of $-1040°$ and therefore corresponds to a 5.1% error. At longer wavelengths the error is reduced. At 350 mμ the errors arising from the 192-, 208-, and 224-mμ Cotton effects are +3.4 (0.2%), −2.0 (0.3%), and −5.1° (0.5%), respectively. The actual rotation at 350 mμ is −184°, so that the error has fallen to 2% at this wavelength. At wavelengths longer than 350 mμ, the error varies slowly reaching a minimum of 1.3% near 500 mμ and increasing to 1.7% by 600 mμ. Thus a multiterm Drude equation (as proposed by Yang[31]) will not yield true rotational strengths. However, the general derivation of a two-term dispersion equation to approximate the visible and near-ultraviolet ORD introduces additional error terms. A particular approximation such as the MTTDE can usually be chosen so that the additional errors introduced minimize the total error and thus the approximation can be used over the range 600–280 mμ despite the inadequacies of the multiterm Drude approximation.

Studies of the Chymotrypsinogen Family. V. The Effect of Small-Molecule Contaminants on the Kinetic Behavior of α-Chymotrypsin[1]

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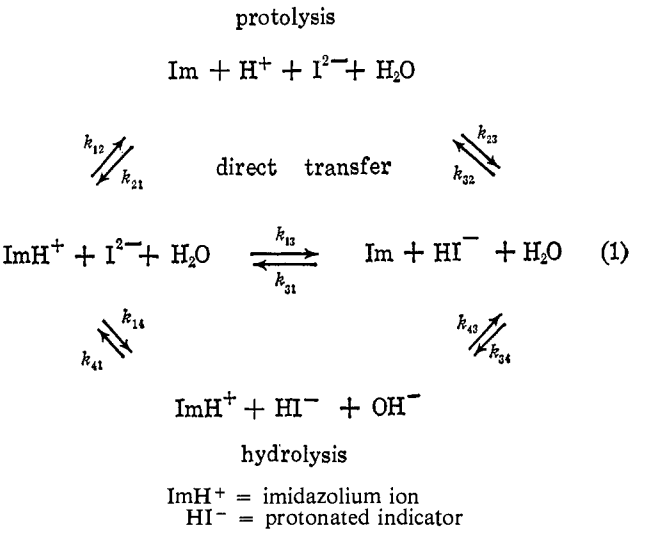
Abstract: All commercial preparations of α-chymotrypsin investigated contained contaminants of one or two types. The first type apparently consists of autolysis products and interferes with quantitative study of the kinetics of chymotryptic catalysis by high-speed methods. The second contaminant, of unknown nature, is tightly bound in such a way as to prevent substrate binding or the binding of competitive inhibitors. Its effect is formally noncompetitive and thus reduces the concentration of participating enzyme. Reliable measurement of the type and extent of contamination is at present possible only with temperature-jump methods but a simple, completely satisfactory purification procedure has been developed. Steady-state kinetics study provides unreliable tests for contamination because of the slow, time-dependent dissociation of the second type of contaminant at low-protein concentrations. The N-*trans*-cinnamoylimidazole test is also unreliable since the contaminants do not block the reaction. Quantitative data previously reported for α-chymotrypsin (CT) as the result of steady-state kinetics investigation must be considered unreliable until verified using pure protein. The dissociation equilibrium constant for indole binding to CT is found to be 2.73×10^{-4} *M* at pH 7.5 and 3°.

During the past several years, relaxation methods have assumed increasing importance as tools in the elucidation of complex enzymic mechanisms. The temperature-jump method, in particular, has been successfully used to determine not only the rate constants in enzymic systems but also to detect the presence of intermediates which would not ordinarily be observable in conventional steady-state kinetics.[2] The method is now proving to be most useful in studies of the active site of chymotrypsin, particularly as regards the protonic ionization of the two imidazole groups in this enzyme.

In a previous communication[3] we reported that the two imidazole groups of α-chymotrypsin (CT), in the presence of a pH indicator (phenol red), are the source of a single large relaxation effect in the neutral pH range. The results of studies of the protonic behavior of these imidazoles as a function of pH, indicator concentration, and enzyme concentration were consistent with the following mechanism.[4]



The over-all relaxation time for the above system is given by eq 2 and 3, derived using a steady-state treatment for $[H^+]$ and $[OH^-]$.

$$\tau^{-1} = \tau_{DT}^{-1} + \tau_{P}^{-1} + \tau_{H}^{-1} \quad (2)$$

(1) This is paper No. 30 from this laboratory. Please request reprint by this number.
(2) G. G. Hammes and P. Fasella, *J. Am. Chem. Soc.*, **84**, 4644 (1962); G. G. Hammes and P. Fasella, *ibid.*, **85**, 3929 (1963); R. Cathou and G. G. Hammes, *ibid.*, **86**, 3240 (1964).
(3) A. Yapel and R. Lumry, *ibid.*, **86**, 4499 (1964).
(4) M. Eigen, G. G. Hammes, and K. Kustin, *ibid.*, **82**, 3482 (1960).

$$\left.\begin{aligned} \tau_{DT}^{-1} &= k_{13}[\overline{(ImH^+)} + \overline{(I^{2-})}] + k_{31}[\overline{(Im)} + \overline{(HI^-)}] \\ \tau_P^{-1} &= \\ &\frac{k_{12}k_{23}[\overline{(ImH^+)} + \overline{(I^{2-})}] + k_{21}k_{32}[\overline{(Im)} + \overline{(HI^-)}]}{k_{23}\overline{(I^{2-})} + k_{21}\overline{(Im)}} \\ \tau_H^{-1} &= \\ &\frac{k_{14}k_{43}[\overline{(ImH^+)} + \overline{(I^{2-})}] + k_{41}k_{34}[\overline{(Im)} + \overline{(HI^-)}]}{k_{41}\overline{(HI^-)} + k_{43}\overline{(ImH^+)}} \end{aligned}\right\} \quad (3)$$

where τ = over-all relaxation time, τ_{DT} = direct transfer relaxation time, τ_P = protolysis relaxation time, and τ_H = hydrolysis relaxation time. The bars over the concentrations indicate equilibrium concentrations at the upper temperature (10°). In the pH range studied, the direct transfer process was found to make the largest contribution to the relaxation time. The concentration of "available" imidazole groups in CT, defined as those groups capable of reacting directly with indicator, was found to be twice the protein concentration, as expected. Both imidazole groups of CT appear to be identical insofar as their ionization behavior is concerned.

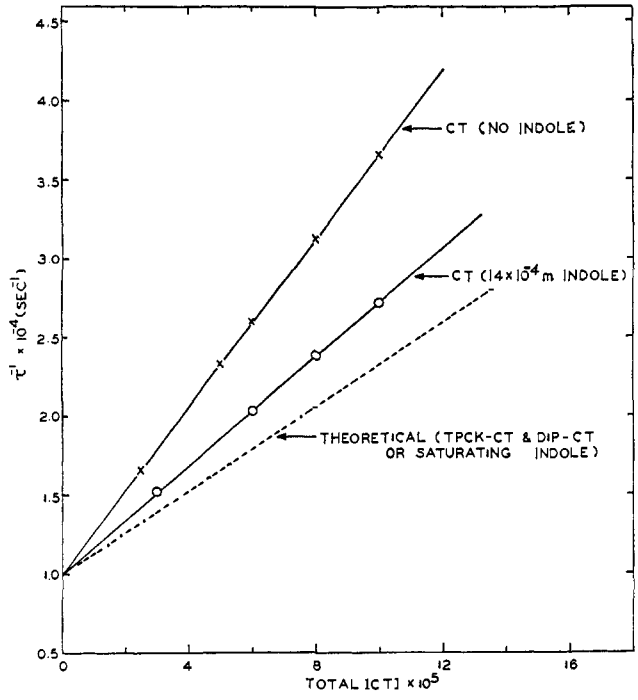


Figure 1. The effect of indole on τ^{-1}; phenol red = 4.6×10^{-5} M, 0.1 M KNO_3, 10°, pH 7.5. All concentrations are initial concentrations.

TPCK-CT, the L-1-tosyl-amido-2-phenylethyl chloromethyl ketone derivative of CT, has only one "available" imidazole group by the temperature-jump method of analysis.[3] This is consistent with the findings of Ong, Schoellmann and Shaw[5-7] who have shown that the "substrate analog," TPCK, reacts with a catalytically functional imidazole, His-57,[8] apparently eliminating its ionization processes at neutral pH values. Aside from the difference in effective concentration of imidazole groups, the experimental results for CT and TPCK-CT were quantitatively identical, thus confirming the fact that the protonic processes measured by the method are essentially identical for the two imidazole groups of CT.

