The Utilization of a Specific Chromogenic Inactivator in an "All or None" Assay for Chymotrypsin*

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The synthesis and properties of a new specific chromogenic inactivator of chymotrypsin are described. The reagent, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate, reacts stoichiometrically with chymotrypsin to yield inactive diphenylcarbamyl chymotrypsin and an equimolar quantity of 3-nitro-4-hydroxybenzoic acid. Since the latter can be estimated colorimetrically, the operational normality of a chymotrypsin solution can be determined directly. The method is not affected by inert protein impurities or by the presence of trypsin and has a precision of approximately ±2%. In the course of the experiments it was shown that diphenylcarbamyl chloride can be used to inactivate impurities of chymotrypsin present in crystalline trypsin preparations.

The purity of an enzyme preparation and hence the operational normality of an enzyme solution cannot be determined by rate studies. Kinetic studies can only relate the activity of a preparation to a standard enzyme preparation of high but unknown purity. The instability of even crystalline enzymes when stored over an extended period of time makes it imperative to know the absolute purity of an enzyme preparation *when used* especially when experiments from which kinetic constants are calculated are being carried out. This fact was brought forcefully to our attention during studies on the inactivation of chymotrypsin (Erlanger and Cohen, 1963), when, using the assay procedure described in this communication, a batch of commercial 3 ×-crystallized α-chymotrypsin was found to be only 70% active enzyme after standing several months in the refrigerator.

The advantages and the requirements of a practical "all or none" assay of α-chymotrypsin were discussed at length by Schonbaum *et al.*, (1961). Their paper described a spectrophotometric assay that exploited the ability of *N-trans*-cinnamoyl imidazole to react specifically with α-chymotrypsin at *p*H 5 to form a relatively stable inactive acylchymotrypsin derivative, the concentration of which could be estimated spectrophotometrically.

This paper describes the utilization of a specific chromogenic inactivator as a reagent for the titration of chymotrypsin. The reagent, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC),[1] is a direct consequence of experiments in which diphenylcarbamyl chloride was shown to be a specific inactivator of chymotrypsin (Erlanger and Cohen, 1963; Erlanger *et al.*, 1963). NCDC hydrolyzes only to a negligible extent at *p*H 7.6 in the absence of enzyme but reacts with chymotrypsin to yield stable inactive diphenylcarbamyl chymotrypsin (Erlanger and Cohen, 1963) and an equimolar quantity of 3-nitro-4-hydroxybenzoic acid (NHB) which, since it is yellow, can be estimated colorimetrically.

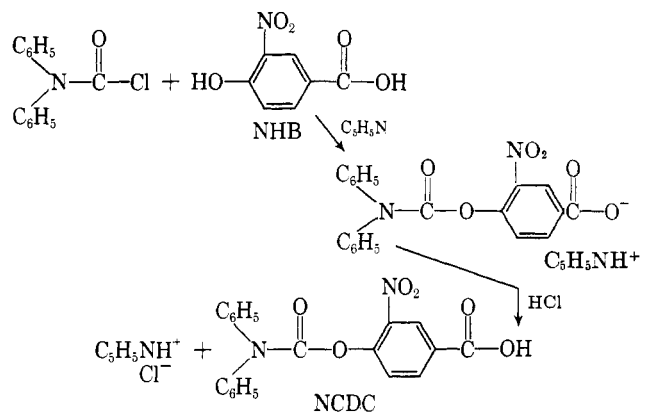
α-chymotrypsin + NCDC ⟶ diphenylcarbamyl-α-chymotrypsin + NHB

Thus the number of moles of NHB released is equivalent to the number of moles of active chymotrypsin. The reaction is not affected by inactive protein or by the presence of trypsin as an impurity.

This paper describes the synthetic methods in detail as well as the assay conditions. In addition, the utility and specificity of the procedure and its merits relative to the method of Schonbaum *et al.* (1961) are discussed.

