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# UPTAKE OF RADIOCALCIUM BY NERVE ENDINGS ISOLATED FROM RAT BRAIN: KINETIC STUDIES

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**1** The uptake of radiocalcium by nerve-ending particles isolated from rat brain was studied *in vitro* by means of a rapid lanthanum quenching technique.

**2** The observed uptake fits a theoretical three-compartment model with two separate uptake phases, a fast, initial phase followed by a late, slow phase. This holds true during control conditions as well as during high-potassium stimulation.

**3** The uptake as a function of the external calcium concentration can be described in terms of Michaelis-Menten kinetics during high-potassium stimulation. Under control conditions the fit is clearly applicable but statistically not as good as during potassium stimulation.

**4** The affinity for the uptake of calcium remains unchanged under control conditions while during high-potassium stimulation the affinity drastically decreases during the late, slow phase of uptake.

**5** During high-potassium stimulation the maximal velocity of calcium uptake is twice that during control conditions. This holds true for both the fast and the slow phases of the uptake.

**6** $Mg^{2+}$ has an inhibitory effect on the uptake, the inhibition being more effective during high-potassium stimulation. Tetrodotoxin has a slight inhibitory effect additional to that exerted by $Mg^{2+}$ during the initial phase of uptake into high potassium stimulated synaptosomes.

## Introduction

The necessity of calcium for the process of release of neural transmitter substances is well documented. Calcium is known to enter the nerve terminal as a consequence of a depolarization-induced increase in the calcium conductance of the presynaptic membrane, resulting in the release of the chemical transmitter at critical sites on the inner face of the presynaptic membrane. This is evident from both electrophysiological studies (Katz & Miledi, 1967; 1969; 1970; Miledi, 1973) and biochemical studies (Llinas & Nicholson, 1975). However the mechanisms underlying the increased calcium conductance in nerve terminals and the physiological regulatory mechanisms involved are still largely unknown.

Pinched-off nerve terminals, i.e. synaptosomes, isolated from brain homogenates have proved an extremely valuable *in vitro* model in the study of the metabolism and function of the central nervous system. Studies conducted by Blaustein and co-workers (Blaustein, 1975; Blaustein & Goldring, 1975; Blaustein & Oborn, 1975) have yielded results of fundamental importance concerning calcium fluxes in synaptosomal preparations. However, it must be kept in mind that the study of calcium fluxes in isolated cell preparations involves a very complicated system, as calcium is present in both bound and exchangeable forms within several extracellular and intracellular compartments (see e.g. Borle, 1975 and references cited).

The present study deals with the uptake kinetics of radiocalcium in nerve endings isolated from rat brain and the effect of the $Mg^{2+}$ ion and tetrodotoxin on this process. The time course, affinity and the saturation characteristics of the uptake process in control and in $K^+$-enriched media are described. The goodness of fit of the results obtained to a closed, three-compartment model is discussed.

## Methods

### *Isolation of synaptosomes*

Synaptosomes were isolated from whole brain homogenates from adult male rats of the Sprague-Dawley strain (180 to 250 g) by differential and density

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gradient centrifugation, essentially according to Gray & Whittaker (1962) as previously described (Jansson, Gripenberg, Härkönen & Korpijoki, 1977). The rats were decapitated under light ether anaesthesia, and the brain, with the exception of the cerebellum and olfactory lobes, was removed, minced and homogenized in ice-cold 0.32 mol/l sucrose containing 0.5 mmol/l ethyleneglycol-bis ($\beta$-aminoethyl ether) *N,N'*-tetra-acetic acid (EGTA) and buffered to pH 7.4 with 20 mmol/l Tris-HCl buffer. All further steps in the isolation procedure were carried out at 4°C. The crude mitochondrial fraction was layered on top of a density gradient made up of 8 ml of 11% Ficoll and 12 ml of 5% Ficoll in 0.32 mol/l sucrose. After centrifugation for 40 min at 20,700 *g* in a swing-out rotor, the cellular material accumulated at the interface of the Ficoll layers was collected and washed in ice-cold incubation medium without added $CaCl_2$. The morphology of the synaptosome fraction was regularly checked by electron microscopy and showed that pinched-off nerve terminals predominated and that extracellular mitochondria were rare.

