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Energy Transduction in Intact Synaptosomes

INFLUENCE OF PLASMA-MEMBRANE DEPOLARIZATION ON THE RESPIRATION AND MEMBRANE POTENTIAL OF INTERNAL MITOCHONDRIA DETERMINED *IN SITU*

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A method is described, based on the differential accumulation of Rb^+ and methyltriphenylphosphonium, for the simultaneous estimation of the membrane potentials across the plasma membrane of isolated nerve endings (synaptosomes), and across the inner membrane of mitochondria within the synaptosomal cytoplasm. These determinations, together with measurements of respiratory rates, and ATP and phosphocreatine concentrations, are used to define the bioenergetic behaviour of isolated synaptosomes under a variety of conditions. Under control conditions, in the presence of glucose, the plasma and mitochondrial membrane potentials are respectively 45 and 148 mV. Addition of a proton translocator induces a 5-fold increase in respiration, and abolishes the mitochondrial membrane potential. The addition of rotenone to inhibit respiration does not affect the plasma membrane potential, and only lowers the mitochondrial membrane potential to 128 mV. Evidence is presented that ATP synthesis by anaerobic glycolysis is sufficient under these conditions to maintain ATP-dependent processes, including the reversal of the mitochondrial ATP synthetase. Addition of oligomycin under non-respiring conditions leads to a complete collapse of the mitochondrial membrane potential. Even under control conditions the plasma membrane ($Na^+ + K^+$)-dependent ATPase is responsible for a significant proportion of the synaptosomal ATP turnover. Veratridine greatly increases respiration, and depolarizes the plasma membrane, but only slightly lowers the mitochondrial membrane potential. High K^+ and ouabain also lower the plasma membrane potential without decreasing the mitochondrial membrane potential. In non-respiring synaptosomes, anaerobic glycolysis is incapable of maintaining cytosolic ATP during the increased turnover induced by veratridine, and the mitochondrial membrane potential collapses. It is concluded that the internal mitochondria must be considered in any study of synaptosomal transport.

When regions of the brain are homogenized under conditions of moderate shear, a high proportion of the nerve endings avoid rupture and can be isolated as synaptosomes (for reviews see Whittaker, 1969; Rodriguez de Lores Arnaiz & de Robertis, 1972). These organelles have been most thoroughly investigated as systems *in vitro* for the study of neurotransmitter uptake, metabolism and release (for reviews see Baldessarini & Karobath, 1973; Levi & Raiteri, 1976). However, in addition they function as small anucleate cells, retaining the enzymes of glycolysis (Bradford, 1969), usually containing one or more mitochondria (Whittaker, 1969) and possessing extremely active ion transport systems across their plasma membranes (Li & White, 1977).

The present paper attempts to describe the pathways of energy transduction in the intact synaptosome by means of parallel determinations of respiration, ATP and phosphocreatine concentrations, and the membrane potentials across both plasma and inner mitochondrial membranes.

A prediction of the Chemiosmotic Theory (for review see Mitchell, 1976) is that the magnitude of the proton electrochemical potential ($\Delta\mu_{H^+}$) across the inner mitochondrial membrane is the primary determinant of the rate of controlled respiration and of the ability of the mitochondrion to maintain cellular ATP concentrations. Under most conditions *in vitro* the mitochondrial membrane potential

Abbreviations used: $\Delta\mu_{H^+}$, proton electrochemical potential gradient across the inner mitochondrial membrane; $\Delta\psi_m$, membrane potential across the inner mitochondrial membrane; $\Delta\psi_p$, membrane potential across the plasma membrane; Tes, 2-{[2-hydroxy-1,1-bis-(hydroxymethyl)-ethyl]amino}ethanesulphonate; $TPMP^+$, methyltriphenylphosphonium cation.