EXPERIMENTAL

NCDC was prepared by the reaction of diphenylcarbamyl chloride with NHB in pyridine. The first product, isolated in good yield, was the pyridinium salt of NCDC which was easily converted to the acid by dilute hydrochloric acid as shown below:



Preparation of 2-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate.—NHB (Griess, 1887), 2.2 g (12 mmoles), was dissolved in 32 ml of dry pyridine. Stirring was begun and 2.8 g (12 mmoles) of diphenylcarbamyl chloride (Distillation Products, Inc.) was added. The result was a clear solution from which, after about 5 minutes, a crystalline precipitate began to form. After 4 hours of stirring the mixture was cooled in an ice bath and the precipitate was recovered by filtration and washed with petroleum ether; yield 4.05 g. It was recrystallized from methanol; yield 3.15 g; mp 187° (uncorr).

Analysis showed this material to be the pyridinium salt of 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate.

Anal. Calcd for $C_{25}H_{19}N_3O_6$ (457.3): C, 65.67; H, 4.16; N, 9.19. Found: C, 66.02; H, 4.31; N, 8.87.

The above product was suspended in 25 ml of ethanol, warmed into solution, and then brought to *p*H 1–2 (*p*H paper) using 4 N HCl (*ca.* 1.5 ml). Addition of

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[1] Abbreviations used in this work: NCDC, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate; NHB, 3-nitro-4-hydroxybenzoic acid.

30 ml of water yielded a white precipitate of NCDC; yield 2.8 g; mp 204–205 (uncorr). Recrystallization from methanol-water did not change the melting point.

Anal. Calcd for $C_{20}H_{14}N_2O_6$ (378.3); C, 63.48; H, 3.72; N, 7.40. Found: C, 63.27; H, 3.79; N, 7.29.

Spectral Data.—Shown in Figure 1 are the absorption spectra of NHB and NCDC each at 10^{-4} M in 0.05 M Tris-chloride buffer, 0.03 M $CaCl_2$, *p*H 7.6. NHB has a maximum at 410 mμ with an extinction coefficient of 3910. The absorption of NCDC at this wavelength is negligible.

Titration Procedure.—Substrate Solution.—Finely powdered NCDC (26.5 mg) was suspended in 0.2 ml of 0.4 M Tris in a 12-ml conical centrifuge tube. Gentle warming in a water bath resulted in a clear solution to which 1.8 ml of 0.05 M Tris-chloride buffer, *p*H 7.6 was added. This is the substrate stock solution.

Enzyme Solution.—α-Chymotrypsin (*ca.* 36 mg, see Discussion) was dissolved in 1.0 ml of 0.001 N HCl. To a test tube was added 1.5 ml of 0.05 M Tris-chloride buffer, *p*H 7.6, containing 0.03 M $CaCl_2$. The test tube was placed in a thermostatically controlled bath kept at 37° and 0.2 ml of the chymotrypsin solution was added. The following blank was also set up: 0.2 ml 0.001 M HCl and 1.5 ml 0.05 M Tris-chloride buffer. After the tubes had equilibrated, 0.1-ml aliquots of the substrate solution were added to each tube. The reaction was allowed to proceed for 45 minutes with intermittent swirling of the tubes. A slight precipitate was present during the first 15 minutes of the experiment. This was presumably the calcium salt of NCDC. After 20 minutes, at the most, however, the solution became clear (see Discussion). After 45 minutes, 1.2 ml of 0.05 M Tris-chloride buffer, *p*H 7.6, was added to each tube. The solutions were transferred to cuvets and the absorbance at 410 mμ was determined using a Beckman DU spectrophotometer. Other instruments are just as suitable if they are calibrated with appropriate solutions of NHB. It is important that in each laboratory the extinction coefficient of NHB at 410 mμ be determined in the spectrophotometer or colorimeter to be used.

