Jack Bean Urease (EC 3.5.1.5). Demonstration of a Carbamoyl-Transfer Reaction and Inhibition by Hydroxamic Acids*

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ABSTRACT: A carbamoyl-transfer reaction has been demonstrated in the urease-catalyzed hydrolysis of urea, using a thymol blue buffer to give a sensitive spectrophotometric measure of acidity changes. This demonstration offers further support for the proposition that carbamate is the first product of the urease-catalyzed hydrolysis of urea. The inhibition of urease by acetohydroxamic acid and chloroacetohydroxamic acid has been studied from both directions and found to be completely reversible. Hydroxyurea, itself a substrate, shows a time-dependent inhibition, completely reversible on consumption of hydroxyurea. Electron microscopy suggests that there are six subunits per 483,000 molecular weight species.

Jack bean urease (urea amidohydrolase, EC 3.5.1.5) has a cherished place in the enzymologist's heart, since it was responsible for the death blow to the proposition that the protein was merely a carrier of the catalytic species (Northrop, 1961). Nonetheless, the enzyme remains ill understood and is deserving of a detailed and intensive mechanistic attack.

Sumner (1951) described urease as "absolutely specific" and until recently only two additional substrates, hydroxyurea and dihydroxyurea, have been found for the enzyme (Davidson and Winter, 1963; Fishbein and Carbone, 1965; Fishbein *et al.*, 1965b; Fishbein, 1967). The enzyme is probably the more remarkable for its enormous efficiency. It has a k_{cat} for the hydrolysis of urea which is about two orders of magnitude greater than the k_{cat} for any peptidase in the hydrolysis of other carboxamides. It is certain therefore that the enzyme enjoys efficient chemistry which may well be different from that of the SH proteinases.

The molecular weight of urease is about 480,000 (483,000: Sumner *et al.*, 1938; 473,000: Gorin *et al.*, 1962), but in spite of extensive investigation, the subunit structure of the oligomer is less than clear (Hellerman *et al.*, 1943; Desnuelle and Rovery, 1949; Ambrose *et al.*, 1951; Creeth and Nichol, 1960; Gorin *et al.*, 1962; Reithel *et al.*, 1964; Gorin and Chin, 1965). The species of mol wt 480,000 contains ~47 sulfhydryl groups (Gorin and Chin, 1965) and various workers have estimated that 4–8 of these are essential for activity. If the highest reported value is taken, this indicates a subunit of mol wt ~60,000. While evidence for species of molecular weight less than 480,000 has been obtained under a variety of conditions (Sehgal *et al.*, 1965; Gorin and Chin, 1967; Stewart and Craig, 1966; Blattler *et al.*, 1967), there is no substantial report of an isolated active species of molecular weight less than ~240,000 (Blattler *et al.*, 1967).

Hydroxamic acids have been reported to be highly specific and potent inhibitors of sword bean, jack bean, and bacterial urease (Kobashi *et al.*, 1962, 1966; Gale, 1965; Fishbein and Carbone, 1965; Hase and Kobashi, 1967). Kobashi *et al.* (1962) reported that the inhibition of sword bean urease was reversible, while Fishbein and Carbone (1965) state that the inhibition of jack bean urease by acetohydroxamic acid is "irreversible, complete and probably noncompetitive." Further, Fishbein *et al.* (1965a) have reported that bacterial urease is also irreversibly, noncompetitively inhibited by acetohydroxamic acid. We have therefore reexamined the inhibition of jack bean urease by hydroxamic acids and the initial results of these investigations are reported in this paper.

Sumner *et al.* (1931) first convincingly demonstrated the absence of cyanate and the formation of ammonium carbamate in the products of the urease-catalyzed hydrolysis of urea. Sumner stated: "There is no reason to doubt that ammonium carbamate is an intermediate product of the action of urease on urea, but it is not necessarily the first intermediate product." Later, he positively inclined to the view that the carbamate is synthesized from carbon dioxide and ammonia (Sumner and Somers, 1953).

Support for the view that ammonium carbamate is the first free intermediate comes from the work of Wang and Tarr (1955), Gorin (1959), and Jencks (1963). Such a product logically arises from a carbamoyl-transfer reaction, although attempts to demonstrate the formation of a carbamoyl-enzyme intermediate ($H_2NC(=O)$-urease), which was postulated as early as 30 years ago (Brandt, 1937), have so far failed. In this paper, we also report results which provide additional direct support for a carbamoyl-transfer reaction, by using a thymol blue buffer to give a sensitive spectrophotometric measure of acidity changes in the enzymatic hydrolysis of a limited amount of urea.

* From the Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia. *Received October 24, 1968.* This work was supported by the A. R. G. C. (Australia) and Grant GM 13759 from the Institute of General Medical Sciences of the U. S. National Institutes of Health.

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TABLE I: pH Functions for the Hydrolysis of Urea.

Products	pH Function	Value of the pH Function at pH 9.05 and 25°[a]
$H_2NCOOH + NH_3$	$f_N - f_D$	−0.3886
$CO_2 + 2NH_3$	$2f_N$	+1.2218
$H_2CO_3 + 2NH_3$	$2f_N - f_C$	+0.1738

[a] A value of −1.0 (say) signifies that 1 g-atom of protons is released per mole of urea hydrolyzed.

The question to which we seek an answer is whether urease catalyzes the hydrolysis of urea according to eq 1, in which carbamate is the first product, or according to eq 2, in which CO_2 and NH_3 are the first products.

$$\text{urea} \xrightarrow[H_2O]{\text{urease}} \underset{NH_3}{H_2NCOOH} \xrightarrow{H_2O} \underset{2NH_3}{CO_2} \xrightarrow{H_2O} \underset{2NH_3}{H_2CO_3} \quad (1)$$

$$\text{urea} \xrightarrow[H_2O]{\text{urease}} \underset{2NH_3}{CO_2} \xrightarrow{H_2O} \underset{2NH_3}{H_2CO_3} \quad (2)$$

Hydrolysis of Urea in the Thymol Blue Buffer. The hydrolysis of 1 mole of urea to ($H_2NCOOH + NH_3$), to ($CO_2 + 2NH_3$), or to ($H_2CO_3 + 2NH_3$) at a fixed pH is accompanied by the release or uptake of x g-atoms of hydrogen ion determined by the appropriate pH functions. The fractional dissociation constants of carbonic acid, f_C, and carbamic acid, f_D, are defined by eq 3 and 4, where K_1 and K_2 are the first and second

$$f_C = (1 + 2K_2/[H^+])/(1 + [H^+]/K_1 + K_2/[H^+]) \quad (3)$$

$$f_D = K_4/(K_4 + [H^+]) \quad (4)$$

apparent dissociation constants of carbonic acid and K_4 is the dissociation constant of carbamic acid. The fractional protonation of ammonia, f_N, is defined by eq 5, where K_3 is the dissociation constant of ammonium ion.

$$f_N = [H^+]/(K_3 + [H^+]) \quad (5)$$

The stoichiometry of the formation of the particular products requires the use of the pH functions shown in Table I. Literature values of K_1, K_2, and K_3 are listed in the preceding paper (Blakeley *et al.*, 1969) and the only value available for K_4 is 1.8×10^{-6} M at 0° (Roughton, 1941). The numerical values of the pH functions at pH 9.05 and 25° are also given in Table I.