DIP-CT (diisopropylphosphoryl-CT) was found to be identical in relaxation time behavior with TPCK-CT so that there is only one "available" imidazole in this CT derivative also.[3] Since the DIP group is attached to the acylation-site serine-194,[8] a close approach of Ser-194 to His-57 is possible and may be sufficient to explain the ability of the DIP group to prevent the direct transfer of the His-57 proton to the indicator molecule.

It was further noted[3] that if indole, a good competitive inhibitor of CT, was added to the enzyme-indicator system under a standard set of experimental conditions, the over-all relaxation time, τ, as given by eq 2, increased progressively with increasing indole concentration until τ coincided with the value obtained for TPCK-CT and DIP-CT. Further increases in indole concentration produced no further increases in τ. Indole has no effect on the direct transfer reaction with TPCK-CT. Hence the blocking by indole is effective only at His-57. The binding of indole thus simply covers up the imidazole group of His-57 or causes some type of protective infolding of the imidazole group which reduces its "available" concentration and thus "blocks" the direct transfer reaction of the imidazolium proton to the indicator molecule.

In Figure 1 is shown the behavior of the CT-indicator system in the presence and absence of indole. The presence of 14.0×10^{-4} M indole causes about 70% blocking of His-57. The dashed "theoretical" line is that which would be obtained if only one imidazole group in CT were "available" for participation in the direct transfer reaction.[3] This "theoretical" line, appropriate for phenol red at the stated concentration, is obtained experimentally for TPCK-CT and DIP-CT which have only one such imidazole group. It is also obtained for CT in the presence of about 10^{-2} M indole, under which conditions His-57 is completely blocked.

For the blocking-by-indole experiments shown in Figure 1, Worthington CT (batch No. CDI-6100-1) was used without further purification. Subsequent experiments of this nature with different batches of commercially available chymotrypsin (used without further purification) produced startlingly different results. Some batches showed no blocking by indole even at very high indole concentrations while others showed nearly complete blocking by indole. The relaxation times of various CT batches measured in the absence of indole and under a standard set of experimental conditions also showed considerable variability.

These initial temperature-jump experiments demonstrated the existence of considerable heterogeneity in commercial CT samples, particularly as regards their behavior toward blocking by indole. The purpose of this investigation was to determine the nature of this heterogeneity and to find out whether reproducible CT

(5) G. Schoellmann and E. Shaw, *Biochemistry*, 2, 252 (1963).
(6) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Am. Chem. Soc.*, 86, 1271 (1964).
(7) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Biol. Chem.*, 240, 694 (1965).
(8) B. S. Hartley, Abstracts of the 6th International Congress of Biochemistry, No. IV, New York, N. Y., 1964, pp 253, 254.

samples could be obtained by some standard method of purification.

Experimental Section

A. Temperature-Jump Experiments. The temperature-jump apparatus employed was similar to that described by Eigen and De Maeyer.[9] The cell was of the standard Hammes design (total capacity ~20 ml) with a 1-ml heated volume and a 1-cm heated optical path length. The temperature jump, corresponding to about 7°, was effected by discharging a 0.05 μf high-voltage, low-inductance capacitor (Plastic Capacitors, Inc.), previously charged to 35,000 v, through the test solution. The heating time (~90% of the total temperature rise) was about 1 μsec. The conducting electrolyte was 0.1 *M* KNO_3 in all cases. The initial temperature (3°) of the test solutions was controlled to ±0.2° by passing water from a constant temperature bath through the cell housing. No arcing or cavitation was observed during discharge, and relaxation times as fast as 5 μsec could be easily measured.

The light source was a 100-w General Electric Q6.6A/T3/1CL-100W tungsten iodide lamp with a quartz envelope. The light beam was passed through a Bausch and Lomb grating monochromator equipped with variable entrance and exit slits and focused at the center of the cell containing the test solution with a system of lenses. The change in light intensity occurring after a temperature jump was measured on an RCA 1P28 photomultiplier tube, the output of which was fed into a Tektronix Type 545A oscilloscope equipped with a Type D differential preamplifier. Since a single-beam mode of operation was used, the photomultiplier input to the oscilloscope was balanced with a 12-v storage battery equipped with an appropriate potentiometric circuit. The change in optical density at 558 mμ (absorption maximum of unprotonated phenol red) occurring after a temperature jump was recorded on Polaroid film using a Tektronix C12 oscilloscope camera.

CT samples were obtained from the Worthington Biochemical Corp., Armour and Co., and Gallard-Schlesinger (Pentex) and were stored at 4°. In tests for blocking by indole, these samples were used without further purification. Those batches which were later purified by gel filtration are so noted in the text. Phenol red was obtained from the Hartman-Leddon Co. and was purified by recrystallization according to the method of Orndorff and Sherwood[10] before use. Indole (Mann assayed Lot L1905) and reagent grade KNO_3 were used without further purification. Deionized water, degassed by boiling or aspiration, was used to prepare all solutions.

Generally, 1% stock solutions of CT were prepared and the pH was adjusted to 3.00 with dilute HCl. These solutions were stored at 4° and were used within 3 days. All stock solution concentrations were measured spectrophotometrically on a Cary Model 11 recording spectrophotometer. The following extinction coefficients were used: CT, ϵ_{282} 2.03 M^{-1} cm^{-1}; indole, ϵ_{270} 5.57 × 10^3 M^{-1} cm^{-1}; phenol red, ϵ_{558} 7.07 × 10^4 M^{-1} cm^{-1}. The molecular weight of CT was assumed to be 25,000.

Test solutions were prepared from the stock solutions by pipetting the appropriate amounts of reagent into a 50-ml volumetric flask and then diluting to the mark with deionized water. The pH of the test solutions was adjusted to 7.50 with 0.1 *M* KOH.

B. Steady-State Kinetics. The steady-state enzyme kinetics were followed by potentiometric titration using a Radiometer TTTl automatic titrator in conjunction with a homemade autotitration assembly. The pH of the reaction mixture was maintained at a constant value of 8.00. Helium gas was continuously passed over the reaction mixture and the mixture was stirred with a magnetic stirring bar. The temperature was maintained at 25 ± 0.1°. The final volume of the reaction mixture was always 10 ml. All data were analyzed by weighted least-squares procedures and no deviation from Michaelis–Menten behavior could be detected. The standard substrate, N-acetyl-L-tryptophan ethyl ester (ATrEE), was Mann Assayed Lot J1322 and was used without further purification. CT stock solutions for steady-state experiments were no more concentrated than about 0.05%. The reaction solution contained 10^{-4} *M* tris(hydroxymethyl)aminomethane buffer at pH 8.00 and 0.2 *M* KCl. The N-*trans*-cinnamoylimidazole procedure for CT concentration determinations has been described.[11]

C. Purification and Analytical Procedures. Gel filtration of CT samples was carried out at 4° on a 1.5 × 18 in. column of G-25 (fine) Sephadex which had been previously equilibrated with pH 3.00 HCl solution. Generally, 10 to 12 ml of a 3% solution of CT (pH 3.00) was placed at the top of the column and eluted with pH 3.00 HCl at a flow rate of 20 ml hr. Approximately 5-ml fractions were collected, and the protein concentration was monitored by measuring the optical density at 282 mμ.

Ninhydrin was obtained from the Sigma Chemical Corp. and was used without further purification. Ninhydrin color tests on CT samples were carried out in the following manner. One milliliter of solution was taken from each of the first 40 fractions off the Sephadex column and then treated with ninhydrin solution according to the method of Yemm and Cocking.[12] After ninhydrin color development the resulting solutions were diluted 60 times with ethanol and their optical densities were read at 570 mμ.

Results

A. Temperature-Jump Kinetics. As noted in the introduction, initial experiments with several different CT batches showed considerable variation in the ability of indole to block the direct-transfer reaction. Since a few of these batches were several years old, it was difficult to decide whether this variability was due to aging of the CT, to autolysis of the enzyme with the resulting contamination, to the presence of small amounts of π-CT or δ-CT which had not been completely removed in the commercial purification procedure, or to the presence of other impurities—possibly free amino acids, small peptides, or heavy metal ions.