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($\Delta\psi_m$) is the major contributor to $\Delta\mu_{H+}$ (Mitchell & Moyle, 1969; Nicholls, 1974; Rottenberg, 1975). In addition, the maintenance of a sufficient $\Delta\psi_m$ is essential for mitochondria to regulate the cytosolic free Ca^{2+} concentration (Nicholls, 1978*b*). It follows that it would be highly advantageous to be able to estimate this potential in intact synaptosomes.

In most cells there are two membrane potentials to be considered: that across the plasma membrane ($\Delta\psi_p$) and that across the inner membrane of the internal mitochondria ($\Delta\psi_m$). Thus any technique must be capable of resolving these potentials. Cyanine dye fluorescence is more sensitive at low membrane potentials (Burckhardt, 1977) and thus responds preferentially to the plasma membrane potential rather than to the high mitochondrial membrane potential. The indicator has been used with Ehrlich ascites cells (Laris *et al.*, 1976) and synaptosomes (Blaustein & Goldring, 1975; Sen & Cooper, 1978; Ng & Howard, 1978). Safranine has a linear spectral response to membrane potential (Åkerman & Wikström, 1976) and can provide a qualitative estimate of $\Delta\psi_m$ in intact Ehrlich ascites cells (Åkerman, 1979).

In the present paper the lipophilic cation methyltriphenylphosphonium ($TPMP^+$) is used. $TPMP^+$ distributes according to a Nernst equilibrium across bilayer regions of membranes (Bakeeva *et al.*, 1970). In intact hepatocytes it is accumulated across both the plasma and mitochondrial membranes (Hoek *et al.*, 1979), and its distribution is therefore a function of the magnitude of both membrane potentials. To resolve the contribution of the plasma membrane to the total accumulation of the cation, $\Delta\psi_p$ is required, and is estimated from the diffusion potential of Rb^+ across the plasma membrane.

A preliminary report of part of this work has been published (Scott & Nicholls, 1979).

Experimental

Materials

[^{3}H]Methyltriphenylphosphonium bromide was obtained from New England Nuclear Chemicals G.m.b.H., Dreieich, Germany. All other radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ficoll was obtained from Pharmacia, Uppsala, Sweden, and was exhaustively dialysed against water before use. Methyltriphenylphosphonium bromide, sodium tetraphenylboron, oligomycin, ouabain, veratrine and all enzymes were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Veratrine is a mixture of alkaloids, and the veratridine content was taken to be 25%. All other reagents were of analytical grade. Unless otherwise stated, sodium salts were employed throughout.

Synaptosomes and 'free' brain mitochondria

Synaptosomes and 'free' (non-synaptosomal) mitochondria were prepared from the cerebral cortices of Duncan–Hartley-strain guinea pigs of either sex, aged 4–8 weeks, by a modification of the method of Cotman & Matthews (1971) employing a discontinuous Ficoll gradient exactly as previously described (Nicholls, 1978*a*). The synaptosomal suspension was made up to 30ml, per two animals, with 320mM-sucrose/5mM-Tes/0.5mM-EDTA (pH7.4) at 4°C, and centrifuged in an MSE-25 6×16ml swing-out rotor for 10min at $75000g_{max.}$. The pellet was resuspended in 10ml of 250mM-sucrose/5mM-Tes (pH7.0) at 4°C, and protein was determined by the biuret method (Gornall *et al.*, 1949). The synaptosomal suspension was divided into portions containing 4.5mg of synaptosomal protein each, and centrifuged in an MSE-18 8×50ml angle rotor for 10min at $17000g_{max.}$. The supernatants were decanted and the synaptosomes were stored as pellets at 0°C for not more than 3h until required.

Synaptosomal respiration was determined with a Clark-type oxygen electrode in an incubation chamber of 0.25ml capacity. ATP was assayed fluorimetrically by the method of Williamson & Corkey (1969). Phosphocreatine was assayed by a modification of the method of Lamprecht *et al.* (1974).

