compound was prepared from isonicotinic acid. The product distilled at 94°/0.15 mm. Hg, uncorrected (lit. (9) b.p. 123 to 123.5°/3 mm. Hg, corrected; lit. (10) b.p. 109°/0.5 mm. Hg, uncorrected).

**3-(Pyrrolidinoformyl)pyridine** (II).—This compound was prepared from nicotinic acid. The product distilled at 114°/0.04 mm. Hg, uncorrected (lit. (10) b.p. 131–133°/0.3 mm. Hg, uncorrected).

The compounds listed in Table II were prepared by the following procedures.

*Procedure A:* **1-Ethyl-3-(N,N-dimethylcarboxamido)piperidine Hydrobromide** (III).—N,N-Dimethylnicotinamide (25 Gm., 0.167 mole) (IV) and ethyl bromide (48.3 Gm., 0.443 mole) were dissolved in 200–300 ml. anhydrous benzene and refluxed for 48–60 hours. The precipitate formed in the reaction was separated and was dissolved in 150–200 ml. distilled water. The aqueous solution was washed with benzene, treated with charcoal, and filtered. The filtrate was subjected to hydrogenation (aqueous or hydroalcoholic solution) in the presence of 1.0 Gm. platinum oxide (Adams' catalyst) at maximum pressures of 45–50 p.s.i. After absorption of hydrogen ceased, the platinum oxide was filtered off and the solvent was removed by azeotropic distillation under reduced pressure with absolute ethanol and anhydrous benzene. The product was purified by recrystallization.

*Procedure B:* **1,2-Bis[3-(N,N-dimethylcarboxamido)piperidino]ethane Dihydrobromide** (VI).—N,N-Dimethylnicotinamide (52.1 Gm., 0.347 mole) (IV) and 1,2-dibromoethane (32.6 Gm., 0.174 mole) were dissolved in 200 ml. anhydrous benzene and refluxed for 53–62 hours. The precipitate formed during the reaction was then treated as described under *Procedure A*.

*Procedure C:* **1-Ethyl-3-(N,N-diethylcarboxamido)-1,2,5,6-tetrahydropyridine Hydrochloride** (XVIII).—1 - Ethyl - 3 - (N,N - diethylcarboxamido)pyridinium bromide (XVII) (2) was prepared by refluxing N,N-diethylnicotinamide (40 Gm., 0.224 mole) (XVI) and ethyl bromide (61 Gm., 0.560 mole) in 250 ml. anhydrous benzene for 48 hours. Excess alkyl halide and solvent were removed by decantation. The residual oil was dissolved in 150 ml. water, the aqueous solution was washed with benzene, and the water was removed under reduced pressure. Last traces of moisture were removed by azeotropic distillation with benzene. The oily product (60 Gm., 0.209 mole) was dissolved in 400 ml. dry methanol and cooled in an ice bath. Sodium borohydride (31.7 Gm., 0.838 mole) was added with stirring to the cold solution over 30 minutes; subsequently the solution was stirred an additional 30 minutes. Methanol was removed by distillation under reduced pressure to give a spongy yellow residue which was dissolved in 230 ml. water and saturated with potassium carbonate. The mixture was extracted with ether, the combined ether extracts were dried over anhydrous potassium carbonate, filtered, and the ether was removed by distillation. The oily residue was purified by conversion to the hydrochloride (anhydrous HCl in anhydrous ether) with or without prior fractionation under reduced pressure. Residual traces of moisture were removed from the oily hydrochloride by azeotropic distillation with benzene. The salt was then purified by recrystallization from ethanol-ethyl acetate.