When the system is buffered by an indicator only, changes in acidity may be observed directly in the absorbance of the buffer (Eigen *et al.*, 1960). Under suitable experimental conditions, the hydrolysis of urea catalyzed by urease can be approximated by two sequential first-order reactions

$$A \xrightarrow{k'} B \xrightarrow{k''} C \quad (6)$$

where A represents *either* urea or ($H_2NCOOH + NH_3$), B represents ($CO_2 + 2NH_3$), and C represents ($H_2CO_3 + 2NH_3$). The effective molar absorptivity of each species (A, B, or C) is that of the buffer anion (at a wavelength where the buffer acid has a negligible absorbance) multiplied by the pH function for the formation of that species from neutral urea; thus the effective molar absorptivity of a species can in principle be positive, negative, or zero. Bender and Zerner (1962) showed that if $\epsilon_A < \epsilon_B > \epsilon_C$, then the absorbance *vs.* time curve will in general exhibit a maximum followed by an exponential decay. The equation for the time of maximum absorbance reduces in the present system to

$$t_{max} = \ln [1 + (f_N + f_D)(k' - k'')/k''f_C]/(k' - k'')$$

when A is ($H_2NCOOH + NH_3$) and to

$$t_{max} = \ln [1 + 2f_N(k' - k'')/k''f_C]/(k' - k'')$$

when A is urea.

The change in absorbance (Δ absorbance) due to chemical reactions is

$$\Delta \text{ absorbance} = \epsilon A_0[(f_N - f_D)A/A_0 + 2f_N B/A_0 + (2f_N - f_C)C/A_0]$$

for eq 1, and

$$\Delta \text{ absorbance} = \epsilon A_0[2f_N B/A_0 + (2f_N - f_C)C/A_0]$$

for eq 2, where A_0 is the initial concentration of urea and ϵ is the molar absorptivity of the basic form of thymol blue at 648 mμ. The dimensionless concentration parameters A/A_0, B/A_0, and C/A_0 at a given time may be determined from the general solution for two sequential first-order reactions (Frost and Pearson, 1961) and particular values of k' and k''. Application of this theory allows a ready distinction between the hydrolysis of urea according to eq 1 or 2.

Because hydroxyurea is both a substrate and also produces a time-dependent inhibition, its hydrolysis was also studied. The hydrolysis of hydroxyurea according to eq 7 requires a new term, f_P, given by eq 8,

$$H_2NC(=O)—NHOH + 2H_2O \longrightarrow NH_3 + NH_2OH + H_2CO_3 \quad (7)$$

$$f_P = [H^+]/(K_5 + [H^+]) \quad (8)$$

where K_5 is the dissociation constant of hydroxylammonium ion (1.07×10^{-6} M at 25°: Bissot *et al.*, 1957). For each mole of hydroxyurea hydrolyzed $-(f_N + f_P - f_C)$ gram-atoms of hydrogen ion are produced at a given

pH. Carbamic or hydroxycarbamic acid production would each have its own particular pH function; however, on the concentration and time scale of the experiment performed, there would be little carbamate present.

Experimental Section

Materials. Urease was prepared, stored, and assayed as previously described (Blakeley *et al.*, 1969). A portion was dialysed into the thymol blue buffer (see below) giving a stable, fully active stock solution containing 8.5×10^3 IU/ml. The activity of urease is essentially unaffected by 0.4 mM thymol blue when assayed against 50 mM urea at pH 9.0 and 38°.

Urea was recrystallized from 70% ethanol (Steinhardt, 1938). Hydroxyurea was recrystallized to constant melting point from warm methanol, giving colorless crystals with mp 136.4–136.8° dec, lit. (Boyland and Nery, 1966) mp 140–142° dec.

Glassware was cleaned in chromic acid, rinsed thoroughly with distilled water, and steamed or boiled in distilled water.

Acetohydroxamic acid was prepared by the method of Fishbein *et al.* (1965b) and after three crystallizations from ethyl acetate had mp 87–88°, lit. (Fishbein *et al.*, 1965b) mp 88°. α-Chloroacetohydroxamic acid was prepared by the procedure of Jones and Werner (1917) and crystallized from acetic acid–chloroform and then from ethyl acetate, giving mp 106–107°, lit. (Jones and Werner, 1917) mp 108–108.5°. The material was used immediately after recrystallization. The hydroxamic acids were characterized by means of a modified Hestrin procedure (Bernhard *et al.*, 1964); the colored species from acetohydroxamic acid had a molar absorptivity of 1025 and that from chloroacetohydroxamic acid had a molar absorptivity of 928.

p-Nitrophenyl *N*-hydroxycarbamate was prepared from distilled *p*-nitrophenyl chloroformate (Anderson and McGregor, 1957) and hydroxylamine hydrochloride in moist ether in the presence of potassium carbonate. After evaporation of the ether phase, the material was recrystallized from warm chloroform and from unheated ethyl acetate–petroleum ether (bp 40–60°), giving colorless crystals with mp 122–123° dec. The infrared spectrum in a KBr pellet resembles that of *p*-nitrophenyl carbamate (J. K. Stoops, unpublished results) in the region 250–1800 cm^{-1} but in addition has major peaks at 1510 and 1276 cm^{-1}. The hydroxycarbamate has an intense broad absorption at 3330 cm^{-1}. *Anal.* Calcd for $C_7H_6N_2O_5$: C, 42.43; H, 3.05; N, 14.14. Found: C, 42.79; H, 3.40; N, 13.58.[1]

