A COLORIMETRIC METHOD FOR THE DETERMINATION OF CHYMOTRYPSIN ACTIVITY*

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The L isomers of esters, amides, and hydrazides of the general formula $RCONHCHR_1COR_2$ where R = CH_3—, C_6H_5—, etc., R_1 = $C_6H_5CH_2$—, *p*-HO—$C_6H_4CH_2$—, etc., and R_2 = —OCH_3, —NH_2, or —$NHNH_2$, are known to be hydrolyzed by chymotrypsin (1–6). It has now been found that the corresponding hydroxamides, $RCONHCHR_1CONHOH$, where R_1 = $C_6H_5CH_2$—, are also hydrolyzed by this enzyme (Table I). Aside from the obvious usefulness of the above hydroxamides in the further definition of chymotrypsin activity we wish to point out that these latter substrates provide the basis for a simple, sensitive, and rapid colorimetric method for the determination of chymotrypsin activity which can be extended to a number of other proteolytic enzymes.

It is well known that acylhydroxamides, *i.e.* hydroxamic acids, react with ferric ion, in acidic solutions, to give characteristic deep red colored coordination compounds (7) and it has been found that the above reaction can be used in a quantitative way to determine the rate of disappearance of hydroxamides of the general formula, $RCONHCHR_1CONHOH$, when these latter compounds are being hydrolyzed by a proteolytic enzyme, in this case chymotrypsin. In practice an aliquot portion of the enzymatic reaction mixture is added to a standard acidic aqueous-methanol solution of ferric chloride, the intensity of the color is observed in a photoelectric colorimeter, and the amount of hydroxamic acid present is estimated from a previously determined calibration curve.

In addition to their offering the basis for a colorimetric procedure the hydroxamides of the general formula $RCONHCHR_1CONHOH$ possess other characteristics that make them desirable substrates. For compounds of the type $RCONHCHR_1COR_2$, where R = CH_3— or C_6H_5— and R_1 = $C_6H_5CH_2$—, the rate of hydrolysis, by chymotrypsin, of the corresponding esters, amides, and hydroxamides, *i.e* R_2 = —OCH_3, —NH_2, and —NHOH when R and R_1 are invariant, is in the order ester > hydroxamide >

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amide (4, 8). Thus the hydroxamides are hydrolyzed sufficiently rapidly to permit their use with relatively low enzyme concentrations and to allow the attainment of saturation of the enzyme at relatively low substrate concentrations. These factors, coupled with the greater sensitivity of the colorimetric method, make it possible to study the hydrolytic reaction at initial substrate concentrations of less than 2.5 micromoles per ml. with a greater precision than is practicable with titrimetric methods. A further desirable characteristic of the hydroxamide type of substrate is based upon the fact that these compounds are acids and, even though their acid

TABLE I

*Hydrolysis of Acetyl- and Benzoyl-L-phenylalaninhydroxamides by Chymotrypsin**

Substrate	S_0†	E_0‡	Buffer concentration§	Analytical method	Per cent hydrolysis in		
					20 min.	40 min.	60 min.
Acetyl-DL-	5	0.15	0.04	A	34	53	69
"	5	0.15	0.04	B	35	52	67
"	2.5	0.15	0.02	A	40	69	84
"	2.5	0.15	0.02	B	40	70	82
Benzoyl-DL-	2.5‖	0.15	0.02	A	*Ca.* 80% in 7 min.		
"	2.5	0.03	0.02	"	58	76	80

* At 25° and pH 7.9 (tris(hydroxymethyl)aminomethane-hydrochloric acid buffer).

† Initial concentration of L isomer in micromoles per ml. of reaction mixture.

‡ Initial enzyme concentration in mg. of protein nitrogen per ml. of reaction mixture.

§ Molar concentration of tris(hydroxymethyl)aminomethane in reaction mixture.

‖ As sodium salt.

strength is not great (7), it is possible to use the corresponding salts with those enzymes whose pH optima are greater than 7 in the event that the hydroxamides *per se* are relatively insoluble in water. It should be pointed out that if this latter procedure is adopted, and particularly if an alkali metal salt is used, care must be taken to provide a system of adequate buffering capacity.

The advantages of the above colorimetric method for the determination of proteolytic activity over other colorimetric methods (9, 10) are obvious when it is realized that the former method is based upon the use of specific substrates of known structure, whereas the latter are dependent upon relatively non-specific substrates of unknown structure.

In the present study the DL-hydroxamides have been used as substrates. In view of the fact that it is known that in other cases the D isomer can competitively inhibit the hydrolysis of the L isomer and that this effect is maximal at apparent enzyme saturation (11), it is recommended that in the application of the above colorimetric method the pure L isomer be used as a substrate until sufficient data are available to evaluate the magnitude of the various rate and inhibition constants. Such studies are now in progress.

EXPERIMENTAL[1]

*Acetyl-*DL*-phenylalaninhydroxamide*—To a solution of 12.5 gm. of 2-methyl-4-benzyl-5-oxazolone, b.p. 115–116° at 1 mm. (12) in 15 ml. of anhydrous ether, were added, with vigorous shaking and cooling in an ice-salt bath, 65.5 ml. of a 5 per cent anhydrous methanol solution of hydroxylamine. The reaction mixture was allowed to stand overnight at room temperature, filtered, the filtrate evaporated to dryness, the syrupy residue extracted with four 225 ml. portions of hot ethyl acetate, and the filtered ethyl acetate extract concentrated *in vacuo* to 150 ml. After standing for 20 hours, the last three at 0°, the crystalline precipitate was collected and dried to give 8.17 gm. of acetyl-DL-phenylalaninhydroxamide, m.p. 120–125°, with softening at 110°. After two recrystallizations from ethyl acetate, the product melted at 131–132° with decomposition.

