# THE FICIN-CATALYSED HYDROLYSIS OF *p*-NITROPHENYL HIPPURATE. DETAILED KINETICS INCLUDING THE MEASUREMENT OF THE APPARENT DISSOCIATION CONSTANT FOR THE ENZYME-SUBSTRATE COMPLEX

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## 1. Introduction

It seems likely that high-resolution kinetic studies, involving the determination of the various intermediates in enzyme-catalysed reactions and their rates of interconversion under different experimental conditions, will eventually provide the necessary background for an understanding of structure-function relationships in catalytic proteins. In the present communication the basis of such a study is presented for the ficin-catalysed hydrolysis of *p*-nitrophenylhippurate.

Several authors have presented evidence [1–4] that ficin and papain, like α-chymotrypsin [5,6], catalyze the hydrolysis of low-molecular weight substrates via a mechanism involving a minimum of two intermediates eq. (1)

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} EP'_2 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftarrows}} E + P_2 \quad \overset{+P_1}{\ldots\ldots} \qquad (1)$$

In the case of α-chymotrypsin-catalyzed reactions the intermediate $EP'_2$ is considered to be an ester, formed between the acyl moiety of the substrate and a serine hydroxyl group [7], whereas the thiol proteases, convincing evidence has been presented [3] that $EP'_2$ is a thioacyl-enzyme intermediate.

The present investigation was initiated to test the applicability of model (1) to the ficin-catalyzed hydrolysis of *p*-nitrophenylhippurate (PNPH) under a wide variety of enzyme and substrate concentrations and to determine the values of individual rate constants.

## 2. Materials and methods

Ficin was prepared as described in a previous communication [8]. The concentration of enzyme solutions was determined spectrophotometrically at 280 nm employing a molar extinction coefficient of $\epsilon_{280} = 46{,}000$ (ref. [9]).

*p*-nitrophenylhippurate. The preparation of PNPH m. pt. 170–171° has also been described previously [8].

The Ficin-catalyzed hydrolysis of PNPH was followed spectrophotometrically at 325 or 400 nm employing a Durrum-Gibson stopped-flow machine (Durrum Instruments Corporation U.S.) at high enzyme concentrations and a Cary automatic-recording spectrophotometer at low enzyme concentrations. In the stopped-flow experiments the enzyme syringe containing sufficient acetic acid/sodium acetate buffer

Abbreviations used: PNPH: *p*-nitrophenylhippurate; Cbz-Gly-ONP: Carbobenzoxyglycine *p*-nitrophenyl ester.

to give the required pH value and $I$, 0.1 after a 1:1 dilution and sufficient EDTA to give a final concentration of 0.5 mM. The substrate syringe contained PNPH in a 6% v/v mixture of acetonitrile and water. All experiments were performed at 25° with a final concentration of acetonitrile not greater than 3.3% v/v.

Under conditions where $[S_o] \gg [E_o]$ the ficin-catalysed hydrolysis of PNPH closely followed Michaelis-Menten kinetics. Steady-state kinetics parameters ($K_m$ and $V_m = k_{cat}[E_o]$) were evaluated from complete progress curves employing the integrated form of the Michaelis-Menten equation:

$$\frac{[P_1]}{\log_{10}\left\{\frac{[S_o]}{([S_o]-[P_1])}\right\}} = k_{cat}[E_o]\,\frac{t}{\log_{10}\left\{\frac{[S_o]}{([S_o]-[P_1])}\right\}} - 2.303\,K_m \ldots \qquad (2)$$

Plots of

$$\frac{[P_1]}{\log_{10}\left\{\frac{[S_o]}{([S_o]-[P_1])}\right\}}$$

against

$$\frac{t}{\log_{10}\left\{\frac{[S_o]}{[S_o]-[P_1]}\right\}}$$

gave straight lines from which values of $k_{cat}$ and $K_m$ were evaluated. This method is only applicable if the enzyme retains full activity throughout the reaction and products do not interfere, conditions which were satisfied in the present study since addition of a second aliquot of substrate to a spent reaction mixture gave a second progress curve indistinguishable from the first.