Thymol Blue Buffer. Thymol Blue (British Drug Houses) was recrystallized from acetic acid (Kolthoff and Rosenblum, 1937) and dried *in vacuo* over P_2O_5 and KOH. At 648.0 mμ, the molar absorptivity, ε, was 5783 in 0.1 N sodium hydroxide and <1 in 0.1 M acetate at pH 5. The thymol blue buffer contained 6.06×10^{-4} M thymol blue, 0.1 M KCl, 5 μM dithiothreitol, 5 μM EDTA, and sufficient sodium hydroxide to give a pH of 8.926 (measured on a Radiometer PHM 4d pH meter). The absorbance at 648.0 mμ of this solution was 1.894, which corresponds to pK'_a of 8.857 for thymol blue, lit. (Kolthoff and Rosenblum, 1937) 8.91. The buffer was quite sensitive to absorption of carbon dioxide since in 2 days of use the pH of the stock solution dropped to 8.750 with a corresponding decrease in absorbance. Aliquots (3.0 ml) of the thymol blue buffer were observed in a Cary 14 spectrophotometer whose cell compartment was thermostated at 25 ± 0.1°. The absorbance of an undisturbed solution was essentially stable for several minutes. However, stirring with a clean, flat-ended glass rod caused an absorbance decrease of 0.031 ± 0.01 absorbance unit, owing, evidently, to uptake of carbon dioxide. This stirring effect and the appropriate dilution factors were taken into account in all measurements of absorbance change upon addition of a reagent. Even though the thymol blue buffer sometimes decreased slowly in absorbance over periods of 10–20 min, the major aspects of the slower kinetic runs are adequately described. The introduction of HCl to a final concentration of 14–27 μM (by means of a 25- or 50-μl aliquot of 1.75 mM standard acid) gave greater than 95% of the expected decreased in absorbance. Similarly, addition of sodium hydroxide to a final concentration of 65 μM gave at least 90% of the expected increase in absorbance. The presence of urease, urea, or hydroxyurea did not affect the sensitivity of the system to addition of standard sodium hydroxide. The pH of the system was evaluated from the absorbance, total concentration, and pK_a' of thymol blue.

Hydrolysis of Urea and Hydroxyurea by Urease in the Thymol Blue Buffer. Aliquots (3.0 ml) of the thymol blue buffer were thermostated at 25 ± 0.1° in the cell compartment of the spectrophotometer. An aliquot of urease in thymol blue buffer was added on a flat-ended glass stirring rod, and the pH was adjusted by addition of an aliquot of standard sodium hydroxide. An aliquot of urea (or hydroxyurea) in boiled-out distilled water was used to initiate the reaction. After the reaction was complete, an aliquot of standard HCl was added, giving an absorbance decrease consistent with the buffer capacity of the products at the appropriate pH values. Changes in absorbance were related to the various products of hydrolysis of urea (or hydroxyurea) by means of the pH functions defined earlier.

Inhibition of Urease by Acetohydroxamic Acid, Chloroacetohydroxamic Acid, and Hydroxyurea. Urease (final concentration, $2.9–4.6 \times 10^{-10}$ M) was equilibrated at 38° in 3.00 ml of 0.02 M phosphate buffer (pH 7.0), 1 mM in EDTA and 1 mM in mercaptoethanol. Urease activity was measured by assay of suitable aliquots (25–200 μl) (Blakeley *et al.*, 1969). Inhibition studies were initiated by the addition of an aliquot of an aqueous stock solution of hydroxamic acid. The enzyme activity, which decreased to an equilibrium value, was assayed continually. In the assay procedure, the urease and inhibitor were diluted 50–400-fold, at which concentrations the rate of inhibition was insignificant on the time scale of the assay. During the

[1] Microanalyses were performed by the Australian Microanalytical Service, Melbourne, Victoria.

assay, the pH-Stat trace showed a slight increasing activity owing to the dissociation of the enzyme–inhibitor complex, but the measured initial rate was not affected by inclusion in the assay of chloroacetohydroxamic acid at the concentration used in the inhibition experiment. This demonstrates that dissociation of the enzyme–inhibitor complex during the assay does not significantly affect the measured initial rate. While single determinations were made during the approach to equilibrium, replicate assays of the uninhibited enzyme and the systems at equilibrium were generally made. The uninhibited enzyme suffered no detectable loss of activity (±1%) at 38° in 25 hr.

In a separate series of experiments, urease was inhibited by relatively high levels of hydroxamic acid (~125 μM). When the activity had decreased to the equilibrium value, the solution was gel filtered at 4° on a column (1 × 15 cm) of Sephadex G-25 which had been equilibrated with the phosphate buffer described previously. An effluent fraction containing enzyme–inhibitor complex of suitable activity was selected and equilibrated at 38° and the increase in activity was followed by periodic assay.

The reaction of hydroxyurea (3.22×10^{-4} M) with urease (5.2×10^{-10} M) was studied under the same conditions and with the same assay procedures as the inhibition by hydroxamic acids.

Gel Filtration on Sephadex G-200. A column (1.1 × 45 cm) of Sephadex G-200 was equilibrated at 4° with 0.002 M phosphate buffer (pH 7.1) 2 μM in dithiothreitol, 10 μM in EDTA, and 10 μM in mercaptoethanol at a flow rate of 10 ml/hr; 2 ml of a solution containing 0.012% Blue Dextran 2000 (Pharmacia, Sweden), 0.70 μg/ml of pig liver carboxylesterase (Horgan *et al.*, 1969a), and 2.0 μg/ml of urease (specific activity 4400) were applied to the column within 40 min of dilution from concentrated stock enzyme solutions. Fractions of 1.3 ml were collected in preweighed tubes. Blue Dextran was measured at 620 and 260 mμ using the 0–0.1-absorbance slide wire of a Cary 14 spectrophotometer. Activity of the esterase was determined by spectrophotometric assay at 272.5 mμ with 5×10^{-4} M phenyl butyrate in 0.05 M Tris buffer (pH 7.5) and of urease as previously described (Blakeley *et al.*, 1969). The recoveries of activity were 83% for the esterase and 82% for urease. A second run was made with the application of 2 ml of a solution containing 6.4 mg/ml of urease and 0.08% Blue Dextran. The absorbance of the effluent fractions was measured at 620, 280, and 260 mμ and the A_{280} and A_{260} due to protein were calculated after correction for the A_{280} and A_{260} of the Blue Dextran.

