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β-SECONDARY DEUTERIUM ISOTOPE EFFECT AND SOLVENT ISOTOPE EFFECTS IN CATALYSIS BY SUBTILISIN BPN'

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Subtilisin BPN' catalyzes the hydrolysis in protium and deuterium oxides of p-$NO_2C_6H_4OCOCL_2NHCO_2CH_2C_6H_5$ (L = H, D) in the pH(D) range 5·0–8·5 (H_2O) and 5·4–9·0 (D_2O), according to simple Michaelis–Menten kinetics. The parameter k_{cat}/K_m exhibits pH(D) inflection points of 7·17 ± 0·05 (H_2O) and 7·69 ± 0·08 (D_2O), and k_{cat} shows 6·88 ± 0·05 (H_2O) and 7·50 ± 0·07 (D_2O). The 'normal' ΔpK values of 0·5–0·6 indicate no unusual effects of D_2O on enzyme properties. The solvent isotope effects (H_2O/D_2O) on the limiting values of the rate constants at high pH(D) are 1·13 ± 0·07 for k_{cat}/K_m and 1·29 ± 0·05 for k_{cat}. These small effects indicate no more than minor contributions of general acid–base catalysis for rate-limiting events for either k_{cat}/K_m or k_{cat}. The β-deuterium secondary isotope effects (2H/2D) are roughly estimated by extrapolation as 0·95 ± 0·01 for k_{cat}/K_m, corresponding to substantial tetrahedral character in the transition state, and 1·03 ± 0·03 for k_{cat}, consistent with no tetrahedral character. Models consistent with these results have as rate-limiting events for k_{cat}/K_m nucleophilic attack by active-site imidazole and for k_{cat}, among other possibilities, the release of carboxylate product from the imidazolium form of the enzyme.

INTRODUCTION

The participation of general acid–base catalysis in the catalytic action of serene proteases can be investigated by the use of solvent isotope effects.[1–4] When an exchangeable proton is involved in acid–base catalysis, reactions are commonly 2–4 times faster in H_2O than in D_2O.

β-Deuterium secondary isotope effects (βDIE) can be used to probe the related question of how much rehybridization has occurred in the carbonyl group of the substrate at the catalytic transition state.[5–8] In the formation of the expected quasi-tetrahedral transition state for acyl transfer, hyperconjugation from the β-CH(D) bonds into the carbonyl π orbital is reduced. This leads to an increase of electron density in the β-CH(D) bonds and to a strengthening of the bonds, and thus to an inverse isotope effect.[9] For βDIEs, a limiting value of $k(H)/k(D) = 0·955$ per deuterium can be estimated from the equilibrium effect for ketone hydration,[5] where a completely tetrahedral structure is achieved in the product. Transition states with intermediate degrees of rehybridization are expected to show intermediate isotope effects. A quantity ↥ can be defined[5] by equation (1) to relate, at least roughly, the measured βDIE $[k(H)/k(D)]$ to the extent of tetrahedral character developed at the transition state:

$$k(H)/k(D) = [K(H)/K(D)]^{\uparrow} \quad (1)$$

where $K(H)/K(D)$ is 0·955 per deuterium.

Similarly, ↥ values may be obtained from α-D isotope effects in acyl transfer reactions of formyl substrates.[10,11] ↥ values calculated from α-D and from β-D effects in similar acyl-transfer reactions agree with each other.[3,5] In the model reactions most relevant for enzyme-catalyzed acyl transfer, ↥ values are between 0·58 and 0·66 for four cases of protolytically catalyzed attack of water on ester carbonyl.[5] For deacylation for chyomtrypsin and elastase, various substrates yield ↥ between 0·27 and 0·84.[3] This paper extends these studies to the bacterial enzyme subtilisin BPN', which, in spite of a preference for hydrophobic side chains at the S_1 sub-site, has a broad specificity.

RESULTS

pH and pD dependence

For hydrolysis of p-$NO_2C_6H_4OCOCH_2NHCbz$ by subtilisin BPN', the values of k_{cat}/K_m, k_{cat} and K_m at various pH(D) are shown in Tables 1 (H_2O) and 2 (D_2O).

