THE USE OF A POTASSIUM ION SELECTIVE ELECTRODE FOR MEASURING KINETIC PARAMETERS OF ENZYMATIC HYDROLYSIS OF BUTYRYLCHOLINE

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The suitability of a Crytur 19–15 potassium ion selective electrode for indication of the course of enzymatic hydrolysis of butyrylcholine is demonstrated. The electrode can be also used at high initial concentrations of substrate. Procedures for experimental data handling are suggested.

A typical substrate electrode is the electrode after Baum[1] which is selective for choline esters. It is based on a liquid membrane containing a 5% solution of acetylcholine tetra-(*p*-chlorophenyl)borate in 3-*o*-nitroxylene. This electrode has been employed with success for the determination of the Michaelis constant K_m and the maximum hydrolysis rate v for acetylcholinesterases[2] and cholinesterases[3].

The basic shortcoming of substrate electrodes in kinetic measurements is their poorer sensitivity at higher concentrations of the esters used as substrates (above 1 mmol l^{-1}). This precludes the use of such electrodes for the investigation of the effect of irreversible inhibitors where the substrate concentrations are an order of magnitude higher[4]. Moreover, reversible inhibitors of cholinesterases that possess quaternary or tertiary nitrogen atoms in their molecules can be expected to interfere at low substrate concentrations.

In this work, the possibility of employing a commercial Crytur 19–15 potassium ion selective electrode for monitoring the enzymatic hydrolysis of butyrylcholine with cholinesterase is examined and the effect of higher substrate concentrations on its response during the hydrolysis is investigated.

EXPERIMENTAL

Chemical and Apparatus

Butyrylcholine iodide (henceforth BuCholI), m.p. 87–91°C (Lachema, Brno) was dried over P_2O_5 24 h before use. Lyofilized concentrate of horse plasma (Institute of Sera and Vaccines, Prague) was used as the enzyme.

The solutions were prepared using a buffer solution made up of triethanolamine hydrochloride whose pH was adjusted with 0·1M-NaOH to pH 7·6 at 30°C; the procedure was controlled with

an MV 870 pH-meter (Präcitronic, G.D.R.) equipped with a GA 50 N glass electrode. Kinetic measurements were performed on a device set up of a U-15 ultrathermostat (MLW Medingen, G.D.R.), a G-1002·500 digital voltmeter and a G-3287·500 RET printer (Funkwerk, G.D.R.). Absorbances were measured on a Spekol 11 spectrophotometer (Carl Zeiss, Jena, G.D.R.).

Potential measurements were made in 10 ml of solution of BuCholI at the desired concentration, to which 1 ml of enzyme solution in TRIS buffer (pH 7·6) was added after the potential established. The indicator electrode was a Crytur 19—15 potassium ion selective electrode (K-ISE), the reference electrode was an RCE 102 saturated calomel electrode (Monokrystaly, Turnov, Czechoslovakia) filled with 1M-$NaNO_3$.

Prior to each measurement, the potassium electrode was activated with the butyrylcholinium cation–tetraphenylborate anion associate, namely so that four potentiometric titrations of BuCholI solution with sodium tetraphenylborate in TRIS buffer (pH 7·6) were repeatedly indicated with it.

Potentiometric Titrations

A solution (5 ml) of BuCholI or choline chloride (henceforth CholCl) at a concentration of 10 mmol l^{-1} was pipetted into a beaker and 10 ml of TRIS buffer pH 7·6 and 5 ml of distilled water were added. The measuring cell was submerged and the solution, constantly stirred on an electromagnetic stirrer, was titrated with sodium tetraphenylborate (60 mmol l^{-1}). The electromotive voltage of the cell was measured after each addition of titrant. In this manner, the mean value of the potential range (ΔE) and the slope of the titration curve in the titration end point ($\Delta E/\Delta V$) were obtained.

For determining the dependence of the potential of the K-ISE on the concentration of BuCholI or CholCl, series of solutions of the substances covering the region of 10—0·01 mmol l^{-1} in TRIS buffer were prepared. Between the potential measurements the electrodes were rinsed with distilled water and dried with a filter paper. The measurements were performed repeatedly.

Determination of Enzyme Activity

The enzyme activity was determined at 30°C by the method suggested by Ellman and coworkers[5], based on measurement of the absorbance increase at 412 nm. Reaction mixture was made up from 1 ml of 0·2M-TRIS buffer (pH 7·6) containing 5,5′-dithiobis(2-nitrobenzoic) acid (0·25 mmol l^{-1}), 0·2 ml of substrate solution (1 mg ml^{-1}) in TRIS buffer, 0·1 ml of enzyme solution (300 μg ml^{-1}), also in TRIS buffer, and 0·7 ml of redistilled water. The mean activity was 507 ncat per mg of weighed-in enzyme.

