The Role of Methionine in α -Chymotrypsin-Catalysed Reactions

By J. R. KNOWLES

Dyson Perrins Laboratory, University of Oxford

(Received 23 July 1964)

1. The reaction of α -chymotrypsin with sodium periodate at pH 5.0 has been investigated. The enzyme consumes 2 moles of periodate/mole, and there is a concomitant fall in enzymic activity (with respect to L-tyrosine ethyl ester) to 55% of that of the native enzyme. After 3 hr. no further change is observed in periodate uptake or in catalytic activity. 2. The oxidized enzyme is a homogeneous preparation of partially active chymotrypsin. 3. In the oxidized enzyme, one of the two methionine residues in the molecule has been converted into its sulphoxide. It is this reaction only that is responsible for the loss of activity. 4. The rate constants for the enzyme-catalysed acylation and deacylation reactions are unaltered by oxidation of the enzyme, both for a non-specific substrate (*p*-nitrophenyl acetate), and for three specific substrates: *N*-acetyl-L-tryptophan ethyl ester, *N*-acetyl-L-tryptophanamide and *N*-acetyl-L-valine ethyl ester. 5. The K_m values for the aromatic substrates with the oxidized enzyme are twice those with the native enzyme. No change in Michaelis constant is seen for the non-aromatic substrate *N*-acetyl-L-valine ethyl ester. 6. The evidence points to the oxidized methionine residue in the modified enzyme being situated in the locus of the active site at which aromatic (or bulky) side chains of the substrates are bound.

Most experiments involving chemical modification of amino acid residues at active sites of enzymes result in the production of enzymically inactive material. For example, the complete reaction of α -chymotrypsin with a wide variety of reagents, including phosphoryl halides, sulphonyl halides, carbamoyl halides, halo ketones, acid anhydrides and ultraviolet irradiation, results in the destruction of enzymic activity. The possible deductions from total-inactivation experiments are normally limited to statements that the modified amino acid residue is either directly involved in the catalytic mechanism, that it is close enough to the active site sterically to block substrate-binding or reaction, or that the critical tertiary folding around the active site has been modified sufficiently to abolish the activity. If an enzyme can be minimally modified by a chemical reagent so as to decrease the activity (but not destroy it), studies on the residual activity may define the function of the modified residue, as distinct from simply defining its existence at the active site. The usefulness of this approach is nicely exemplified by the work of Vallee and his co-workers on carboxypeptidase A (Vallee, 1964).

A report by Jansen, Curl & Balls (1951) on the reaction of α -chymotrypsin with periodate appeared to satisfy the requirement for a modified but partially active enzyme. The present paper reports a more detailed study of this reaction, and

the results of some experiments designed to delineate the function of the modified residue in the oxidized enzyme.

MATERIALS

Three-times-crystallized α -chymotrypsin (lot no. CDI 6037-38) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Assays of purity, moisture content etc. are given below.

Sodium metaperiodate was AnalR grade from British Drug Houses Ltd., Poole, Dorset.

L-Tyrosine ethyl ester was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A.

N-Cinnamoylimidazole was prepared according to the method of Schonbaum, Zerner & Bender (1961), and had m.p. 133–133.5° [Schonbaum *et al.* (1961) give 133–133.5°] (Found: C, 72.6; H, 5.2; N, 14.4. Calc. for $C_{12}H_{10}N_2O$: C, 72.7; H, 5.1; N, 14.2%).

N-Acetyl-L-tryptophan was prepared according to the method of du Vigneaud & Sealock (1932), and had m.p. 189–190.5°, $[\alpha]_D^{25} + 29.0 \pm 0.05^\circ$ (c 1 in aq. 40 mM-NaOH). du Vigneaud & Sealock (1932) give m.p. 189–190°, $[\alpha]_D^{25} + 29^\circ$ (c 1 in aq. NaOH).

N-Acetyl-L-tryptophanamide was purchased from Yeda Research and Development Co., Israel (lot no. TR17), and had m.p. 197°, $[\alpha]_D^{25} + 19.5^\circ$ (c 2 in methanol). Huang & Niemann (1951) give m.p. 192–193°, $[\alpha]_D^{25} + 20^\circ$ (c 2 in methanol).

N-Acetyl-L-tryptophan ethyl ester was prepared by the method of Spies (1948), and had m.p. 109.5–110°, $[\alpha]_D^{25} + 7.1 \pm 0.02^\circ$ (c 6 in ethanol). Zerner, Bond & Bender (1964)

give m.p. 110–111.5°, $[\alpha]_D^{24} + 7.1^\circ$ (c 6 in ethanol) (Found: C, 65.2; H, 6.4; N, 10.1. Calc. for $C_{15}H_{18}N_2O_3$: C, 65.7; H, 6.6; N, 10.2%).

N-Acetyl-L-valine ethyl ester was prepared according to the method of Waite & Niemann (1962), and had m.p. 31.5–34.5°, $[\alpha]_D^{25} - 51.7 \pm 0.15^\circ$ (c 2 in water). Waite & Niemann (1962) give m.p. 32.5–34.2°, $[\alpha]_D^{25} - 50.3^\circ$ (c 4.5 in water) (Found: C, 57.5; H, 9.0; N, 7.8. Calc. for $C_9H_{17}NO_3$: C, 57.7; H, 9.0; N, 7.5%).

p-Nitrophenyl acetate was given by Mr A. Williams, and had m.p. 79.5°. Kezdy & Bender (1962) give m.p. 79.5–80.0°.

Acetonitrile was purified by the method of Cooper & Waters (1964).

All buffers were made up with AnalR reagents and freshly distilled water. pH measurements were made on a Radiometer instrument TTT1c with scale-expander PHA630, standardized against British Drug Houses Ltd. standard buffer solutions.

METHODS

Periodate estimation. Periodate uptake was followed by spectrophotometric estimation of the single equivalent of I_2 liberated on the addition of excess of aq. KI solution to the periodate solution in borate buffer, pH 8.1 (Müller & Friedberger, 1902). Since protein interferes with the assay (by reacting with the I_2 formed; see Glazer & Sanger, 1964; Dube, Roholt & Pressman, 1963), it was removed by precipitation with methanol followed by centrifugation. A typical uptake curve was obtained as follows. To 0.5 ml. of 2 mM-enzyme solution in 0.1 M-acetate buffer, pH 5.0, was added at 0°, in the dark, 0.5 ml. of aq. 10 mM-sodium metaperiodate. At suitable intervals, a 50 μ l. sample was withdrawn, 0.95 ml. of pure methanol added, and the solution mixed and then centrifuged for 30 sec. The clear supernatant was poured off, and 0.8 ml. of it pipetted into a stoppered 1 cm. optical cell. Then 1.5 ml. of 0.1 M-borate buffer, pH 8.1, was added, followed by 0.2 ml. of aq. 2% (w/w) KI solution. The extinction at 352 m μ was measured on a Unicam SP.500 spectrophotometer. Extinctions corresponding to initial periodate concentrations were obtained by exactly similar assays in the absence of protein.

