was taken up in absolute alcohol and treated with anhydrous ether. There was obtained 520 mg. (84%) of the carbinol (III, R = R′ = OCH_3), m.p. 147° (dec.).

Anal. Calcd. for $C_{14}H_{18}O_6$: C, 59.56; H, 6.43. Found: C, 59.53; H, 6.57.

3-Carboxy-2-hydroxymethyl-1-keto-6,7-methylenedioxy-1,2,3,4-tetrahydronaphthalene (1.0 g.) in 75 ml. of ethanol was shaken with hydrogen at 60° and 4 atmospheres in the presence of 200 mg. of 5% palladium on alumina for 60 minutes, at the end of which time one equivalent of hydrogen had been absorbed and the reaction had stopped. The oil remaining after removal of the catalyst and evaporation of the solvent was crystallized from 10% aqueous ethanol to give 0.8 g. (80%) of the carbinol (III, R,R′ = O-CH_2O-), m.p. 157° (dec.).

Anal. Calcd. for $C_{13}H_{14}O_6$: C, 58.64; H, 5.30. Found: C, 58.76; H, 5.47.

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NOTRE DAME, INDIANA

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC.]

Magnamycin. A New Antibiotic[1,2]

BY RICHARD L. WAGNER, F. A. HOCHSTEIN, KOTARO MURAI, N. MESSINA AND PETER P. REGNA

RECEIVED MAY 25, 1953

The new antibiotic Magnamycin has been isolated from fermentation broths by solvent extraction and purified by repeated crystallization. The high purity of the weak base obtained by this procedure has been demonstrated by solubility analysis, by countercurrent distribution studies and by paper chromatography. The chemical and physical properties of Magnamycin, of its carbonyl derivatives and its tetrahydro derivative suggest the presence of both saturated and α,β-unsaturated carbonyl systems. Alkaline degradation of the antibiotic has yielded acetic acid, isovaleric acid and dimethylamine. Mild acid hydrolysis cleaves Magnamycin into two fragments, namely, the isovalerate ester of a $C_7H_{14}O_4$ compound, and a crystalline solid, the formula of which is probably $C_{29-30}H_{47-49}NO_{12}$.

Magnamycin[3] is a new antibiotic elaborated by strains of the microörganism *Streptomyces halstedii*. The antibiotic may be isolated from filtered fermentation broth by simple extraction with a water-immiscible solvent. The crude product obtained on concentration of the solvent extracts can be purified by crystallization from alcohol–water mixtures. The purity of Magnamycin obtained by repeated crystallizations from ethanol has been shown by solubility analysis to be 99.2 ± 0.1%. Both countercurrent distribution and paper chromatography studies confirm the homogeneity of this material.

On the basis of analyses of Magnamycin and its crystalline derivatives, the antibiotic has been assigned a tentative empirical formula $C_{41-42}H_{67-69}NO_{16}$. The optically active substance $[\alpha]^{25}D$ −58.6° (*c* 1%, chloroform) crystallizes from ethanol as colorless laths. It is readily soluble in most organic solvents but virtually insoluble in hexane and in water. Although Magnamycin is a weak base, pK_b 7.2, it forms stable salts with mineral acids. The water-soluble hydrochloride as well as the relatively insoluble periodate have been prepared.

Crystalline Magnamycin shows no signs of decomposition after storing for several months in the dark at room temperature. Although aqueous solutions of the new antibiotic between *p*H 5–7 show no loss in microbiological activity after 11 days at 25°, more acid solutions, *p*H 3, and more basic solutions, *p*H 9, are half-inactivated in the same period.

Although Magnamycin contains nitrogen, it gives negative ninhydrin and Van Slyke nitrogen tests, indicating the absence of any primary amine. Fehling and Tollens tests are positive. The presence of carbonyl groupings is suggested by the positive 2,4-dinitrophenylhydrazine test. The unsaturated character of Magnamycin is demonstrated further by the positive Baeyer permanganate test and the ready decolorization of bromine. The ceric nitrate test shows the presence of hydroxyl groups, while the negative ferric chloride test shows them to be non-phenolic. The boric acid test suggests that there are no suitable oriented adjacent hydroxyl groups. In strong mineral acids, *e.g.*, 2–6 *N*, Magnamycin develops a characteristic deep violet color which slowly fades to a red-brown. This characteristic reaction in aqueous acid interferes with many standard diagnostic color tests.