In an attempt to set up some criterion of purity, the relaxation times of eleven different batches of commercial CT were checked under the following standard set of conditions: 8.0 × 10^{-5} *M* CT (used without further purification), 4.6 × 10^{-5} *M* phenol red, 0.1 *M* KNO_3, 10° (upper temperature-jump temperature), pH 7.50. Each batch was checked both in the absence of indole and in the presence of ~5 × 10^{-3} *M* indole, which should correspond to about 90% blocking based on a dissociation K_i of 2.7 × 10^{-4} *M* for indole. The results of these measurements are given in Table I.

Each relaxation time (τ) listed in Table I is the mean value of at least six separate relaxation spectra, the individual precision of which was generally within ±3%. The mean value of τ listed for each batch could be reproduced to ±1% or better in successive experiments. The age of a batch, as listed in Table I, is the approximate length of time which had elapsed since that particular batch was received in this laboratory from the supplier until the temperature-jump experiments were run. Two of the batches (Worthington CDI-6068-9 and Armour Y-9401) were available in such short supply that only one check experiment could be performed on each. In these two cases the experiment was carried out in the presence of indole.

There are several interesting points to be noted in Table I. (a) Five of the batches (Worthington CDI-6084-5, CDI-6100-1, CDI-6112-3, and CDI-6121-2, and Pentex B2842) had τ^{-1} values equal to 3.12 (±0.03) × 10^4 sec^{-1} in the absence of indole. All of these batches, with the exception of CDI-6084-5, showed at least partial blocking by indole, as evidenced by the increase in τ when indole was added to

(9) M. Eigen and L. DeMaeyer, "Techniques of Organic Chemistry," Vol. VIII, Part II, A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1963.

(10) W. R. Orndorff and F. W. Sherwood, *J. Am. Chem. Soc.*, **45**, 486 (1923).

(11) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(12) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).

Table I. Comparison of Relaxation Times for Various CT Batches[a]

	Company	Batch no.	[Indole]^0 $\times 10^4$ M	$\tau \times 10^6$, sec	$\tau^{-1} \times 10^{-4}$, sec^{-1}	Age
1.	Worthington	CDI-6068-9	48.8	25.1	3.99	3 year
2.	Worthington	CDI-6070-1	...	24.9	4.01	3 year
	Worthington	CDI-6070-1	48.8	24.9	4.01	3 year
3.	Worthington	CDI-6084-5	...	32.1	3.11	1 month
	Worthington	CDI-6084-5	...	32.0	3.12	1.5 year
	Worthington	CDI-6084-5	48.8	32.1	3.11	1.5 year
4.	Worthington	CDI-6100-1	...	32.1	3.11	1 month
	Worthington	CDI-6100-1	14.0	42.0	2.38	1 month
5.	Worthington	CDI-6112-3	...	31.7	3.15	1 month
	Worthington	CDI-6112-3	48.8	43.3	2.31	1 month
6.	Worthington	CDI-6114-5	...	28.3	3.53	1 month
	Worthington	CDI-6114-5	38.2	28.8	3.47	1 month
7.	Worthington	CDI-6121-2	...	32.0	3.12	1 month
	Worthington	CDI-6121-2	50.0	43.0	2.33	1 month
8.	Worthington	CDC-52[b]	...	18.22	5.49	2 weeks
	Worthington	CDC-52[b]	50.0	20.50	4.88	2 weeks
9.	Armour	Y-9401	40.0	28.3	3.54	3 year
10.	Armour	B-13406	...	30.8	3.25	2 weeks
	Armour	B-13406	50.0	35.5	2.82	2 weeks
11.	Pentex	B-2842	...	31.7	3.15	2 weeks
	Pentex	B-2842	50.0	38.2	2.62	2 weeks

[a] Standard test conditions noted in text. [b] Chromatographically homogeneous.

the test system. τ for batch CDI-6084-5 was unaffected by the presence of 48.8×10^{-4} M indole. (b) Four of the remaining six batches gave τ^{-1} values significantly higher than 3.12×10^4 sec^{-1} in the absence of indole, varying from 3.25×10^4 sec^{-1} (Armour B13406) to 5.49×10^4 sec^{-1} (Worthington CDC-52 (see Table I)). Two of these batches were essentially unaffected by the addition of indole (Worthington CDI-6070-1 and CDI-6114-5) while the other two batches (Worthington CDC-52 (see Table I) and Armour B13406) showed some blocking by indole as evidenced by the longer relaxation time in the presence of indole. The final two batches (Worthington CDI-6068-9 and Armour Y-9401), for which only enough sample was available for one experiment, had τ^{-1} values significantly higher than 3.12×10^4 sec^{-1}, even at high indole concentrations. (c) It was difficult to tell from these experiments whether or not aging of the CT samples was responsible for some of the variation among batches. Although the oldest batches generally gave high values for τ^{-1} (*i.e.*, $>>3.12 \times 10^4$ sec^{-1} in the absence of indole), two of the newest batches (CDI-6114-5 and CDC-52 (see Table I)) also gave very high τ^{-1} values. Furthermore, batch CDI-6084-5 gave a τ^{-1} value in the absence of indole essentially identical with that obtained 1 year earlier with this batch.

Although six of the eleven batches tested showed at least partial blocking by indole, it was still not possible to decide whether "blockable" or "nonblockable" CT was the "normal" state, since the amount of blocking varied from batch to batch. If blockable CT was normal, then the presence of some impurity, probably bound to the enzyme, was preventing the indole from blocking the direct-transfer reaction. If nonblockable CT was normal, then the impurity plus the indole were cooperating in some manner to block the direct-transfer reaction.

B. Gel Filtration. In an attempt to resolve the above dilemma, purification on G-25 (fine) Sephadex was carried out on two representative CT batches: CDI-6114-5, a nonblockable batch with a high τ^{-1} value ($>3.12 \times 10^4$ sec^{-1}), and CDI-6121-2, a partially blockable batch with a normal τ^{-1} value (3.12×10^4 sec^{-1}). A typical chromatogram is shown in Figure 2.

In all chromatograms, only one peak was observed when fractions were monitored at 282 mμ. This peak corresponds to the main CT fraction and always came over in four or five tubes (fractions 8–11), each containing 5 ml, so that the original CT solution placed at the top of the column was diluted by about a factor of 2 after passage through the column. Generally, fractions 8–11 (denoted by the shaded area in Figure 2) were saved and mixed together, and the resultant solution was used in subsequent temperature-jump and steady-state kinetic experiments.

Two batches (CDI-6114-5 and CDI-6121-2) were studied in detail on the temperature-jump apparatus. The same standard concentration conditions noted earlier were used, except that experiments were carried out as a function of indole concentration rather than just at one saturating concentration of indole. The results of these experiments are shown in Figure 3. There are several important effects to be noted in Figure 3. Batch CDI-6114-5, which was originally nonblocking and which had a high τ^{-1} value in the absence of indole, became completely blockable after Sephadex treatment. In the absence of indole, it also gave a "normal" τ^{-1} value of 3.12×10^4 sec^{-1}. Batch CDI-6121-2, which was partially blockable before treatment, became completely blockable after Sephadex treatment. τ^{-1} in the absence of indole also remained unchanged with a value of 3.12×10^4 sec^{-1}. Regardless of its past history, a standard purified CT sample is thus obtained after Sephadex gel filtration. The direct-proton-transfer reaction from one imidazole to the indicator molecule is completely blocked in the presence of a saturating indole concentration and a value of $\tau^{-1} = 3.12 \times 10^4$ sec^{-1} in the absence of indole is obtained under the standard set of test conditions.

The results in Figure 3 suggested that some fairly

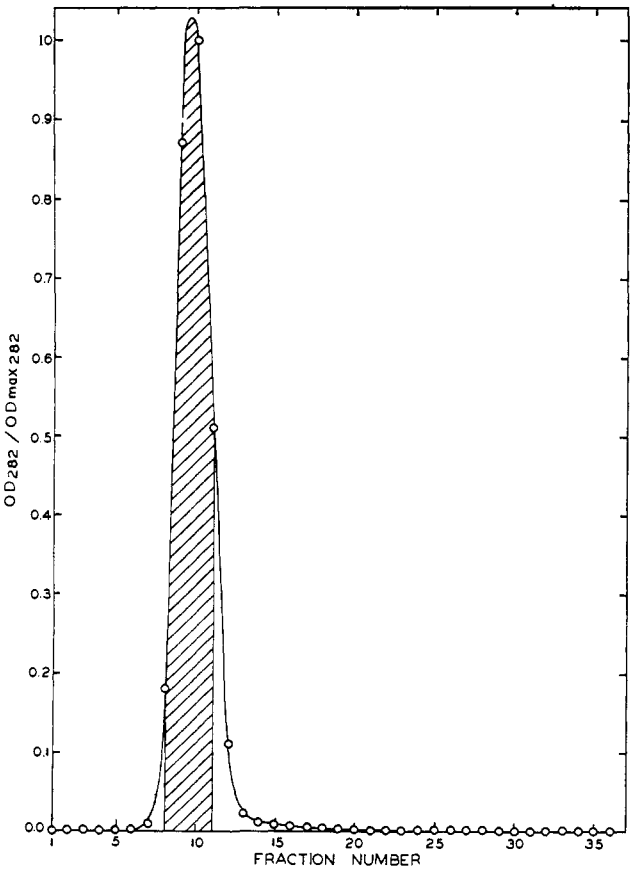


Figure 2. Chromatogram of Worthington CT(CDI-6114-5) on G-25 Sephadex: flow rate = 20 ml/hr. Each fraction contains 5 ml. Optical density measured at 282 mμ. Shaded area corresponds to the fractions used in subsequent temperature-jump and steady-state kinetic experiments.

low molecular weight impurity (<5000 mol wt) was removed from the CT samples upon passage through the Sephadex column.