The following checks on the method were made: (i) excess of iodate does not interfere with the estimation of periodate at pH 8.1; (ii) neither methanol nor acetate (from the buffer) reacts with periodate at pH 5.0; (iii) no periodate is lost during the precipitation of protein; (iv) neither methanol nor acetate reacts with the I_2 produced at pH 8.1 (i.e., the I_2 observed after protein removal is equivalent to the periodate present in the original sample); (v) protein precipitation by the 20-fold excess of methanol is essentially complete, the observed extinction diminishing only by approx. 10% over 30 min. (the time from initial sampling to extinction reading was commonly less than 3 min.); (vi) check estimates of periodate plus iodate were made by adding to 0.8 ml. of the methanol solution, after removal of protein, 1.5 ml. of 2 N-HCl and 0.4 ml. of KI solution. Under these conditions 4 moles of I_2 are liberated/mole of periodate, and 3 moles/mole of iodate. These estimates agreed well with the known concentrations of iodate and remaining periodate. Points on the uptake curve (Fig. 1) are means from two reactions, duplicate samples being taken in each case. Readings from the same run were

within 3%, and those from different runs were within 6%. This is reasonable, since only 0.025–0.015 μ mole of periodate is being assayed.

Preparation of oxidized α -chymotrypsin. Oxidized α -chymotrypsin was prepared as follows. A 1 g. sample of α -chymotrypsin was dissolved in 20 ml. of 0.1 M-acetate buffer, pH 5.0, at 0°, and added to 20 ml. of aq. 10 mM-sodium metaperiodate solution. The mixture was stirred slowly for 3 hr. in the dark at 0°, and solid $(NH_4)_2SO_4$ was then added to give 75% saturation during 1 hr. at 0°. The protein was spun down and dissolved in the minimum of 20 mM- H_2SO_4 , and solid $(NH_4)_2SO_4$ added to induce crystallization. After 2 days at 4° the material had crystallized, the diamond-shaped crystals being identical in form with those obtained from α -chymotrypsin itself under these conditions. The crystalline oxidized α -chymotrypsin was spun down, dissolved in 20 mM- H_2SO_4 and dialysed exhaustively against distilled water for 3 days at 4°. After centrifugation to remove a very slight cloudiness, the material was freeze-dried.

Samples required for assay of enzymic activity during the course of the oxidation were treated as described above, though the crystallization step was omitted in these cases and the $(NH_4)_2SO_4$ was added over 5 min.

Activity assays. The 'all-or-none' assay employed by Schonbaum *et al.* (1961) with *N*-cinnamoylimidazole was used to obtain operational normalities of enzyme solutions, and as a check that no total inactivation was occurring during the oxidation. In general the commercial enzyme contains about 86% (on a weight basis, assuming a molecular weight of 25000) of 'titratable' active sites by this all-or-none assay, a moisture content of 6.9% and an ash weight corresponding to 0.4%. The 'rate' assay employed by Schwert & Takenaka (1955) with L-tyrosine ethyl ester was used as a measure of enzymic activity. For both these methods a Unicam SP.500 spectrophotometer [with a temperature-controlled (Adkins thermostat) cell compartment, fitted with a photomultiplier and coupled to a pen recorder] was used.

Amino acid analyses. Acid-catalysed hydrolysates were obtained by heating 1 ml. of constant-boiling HCl (twice redistilled)/mg. of protein in a sealed tube, O_2 -free, at 110° for 17 hr. Insignificant amounts of cysteic acid and chlorotyrosines were observed. Base-catalysed hydrolysates were obtained by the procedure of Ray & Koshland (1962). The hydrolysates were analysed with a two-column amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex).

Sedimentation. Sedimentation experiments were performed in a Spinco model E analytical ultracentrifuge at 59780 rev./min. at 20°. The proteins were in concentrations of approx. 8 mg./ml. in 0.1 M-acetate buffer, pH 4.85.

Kinetic determinations. The rates of reaction of α -chymotrypsin and oxidized α -chymotrypsin with the following substrates were determined according to the methods described.

(a) *p*-Nitrophenyl acetate. The rate of the enzyme-catalysed hydrolysis of this substrate under conditions of enzyme saturation was followed spectrophotometrically by the method of Kezdy & Bender (1962). A typical run is described. To 3.0 ml. of 0.1 M-phosphate buffer in a 1 cm. quartz cell was added 50 μ l. of substrate solution in acetonitrile. The change in extinction at 400 m μ due to spontaneous hydrolysis of the substrate was followed for

4 min., and 50 μ l. of aqueous enzyme solution was then added. The change in extinction was followed for a further 6 min. Both parts of the extinction-time plot were linear over the range studied. Enzyme concentrations were calculated from the immediate increase in extinction on the addition of enzyme, assuming ϵ_{400} to be 18350 and pK to be 7.04 for *p*-nitrophenol (Keddy & Bender, 1962). These concentrations agreed very closely with those obtained by the titration procedure of Schonbaum *et al.* (1961) with *N*-cinnamoylimidazole, and with the concentrations calculated on a weight basis, assuming that 86% of the preparation as weighed out is active. Extinction-time curves were obtained in the Unicam SP.500 spectrophotometer.

(b) *N*-Cinnamoylimidazole. The acylation of α -chymotrypsin and of oxidized enzyme by this reagent was followed spectrophotometrically at 355 $m\mu$ according to the method of Schonbaum *et al.* (1961). Low concentrations of reagent (approx. 10 μ M) and high instrument sensitivities were employed to detect the difference in acylation rates.

(c) *N*-Acetyl-L-valine ethyl ester. The kinetics of hydrolysis of this ester catalysed by α -chymotrypsin and by oxidized enzyme were examined with a Radiometer Titrator (TTT1c) coupled to a Titrigraph (SBR2c). The titration cells were 10 ml.-capacity scintillation bottles, which fitted snugly into a thermostatically controlled jacket. A fresh bottle was used for each run. The cell was covered by a polythene cap fitted with the two miniature electrodes (Radiometer G222B and K4312), the titration-base feed capillary, the nitrogen inlet and a 1 mm. hole for the strong-base feed capillary. The electrode assembly could be raised from and lowered into the titration cell rapidly on a ratcheted rack and pinion. Stirring was achieved magnetically. Nitrogen was blown over the surface of the reaction solution, having been bubbled through distilled water in a thermostatically controlled container. A typical run was performed as follows. The titration cell (commonly containing 3.0 ml. of substrate solution and 0.5 ml. of 0.9 M-NaCl solution), which had been thermostatically controlled at the temperature of the run for at least 10 min., was transferred to the jacket, and 1 ml. of enzyme solution, also thermostatically controlled at the temperature of the run, was added. The final volume in the cell was thus 4.5 ml. The electrode assembly was lowered, stirring started, and strong base (1.0 N- or 0.1 N-NaOH) was added through the strong-base feed capillary with the Titrator coupled to a magnetic valve (Radiometer MNV1c) which controlled the addition of strong base. In this way the pH of the solution was brought up to the pH required for the titration quickly and accurately (the pH of aqueous solutions of α -chymotrypsin is between 3 and 5). This method is to be preferred to (a) storing the enzyme at the pH of the run, since α -chymotrypsin is denatured irreversibly fairly rapidly at alkaline pH values (Bender, Clement, Keddy & Heck, 1964a) and later runs will be in error if denaturation has been significant, or (b) raising the pH of the enzyme solution before addition by using a different meter. The method described eliminates inconsistencies between meters, and avoids the wastage of enzyme consequent on raising the pH of enzyme solutions batchwise. When the pH of the solution was that required, the Titrator was coupled to a 0.5 ml. syringe burette (Radiometer SBU1a with B101/0.5) (containing 0.01 N-NaOH, standardized against potassium hydrogen phthalate) and the Titrigraph, for pH-stat operation. The time taken from

initial enzyme addition to the start of the titration proper was commonly less than 30 sec. In every experiment the reaction time was at least 8 min.