Several biologically active derivatives of Magnamycin have been prepared. A diacetyl derivative is formed on acetylation in acetic anhydride–pyridine. An oxime and a thiosemicarbazone have been prepared by standard procedures. The hydrogenation of Magnamycin over palladium–charcoal catalyst in ethanol solution results in the prompt absorption of two moles of hydrogen and yields a crystalline tetrahydro derivative which also forms a diacetate on acetylation.

Alkaline hydrolysis of Magnamycin yields one mole each of acetic and isovaleric acids and dimethylamine. Controlled mild acid hydrolysis results in hydrolytic cleavage of the Magnamycin molecule into two fragments. The first product, a viscous oil, with the empirical formula $C_{12}H_{22}O_5$, contains the isovaleric acid of Magnamycin. The second fragment, a crystalline solid, is a weak base which appears to have the empirical formula $C_{29-30}H_{47-49}NO_{12}$. Although analyses of the compound itself have not been entirely satisfactory, the diacetate is readily purified, and its analyses are consistent with those calculated for the C_{29}

(1) Magnamycin is a Chas. Pfizer and Co. trademark for the antibiotic carbomycin.

(2) Presented before the Division of Medicinal Chemistry at the 123rd Meeting of the American Chemical Society, Los Angeles, Calif., March, 1953.

(3) F. W. Tanner, A. R. English, T. M. Lees and J. B. Routien, *Antibiotics and Chemotherapy*, **2**, 441 (1952).

formula. This crystalline basic substance, isolated from the hydrolytic reaction, has infrared and ultraviolet spectra nearly identical with those of the parent compound.

The nature of certain structural units in Magnamycin other than those identified by degradation is apparent from an examination of the spectral and hydrogenation data. The ultraviolet absorption spectrum of Magnamycin (Fig. 1) in 95% ethanol solution shows a strong absorption peak at λ238 mμ ($E^{1\%}_{1cm}$ 185) together with a much weaker peak at λ327 mμ ($E^{1\%}_{1cm}$ 0.9). The position and intensity of this pair of peaks are characteristic of α,β-unsaturated ketones.[4] Furthermore, this assignment is consistent with the presence of absorption peaks at 5.95 and 6.15 μ in the infrared spectrum of Magnamycin measured in chloroform solutions (Fig. 2). The strong peak at 5.8 μ must arise from isovalerate and acetate ester carbonyl absorption and from saturated carbonyl groups in the molecule, while the weak absorption band at 10.25 μ appears, like the 6.15 μ band, to arise from —C=C— absorption. Also in accord with the α,β-unsaturated carbonyl system in Magnamycin is the disappearance of the 5.95 and 6.15 μ bands as well as the 10.25 μ band on reduction to tetrahydromagnamycin.

Thiosemicarbazones of α,β-unsaturated ketones are reported[5] to show absorption maxima at 302 mμ. The thiosemicarbazone of Magnamycin, however, exhibits no peak at 302 mμ, but instead shows maxima at 235 and 270 mμ (Fig. 1) characteristic of saturated thiosemicarbazones. The oxime resulting from the condensation of hydroxylamine with Magnamycin appears also to involve a saturated carbonyl, since the derivative shows an ultraviolet spectrum virtually identical to that of the parent substance. In contrast, α,β-unsaturated oximes are reported to show no absorption in the 325 mμ region.[5] The similarity of both the ultraviolet and infrared spectra of the crystalline acid degradation fragment $C_{29-30}H_{47-49}NO_{12}$ to those of Magnamycin suggests that both the saturated and the α,β-unsaturated carbonyl groups are contained in this portion of the molecule. Further work on the degradation and structure of Magnamycin will be reported later.

Experimental

Isolation of Crude Magnamycin.—Ten liters of Magnamycin fermentation broth (100 γ/ml.) was acidified to *p*H 3.0 with concentrated hydrochloric acid and stirred for one hour with 200 g. of Super-cel. After filtering on a buchner funnel, the clarified broth was extracted twice with 2.5 l. of methyl isobutyl ketone. The combined methyl isobutyl ketone extracts were evaporated *in vacuo* to 500 ml. The antibiotic was extracted with dilute sulfuric acid (*p*H 2.0) and the aqueous phase was washed several times with benzene. The solution was adjusted to *p*H 6.5 by the slow addition of 10% sodium hydroxide, repeatedly extracted with ether, the ether phase dried over sodium sulfate and evaporated to yield crystalline Magnamycin (3.0 g.): yield 30%; microbiological potency 800 γ/mg.