## 3. Results

The kinetic data collected for the ficin-catalysed hydrolysis of PNPH may be separated into three categories. The first is when initial enzyme concentration ($[E_o]$) is much greater than that of the substrate ($[S_o]$) and the second when $[S_o] \gg [E_o]$. For these conditions the differential equations arising from scheme (1) may be solved analytically. This is not the case for the third category where $[E_o] \approx [S_o]$, when recourse to analogue or numerical methods becomes necessary. In the present study it was elected to extract values of $k_{-1}/k_{+1}$ (i.e. $K_s$), $k_{+2}$ and $k_{+3}$ from data falling into the first two categories and to set these values into an analogue computer programme in order to test the fit of the data to the model under conditions where $[E_o] \approx [S_o]$.

### 3.1. $[E_o] \gg [S_o]$

A typical progress curve for the release of *p*-nitrophenol ($P_1$) from PNPH under these conditions is given in fig. 1 and first-order plots from this and other traces for different enzyme concentrations and pH values in figs. 2 and 3. It is noteworthy that no acceleration of *p*-nitrophenol release is observed in the early phases (fig. 1) and that the process is first-order for greater than 90% of the reaction. Furthermore, as shown below, the variation of the observed first-order rate constant ($k_1^{obs}$) with enzyme concentration is hyperbolic, with a half-maximal value of $k_1^{obs}$ when $[E_o] \approx 2 \times 10^{-4}$ M. The foregoing observations lead to the conclusion that $k_{-1} \neq 0$. Thus, the formation of ES from E and S must be a very rapid equilibration process, reaching completion in the dead time of the apparatus (about 3 msec). Given that

$$\frac{k_{-1}}{k_{+1}} \approx 2 \times 10^{-4},$$

lower limits of $k_{+1}$ and $k_{-1}$ may be set at about $10^6$ $M^{-1}$ $sec^{-1}$ and 200 $sec^{-1}$ respectively. The value of $k_{+2}$ at pH 5.9 is about 40 $sec^{-1}$ (*vide infra*) so that $k_{-1} > 5k_{+2}$ and the condition $k_{-1} \gg k_{+2}$ is satisfied. Hence the formation of ES may be regarded as essentially a pseudo-equilibrium process. A further simplyfying condition arises from the observation that incorporation of low concentrations (up to $6.0 \times 10^{-5}$ M) of *p*-nitrophenol in reaction mixtures did not affect the rate of release of $P_1$ from PNPH. Hence for scheme (1) we may write $k_{-2} \approx 0$.

Given the foregoing simplifications, the first-order rate constant for the rate of release of $P_1$ from PNPH

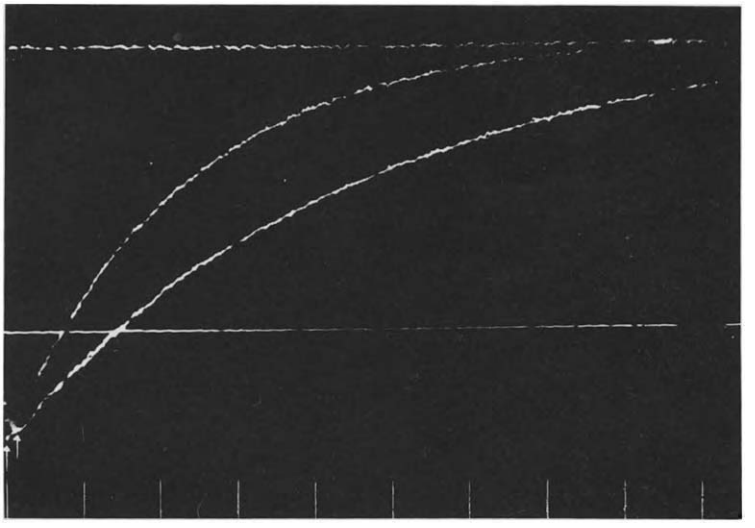

Fig. 1. Typical curve for the ficin-catalysed hydrolysis of PNPH, when $[E_0] \gg [S_0]$. Ficin was $3.14 \times 10^{-5}$ M and PNPH $0.5 \times 10^{-5}$ M in acetate buffer pH 5.9, $I$ 0.1 at 25°. The reaction mixture also contained $5 \times 10^{-5}$ M EDTA. Since the total absorbancy change corresponds to 0.041, the ordinate, which is linear in transmission, may be taken as linear in *p*-nitrophenol concentration. The time scales for the two traces are 50 and 100 msec per division respectively.