Since the Titrigraph traces are slightly curved, initial velocities are difficult to estimate visually with much precision. A computer programme has been written (in Autocode, for a Ferranti Mercury digital computer) along the general lines of those of Abrash, Kurtz & Niemann (1960) and of Elmore, Kingston & Shields (1963). From the experimental results (the co-ordinates of at least nine points on a titration curve) the coefficients of the polynomial best fitting these results are computed. The value of the gradient at zero time is then readily calculated. Unlike the authors mentioned above, I do not use Student's *t* test to decide on the significance of the coefficient for the *n*th-order term, but limit the polynomial to one of order two. By so doing the restriction is placed on the equation that it will only 'fit' non-re-entrant curves. The plot is curved for a variety of reasons (e.g., product inhibition, autolysis of the enzyme, change of volume, change of ionic strength etc.), but I believe that the arbitrary restriction of the best-fit polynomial to a second-order equation eliminates possibly serious errors in the calculated initial velocities, arising from random fluctuations in the titration curves. With as few as nine points (this is also about the number treated by the two groups cited above) this limitation becomes even more necessary. Ideally, perhaps, one should have an *n*th-order polynomial derived from a large (say, more than 30) number of points for each run, but this, for the 14 or so runs necessary for one Lineweaver-Burk plot, is unduly laborious. The computer programme, having obtained all the initial velocities, now calculates the kinetic parameters of the Michaelis-Menten equation:

$$v_0 = k_0[E_0][S_0]/(K_m + [S_0])$$

from a least-squares treatment of a Lineweaver-Burk plot. All values of $1/v_0$ and $1/[S_0]$ are printed out, with values of k_0 and K_m , and the probable errors in these two parameters (Margenau & Murphy, 1956). As a check against truly errant points in the Lineweaver-Burk plot (e.g. teleprinter errors, pipetting errors etc.), the programme then calculates the most deviant point, discards it, and recalculates k_0 and K_m and their errors. This process is repeated until the deviation of each point (in $1/v_0$) is less than 5% of the value of $1/v_0$. In this way any really deviant point becomes immediately apparent. Normally, however, the values of k_0 and K_m calculated from all points are accepted (see relevant Lineweaver-Burk plots).

The errors quoted in values of k_0 and K_m are probable errors from the Lineweaver-Burk plot. They are not (and are not meant to be) measures of accuracy, only of the precision of the experimentation.

(d) *N*-Acetyl-L-tryptophanamide. The enzyme-catalysed hydrolysis of this substrate was followed spectrophotometrically at 306 $m\mu$ according to the method of Bender *et al.* (1964a). A Beckman DB recording spectrophotometer (with a thermostatically controlled cell compartment) was used. In the 'reference' beam of the instrument were two 1 cm. cells in tandem, containing respectively 2.8 ml. of 0.1 M-tris-HCl buffer, pH 7.87, with 0.2 ml. of aqueous enzyme solution, and 2.8 ml. of substrate solution in buffer with 0.2 ml. of water. In the 'solution' beam were also two cells in tandem, containing respectively 2.8 ml. of substrate solution in buffer with 0.2 ml. of enzyme solution, and

2.8-ml. of buffer solution with 0.2-ml. of water. The spectrophotometer was run on the 80–100% transmission range. The value of $\Delta\epsilon$ for the conversion of *N*-acetyl-L-tryptophanamide into *N*-acetyl-L-tryptophan (73.8) was taken from Bender *et al.* (1964a). Transmission percentages were converted into extinctions, and plotted out to obtain initial velocities. These values, with the corresponding substrate concentrations, were fed into a computer programme that just performs the least-squares treatment on the Lineweaver-Burk plot (i.e., the latter half of the programme described above) to yield values of k_0 and K_m .

(e) *N*-Acetyl-L-tryptophan ethyl ester. To obtain results under as similar conditions as possible, the method of Zerner *et al.* (1964) for measuring the kinetics of hydrolysis of this substrate was modified to eliminate acetonitrile from the reaction solution. *N*-Acetyl-L-tryptophan ethyl ester (approx. 1 mM) can be dissolved in water or buffer solution by heating at 80° in a water bath for 2 min. The following checks were made to ensure that none of the ester is hydrolysed during the dissolution. A solution of the ester in water (prepared as described above) was brought to pH 8.0 in the pH-stat, and then placed in a boiling-water bath for 10 min. The solution was cooled and then returned to the pH-stat titration cell. Under these conditions less than 2% of the ester was hydrolysed. This was further checked by the addition of 10 μ l. of 1 μ M- α -chymotrypsin solution and measuring the total uptake of base. The base required for complete hydrolysis was within 2% of that calculated on the basis of the known weight of *N*-acetyl-L-tryptophan ethyl ester in solution. Extinction coefficients of *N*-acetyl-L-tryptophan ethyl ester and *N*-acetyl-L-tryptophan at 300 m μ under the conditions used (in 0.1 M-tris-HCl buffer, pH 7.90) were determined in duplicate. These were: *N*-acetyl-L-tryptophan ethyl ester: 567.7, 566.9; *N*-acetyl-L-tryptophan: 779.2, 782.3. The solutions of the ethyl ester were hydrolysed completely to *N*-acetyl-L-tryptophan (10 μ l. of 1 μ M- α -chymotrypsin was added to 10 ml. samples of the ethyl ester and left overnight) to check with the values obtained from the authentic samples of *N*-acetyl-L-tryptophan. This method gave, for *N*-acetyl-L-tryptophan: 782.5, 787.8. The values used were: *N*-acetyl-L-tryptophan ethyl ester: 567; *N*-acetyl-L-tryptophan: 781; i.e., $\Delta\epsilon_{300}$: 214. The kinetic runs were performed on the Unicam SP.500 spectrophotometer as described above. The 75–100% transmission range was used. Instrument drift during the time of a single run was negligible. To 3.0 ml. of substrate solution maintained at a thermostatically controlled temperature in a 1 cm. optical cell was added 10 μ l. of enzyme solution (approx. 10 μ M) in water. Recording was started less than 10 sec. after mixing. Serial dilutions of the enzyme solution were avoided (Jones & Niemann, 1963) and check experiments were carried out to ensure that the $[E_0]$ (determined from v_0) was indeed proportional (at these high dilutions) to the volume of stock solution taken. The extinction-time plots were curved. This is not unexpected since the reaction is so fast, and the ester soluble with such difficulty, that it is impossible to study only the first few percent of the reaction. For the most dilute substrate solutions hydrolysis was essentially complete within 3 min., and even at the highest substrate concentrations about a quarter of the substrate had been used up after this time. The experimental results were therefore fed directly into a computer programme (adapted to accept 75–100% transmission readings) similar to that

used for the runs with *N*-acetyl-L-valine ethyl ester. The initial velocities were thus computed from the 'best-fit' binomial expressing the experimental curve, and these results subjected to a Lineweaver-Burk treatment to obtain k_0 and K_m . Because of the rapidity of the reaction, these results are not as accurate as those obtained for the other substrates.