*Solutions and chemicals*

The composition of the incubation medium used was (mM): NaCl 136, KCl 5.6, $MgCl_2$ 1.3, Tris-HCl buffer (pH 7.4) 20 and glucose 11. In order to retain isoosmolarity of the medium the concentration of $Na^+$ was decreased when that of $K^+$ or $Mg^{2+}$ was increased. When $K^+$ and $Mg^{2+}$ were simultaneously raised, $Na^+$ was lowered to compensate only for the increase in $K^+$. $^{45}CaCl_2$ (specific activity 3.12 Ci/mmol) was obtained from The Radiochemical Centre, Amersham; Ficoll from Pharmacia Fine Chemicals AB, Uppsala, Sweden; EGTA from The Sigma Chemical Co., St. Louis, Missouri, U.S.A., and tetrodotoxin from The Sankyo Co., Tokyo, Japan.

*$^{45}Ca$ uptake studies*

Synaptosomes resuspended at some 5 mg/ml protein were preincubated at 37°C for 10 min in calcium-free medium. After preincubation, the uptake reaction was initiated by adding $CaCl_2$ at a given concentration with a specific $^{45}Ca$ activity of 0.05 mCi/mmol. The uptake of $^{45}Ca$ was terminated by the lanthanum method of van Breemen, Farinas, Casteels, Gerba, Wuytack & Deth (1973). After loading for different periods of time (2 s to 4 min), $^{45}Ca$ uptake was arrested by diluting a 200 µl sample of the synaptosome suspension with 3 ml ice-cold incubation medium, without added calcium or phosphate ions, but supplemented with 0.5 mmol/l $LaCl_3$. In order to achieve satisfactory time resolution of the uptake between 2, 4 and 8 s, 100 µl of synaptosome suspension and 100 µl of incubation medium were carefully mixed and the uptake arrested by diluting the sample with 3 ml of the quenching solution. Extracellularly bound calcium was then displaced by incubating at 0°C for 20 min in the medium containing $La^{3+}$ (see Jansson *et al.*, 1977). Synaptosomes were collected by suction-filtration on Whatman GF/A filter discs ($\phi$ 25 mm). The synaptosomes retained on the filters were then immediately washed with 20 ml fresh, ice-cold La-medium to eliminate unbound radioactivity. After drying, the filters were placed in counting vials containing 10 ml of a scintillation medium prepared according to Anderson & McLure (1973). Radioactivity was determined in a Wallac Decem NTL 314 liquid scintillation counter. The counting efficiency as determined from internal standards was 85.5%. The uptake at zero time, representing the sum of nonspecific adsorption of $^{45}Ca$ synaptosomes, to contaminating particles and to the filters, was estimated by extrapolating the uptake at 2 and 4 s in normal medium back to zero and subtracting the value obtained from the amount of $^{45}Ca$ accumulated during each period of time. In experiments where different concentrations of $^{45}Ca$ were used the zero uptake values were obtained by mixing 100 µl aliquots of synaptosomal suspension with incubation medium containing $^{45}Ca$ previously diluted with 3 ml of the La-medium. In these experiments the samples at each extracellular concentration thus had their own blank values. Proteins were determined according to Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard.

## Results

*Uptake of calcium as a function of time*

The amount of calcium taken up by the synaptosomes at an external concentration of 2.2 mmol/l in control medium increased with time from 0.79 nmol Ca $mg^{-1}$ prot. at 4 s to 7.52 nmol Ca $mg^{-1}$ prot. at 240 s (Figure 1), the uptake being fastest during the initial seconds, i.e. 0.198 nmol Ca $mg^{-1}$ prot. $s^{-1}$, as compared to 0.01 nmol Ca $mg^{-1}$ prot. $s^{-1}$ at 240 s. The uptake of calcium was markedly increased when the $K^+$ concentration in the incubation medium was raised from 5.6 to 40 mmol/l. This effect was instantaneous and most prominent during the initial phase of calcium uptake (Figure 1). The uptake rate during the initial seconds was 0.843 nmol Ca $mg^{-1}$ prot. $s^{-1}$ but declined rapidly and approached the nonstimulated uptake rate of 0.02 nmol Ca $mg^{-1}$ prot. $s^{-1}$ after 120 s. In Table 1 the observed uptake values are compared to uptake values expected, assuming a closed two or three-compartment model having two separate uptake phases. A close correlation to the three-compartment model is evident by visual assessment or by