Electron Microscopy. Silicotungstic acid ($SiO_2 \cdot 12WO_3 \cdot 26H_2O$, Baker Analyzed Reagent) was freshly prepared at ice temperature in 1% (w/v) aqueous solution, adjusted with 1 N carbonate-free sodium hydroxide to pH 7.0 ± 0.1, and centrifuged. Urease of specific activity 4480 was diluted to 0.41 mg/ml in a 0.002 M phosphate buffer (pH 7.1), 0.1 mM in EDTA and 0.1 mM in mercaptoethanol. In rapid sequence, a 100-μl portion of the 1% silicotungstate solution was diluted at 4° with three parts of this phosphate buffer, and 100 μl of the resulting solution was added to 50 μl of the urease solution at ice temperature. Approximately 5 μl of this solution was rapidly spotted on a carbon film on a copper grid which was then blotted with filter paper and evacuated in a Siemens Elmiskop 1A electron microscope within 2 min of mixing the enzyme with the silicotungstate. The essence of a successful experiment is the speed with which these operations are performed. Plates (Ilford N50) were exposed at a magnification of 80,000.

Results

Enzymatic Hydrolysis of Urea in the Thymol Blue Buffer. The total hydrolysis of 117.2 μM urea was observed spectrophotometrically at 25°, using urease at 2.0×10^{-7} M and a thymol blue buffer at a final pH of 9.05 to indicate changes in acidity. A maximum in absorbance occurred 10–11 sec after mixing, followed by a decay to a stable value as shown on the Cary trace in Figure 1. The decay in absorbance had a first-order rate constant of 0.11 sec^{-1}. This agrees within 10% with the rate constant for hydration of carbon dioxide under these conditions (0.120 sec^{-1}, Pinsent *et al.*, 1956a) and reasonably allows the decay of absorbance in Figure 1 to be associated with the hydration of carbon dioxide. The final absorbance value was 115% of the theoretical value, calculated by means of the appropriate pH function (Table I).

When the concentration of urease was decreased 41-fold to 4.9×10^{-9} M, no maximum in absorbance was observed. Instead, the absorbance increased according to a first-order rate law to give a final ab-

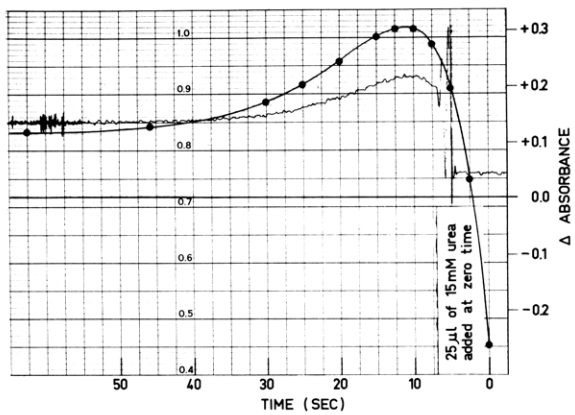

FIGURE 1: Cary 14 trace of the enzymatic hydrolysis of urea in the thymol blue buffer at 25°. [Urea] = 117.2 μM; [urease] = 2.0×10^{-7} M; [thymol blue] = 0.59 mM. The true absorbance at 648.0 mμ of the solution is given by (chart reading + 1.219). The pH ranged from 8.98 (calcd) at zero time, to 9.09 at t_{max}, to 9.05 at the end of reaction. (●—●—●) Points calculated for the system $A \xrightarrow{k'} B \xrightarrow{k''} C$ with $k'' = 0.120$ sec^{-1} and $k'/k'' = 1.2$, where A is ($H_2NCOOH + NH_3$), B is ($CO_2 + 2NH_3$) and C is ($H_2CO_3 + 2NH_3$). The theoretical total absorbance change when urea is hydrolyzed to ($H_2CO_3 + 2\ NH_3$) is $(\Delta A_\infty)_{calcd} = \epsilon(2f_N - f_C)[urea]_0$. The chart reading at zero time was calculated on the basis of the appropriate dilution factor and a decrease of 0.031 absorbance unit due to stirring.

TABLE II: Enzymatic Hydrolysis of Urea in the Thymol Blue Buffer.[a]

[Urea]$_0$ (μM)	10^7[Urease][b] (M)	t_{max} (sec)	$(\Delta A_\infty)_{obsd}/(\Delta A_\infty)_{calcd}$[c] (%)	k_{obsd}[d] (sec^{-1})	k_{H_2O}[e] (sec^{-1})	Final pH[f]
117.2	2.0	10–11[g]	115	0.11	0.120	9.050
117.2	0.049		104	0.0080[h]	0.125	9.071
18.7	2.0	9–12	126[i]	0.11	0.099	8.940
38.1	0.52	17–22	115[i]	0.053	0.103	8.967

[a] The runs were carried out at 25° with a final concentration of thymol blue of 0.59 mM (see Experimental Section). [b] Based on activity measured in the standard assay procedure (Blakeley *et al.*, 1969). [c] See legend to Figure 1. [d] Associated with decreasing absorbance after the maximum had occurred. [e] The rate constant for the hydration of carbon dioxide was calculated at the final pH from data of Pinsent *et al.* (1956a). [f] Evaluated spectrophotometrically. [g] See Figure 1. [h] Associated with absorbance rising slowly to a final value, since no maximum occurred. [i] The small total absorbance change makes these figures approximate.

sorbance value of 104% of theory (Table II). In this case the enzymatic hydrolysis of urea is evidently much slower than subsequent reactions leading to the final products. The observed rate constant was 0.0080 sec^{-1}, from which the value of k_{cat}/K_m may be calculated to be 1.6×10^6 M^{-1} sec^{-1}, using the Michaelis–Menten expression applied at low substrate concentration. This value of k_{cat}/K_m has been approximately confirmed by a pH-Stat study at pH 9 and 25° (R. L. Blakeley and B. Zerner, unpublished results). When the concentration of urea was decreased to 19 μM while the concentration of urease was kept high (2.0×10^{-7} M), the time of maximum absorbance was not changed. Further, the decay of absorbance again had a rate constant consistent with the hydration of carbon dioxide (Table II) although the corresponding absorbance decrease was small (~0.03). When the concentration of enzyme was decreased fourfold (in the presence of 38 μM urea), the time of maximum absorbance doubled and the small absorbance decrease after the maximum had a rate constant (0.053 sec^{-1}) less than that expected for the hydration of carbon dioxide (Table II). Under these conditions $k_{cat}[E]_0/K_m = 0.08$ sec^{-1}, which is entirely consistent with the conclusion that the hydrolysis of urea becomes partially rate limiting at this concentration of urease.