Theory of the method for the simultaneous estimation of the electrical potential across the plasma and mitochondrial membranes of intact synaptosomes

If $TPMP^+$ achieves a Nernst equilibrium across both the synaptosomal and inner mitochondrial membranes, then the total accumulation of the cation within the synaptosome will be a function of both potentials and of the volumes of the cytosolic and mitochondrial matrix compartments. If the activity coefficients for $TPMP^+$ in the extra-cellular, cytosolic and matrix compartments are a_e, a_c and a_m respectively, then the equilibrium concentrations of the cation in the respective compartments will be related to the plasma membrane potential ($\Delta\psi_p$) and mitochondrial membrane potential ($\Delta\psi_m$) thus:

$$\frac{a_c[TPMP^+]_c}{a_e[TPMP^+]_e} = 10^{\Delta\psi_p/60} \quad (1)$$

$$\frac{a_m[TPMP^+]_m}{a_c[TPMP^+]_c} = 10^{\Delta\psi_m/60} \quad (2)$$

However, neither the cytosolic nor matrix concentrations of $TPMP^+$ can be determined directly, as the parameter that is obtained by separating the synaptosomes from the incubation medium is the overall concentration of $TPMP^+$ within the synaptosomes, $[TPMP^+]_s$. This parameter is related to the concentrations of the cations in the cytosol and matrix, and

the respective volumes of the two compartments V_c and V_m, thus:

$$[TPMP^+]_s = \frac{V_c[TPMP^+]_c + V_m[TPMP^+]_m}{(V_c + V_m)} \quad (3)$$

Substituting from eqns. (1) and (2) we obtain:

$$\frac{[TPMP^+]_s}{[TPMP^+]_e} = \frac{a_e \cdot 10^{\Delta\psi_p/60} \cdot \left\{\frac{V_c}{a_c} + \frac{V_m}{a_m} \cdot 10^{\Delta\psi_m/60}\right\}}{(V_c + V_m)} \quad (4)$$

It is apparent that to solve eqn. (4) for $\Delta\psi_m$, the magnitude of $\Delta\psi_p$ is required. As discussed in the Results section, the diffusion potential of Rb^+ across the synaptosomal plasma membrane is an approximate measure of the plasma membrane potential, such that:

$$\frac{[Rb^+]_c}{[Rb^+]_e} \simeq 10^{\Delta\psi_p/60} \quad (5)$$

Substituting eqn. (5) into eqn. (4), solving for $\Delta\psi_m$ and assuming that V_c is much greater than V_m (see below), we obtain:

$$\Delta\psi_m = 60 \log \left\{\frac{V_c \cdot a_m}{V_m}\left(\frac{[Rb^+]_e \cdot [TPMP^+]_s}{[Rb^+]_c \cdot [TPMP^+]_e \cdot a_e} - \frac{1}{a_c}\right)\right\} \quad (6)$$

The practical determination of $[Rb^+]_c$ and $[TPMP^+]_s$

Synaptosomal pellets containing 4.5mg of protein were resuspended in 0.5ml of an air-saturated incubation medium at 30°C and pH7.4, which contained 122mM-NaCl, 3.1mM-KCl, 1.2mM-$MgSO_4$, 1.3mM-$CaCl_2$, 0.4mM-KH_2PO_4, 5mM-$NaHCO_3$, 20mM-Tes, 10mM-D-glucose and 10μM-amino-oxyacetate. The suspension was then immediately transferred to 1ml of incubation medium that contained additionally 5μM-tetraphenylboron and radioisotopes to give a final concentration of 1.3μM-[^{3}H]TPMP$^+$ (0.4μCi/ml of incubation), 40μM-[^{14}C]sucrose (0.4μCi/ml of incubation) and 50μM-^{86}RbCl (0.28μCi/ml of incubation). The suspensions were then incubated in 10ml polycarbonate flasks at 30°C in a shaking water bath, and 200μl portions were withdrawn at defined times and filtered through 0.6μm pore-size cellulose acetate filters (Sartorius, Göttingen, Germany). The cellulose acetate filters were found to retain in excess of 85% of the synaptosomal protein. The slight binding of $TPMP^+$ to the acetate filters was corrected for in each experiment.