Microbiological assays were carried out by a plate assay method similar to that described for streptomycin.[6] *B. subtilis* was used as the test organism, and pure Magnamycin base as the standard.

Purification of Magnamycin.—Crude Magnamycin base (7.50 g.) was suspended in 37 ml. of anhydrous ethanol and shaken for 20 hours at 25°. The insoluble product was filtered, washed with ethanol and dissolved in 25 ml. of methanol. The pale yellow colored solution was treated with charcoal (Darco G-60), filtered and with stirring was slowly diluted with an equal volume of water. Pure Magnamycin base crystallized overnight as colorless laths. After drying for 3 hours at 100°, 1 mm. pressure, the substance melted at 212–214° (dec.) when placed in a bath at 190° whose temperature was rising at 3° per minute; $[\alpha]^{25}D$ −58.6° (*c* 1% in chloroform).

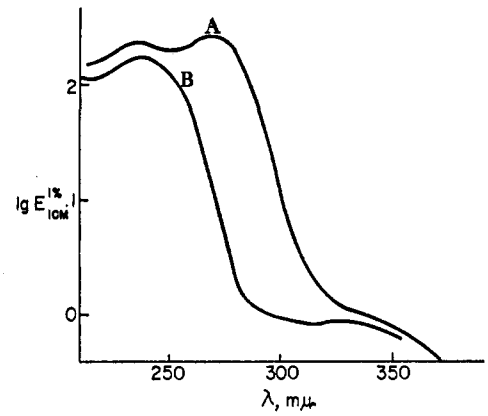

Fig. 1.—Ultraviolet absorption spectra in ethanol: A, Magnamycin thiosemicarbazone; B, Magnamycin.

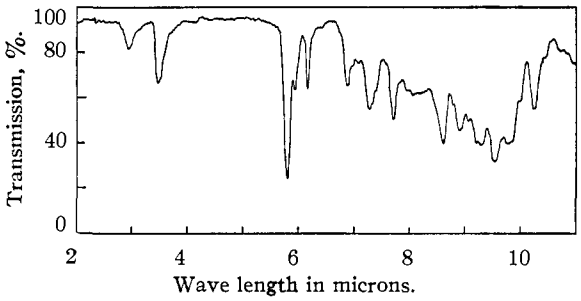

Fig. 2.—Infrared absorption spectrum of Magnamycin.

A solubility analysis[7,8] of this substance in absolute ethanol at 36.60 ± 0.02° showed a purity of 99.2 ± 0.1%. At this temperature, solubility equilibrium was attained within 40 hours. Magnamycin shows no detectable signs of decomposition after 7 days in ethanol solution at 36°. The homogeneity of this Magnamycin sample was demonstrated further by a 60-transfer countercurrent distribution between benzene and *p*H 4.5 sodium acetate buffer. The distribution curve obtained for this material agreed with a calculated curve for a homogeneous substance.[9] Paper chromatography[10] using several systems showed no evidence of inhomogeneity.

In absolute ethanol, this sample of Magnamycin showed λ_{max} 238 mμ ($E^{1\%}_{1cm}$ 185) and λ_{max} 327 mμ ($E^{1\%}_{1cm}$ 0.9). Potentiometric titration in 3:1 dimethylformamide–water showed a neutral equivalent of 836 and pK_b 7.2. The infrared absorption data are reported for chloroform solutions (Fig. 2).

Anal. Calcd. for $C_{41}H_{67}NO_{16}$: C, 59.33; H, 8.14; N, 1.69; 6C-(CH_3), 10.87; N-(CH_3)$_2$, 3.62; OCH_3, 3.75; mol. wt., 830. Calcd. for $C_{42}H_{69}NO_{16}$: C, 59.77; H, 8.24; N, 1.66. Found: C, 59.89; H, 7.96; N, 1.78; C-CH_3, 10.58; N-(CH_3)$_2$, 2.94; OCH_3, 3.75.