The effects of two metal ions, Ca^{2+} and Cu^{2+}, on the indole blocking reaction were studied in some detail with the temperature-jump apparatus, since it seemed possible that one or both of these ions might be responsible for the observed CT contamination. Using "Sephadexed" CT (CDI-6114-5), it was found that neither of these ions prevented the indole blocking reaction even when present in high concentration and were, therefore, not the impurities responsible for the nonblocking. Cu^{2+}, in fact, was found to be a very good blocker of the imidazole-indicator direct-transfer reaction at His-57, and only one Cu^{2+} was bound per CT molecule. His-57 but not His-40 is involved in the binding. Steady-state kinetics revealed a complex inhibition pattern for this ion. Cu^{2+} was found to bind to CT in significant amounts even when present in trace concentrations ($<10^{-5}$ *M*). Quantitative results of this study will be presented in a forthcoming publication.

Although chromatographed CT (Figure 2) shows only one peak when monitored at 282 mμ, this does not eliminate the presence in commercial CT samples of amino acids, peptide fragments, or other small molecules which have no 282-mμ absorption. In order to test for the presence of free amino acids or peptides, the

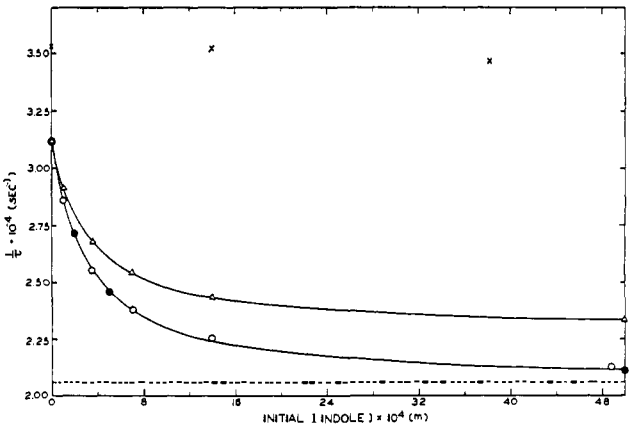


Figure 3. Comparison of the indole "blocking" effect for chromatographed and nonchromatographed CT: ---------, theoretical line for complete blocking; X, CDI-6114-5 before Sephadexing; ○, CDI-6114-5 after Sephadexing; △, CDI-6121-2 before Sephadexing; ●, CDI-6121-2 after Sephadexing; CT = 8×10^{-5} *M*, phenol red = 4.6×10^{-5} *M*, 0.1 *M* KNO_3, pH 7.5, 1.0°. All concentrations are initial concentrations. Solid line (upper curve) calculated for $K_i = 2.77 \times 10^{-4}$ *M* assuming that indole binds only to noncontaminated CT (see Discussion section). Solid line (lower curve) calculated for $K_i = 2.73 \times 10^{-4}$ *M*. K_i values refer to a temperature of 3° (see text).

eluent from a typical chromatogram of 12 ml of 3% CT solution was subjected to ninhydrin analysis as described in the Experimental Section. The results of a typical analysis of batch CDI-6114-5 are shown in Figure 4.

The first peak, with maximum optical density in fraction 11, corresponds to the 282-mμ absorption of purified CT shown in Figure 2 and indicates the expected weak ninhydrin reaction with the purified enzyme. The second peak is undoubtedly due to the presence of the impurity or impurities responsible for the prevention of blocking by indole and for the abnormally high τ^{-1} value obtained with this batch. Since this chromatogram showed no substantial 282-mμ absorption in the fraction range of the second peak, the presence of tyrosine- or tryptophan-containing amino acids or peptides is excluded. Ultraviolet spectra of fraction 19 (with no ninhydrin added) from 380 to 230 mμ, corresponding to the maximum in the second peak, showed no absorption bands except those associated with the weak background absorbance of CT still present in the eluent. Although the impurity or impurities may absorb in the very deep ultraviolet, the apparent lack of any ultraviolet absorption may be due to the fact that they are present in very low concentration in the eluent or have small extinction coefficients. The strong ninhydrin color reaction, however, suggests that at least part of the CT contamination is due to one or more amino acids or peptides. For the chromatogram shown in Figure 4, a rough estimate using leucine equivalents indicates ~4 to 6 contaminating amino acids per CT molecule. The contaminant or contaminants contained in the second peak have been arbitrarily called substance "T", and will be referred to as such from here on.

As a check to be sure that the contaminant causing the "nonblocking" in batch CDI-6114-5 was contained in the second peak, a separate chromatogram was run and the two peaks were isolated. Fractions 17–19 were mixed together and assumed to contain

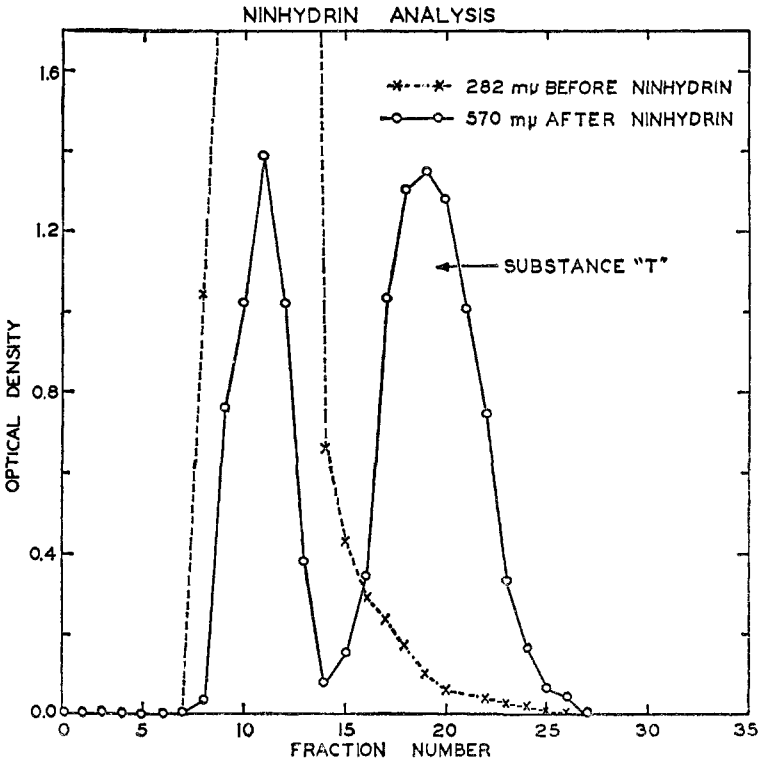


Figure 4. Ninhydrin color test on CT (CDI-6114-5). Solid line gives the optical density at 570 mμ after reaction with ninhydrin. Dashed line gives optical density at 282 mμ before addition of ninhydrin.

substance T. The following standard test solution containing 10 ml of substance T per 50 ml of solution was prepared: 8.0×10^{-5} *M* CT (Sephadexed CDI-6114-5), 4.6×10^{-5} *M* phenol red, 0.1 *M* KNO_3, 14×10^{-4} *M* indole, pH 7.50. Temperature-jump analysis of this sample showed $\tau^{-1} = 3.53 \times 10^4$ sec^{-1}, which is identical with the value obtained in the presence of indole before Sephadex treatment. A control experiment without added substance T showed complete blocking by indole. Although the exact agreement in τ^{-1} values is probably coincidental, the fact that the Sephadex-treated CT (upon addition of substance T) is rendered nonblockable and that τ^{-1} reaches its original abnormally high value clearly demonstrates that the contaminating substances are removed from the CT by Sephadex treatment and that these contaminants are indeed contained in the second peak shown in Figure 4.