testing using one sample Student's $t$ test for the mean in both control and high-potassium media. The numerical values of the constants C and $\lambda$ of the theoretical curve

$$f(t) = \frac{C_1}{\lambda_1}(1 - e^{-\lambda_1 t}) + \frac{C_2}{\lambda_2}(1 - e^{-\lambda_2 t})$$

are given in Table 2. $C_1$ and $C_2$ can be considered as two different cellular compartments, $\lambda_1$ and $\lambda_2$ can be taken to represent the influx rate constants of the fast, initial and the late, slow phases, respectively. As can be seen in Table 2, high-potassium stimulation increases $C_1$ by a factor of 4.2 and $C_2$ by factor of 4.7 as compared to the control medium; the values of $\lambda_1$ and $\lambda_2$ can also be seen to be 2.3 and 6.7 times greater than the respective values in the control medium. In fitting this curve the uptake values up to 30 s are taken to represent the fast phase and uptake values after 30 s to represent the late, slow phase. The methods for obtaining the best fit, based on experimental data and computer programme used are given in the Appendix.

The higher uptake of $Ca^{2+}$ than that mentioned in earlier papers (see for example Blaustein 1975; Blaustein & Goldring, 1975; Blaustein & Oborn, 1975; Goddard & Robinson, 1976) is simply taken to reflect methodological differences between the present and earlier studies; higher incubation temperature, quenching agent, i.e. $LaCl_3$ versus EGTA and isolation procedure. The possible efflux of calcium ions during the uptake phase remains unresolved but is probably of low magnitude because of the high transmembrane concentration gradient favouring influx of calcium ions.

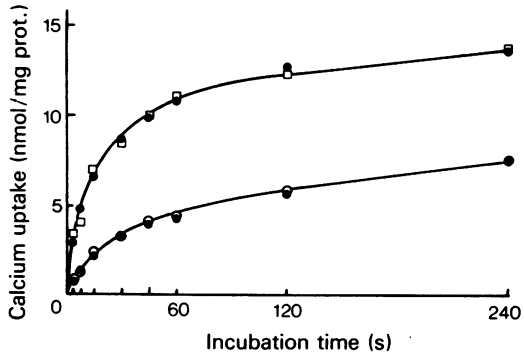


**Figure 1** Time course of calcium uptake in control (○) and 40 mmol/l $K^+$ media (□). Values are means from 6 to 9 experiments. The black dots in both curves represent the theoretical values obtained assuming a closed, three-compartment system. The smooth curve has been drawn visually; s.e. mean fall within symbols.

*Calcium uptake as a function of the external calcium concentrations*

The velocity of calcium uptake during the initial phase, i.e. the first 15 s, increased with increasing external calcium concentration in both the control as well as in 40 mmol/l $K^+$ medium. The uptake rates at external $Ca^{2+}$ concentrations of 0.44 and 6.6 mmol/l were 0.11 and 0.41 nmol Ca $mg^{-1}$ prot. $s^{-1}$, respectively, in the control medium with corresponding values of 0.28 and 0.75 nmol Ca $mg^{-1}$ prot. $s^{-1}$ for $K^+$-depolarized synaptosomes. The rate of uptake between 15 and 120 s, corresponding mainly to the

**Table 1** Comparison of the observed calcium uptake values with the theoretical values expected assuming the two and three-compartment models

| *Time (s)* | *Ca uptake in control medium* | *Expected in 3-compart.* | *Expected in 2-compart.* | *Ca uptake in 40 mmol/l $K^+$* | *Expected in 3-compart.* | *Expected in 2-compart.* |
|---|---|---|---|---|---|---|
| 4 | 0.79 ± 0.13 (9) | 0.77 | 0.51* | 3.37 ± 0.32 (9) | 2.85 | 2.01*** |
| 8 | 1.22 ± 0.16 (6) | 1.39 | 0.98 | 4.02 ± 0.23 (9) | 4.70** | 3.70 |
| 15 | 2.39 ± 0.28 (7) | 2.22 | 1.73* | 7.04 ± 0.30 (8) | 6.64 | 6.03** |
| 30 | 3.23 ± 0.25 (9) | 3.33 | 3.04 | 8.36 ± 0.51 (6) | 8.70 | 9.16 |
| 45 | 4.11 ± 0.25 (7) | 3.98 | 4.03 | 9.98 ± 0.41 (9) | 9.89 | 10.8 |
| 60 | 4.35 ± 0.40 (7) | 4.45 | 4.78 | 11.1 ± 0.59 (7) | 10.8 | 11.6 |
| 120 | 5.76 ± 0.39 (7) | 5.74 | 6.35 | 12.3 ± 0.56 (9) | 12.7 | 12.5 |
| 240 | 7.52 ± 0.61 (9) | 7.53 | 7.03 | 13.7 ± 0.75 (9) | 13.6 | 12.6 |