Magnamycin Hydrochloride.—Magnamycin (4.30 g.) was finely powdered and suspended in 4.50 ml. of 1.186 *N* hydrochloric acid. After stirring for 30 minutes, the cooled suspension was filtered and washed with cold water. The crystalline Magnamycin hydrochloride (3 g.) was dried *in*

(4) R. B. Woodward, THIS JOURNAL, **63**, 1123 (1941).
(5) L. K. Evans and A. E. Gillam, *J. Chem. Soc.*, 565 (1943).
(6) Federal Register, **17**, 6579 (July 18th, 1952).
(7) H. K. Zimmerman, *Chem. Revs.*, **51**, 25 (1952).
(8) J. B. Dalton and C. L. A. Schmidt, *J. Biol. Chem.*, **103**, 549 (1933).
(9) L. C. Craig, *Anal. Chem.*, **22**, 1346 (1950).
(10) P. P. Regna and I. A. Solomons, *Ann. N. Y. Acad. Sci.*, **53**, 229 (1950).

vacuo at 50° for 24 hours; m.p. 149–150° when placed in a bath at 146° and the temperature raised 1° per minute.

Anal. Calcd. for $C_{41}H_{67}NO_{16}\cdot HCl$: C, 56.83; H, 7.91; N, 1.62; Cl, 4.09. Calcd. for $C_{42}H_{69}NO_{16}\cdot HCl$: C, 57.29; H, 8.01; N, 1.59; Cl, 4.02. Found: C, 56.72; H, 7.87; N, 1.51; Cl, 4.02.

Magnamycin Periodate.—Magnamycin (1.0 g.) was dissolved in 20 ml. of 0.2 *N* sulfuric acid. To the well stirred solution was added 20 ml. of 0.388 *N* periodic acid. The white crystalline precipitate which formed was filtered, washed well with water and dried *in vacuo* for 24 hours: yield 0.95 g. The periodate salt was recrystallized twice from acetone–water mixtures and dried *in vacuo* at 100° for three hours; m.p. 134–135° (dec.).

Anal. Calcd. for $C_{42}H_{69}NO_{16}\cdot HIO_4$: C, 48.69; H, 6.81; N, 1.35; I, 12.25. Calcd. for $C_{41}H_{67}NO_{16}\cdot HIO_4$: C, 48.19; H, 6.71; N, 1.37; I, 12.43. Found: C, 48.65; H, 6.81; N, 1.26; I, 11.96.

Diacetylmagnamycin.—A solution of 2 g. of Magnamycin in 40 ml. of acetic anhydride and 0.1 ml. of pyridine was held at room temperature for 24 hours, then poured onto 200 g. of ice. After two hours, the aqueous solution was adjusted to *p*H 5, filtered and the crude product (1.67 g.) was recrystallized three times from aqueous acetone and dried *in vacuo* at 100° for three hours; m.p. 149–150°, $[\alpha]^{25}D$ −81° (*c* 1% in chloroform). A standard acetyl determination showed 3.88 equivalents of volatile acids per mole of diacetylmagnamycin (calcd. 4 equivalents). Titration in chloroform solution with perchloric acid in dioxane showed an equivalent weight of 919.

Anal. Calcd. for $C_{45}H_{71}NO_{18}$: C, 59.12; H, 7.83; N, 1.53. Calcd. for $C_{46}H_{73}NO_{18}$: C, 59.53; H, 7.93; N, 1.51. Found: C, 59.44; H, 7.86; N, 1.52.

Magnamycin Oxime.—Magnamycin (2.98 g.) and hydroxylamine hydrochloride (0.242 g.) were dissolved in 1 ml. of pyridine and 40 ml. of ethanol. The solution was heated under reflux for four hours, allowed to stand 18 hours at room temperature and evaporated to dryness under a slow stream of nitrogen. The residue was triturated with water, filtered, washed thoroughly and dried. The filtrate was allowed to stand for 24 hours and a second crop was recovered and treated as described above: over-all yield 0.91 g. The crude derivative was recrystallized twice from chloroform–hexane mixtures and dried *in vacuo* at 100°; m.p. 198–199° (dec.).

Anal. Calcd. for $C_{42}H_{70}N_2O_{16}$: C, 58.72; H, 8.21; N, 3.26. Calcd. for $C_{41}H_{68}N_2O_{16}$: C, 58.27; H, 8.11; N, 3.32. Found: C, 58.51; H, 8.06; N, 3.14.