Some spontaneous hydrolysis occurred and albumin, added to stabilize the subtilisin, catalyzed substrate hydrolysis to a minor extent. Solvent isotope effects for the spontaneous and albumin-catalyzed hydrolyses of the substrate were 1·37 ± 0·03 and 1·35 ± 0·05, respectively, and were independent of pH. The data in Tables 1 and 2 (which have been corrected and therefore refer to the enzyme-catalyzed reaction alone) are described by the equation

$$k = k^{lim}K_a/(K_a + a_L) \quad (2)$$

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Table 1. Kinetic parameters[a] for solvolysis of *p*-$NO_2C_6H_4OCOCH_2NHCbz$ by subtilisin BPN' at 25·00 ± 0·02 °C and $\mu = 0{\cdot}27$ M[b] as a function of pH

pH	k_{cat} (s^{-1})	$10^{-4}k_{cat}/K_m$ ($l\,mol^{-1}s^{-1}$)	10^4K_m (M)
5·00	0·986	0·444	2·22
5·00	1·115	0·484	2·31
5·60	3·550	2·030	1·74
5·62	3·278	1·820	1·80
6·01	9·400	4·990	1·90
6·01	11·240	5·323	2·11
6·40	24·800	10·40	2·39
6·48	29·540	17·42	1·70
6·84	46·68	32·39	1·44
7·23	68·33	54·10	1·26
7·61	90·53	79·00	0·90
7·61	97·43	75·47	1·29
8·02	92·08	104·30	0·88
8·03	99·80	100·50	0·99
8·52	92·70	95·17	0·97

[a] Kinetic parameters were obtained from Eadie–Hofstee plots and all have errors less than ±5%.
[b] With $KHPO_4$ and K_2HPO_4 above pH 6; with CH_3CO_2K (0·1 M) and KCl below pH 6.

Table 2. Kinetic parameters[a] for solvolysis of *p*-$NO_2C_6H_4O_2CCH_2NBCbz$ by subtilisin BPN' at 25·00 ± 0·02 °C in D_2O and $\mu = 0{\cdot}27$ M[b] as function of pD

pD	k_{cat} (s^{-1})	$10^{-4}k_{cat}/K_m$ ($l\,mol^{-1}s^{-1}$)	10^4K_m (M)
5·40	0·623	0·479	1·31
5·40	0·587	0·572	1·03
6·50	6·030	5·700	1·06
6·50	7·290	5·90	1·24
6·92	17·72	11·77	1·50
7·35	28·30	21·41	1·34
7·35	31·85	29·00	1·10
7·85	53·60	45·60	0·81
8·08	64·50	84·37	0·76
8·12	76·20	72·55	1·05
8·54	76·17	83·18	0·92
9·02	75·30	85·78	0·88

[a,b] See footnotes to Table 1.

where k^{lim} is the limiting value of the particular rate constant, K_a is an apparent ionization constant and a_L is the hydrogen-ion activity expressed in molar units for both H_2O and D_2O.

Nonlinear least-squares fit[3] of the data yields the following:

$$10^{-6}(k_{cat}/K_m)^{lim} = 1{\cdot}10 \pm 0{\cdot}035\ l\,mol^{-1}s^{-1} \quad (H_2O)$$
$$10^{-6}(k_{cat}/K_m)^{lim} = 0{\cdot}99 \pm 0{\cdot}053\ l\,mol^{-1}s^{-1} \quad (D_2O)$$
$$10^8K_a = 6{\cdot}81 \pm 0{\cdot}82\ (H_2O)\ \text{for}\ k_{cat}/K_m$$
$$10^8K_a = 2{\cdot}02 \pm 0{\cdot}39\ (D_2O)\ \text{for}\ k_{cat}/K_m$$
$$(k_{cat})^{lim} = 103{\cdot}2 \pm 2{\cdot}7\ s^{-1}\ (H_2O)$$
$$(k_{cat})^{lim} = 80{\cdot}0 \pm 4{\cdot}0\ s^{-1}\ (D_2O)$$
$$10^8K_a = 13{\cdot}0 \pm 1{\cdot}5\ (H_2O)\ \text{for}\ k_{cat}$$
$$10^8K_a = 3{\cdot}14 \pm 0{\cdot}54\ (D_2O)\ \text{for}\ k_{cat}$$

The solvent isotope effects in the now customary notation[12] are

$$^{DOD}(k_{cat}/K_m)^{lim} = 1{\cdot}13 \pm 0{\cdot}07$$
$$^{DOD}(k_{cat})^{lim} = 1{\cdot}29 \pm 0{\cdot}053$$

The values of pK_a for k_{cat}/K_m are 7·17 ± 0·05 (H_2O) and 7·69 ± 0·08 (D_2O) and for k_{cat} are 6·88 ± 0·05 (H_2O) and 7·50 ± 0·07 (D_2O). Figures 1 and 2 show the pH(D) rate profiles.