RESULTS

Oxidized α -chymotrypsin. The uptake of periodate with time, and the concomitant loss of enzymic activity (measured against L-tyrosine ethyl ester), are shown in Fig. 1. Both periodate consumption and loss of activity occur rapidly, and after about 2 hr. the reaction is complete. After this time, 1.80 moles of periodate have been consumed/mole of enzyme, and the enzymic activity has fallen to 55% of its original value. No further change in either parameter is apparent even after 20 hr., though there is still present in the solution a threefold excess of periodate. With allowance for the moisture content of the α -chymotrypsin used, the periodate consumption amounts to very nearly 2 moles of periodate/mole of enzyme. By the 'all-or-none' assay (Schonbaum *et al.* 1961) oxidized α -chymotrypsin contained as much active material as the parent protein, within the experimental limits of the method (2–3%). Yet by the 'rate' assay (Schwert & Takenaka, 1955) the oxidized enzyme was 55% as active as α -chymotrypsin itself. Since periodate uptake ceases after about 2 hr., it is extremely improbable that any enzyme remains unmodified in oxidized α -chymotrypsin, and from the activity assays it appears that no total inactivation occurs. The behaviour of oxidized α -chymotrypsin during sedimentation

Fig. 1. Esterase activity (●) and periodate uptake (○) as a function of time during the oxidation of α -chymotrypsin (1 mM) with periodate (5 mM) in 0.1 M-acetate buffer, pH 5.0, at 0°. Other conditions are given in the text.

in the ultracentrifuge is identical with that of the parent enzyme.

On analysis of the acid-catalysed hydrolysates, no significant differences could be detected between oxidized α -chymotrypsin and the parent enzyme (see Table 1). On analysis of the base-catalysed hydrolysates, however, it appeared that a single methionine has been converted into the sulfoxide in oxidized α -chymotrypsin. A small quantity of the sulphone is also apparent. The sulfoxides and sulphone were identified by the positions of emergence from the long column of the amino acid analyser. The stereochemical integrity of the sulfoxide in intact oxidized α -chymotrypsin is not known. Even if the sulfoxide is not racemic in the protein, it is naturally racemized very rapidly under the conditions of baryta-catalysed hydrolysis, and two peaks of equal area appear on the analyser trace. The absence of sulfoxide in acid-catalysed hydrolysates of oxidized proteins has been explained by Ray & Koshland (1962) and by Floyd, Cammaroti & Lavine (1963), who found that methionine sulfoxide reverts to the parent compound under the conditions of acid hydrolysis.

The conversion of 1 mole of methionine into its sulfoxide requires only 1 mole of periodate, and yet α -chymotrypsin consumes 2 moles of oxidant/mole. To discover the relationship between periodate uptake, oxidation of methionine and loss of activity, 'rate' assays were performed on samples of α -chymotrypsin that had been allowed to react (for 6 hr. at 0°) with periodate in concentrations 0.5, 0.75, and 1.0 times the molar amount of enzyme. Duplicate analyses of the base-catalysed hydrolysates of the materials from separate 0.75:1.0 (periodate: α -chymotrypsin) reactions were also performed. The results are shown in Fig. 2. It appears that, of the 2 moles of periodate consumed/mole, 1 mole/mole is oxidizing some (as yet unidentified) species on which the catalytic activity is not dependent, and that concomitantly with this occurs the slower oxidation of methionine. That is (though the evidence is limited to that expressed in Fig. 2), it is only the oxidation of methionine that is responsible for the fall in activity.

There now follows the results of a comparison of α -chymotrypsin with the oxidized enzyme with respect to the behaviour of these catalysts with

Table 1. *Results of amino acid analyses of α -chymotrypsin and oxidized α -chymotrypsin*

Experimental details are given in the text. The results were calculated as follows: for acid-catalysed hydrolysates, the values (in μ moles) of aspartic acid+arginine were assumed to be $21+3=24.0$; for base-catalysed hydrolysates the values of phenylalanine were assumed to be 6.0. Each analysis is the mean of two separate hydrolysis experiments. The results for acid-catalysed hydrolysates are uncorrected, so that values for threonine, serine, cystine/2, tryptophan and valine are low compared with the corrected values. The analyser colour yields for tryptophan, methionine sulfoxide and methionine sulphone were not known with the accuracy of the others.

	Amino acid content			
	Acid-catalysed hydrolysate		Base-catalysed hydrolysate	
	α -Chymotrypsin	Oxidized α -chymotrypsin	α -Chymotrypsin	Oxidized α -chymotrypsin
Lys	13.5	13.6	—	—
His	2.2	2.3	—	—
Arg	3.4	3.5	—	—
Trp	—	—	7.7	7.7
Asp	20.6	20.5	—	—
Thr	20.7	20.7	—	—
Ser	23.6	23.8	—	—
Glu	14.2	14.3	—	—
Pro	9.4	9.2	9.0	9.1
Gly	22.8	23.0	—	—
Ala	20.0	20.2	—	—
Cys/2	8.6	8.5	—	—
Val	19.1	18.9	—	—
Met	1.9	2.0	2.0	1.0
Ile	7.8	7.9	—	—
Leu	17.4	17.3	17.3	17.5
Tyr	3.8	3.7	4.3	4.5
Phe	5.7	5.7	6.0	6.0
Met sulfoxides	—	—	—	0.84
Met sulphone	—	—	—	0.11

Fig. 2. Relationship between esterase activity (●) and oxidation of methionine residues (○) of α -chymotrypsin after treatment with different molar proportions of periodate in 0.1M-acetate buffer, pH 5.0, at 0° for 6 hr. Other details are given in the text.