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# Sensitive and Reproducible Assay Method for Chymotrypsin

By B. L. KABACOFF, M. UMHEY, A. WOHLMAN, and S. AVAKIAN

**A new assay method for chymotrypsin was developed. This method, based on N-acetyl-L-tyrosine ethyl ester (ATEE), was found to be ten times as sensitive as the proposed N.F. assay method for chymotrypsin. In the new method described, the residual substrate is measured by a single colorimetric determination. The method is convenient and highly reproducible.**

THE CLASSICAL assay methods for proteolytic activity such as hemoglobin digestion, casein digestion, and milk clotting, although convenient and simple, lack specificity. N-acetyl-L-tyrosine ethyl ester (ATEE) is a specific substrate for chymotrypsin. The proposed N.F. method for the analysis of chymotrypsin, based on the work of Schwert and Takenaka (1), utilizes ATEE as the enzyme substrate. The rate of hydrolysis of this substrate in pH 7.0 buffer is followed spectrophotometrically at 237 mμ. Readings are taken every 30 seconds for a period of 4 minutes. The temperature must be kept within ±0.1° of 25.0°.

The above method has certain drawbacks which limit its practical application. Elaborate equipment is required to maintain the cell compartment of the spectrophotometer at the exact temperature speci-

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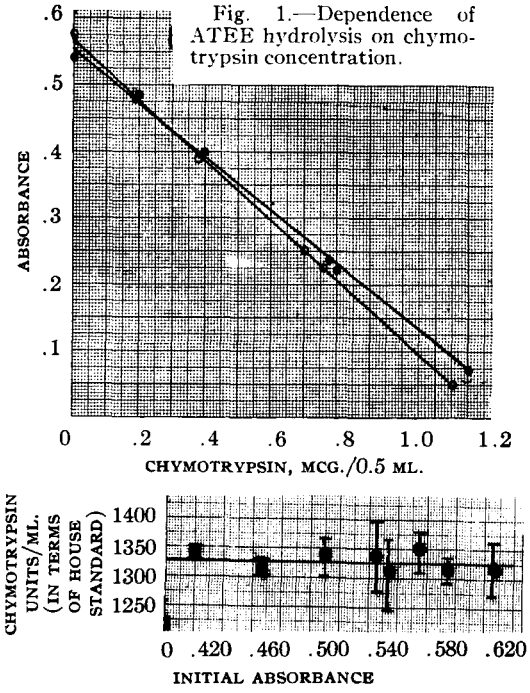


Fig. 1.—Dependence of ATEE hydrolysis on chymotrypsin concentration.

Fig. 2.—Effect of variation of initial ATEE concentration on the assay.

fied. The manipulations involved make readings at intervals of exactly 30 seconds difficult. Variable results may often be obtained when ATEE from different sources is used. Even when the variables are carefully controlled, results are often erratic. The present study was undertaken to devise a new assay method for chymotrypsin based on ATEE and to evaluate its reproducibility and convenience. In this method, the residual ATEE after incubation with the enzyme is measured by reacting it with alkaline hydroxylamine, thus forming the hydroxamic acid (2). The hydroxamic acid is then acidified and reacted with ferric ion to produce a color which is read on a spectrophotometer.

## EXPERIMENTAL

The chymotrypsin used in the study was obtained from Princeton Laboratories, Princeton, N. J., and the Worthington Biochemical Corporation, Freehold, N. J. The enzymes from both sources were crystallized. The ATEE was obtained from Mann Research Laboratories, New York, N. Y., Calbiochem, Los Angeles, Calif., and K & K Laboratories, Jamaica, N. Y. The chymotrypsin used as the standard had an assay of 1290 proposed N.F. units/mg. All of the samples utilized in this study were assayed against this standard.

**Reagents.**—All solutions were prepared with distilled water and reagent grade materials. Hydroxylamine hydrochloride, 139 Gm./L., and sodium hydroxide, 140 Gm./L., were used. Alkaline hydroxylamine solution is prepared by mixing equal volumes of the above solutions just prior to use. Hydrochloric acid (0.001 *N* and 4.0 *N*), phosphate buffer of 1.09 Gm. anhydrous potassium dihydrogen phosphate, and 2.27 Gm. anhydrous disodium hydrogen phosphate diluted to 100.0 ml. (the pH of this solution should be 7.0 ±0.1) were employed. The substrate solution was 115 mg. of N-acetyl-L-tyrosine ethyl ester in 50.0 ml. of phosphate buffer. Shake well to dissolve and filter through Whatman No. 42 filter paper (prepare fresh daily). Ferric chloride (hexahydrate), 189 Gm./L.