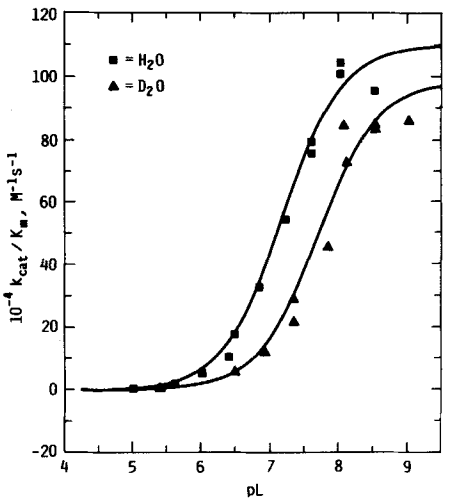


Figure 1. pL–rate profiles for k_{cat}/K_m in the subtilisin BPN'-catalyzed hydrolysis of *p*-$NO_2C_6H_4OCOCH_2NHCbz$

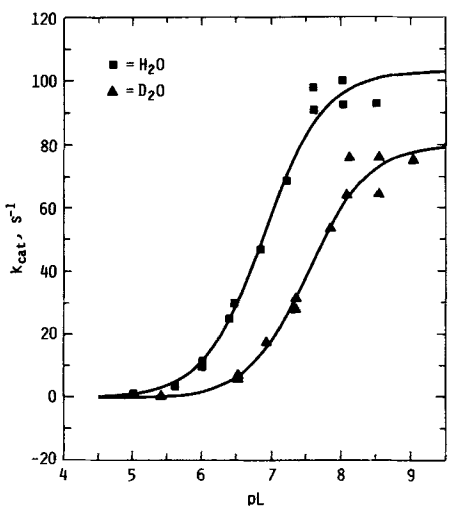


Figure 2. pL–rate profiles for k_{cat} in the subtilisin BPN'-catalyzed hydrolysis of *p*-$NO_2C_6H_4OCOCH_2NHCbz$

β-Deuterium isotope effects

Table 3 gives the mean ratios of initial rates of reactions of $NO_2C_6H_4OCOCH_2NHCbz$ and $NO_2C_6H_4OCOCD_2NHCbz$ with subtilisin BPN′ at a series of substrate concentrations. The isotope effect appeared to be moving towards 1·0 or larger as the degree of saturation increases. It seemed worthwhile to estimate the isotope effect at unit saturation [$^{2D}k_{cat}$] by extrapolation, even though it is obvious that the estimate will not be very precise. The individual measurements have standard deviations of 1–3·2% and it was not possible to make measurements above 44% saturation because of the limited solubility of the substrate.

The observed isotope effects $k(2H)/k(2D)$ can be thought of[13] as weighted averages of the averages of the isotope effects on k_{cat} and k_{cat}/K_m, as follows:

$$1/k(2D) = [1/k_{cat}(2D)] + \{1/[Sk_{cat}/K_m(2D)]\} \quad (3)$$

then,

$$k(2H)/k(2D) = \frac{k(2H)}{k_{cat}(2D)} + \frac{k(2H)}{Sk_{cat}/K_m(2D)} \quad (4)$$

or,

$$k(2H)/k(2D) = \frac{k(2H)}{k_{cat}(2H)} \cdot \frac{k_{cat}(2H)}{k_{cat}(2D)} + \frac{k(2H)}{k_{cat}/K_m(2H)} \cdot \frac{k_{cat}/K_m(2H)}{k_{cat}/k_m(2D)} \quad (4a)$$

$$k(2H)/k(2D) = F_{ES}^{2D}k_{cat} + (1 - F_{ES})^{2D}(k_{cat}/K_m) \quad (5)$$

where:

$$F_{ES} = k(2H)/k_{cat}(2H) = S/(S + K_m^{2H})$$
$$1 - F_{ES} = k(2H)/(k_{cat}/K_m(2H) = K_m^{2H}/(S + K_m^{2H})$$

Note that the factor F_{ES} depends on K_m *for the protiated substrate only.*[14] In the following treatment, K_m was taken as 0·22 mM, a mean value which we have established to within a standard deviation of 8% by repeated determinations. Changes of 10% in K_m (0·20 and 0·24 mM) led to changes of less than 1% in the calculated isotope effects, as expected from the known[14] insensitivity of this treatment to the exact value employed for K_m.