Time Course of the Reaction.—Figure 2 shows the progress of the reaction between chymotrypsin and NCDC at the above concentrations. The assay procedure was carried out in a Beckman DU spectrophotometer with the temperature controlled at 37°. The absorbance values are higher by a factor of 1.67 than the values obtained using the assay procedure described previously, since the final addition of 1.2 ml 0.05 M Tris buffer, *p*H 7.6, was not made. The curve illustrates, however, that a plateau is reached in about 22–25 minutes if sufficient active enzyme is present to give a final optical density at 410 mμ of about 0.300 (when final volume is 3.0 ml).

Results of Representative Runs.—The results reported here are calculated as per cent purity but, if desired, can be calculated as normality. The purity of an enzyme preparation was calculated as follows:

(1) A molecular weight of 25,000 was assumed (Wilcox *et al.*, 1957). If the operational normality of a chymotrypsin solution is to be calculated, it is, of course, not necessary to assume a molecular weight. The normality is simply equivalent to the normality of the NHB formed by the reaction.

(2) The moisture content of the enzyme preparation was not considered. Therefore per cent purity, as calculated, represents the grams of active chymotrypsin (mw 25,000) present in 100 g of a preparation as received.

(3) The assay of 7.2 mg of 100% pure chymotrypsin would yield a final concentration of NHB of 9.59×10^{-4} M. Since the extinction coefficient of NHB is 3910 an absorbance of 0.375 would be obtained. Therefore per cent purity = observed absorbance × 100/0.375.

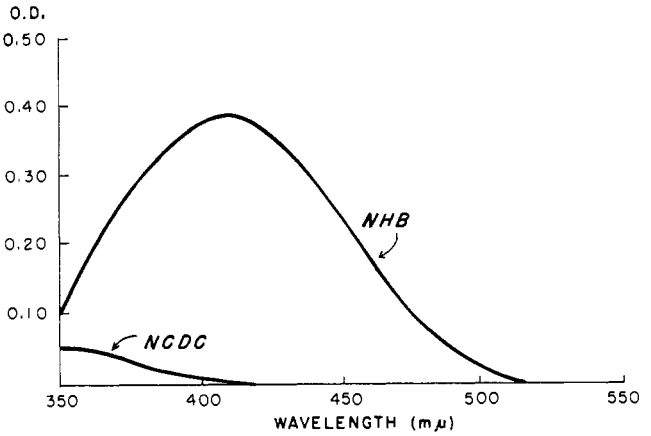

FIG. 1.—Absorption spectra of NCDC and NHB, both at 10^{-4} M in 0.05 M Tris buffer, 0.03 M $CaCl_2$, *p*H 7.6

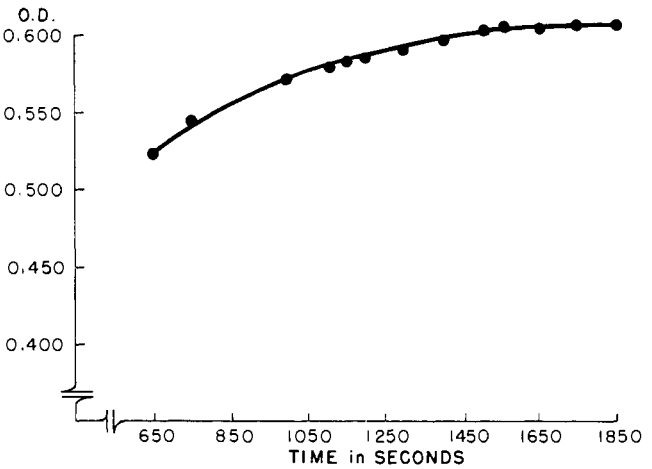

FIG. 2.—Time course of the reaction between NCDC and α-chymotrypsin. Details in text.

The results of a number of runs using commercial 3×-crystallized α-chymotrypsin showed that a precision of ±2% was attainable. Two samples, tested immediately upon receipt, assayed as 89.7 and 90.4% pure, respectively. A preparation which had been stored in the refrigerator for 2 years and used intermittently was found to be only 66.0% active enzyme. Preparations stored in the refrigerator for several months were found to be between 80 and 84% active.