The mathematical form of the theoretical two-compartment system is $f(t) = \frac{C}{\lambda}(1 - e^{-\lambda t})$.

The deviation of the expected theoretical values from those observed is tested statistically using one sample Student's $t$ test for the mean. *Signifies almost significant ($P < 0.1$), **significant ($P < 0.05$) and ***highly significant ($P < 0.005$). Calcium uptake values are in nmol Ca $mg^{-1}$ prot. ± s.e. mean. Number of experiments in parentheses.

late, slow phase was likewise dependent on the extracellular calcium concentration. In the control medium, at calcium concentrations of 0.44 and 6.6 mmol/l the uptake rates were 0.01 and 0.07 nmol Ca $mg^{-1}$ prot. $s^{-1}$, respectively. In 40 mmol $l^{-1}$ $K^+$ medium the corresponding values were 0.01 and 0.09 nmol Ca $mg^{-1}$ prot. $s^{-1}$. Thus, regardless of the $Ca^{2+}$ concentration used, the stimulatory effect of $K^+$ on $Ca^{2+}$ uptake was transient in nature.

Double reciprocal plots of the rates of calcium uptake versus the concentration of calcium in the incubation medium showed a better linearity in the 40 mmol/l $K^+$ medium than in the control medium. This holds for the fast, initial phase (Figure 2a) as well as the late, slow phase (Figure 2b). As shown in Table 3, high-potassium stimulation caused a twofold increase in the maximal $Ca^{2+}$ uptake velocity, $V_{max}$, both during the initial and the late phase. Blaustein (1975) has previously reported that the $V_{max}$ for $Ca^{2+}$ uptake also increases with increasing external $K^+$ concentration. As shown in Table 3, the half-saturation constant of the Ca uptake, $K_m$, in control medium remains unchanged at 1.11 mmol/l during the initial and late phases, while in the high-potassium medium the $K_m$ value was four times higher in the late phase than in the initial phase (0.83 and 3.30 mmol/l, respectively). According to Blaustein (1975) $K_m$ was not affected by increased external $K^+$ concentration when using short, i.e. 30 s, incubation times. The present $K_m$ values are of the same order of magnitude as the value of 0.8 mmol/l obtained earlier (Blaustein, 1975).