Fig. 3. Steady-state portion of the hydrolysis of *p*-nitrophenyl acetate by α -chymotrypsin (●) and by oxidized α -chymotrypsin (○) at high substrate concentration in aq. 1.6% (v/v) acetonitrile, pH 7.60, at 25°. The substrate concentrations ($[S_0]$) tested were in the range 0.171–0.854 mM, and the enzyme concentrations ($[E_0]$) used were $7.71 \mu\text{M}$ for α -chymotrypsin and $6.54 \mu\text{M}$ for oxidized α -chymotrypsin. The velocities are corrected for the spontaneous hydrolysis of substrate under the conditions indicated. Other details are given in the text.

three 'specific' substrates and two 'non-specific' substrates, the interactions of which with α -chymotrypsin have been well characterized.

Reaction with *p*-nitrophenyl acetate. These reactions were carried out in aq. 1.6% (v/v) acetonitrile at pH 7.60 and 25°. To ensure saturation of the enzyme, substrate concentrations were never less than 25 times the enzyme concentration. Runs with both α -chymotrypsin and oxidized enzyme were made at three different high substrate concen-

Table 2. Hydrolysis of *p*-nitrophenyl acetate by α -chymotrypsin and oxidized α -chymotrypsin

Details are given in the text.

Enzyme	$10^8 V$ (M sec. ⁻¹)	$[E_0]$ (μM)	$10^3 k_{+3}$ (sec. ⁻¹)
α -Chymotrypsin	3.24	7.71	4.20
Oxidized α -chymotrypsin	2.74	6.54	4.19

trations, since even under conditions of $[S_0] \gg [E_0]$ and, in fact, $[S_0] \gg K_m$, (where $[S_0]$ and $[E_0]$ are initial concentrations of substrate and enzyme respectively), Kezdy & Bender (1962) have found v_0 (the initial velocity) to be dependent on $[S_0]$. These authors have shown that the true rate constant for the enzymic deacylation can be obtained by plotting v_0 against $[S_0]$ at high values of $[S_0]$. The intercept on the v_0 axis is equal to the true V , i.e., $k_{+3}[E_0]$, uncomplicated by the anomalous non-enzymic reaction that results in the observed $[S_0]$ -dependence. The results of these experiments are shown in Fig. 3, each point being the mean from two experiments.

A least-squares treatment gives the values of V in Table 2. Also in Table 2 are the values of $[E_0]$ calculated from the initial 'burst' in extinction on the addition of enzyme, which corresponds to an internal 'all-or-none' titration of active sites. Table 2 shows that the values of k_{+3} (the deacylation rate constant) are identical within experimental error. Kezdy & Bender (1962) obtained k_{+3} values of $3.2 \times 10^{-3} \text{sec.}^{-1}$ at pH 7.2 and $5.3 \times 10^{-3} \text{sec.}^{-1}$ at pH 7.8.

Reaction with *N*-cinnamoylimidazole. With dilute solutions of reagent and enzymes, to produce changes in extinction of only about 0.08 unit, the rate of acylation of oxidized α -chymotrypsin was significantly lower than that of α -chymotrypsin at pH 5.02 (0.1M-acetate buffer) in aq. 3.2% (v/v) acetonitrile at 25°. The resulting curves are shown in Fig. 4.

Reaction with *N*-acetyl-L-valine ethyl ester. These reactions were done in aqueous solution at pH 7.90 in 0.1M-sodium chloride at 25°. The rates of the substrate and enzyme blank reactions were negligible. Enzyme concentrations were taken as 86% of the amount weighed out, a molecular weight of 25000 being assumed. Values of the parameters k_0 and K_m for α -chymotrypsin and the oxidized enzyme are given in Table 3. The Lineweaver-Burk plots leading to these values are shown in Fig. 5. The values of these parameters for the α -chymotrypsin-catalysed reaction were 0.160sec.^{-1} and 95mM ; Waite & Niemann (1962) give 0.129sec.^{-1} and 110mM respectively. The degree of hydrolysis during a run was always less than 3.5%.

Reaction with *N*-acetyl-L-tryptophanamide. The results of the enzyme-catalysed hydrolysis of this substrate are shown in Table 3. The corresponding Lineweaver-Burk plots are given in Fig. 6. The reactions were done in 0.1M-tris-hydrochloric acid buffer, pH 7.87, at 25°. The extent of hydrolysis during a run was always less than 1.5%. The values of k_0 and K_m obtained for the α -chymotrypsin-catalysed reaction (0.0468sec.⁻¹ and 4.8mm) compare well with the results of Bender *et al.* (1964a) of 0.0437sec.⁻¹ and 4.81mm respectively at pH 8.0.

Reaction with *N*-acetyl-L-tryptophan ethyl ester. The kinetics of the hydrolysis of this ester catalysed

by α -chymotrypsin and the oxidized enzyme were studied in aqueous solution at pH 7.90 (0.1M-tris-hydrochloric acid buffer) at 25°. The Lineweaver-Burk plots are shown in Fig. 7, and the values of k_0 and K_m in Table 3. The values obtained for the α -chymotrypsin-catalysed reaction (49.0sec.⁻¹ and 0.117mm) compare well with those of Bender *et al.* (1964a) of 44.9sec.⁻¹ and 0.123mm respectively at pH 8.18 in aq. 0.81% (v/v) acetonitrile.

DISCUSSION

Methionine has been implicated in the catalytic action of α -chymotrypsin by several workers. This has derived from photo-oxidation studies (Ray,

Fig. 4. Acylation of α -chymotrypsin (—) and of oxidized α -chymotrypsin (---) with *N*-cinnamoylimidazole in aq. 3.2% (v/v) acetonitrile, pH 5.02, at 25°. Other details are given in the text. Spontaneous hydrolysis (in the absence of enzyme) (····) is also shown.

Fig. 5. Lineweaver-Burk plot for the hydrolysis of *N*-acetyl-L-valine ethyl ester by α -chymotrypsin (●) and by oxidized α -chymotrypsin (○) in 0.1M-NaCl, pH 7.90, at 25°. The substrate concentrations ($[S_0]$) tested were in the range 6.46–77.6mm, and the enzyme concentrations ($[E_0]$) used were 98 μ M for α -chymotrypsin and 100.7 μ M for oxidized α -chymotrypsin. Other details are given in the text.

Table 3. Comparison of kinetic parameters of reactions catalysed by α -chymotrypsin and oxidized α -chymotrypsin

Experiments with *N*-acetyl-L-valine ethyl ester were carried out in 0.1M-NaCl, pH 7.90, at 25°, those with *N*-acetyl-L-tryptophanamide in 0.1M-tris-HCl buffer, pH 7.87, at 25°, and those with *N*-acetyl-L-tryptophan ethyl ester in 0.1M-tris-HCl buffer, pH 7.90, at 25°. Other details are given in the text.