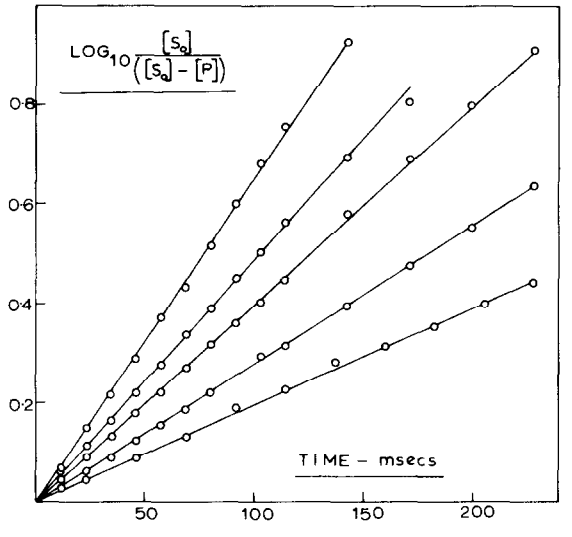


Fig. 2. First-order logarithmic plots at pH 5.90 for different ficin concentrations ($[E_0] \gg [S_0]$). The curves from the top downwards are for $[E_0]$ = 0.152, 0.094, 0.073, 0.048, 0.031 and 0.021 mM. Substrate was 5 $\mu$M throughout and other conditions as in fig. 1.

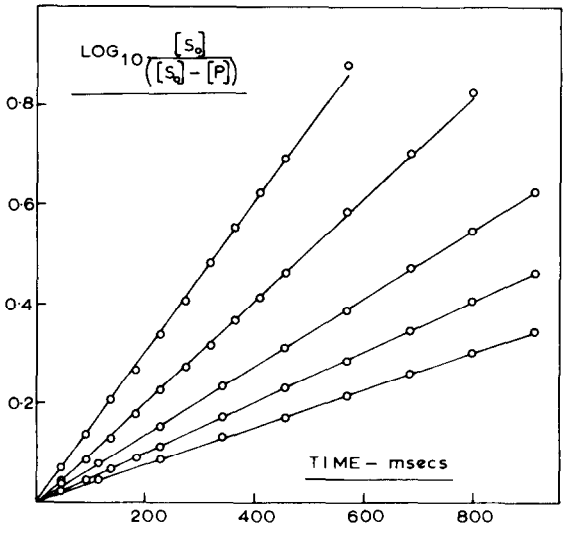


Fig. 3. First-order logarithmic plots at pH 3.90 for different ficin concentrations ($[E_0] \gg [S_0]$). Apart from the pH value conditions were the same as in figs. 1 and 2. Enzyme concentrations were 0.15 (top curve), 0.089, 0.054, 0.036 and 0.024 mM.

when $[E_o] \gg [S_o]$ is given by (ref. [10]):

$$k_1^{obs} = \frac{k_{+2}\,[E_o]}{K_s + [E_o]} \quad \ldots\ldots \qquad (3)$$

where

$$K_s = \frac{k_{-1}}{k_{+1}} \text{ if } k_{-1} \gg k_{+2} \text{ and } k_{-2} = 0\,.$$

Eq. (3) gives on rearrangement:

$$\frac{1}{k_1^{obs}} = \frac{K_s}{k_{+2}} \times \frac{1}{[E_o]} + \frac{1}{k_{+2}} \quad \ldots\ldots \qquad (4)$$

so that a plot of $1/k_1^{obs}$ against $1/[E_o]$ should be linear so long as $k_1^{obs}$ is hyperbolically dependent on $[E_o]$. Plots of this nature at two pH values are given in fig. 4 and the values of $K_s$ and $k_{+2}$ calculated from them in table 1.