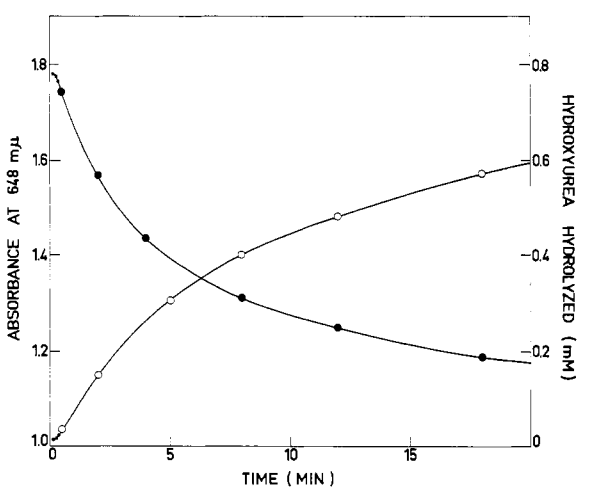

FIGURE 2: Enzymatic hydrolysis of hydroxyurea in the thymol blue buffer at 25°. [Hydroxyurea] = 1.10 mM; [urease] = 2.0×10^{-7} M; [thymol blue] = 0.59 mM. (●—●—●) Absorbance at 648.0 mμ. (○—○—○) Concentration of hydroxyurea that has been hydrolyzed, calculated from (Δ absorbance)/$\epsilon(f_N + f_P - f_C)$. The absorbance at zero time was based on a linear extrapolation of the absorbance after an apparent induction period of about 15 sec and agreed with that expected for dilution and stirring upon addition of hydroxyurea. An appreciable decrease in absorbance (and pH) occurred as the hydroxyurea hydrolyzed, so that individual terms in the pH function were evaluated at each pH. For example, $(f_N + f_P - f_C) = -0.340$ at zero time and -0.184 at 20 min, with corresponding pH values of 8.90 and 8.57, respectively.

Enzymatic Hydrolysis of Hydroxyurea in the Thymol Blue Buffer. The partial hydrolysis of 1.10 mM hydroxyurea was observed spectrophotometrically in the same manner as the hydrolysis of urea. For hydroxyurea, however, there was no maximum in absorbance, but rather the absorbance (and pH) decreased slowly (and continuously) after an apparent induction period of ~15 sec (Figure 2). Because of the long time scale of this experiment, the hydroxyurea was assumed to be completely hydrolyzed to ($H_2CO_3 + NH_2OH + NH_3$), and the amount of hydroxyurea used up was calculated according to the pH function already described.

The rate of hydrolysis of hydroxyurea decreased continuously with time. The decrease is not due to loss of substrate, since addition of more hydroxyurea at 27 min had little effect on the rate of absorbance change. After 15-min reaction with hydroxyurea, the urease was only 20 ± 5% active when assayed against urea on a pH-Stat at pH 7 and 38°. The rate of hydrolysis of hydroxyurea shown in Figure 2 also decreases to about 20% of its initial value in 15 min.

Inhibition of Urease by Hydroxamic Acids. The loss of activity of urease in the presence of acetohydroxamic acid and chloroacetohydroxamic acid is shown in Figure 3. The rate of attainment of and the final extent of inhibition increased with the concentration of the hydroxamic acid. This suggested that the inhibition involved a slowly attained equilibrium as shown in eq 9,

TABLE III: Observed Rate Constants in the Inhibition of Urease by Hydroxamic Acids.[a]

Inhibitor	10^5[Inhibitor] (M)	$10^3 k_{obsd}$ (sec^{-1})	Act. at Equilibrium (%)
Acetohydroxamic acid	0.50	1.18	65.5
	2.50	1.80	33
	4.68	2.63	24.6
	8.96	4.72	20
	12.6	6.1	18
Chloroacetohydroxamic acid	2.30	6.8	62.5
	4.06	9.2	47
	6.48	12.8	40
	12.2	21	32

[a] The conditions are given in the legend to Figure 3 and in the Experimental Section.

TABLE IV: Rate and Equilibrium Constants Associated with the Inhibition of Urease by Hydroxamic Acids.[a]

	Equilibrium Inhibition[b]			Isolated Complex[c]
Inhibitor	$10^5 K$ (M)	k_{+1} (M^{-1} sec^{-1})	$10^3 k_{-1}$ (sec^{-1})	$10^3 k_{-1}$ (sec^{-1})
Acetohydroxamic acid	1.98	42	0.83	0.7_0
α-Chloroacetohydroxamic acid	2.39	144	3.44	$4._1$

[a] Conditions: see legend to Figure 3 and Experimental Section. [b] k_{+1} and k_{-1} were obtained from the slopes and intercepts, respectively, in Figure 4, and $K = k_{-1}/k_{+1}$. [c] k_{-1} as determined from the rate of spontaneous reactivation at 38° of the enzyme–inhibitor complex isolated by gel filtration at 4°.

where I is the hydroxamic acid, E is the enzyme, and EI is an enzymatically inactive complex.

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \qquad (9)$$

Since the concentration of the inhibitor is essentially constant, this can be treated as a simple first-order approach to an equilibrium (Frost and Pearson, 1961) for which $\ln([E]_t - [E]_{equil}) = -k_{obsd}t + c$, with $k_{obsd} = k_{-1} + k_{+1}[I]$. The data gave satisfactory first-order plots and the results are summarized in Table III.

A graph of k_{obsd} *vs.* [I] is linear for both inhibitors (Figure 4), as required by eq 9. The values of k_{+1} and k_{-1} are listed in Table IV along with K, the dissociation constant of the enzyme–inhibitor complex. The value of K (determined from residual activity after a suitable time of reaction) was not affected by a decrease in the mercaptoethanol concentration from 1 to 0.1 mM.

As a check on this interpretation, the inhibited urease was separated from excess inhibitor by gel filtration at 4° and its spontaneous reactivation at 38° was observed. The reactivation was a first-order process, and the values of k_{-1} found by this method are also shown in Table IV. The agreement of k_{-1} determined by the two methods validates the kinetic interpretation of the inhibition by hydroxamic acids.

Urease which had reached its maximum extent of inhibition (<30% residual activity) at a particular level of inhibitor was dialyzed exhaustively at 38° against 0.02 M phosphate (pH 7.0), 1 mM in EDTA and in mercaptoethanol. The recovery of activity was >95% for both hydroxamic acids. An appropriate control showed a loss of activity (6.6%), part of which may be due to dilution.