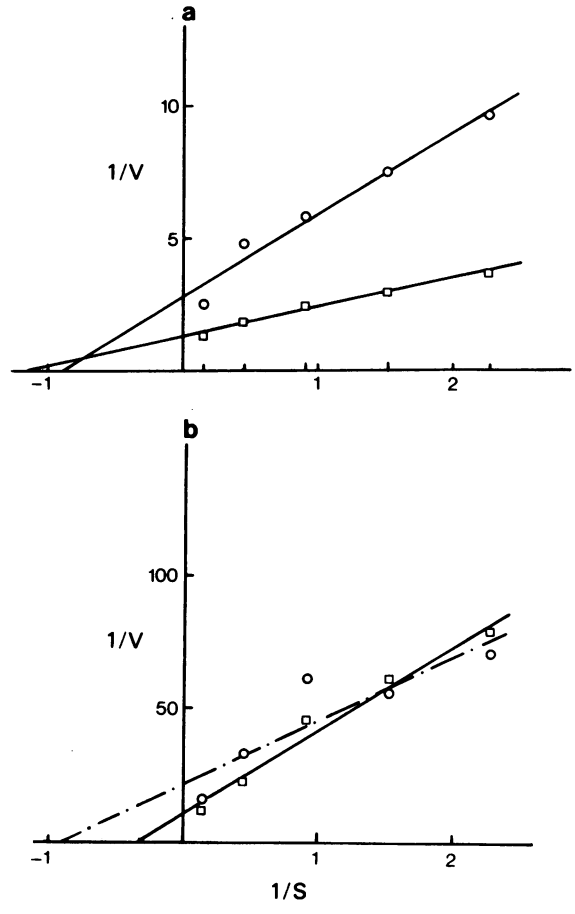


**Figure 2** Lineweaver-Burk plots of calcium uptake. Ordinates 1/V, where V is amount of calcium taken up per s, abscissae 1/S, where S is the concentration of calcium in the medium: (a) represents uptake during the first 15 s while (b) represents uptake between 15 and 120 s. Each point is the mean value obtained from 5 experiments: (○) control; (□) $K^+$-stimulated. The regression lines were obtained by the method of least squares. In (a) the r values are 0.967 for $K^+$-rich medium and 0.780 for control. The r values in (b) are 0.902 in $K^+$-depolarized medium and 0.735 for control.

**Table 2.** The numerical values of the constants of the equation describing the theoretical, closed, three-compartment system

| | *Control* | *$K^+$-stimulated* |
|---|---|---|
| $C_1$ | 0.18 | 0.75 |
| $C_2$ | 0.03 | 0.14 |
| $\lambda_1$ | 0.06 | 0.14 |
| $\lambda_2$ | 0.003 | 0.02 |

**Table 3.** The values of $K_m$ and $V_{max}$ obtained from the plots in Figures 2(a) and (b)

| | *Initial phase* (0–15 s) | | *Late phase* (15–120 s) | |
|---|---|---|---|---|
| | *Control* | *$K^+$-stimulated* | *Control* | *$K^+$-stimulated* |
| $K_m$ | 1.11 | 0.83 | 1.11 | 3.33 |
| $V_{max}$ | 0.35 | 0.76 | 0.05 | 0.10 |

$K_m$ is the external calcium concentration in mmol/l at which $\frac{1}{2}V_{max}$ occurs. $V_{max}$ represents the maximal uptake velocity of calcium in nmol Ca $mg^{-1}$ prot. $s^{-1}$.

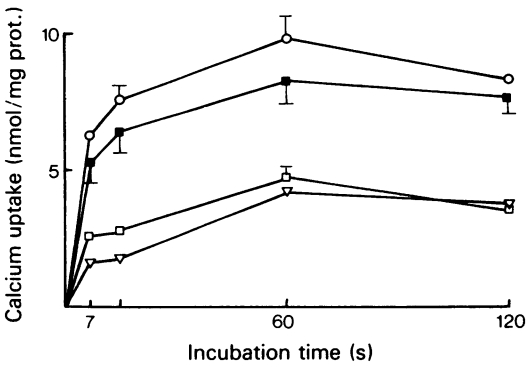


**Figure 3** The effects of $Mg^{2+}$ (□) and tetrodotoxin (■) on $K^+$-induced calcium uptake (○), obtained by subtracting uptake values in the control medium from those in $K^+$-rich medium; (▽) uptake with $Mg^{2+}$ plus tetrodotoxin. Means from 2–3 paired experiments; vertical lines show s.e. mean.

*Effect of tetrodotoxin (TTX) and $Mg^{5+}$ on calcium uptake*

$Mg^{2+}$ at 7.5 mmol/l lowered the high $K^+$-induced calcium uptake to 48 to 36% of the control values, as shown in Figure 3. TTX at 1 μg/l had a slight inhibitory effect of its own on high $K^+$-induced uptake and together with $Mg^{2+}$, TTX showed an additional inhibitory effect during the initial phase of uptake; at 7 and 15 s the uptake values were 25 and 22% of the control values. In the control incubation medium with a $K^+$ concentration of 5.6 mmol/l, $Mg^{2+}$ inhibited calcium uptake by 15 to 25% while TTX itself had no effect, nor did it increase $Mg^{2+}$-induced inhibition. These results fit those obtained by Blaustein (1975) who found that TTX had no effect upon longer (i.e. 2 min) incubations.