On the basis of the temperature-jump results, it is clear that substance T can consist of at least two classes of contaminants. The first class, class I, increases τ^{-1} to abnormally high values under standard experimental conditions. The second class, class II, prevents the indole blocking reaction. Each class may itself, of course, contain more than one component. On this basis, batch CDI-6114-5 contains high concentrations of both class I and class II contaminants while Armour B-13406 contains both classes of impurities in lower concentrations. Batch CDI-6084-5 contains only class II contaminants in high concentration while CDI-6121-2 contains class II contaminants in much lower concentration. The analysis can be easily extended to the other batches tested and shows that the relaxation behavior of any given batch is uniquely determined by the relative amounts of class I and class II contaminants present in that batch.

It is possible to calculate the approximate amount of blockable CT present in any given commercial batch by comparing its τ^{-1} value at high indole concentration (and under the standard test conditions) with the τ^{-1} value obtained for Sephadexed CT (completely blocking) under the same conditions. τ^{-1} in the absence of indole, however, must not be too much larger than 3.12×10^4 sec^{-1}, since the limiting theoretical value[13] of 2.06×10^4 sec^{-1} (obtained for purified CT with one imidazole completely blocked) only applies in this case. The percentage blocking material in the CT batches tested by the temperature-jump technique is shown in Table II for those batches where such a calculation was possible.

Table II. Percentage "Blocking" CT in Several Commercial CT Batches

	Company	Batch no.	Blocking, %
1.	Worthington	CDI-6070-1	0
2.	Worthington	CDI-6084-5	0
3.	Worthington	CDI-6100-1	85
4.	Worthington	CDI-6112-3	81-84
5.	Worthington	CDI-6114-5	<1
	Worthington	CDI-6114-5 (Sephadexed)	100
6.	Worthington	CDI-6121-2	80
	Worthington	CDI-6121-2 (Sephadexed)	100
7.	Armour	B-13406	~30–40
8.	Pentex	B-2842	~50

C. Steady-State Kinetics. Although the effects of substance T on the temperature-jump kinetics are dramatic and at present are the most reliable and accurate way to test for contamination, tests were also carried

(13) See ref 3 and the introduction to this paper.

out using conventional steady-state kinetics to determine the influence of substance T on the enzymic rate parameters. Experiments with ATrEE were carried out both in the presence and absence of indole. CT concentrations were varied from 3.0×10^{-8} to 6.0×10^{-8} M, and substrate concentrations from 4.0×10^{-5} to 4.0×10^{-4} M. Indole concentrations were on the order of 10^{-4} M. Bender, *et al.*,[14] have shown that the reaction of CT with good ester substrates passes through an acyl–enzyme intermediate, and that the slow step at high substrate concentration is the deacylation of this intermediate. All data could then be analyzed in terms of the simple Hartley–Kilby mechanism (eq 4) with zero-time, steady-state velocity v_i (eq 5). The total enzyme concentration is E_0,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + \text{products} \qquad (4)$$

$$v_i = \frac{k_3 E_0 S_0}{S_0 + K_m} \qquad (5)$$

and the initial substrate concentration is S_0. The initial velocity for competitive inhibition of CT by indole is given by eq 6 where K_i is the dissociation inhibition constant.

$$v_i = \frac{k_3 E_0 S_0}{S_0 + K_m\left(1 + \frac{[I]}{K_i}\right)} \qquad (6)$$

Values of all rate parameters were obtained using a weighted least-squares procedure.

The results of a set of experiments on both blockable and nonblockable CT batches are given in Table III. Batch CDI-6114-5 (a nonblockable batch) shows a substantial increase in k_3 after Sephadex purification. Batch CDI-6121-2 (a highly blockable batch) has a k_3 value much closer to the value obtained for Sephadexed CDI-6114-5 (completely blockable) than to that obtained for non-Sephadexed CDI-6114-5 (nonblockable), in agreement with the temperature-jump results which showed that CDI-6121-2 was substantially less contaminated by substance T than CDI-6114-5. K_m values for both Sephadexed and non-Sephadexed CT batches were essentially identical ($9.5 (\pm 0.4) \times 10^{-5}$ M): thus, this parameter appears to be unaffected by the presence of substance T. After Sephadex treatment, batch CDI-6121-2 had rate parameters essentially identical with those obtained for chromatographed CDI-6114-5. It is also interesting to note that the K_i values for indole inhibition are identical (within experimental error) for both chromatographed and nonchromatographed CT samples. The K_i values reported in Table III are in good agreement with those previously reported by Niemann, *et al.*,[15] at pH 7.9.

A comparable series of steady-state experiments using N-acetyl-L-tyrosine ethyl ester (ATyEE) and N-acetyl-L-phenylalanine methyl ester (APhME) as substrates and including several other batches listed in Table I gave results similar to those in Table III. Nonblockable CT batches generally gave lower k_3 values than blockable batches or batches which had been rendered totally blockable by chromatography on Sephadex, while K_m values before and after Sephadex treatment remained unchanged.

The steady-state results in Table III also show that dilution is not particularly effective in removing substance T from contaminated CT samples—especially when it is present in high concentration—*e.g.*, CDI-6114-5. All temperature-jump experiments were carried out at a CT concentration of 8.0×10^{-5} M; those used in steady-state experiments were more than 1000 times lower. The steady-state kinetic values for k_3 were, nonetheless, significantly affected by the presence of substance T. It is impossible, however, to make quantitative comparisons between batches on the basis of the steady-state kinetic data. Although the major contaminants in substance T, *i.e.*, those which prevent blocking by indole, appear to have an unusually high affinity for CT, a slow time-dependent amount of dissociation occurs in dilute stock solutions and in solutions used for the steady-state experiments. This is demonstrated by the nearly identical k_3 values obtained for CDI-6121-2 (~80 per cent blocking) both before and after Sephadex treatment. On the basis of the temperature-jump data, approximately a 20% decrease in the apparent k_3 would have been expected for the non-Sephadexed enzyme as compared to the Sephadexed enzyme (see Discussion section), whereas the measured decrease is only about 3%. Dissociation of the contaminant on dilution is obviously far from complete, particularly with a nonblockable batch like CDI-6114-5, although complete dissociation can presumably be effected by long standing in dilute solution. More precise knowledge of the binding characteristics of the impurities in substance T to CT can only be obtained when they can be put into solution under controlled concentration conditions. This must await their positive identification. Consistent values for k_3 are obtained only after the contaminants are removed by gel filtration on G-25 Sephadex.

Stimulated by Bender's thoughtful criticisms of the low levels of steady-state inhibition observed with substance T, an additional set of kinetic experiments was carried out in which high concentrations of contaminants were added to reaction mixtures. Since traces of chymotrypsin and large autolysis products of CT could not easily be separated from the contaminants with a short column, CT purification was carried out using a G-25 Sephadex column 1 m in length under conditions otherwise identical with those previously described. Batch CDI-6114-5 (nonblockable with a high τ^{-1} value) was used for these experiments. Higher loading with the same flow rate used previously was possible with this longer column so that 10 ml of a 5% protein solution was usually added to the top of the column to initiate a separation experiment. The eluent fractions could be quickly and conveniently analyzed by simple conductometry and the results are shown in Figure 5.

A large peak in conductance appears between fractions 50 and 69 and measures the presence of inorganic ions as well as any other contaminants having a high conductivity. As a check to see whether the contents of these tubes contained the components of substance T responsible for the enzymic inhibition noted previously, a series of steady-state kinetic studies was carried out with selected samples in this range of

(14) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3669 (1964).
(15) R. A. Wallace, A. N. Kurtz, and C. Niemann, *Biochemistry*, **2**, 824 (1963).