Inhibition of Urease by Hydroxyurea. The time course of the inhibition of urease (5.2×10^{-10} M) by 3.22×10^{-4} M hydroxyurea is shown in Figure 5. The urease recovered full activity without dialysis.

Gel Filtration on Sephadex G-200. Urease was gel filtered at high and low concentrations on Sephadex G-200 using pig liver carboxylesterase at low concentration and Blue Dextran as markers. The carboxylesterase was eluted in two partially resolved peaks with V_e/V_0 ratios of 1.27 and 1.61 corresponding to species with molecular weights ~160,000 and ~80,000, respectively (Horgan *et al.*, 1969). Urease was eluted in a single symmetrical peak with a V_e/V_0 of 1.10 and 1.05 at high and low concentrations, respectively. At the higher concentration, urease has a sedimentation coefficient of 18.8 S, which corresponds to the 483,000 molecular weight species of Sumner *et al.* (1938).

Electron Microscopy. Electron micrographs of urease showed negatively stained molecules, as in Figure 6A, and molecules slightly darker than the unstained background, as in Figure 6B. Whether or not the molecules were embedded in the negative stain, they frequently appeared to be separated into two halves (Figure 6A). Triangular forms occurred frequently; however, these

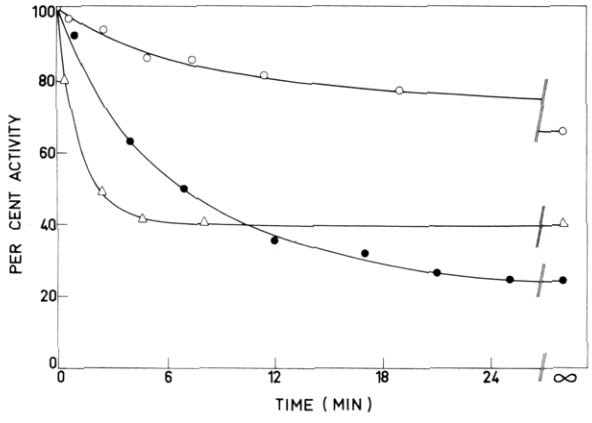

FIGURE 3: Inhibition of urease by hydroxamic acids at 38° in 0.02 M phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM mercaptoethanol with urease at 2.9–4.6 $\times$ 10^{-10} M. (○—○—○) Acetohydroxamic acid (0.50 $\times$ 10^{-5} M); terminal points taken at 64 and 90 min. (●—●—●) Acetohydroxamic acid (4.68 $\times$ 10^{-5} M); terminal points taken at 25 min and 24 hr. (△—△—△) Chloroacetohydroxamic acid (6.48 $\times$ 10^{-5} M); terminal points taken at 7.5 and 30 min.

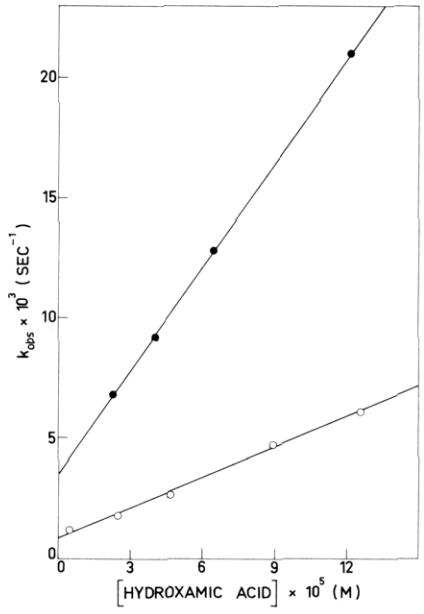

FIGURE 4: Dependence of k_{obsd} for inhibition of urease on the concentration of hydroxamic acid (see Figure 3 and text). (●—●—●) Chloroacetohydroxamic acid and (○—○—○) acetohydroxamic acid.

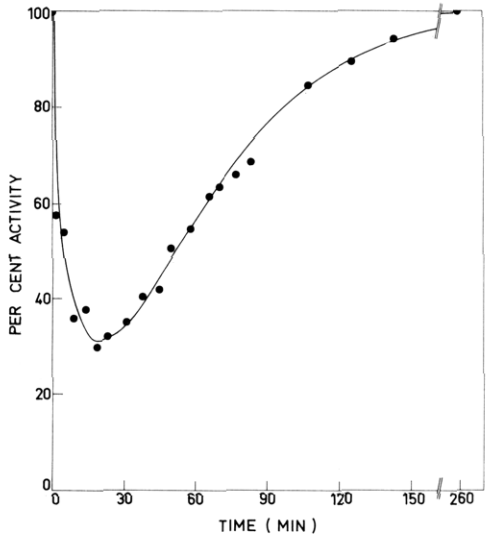

FIGURE 5: Activity of urease (5.2 $\times$ 10^{-10} M) in the presence of hydroxyurea (initially 3.22 $\times$ 10^{-4} M) as a function of time. The conditions are the same as those of Figure 3.

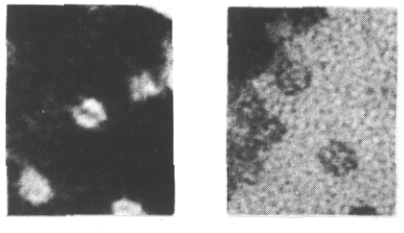

FIGURE 6: Electron micrographs of urease (×375,000).

were resolved only in phosphotungstate stain (R. L. Blakeley and B. Zerner, unpublished results), and in the silicotungstate stain no idea of subunit structure could be obtained from them. Many molecules tended to aggregate in an apparently planar fashion resulting in an unresolved, apparently hexagonal symmetry. In a small proportion of molecules there was clear evidence for subunits (Figure 6B).