Substrate	Enzyme	k_0 (sec. ⁻¹)	K_m (mm)
<i>N</i> -Acetyl-L-valine ethyl ester	α -Chymotrypsin	0.160 $\pm$ 0.005	95 $\pm$ 3
	Oxidized α -chymotrypsin	0.174 $\pm$ 0.006	96 $\pm$ 4
<i>N</i> -Acetyl-L-tryptophanamide	α -Chymotrypsin	0.0468 $\pm$ 0.0001	4.8 $\pm$ 0.2
	Oxidized α -chymotrypsin	0.0476 $\pm$ 0.0001	10.6 $\pm$ 0.4
<i>N</i> -Acetyl-L-tryptophan ethyl ester	α -Chymotrypsin	49.0 $\pm$ 4.2	0.117 $\pm$ 0.030
	Oxidized α -chymotrypsin	51.3 $\pm$ 2.4	0.206 $\pm$ 0.020

Fig. 6. Lineweaver-Burk plot for the hydrolysis of *N*-acetyl-L-tryptophanamide by α -chymotrypsin (●) and by oxidized α -chymotrypsin (○) in 0.1M-tris-HCl buffer, pH 7.87, at 25°. The substrate concentrations ($[S_0]$) tested were in the range 0.843–11.8 mM, and the enzyme concentrations ($[E_0]$) used were 59.3 μ M for α -chymotrypsin and 68.0 μ M for oxidized α -chymotrypsin. Other details are given in the text.

Fig. 7. Lineweaver-Burk plot for the hydrolysis of *N*-acetyl-L-tryptophan ethyl ester by α -chymotrypsin (●) and by oxidized α -chymotrypsin (○) in 0.1M-tris-HCl buffer, pH 7.90, at 25°. The substrate concentrations ($[S_0]$) tested were in the range 0.1397–1.076 mM, and the enzyme concentrations ($[E_0]$) used were 0.01648 μ M for α -chymotrypsin and 0.0329 μ M for oxidized α -chymotrypsin. Other details are given in the text.

Latham, Katsoulis & Koshland, 1960; Ray & Koshland, 1960; Schachter & Dixon, 1962, 1964), from hydrogen peroxide oxidation (Koshland, Strumeyer & Ray, 1962) and from alkylation with iodoacetic acid (Koshland *et al.* 1962) or with α -bromo ketones (Lawson & Schramm, 1962; Schramm & Lawson, 1963). In most of these cases activity losses of 50% and more have been observed. An effect on the hydrogen peroxide reaction has been noted by Schachter, Halliday & Dixon (1963), who found that there is a greater loss of activity when hydrogen peroxide reacts with α -chymotrypsin in the presence of specific substrates of the enzyme. Though the activity loss when periodate reacts with α -chymotrypsin in the presence of such substrates has not been measured, there appears to be no difference in the amount (or apparently in the rate, though Fig. 1 shows that the method does not allow accurate rate measurement) of periodate consumed by the enzyme in the presence of 5 mM-*N*-acetyl-L-tryptophan (which is a good competitive inhibitor of α -chymotrypsin; Foster, Shine & Niemann, 1955). This is not unexpected if the explanation given by Schachter *et al.* (1963) of the effect of specific substrates on the peroxide reaction is correct. These authors postulate the formation of the peracid of the acyl moiety of the substrate, as a result of hydrogen peroxide acting as a water analogue in the deacylation step. Such a reaction when periodate is used is not possible.

Various attempts were made to reduce the sulphoxide in oxidized α -chymotrypsin back to methionine, and regenerate a fully active enzyme. The method of Iselin (1961) involves incubation at 50° with thioglycolic acid, and gives excellent results with methionine peptides. Thioglycolic acid, cysteine and 2-mercaptoethanol were separately incubated at 4° with the oxidized enzyme at various pH values for up to 5 days. In no case was any increase in activity noticed, and in most cases there was a loss of activity of about 10%. Incubation at higher temperatures commonly led to irreversible precipitation of the protein.

It seems likely that the methionine residue oxidized in the present work is the same one that Schachter & Dixon (1964) have shown to be oxidized by hydrogen peroxide and by methylene blue-sensitized photo-oxidation. This methionine residue (no. 192; Hartley, 1964) is only three residues away from the serine residue that is acylated by such reagents as di-isopropyl phosphorofluoridate, and by substrates of α -chymotrypsin during the catalytic reaction. Since free methionine reacts with periodate almost instantaneously at room temperature, the modification in α -chymotrypsin of only one of the two residues in the molecule supports the view of Koshland *et al.* (1962) and Ray & Koshland (1960) that one

methionine residue is 'accessible' and the other one 'buried'.

It is tempting to suggest that the decrease in enzyme activity observed in the present work is due to a direct effect of oxidation of methionine, rather than a second-order effect on the tertiary structure of the enzyme as a whole. The kinetic and specificity evidence that follows substantiates this rather fine distinction.

The existence of a minimally modified, partially active, enzyme makes it feasible to study the detailed effect of the modification by looking at the nature of the residual activity. Niemann and his co-workers (see, e.g., Hein & Niemann, 1961, 1962) have usefully obtained a great deal of information about the specificity of α -chymotrypsin, and Bender and his co-workers (see, e.g., Zerner & Bender, 1964) have discussed the meaning of the kinetic constants obtained from Michaelis-Menten breakdowns of the reaction-rate data, and the dependence of these constants on the rate-determining step of the catalytic reaction. Since these data are available, experiments can be devised to discover in what way the activity of a modified α -chymotrypsin has been altered, with the intention of defining the function of the modified residue, and with the hope of casting some light on the general questions of mechanism and specificity.

The evidence that the hydrolytic reactions catalysed by α -chymotrypsin proceed via the formation of an acyl-enzyme intermediate seems now to be compelling, both for many non-specific and, more importantly, for specific substrates (for references, see Bender & Kezdy, 1964). The present results are discussed in terms of the kinetic scheme:

where the formation of a loose 'Michaelis-Menten' complex is succeeded by acylation to an acyl-enzyme, which is deacylated to yield the enzyme and the acid moiety of the substrate.

From a Michaelis-Menten breakdown of the reaction rates, according to $v_0 = k_0[\text{E}_0][\text{S}_0]/(K_m + [\text{S}_0])$, the observed quantities k_0 and K_m are obtained. The mechanism of eq. (1) leads to the relationships:

$$k_0 = k_{+2}k_{+3}/(k_{+2} + k_{+3})$$

$$\text{and } K_m = (k_{-1} + k_{+2})k_{+3}/k_{+1}(k_{+2} + k_{+3})$$

When acylation is genuinely rate-determining, k_0 becomes k_{+2} , and K_m becomes K' [K_m is here the operational parameter; K' is defined as the theoretical quantity $(k_{-1} + k_{+2})/k_{+1}$, which is sometimes called K_m in the American literature]; and if deacylation is much the slower step, $k_0 = k_{+3}$, and

$K_m = K' \cdot k_{+3}/k_{+2}$. These facts make simple comparisons of K_m values (as if they were K' values) dangerous if not meaningless, if it is not certain what the rate-determining step is. For specific substrates, then, the rate-determining step must be identified. If a series of acid derivatives of widely different base-catalysed hydrolysis rates have the same k_0 values, then a common intermediate is implied, and deacylation can be assumed to be rate-determining. This approach has been used by Zerner & Bender (1963) for the *N*-acetyl-L-tryptophan derivatives. It is clear from their results that, for the methyl, ethyl and *p*-nitrophenyl esters, deacylation is rate-limiting, and that, for the amide, acylation is the slow step. This conclusion has been confirmed by direct observation (at low pH) of the 'burst' of *p*-nitrophenol from the *N*-acetyl-L-tryptophan *p*-nitrophenyl ester- α -chymotrypsin system under conditions when $[\text{S}_0] > [\text{E}_0]$ (Kezdy, Clement & Bender, 1964). Therefore for two of the specific substrates (the tryptophan derivatives) used in the present work the significance of the observed kinetic constants is known. For the third specific substrate, *N*-acetyl-L-valine ethyl ester, the rate-determining step cannot yet be defined, though knowledge of the actual k_{+2}/k_{+3} ratio for this ester is not crucial to the present discussion, as shown below.