Table I.—Comparison of ATEE from Different Sources

| | Chymotrypsin Activity, Proposed N.F. Units/mg. | | |
|---|---|---|---|
| | Mann | Calbiochem | K & K |
| CTD-5 | 1250 ± 41 (4)[a] | 1290 ± 69 (4)[a] | 1230 ± 43 (2)[a] |
| CTD-7 | 1340 ± 13 (4)[a] | 1340 ± 26 (4)[a] | 1330 ± 20 (2)[a] |

[a] Number of determinations.

**Method.**—A solution of chymotrypsin sample in 0.001 *N* hydrochloric acid is prepared so that it contains 1.5 to 2.0 proposed N.F. units (1.2 to 1.6 mcg.) of chymotrypsin per ml. A solution using chymotrypsin of known potency (standard) is also prepared.

One-milliliter aliquots of the substrate solution are placed into each of three test tubes immersed in a water bath maintained at 25 ±0.5°. To one tube, 0.5 ml. of the sample solution is added. To the second tube, 0.5 ml. of the standard is added. To the third tube, 0.5 ml. of 0.001 *N* HCl is added. The exact time of each addition is noted. It is advisable to stagger the additions 1 minute apart.

Exactly 50 minutes after the addition of enzyme solution or 0.001 *N* HCl to the test tubes, the reaction is stopped by the addition of 2.0 ml. of alkaline hydroxylamine solution. Two minutes later, 1.0 ml. of 4 *N* hydrochloric acid is added to each tube.

The color is then developed by the addition of 1.0 ml. of ferric chloride solution to each tube. The absorbances of the solutions (unknown, standard, 0.001 *N* HCl) are read against distilled water at 540 mμ in a 1-cm. cell. These readings should be made within 20 minutes after the ferric chloride addition. The absorbances of the unknown and standard solutions are each subtracted from that of the 0.001 *N* hydrochloric acid. These differences in absorbance are proportional to the activities of the enzyme solutions. Samples should be run in duplicate or triplicate.

After the addition of each reagent, the tubes should be agitated vigorously. If this precaution is not taken, the absorbance may be erroneously high. The quality of the substrate may be checked prior to the assay by adding all the reagents as stated above using 0.001 *N* hydrochloric acid instead of

Table II.—Variation Within a Single Assay

| Sample | Proposed N.F. Units/mg. | Mean | S. E. | Coefficient of Variation % |
|---|---|---|---|---|
| CTD-5 | 1320 | | | |
| | 1260 | 1270 | ±23 | 1.8 |
| | 1230 | | | |
| CTD-7 | 1370 | | | |
| | 1320 | 1330 | ±10 | 0.8 |
| | 1310 | | | |
| 6017C | 1280 | | | |
| | 1270 | 1260 | ±32 | 2.5 |
| | 1220 | | | |
| 6030C | 1240 | | | |
| | 1240 | 1210 | ±52 | 4.2 |
| | 1150 | | | |

TABLE III.—VARIATION AMONG SEVERAL ASSAYS

| | Chymotrypsin Activity, Proposed N.F. Units/mg. | | | |
|---|---|---|---|---|
| | CTD-5 | CTD-7 | 6017C | 6030C |
| 9-21-62 | 1270 | 1330 | 1340 | 1280 |
| 9-24-62 | 1250 | 1300 | 1250 | 1110 |
| 9-25-62 | 1220 | 1340 | 1280 | 1180 |
| 10- 1-62 | 1290 | 1350 | 1240 | 1190 |
| 10- 9-62 | 1280 | 1340 | 1200 | 1210 |
| 10-13-62 | 1210 | 1320 | 1260 | 1210 |
| Mean | 1250 | 1330 | 1260 | 1200 |
| Av. Deviation from Mean | 27 (2.2%) | 13 (1.0%) | 22 (1.7%) | 37 (3.1%) |

chymotrypsin solution. Do not incubate but read the absorbance immediately. The absorbance should be between 0.400 and 0.650.