The ultraviolet spectrum was determined in 95% ethanol: λ_{max} 238 mμ ($E^{1\%}_{1cm}$ 193) and λ_{max} 325 mμ ($E^{1\%}_{1cm}$ 1.0).

Magnamycin Thiosemicarbazone.—Magnamycin (6.0 g.) and thiosemicarbazide (0.65 g.) were dissolved in 80 ml. of ethanol. The solution was heated under reflux for four hours, chilled in ice and the crystalline product, recovered by filtration, was washed with cold ethanol and dried: yield 2.9 g. The crystalline derivative was twice recrystallized from ethanol and dried *in vacuo* at 56°; m.p. 172–173° (dec.). The absorption spectrum (Fig. 1) of Magnamycin thiosemicarbazone in 95% ethanol shows λ_{max} 235 mμ ($E^{1\%}_{1cm}$ 241) and λ_{max} 270 mμ ($E^{1\%}_{1cm}$ 286).

Anal. Calcd. for $C_{42}H_{70}N_4O_{15}S$: C, 55.86; H, 7.81; N, 6.20; S, 3.55. Calcd. for $C_{43}H_{72}N_4O_{15}S$: C, 56.31; H, 7.91; N, 6.11; S, 3.50. Found: C, 55.76; H, 7.62; N, 5.81; S, 3.49.

Tetrahydromagnamycin.—Magnamycin (5.0 g.) was hydrogenated over 1.5 g. of 5% palladium-on-charcoal catalyst in 200 ml. of ethanol at 27°. The Magnamycin absorbed 1.95 moles of hydrogen per mole of antibiotic during a period of two hours. The catalyst was removed by filtration and the filtrate evaporated to dryness. The anhydrous product was dissolved in 100 ml. of isopropyl alcohol, 350 ml. of hexane added and the tetrahydro derivative was allowed to crystallize during a three-day period. Repeated recrystallizations from isopropyl alcohol–hexane gave a product which melted consistently at 121–122° but which did not analyze satisfactorily. The ultraviolet spectrum in ethanol shows only weak carbonyl absorption at 280 mμ.

Diacetyltetrahydromagnamycin.—Tetrahydromagnamycin (2.5 g.) was dissolved in 25 ml. of pyridine and 30 ml. of acetic anhydride. The solution was allowed to stand for 17 hours, evaporated to dryness *in vacuo* under a slow stream of nitrogen, dissolved in water and neutralized to *p*H 6.5. The crude precipitate was filtered, triturated in water and the derivative was recrystallized from acetone–ether mixtures and finally from ether: m.p. 179–181° (dec.). A standard acetyl determination showed 4.0 equivalents of volatile acids per mole of diacetyltetrahydromagnamycin (calcd. 4 equivalents).

Anal. Calcd. for $C_{45}H_{75}NO_{18}$: C, 58.87; H, 8.24; N, 1.53. Calcd. for $C_{46}H_{77}NO_{18}$: C, 59.27; H, 8.33; N, 1.50. Found: C, 59.14; H, 8.21; N, 1.52.

Alkaline Hydrolysis of Magnamycin.—Magnamycin (10 g.) was dissolved in 100 ml. of ethanol and 200 ml. of 20% potassium hydroxide and heated under reflux for three days in a slow stream of nitrogen. The volatile base was absorbed in standard hydrochloric acid; a total of 0.31 equivalent of base was liberated per mole of Magnamycin. (Hydrolysis with stronger alkali has yielded 0.76 equivalent of volatile base per mole of Magnamycin.) The volatile base was identified as dimethylamine through its hydrochloride and by the preparation of N,N-dimethyl-*p*-bromobenzenesulfonamide, m.p. 93–94°; mixed melting point with an authentic sample was not depressed.