The filters were added without washing to a scintillant containing (per litre) 310ml of Triton X-100, 630ml of toluene, 60ml of water and 5g of 5-diphenyloxazole. After allowing time for the synaptosomes to lyse, the vials were counted simultaneously for ^{3}H, ^{14}C and ^{86}Rb radioactivities in an Intertechnique type 32 liquid-scintillation counter. Portions of the incubation medium before filtration were also counted for radioactivity under identical conditions.

The apparent spaces for $^{86}Rb^+$ and [^{3}H]$TPMP^+$ within the synaptosomes were calculated as previously described (Nicholls, 1974) after allowing for the extra-synaptosomal fluid entrapped on the filter (calculated from the [^{14}C]sucrose radioactivity counts). In a typical filtration of 0.6mg of synaptosomal protein the following apparent spaces were obtained: $^{86}Rb^+$, 13μl; [^{14}C]sucrose, 6μl; [^{3}H]-$TPMP^+$, 58μl. The accumulation ratios for the ions were then calculated by dividing the apparent spaces by the water-permeable inulin-impermeable space for the amount of synaptosomes on the filter.

The ^3H_2O-permeable hydroxy[^{14}C]methylinulin-impermeable space of the synaptosomal preparation was determined by suspending a synaptosomal pellet in an incubation medium that contained additionally ^3H_2O (5μCi/ml of incubation) and hydroxy[^{14}C]-methylinulin (1μCi/ml of incubation). Portions (400μl) of the incubation were layered into Eppendorf centrifuge tubes containing 150μl of 1M-$HClO_4$ and 400μl of a mixture of 60% (v/v) Dow Corning 550 silicone fluid and 40% (v/v) dinonyl phthalate. The tubes were centrifuged for 60s on an Eppendorf model 5412 bench centrifuge. Portions of the supernatant and neutralized infranatant were counted for radioactivity. The impermeable space was calculated to be 3.23μl/mg of protein, in good agreement with published values (Marchbanks, 1975).

The contribution of the matrices of the internal mitochondria to the total synaptosomal volume is difficult to estimate with any degree of precision. Of the total synaptosomal protein from the present preparation, 12% can be recovered as synaptosomal mitochondria after digitonin disruption (Nicholls, 1978*a*), and as the matrix volume of guinea-pig cerebral-cortical mitochondria in an NaCl-based medium has been found to be 0.65μl/mg of protein (Nicholls, 1978*a*), we adopt a value of 0.08μl/mg of synaptosomal protein for the volume of the included matrices. It should be noted that a 2-fold error in this estimate would only alter $\Delta\psi_m$ by 18mV.

Determination of $^{86}Rb^+$ and [^{3}H]$TPMP^+$ accumulation by isolated mitochondria

'Free' mitochondria obtained from the pellet of the discontinuous Ficoll density gradient were incubated for 3min at a protein concentration of 1mg/ml of incubation in a medium containing, at 30°C and pH7.0, 75mM-NaCl, 10mM-Tes, 16μM-albumin (bovine, fraction V from Sigma), 2mM-succinate, 0.2mM-ATP, 1μg of oligomycin/ml, 0.5μM-valinomycin, 3μM-tetraphenylboron, 1μM-[^{3}H]$TPMP^+$ (0.4μCi/ml), 40μM-[^{14}C]sucrose (0.4μCi/ml), and

50 μM-^{86}RbCl (0.28 μCi/ml). The mitochondria were then filtered through 0.6 μm pore-size cellulose acetate filters, and the remainder of the experiment was performed precisely as described for intact synaptosomes.

