

5. Release of these materials from mycelium treated with fatty acid was greatest at acid pH values, but change of pH had no significant effect upon the release by detergents.

6. Incubation of mycelial pads of these fungi in the presence of arsenite leads to the accumulation of pyruvate and  $\alpha$ -oxoglutarate together with traces of an unidentified  $\alpha$ -keto acid. The yield of  $\alpha$ -keto acids is increased by the presence of glucose in the medium.

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## The Mechanism of the Reaction of Chymotrypsin with *p*-Nitrophenyl Acetate

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Hammond & Gutfreund (1955) have concluded from studies of the hydrolysis of acetyl-L-phenylalanine ethyl ester catalysed by chymotrypsin that at least two steps occur during this reaction: first, an initial rapid adsorption of the substrate, and, secondly, a reaction involving an imidazole group of the enzyme and the carbonyl group of the substrate. Kinetic evidence showed that this latter reaction is rate-determining. Schaffer, May & Summerson (1953) have shown that during the inactivation of chymotrypsin with alkyl phosphates the hydroxyl group of one serine of the enzyme is phosphorylated. It remained to be decided whether

the participation of the serine residue is restricted to the reactions involving irreversible inhibition of the enzyme or whether this group is also involved in a relatively rapid reaction step preceding or succeeding the rate-determining reaction with the imidazole group.

Hartley & Kilby (1952, 1954) found that chymotrypsin catalyses the hydrolysis of *p*-nitrophenyl acetate and that during the course of this reaction there occurs initially a rapid liberation of one mole of *p*-nitrophenol/mole of chymotrypsin followed by a slow hydrolysis. It has recently been shown by one of us (Gutfreund, 1955) that the initial rapid reaction can be followed by a stopped-flow method, and it was suggested that a detailed investigation of this

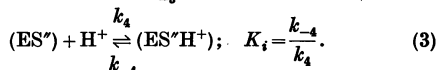
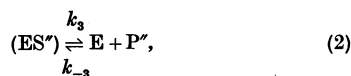
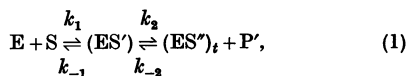
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initial reaction would give information about a reaction step which might involve the acylation of the catalytic site of hydrolytic enzymes.

The studies of the reaction of *p*-nitrophenyl acetate with chymotrypsin described in this paper were designed to show first whether the enzyme does in fact become acylated during the course of the catalysis, and secondly whether the pH dependence of the different steps permits one to draw any further conclusions about the particular groups of the enzyme involved in each of them.

### THEORETICAL

The experimental results to be given below require for their detailed description a kinetic scheme involving two different enzyme-substrate complexes:



The formation of the second complex is accompanied by the liberation of the product  $P'$  (*p*-nitrophenol). This complex exists in two forms,  $(ES'')$  and  $(ES''H^+)$ , only the former of which is capable of decomposing to give the product  $P''$  (acetate) with regeneration of enzyme.

In the early stages of the reaction we may assume that

$$k_2[ES'] \gg k_{-2}[ES''] [P'] \quad \text{and} \quad k_3[ES''] \gg k_{-3}[E] [P''],$$

where the quantities in square brackets represent the molar concentration of the corresponding species. If an adequate concentration of substrate is present, we may also employ the approximation introduced by Gutfreund (1955) and set  $[S] = [S]_0$ , where  $[S]_0$  is the initial substrate concentration. The steady-state approximation applied to  $(ES')$  then leads to the result

$$\{(k_2 + k'_2)[S]_0 + k'_3 K_m\} [E] = k'_3 K_m [E]_0 + \frac{k_2 K_m [S]_0 [E]_0}{K_m + [S]_0} \exp \left[ - \left\{ \frac{(k_2 + k'_2)[S]_0 + k'_3 K_m}{K_m + [S]_0} \right\} t \right], \quad (4)$$

where  $[E]_0 = [E] + [ES'] + [ES'']$ , is the total enzyme concentration,  $K_m = (k_{-1} + k_2)/k_1$ ,  $k'_3 = k_3 K_t / \{K_t + [H^+]\}$ , and  $t$  is the time. The rates of appearance of the products are

$$V = \frac{d[P']}{dt} = \frac{k_2}{K_m} [E] [S]_0, \quad (5)$$

$$\text{and} \quad \frac{d[P'']}{dt} = k'_3 [E]_0 - k'_3 \frac{K_m + [S]_0}{K_m} [E]. \quad (6)$$