For the reagent *N*-cinnamoylimidazole, which can be treated as an essentially irreversible acylating agent of α -chymotrypsin, the initial reaction velocity will be proportional to k_{+2}/K' . As shown in Fig. 4, no attempt was made in this case to extract quantitative estimates of initial rates from the experimental results. For *p*-nitrophenyl acetate it is known (Hartley & Kilby, 1952, 1954) that the turnover rate of the hydrolysis under conditions of $[\text{E}_0] \ll [\text{S}_0]$ is a measure of the rate-limiting deacylation of the acetyl-enzyme (Kezdy & Bender, 1962).

It is now possible to discuss the results obtained with these various substrates with α -chymotrypsin and the oxidized enzyme, as far as possible in terms of the effect on the individual rate constants of eqn. 1. The results can be summarized as follows: (1) the rate of acylation of oxidized α -chymotrypsin by *N*-cinnamoylimidazole is significantly lower than that of α -chymotrypsin; (2) the deacylation rates of acetyl- α -chymotrypsin and acetyl-(oxidized α -chymotrypsin) (k_{+3}) are identical; (3) the k_0 values for *N*-acetyl-L-tryptophanamide (k_{+2}) are identical, but the K_m value (K') for oxidized α -chymotrypsin is about twice that for α -chymotrypsin; (4) the k_0 values for *N*-acetyl-L-tryptophan ethyl ester (k_{+3}) are identical, but the K_m value ($K' \cdot k_{+3}/k_{+2}$) for oxidized α -chymotrypsin is about twice that for α -chymotrypsin; (5) the k_0 values and K_m values for *N*-acetyl-L-valine ethyl ester are the same for oxidized

α -chymotrypsin and α -chymotrypsin; [(6) the rate of reaction of L-tyrosine ethyl ester with oxidized α -chymotrypsin is 55% of that with α -chymotrypsin].

The experiments with *p*-nitrophenyl acetate and with *N*-acetyl-L-tryptophan ethyl ester each demonstrate that oxidation of α -chymotrypsin has not affected the rate of deacylation either of 'non-specific' acetyl- α -chymotrypsin or of 'specific' *N*-acetyl-L-tryptophanyl- α -chymotrypsin. Moreover, the k_0 values for *N*-acetyl-L-tryptophanamide show that the rate of acylation is also unaffected, at least for this substrate. The fact that k_0 for *N*-acetyl-L-valine ethyl ester is unaltered is further support for the postulate that oxidation of the enzyme affects neither of the catalytic (as distinct from the binding) rate constants. Koshland *et al.* (1962) have reported that α -chymotrypsin oxidized with hydrogen peroxide shows no change in k_0 for *N*-acetyl-L-tyrosine ethyl ester (for which k_0 almost certainly equals k_{+3}), yet K_m is raised threefold compared with unoxidized enzyme. Moreover, Lawson & Schramm (1962) found that a more bulky methionine modification [the conversion of a methionine residue into its *S*-(acetyl α -aminoisobutyric acid) sulphonium salt] led to an 11-fold increase in K_m (for *N*-acetyl-L-tyrosine ethyl ester). For *N*-acetyl-L-tryptophanamide ($K_m = K'$) there is an approximately twofold increase in K_m on oxidation of the enzyme. Paralleling this, the K_m (i.e., $K' \cdot k_{+3}/k_{+2}$) for *N*-acetyl-L-tryptophan ethyl ester with oxidized α -chymotrypsin is about twice that with α -chymotrypsin. As shown above, k_{+2} and k_{+3} (for *N*-acetyl-L-tryptophan derivatives, at least) are unaltered on oxidation of the protein, so that change in $K' \cdot k_{+3}/k_{+2}$ must be due to a change in K' . It is natural to extend this to the results for the hydrolysis of L-tyrosine ethyl ester, and ascribe the fall in rate with oxidized α -chymotrypsin (to 55% of that with α -chymotrypsin) to an effect on K' . However, Dixon and co-workers (reported in a footnote by Schachter & Dixon, 1964) find that their preparation of oxidized α -chymotrypsin does not show an increase in K_m towards *N*-acetyl-L-tryptophan ethyl ester at pH 8.0. This result does not fit into the picture that emerges from the present work and that of Koshland *et al.* (1962) and Lawson & Schramm (1962). No simple explanation is apparent, though there may be real differences in the behaviour of the hydrogen peroxide- and periodate-modified enzymes.

Thus far, the differences in activity of α -chymotrypsin and the oxidized enzyme that have been mentioned are those with 'good' specific substrates, i.e., derivatives of the aromatic amino acids tryptophan and tyrosine. Definitions of 'goodness' have to be limited to comparative statements,

e.g. that derivatives of aromatic acids generally appear to have lower K_m values and higher k_0 values than their aliphatic counterparts, and classification of the 'specificity' of a substrate must rely on postulates as to the existence of specific interactions (e.g., at each of the three 'loci' of the model of Hein & Niemann, 1961, 1962) between substrate and enzyme, reflected in the value of K_m . By any criterion of 'goodness' or of specificity, however, valine compounds are less 'good' and less specific substrates of α -chymotrypsin than tryptophan derivatives. It is therefore significant that the K_m values for the hydrolysis of *N*-acetyl-L-valine ethyl ester by α -chymotrypsin and the oxidized enzyme are the same. The conclusion that this leads to is that oxidation of α -chymotrypsin results in higher K' values for 'good', aromatic, substrates, but not for less 'demanding' substrates such as *N*-acetyl-L-valine ethyl ester. Since there are strong indications (see Zerner & Bender, 1964) that $K' = K$, (i.e. k_{-1}/k_{+1}) because $k_{+2} \ll k_{-1}$, this is equivalent to saying that oxidation of the methionine residue in α -chymotrypsin affects the binding of aromatic amino acid derivatives but not of valine derivatives. According to the Hein-Niemann model, one could say that the methionine residue that has been altered in oxidized α -chymotrypsin lies in the ρ_2 -locus, i.e., the locus in which is bound the aromatic side chains of the 'good' substrates. [The fall in activity was ascribed to oxidation of methionine on the basis of activity assays with L-tyrosine ethyl ester. The differences in activity with respect to other substrates (e.g., *N*-acetyl-L-tryptophan ethyl ester, *N*-acetyl-L-tryptophanamide) need not be due to oxidation of methionine, but could arise from other changes in the enzyme. This caveat is a necessary one, though the more economical hypothesis is made here.] The decrease of the acylation rate observed with *N*-cinnamoylimidazole may well arise from an increase in K' (the initial rate being proportional to k_{+2}/K') due to the effect on the binding of the cinnamoyl side chain in the ρ_2 locus.