NCDC and Trypsin.—Curve A of Figure 3 represents the results of several assay runs using trypsin (Worthington 2× crystallized, lyophilized) as the enzyme. Various incubation times were studied. It should be noted that hydrolysis is occurring but that there is an initial rapid reaction followed by a slower one. That the rapid reaction is due to the presence of a small amount of chymotrypsin in the trypsin preparation is shown by the following experiment:

Trypsin (58.1 mg) was dissolved in 1.6 ml of 0.05 M Tris-chloride buffer, *p*H 7.6. To this solution was added 0.2 ml of a stock solution containing 5.2 mg diphenylcarbamyl chloride in 25 ml of isopropanol. The slightly turbid solution was incubated at 25° for 10 minutes and then centrifuged clear. This process represented a reaction between a 1.4×10^{-3} M solution of trypsin and approximately one-tenth the quantity

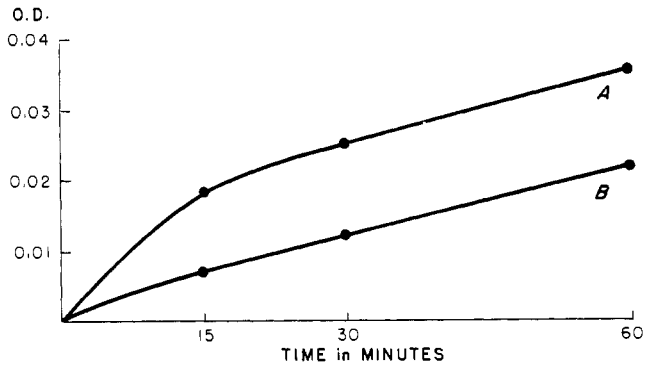

FIG. 3.—Time course of the reaction between NCDC and trypsin. Curve *A*, untreated crystalline trypsin preparation: 1.5 ml 0.05 M Tris-chloride buffer containing 0.03 M $CaCl_2$, 0.2 ml of a trypsin solution containing 36.0 mg/ml 0.001 M HCl, and 0.1 ml of NCDC solution containing 13.1 mg/ml prepared as described in the experimental section. At the designated times 1.2 ml of 0.05 M Tris buffer (no Ca^{2+}) was added and the absorbance was determined. All buffers were *p*H 7.6. Curve *B*, crystalline trypsin treated with diphenylcarbamyl chloride as described in text.

of diphenylcarbamyl chloride and, according to the previously reported kinetic studies (Erlanger and Cohen, 1963), would result in the complete inactivation of any chymotrypsin present in quantities below approximately 5%. The supernatant solution was then clarified by centrifugation and assayed as before (see legend to Fig. 3). The results are shown in curve B of Figure 3. The initial fast reaction is no longer occurring and from the difference in absorbance between curves A and B after 60 minutes we can calculate that our trypsin preparation contained about 2% chymotrypsin. We can conclude also that NCDC can react with trypsin but at a rate which is about two to three orders slower than its reaction with chymotrypsin. Exact values cannot be ascertained because the early stages of the reaction with chymotrypsin are obscured by turbidity.

This experiment, incidentally, demonstrates that diphenylcarbamyl chloride can be used to inactivate the chymotrypsin always present to some extent in commercial preparations of crystalline trypsin.

DISCUSSION

The experimental data show that NCDC can be used in a rather specific "all or none" titration of chymotrypsin. This type of assay procedure has a number of very important advantages over the usual kinetic assay methods, namely: (a) Reproducibility of results in different laboratories does not require stringent attention to details such as concentration, temperature, etc. (b) Assuming the existence of one active site (Balls and Jansen, 1952) one can estimate directly the actual operational normality of a chymotrypsin solution and, as well, the absolute purity of a chymotrypsin preparation. (c) Modification of the enzyme, either chemically or physicochemically, although it can modify the reaction rates, will not affect an "all or none" assay.