Measurements of the rate of appearance of  $P'$  at small values of  $t$  can be employed to evaluate

$$k = \frac{(k_2 + k'_2)[S]_0 + k'_3 K_m}{K_m + [S]_0}. \quad (7)$$

If such measurements are carried out at sufficiently high values of  $[S]_0$  so that  $(k_2 + k'_2)[S]_0 \gg k'_3 K_m$ ,  $(k_2 + k'_2)$  and  $K_m$

can be evaluated in the usual way by means of a Lineweaver & Burk (1934) or an Eadie (1942) plot of the data for experiments at various values of  $[S]_0$ .

In determining the rate of appearance of  $P'$  at small values of  $t$ , we have employed the integral of equation (5), which is an expression of the form

$$[P'] = At + B(1 - e^{-kt}), \quad (8)$$

where  $k$  is the quantity to be determined. It should be noted that, in the actual experiments,  $A$  in this equation includes contributions from spontaneous hydrolysis of the substrate and electrical drift as well as from  $k_3$ . Data conforming to an equation of this form can best be handled by an elaboration of the procedure suggested by Guggenheim (1926). Readings  $r_1$ ,  $r_2$  and  $r_3$  (where  $r$  is any quantity linearly dependent on  $[P']$ ) are made at times  $t_1$ ,  $t_1 + \Delta t$ , and  $t_1 + 2\Delta t$ .  $\Delta t$  should be three or four times the half-time of the reaction. It can be shown that if  $\log |r_1 + r_3 - 2r_2|$  is plotted against  $t_1$  for a series of such sets of readings (the series of  $t_1$  values should cover one or two half-times of the reaction), a straight line of slope  $-k/2.303$  is obtained. It should be emphasized that it is impossible to prove the unique adherence of experimental data of usual accuracy to an equation of the form of (8) because the equation contains three adjustable parameters. Our data gave satisfactory linear plots in all cases, but we recognize that this in itself does not show that our proposed reaction scheme is correct. As discussed below, much more convincing evidence is obtained from the variation of  $k$  with  $[S]_0$ .

Observations of the kinetics of hydrolyses catalysed by chymotrypsin have been in the past usually carried out at times long enough so that the exponential term in equation (4) can be neglected. In these circumstances

$$V = \frac{d[P']}{dt} = \frac{d[P'']}{dt} = \frac{k_2 k'_3 [S]_0 [E]_0}{(k_2 + k'_2)[S]_0 + k'_3 K_m}. \quad (9)$$

Application of the Lineweaver-Burk or Eadie plots to data at various values of  $[S]_0$  will then give the apparent constants

$$K_m (\text{app.}) = \frac{k'_3}{k_2 + k'_2} K_m \quad (10)$$

and

$$\frac{1}{k'_3 (\text{app.})} = \frac{1}{k_2} + \frac{1}{k'_2}. \quad (11)$$

Equations (10) and (11) illustrate that care must be exercised in the interpretation of kinetic data for complex reactions. In particular, it is almost always assumed that one particular step is rate-determining for the overall process, whereas, as shown by equation (11), it may well happen that an apparent rate constant is actually a composite of two or more true rate constants. It will be shown below that with *p*-nitrophenyl acetate  $k_2 \gg k_3$ , so that in this particular case

$$K_m (\text{app.}) = \frac{k_3}{k_2} K_m \frac{K_t}{K_t + [H^+]} \quad (12)$$

and

$$k'_3 (\text{app.}) = k_3 \frac{K_t}{K_t + [H^+]}. \quad (13)$$

In our measurements of the rate of the reaction of equation (2),  $k_3 [S]_0 \gg k'_3 K_m$ ; in these circumstances

$$V = k_3 [E]_0 \frac{K_t}{K_t + [H^+]}. \quad (14)$$

## EXPERIMENTAL

**Buffers.** Except where otherwise noted, all reaction solutions contained phosphate buffer at 0.05M total phosphate concentration made up from  $H_3PO_4$  and NaOH.