Bender, Kezdy & Gunter (1964b) have proposed a useful separation of the specificity concept into 'binding' specificity and 'kinetic' specificity. The simplest hypothesis is that the same limited number of amino acid residues at the active site are responsible for both types of specificity. Yet the present results imply that there are genuine differences between these specificities, since binding efficiency has been altered without affecting the rates of the subsequent catalytic steps.

In the absence of more data, it is difficult to say whether the effect of converting a methionine residue into its sulfoxide is in origin steric, polar, or both. It seems likely, however, that it is largely a steric effect, since, whereas oxidation leads to a

two- to three-fold increase in K_m for aromatic substrates, sulphonium salt formation (with a bulky group; Lawson & Schramm, 1962) leads to an 11-fold increase. Thus it is possible that, in the present experiments, the isopropyl side chain of valine is not large enough to interact with the sulfoxide group in oxidized α -chymotrypsin, whereas the greater binding requirements of the aromatic substrates do result in an unfavourable interaction. If this thesis is correct, it may be relevant that trypsin, which catalytically is very closely similar to α -chymotrypsin (Bender & Kaiser, 1962) but which differs in not being specific for aromatic amino acid derivatives, has no methionine residue (actually a glutamine residue) in the position corresponding to the methionine residue at position no. 192 (Hartley, 1964) in α -chymotrypsin.

The author is grateful to Dr Myron L. Bender for copies of papers by him and his co-workers before publication, to Dr D. B. Hope for the amino acid analyses, and to Dr W. E. van Heyningen for the sedimentation experiment. This work was supported in part by the Department for Scientific and Industrial Research.

REFERENCES

- Abrash, H. I., Kurtz, A. N. & Niemann, C. (1960). *Biochim. biophys. Acta*, **45**, 378.
- Bender, M. L., Clement, G. E., Kezdy, F. J. & Heck, H. d'A. (1964a). *J. Amer. chem. Soc.* **86**, 3680.
- Bender, M. L. & Kaiser, E. T. (1962). *J. Amer. chem. Soc.* **84**, 2556.
- Bender, M. L. & Kezdy, F. J. (1964). *J. Amer. chem. Soc.* **86**, 3704.
- Bender, M. L., Kezdy, F. J. & Gunter, C. R. (1964b). *J. Amer. chem. Soc.* **86**, 3714.
- Cooper, T. A. & Waters, W. A. (1964). *J. chem. Soc.* p. 1538.
- Dube, S. K., Roholt, O. A. & Pressman, D. (1963). *Fed. Proc.* **22**, 245.
- du Vigneaud, V. & Sealock, R. R. (1932). *J. biol. Chem.* **96**, 511.
- Elmore, D. T., Kingston, A. E. & Shields, D. B. (1963). *J. chem. Soc.* p. 2070.
- Floyd, N. F., Cammaroti, M. S. & Lavine, T. F. (1963). *Arch. Biochem. Biophys.* **102**, 343.
- Foster, R. J., Shine, H. J. & Niemann, C. (1955). *J. Amer. chem. Soc.* **77**, 2378.
- Glazer, A. N. & Sanger, F. (1964). *Biochem. J.* **90**, 92.
- Hartley, B. S. (1964). *Nature, Lond.*, **201**, 1284.
- Hartley, B. S. & Kilby, B. A. (1952). *Biochem. J.* **50**, 672.
- Hartley, B. S. & Kilby, B. A. (1954). *Biochem. J.* **56**, 288.
- Hein, G. E. & Niemann, C. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1341.
- Hein, G. E. & Niemann, C. (1962). *J. Amer. chem. Soc.* **84**, 4495.
- Huang, H. T. & Niemann, C. (1951). *J. Amer. chem. Soc.* **73**, 1541.
- Iselin, B. (1961). *Helv. chim. acta*, **44**, 61.
- Jansen, E. F., Curl, A. L. & Balls, A. K. (1951). *J. biol. Chem.* **189**, 671.
- Jones, J. B. & Niemann, C. (1963). *Biochemistry*, **2**, 498.
- Kezdy, F. J. & Bender, M. L. (1962). *Biochemistry*, **1**, 1097.
- Kezdy, F. J., Clement, G. E. & Bender, M. L. (1964). *J. Amer. chem. Soc.* **86**, 3690.
- Koshland, D. E., Strumeyer, D. H. & Ray, W. J. (1962). *Brookhaven Symp. Biol.* **15**, 101.
- Lawson, W. B. & Schramm, H. J. (1962). *J. Amer. chem. Soc.* **84**, 2017.
- Margenau, H. & Murphy, G. M. (1956). *The Mathematics of Physics and Chemistry*, 2nd ed., chapter 13. Princeton: D. Van Nostrand Co.
- Müller, E. & Friedberger, O. (1902). *Ber. dtach. chem. Ges.* **35**, 2652.
- Ray, W. J. & Koshland, D. E. (1960). *Brookhaven Symp. Biol.* **13**, 135.
- Ray, W. J. & Koshland, D. E. (1962). *J. biol. Chem.* **237**, 2493.
- Ray, W. J., Latham, H. G., Katsoulis, M. & Koshland, D. E. (1960). *J. Amer. chem. Soc.* **82**, 4743.
- Schachter, H. & Dixon, G. H. (1962). *Biochem. biophys. Res. Commun.* **9**, 132.
- Schachter, H. & Dixon, G. H. (1964). *J. biol. Chem.* **239**, 813.
- Schachter, H., Halliday, K. A. & Dixon, G. H. (1963). *J. biol. Chem.* **238**, pc3134.
- Schonbaum, G. R., Zerner, B. & Bender, M. L. (1961). *J. biol. Chem.* **236**, 2930.
- Schramm, H. J. & Lawson, W. B. (1963). *Hoppe-Seyl. Z.* **332**, 97.
- Schwert, G. W. & Takenaka, Y. (1955). *Biochim. biophys. Acta*, **16**, 570.
- Spies, J. R. (1948). *J. Amer. chem. Soc.* **70**, 3717.
- Vallee, B. L. (1964). *Fed. Proc.* **23**, 8.
- Waite, H. R. & Niemann, C. (1962). *Biochemistry*, **1**, 250.
- Zerner, B. & Bender, M. L. (1963). *J. Amer. chem. Soc.* **85**, 356.
- Zerner, B. & Bender, M. L. (1964). *J. Amer. chem. Soc.* **86**, 3669.
- Zerner, B., Bond, R. P. M. & Bender, M. L. (1964). *J. Amer. chem. Soc.* **86**, 3674.