As predicted from eq. (3) $k_1^{obs}$ was found to be independent of substrate concentration in the range $[S_o] = 0.5-2.5 \times 10^{-5}$ M so long as the condition $[E_o] \gg [S_o]$ was satisfied. It is possible that the hyperbolic nature of $k_1^{obs}$ against $[E_o]$ plots (i.e. linear double reciprocal plots) could arise from a concentration-dependent aggregation of the enzyme to give a complex with diminished catalytic activity. This possibility was explored by the inclusion of $0.5 \times 10^{-4}$ M iodoacetamide-treated ficin [11] in reaction mixtures containing $0.5 \times 10^{-4}$ M active enzyme and $0.5 \times 10^{-5}$ M PNPH. It was found that progress curves with and without inactivated enzyme were not significantly different, so that complications arising from protein aggregation are unlikely to account for the experimental observations.

### 3.2. $[S_o] \gg [E_o]$

It was originally shown by Gutfreund and Sturtevant [12] that when this condition holds for scheme

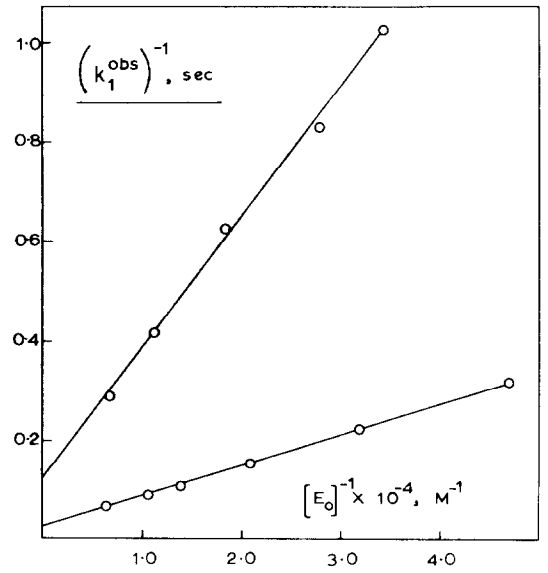


Fig. 4. Double reciprocal plots according to eq. (4) of data from figs. 2 and 3. The upper curve is for pH 3.90 and the lower for pH 5.90. The values of $k_{+2}$ and $K_s$ derived from these plots are given in table 1.

(1) then the concentration of $P_1$ at time $t$ in the early stages of the reaction, where free substrate concentration approximates to its initial value, is given by:

$$[P_1] = \frac{k_{cat}\,[E_o]\,t}{\left\{1 + \frac{K_m}{[S_o]}\right\}} + \left[\frac{\frac{k_{+2}}{(k_{+2}+k_{+3})}}{\left\{1 + \frac{K_m}{[S_o]}\right\}}\right]^2 [E_o](1-e^{-kt}) \quad \ldots\ldots \qquad (5)$$

Table 1

Kinetic parameters for the ficin-PNPH system obtained under the condition $[E_o] \gg [S_o]$.

| pH | $K_s \times 10^4$ M | $k_{+2}$ $sec^{-1}$ | $k_{+2}/K_s \times 10^{-5}$ $M^{-1}$ $sec^{-1}$ | $k_{cat}/K_m \times 10^{-5}$ $M^{-1}$ $sec^{-1}$ |
|---|---|---|---|---|
| 5.90 | 2.8 ± 0.4 | 44.0 ± 4.0 | 1.57 ± 0.4 | 1.34 ± 0.17 |
| 3.90 | 2.0 ± 0.4 | 7.8 ± 1.3 | 0.39 ± 0.13 | 0.46 ± 0.07 |

The values of $K_s$ and $k_{+2}$ were obtained as described in the text. The ratio of $k_{cat}/K_m$ is included for comparison with the $k_{+2}/K_s$ ratio. Acetate buffers were used throughout, $I$ 0.1 at 25°.

where

$$k_{cat} = \frac{k_{+2} \times k_{+3}}{(k_{+2} + k_{+3})} \cdots\cdots$$

$$K_m = K_s \times \frac{k_{+3}}{(k_{+2} + k_{+3})} \cdots\cdots \quad (6)$$

and

$$k = \frac{k_{+2} + k_{+3}\,(1 + \dfrac{K_s}{[S_o]})}{\left\{1 + \dfrac{K_s}{[S_o]}\right\}} \cdots\cdots \quad (7)$$

When the value of $t$ becomes large the exponential in (5) vanishes and the only time-dependent term is linear in $t$. The variation of this steady-state rate with substrate concentration may then be used to evaluate $k_{cat}$ and $K_m$. These steady-state kinetic parameters, evaluated as indicated in section 2, are given in table 2.