Discussion

Enzymatic Hydrolysis of Urea in the Thymol Blue Buffer. Urease catalyzes the hydrolysis of urea according to eq 1, where carbamate is the initial product. The evidence which supports eq 1 and rules out an alternative pathway involving carbon dioxide as the initial product (eq 2) is now summarized: (1) The over-all absorbance change (115%, 104% measured) at the end of the reaction is of the order of 100% of the theoretical (Table II) for complete conversion of urea into (H_2CO_3 + $2NH_3$). This is a sensitive test of the definition and accuracy of the system, since a difference of 0.01 of a pH unit produces a 7% change in the calculated absorbance change. (2) The calculated curve in Figure 1 is derived from eq 6, where A is (H_2NCOOH + NH_3) assumed to be formed instantly from the urea, B is (CO_2 + $2NH_3$), and C is (H_2CO_3 + $2NH_3$). The curve is based on $k'' = 0.12$ sec^{-1} (Pinsent *et al.*, 1956a) at the final pH of 9.05, $k'/k'' = 1.2$, pH functions evaluated at pH 9.05 and 25°, and the calculated concentrations of each species at each time. The agreement between calculated and observed curves would be somewhat better if the pH functions had been evaluated at each pH, *i.e.*, at each absorbance value. (3) From the conditions obtaining in the experiment in Figure 1, it is possible to calculate a first-order rate constant ($k_{cat}[E]_0/K_m$) of 0.32 sec^{-1} for the loss of urea. The assumption that carbamate is *instantly* produced is

therefore a poor approximation, since its rate constant of formation is about twice that deduced for its decomposition ($k' = 1.2$ and $k'' = 0.14$ sec^{-1}; Figure 1). This noninstantaneous formation of carbamate would also contribute to the discrepancy between the observed and calculated curves (Figure 1).

On the other hand, if carbamate were not formed, but instead ($CO_2 + 2NH_3$) were the initial product (eq 2), the maximum value of k' that could account for the observed time of maximum absorbance is ~0.12 sec^{-1}. This is well below the rate constant for disappearance of urea and hence excludes the direct formation of carbon dioxide from urea by the action of urease. (4) At pH 9.05 and 25°, the hydration of carbon dioxide has a rate constant $k_{H_2O} = 0.12$ sec^{-1} (Pinsent *et al.*, 1956a), and carbon dioxide reacts with ammonia to form carbamate with a first-order rate constant $k_{NH_3} = 4.365 \times 10^2(1 - f_N)(\Sigma[NH_3])$ sec^{-1} (Pinsent *et al.*, 1956b), where $\Sigma[NH_3]$ is the total molar concentration of ammonia. The initial concentration of urea was 117.2 μM in the experiment in Figure 1, which gives the value $k_{NH_3} = 0.040$ sec^{-1} when the urea is totally hydrolyzed. Thus k_{NH_3}/k_{H_2O} is 0.33 *at the most*, and to a first approximation, no more than 25% of any initially formed carbon dioxide could be converted into carbamate. Actually, the following equilibrium would be set up on a time scale comparable with that of hydration of carbon dioxide:

$$H_2NCOO^- + H^+ \underset{\text{pH 9.05}}{\overset{K'}{\rightleftharpoons}} H_3N + CO_2$$

Here $K' = (CO_2)(NH_3)/(H_2NCOO^-) = 5.1 \times 10^{-4}$ M, evaluated from rate constants in the two directions at pH 9.05 and 0° (Roughton, 1941; Pinsent *et al.*, 1956b).

If this equilibrium constant holds at 25°, then the ratio $(H_2NCOO^-)/(CO_2)$ would have a maximum value of 0.18 at equilibrium. Thus the maximum carbamate would be about 15% of the total carbon dioxide formed. The rate of attainment of equilibrium would be determined largely by the rate constant for decomposition of carbamate at pH 9.05, which is 0.038 sec^{-1} at 0° (Roughton, 1941) and would be 0.22 sec^{-1} at 25° if it had the same temperature dependence as k_{NH_3}. It is evident from these considerations that there is very little conversion of carbon dioxide into carbamate under the conditions of Figure 1, validating the previous kinetic interpretations. They are also supported by the fact that changing the concentration of urea from 117.2 to 19 μM does not affect the time of maximum absorbance.

The previous work of Sumner *et al.* (1931) at pH ~9 showed the formation of appreciable amounts of carbamate in the enzyme-catalyzed hydrolysis of urea. However, at worst, carbamate would have a half-life of less than 10 sec under the conditions of these workers in the absence of ammonia. Thus it appears that even if carbon dioxide were the initial product of enzymatic hydrolysis of urea, the high concentrations of carbamate would have been found because the large initial concentrations of urea would have rapidly produced a high concentration of ammonia. Gorin (1959) appears to have removed this possibility by using carbonic anhydrase to facilitate the hydration of carbon dioxide. The present work constitutes a more direct demonstration that carbamate is the initial product.

Carbamoyl-Transfer Reaction. The first chemical evidence for a carbamoyl-transfer reaction in the urease-catalyzed hydrolysis of urea was published by Fearon (1936), who demonstrated the formation of biurea during the enzymatic hydrolysis of urea in the presence of semicarbazide. Fearon's result is reasonably interpreted if semicarbazide acts as a nucleophile toward the carbamoyl group of a transient enzymatic species. We are currently checking Fearon's experiments and are investigating the use of other potential trapping agents. While the present results provide good evidence for the transfer of a carbamoyl group to water during the enzymatic hydrolysis of urea, it must be noted that we have obtained no evidence for a carbamoyl-enzyme intermediate ($H_2NC(=O)$-urease) as a *stable* species, although, of course, the above result is not inconsistent with the transient formation of such a species.

Inhibition by Hydroxamic Acids. The present work establishes that inhibition of urease by acetohydroxamic acid and chloroacetohydroxamic acid is fully reversible, in agreement with the report of Kobashi *et al.* (1962), but in contrast to that of Fishbein and Carbone (1965). Formally, at least, the inhibition is competitive, but the magnitude of the dissociation constant of the enzyme–inhibitor complex (~2×10^{-5} M) and the low rate constants for reactivation suggest that a reversible chemical reaction may be involved. These aspects are being further explored.

Because of the strong inhibition by hydroxamic acid, it was hoped that a suitably activated ester group incorporated into a hydroxamic acid molecule might prove to be a substrate for urease. Accordingly *p*-nitrophenyl *N*-hydroxycarbamate was prepared. However, at pH 6 and 25°, urease at 0.34 mg/ml has no detectable effect in 4 min on the slow spontaneous rate of hydrolysis of this compound, whereas under the same conditions urea would have a half-life of ~0.25 sec.

Inhibition by Hydroxyurea. The inhibition of urease by hydroxyurea is complicated by the fact that hydroxyurea is a substrate. While the initial decrease in activity caused by hydroxyurea resembles that of acetohydroxamic and chloroacetohydroxamic acids, the urease inhibited by hydroxyurea slowly regains full activity (Figure 5) as hydroxyurea is depleted.

Since hydroxyurea was slow to reach its maximum extent of inhibition, it seemed possible that its enzymatic hydrolysis might also depend upon this prior, slow association as an inhibitor. The thymol blue buffer was used to monitor the rate of hydrolysis of hydroxyurea according to eq 7.