RESULTS AND DISCUSSION

Direct potentiometric measurements were carried out to obtain the dependences of the cell voltage on the logarithms of concentrations of BuCholI and CholCl. The slopes, $W = \Delta u/\Delta \log c$, for voltages u in mV and concentrations c in mol l^{-1}, were 55·5 for BuCholI and 20·1 for CholCl. The lower concentration limits of the regions of linearity of the dependences were 0·025 mmol l^{-1} for BuCholI and 0·091 mmol l^{-1} for CholCl.

The titration curves of the two quaternary ammonium bases titrated with sodium tetraphenylborate were evaluated, the mean potential ranges and slopes of the titra-

tion curves in the titration end points for BuCholI and CholCl were $\Delta E = 125{\cdot}3$ and 45·1 mV and $\Delta E/\Delta V = 397$ and 227 mV ml^{-1}, respectively. It is clear that the sensitivity of the K-ISE used is considerably higher for the butyrylcholinium cation.

The interfering effect of CholCl was tested by direct potentiometric measurements on BuCholI solution (2 mmol l^{-1}) containing various amounts of the interferent. The electrode potential was only found affected if CholCl was present in a 30-fold excess with respect to BuCholI.

The response time of the K-ISE was measured after adding 0·1 ml of BuCholI solution (10 mmol l^{-1}) to solutions of this substance at concentrations from 5 to 0·5 mmol l^{-1}. The response time did not exceed 20 s even in solutions whose concentration approached 0·5 mmol l^{-1}.

The principal problem in monitoring the course of enzymatic hydrolysis with substrate ion selective electrodes lies in the conversion of the data measured to the molar rates of hydrolysis.

The time course of the K-ISE potential is shown in Fig. 1. Readings were made periodically in 10 s intervals for 1 to 3 min until a steady potential established. The data were processed by the adapted VLNA program[6] on a PMD-85 computer. This program, suited to the determination of potentiometric titration end points, fits the data set with three straight lines, seeks their points of intersection (X1, X2), calculates the potential change and the time change (ΔY, ΔX) corresponding to the maximum potential change, and determines the slope of the central straight line ($\Delta Y/\Delta X$).

The potential increase was observable in 10 to 20 s (point X1) for substrate concentrations of 0·4 to 1 – 3 mmol l^{-1} (according to the concentration of enzyme). This can be explained in terms of delay of response of the K-ISE. The delay is longer

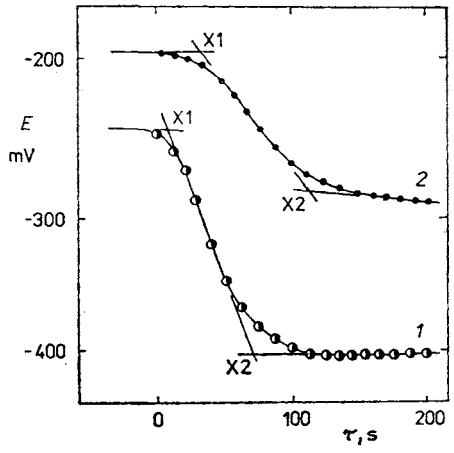

FIG. 1

Potential change of Crytur 19—15 potassium ion selective electrode during enzymatic hydrolysis of butyrylcholine at enzyme concentration $c_E = 296{\cdot}5$ μg ml^{-1}. Concentration of butyrylcholine (mmol l^{-1}): *1* 0·617, *2* 2·468

at higher concentrations, which is apparently related with the lowering sensitivity of the electrode. The expected potential change only appears after a portion of the substrate has reacted. The data are given in Table I.

From among the quantities evaluated, only *X2* exhibited a linear dependence. This provides a total time of hydrolysis (τ) to the final substrate concentration of BuCholI of about 0·07 mmol l^{-1}, corresponding to the sensitivity of the K-ISE to the substrate. Theoretically, τ can be expressed by the adapted Michaelis–Menten equation

$$\tau = \tau_0 + \tau_1 = ([S]_0 - [S]_1)/v + (K_m/v) \ln ([S]_1/7 \,.\, 10^{-5}) , \qquad (1)$$

where τ_0 (described by the first right-hand term) is the time for which the hydrolysis proceeds at the maximum rate (zeroth order reaction), τ_1 (described by the second right-hand term) is the time for which the hydrolysis proceeds as a first order reaction, $[S]_0$ is the initial substrate concentration, $[S]_1$ is the approximate concentration of substrate at which the zeroth order reaction transforms into the first order reaction, K_m is the Michaelis constant and v is the maximum rate in the experiment.

