμ, ε 17,100, C, 76.56; H, 9.17, which was shown to be, by paper chromatography, a mixture of readily interconvertible 20α and 20β alcohols, that had little tendency to react in the tautomeric 18-hydroxyprogesterone form.[6] However, oxidation of XV with chromic acid in pyridine gave 3,20-diketo-4-pregnen-18-oic acid (XVI), m.p. 225–227°, λ^{KBr}_{max} 2.94, 5.70, 6.00, 6.20 μ and 240.2 mμ, ε 17,200, C. 73.07; H, 7.98, existing as the tautomeric hydroxy-lactone; the neutral fraction contained, besides much starting material, 3,20-diketo-4-pregnen-18-al (XVII) m.p. 139–142°, λ^{KBr}_{max} 3.67, 5.83, 6.00, 6.22 μ and 240.4 mμ, ε 17,000.

In order to establish the structure of the enol ether (XIV), the product was treated with osmium tetroxide, yielding 20,21-dihydroxy-18,20-epoxy-4-pregnen-3-one (XVIII),[6,7] m.p. 191–195°, λ^{KBr}_{max} 2.90, 6.00 and 6.20 μ, C, 72.72; H, 8.75, which reacted with lead tetraacetate, affording 18-hydroxy-3-keto-4-etien-20-oic acid 20,18-lactone (XIX), m.p. 227–231°, λ^{KBr}_{max} 5.62, 5.99 and 6.19 μ (lit.[7] m.p. 221–224°). This product was identical with a lactone isolated in these laboratories during experiments involving the perfusion of desoxycorticosterone through beef adrenals.[8]

(4) H. C. Brown and B. C. Subba Rao, THIS JOURNAL, **78**, 5694 (1956).

(5) Rotations were determined in commercial acid-free chloroform.

(6) Acetylation of XVIII with acetic anhydride in pyridine overnight—conditions which did not essentially affect XV—gave a monoacetate, m.p., 158–159°, λ^{KBr}_{max} 2.90, 5.77 5.99 and 6.22 μ.

(7) *Cf.* F. W. Kahnt, R. Neher and A. Wettstein, *Helv. Chim. Acta*, **38**, 1237 (1955), who claimed to have isolated 18,21-dihydroxy-4-pregnen-3,20-dione from a mixture obtained by incubation of desoxycorticosterone with adrenal homogenates.

(8) Dr. J. S. Mihina, private communication.

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AN ENZYMATIC CHLORINATION REACTION

Sir:

Despite the frequent occurrence of chlorine-containing natural products, the biological mechanism for the formation of the carbon–chlorine bond is unknown. We wish to report the synthesis of the carbon–chlorine bond catalyzed by an enzyme (or enzyme system) from *Caldariomyces fumago*. The major chlorine-containing metabolite excreted by this organism is caldariomycin, which has been identified tentatively as 2,2-dichloro-1,3-cyclopentanediol[1].

Acetone-dried mycelial powders of this mold catalyze the conversion of chloride ion (Cl^{36}) into an ether extractable organic form when supplemented with β-ketoadipic acid (Table I). The requirement for β-ketoadipic acid is specific; a number of related β-keto carboxylic acids as well as other common metabolic intermediates have been tested and do not show significant activity. Table I also illustrates the enzymatic nature of this reaction since heat denatured mycelial powders do not catalyze the reaction.

TABLE I

THE ENZYMATIC CONVERSION OF CHLORIDE ION TO AN ETHER EXTRACTABLE ORGANIC FORM

The complete system contained 500 μmoles of potassium phosphate buffer, *p*H 6.0; 10 μmoles of potassium Cl^{36} (specific activity of 8900 c.p.m./μmole); 100 μmoles of potassium β-ketoadipate and 100 mg. of acetone-dried mycelial powder in a total volume of 5 ml. Following 1 hour of aerobic incubation at 25°, the reaction mixture was acidified to *p*H 3 with 7*N* sulfuric acid and the aqueous phase extracted twice with 2 volumes of diethyl ether. The ether extract was dried over sodium sulfate, concentrated on a steam-bath under nitrogen, plated and counted in a gas flow counter.

	Additions	Cl^{36} Incorporation c.p.m.	μmoles
1	Complete	890	0.1
2	1 minus β-ketoadipate	30	0.003
3	1 minus Cl^{36}	0	0
4	1 with heat denatured mycelial powder	0	0

Large scale incubations allowed the accumulation of approximately 5 mg. of the radioactive, enzymatically synthesized compound. The behavior of this unknown compound on a Dowex-1 column (formate phase) indicated it to be a weaker acid than β-ketoadipic acid. The organically

(1) P. W. Clutterbuck, S. L. Mukhopadhyay, A. E. Oxford, and H. Raistrick, *Biochem. J.*, **34**, 664–677 (1940).

bonded Cl^{36} was stable to 1 N sulfuric acid and labile to 1 N sodium hydroxide at room temperature. These features strongly suggested that the unknown acid was a decarboxylation product of β-ketoadipic acid and that the chlorine was situated on a carbon alpha to the ketone. Assuming a beta decarboxylation, these considerations limited the possibilities to either δ-chlorolevulinic acid or β-chlorolevulinic acid. Consequently dimethyl α-chloro-β-ketoadipate was synthesized by the chlorination of dimethyl β-ketoadipate with 1 equivalent of sulfuryl chloride according to the method of Allihn for the chlorination of ethyl acetoacetate.[2] This intermediate was hydrolyzed and decarboxylated in 7 N sulfuric acid to yield δ-chlorolevulinic acid (m.p. 73–73.5°). *Anal.* Calcd. for $C_5H_7O_3Cl$: C, 39.88; H, 4.69; Cl, 23.55. Found: C, 39.92; H, 4.60; Cl, 23.43. The position of the chlorine in the synthetic product was established by displacement of the chlorine with hydroxide ion and then periodate oxidation to yield formaldehyde which was isolated as the dimedone derivative (m.p. 191°).