**Enzyme.** The  $\alpha$ -chymotrypsin was a preparation, kindly supplied by Dr B. S. Hartley, that had been recrystallized several times.

**Substrate.** The *p*-nitrophenyl acetate was generously supplied to the authors by Dr W. N. Aldridge.

**Solvent.** Most of the experiments were performed in 20% (v/v) isopropyl alcohol solutions, in order to increase the solubility of the substrate.

**Stopped-flow technique.** The Gibson (1952) stopped-flow apparatus was used to measure the changes in light-absorption accompanying the second reaction in equation (1). The apparatus, as modified by Gutfreund (1955), was further modified by inclusion of electrical heating and a very rapidly responding thermocouple immersed in the fluid just beyond the observation cell, with which a positive measure of the actual reaction temperature was obtained. All experiments in the stopped-flow apparatus were performed at  $25.0 \pm 0.2^\circ$ .

The half-times of the reactions observed here were of the order of 1 sec., so that it was convenient to employ a pen-and-ink recorder in place of oscillographic recording. For this purpose, the output of a cathode-follower stage after the photomultiplier tube was amplified by a stable d.c. amplifier and fed to a Type BL 201 (Brush Electronics Co.,

Cleveland, Ohio) recorder with a chart speed of 5 or 25 mm./sec. It was ascertained that the amplifying and recording system was accurately linear, and, by means of experiments on the charging of a known capacitance through a known resistance, that the speed of response was adequate for the present purpose.

## RESULTS

**Evaluation of  $k_2$ .** The reaction described by equation (1) was studied by following the liberation of *p*-nitrophenol at times sufficiently small so that the exponential in equation (4) could be evaluated. These rate measurements were carried out by the stopped-flow technique described above. A typical experiment is represented by the modified Guggenheim plot (see p. 657) in Fig. 1. Fig. 2 shows a Lineweaver & Burk (1934) plot of rate measurements on this time scale. These values were obtained at pH 6.45 and 7.7 in solutions which were 0.05M with respect to sodium phosphate and contained 20% (v/v) of isopropyl alcohol. The range of  $[S]_0$ , the initial concentration of *p*-nitrophenyl acetate, was  $0.25 \times 10^{-3}M$  to  $5 \times 10^{-3}M$ .

The reciprocal of the limiting velocity  $1/V_{max}$  is obtained from the evaluation of the intercept at  $1/[S]_0 = 0$  of a plot of  $1/[S]_0$  against  $1/V$  (Fig. 2). The least-square calculations give at pH 7.75  $V_{max} = 3.3 \pm 0.3$  (s.e.)  $sec^{-1}$  and at pH 6.45

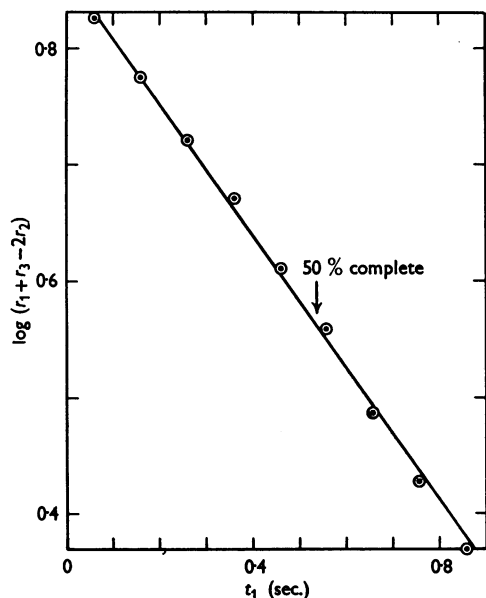


Fig. 1. A plot to illustrate the modified Guggenheim method of evaluating the rate constants of the acylation reaction. The symbol  $r$  is a measure in arbitrary units of the concentration of *p*-nitrophenol. The reaction mixture contained 0.05M sodium phosphate, pH 7.75, 20% (v/v) isopropyl alcohol, and 1.15 mg. of chymotrypsin/ml.; the initial concentration of *p*-nitrophenyl acetate was  $5 \times 10^{-3}M$ .