Table III. Effect of Substance T on Steady-State Rate Parameters[a]

CT batch no.	$K_m \times 10^5$ M	k_3, sec^{-1}	$K_i \times 10^4$ M
1. Worthington CDI-6114-5 (not Sephadexed)	9.2 ± 0.4	28.0 ± 1.05	6.9 ± 0.1
2. Worthington CDI-6114-5 (Sephadexed)	9.9 ± 0.5	45.5 ± 0.9	6.8 ± 0.2
3. Worthington CDI-6121-2 (not Sephadexed)	9.6 ± 0.3	44.0 ± 0.9	6.6 ± 0.2
4. Worthington CDI-6121-2 (Sephadexed)	9.6 ± 0.4	45.9 ± 0.8	6.6 ± 0.2

[a] S_0 varied from 4.0×10^{-5} to 4.0×10^{-4} M. E_0 varied from 3.0×10^{-8} to 6.0×10^{-8} M. [indole] varied from 4.0×10^{-4} to 8.0×10^{-4} M. Temperature = 25°, pH 8.00. All experiments done in the presence of 0.2 M KCl and 10^{-4} M tris(hydroxymethyl)aminomethane buffer.

fractions. A 6-ml amount of contaminated eluent was added to the standard reaction mixture as a substitute for water. In no case was any enzymic inhibition observed with these fractions. However, the added contaminants did exhibit considerable buffering capacity in the pH 8 region. This behavior would be characteristic of small impurities containing α-amino groups so that the contaminants present in fractions 50 to 69 are probably class I contaminants. It is also likely that these class I impurities are responsible for the strong ninhydrin reaction noted previously.

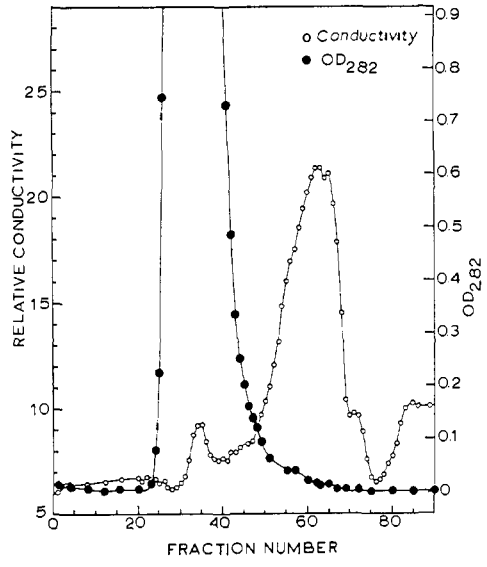


Figure 5. Chromatogram of Worthington CT (CDI-6114-5) on G-25 Sephadex. Column length was 1 m; flow rate = 20 ml/hr. Each fraction contains 5 ml. Left ordinate gives relative conductance of fractions on an arbitrary scale. Right ordinate gives OD_{282} for the same fractions and is a measure of the CT concentration in each fraction.

Although no enzymic inhibition was observed up to fraction 69, experiments identical with those described above showed that eluent fractions beginning with 70 exhibited a strong inhibitory effect on chymotryptic hydrolysis of substrates. The inhibitory action of the contaminants in these tubes started sharply at fraction 70 where the small conductance shoulder begins and continues to at least fraction 93. We have not yet analyzed fractions beyond this point, but the contents of tube 93 is even more inhibitory than those of tubes 70 to 81. From tube 70 onward there is no detectable buffering power of the contaminants. It is most probable that the contaminants in these tubes are responsible for the nonblocking effect noted in temperature-jump studies, and as such are class II contaminants. The longer Sephadex column thus has the distinct advantage of not only separating all substance T impurities from CT but also of separating class I and class II contaminants from each other.

The results of a typical experiment designed to demonstrate the inhibitory effect of the class II contaminants are shown in Figure 6. At time zero in this figure, the reaction mixture (total volume 10 ml) contains 6 ml of the combined eluent from tubes 80 to 81 in addition to 1.0×10^{-4} M Tris buffer and 0.2 M KCl. At point 1, Sephadexed CT was added to a final concentration of 2.0×10^{-7} M and allowed to incubate for 3 min. At point 2, the substrate, N-acetyl-L-tryptophan ethyl ester, was added to a final concentration of 1.66×10^{-4} M to initiate the hydrolytic reaction. At point 3, an additional aliquot of purified enzyme equal in concentration to the initial amount was added. The rapidly rising curve at point 2 is a control experiment identical in concentration with that described above except that water is substituted for the class II contaminant.

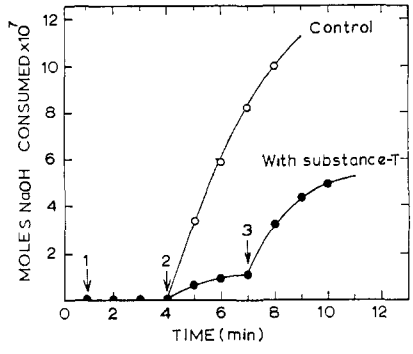


Figure 6. Inhibitory effect of class II contaminants in substance T as demonstrated by steady-state kinetics. At time zero the reaction mixture contains 6 ml of the eluent from tubes 80 and 81, 1.0×10^{-4} M Tris buffer, and 0.2 M KCl. The total volume is 10 ml; pH 8.00; 25°. After 1 min 50 μl of 4.86×10^{-6} M Sephadexed CT is added and allowed to incubate for 3 min. After 4 min 2.0 ml of 1.0×10^{-3} M N-acetyl-L-tryptophan ethyl ester was added to initiate the enzymic reaction. After 7 min an additional 50 μl of 4.86×10^{-6} M Sephadexed CT was added. Ordinate expresses the moles of NaOH consumed during the time course of the enzymatic reaction. The fast-rising curve is a control experiment in which water is substituted for the contaminated eluent.

The strong inhibition produced by the class II contaminants is very evident, especially when the enzyme is

Table IV. N-*trans*-Cinnamoylimidazole Titration of Pure and Contaminated CT Batches[a]

Batch no.	[CT], *M*: A. By spectrophotometric determination	B. By titration	% active sites (B/A)
1. CDI-6114-5 (not Sephadexed)	1.61×10^{-3}	1.50×10^{-3}	93.2
2. CDI-6121-2 (not Sephadexed)	1.76×10^{-3}	1.66×10^{-3}	94.3
3. CDI-6121-2 (Sephadexed)	1.46×10^{-3}	1.40×10^{-3}	95.8

[a] Concentrations listed in table are calculated original stock solution concentrations. Original stock solutions were diluted by a factor of 30 in the titration experiments and by a factor of 120 in the spectrophotometric determinations.

allowed to incubate for a few minutes prior to the addition of substrate. At point 3, where the addition of enzyme was not preceded by an incubation period, a distinct time dependence leading to nearly total inhibition of the added protein is demonstrated and is consistent with a limited series of incubation studies, all of which show that the combination of class II contaminants with CT is very slow.

Inhibition, however, can be easily detected without incubation. For example, using the standard Determatube CT assay procedure (Worthington Biochemicals Corp.) in which N-acetyl-L-tyrosine ethyl ester is the substrate, 60% inhibition was observed immediately when Sephadexed enzyme and dry Determatube contents were added to tube 82. The analytical method is spectrophotometric and, although no systematic series of comparisons was carried out, when large concentrations of class II contaminants are present, both spectrophotometric and titrimetric methods demonstrate very large degrees of inhibition. It should be remembered that the experimental chromatographic procedures greatly concentrate the contaminants relative to their customary concentrations in commercial chymotrypsin preparations. Thus, although relatively small inhibition will be observed in the course of more normal experiments, this group of experiments does demonstrate conclusively that contaminants of class II are inhibitory and may thus introduce small but serious quantitative errors in the values of kinetics parameters obtained using nonpurified material.

Since a number of investigators in the CT field determine their enzyme concentrations by direct titration of the CT "active sites" with N-*trans*-cinnamoylimidazole, it was expedient to determine whether or not this method could be used to distinguish between contaminated and pure CT. Batches CDI-6114-5 (nonblockable) and CDI-6121-2 (~80% blockable) were titrated with N-*trans*-cinnamoylimidazole using method A of Schonbaum, Zerner, and Bender.[11] CT concentrations were first determined spectrophotometrically at 282 mμ as described in the Experimental Section and then redetermined in terms of "active site" concentration using the N-*trans*-cinnamoylimidazole titration method. The results of these experiments are shown in Table IV. It is obvious that the contaminants cannot be detected by the N-*trans*-cinnamoyl titration, since nearly identical concentrations of "active sites" were obtained with contaminated and purified material. Non-Sephadexed CDI-6114-5, in particular, would have been expected to show a significant decrease in "active sites" by the titration method had substance T been detectable by this procedure. There is a small reduction in "active site" concentration with contamination but considering the high protein concentrations ($\approx 6 \times 10^{-5}$ *M*) at which this test was carried out, no reduction in "active sites" of the size expected if substance T could block the reaction with N-*trans*-cinnamoylimidazole was obtained. Sephadex-treated preparations gave 95 → 100% "active" CT by the titration method.