Analysis—$C_{11}H_{14}O_3N_2$ (222). Calculated, C 59.4, H 6.3, N 12.6
Found, " 59.2, " 6.3, " 12.6

*Benzoyl-*DL*-phenylalaninhydroxamide*—To a solution of 14 gm. of benzoyl-DL-phenylalanine methyl ester in 50 ml. of anhydrous methanol were added 24.5 ml. of a 10 per cent methanol solution of hydroxylamine. The solution was cooled to 0°, 49.4 ml. of N methanolic sodium methoxide were added, and the reaction mixture allowed to stand at room temperature for 48 hours. The precipitated solid was dissolved by warming the solution to 40°. The solution was cooled to 0°, acidified to Congo red with 6 N hydrochloric acid, evaporated to dryness *in vacuo*, and the residue dried over solid sodium hydroxide and extracted with a total of 700 ml. of hot ethyl acetate. The extract was cooled, and the crystalline precipitate collected and dried to give 10.8 gm. of benzoyl-DL-phenylalaninhydroxamide, m.p. 157–158° with decomposition. A second crop of 1.2 gm., m.p. 156–157° with decomposition, was obtained from the mother liquor. A portion of the above product was recrystallized from *n*-butanol

[1] All the melting points are corrected.

to give the hydroxamide, m.p. 158–159° with decomposition and with preliminary softening at 152°.

Analysis—$C_{16}H_{16}O_3N_2$ (284). Calculated, C 67.6, H 5.7, N 9.9
Found, " 67.7, " 5.6, " 9.8

Method A—A 1.0 ml. aliquot of the enzymatic digest was added to 1.0 ml. of 36 per cent aqueous formaldehyde, previously adjusted to pH 7.0, and the solution titrated potentiometrically with 0.01 N aqueous sodium hydroxide (13).

Method B—A standard ferric chloride solution was prepared as follows: 0.50 gm. of reagent grade anhydrous ferric chloride was dissolved in 80 ml. of 1.0 N hydrochloric acid and the volume of the solution made up to 100 ml. with absolute methanol. A 1.0 ml. aliquot of the enzymatic digest was added to 5.0 ml. of absolute methanol and 1.0 ml. of standard ferric chloride solution, contained in a calibrated colorimeter tube, and the volume of the solution made up to 10.0 ml. by the addition of water. The contents of the tube were shaken and the intensity of the color determined with a Klett-Summerson photoelectric colorimeter equipped with a green filter. Solutions containing varying amounts of substrate in either 0.02 M or 0.04 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer were used for the preparation of a standard calibration curve. The values observed for the two buffer concentrations were identical and it was found that for concentrations of hydroxamide between the limits of 1 and 10 micromoles per ml. of original solution the colorimeter readings were directly proportional to the concentration of hydroxamide. The presence of enzyme in concentrations up to 0.15 mg. of protein nitrogen per ml. of original solution had no demonstrable effect upon the intensity of the color at any given hydroxamide concentration. However, at high concentrations of hydroxamide it was observed that the intensity of the color slowly faded on standing and because of this phenomenon the time interval between the time of mixing and the time of reading was standardized at 90 seconds. The experimental conditions specified for the color development were selected with regard to their suitability for the attainment of the following objectives; *i.e.*, immediate cessation of enzymatic activity, avoidance of the precipitation of substrate or hydrolysis products, and the development of maximal color intensity.

Enzyme Experiments—The chymotrypsin was an Armour preparation and the data obtained are given in Table I. The conventional controls, *i.e.* substrate alone and enzyme alone, were performed coincidentally with each separate experiment.

SUMMARY

It has been observed that the acetyl- and benzoyl-L-phenylalaninhydroxamides are hydrolyzed by chymotrypsin, and a colorimetric method for the determination of chymotrypsin activity based upon the use of hydroxamides as substrates has been described.

BIBLIOGRAPHY

1. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118**, 405 (1937).
2. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **145**, 253 (1942).
3. Kaufman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.*, **177**, 793 (1949).
4. Snoke, J. E., and Neurath, H., *Arch. Biochem.*, **21**, 351 (1949).
5. Kaufman, S., and Neurath, H., *Arch. Biochem.*, **21**, 437 (1949).
6. MacAllister, R. V., and Niemann, C., *J. Am. Chem. Soc.*, **71**, 3854 (1949).
7. Sidgwick, N. V., The organic chemistry of nitrogen, edited by Taylor, T. W. J., and Baker, W., Oxford (1937).
8. Iselin, B. M., Huang, H. T., MacAllister, R. V., and Niemann, C., *J. Am. Chem. Soc.*, in press.
9. Anson, M. L., *J. Gen. Physiol.*, **22**, 79 (1938).
10. Hawk, P. B., and Bergeim, O., Practical physiological chemistry, Philadelphia, 11th edition, 287 (1937).
11. MacAllister, R. V., Harmon, K. M., and Niemann, C., *J. Biol. Chem.*, **177**, 767 (1949).
12. Bergmann, M., Stern, F., and Witte, C., *Ann. Chem.*, **449**, 277 (1926).
13. Iselin, B. M., and Niemann, C., *J. Biol. Chem.*, **182**, 821 (1950).