The values of $K_m$ and $k_{cat}$ taken in conjunction with the values of $K_s$ and $k_{+2}$ of table 1, enable calculation of a value for $k_{+3}$ using eq. (6). The values so obtained at pH 3.9 and 5.9 are given in table 2.

From eq. (6) it is apparent that:

$$\frac{k_{cat}}{K_m} = \frac{k_{+2}}{K_s} \cdots\cdots \quad (8)$$

So that, if eq. (1) holds, the ratio of $k_{cat}/K_m$ derived from the steady-state conditions should be identical to the value of the ratio of $k_{+2}/K_s$ obtained from data where $[E_o] \gg [S_o]$. That this is so within experimental error is apparent in table 1.

Eq. (5) predicts that, when $[S_o] \gg [E_o]$, a burst of $p$-nitrophenol would be released in the early stages of the reaction and that the amplitude of the burst (A) will be:

$$A = \left[\frac{\dfrac{k_{+2}}{(k_{+2} + k_{+3})}}{\left\{1 + \dfrac{K_m}{[S_o]}\right\}}\right]^2 \times [E_o] \cdots\cdots \quad (9)$$

and that the half-life of the burst should be $(\log_e 2)/k$. Substituting the appropriate values from tables 1 and 2 in eqs. (5) and (9) it may be calculated that, at pH 5.9 with a substrate concentration of $1.0 \times 10^{-4}$ M, there should be a burst of $p$-nitrophenol equivalent to about 30% of the enzyme concentration and with a half-life of 35 msec. The experimental values, calculated from the trace shown in fig. 5(a), were a burst of 22% and a half-life of 35 msec, in fair agreement with the predicted values. At pH 3.9 however the burst would only be circa 3% and so virtually undetectable. Inspection of fig. 5(b) reveals that, as predicted, there is a negligible burst at this pH value.

### 3.3. $[E_o] \approx [S_o]$

Under this condition no direct analytical solution of the differential equations arising from (1) are possible. Thus values of $K_s$, $k_{+2}$ and $k_{+3}$ from the experiments where $[E_o] \gg [S_o]$ and $[S_o] \gg [E_o]$ were set in an analogue computer programme and the curves obtained at the appropriate $[E_o]$ and $[S_o]$ concentrations com-

Table 2

Steady-state kinetic parameters for the ficin-PNPH system and calculated values for the "deacylation" rate-constant.

| pH | $K_m^{obs} \times 10^5$ M | $k_{cat}$ sec$^{-1}$ | $k_{+3}^{calc}$ sec$^{-1}$ | $K_m^{calc} \times 10^5$ M |
|---|---|---|---|---|
| 5.90 | 5.3 ± 0.4 | 7.1 ± 0.3 | 8.5 ± 2.4 | 4.5 |
| 3.90 | 10.7 ± 0.6 | 5.0 ± 0.3 | 13.9 ± 6.9 | 12.8 |

The observed steady-state kinetic parameters were determined as described in section 2 and the values of $k_{+3}^{calc}$ and $K_m^{calc}$ as described in section 3. Condisitions are as for table 1.

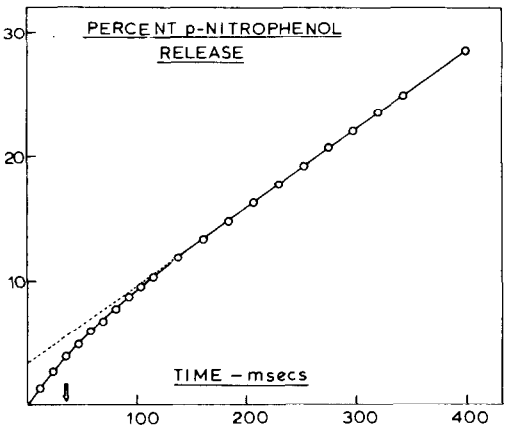


Fig. 5a.