One further set of experiments was carried out in which Sephadexed CT was allowed to incubate for several minutes with high concentrations of class II contaminants obtained from the chromatogram shown in Figure 5. N-*trans*-Cinnamoylimidazole titration of these solutions, which contained class II contaminants in amounts giving almost total inhibition as judged by assay with conventional substrates, showed an extremely high percentage of active sites (>90%). Thus N-*trans*-cinnamoylimidazole reacts with chymotrypsin even when class II contaminants are present in excess. Whatever the explanation for this remarkable behavior may be, this reagent fails as a test for active enzyme when class II contaminants are present.

We have seen that class II contaminants leave a long G-25 Sephadex column well after class I contaminants and very far after the protein. They must thus have small molecular weights. In addition, we have seen that class II contaminants do not themselves block the imidazole of His-57 since these contaminants have no effect on the CT relaxation times in the absence of indole. For example, batch CDI-6084-5 (a completely nonblocking batch with normal τ^{-1} values) gave a τ^{-1} *vs.* protein concentration plot identical with one obtained with Sephadexed CT at pH 7.5. The class II contaminants thus affect neither the His-57 direct-transfer reaction nor the indicator ionization itself. Class I contaminants, of course, will always decrease the relaxation time if present in detectable concentrations since the ionization of their amino groups will contribute to the CT protonic relaxations. Class II contaminants are efficient inhibitors of the catalytic process through a binding reaction with protein which is slow in both forward and reverse directions.

Under our usual sets of experimental conditions we find that the contaminants have negligible effects on both optical rotatory dispersion patterns and thermodynamic changes in transition I for CT when present in concentrations now found in commercial preparations. ORD parameters measured under a variety of concentrations and pH conditions and from 300 to 500 mμ for batch CDI-6114-5[16] were identical both before and after Sephadex treatment. At pH 2.5 (HCl) and 27°, for example, $-[\alpha]_{350}$ for all samples tested was 290 ± 5° while at pH 7.0 and 27° in the presence of 0.05 *M* phosphate buffer $-[\alpha]_{350}$ was 259 ± 4°. The ORD

(16) Additional samples of this batch were obtained from a number of other laboratories around the country, and they gave results identical with our sample of the batch in all types of test experiments for purity. This demonstrates that the batch itself was contaminated, rather than just a few isolated samples of the batch.

errors are somewhat too large to exclude a small effect of contaminants but a very large number of experiments would be necessary to demonstrate any such effect.

Within usual experimental errors transition I at pH 2.00 (±0.05) demonstrated identical transition temperatures (T_0 = 32.5 ± 0.2°), enthalpy changes ($\Delta H°$ = 89 ± 5 kcal/mole), and entropy changes ($\Delta S°$ = 291 ± 15 eu) for both Sephadexed and non-Sephadexed CDI-6114-5 and several other batches tested.

Although further studies of ORD or transition I data with non-Sephadexed material would be unwise, our results provide no reason to doubt the quantitative reliability of data obtained in such experiments with contaminated batches.

Discussion

Contamination and Purification. All commercial CT preparations contain impurities. The contaminants are of at least two classes. The first class yields high values of τ^{-1} but does not prevent indole blocking. These contaminants have high conductivities and have no detectable inhibitory effect on the enzyme. They are probably amino acids or peptides produced by autolysis and may be of little concern except in studies of the protonic processes of the enzyme. The major effect of the member or members of the second class is to prevent blocking by indole and related compounds. They exhibit a strong inhibiting effect on the catalytic activity of the enzyme and have relatively low conductivities. Members of this class are dissociated in a slow and incomplete fashion at low protein concentration so as to produce spurious values of k_3 with ester substrates and thus highly unreliable values of ΔH_3^* and ΔS_3^*. The contaminants of this class promise to be a useful source of information regarding the catalytic process with CT. However, at present they provide an important limitation to the interpretation of data obtained with contaminated CT. Fortunately, all types of contaminant are easily removed using the Sephadex procedure given in the Experimental Section. Positive evidence of the efficacy of this treatment is at present obtainable only from temperature-jump studies. Steady-state kinetic values for K_m and k_3 may also be used as a check for the presence of impurity. If, under the standard conditions described in the text, K_m = 9.5 (±0.4) × 10^{-5} and k_3 = 45.5 (±0.4) sec^{-1}, the contaminants may have been removed. Because of the slow dissociation of the contaminant of the second class at low enzyme concentrations, steady-state kinetic parameters agreeing with these values do not necessarily prove that a CT sample is pure. However, if k_3 and K_m differ significantly from these values, either small contaminants remain or there is additional contamination with other types of CT.

Temperature-jump tests for CT purity are simpler, more convenient, and more reliable when equipment is available. Under the standard set of experimental conditions noted in the text, experiments should be run on a CT batch both in the absence of indole and in the presence of a saturating concentration of indole (~5.0 × 10^{-3} to 10^{-2} *M*). A high τ^{-1} value (≫3.12 × 10^4 sec^{-1}) in the absence of indole indicates the presence of contaminants of the first class. The extent of the prevention of blocking by indole measures the amount of contaminants of the second class present. Available evidence suggests that these tests are independent of each other, but experiments with known concentrations of contaminants of the second class are necessary to confirm this independence. As an example, Armour B13406 and Worthington CDC-52 (see Table I) gave high τ^{-1} values but also showed some blocking by indole. Worthington CDI-6114-5 (no Sephadex treatment), however, also gave a high τ^{-1} value but showed no blocking by indole. Worthington CDI-6121-2 gave the normal τ^{-1} value and was 80% blocked by indole. Worthington CDI-6084-5 gave a normal τ^{-1} value and showed no blocking by indole. It is probable that one particular class of impurities (class II) is responsible for the prevention of blocking by indole, and one or more other impurities (class I) are responsible for the increase in τ^{-1} values above the normal value of 3.12 × 10^4 sec^{-1}. The high τ^{-1} values suggest the presence of imidazole groups or α-amino groups in the first class of contaminants as does the buffer effect of class I contaminants in the pH 8 region.

The failure of the N-*trans*-cinnamoylimidazole test to detect class II contaminants is less distressing than might appear since we have never found the Sephadex treatment to fail. Indeed, it is quite remarkable that the many different batches of material, including samples from several suppliers, always gave identical results in the temperature-jump tests and in the steady-state studies after Sephadex purification. Protein heterogeneity, if it exists, must not appear in these tests. Thus, until a more convenient test for contamination can be provided, it is reasonable to proceed on the assumption that CT treated in the manner described is clean. Furthermore, our results to the present indicate that once this procedure has been carried out, the second class of contaminant does not reappear with storage. The first class probably contains hydrolysis and autolysis products which will always accumulate in time except on cold storage of water-free material.

An implication of our results is that contaminants of the second class are introduced during preparation, and since the chymotrypsins are usually prepared by activation of chymotrypsinogen, it is possible that all chymotrypsins as well as the zymogen contain contaminants in varying proportions. An improbable alternative is that this type of contaminant is one or both of the two peptides, Ser-Arg and Thr-Asn, produced on activation of chymotrypsinogen to α-chymotrypsin.

That the observed contamination is due to π-CT or δ-CT, two of the intermediates formed in the preparation of CT from chymotrypsinogen, is unlikely since G-25 Sephadex (molecular weight exclusion limit 5000) would not be able to separate these intermediates from CT—all having molecular weights close to 25,000.

Conventional commercial purification procedures—dialysis and lyophylization—are not particularly effective in removing the bound contaminant although prolonged and extensive dialysis might eventually remove most of the impurities. The steady-state kinetic studies demonstrate that dilution is not an effective means of removing the nonblocking impurity.

Precautions should always be taken so that experimental work is carried out under conditions such that the autolysis of CT will be minimized. It is well

known that CT autolyzes quite rapidly at neutral pH values, particularly at high concentrations and at temperatures above 25°. In our experience, we have found that autolysis products may accumulate over a period of days at pH's as low as 3.00 and temperatures as low as 3°, even in fairly dilute solutions. It is probable that autolysis also occurs in the moist solid for CT samples having even a relatively low water content. Chromatography at pH's near neutrality should particularly be avoided since it may lead to more contamination than is removed. We have found that some commercial CT batches chromatographed near neutrality contain at least 30% autolysis products. These products are usually too small to be detected in starch–gel measurements but can give very unreliable catalytic data. Erlanger, *et al.*,[17] have provided evidence for the heterogeneity of CT at higher pH values. This heterogeneity may exist, but we have not been able to detect it in the presence of autolysis products and aggregation products.