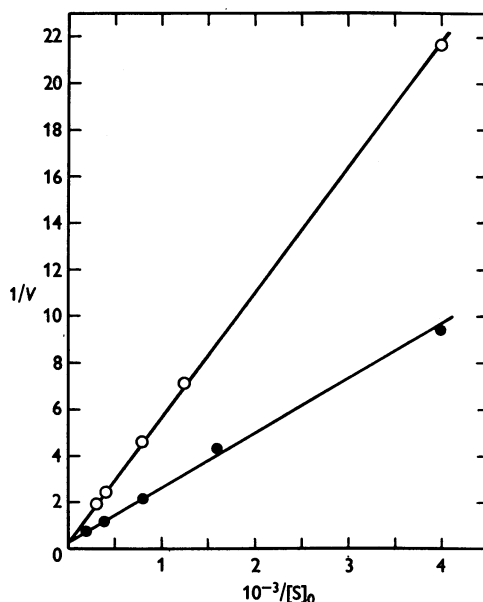


Fig. 2. Effect of  $[S]_0$ , initial concentration of *p*-nitrophenyl acetate, on the velocity ( $V$ ) of the initial liberation of *p*-nitrophenol in the hydrolysis of *p*-nitrophenyl acetate catalysed by chymotrypsin at pH 6.45 (O) and pH 7.75 (●). The reaction mixture was 0.05M in sodium phosphate and contained 20% (v/v) isopropyl alcohol. The enzyme concentration was 1.15 mg. of chymotrypsin/ml.

$V_{\max.} = 3.0 \pm 0.3 \text{ sec.}^{-1}$ . The  $K_m$  values are  $7.7 (\pm 0.8) \times 10^{-3}$  and  $1.6 (\pm 0.16) \times 10^{-2}$  at pH 7.75 and 6.45 respectively. This is a first-order reaction and is therefore independent of enzyme concentration; so in this case the mean value for  $V_{\max.}$  is taken as the first-order rate constant  $k_2 = 3.15 \text{ sec.}^{-1}$ .

*Evaluation of  $k_3$ .* The reaction of equation (2) was studied by following the rate of formation of *p*-nitrophenol at times large enough so that the exponential in equation (4) could be neglected. For this purpose we used a Unicam spectrophotometer at 27°. The reference solution was identical with the reacting solution except that it contained no enzyme; in this way no separate correction need be applied for the spontaneous hydrolysis of *p*-nitrophenyl acetate. Rate measurements were made over the range of pH 6.45 to 7.75: reaction mixtures were 0.05 M in sodium phosphate and contained 20% (v/v) isopropyl alcohol; the initial concentration of *p*-nitrophenyl acetate was in each case  $2.5 \times 10^{-3} \text{ M}$ , and the enzyme concentration 0.115 mg./ml. Fig. 3 gives the results of measurements of the rates ( $V$ ) of hydrolysis in the form of a plot of  $1/V$  against  $[\text{H}^+]$ . The turnover number or rate constant  $k_3 = 2.54 \times 10^{-2} \text{ sec.}^{-1}$  has been calculated from the extrapolated value

$$1/V = 0.079 \times 10^8 \text{ sec. M}^{-1} \text{ at } [\text{H}^+] = 0,$$

with the molecular weight 23 000 for chymotrypsin.