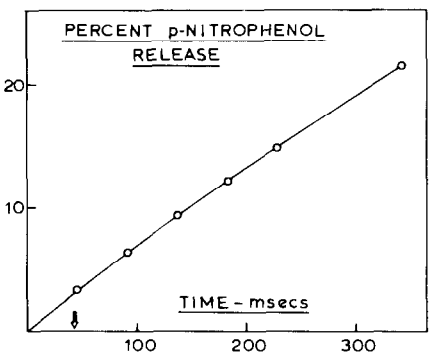


Fig. 5b.

Progress curves for the ficin-catalysed release of *p*-nitrophenol from PNPH under the condition $[S_o] \gg [E_o]$. Both curves are for 0.1 mM substrate but in fig. 5a ficin was 15.5 $\mu$M and pH 5.90 whereas in fig. 5b ficin was 30.6 $\mu$M and pH 3.90. All other conditions as in fig. 1. The ordinate represents the percent of *p*-nitrophenol released from PNPH at different times (100% is equivalent to 0.1 mM).

pared with experimental curves. Comparisons of experimental and calculated curves so obtained are given in figs. 6 and 7.

The value of $K_s$ used for obtaining the optimum fit for the curves at pH 5.90 was $3.9 \times 10^{-4}$ M whereas the value calculated from the studies with $[E_o] \gg [S_o]$ was $2.8 \times 10^{-4}$ M (table 1). Given the errors implicit in the measuring of the absolute enzyme con-

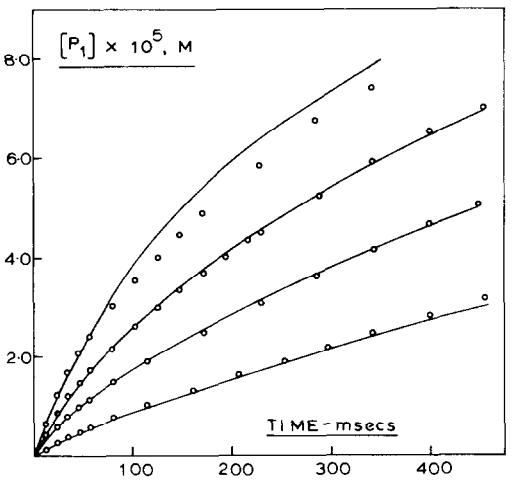


Fig. 6. Comparison of observed and computed curves for the time-course of *p*-nitrophenol release under conditions where $[E_o] \approx [S_o]$. The solid lines represent the concentration of *p*-nitrophenol at different times obtained from an analogue computer programme with $k_{+1} = 2.56 \times 10^6$ $M^{-1}$ $sec^{-1}$, $k_{-1} = 10^3$ $sec^{-1}$, $k_{+2} = 40$ $sec^{-1}$ and $k_{+3} = 8.3$ $sec^{-1}$. The circles denote experimental curves at pH 5.90. The ficin concentrations were 75.6 (top trace), 47.2, 29.4 and 15.5 $\mu$M and the substrate 100 $\mu$M throughout. These values were set in the analogue computer programme to obtain the curves shown.

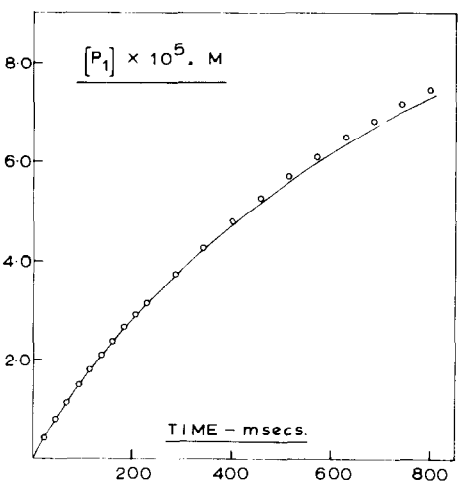


Fig. 7. Observed and calculated time courses for *p*-nitrophenol release from PNPH when $[E_o] \approx [S_o]$ at pH 3.90. The solid line represents the curve computed with $k_{+1} = 5.0 \times 10^6$ $M^{-1}$ $sec^{-1}$, $k_{-1} = 10^3$ $sec^{-1}$ (i.e. $K_s = 2.0 \times 10^{-4}$ M), $k_{+2} = 6.58$ $sec^{-1}$ and $k_{+3} = 13.9$ $sec^{-1}$ (cf. the values in table 1 and 2). The circles represent experimental points with 84.1 $\mu$M ficin and 100 $\mu$M PNPH. These values were set in the analogue computer programme to obtain the computed curve.

centration, the difference in these values is considered to be within experimental error, so that there is fair agreement between calculated and experimental curves at pH 5.90 and 3.90.