The Contaminant–Protein Reaction. The steady-state kinetics parameters (Table III) show that the second class of substance T contaminants behaves as a noncompetitive inhibitor, *i.e.*, both slope and intercept of Lineweaver–Burk plots are increased by the same factor relative to the results obtained with pure CT. The K_m values are thus essentially independent of the amount of contamination. The fact that the contaminant is readily removed by Sephadex demonstrates that the interaction with the protein is reversible though the steady-state kinetics results demonstrate that removal is slow. According to a theory of Morales[18] noncompetitive behavior can appear with moderate or small free energies of binding and rapid equilibration of the enzyme inhibitor reaction only if K_m is a thermodynamic quantity. Such is known not to be the case for the ATrEE–CT system.[14] Hence the apparent noncompetitive behavior of the second-class contaminant must be due to the slow dissociation rate of the contaminant–CT complex. It is also obvious that the complex has unusually high stability. These conclusions do not, of course, prove that the contaminant, the substrate, and indole are all bound at the same site on the protein in the same way. It appears reasonable to assume that the substrate and indole do occupy parts of the same site and since the contaminant prevents normal substrate binding either by occupying the substrate site or by a linkage between contaminant site and substrate site, the contaminant also prevents indole binding. If the latter is true, it should be possible to find conditions for temperature-jump studies which would lead to detectable quantitative differences depending on the interaction of indole with contaminant. These conditions are unfortunately not easy to obtain, but it is significant that the χ-squares fit of the indole binding data for CDI-6121-2 (not Sephadexed) analyzed on the basis that contaminant prevents indole binding is substantially lower than the value obtained on the assumption that it does not prevent indole binding but only prevents the indole–imidazole interaction. It is also noteworthy that the indole inhibition constant (K_i) is independent of the degree of contamination (Table III) which is to be expected, since the net effect of the contaminant is to reduce the participating concentration of CT molecules. There thus seems to be little question about the mechanism of the interaction though we cannot distinguish between a single binding site and a pair of linked sites.

The most noteworthy characteristic of the binding of the class II contaminant is that neither imidazole group is blocked from participation in the direct-transfer reaction with indicator. This difference in behavior between the contaminant and indole may be a relatively uninteresting consequence of differences in geometry of the two molecules. However, it is possible that the difference may be a consequence of profound variation in the binding reactions. Vaslow and Doherty[19] distinguished two kinds of binding reactions with remarkably different thermodynamic characteristics. We have extended the pH range beyond that explored by these authors to confirm the general pattern of their results and also to show that the binding of indole, for example, is accompanied by remarkably large thermodynamic changes which can only represent large changes in the conformation of the protein. Indole binding is thus an example of one kind of reaction. Presumably these changes are responsible for the loss of the imidazole–indicator direct-transfer reaction at His-57. On the other hand, there are small molecules which are competitive inhibitors but are bound in such a way as to produce small enthalpy and entropy changes and thus presumably small changes in the protein. We shall report on these matters and their relevance to the catalytic process soon, but we note that the contaminant which prevents blocking by indole is probably of the latter type and not of the indole type. Thus, the binding of this contaminant would not involve any major displacement of His-57, whereas indole binding forces this imidazole group to take a buried position even if the indole group is not bound very near to His-57. We have found a strong linkage between the protonic processes of this imidazole group and indole binding.

The insensitivity of the N-*trans*-cinnamoylimidazole (CinIm) reaction to the presence of contaminants is both puzzling and interesting. There are several possible explanations for this behavior: (a) CinIm is able to displace the tightly bound contaminant; (b) CinIm is not bound in its Michaelis–Menten complex at the same site as either more normal substrates or indole; (c) CinIm can by-pass the Michaelis–Menten complex step and form an acyl-enzyme directly. In view of the fact that even *p*-nitrophenyl acetate forms the acyl-enzyme *via* a Michaelis–Menten complex (c) appears unreasonable, although the experiments with high class II contaminant concentration seem to favor alternative c. It is thus not possible at this time to make any selection from among the above alternatives. Presumably this problem like many others remaining in the present work will be susceptible to solution when the second-class contaminant is identified and can be added under stoichiometric control to the reacting CT system.

Indole Binding. The first-order binding of indole to CT can be represented by eq 7

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$$CTI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CT + I \qquad (7)$$

$$K_i = \frac{k_1}{k_{-1}} = \frac{[CT][I]}{[CTI]}$$

where [I] is the free indole concentration, [CTI] is the concentration of complex formed, [CT] is the concentration of uncomplexed CT, and K_i is the dissociation

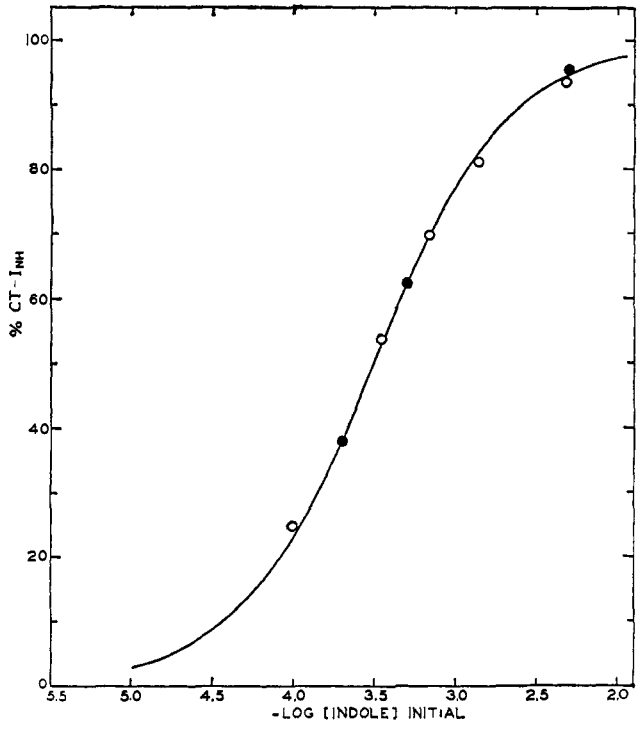

Figure 7. First-order binding of indole to purified CT. Experimental conditions: 8×10^{-5} *M* CT, phenol red = 4.6×10^{-5} *M*, 0.1 *M* KNO_3, pH 7.5, 10° (upper temperature-jump temperature). Solid line gives theoretical curve for $K_i = 2.73 \times 10^{-4}$ *M*, measured at 3° (see Discussion section). Points are calculated from experimental data: ○, CDI-6114-5 after Sephadexing; ●, CDI-6121-2 after Sephadexing.

inhibition constant. The temperature-jump kinetic results shown in Figure 3 allow a calculation of the indole inhibition constant for purified CT at pH 7.50. Using values of $\tau^{-1} = 3.12 \times 10^4$ sec^{-1} for pure CT in the absence of indole and $\tau^{-1} = 2.06 \times 10^4$ sec^{-1} (theoretical[13]) for the pure CT–indole complex (CT with one imidazole completely blocked), the data in the lower curve of Figure 3 (Sephadexed CDI-6114-5 and Sephadexed CDI-6121-2) could be analyzed according to eq 7 using a least-squares procedure to calculate K_i. The results of this calculation are shown in Figure 7. The least-squares value for K_i determined from the data was 2.73 (±0.06) × 10^{-4} *M* at pH 7.50. It should be pointed out that this K_i value refers to a temperature of 3° (lower temperature-jump temperature) rather than to 10° (upper temperature-jump temperature). All of our experimental evidence to date suggests that the dissociation rate of indole from CT is extremely slow so that the effective amount of indole bound to CT after a temperature jump from 3 to 10° remains essentially unchanged for at least a period of about 5 msec or longer. Since the relaxation of the protonic processes which are monitored (and whose lengthened relaxation times are a measure of the amount of indole bound to CT) is over in about 100 μsec, it is evident that the measured K_i values must refer to the lower temperature-jump temperature rather than the upper. Figure 7 also demonstrates that the binding of indole to CT is completely first order in indole concentration and that the binding is affecting only one of the CT imidazole groups (His-57).

Conclusion. Tests to determine the identity of the contaminants of class II are under way and the results of these tests will be presented in a forthcoming publication.

For the present time, we would strongly recommend that all users of chymotrypsin purify their samples before use by gel filtration on G-25 Sephadex in the manner described, particularly since no commercial sample tested showed complete freedom from contamination. At this time, we must conclude that previously reported quantitative information obtained for CT reactions by steady-state kinetics studies are to be considered unreliable unless adequate purification procedures have been employed. Our results thus far suggest that, in particular, temperature and pH studies of kinetic parameters are likely to be most in error as a result of the contaminants.

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