## DISCUSSION

The most important conclusions from our extension of the studies of Hartley & Kilby (1954) of the hydrolysis of *p*-nitrophenyl acetate catalysed by chymotrypsin are that the reaction proceeds via three distinguishable steps with rate constant  $k_1$ ,  $k_2$  and  $k_3$  [see equations (1)–(3)]. The rate constant  $k_1$  is too large to be measured by the method employed here,  $k_2$  is  $3.02 \text{ sec.}^{-1}$  at 25° and  $k_3$  is  $2.54 \times 10^{-2} \text{ sec.}^{-1}$  at 27°. Furthermore we have shown that the step involving  $k_3$  has a pH dependence similar to that of the usual overall rate of hydrolysis reactions catalysed by chymotrypsin. The value of

$$K_i = 0.52 \times 10^{-7} \text{ M},$$

for the dissociation constant of the basic group involved in the reaction step characterized by  $k_3$ , is somewhat lower than the value  $K_i = 1.4 \times 10^{-7} \text{ M}$  obtained by Hammond & Gutfreund (1955) for the dissociation constant of the catalytic site in the hydrolysis of acetyl-L-tyrosine ethyl ester catalysed by chymotrypsin. However, the different solvent and electrolyte composition of the reaction mixtures in the two investigations do not allow such a detailed comparison of the results. The rate measurements for the study of the effect of pH on  $k_3$  were all carried out at one substrate concentration ( $[\text{S}]_0 = 2.5 \times 10^{-3} \text{ M}$ ). It is justifiable to assume zero-order kinetics under these conditions, since the Michaelis constant  $K_{m(\text{app.})}$  for this reaction [see equations (10)–(12)] can be evaluated, and is always smaller than  $1.35 \times 10^{-4} \text{ M}$ .

In recent summaries of all the available information about the catalytic site of chymotrypsin (Hammond & Gutfreund, 1955; Jandorf, Michel, Schaffer, Egan & Summerson, 1955), it has been pointed out that an imidazole group of histidine is essential for the rate-determining step. Balls & Jansen (1952) discovered that during the irreversible inhibition of chymotrypsin with diisopropyl fluorophosphate (diisopropyl phosphorofluoridate) a single diisopropyl phosphate group is attached to each chymotrypsin molecule. Subsequently, Schaffer *et al.* (1953) isolated diisopropyl phosphoserine from the enzymic hydrolysate of chymotrypsin inhibited with diisopropyl fluorophosphate. Up to now there has been considerable doubt whether this information proved that serine is involved in one of the reaction steps of hydrolysis catalysed by chymotrypsin or whether the presence of phosphoserine was due to an artifact of the reaction with this type of inhibitor or to an acyl migration from an imidazole group of histidine to a hydroxyl group of serine.

In our reaction scheme [equations (1), (2) and (3)] ( $\text{ES}'$ ) is a rapidly formed complex and ( $\text{ES}''$ ) is the enzyme containing one acetyl group. The evidence

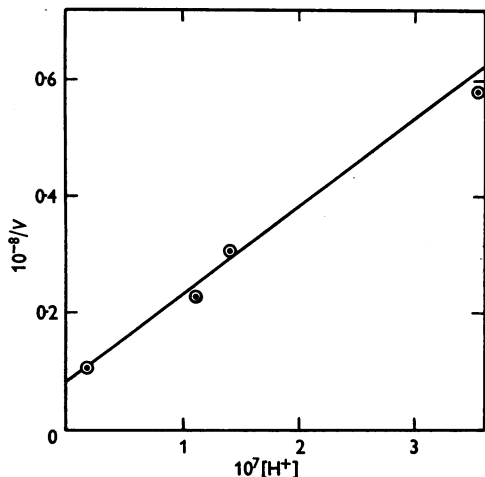


Fig. 3. A plot of  $\text{H}^+$  concentration against the reciprocal of the velocity  $V$  of the final step of the hydrolysis of *p*-nitrophenyl acetate catalysed by chymotrypsin. The reaction mixture was 0.05 M in sodium phosphate and contained 20% (v/v) isopropyl alcohol. The initial concentration of *p*-nitrophenyl acetate was  $2.5 \times 10^{-3} \text{ M}$  and the enzyme concentration 1.115 mg. of chymotrypsin/ml.

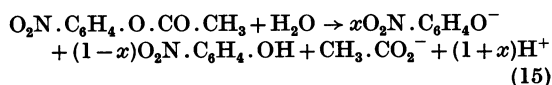
for the existence of (ES') is provided by the fact that the formation of (ES') cannot be described by second-order kinetics, but requires the Michaelis-Menten interpretation. The evidence available at present makes it appear very probable that in (ES') the hydroxyl group of a serine residue is acetylated.