## 4. Discussion

The results indicate that the kinetic data for the ficin-catalysed hydrolysis of PNPH may be consistently fitted to the simple three-step mechanism (1), over a wide range of enzyme and substrate concentrations at both pH 5.90 and pH 3.90. Nevertheless it should be emphasized that this scheme is only minimal and other, more complex models may apply as long as certain conditions are satisfied. Thus a scheme such as:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} ES' \xrightarrow{k_{+3}} EP_2 \xrightarrow[+P_1]{k_{+4}} E + P_2 \ldots\ldots \quad (10)$$

is consistent with the data but only if equilibration of ES′ with E and S occurs within 3 msec. There could also be other intermediates between $EP_2$ and products but these could only exist at very low concentrations e.g. if the rate of their formation from $EP_2$ is slow compared with the rate of their breakdown. The enzyme-hippuric acid acid complex is also neglected since the observation that 0.1 mM hippurate does not affect the kinetics means that, in these experiments, this intermediate is not present at significant concentrations.

It is of interest to compare the results of the present study with those obtained by Hubbard and Kirsch [13] for the papain-catalysed hydrolysis of some nitrophenyl esters of carbobenzoxyglycine under the condition $[E_o] \gg [S_o]$. These authors noted that the observed first-order rate constant for the papain-catalysed release of nitrophenol from these substrates was linearly dependent on enzyme concentration. This implies that for these substrates $K_s \gg [E_o]$ so that $k_{+2}$ and $K_s$ may not be evaluated separately (see eq. (3)). However it is noteworthy that the value of

$$\frac{k_{+2}}{K_s} = 2.75 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$$

at pH 6.8 for the hydrolysis of Cbz-Gly-ONP by papain is similar to that for ficin with PNPH as substrate (ca $1.3 \times 10^5$ $M^{-1}$ $sec^{-1}$ at pH 5.9).

A further study of the hydrolysis of a *p*-nitrophenyl ester substrate under conditions where $[E_o] \gg [S_o]$ was by Kèzdy and Bender [10]. These authors followed the rate of chymotrypsin-catalysed release of *p*-nitrophenol from *p*-nitrophenyl acetate and applied the double reciprocal plot (4) to their data to obtain values of $K_s = 3.7 \times 10^{-4}$ M and $k_{+2} = 0.18$ $sec^{-1}$ at pH 5.91 and 25°. However Faller and Sturtevant [14] have subsequently investigated the same reaction and claim that acylation of chymotrypsin by *p*-nitrophenol acetate "follows second order kinetics rather than Michaelis-Menten kinetics as previously reported". In view of this consideration and the fact that *p*-nitrophenyl acetate bears little resemblance to "normal" chymotrypsin substrates, the present study represents the first direct measurement of the apparent dissociation constant for the complex between a proteolytic enzyme and a *p*-nitrophenyl ester substrate.

A consideration of the values of kinetic parameters for the ficin-catalysed hydrolysis of PNPH at pH 3.9 and 5.9 (tables 1 and 2) suggest the following conclusions. Firstly that $K_s$ seems to be essentially pH-independent in this pH range. Secondly, $k_{+2}$ decreases by a factor of circa 6 in going from pH 5.9 to 3.9, thereby implicating in the acylation reaction a prototropic group which undergoes ionisation between these pH values. The third and perhaps most significant conslusion is that the value of $k_{+3}$ does not decrease significantly at low pH values and may even increase slightly. Since the prototropic group involved in "acylation" would also be expected to be involved in "deacylation", the foregoing observation suggests that its $pK_a$ value undergoes a considerable shift on going from the ES to the $EP_2$ intermediate.

The present study indicates that it is possible to evaluate directly the kinetic parameters for the interconversion of all kinetically significant intermediates in the ficin-PNPH system. The evaluation of these parameters under a wide variety of experimental conditions is in progress.

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