Alcohol was distilled from the alkaline hydrolysate and the aqueous residue was acidified with dilute sulfuric acid. Steam distillation yielded 1.5 equivalents of volatile acids, which were converted (*via* their sodium salts) to *p*-bromophenacyl esters. The mixed esters were sublimed *in vacuo* and separated by crystallization from ethanol–water mixtures. The less soluble product was identified as the *p*-bromophenacyl ester of isovaleric acid; m.p. 66–67°. Its melting point was not depressed by mixing with an authentic sample and its identity was confirmed by analysis and by comparison of infrared spectra. The mother liquor of the isovaleric acid derivative yielded crude *p*-bromophenacyl acetate which was purified by sublimation *in vacuo* and crystallization. The identity of the *p*-bromophenacyl acetate was confirmed by analysis and by comparison of its infrared spectrum with an authentic sample.

Acid Hydrolysis of Magnamycin.—Magnamycin (3.10 g.) was dissolved in 25 ml. of 0.25 *N* hydrochloric acid and the clear solution held at 5° for three days. The aqueous solution was extracted with seven 25-ml. portions of ether; the ether layers were combined and dried, concentrated and the residue fractionally distilled at 1.1 mm. pressure to yield 0.6 g. of a viscous colorless oil, $n^{25}D$ 1.4630, $[\alpha]^{25}D$ −73° (*c* 2% in chloroform).

Anal. Calcd. for $C_{12}H_{22}O_5$: C, 58.50; H, 9.00. Found: C, 58.59; H, 8.94.

The ester had a saponification equivalent of 250 (theory 246). Isovaleric acid was isolated from the alkaline hydrolysate and identified through its *p*-bromophenacyl ester, m.p. 66–67°; a mixed melting point with an authentic sample was not depressed. The infrared spectrum of the original oil shows OH absorption at 2.8–2.9 μ and ester carbonyl absorption at 5.8 μ. The ester shows no appreciable absorption in the ultraviolet.

The aqueous phase remaining after ether extraction of the ester was adjusted to *p*H 10 and the amorphous precipitate (1.2 g.) which crystallized after standing for several hours was removed by filtration. Repeated crystallizations from ethanol yielded a small quantity of a pure crystalline product melting at 190–192° (dec.) when the substance was placed in a bath at 160° with temperature rising 4 to 5° per minute.

Anal. Calcd. for $C_{29}H_{47}NO_{12}$: C, 57.89; H, 7.87; N, 2.33; OCH_3 (1) 5.16. Calcd. for $C_{30}H_{49}NO_{12}$: C, 58.52; H, 8.02; N, 2.28; OCH_3 (1) 5.04. Found: C, 58.31; H, 7.85; N, 2.23; OCH_3, 5.27.

This substance is optically active $[\alpha]^{25}D$ −16° (*c* 2.5% in chloroform); potentiometric titration in 3:1 dimethylformamide–water shows a neutral equivalent of 620 and pK_b 5.8. The infrared spectrum differs from that of Magnamycin noticeably in the greater intensity of the 5.95 and 6.15 μ bands, but is otherwise extremely similar. Its ultraviolet spectrum in 95% ethanol shows peaks at λ 240 mμ ($E^{1\%}_{1cm}$ 228) and λ 330 mμ ($E^{1\%}_{1cm}$ 0.8).

Acetylation in pyridine–acetic anhydride at 25° yielded a diacetyl derivative melting at 199–200° (dec.) after three recrystallizations from ethanol–water.

Anal. Calcd. for $C_{33}H_{51}NO_{14}$: C, 57.79; H, 7.50; N, 2.04; $COCH_3$, 18.83. Calcd. for $C_{34}H_{53}NO_{14}$: C, 58.35; H, 7.63; N, 2.00; $COCH_3$, 18.45. Found: C, 57.77; H, 7.35; N, 2.00; $COCH_3$, 18.40.

Acknowledgments.—We are indebted to Mr. G. B. Hess for the titration and spectrophotometric data, to Dr. J. Means and Mr. T. Toolan for analyses and to Drs. T. Lees and R. Ottke for paper chromatographic studies. Mr. F. Leghorn developed the microbiological assay method and carried out the assays. We should also like to express our appreciation to Mr. M. Noseworthy, Miss J. Salvo and Mr. J. Catania for their technical assistance.

BROOKLYN 6, N. Y.