The step represented by equation (2) is intramolecularly catalysed by an imidazole residue, which must be in its uncharged form to be effective. The rate of this step, which is frequently the overall rate-determining step, thus depends on pH in the manner demonstrated by Hammond & Gutfreund (1955). The step characterized by the rate constants  $k_2$  and  $k_{-2}$  may also be affected by intramolecular catalysis; the fact that its rate is independent of pH over the range 6.45-7.75 would indicate that the group causing the catalysis is unaffected in this range by changes in pH. This group might thus be either a carboxylate ion or an undissociated basic group.

The experiment illustrated in Fig. 4 constitutes further support for the mechanism outlined in equations (1)-(3). In this experiment *p*-nitrophenol served as the only buffer present. If the acylation step involves either an SH or an OH group in the enzyme, there will be liberated one molecule of *p*-nitrophenol/molecule of enzyme acetylated, and at pH 6.6 part of this would be in the form of the coloured nitrophenoxide ion. On the other hand, if the acylation involves a basic group, there will be liberated, in addition to the *p*-nitrophenol, an amount of H<sup>+</sup> ion corresponding to the degree of dissociation of the basic group involved. In view of the fact that at pH 6.6 all basic groups

except that of histidine would be essentially undissociated, and that the rate of the acylation has been shown to be independent of pH, the increase of absorption observed in the early part of the reaction may be taken as direct evidence that the acylation involves either an SH or an OH group.

The overall hydrolysis reaction at pH 6.6 is



The protons liberated in the hydrolysis combine with the buffer present, in this case *p*-nitrophenol, so that, as long as the buffer concentration is not appreciably changed by the reaction, exactly one molecule of nitrophenoxide ion is removed from the system for each molecule of substrate hydrolysed. This process is demonstrated by the decrease in absorption observed after the completion of the fast acylation. The relatively high slope of the last step in the reaction is due to the fact that it corresponds to the removal of fully coloured ions, whereas in the rapid step only a small fraction of the liberated nitrophenol is in the form of the coloured ions.

The rate constant of the fast step is found to be 0.20 sec.<sup>-1</sup>, which is approximately one-half that observed at the same substrate concentration in phosphate buffer at nearly the same pH. This decrease in rate is very probably the result of competitive inhibition by the relatively large concentration of *p*-nitrophenol present. The following argument eliminates the possibility that it is to any important extent due to reversal of the acylation step. The method of calculation of the rate constant for this step leads actually to  $k_2 + k_{-2}[P']$ , since the reverse reaction may be considered as first-order in cases where P' remains essentially constant, as in the experiment under discussion. Increasing [P'] should thus actually have increased the observed apparent first-order rate constant. Further measurements would be required to eliminate the possibility that a small increase due to this cause was overwhelmed by the effect of competitive inhibition.

We believe that the kinetic scheme proposed here is an adequate foundation for explanation of all known facts concerning hydrolyses catalysed by chymotrypsin and that the evidence presented in this paper proves that the a covalent bond between enzyme and substrate is formed to yield the acylated complex (ES').

## SUMMARY

1. It is shown that the kinetics of the hydrolysis of *p*-nitrophenyl acetate catalysed by chymotrypsin can be fully described by a mechanism involving three distinct steps. The first step, which involves the rapid adsorption of the substrate on the enzyme,

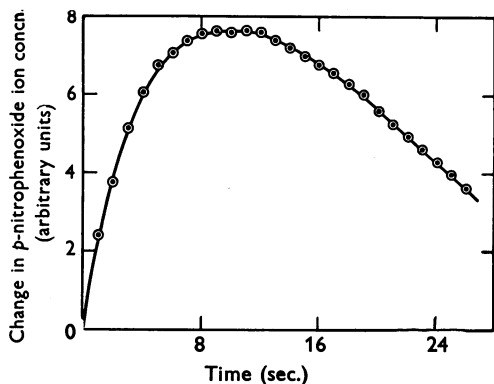


Fig. 4. Variation with time of the concentration (in arbitrary units) of *p*-nitrophenoxide ion during the hydrolysis of *p*-nitrophenyl acetate catalysed by chymotrypsin in 10<sup>-3</sup>M *p*-nitrophenol, pH 6.6. The curve includes the initial rapid liberation of *p*-nitrophenol and a small fraction of the subsequent slow step. The reaction mixture contained 20% (v/v) isopropyl alcohol, and 1.15 mg. of chymotrypsin/ml.; the initial substrate concentration was 2.5 × 10<sup>-3</sup>M.