Calculations and a comparison with the data measured at the enzyme concentration $c_E = 236{\cdot}8$ μg ml^{-1} (Table I) revealed that observable course of the hydrolysis as a zeroth order reaction was only apparent at a concentration of 2·84 mmol l^{-1} and represented 9·8% of the total reaction time. At the highest measured concentration of BuCholI in this series, 3·98 mmol l^{-1}, the zeroth order reaction participated 14·5% in the total time of hydrolysis.

Based on the total time of hydrolysis measured (in s), the average hydrolysis rate, in mol l^{-1} min^{-1}, was calculated as

$$\langle v \rangle_{hyd} = ([S]_0/\tau) \,.\, 60 \,. \qquad (2)$$

For a conversion of the data measured during the hydrolysis monitored with the use of substrate ion selective electrodes, Eq. (*3*) after Baum and Ward[3], based on the adapted Nernst equation, was largely used:

$$du/d\tau = 2{\cdot}303 \, (W/[S]_0) \, (d[S]_0/d\tau) \,. \qquad (3)$$

The hydrolysis rates so obtained, denoted $v_{hyd,B}$, are given in Table I. In contrast to the procedure[3] where the slope $du/d\tau$ was obtained by reading the potential change in the starting several tens of seconds, in this work the $du/d\tau$ value was represented by the slope of the central straight line as provided by the VLNA program. This enabled the relation to be also employed at high substrate concentrations.

An alternative procedure for the calculation of the hydrolysis rate consisted in the inclusion of the difference of two states, before and after the hydrolysis, with K-ISE

TABLE I

Data of hydrolysis of butyrylcholine iodide at various concentrations of cholinesterase (c_E)

Substrate concentration mmol l^{-1}	No. of measurements	*X1* s	*X2* s	ΔY mV	$\Delta Y/\Delta X$ mV min^{-1}	$v_{hyd,S}$ mmol l^{-1} min^{-1}	$v_{hyd,B}$ mmol l^{-1} min^{-1}	$\langle v \rangle_{hyd}$ mmol l^{-1} min^{-1}
			$c_E = 296{\cdot}5$ μg ml^{-1}					
0·617	7	4·33	79·21	81·42	65·44	0·417	0·316	0·467
1·236	6	19·41	95·27	84·94	67·69	0·736	0·653	0·780
2·468	6	36·81	117·38	83·13	62·00	1·156	1·197	1·262
4·936	6	67·17	186·13	85·69	43·22	1·519	1·669	1·591
7·404	7	92·46	245·04	85·84	33·87	1·739	1·962	1·815
9·872	6	128·52	284·62	73·86	28·49	1·696	2·201	2·081
12·341	6	168·16	339·91	67·89	23·42	1·527	2·290	2·178
			$c_E = 370{\cdot}6$ μg ml^{-1}					
0·456	4	13·77	68·06	28·15	36·31	0·156	0·166	0·402
0·726	4	11·75	73·75	43·98	43·02	0·350	0·324	0·591
0·908	3	−2·10	78·61	72·00	46·68	0·780	0·465	0·693
2·453	5	33·15	111·15	72·79	56·66	1·540	7·424	1·300
4·906	5	75·09	179·91	72·26	42·58	1·957	2·212	1·636
			$c_E = 236{\cdot}8$ μg ml^{-1}					
0·568	3	−12·68	82·78	81·03	50·89	0·564	0·306	0·413
1·136	3	21·74	97·74	77·45	45·35	0·784	0·546	0·697
1·704	3	24·91	116·45	71·61	43·60	0·940	0·787	0·878
2·840	3	35·61	157·85	76·57	37·58	1·350	1·130	1·079
3·977	3	52·50	185·41	71·38	35·84	1·567	1·509	1·287

potentials u_1 and u_2 (the hydrolysis rate in this approach is denoted $v_{hyd,S}$):

$$u_2 - u_1 = (RT/F) \ln \{([S]_0 - v_{hyd,S}\tau)/[S]_0\} \quad (4)$$

from which the hydrolysis rate is

$$v_{hyd,S} = [S]_0 [\exp (\Delta u/W . 2{\cdot}303) - 1]/\tau . \quad (5)$$

The observed and calculated hydrolysis rates $v_{hyd,S}$, $v_{hyd,B}$ and $\langle v \rangle_{hyd}$ given in Table I, exhibited a hyperbolic dependence on the concentration of substrate, consistent with the Michaelis–Menten equation. An example of this dependence is shown in Fig. 2.

The dependences were linearized according to Hofstee, and the correlation coefficients were calculated[6,7]. The Michaelis constant was determined using the method by Eisenthal and Cornish-Bowden[8], where a simple computer program was set up for the calculation of the points of intersection of the straight lines. The mean coordinates of the points of intersection were evaluated statistically. Based on the Grubs test, outliers were eliminated and the confidence intervals of the mean were determined[6]. The values of the Michaelis constant and the highest rates of the enzymatic hydrolysis obtained by the two methods are given in Table II. The data demonstrate that comparable results in the various series emerge from calculations using the average rate $\langle v \rangle_{hyd}$; here the Michaelis constant values lie within the limits of 1·91 to 2·84 mmol l^{-1} with a dispersion of up to 20% rel.