The enzymatically synthesized chlorine containing acid and the synthetic product were identical as shown by constant specific radioactivity on repeated crystallization (synthetic δ-chlorolevulinic acid (24.74 mg.) was added to a purified preparation of the unknown radioactive acid (24,000 + 1,000 c.p.m.) the specific activity (c.p.m./mg.) after successive crystallizations from hexane-ether was 770, 1016, 1081 and 1021). In addition, paper chromatography of the natural and synthetic compounds revealed identical behavior in several solvent systems. Chromatography of the natural and synthetic compounds on silicic acid yielded fractions having a constant ratio of weight to radioactivity.

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(2) F. Allihn, *Ber.*, **11**, 567 (1878).

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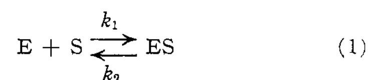
ACTIVATION OF AN ENZYME CATALYZED REACTION BY EXCESS SUBSTRATES

Sir:

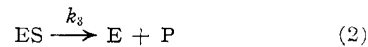
In the course of an investigation of the kinetics of the α-chymotrypsin catalyzed hydrolysis of methyl aceturate, in aqueous solutions at 25.0° and pH 7.90 and 0.50 M in sodium chloride, we have encountered what appears to be the first example of activation of an enzyme catalyzed reaction, of the type $E + S \rightleftarrows ES \rightarrow E + P$, by excess substrate.

Methyl aceturate is sufficiently soluble in water to permit the examination of its hydrolysis in the presence of α-chymotrypsin over a wide range of substrate concentrations. For the case at hand such reactions were followed with a pH-stat[1] under conditions where $[E] = 4.54 \times 10^{-5}\ M$[2] and $[S]_0$, was varied from $5.0 \times 10^{-3}\ M$ to $6.0 \times 10^{-1}\ M$. When the results of these experiments were presented in a v_0 *vs.* $v_0/[S]_0$ plot,[3,4] it became apparent that the plot consisted of two essentially linear segments, of differing slope and intercept, and a non-linear connecting segment. The slope and intercept of the linear segment associated with values of $[S]_0 = 5.0 \times 10^{-3}\ M$ to $3.0 \times 10^{-2}\ M$ led to values of $K_S = 1.0 \times 10^{-2}\ M$ and $k_3 = 1.2 \times 10^{-4}\ M$/min./mg. protein nitrogen per ml. The slope and intercept of the other linear segment, associated with values of $[S]_0 = 5.0 \times 10^{-2}\ M$ to $6.0 \times 10^{-1}\ M$, gave values of $K_S = 4.1 \times 10^{-1}\ M$ and $k_3 = 1.0 \times 10^{-3}\ M$/min./mg. protein nitrogen per ml.

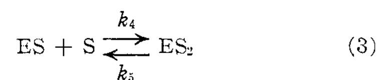
For a system represented by equations 1 to 4, inclusive, it can be shown that the rate equation for such a representation is given by equation 5



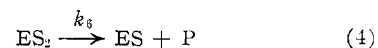
$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \quad (1)$$



$$ES \xrightarrow{k_3} E + P \quad (2)$$



$$ES + S \underset{k_5}{\overset{k_4}{\rightleftarrows}} ES_2 \quad (3)$$



$$ES_2 \xrightarrow{k_6} ES + P \quad (4)$$

$$v_0 = \{(K_S''k_3 + k_6[S]_0)[E][S]_0\}/\{K_S''[S]_0 + [S]_0^2 + K_S'K_S''\} \quad (5)$$

where $K_S' = (k_2 + k_3)/k_1$ and $K_S'' = (k_5 + k_6)/k_4$.

With $K_S' = 1.0 \times 10^{-2}\ M$, $K_S'' = 4.1 \times 10^{-1}\ M$, $k_3 = 1.2 \times 10^{-4}\ M$/min./mg. protein nitrogen per ml. and $k_6 = 1.0 \times 10^{-3}\ M$/min./mg. protein nitrogen per ml. it was found that equation 5 provided a satisfactory quantitative description of the dependence of v_0 upon $[S]_0$ over the 120-fold range of substrate concentration that had been studied. Therefore, we conclude that the α-chymotrypsin catalyzed hydrolysis of methyl aceturate may be represented by equations 1 to 4, inclusive, and that evidence has been obtained with respect to the formation of an ES_2 complex that is capable of yielding reaction products at a greater rate than the corresponding ES complex. That is, activation by excess substrate has been demonstrated.

The extension of these studies to systems involving two different substrates, in order to explore the possibility of demonstrating synergism in an isolated enzyme system, and to systems involving a substrate and a substrate analog that is incapable of yielding reaction products, in order to investigate the possibility of observing inhibition at low concentrations of the analog and activation at high concentrations, is in progress.

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(1) T. H. Applewhite, R. B. Martin and C. Niemann, THIS JOURNAL, **80**, 1457 (1958).

(2) Based upon an assumed molecular weight of 22,000 and a nitrogen content of 16.0% for monomeric α-chymotrypsin.

(3) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).

(4) All values of v_0 were corrected for the non-enzyme catalyzed hydrolysis of the substrate.