## RESULTS

**Dependence of ATEE Hydrolysis on Chymotrypsin Concentrations.**—Figure 1 gives the absorbance obtained after incubation of varying concentrations of chymotrypsin with substrate solution in two typical experiments. Each point on the curves represents duplicate determinations. It can be seen that the relationship is linear up to 1.2 mcg./0.5 ml. Since the absorbance, which is a measure of ATEE present, fell from approximately 0.55 to 0.06, we may say that about 90% of the substrate was hydrolyzed by this concentration of enzyme.

**Effect of Variation of Initial ATEE Concentration on the Assay.**—Eight solutions of ATEE (from three different suppliers) were made up at various times and were used in assaying lot CTD-7 of chymotrypsin. Although the same amount of ATEE was weighed out, the resulting solution contained variable concentrations of intact ester as evidenced by differences in the absorbances obtained after ferric chloride addition. Because of the slow solubility of ATEE, varying amounts of the ester remained undissolved and were filtered out. Prolonged attempts to effect complete solution of ATEE may result in a variable degree of hydrolysis of the ester.

It can be seen from Fig. 2 that a wide variation of initial ATEE concentration produces no appreciable effect on the assay.

**Effect of ATEE from Different Sources on the Assay.**—Substrates from three sources were compared using two lots of chymotrypsin (Table I). The greatest variation obtained was that between the Mann and K & K product using CTD-5. This difference was not statistically significant ($P>0.2$).

**Variation Within a Single Assay.**—Four lots of chymotrypsin were assayed in triplicate. In each case, three aliquots from a single dilution were incubated against the same substrate solution. The results given in Table II are typical. It can be seen that the reproducibility is quite satisfactory.

**Variation Among Several Assays.**—Four lots of chymotrypsin were assayed in duplicate or triplicate at various times over a period of a few weeks. The results are given in Table III. The average error (deviation from the mean) ranges from 1.0 to 3.1%.

## DISCUSSION

In the proposed N.F. assay method for chymotrypsin, the ratio of substrate to enzyme is relatively small ($2.5 \times 10^4$ on a molar basis). The resulting reaction is frequently not zero order with respect to substrate. As a result, the fall in absorbance is often not linear. In the present study, the ratio of substrate to enzyme is ten times that found with the N.F. method. Therefore, the variable hydrolysis which occurs during the preparation of ATEE solutions is negligible. As was shown in Fig. 2, the initial concentration of ATEE may vary over a wide range without affecting the accuracy of the results. In addition, we found that during the enzymatic reaction, linearity exists even when 90% of the ATEE has been hydrolyzed.

The separate 4-minute incubation required for each sample analyzed by the proposed N.F. method, requires special equipment to preclude temperature variations greater than $\pm 0.1°$. It was found that since with our method, the standard and sample are incubated together for 50 minutes, an ordinary water bath (which may fluctuate within a range of $\pm 0.5°$) was adequate.

In the method under consideration, the absorbance of each sample must be read only once, within 20 minutes after the color development. This is in direct contrast to the repeated 30-second interval readings required with the proposed N.F. method. Additionally, any instability in the spectrophotometer, which is insufficient to interfere with the usual type of assay, will result in a significant error in results obtained by the N.F. method.

The sensitivity of the hydroxamate method as described here is approximately ten times that of the proposed N.F. method. Therefore, any possible interference resulting from other ingredients in chymotrypsin formulations would be greatly minimized by the dilution. At the concentrations utilized in this study, it was found that a 50-minute incubation period provided accurate and reproducible results. When working with lower concentrations, a longer period of incubation may be desirable. Conversely, with larger concentrations, an incubation period of less than 50 minutes may prove satisfactory.

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