## Discussion

Comparing the observed uptake values with those calculated from the two and three-compartment models shows that the uptake process more closely fits the latter. The present results do not, however, allow any definite statistical verification of the interpretation, that there are indeed three compartments although as judged from Figure 1 and Table 1, this seems justified. Further, the interpretation of the uptake values obtained from the fitting of the data to the three-compartment model is an agreement with studies on calcium uptake by whole kidney cell preparations (Borle, 1975).

As to the effect of high-potassium stimulation on calcium uptake during the initial, fast phase, it is evident that the following changes take place as compared to control uptake. The $K_m$ value decreases by a factor of 1.3, i.e. the calcium affinity of the calcium binding or 'transport' sites increases, and the maximal velocity of the uptake, $V_{max}$, increases by a factor of 2.2 (Figure 2 and Table 3). The changes in the numerical values of the constants of the theoretical three-compartment system seen during the same time period are such that the theoretical cellular compartment $C_1$ is enlarged by a factor of 4.2 while $\lambda_1$, which tentatively represents the flux rate constant, increases by a factor of 2.3. Altogether these changes can be taken to suggest that during high-potassium stimulation in the initial phase either the transport characteristics of pre-existing transport sites or ion channels in the synaptic membrane are altered or that a new population of transport sites or channels is unmasked or activated.

The most striking differences between the fast, initial and the late, slow phase of calcium uptake is the decrease in the velocity of uptake. Both maximal velocities and flux rates are reduced to about one seventh of their values during the initial phase. This is the case both during control conditions and during high-potassium stimulation, and indicates that some degree of saturation is being approached, i.e. that some compartment involved in the uptake process is reaching a 'filled' state, or that the steric conformation of the $Ca^{2+}$ channels in the presynaptic membrane has changed so that the channels have become non-conducting. The affinity, $K_m$, of the calcium binding or transport sites is of the same magnitude during the initial and the late phases during control conditions. During high-potassium stimulation, however, the fourfold increase in the $K_m$ value from the initial to late phases is striking. These observations on presynaptic nerve-endings may be analogous to the inactivation of specific, late $Ca^{2+}$ channels seen upon prolonged depolarization in the squid giant axon as shown by Baker, Meves & Ridgway (1973).

Only a minor part of the calcium uptake seems to involve sodium channels since TTX, which effectively blocks the increased sodium permeability associated with the nerve action potential (Narahashi, Moore & Scott, 1964) had no effect on calcium uptake during control conditions and only a minor effect during high-potassium stimulation (Figure 3). Further, the good linear fit ($r = 0.967$) of the high-potassium stimulated calcium uptake during the initial phase favours the assumption of a single population of channels or transport sites being involved during this period. This is in accordance with the calcium influx through specific gated channels during the action potential of squid giant axon (Baker, Hodgkin & Ridgway, 1971).

What, then, are the routes by which calcium enters

the synaptosomes? The facts that under control conditions $Mg^{2+}$ has only a moderate effect and TTX no effect of its own nor any potentiating effect on the slight $Mg^{2+}$ inhibition suggests that under these conditions calcium enters the synaptosomes through non-sodium and non-specific 'leaky' channels. In agreement with this suggestion is the fact that in the Lineweaver-Burk plots under control conditions, the uptake as a function of external $Ca^{2+}$ concentrations deviates considerably more from a straight line than during high-potassium stimulation. In interpreting the goodness of fit to the regression lines in the Lineweaver-Burk plots, however, it must be kept in mind that during control conditions the lower numerical uptake values tend to produce greater statistical variation in the 1/V values plotted in Figure 2a. The involvement of specific $Ca^{2+}$ channels in the uptake seen during high-potassium stimulation is consistent with the good linearity in the Lineweaver-Burk plots and the better inhibitory effect of $Mg^{2+}$, a known $Ca^{2+}$ antagonist in transmitter release (see Dodge & Rahamimoff, 1967). The partial entry of calcium through sodium channels during depolarization is, however, indicated by the additional inhibition of the $Mg^{2+}$ inhibition by TTX during the initial phase.

The present study lends further support to the use of synaptosomes as valuable *in vitro* models in the study of the complicated processes involved in the release of transmitter substances at synapses. The effect of prostaglandin $E_1$, dopamine and cyclic adenosine 3',5'-monophosphate, agents known to modulate transmitter release, on synaptosomal calcium uptake is described in the following paper.

## APPENDIX

E. Heinonen & E. M. Tolppanen

This appendix summarizes the methods used in the preceding paper for estimating the kinetic constants of the biochemical process under consideration.