A linear least-squares regression of the 44 ratios of initial velocities at different F_{ES} were fitted to equation (6) [a rearranged form of equation (5)].

$$^{2D}k = {}^{2D}(k_{cat}/K_m) + [{}^{2D}k_{cat} - {}^{2D}(k_{cat}/K_m)]\,F_{ES} \quad (6)$$

As already emphasized, F_{ES} is limited to values of 0·44 and lower and owing to the scatter at each F_{ES} it is therefore not possible to evaluate the extrapolated isotope effects on k_{cat} with sufficient confidence to base strong mechanistic conclusions on its exact values, although the value of $^{2D}(k_{cat}/K_m)$ is obtained with good precision. The intercept value at $F_{ES} = 0$ gives the isotope effect $^{2D}(k_{cat}/K_m)$ and extrapolation to $F_{ES} = 1·0$ yields the isotope effect for $^{2D}k_{cat}$. The slope of equation (6) gives the difference between the two isotope effects and is equal to +0·080 [standard deviation (SD) = 0·036]. This indicates at the 95% (or better) confidence level that $^{2D}k_{cat}$ is larger than $^{2D}(k_{cat}/K_m)$. (A direct assessment of the significance of this difference can be made through statistical F and t tables.[15] F values were 4·95 for 42 degrees of freedom and the lack of fit 1·32 for 5 degrees of freedom, which indicates the 95% confidence level in the goodness of fit. Similarly, calculated t values give the 96·5% confidence level if the slope is allowed to take positive or negative values and the 99% confidence level if the slope can have only positive values.) The isotope effects obtained from the intercepts are $^{2D}(k_{cat}/K_m) = 0·947$, SD = 0·012, and $^{2D}k_{cat} = 1·03$, SD = 0·03. These experiments were conducted at pH 6·00, where the correction for background hydrolysis was less than 1%. The βDIE for the nonenzymic hydrolysis of p-$NO_2C_6H_4OCOCL_2NHCbz$ at pH 6·00 in 0·2 M phosphate buffer was $^{2D}k = 0·939 \pm 0·013$.

Table 3. Secondary isotope effects for p-$NO_2C_6H_4OCOCH_2NHCbz$. as a function of substrate concentration for subtilisin BPN′ at pH 6·00, $\mu = 0·27$ M and 25·00 ± 0·02 °C[a]

10^4[$NO_2C_6H_4OCOCL_2NHCbz$] (M)	F_{ES}[b]	$k(2H)/k(2D)$	Number of ratios
0·439	0·166	0·957 ± 0·020	10
0·658	0·230	0·969 ± 0·020	7
0·877	0·285	0·982 ± 0·025	4
1·096	0·333	0·963 ± 0·032	6
1·316	0·374	0·991 ± 0·023	5
1·535	0·411	0·985 ± 0·032	5
1·754	0·444	0·979 ± 0·010	7

[a] Phosphate buffer, 0·20 M; subtilisin, $9·93 \times 10^{-9}$ M.
[b] Fraction of enzyme saturated = [S]/(K_m + [S]); K_m = 0·22 mM.

DISCUSSION

pH and pD dependence for k_{cat}/K_m and k_{cat} for subtilisin BPN' with p-$NO_2C_6H_4OCOCH_2NHCbz$