The data in Figure 2 show that the rate of hydrolysis of hydroxyurea is greatest immediately upon mixing it with urease and then decreases continuously. The decrease in rate of hydrolysis of hydroxyurea was shown not to be due to substrate depletion, and it was paralleled by loss of activity of the enzyme toward urea. This is consistent with the results of Fishbein *et al.* (1965b)

and Fishbein and Carbone (1965) who used a discontinuous assay system. It therefore appears that hydroxyurea is initially a substrate and only slowly brings about inhibition. It is possible that the inhibition is produced by the product of a reaction between hydroxyurea and an enzymatic or other species formed during its hydrolysis. This would require, of course, that the postulated product be a substrate for urease, since the enzyme regains full activity on depletion of hydroxyurea. Alternately, and more simply, the inhibition may be akin to that observed with hydroxamic acids and may therefore arise from hydroxyurea itself. These aspects require further exploration.

Number of Subunits. Qualitative analysis of urease by the Edman and dansylation procedures *indicates* that asparagine is the N-terminal amino acid (J. A. Hinds and B. Zerner, unpublished results) (*cf.* Thompson, 1954).[2] This suggests that the species of mol wt 483,000 is composed of similar subunits. Previous work involving active enzyme has indicated four or eight essential SH groups in this species (Ambrose *et al.*, 1951; Gorin and Chin, 1965). In addition, Kobashi *et al.* (1966) on the basis of inhibition by hydroxamic acids have suggested that the number of active sites in the 483,000 molecular weight species of sword bean urease is 2. There is one previous report, based on ultracentrifugation in 6 M guanidinium chloride (and in which ribonuclease was used as a control protein), which is consistent with six subunits of mol wt ~80,000 (Reithel *et al.*, 1964). Electron microscopy tends to support this result. While it is not possible at present to decide the geometry of the hexameric structure, all electron micrographs so far obtained are not inconsistent with the stacking of two triangular species of mol wt 240,000, or (at least under the conditions of microscopy) the planar arrangement of two such species into a distorted hexagonal form.

While a fully active species of mol wt 240,000 is stable in high concentration at pH 9 (Blattler *et al.*, 1967), no such species was formed *rapidly* at pH 7 with the concentration of urease at only 2 μg/ml under the conditions of the gel filtration experiment.

Other experiments (J. A. Hinds and B. Zerner, unpublished results) have confirmed the observation that the "active site SH groups" are slow to react with *N*-ethylmaleimide (Gorin and Chin, 1965). However, using the Ellman (1959) titration, we have so far been unable to define unequivocally the number of "essential SH groups" in the 483,000 molecular weight species.

The mechanism of action of this enzyme is still unknown, and results obtained during the course of the present investigation probably ask more questions than they answer. Why, for example, are the "active site SH groups" so unreactive? What are the real determinants of a substrate? Why is the enzyme so highly efficient? What is the nature of the interaction with hydroxamic acids? These and other questions lead us to further work on this remarkable protein.

[2] While asparagine is clearly indicated, it must be emphasized that as yet unexplainable quantities of glutamic acid, glycine, and alanine appear when this "pure" protein is analyzed by the Edman procedure.

Acknowledgments

We acknowledge with thanks the indefinite loan of a Cary 14 spectrophotometer from the Wellcome Trust, London. We thank Mr. John Hardy for his assistance with the electron microscopy and Mr. A. J. Anderson and Dr. J. K. Stoops for experimental assistance. A sample of hydroxyurea was kindly provided by the Squibb Institute for Medical Research.

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Carboxylesterases (EC 3.1.1). A Large-Scale Purification of Pig Liver Carboxylesterase*

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ABSTRACT: Two procedures for the large-scale purification of pig liver carboxylesterase are described. They start from chloroform–acetone powders of minced pig liver and involve ammonium sulfate fractionation, chromatography on CM-cellulose and CM-Sephadex, and gel filtration. These procedures produce an enzyme of hitherto unobtained purity. The yield of enzyme is about 250 mg from 800 g of powder.

This is the first of a series of papers concerned with the purification, properties, and mechanism of action of carboxylesterases (carboxylic ester hydrolases, EC 3.1.1) from various animal and plant sources.

There are many reports in the literature on the preparation of mammalian liver carboxylesterases.[1] However, none of the reported procedures yields a large amount of homogeneous enzyme. Burch (1954) reported one of the more successful purifications of horse liver carboxylesterase (EC 3.1.1.1) which used an acetone-dried powder of horse liver as the source of the enzyme. Electrophoresis indicated that the protein was ~95% homogeneous. However, it was contaminated by hematin.

The pig liver enzyme has received most attention. Adler and Kistiakowsky (1962) were the first to report the preparation of a homogeneous sample of the enzyme. However, in our hands this procedure failed to yield a satisfactory preparation. Kibardin (1962a) has also reported the preparation of a "homogeneous" enzyme which he succeeded in resolving into three components (Kibardin, 1962b). His work provides the first real indication of the difficulties encountered in the purification of these enzymes.

Krisch (1963) made a significant advance on previous work. Using an isolated microsomal fraction as starting material, he succeeded in crystallizing the enzyme. However, the reported yield was low (14 mg) and the method does not permit easy scaling up because of the prohibitive times of ultracentrifugation involved.

As part of a broadly based program concerned with the mechanism of action of hydrolytic enzymes, the carboxylesterases were of immediate interest because of their reported similarities to the serine proteinases. For this reason, we required large-scale preparations of highly purified enzymes.

* From the Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia. *Received October 24, 1968.* This work was supported in part by the National Health and Medical Research Council (Australia), the A. R. G. C. (Australia), and Grant GM 13759 from the Institute of General Medical Sciences of the U. S. National Institutes of Health. Preliminary communications: Horgan *et al.* (1966a,b).

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[1] For previous work on these enzymes, see (a) pig: Rozengart *et al.* (1952), Adler and Kistiakowsky (1961, 1962), Kibardin (1962a,b), Krisch (1963, 1966), Boguth *et al.* (1965), Barker and Jencks (1967), and Levy and Ocken (1967); (b) horse: Boursnell and Webb (1949), Connors *et al.* (1950), Burch (1954), and Hofstee (1967); (c) ox: Webb (1948), White (1956), Kirkland (1963), and Benöhr and Krisch (1967a,b); (d) chicken: Drummond and Stern (1961); and (e) sheep: Baker and King (1935).