The choice of NCDC as a reagent was influenced by its solubility in water at *p*H 6 and above and by the fact that it reacts with chymotrypsin at a reasonable rate without any turnover to yield a colored byproduct. As mentioned in a previous paper (Erlanger and Cohen, 1963), *p*-nitrophenyl-*N*,*N*-diphenylcarbamate (Immelman, 1949) also reacts with chymotrypsin. However, its low solubility in aqueous solution prevented its use as a reagent.

The high specificity of NCDC for chymotrypsin makes it possible to assay mixtures of chymotrypsin and trypsin, a concentration of the latter of as much as 50% introducing no more than a 1–2% error. Certain precautions must be observed, however, to assure accurate results: (a) Enough active chymotrypsin should be present to produce an optical density of at least 0.275. Too low a concentration of enzyme may slow the reaction rate to the extent that the reaction is not sufficiently complete within 45 minutes. If, after an initial experiment, the optical density is found to be below 0.275, the quantity of the enzyme preparation should be increased accordingly. (b) The presence of $CaCl_2$ at the concentration stated in the experimental section is of crucial importance. Higher concentrations yield turbid solutions at the end of the run due to the presence of the insoluble Ca^{2+} salt of NCDC. In the absence of calcium the assay yields low results. This is apparently due to the presence of at least two species of chymotrypsin in preparations of 3×-crystallized α-chymotrypsin, including a fraction requiring the presence of Ca^{2+} for stability at 37°. This finding is discussed in detail in another communication.

The question arises as to the relative merits of this assay procedure and the one reported earlier by Schonbaum *et al.* (1961). Similar quantities of enzyme are required in both assays (*ca.* 5–10 mg). Both are reproducible to within ±1.5–2%. Neither requires precise control of temperature or reactant concentration. The NCDC assay method, however, has the following advantages: (a) No organic solvent is required to keep the reactants in solution. (b) Unlike the procedure of Schonbaum *et al.* (1961), which relies upon the measurement of difference spectra, the NCDC assay produces a visible color *de novo*. No errors due to high blanks are possible nor is it necessary to correct for absorption by other reaction products. (c) Careful control of *p*H is not necessary since no measurable turnover occurs over a wide range. The procedure of Schonbaum *et al.* (1961) is carried out at *p*H 5.05. At this *p*H *trans*-cinnamoyl chymotrypsin is stable but *trans*-cinnamoyl imidazole (*pK* 3.65) is not completely in the basic form. If the *p*H is raised to 5.6 to eliminate this problem, deacylation of the acyl enzyme becomes a significant factor. (d) The NCDC assay procedure can be operated with less sophisticated equipment since readings are made at 410 mμ against a negligible blank rather than at 310 mμ or 335 mμ against a rather high blank. Schonbaum *et al.* (1961) used a recording spectrophotometer.

The NCDC procedure has two disadvantages. First of all, 45 minutes at 37° are required for the assay. The possibility of enzyme denaturation therefore arises. We have found, however, that at 0.03 M Ca^{2+} concentration, denaturation of α-chymotrypsin is negligible. This will be discussed in more detail in a future publication. A second disadvantage is the presence of turbidity during the early stages of the reaction. If sufficient enzyme is present, however, this turbidity disappears by the time the optical density reaches a value of 0.250.

In conclusion, the direct titration of chymotrypsin by NCDC is simple, practical, and reproducible. It is being utilized in this laboratory in experiments which may lead to a clearer understanding of the mechanism of the catalytic activity of the enzyme.