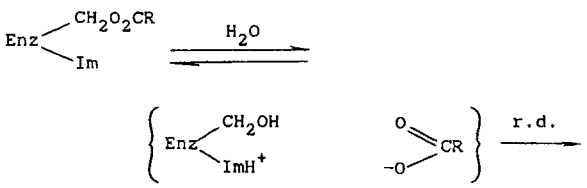
The pK value from the pH–rate profile for k_{cat}/K_m in H_2O, 7·17 ± 0·05, is in good agreement with a value of 7·15 reported for subtilisin BPN' with various substrates by Philipp *et al.*[16] Polgar[17] found 7·20 for subtilisin Carlsberg with p-$NO_2C_6H_4OCOCH_2NHCbz$. Our pK value in D_2O is 7·69 ± 0·08, yielding a difference between H_2O and D_2O of $\Delta pK = 0·52 \pm 0·09$. The results for k_{cat} (6·88 ± 0·05 in H_2O, 7·50 ± 0·07 in D_2O) give $\Delta pK = 0·62 \pm 0·09$. These values approximate the expected[18] solvent isotope effect for ionization of an acid in this pK_a range. They indicate that neither the free enzyme (the ionizing entity for k_{cat}/K_m) nor the acyl enzyme (probably the ionizing entity for k_{cat}, for which deaclyation is likely[19] to be rate determining with this substrate) undergoes any unusual structural alteration in D_2O. This in turn means that the solvent isotope effects on the limiting values of k_{cat} and k_{cat}/K_m ought to be directly interpretable in mechanistic terms without reference to possible conformational or other changes induced by D_2O. The effects of 1·1–1·3 are much smaller than other serine proteases exhibit when general acid–base catalysis is important in the rate-determining steps.[1-4] Then ^{DOD}k is typically 2·4 or greater. The small effects observed here therefore suggest that such catalysis is at best a minor component of the rate-limiting process.

β-Deuterium isotope effects

The isotope effect on k_{cat}/K_m is 0·95, which corresponds to substantial tetrahedral character ($\hat{t} \approx 0·6$). This kinetic term corresponds to acylation, and whereas it would not be surprising for carbonyl attack to be rate limiting for acylation (consistent with $\hat{t} \approx 0·6$), it is striking that the attack does not seem to be general acid–base catalyzed [$^{DOD}(k_{cat}/K_m) = 1·1$]. A small solvent isotope effect (1·23) was also reported for k_{cat}/K_m of this same substrate with subtilisin Carlsberg.[17,20] Five aryl esters of *N*-(methoxycarbonyl)-L-Phe, that satisfy the preference of subtilisin BPN' at the S_1 subsite,[19] were studied recently by Matta and Andracki.[21] Their kinetic data seem to indicate the initial steps to be rate determining at low substrate concentration without noticable sensitivity to the nature of the leaving group. The solvent isotope effects for the k_{cat}/K_m term are small, 0·9 ± 1·3 for leaving groups with electronic properties between *p*-nitrophenyl and *p*-methoxylphenyl, whereas Matta *et al.*[22] reported solvent isotope effects of 2–3 for the same enzyme and other phenyl esters. All these processes, however, follow the same pH dependence corresponding to the uncomplexed enzyme. Matta and Andracki[21] interpreted their data by proposing an isotopically insensitive step, a conformational change, to be rate determining in the course of acylation of the aryl esters of *N*-(methoxycarbonyl)-LPhe. A rate-limiting conformational change by itself cannot explain the acylation of subtilisin BPN' by $NO_2C_6H_4OCOCH_2NHCbz$, since it would not be consistent with the approximately 60% tetrahedral character derived from the β-deuterium isotope effect on k_{cat}/K_m.

The variation of solvent isotope effect with substrate structure suggests that the involvement of rate-limiting general catalysis in these systems is a sensitive function of substrate structure. Earlier investigators[23] suggested that certain substrates exhibiting small solvent isotope effects in their reactions with chymotrypsin were undergoing a nucleophilic attack on substrate carbonyl by histidine rather than serine. A later rearrangement to the acylserine species would then permit normal behaviour in deacylation. Such an attack by histidine, without general catalysis, could explain the presence of a βDIE and the absence of a solvent isotope effect for k_{cat}/K_m in the present case. A mechanism involving nucleophilic attack by histidine instead of general base catalysis could probably be promoted by a different conformational arrangement to that which is occurring with substrates that more nearly mimic the natural substrates. When activated esters with good leaving groups are involved, nucleophilic attack by histidine may be preferred over the histidine-catalyzed attack by serine.

The k_{cat} process which, as already mentioned, presumably represents deaclyation, exhibits neither a solvent isotope effect of a magnitude consistent with general catalysis nor a β-D effect indicative of other than trigonal carbonyl. Any number of models may fit the data; obvious ones involve conformational changes. One possibility is that a product-release step, which is in effect an ion-pair dissociation, limits the rate:



A small normal β-D isotope effect similar to that on carboxylic acid ionization and related reactions (about 1% per deuterium)[7,24] would be expected in this case.