is too fast to be measured by the methods used. The second step, which involves the acylation of the enzyme and the concomitant liberation of *p*-nitrophenol, is characterized by the rate constant  $k_2 = 3.15 \text{ sec.}^{-1}$ . The third step involves the liberation of acetate and reactivation of the enzyme; the rate of this reaction is defined by  $k_3 = 0.0254 \text{ sec.}^{-1}$ .

2. The rate constant  $k_2$  for the second step is, within experimental error, the same at pH 6.45 and pH 7.75; from this and other available evidence it is suggested that it involves the acetylation of the OH group of a serine residue in the enzyme. The third step has a pH dependence similar to that previously found for the overall rate of reactions catalysed by chymotrypsin, and it is suggested that it involves the imidazole group of a histidine residue of the enzyme.

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## Biochemical Changes Occurring during Sporulation in *Bacillus* Species

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We have shown that spore germination in *Bacillus* species is accompanied by the excretion of calcium dipicolinate (Powell & Strange, 1953; Powell, 1953) and a non-dialysable peptide containing  $\alpha\epsilon$ -diaminopimelic acid (DAP), glutamic acid, alanine, and the acetyl derivatives of glucosamine and an unidentified amino sugar (Strange & Powell, 1954). This peptide is probably derived from the spore coat (Strange & Dark, 1956). We have now studied the synthesis of dipicolinic acid and the accumulation of calcium by sporulating cells of *Bacillus cereus* and *B. subtilis*. It seems possible that dipicolinic acid is derived from DAP by deamination followed by ring closure and dehydrogenation, especially since conversion of lysine into pipecolic acid has been demonstrated (Rothstein & Miller, 1953; Lowy, 1953). Evidence supporting this possibility has recently been supplied by Perry & Foster (1955). These authors isolated [ $^{14}\text{C}$ ]dipicolinic acid from spores produced 'endogenously' in a medium containing totally labelled [ $^{14}\text{C}$ ]DAP and found that it was significantly more radioactive than any of the twelve amino acids also isolated. We have been unable to demonstrate deamination or oxidation of DAP by sporulating cells of *B. cereus* or any significant change in the amount of DAP present in the cell

during sporulation. We found, however, that during sporulation an apparent change in distribution of DAP and of hexosamine between the soluble and insoluble fractions of the cell occurred. This change, and its connexion with the excretion of the spore peptide during germination, will be discussed.

#### METHODS

For observations on the accumulation of dipicolinic acid and calcium during sporulation, the laboratory strain of *B. cereus* was grown in a potato-extract medium (Robinow, 1951) enriched with 1/10 vol. of a solution containing acid and trypsin-hydrolysed casein, yeast extract, glycerophosphate and glutamine (CCY) (Gladstone & Fildes, 1940). Cultures were grown in 11. flasks containing 200 ml. of medium inoculated with  $10^8$  spores/ml. and shaken during incubation. Cells were harvested at various stages of growth and sporulation, washed once with 0.9% (w/v) NaCl, then suspended in water and freeze-dried. Suspensions containing 2 mg. dry wt. of cells/ml. were disintegrated in water for 45-60 min. at 4° in the Mickle (1948) tissue disintegrator with Ballotini beads, size 12. The clear cell extract obtained by centrifuging at 6000 g, was deproteinized with 2.5% (w/v)  $\text{HClO}_4$ , then neutralized and buffered with 50 mM sodium potassium phosphate, pH 7.3. At this stage the volume of extract was adjusted so that 1 ml. contained the soluble fraction from 1 mg. of cells. The ultraviolet