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A Chromatographic Study of Phosphoglucomutase: Separation of Phospho- and Dephospho-Enzyme Forms*

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A chromatographic method has been developed which permits the isolation of phosphorylated and dephosphorylated enzymatically active forms of phosphoglucomutase. The enzyme, obtained from rabbit muscle, is chromatographed at *p*H 7 on carboxymethyl-cellulose, eluting with a linear gradient of sodium phosphate buffer. Inactive protein, if present, passes through the column and is separated from the active enzyme forms which are subsequently eluted. Chromatographed enzyme can be stored at approximately 0° as a slurry in *p*H 5–buffered ammonium sulfate; or, alternatively, in liquid nitrogen as frozen pellets of neutral buffered solutions. That a homogeneous enzyme of high purity can be obtained by such chromatography was evidenced by the constant specific enzymatic activity exhibited by the fractions belonging to a single peak, by the enzyme's subsequent elution as a single peak upon repeated chromatography, and by its homogeneity in equilibrium ultracentrifugation. The phosphorylated and dephosphorylated nature of the separated phosphoglucomutase components was identified by ^{32}P-substrate labeling experiments. Amino acid analyses were performed, and the composition of the chromatographed phosphoenzyme was compared with that published for phosphoglucomutase in earlier reports.

Recent studies concerning the relationship of structural characteristics of phosphoglucomutase to its activity (Koshland *et al.*, 1962), in some cases involving rather small but measurable changes, emphasized the desirability of finding a reproducible method for obtaining enzyme samples of the highest purity. Moreover, according to the mechanism of action proposed by Najjar and Pullman (1954), this muscle enzyme must exist in two catalytically active forms, one a phosphoprotein and the other a nonphosphorylated species. A separation of these two forms would be important not only for the study of this enzyme, but also as an indication that other enzymes existing in two catalytically active forms might be separable chromatographically.

Earlier work had indicated that use of CM-cellulose[1] offered a suitable means of chromatographing the enzyme (Ray and Koshland, 1962). The present communication describes the development of a method to achieve this separation and some of the properties of rabbit muscle phosphoglucomutase.

EXPERIMENTAL

Enzyme Preparation.—Enzyme used in this study was prepared from rabbit skeletal muscle according to the method of Najjar (1955), with the following modifications. In many instances when storage was desirable, freshly ground muscle in individual polyethylene bags was frozen at −25°. The frozen muscle was allowed to defrost overnight at 4° and was then extracted for 40 minutes with 5 volumes of cold deionized water. The extract was adjusted to *p*H 5.0 with 1.0 M acetic acid, heated to 65°, and cooled to 4° by immersing the flask in an ethylene glycol–water bath at −10°. The mixture was adjusted to 25% saturation by addition of solid ammonium sulfate prior to centrifuging the heat-coagulated protein, a modification similar to that recently suggested by Najjar (1962). The supernatant was then adjusted to 65% saturation, and after standing overnight at 4° the precipitated enzyme was collected by centrifugation. The protein was then dissolved in an equal volume of sodium acetate, heated to 63° for 3 minutes, and centrifuged, and the supernatant was adjusted to a protein concentration of 5 mg/ml. The solution was brought to 50% saturation with ammonium sulfate and the sedimented protein was removed by centrifuging. After dropwise addition of saturated ammonium sulfate to 60% saturation over a period of 2–3 hours, the solution was allowed to settle overnight in the cold prior to centrifuging the crystallized protein.

The crystallized enzyme was stored at 0–4° as a suspended slurry in 65% saturated ammonium sulfate, 0.15 M in sodium acetate buffer, *p*H 5.0.

Enzyme Assay.—The assay method employed was essentially that described by Najjar (1955), except that the incubation mixture contained histidine (final concentration 0.04 M) and Tris (0.013 M) buffer rather than cysteine. No preactivation of the enzyme samples was employed. The unit of activity was that defined by Najjar, viz., that amount of phosphoglucomutase which catalyzes the formation of 1 mg of acid-labile P in 5 minutes' incubation at 30°.

* A preliminary account of some of these findings has been presented (Horton *et al.*, 1963). Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission. Supported in part by a grant from the National Science Foundation.

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[1] Abbreviations used in this work: CM-, carboxymethyl-; Tris, tris(hydroxymethyl)aminomethane.