The overall picture for p-$NO_2C_6H_4OCOCH_2NHCbz$ with subtilisin BPN' is therefore one in which an unusual mechanism of acylation may be involved, and in which an unusual step in deaclyation may limit the rate. These facts are particularly striking in view of the large magnitude of catalysis being effected: k_{cat}/K_m is ca 10^6 l mol^{-1} s^{-1} (1500 fold greater than the second-order rate constant for hydroxide attack), while k_{cat} is

$10^2\ s^{-1}$. These are among the largest parameters each reported for phenyl ester substrates of subtilisin Carlsberg.[19] One possible factor which might be at work in the acylation step is insertion of the *p*-nitrophenyl group into the 'specificity pocket' of the active site. This could maintain strong transition-state binding while producing nucleophilic attack by the wrong functional group.

EXPERIMENTAL

Materials and solutions. Buffer salts and solvents were as described previously.[2] The acquisition and synthesis of p-$NO_2C_6H_4OCOCL_2NHCbz$ (L = H, D) has also been reported previously.[3] Subtilisin BPN′ Type VII (E.C. 3.4.21.14) was obtained from Sigma as a crystallized and lyophilized powder with an indicated activity of 12·2 units mg^{-1}. A 7×10^{-6} M stock solution of subtilisin in 70% glycerol and 30% 0·2 M phosphate buffer (pH 6·5) was stored at −70 °C in small vials. Active-site titration of the stock solution showed the commercial material to be 90 ± 2% active subtilisin. Dilutions of this stock solution with the appropriate buffer were made fresh for each experiment. To stabilize enzyme stock solutions further through a working period, 1 mg ml^{-1} of bovine serum albumin was added to the diluted enzyme stock solution. Phosphate buffers were used in calculated concentrations (0·09–0·20 M) to give an ionic strength of 0·27 M in all runs. Acetate buffer (μ = 0·27 with KCl) was used at pH = 5·0.

Kinetic procedures. All reaction rates were measured spectrophotometrically under zero-order conditions. Reactions were monitored at 347·5 nm, the isosbestic point of *p*-nitrophenoxide and *p*-nitrophenol. The molar absorptivity was determined from seven solutions buffered at pH 2–12. A Beer's law plot constructed from data at pH 7·00 gave a molar absorptivity of 5500 ± 99 l mol^{-1} cm^{-1} for *p*-nitrophenol and *p*-nitrophenoxide and 330 ± 19 l mol^{-1} cm^{-1} for the ester. There is evidence for the lack of solvent isotope effect on the molar absorptivity of *p*-nitrophenoxide.[2] To obtain optimum reproducibility, all components of the reaction mixture were mixed and then pipetted into cuvettes. Buffer was added to bring the total volume to 1 ml, including the volume of an aliquot of substrate stock solution in acetonitrile. Solutions were brought to thermal equilibrium in a jacketed cell holder in the cell compartment of a Cary-16 or Cary-118 spectrophotometer. The temperature was maintained by water circulated from a Lauder 4KR bath, was monitored by a digital thermometer and was recorded electronically from a thermistor probe in an adjacent cell. Injection of 50 μl or less of a 3×10^{-3} M solution of the substrate in acetonitrile initiated the reaction. Runs of isotopic substrates were conducted in alternation at each substrate concentration. Rates in H_2O and D_2O were measured with use of the same enzyme stock solution at each pH, within 48 h of each other. Data acquisition and analysis methods are detailed elsewhere.[2]

Background correction. The total velocity in a particular isotopic solvent mixture, V_T, is the sum of enzymic (V_E) and non-enzymic (V_N) contributions. The background reaction was assumed to be first order in substrate:

$$V_T = V_E + V_N = V_E + k_{obs}[S]$$

Values of k_{obs} were determined in the absence of enzyme and under conditions identical with those of the enzymic reactions of interest including the presence of albumin in the appropriate concentration (3% of a 1 mg ml^{-1} solution). The magnitude of the correction reached 20% at pH 8·5 and dropped below 1% at pH 6·5.

Nonlinear least-squares fit of the parameters from Tables 1 and 2 was performed by the use of BMDPAR.[25]

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