The solution to the problem is general in nature and thus a programme written in standard FORTRAN IV language may be used in various other biochemical analyses where it is necessary to fit data to a double exponential theoretical curve. This programme was originally developed and tested on a small POP-11/03 LABORATORY computer. The full print-out of the programme may be obtained from the authors on request.

The method for estimating the kinetic constants from data derived from tracer studies of biochemical processes using a three-compartment model is given by Robertson, Tosteson & Gamble (1957). Borle (1975) describes, in his paper, a manual method of fitting a theoretical double exponential curve to his experimental data and subsequantly computing the estimates for the kinetic constants using a computer programme (Borle, 1975). Our own experience with this type of manual curve fitting, however, shows that large errors may be generated due to inherent inaccuracies in the human process. Small subjective variations in the fitting may cause appreciable deviations in the final results.

The uptake of one compartment in a three-compartment system is generally given in the rate equation form (Borle, 1975):

$$\frac{d(R_{1+3})/E}{dt} = -C_1 e^{-\lambda_1 t} - C_2 e^{-\lambda_2 t}$$

where $R$ is the amount of $^{45}Ca$ in the cellular compartments, $E$ is the specific activity, $C(i)$ and $\lambda(i)$ $(i = 1,2)$ are computed by fitting the theoretical curve to experimental data.

This equation can also be formulated in the uptake form for the straightforward treatment of actual observations. By integrating, and bearing in mind that $(R_{1+3})/E = 0$ at zero time we get:

$$\frac{R_{1+3}}{E} = \frac{C_1}{\lambda_1}(1 - e^{-\lambda_1 t}) + \frac{C_2}{\lambda_2}(1 - e^{-\lambda_2 t})$$

From this, a formula for uptake at the equilibrium at infinite time can be derived:

$$I = \frac{C_1}{\lambda_1} + \frac{C_2}{\lambda_2}.$$

The initial values are estimated using the following algorithm:

(1) Using the actual observation or an estimate of the uptake at equilibrium (at infinite time), the following simple transformation is performed on the observations:

$$Y_i' = I - Y_i$$

Where $I$ is the uptake at infinite time, $Y_i$ are the untransformed uptake values, $Y_i'$ are the tranformed uptake values.

(2) If $\lambda_1 \gg \lambda_2$, then for observations $(t > t_1)$, a good approximation of the parameters $C_2$ and $\lambda_2$ ($C_2'$ and $\lambda_2'$ respectively for the slow phase) is given by the simple exponential equation:

$$f(t) = \frac{C_2'}{\lambda_2'} e^{-\lambda_2' t}$$

to which the experimental data are fitted for points $(t > t_1)$. $t_1$ is an empirically derived time point, after which the fast phase of the process does not significantly contribute to the total uptake. The experimental data are fitted to the total uptake for points $(t > t_1)$.

(3) The theoretical curve for the slow phase thus obtained is then subtracted from the original experi-

mental observations before time $t_1$ to get the uptake attributable to the fast phase of the system.

(4) A single exponential curve is fitted again on these points to get the initial estimates for the parameters of the fast phase.

Using this algorithm a sufficiently good estimate is usually obtained. In practice, we have found that a sufficient number of observations must be made for both the fast phase and the slow phase. In our case, four observations for both phases were enough.

Taylor's Linear Least-Squares Differential-Correction Technique (McCalla, 1967) is used in the computation of the curve fit. This method uses the partial differentials of the uptake function, which must be given. The partial differentials are as follows:

$$\frac{\delta f_i}{\delta C_i} = \frac{1}{\lambda_i}(1 - e^{-\lambda_i t}); \quad (i = 1,2)$$

$$\frac{\delta f_i}{\delta \lambda_i} = \frac{C_i}{\lambda_i}(e^{-\lambda_i t} + \lambda_i e^{-\lambda_i t} - 1); \quad (i = 1,2)$$

The Differential-Correction method employs an iterative correction term for the estimates of unknown variables $C_i$ and $\lambda_i$. When the correction term becomes smaller than a given constant the loop is terminated. The algorithm is also aborted upon divergence of the correction term. A detailed description of Taylor's Linear Least-Squares Differential-Correction method is given by McCalla (1967).

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