The dispersion could be reduced to below 10% rel. by increasing the number of measurements repeated. The computer program used, seeking for the optimum three straight lines by the least squares method, does not allow the precision to be additionally improved; a small electrode drift during the measurement brings about a small shift of the point intersection, whereupon the results are affected rather markedly. This is apparently the cause of the fact that the use of the rates $v_{hyd,S}$ and $v_{hyd,B}$, which are considerably more dependent on the measurement and evaluation of the potential change, results in a considerably higher dispersion of data.

By using the highly accurate method by Ellman and coworkers[5] measuring directly the concentration of the thiol released, Augustinsson and coworkers[9] attained a precision of ±6·5% rel. of the Michaelis constant for butyrylcholine and ±11·1% for thiophenyl acetate. When measuring with substrate electrodes, the electrode potential can be expected to be affected not only by the released choline as such but also by its inhibiting effect on cholinesterase[10], particularly in the final stage of hydrolysis. The authors[9,10] also advise the use of a different form of the Michaelis–Menten equation for the description of the hydrolysis with cholinesterase, whereby a higher precision can be attained.

TABLE II

Values of kinetic parameters K_m (mmol l^{-1}) and v (mmol l^{-1} min^{-1}) of hydrolysis of butyrylcholine at various concentrations of cholinesterase (c_E)

Rate type	Correlation coefficient[a]	K_m[a]	v[a]	K_m[b]	v[b]
		$c_E = 296{\cdot}5$ μg ml^{-1}			
$v_{hyd,S}$	0·9612	2·24 ± 0·88	2·08 ± 0·37	2·49 ± 0·44	2·27 ± 0·21
$v_{hyd,B}$	0·9513	5·09 ± 1·74	3·32 ± 0·67	4·78 ± 0·70	3·19 ± 0·16
$\langle v \rangle_{hyd}$	0·9865	2·84 ± 0·42	2·61 ± 0·19	2·93 ± 0·32	2·66 ± 0·14
		$c_E = 370{\cdot}6$ μg ml^{-1}			
$v_{hyd,S}$	0·9412	—	—	—	—
$v_{hyd,B}$	0·9402	—	—	—	—
$\langle v \rangle_{hyd}$	0·9614	2·28 ± 0·34	2·44 ± 0·24	1·82 ± 0·48	2·13 ± 0·38
		$c_E = 236{\cdot}8$ μg ml^{-1}			
$v_{hyd,S}$	0·9514	1·60 ± 1·31	2·03 ± 0·85	—	—
$v_{hyd,B}$	0·9486	7·18 ± 3·08	4·10 ± 1·35	7·51 ± 2·18	4·73 ± 1·51
$\langle v \rangle_{hyd}$	0·9816	2·03 ± 0·44	1·91 ± 0·24	2·17 ± 0·36	2·01 ± 0·23

[a] Evaluated according to Hofstee; [b] evaluated according to Eisenthal and Cornish-Bowden.

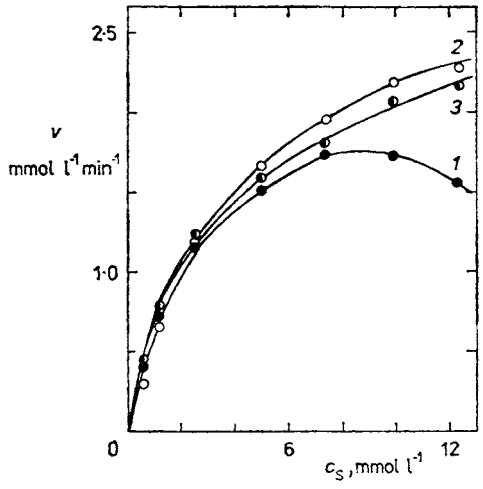

FIG. 2

Dependence of hydrolysis rates $v_{hyd,S}$ (*1*), $v_{hyd,B}$ (*2*) and $\langle v \rangle_{hyd}$ (*3*) on substrate concentration; enzyme concentration $c_E = 296{\cdot}5$ μg ml^{-1}

The difference between the observed value of the Michaelis constant and the value of 0·77 mmol l^{-1} reported by Brestkin and coworkers[11] may be also due to a delay in the electrode response in the final stage of the hydrolysis, where the concentration of butyrylcholine is very low and the cholinium cation is probably extracted into the membrane phase. This delay is relatively longest during experiments with the lowest initial concentration of substrate, giving rise to an increase in both the Michaelis constant and the maximum rate.

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