[CONTRIBUTION NO. 1793 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-hexahydrophenylalaninamide in Aqueous Solutions at 25° and *p*H 7.9[1]

BY ROBERT R. JENNINGS[2] AND CARL NIEMANN[3]

RECEIVED APRIL 10, 1953

On the basis of the respective K_S and k_3 values of acetyl-L-phenylalaninamide and of acetyl-L-hexahydrophenylalaninamide it has been concluded that the affinity of α-chymotrypsin for the former specific substrate is no greater and is probably less than for the latter. In the course of this study a rational procedure has been developed for the determination of the initial velocities of certain enzyme-catalyzed reactions and the scope and limitations of an alternative method for the determination of the K_S and k_3 values of comparable enzymatic systems in which the initial velocities need not be determined has been examined.

In view of the tendency to associate α-chymotrypsin activity with specific substrates possessing aromatic side chains,[4-9] and to consider that the aromatic character of these side chains is, in some way, important for α-chymotrypsin activity[7-9] it was thought worthwhile to compare the behavior of a specific substrate such as acetyl-L-phenylalaninamide[10] with that of its non-aromatic analog, *i.e.*, acetyl-L-hexahydrophenylalaninamide. A preliminary investigation revealed that the latter compound was hydrolyzed in the presence of α-chymotrypsin and that the α-chymotrypsin-catalyzed hydrolysis of this specific substrate at 25° in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer appeared to be optimal, with respect to the *p*H of the above reaction medium, in the region of *p*H 7.9 ± 0.1, *cf.* Fig. 1. Since this behavior was identical with that observed for the comparable reaction with acetyl-L-phenylalaninamide,[10] the above conditions were selected for a more detailed study of the kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-hexahydrophenylalaninamide.

It was noted previously[10] that with a specific substrate such as acetyl-L-phenylalaninamide operational difficulties are aggravated because of the relatively slow rate of hydrolysis of the specific substrate. Anticipating comparable difficulties with acetyl-L-hexahydrophenylalaninamide, it was decided to consider, in a general way, the use of improved techniques for the evaluation of the kinetic constants rather than to study another pair of specific substrates wherein the behavior of the non-aromatic member of the pair could be complicated by the possible presence of several stereoisomers.

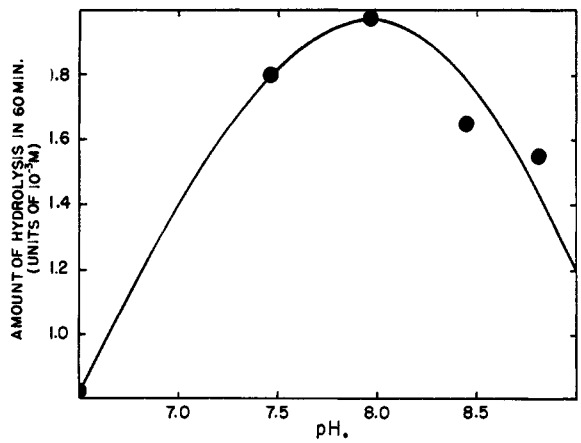

Fig. 1.—*p*H–activity relationship of the system α-chymotrypsin–acetyl-L-hexahydrophenylalaninamide in aqueous solutions at 25° and 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

For the system

$$E_t + S_t \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_t + P_{1t} + P_{2t} \ldots \quad (1)$$

where $K_S = (k_2 + k_3)/k_1$, and when zone A conditions[11-13] are satisfied, the kinetic constants K_S and

(1) Supported in part by a grant from Eli Lilly and Co.
(2) United States Rubber Co. Postgraduate Fellow, 1952–1953.
(3) To whom inquiries regarding this article should be sent.
(4) M. Bergmann and J. S. Fruton, *Advances in Enzymology*, **1**, 63 (1941).
(5) J. S. Fruton in D. E. Green "Currents in Biochemical Research," Interscience Publishers, Inc., New York, N. Y., 1946, p. 123, *et seq.*
(6) J. S. Fruton, *Advances in Protein Chemistry*, **5**, 1 (1949).
(7) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).
(8) E. L. Smith in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. I, Academic Press, Inc., New York, N. Y., 1951, p. 793, *et seq.*
(9) H. Neurath in E. S. Guzman-Barron, "Modern Trends in Physiology and Biochemistry," Academic Press, Inc., New York, N. Y., 1952, p. 453 *et seq.*
(10) H. T. Huang, R. J. Foster and C. Niemann, THIS JOURNAL, **74**, 105 (1952).
(11) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).
(12) A. Goldstein, *ibid.*, **27**, 529 (1944).
(13) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).