Results

The accumulation of Rb^+ by synaptosomes

The electrical permeability of the plasma membrane of most eukaryotic cells to Na^+ and K^+ is low in relation to the cellular content of the ions, with the result that the half-time for the exchange across the plasma membrane may be several hours. In contrast the synaptosome, with highly active plasma membrane transport processes and a high surface-to-volume ratio, can very rapidly alter the distribution of Na^+ and K^+ across the plasma membrane in response to stimuli that might be expected to alter the plasma membrane potential (Li & White, 1977). This raises the possibility of monitoring transient changes in plasma membrane potential by following the ion distribution across the membrane. A precise quantification of the plasma membrane potential from the ionic gradients is complex and requires the application of the constant-field equation (Goldman, 1943; Hodgkin & Katz, 1949). However, the synaptosomal plasma membrane appears to be typical of excitable membranes under polarized conditions in that the permeability to K^+ is greatly in excess of the Na^+ permeability (Keen & White, 1971). It follows therefore that the steady-state distribution of K^+ across the membrane is close to thermodynamic equilibrium, even when the $(Na^+ + K^+)$-dependent ATPase is operative and the ions are continuously cycling across the membrane (Fig. 1). Therefore the plasma membrane potential may be approximated to by the K^+ diffusion potential calculated from the steady-state K^+ distribution. As Rb^+ can substitute for K^+ for uptake by the $(Na^+ + K^+)$-dependent ATPase, and as the permeability of the membrane for Rb^+ is as high as for K^+ (Keen & White, 1971; Blaustein & Goldring, 1975), it follows that the Rb^+ distribution may also be employed to estimate $\Delta\psi_p$.

Although this estimation has limitations, particularly when the Na^+ permeability is enhanced (see Fig. 10), it is of sufficient accuracy when used in concert with $TPMP^+$ to be able to distinguish an efflux of $TPMP^+$ as a result of a depolarization of the plasma membrane from one that is a consequence of a depolarization of the inner membrane of the internal mitochondria. Only in the former case will $TPMP^+$ efflux be accompanied by a proportionate efflux of Rb^+.

The accumulation of $^{86}Rb^+$ by synaptosomes is examined in Fig. 2(*a*). By 9 min an Rb^+ diffusion potential of 47 mV is obtained, which is within 4 mV of the final steady state. This time course reflects not only the time required for the Rb^+ to equilibrate across the membrane, but also the time for the synaptosome itself to establish its steady-state ionic gradients after storage in sucrose at 0°C. In 19 determinations, the Rb^+ diffusion potential attained after 8 min incubation was 45 ± 2.4 mV (mean ± S.D.)

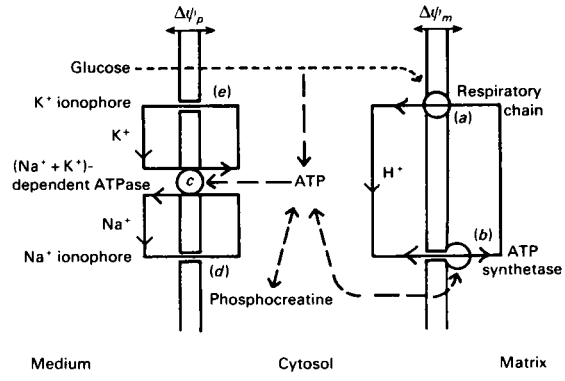

Fig. 1. *Schematic representation of the major pathways of energy transduction in intact synaptosomes metabolizing glucose*

(*a*) Site of inhibition by rotenone; (*b*) site of inhibition by oligomycin; (*c*) site of inhibition by ouabain; (*d*) site of activation by veratridine; (*e*) activity increased by valinomycin.

In the absence of an ionophore such as valinomycin, the internal mitochondria possess no electrical permeability to Rb^+ and so will not accumulate the cation. However, even if valinomycin were added to synaptosomes under initial incubation conditions, the high cytosolic K^+ concentration would depolarize the mitochondrial inner membrane (Mitchell & Moyle, 1969; Nicholls, 1974) and prevent the establishment of a significant mitochondrial Rb^+ gradient. The addition of valinomycin will also greatly increase the electrical permeability of the plasma membrane to Rb^+. If Rb^+ is not at electrochemical equilibrium across the plasma membrane before addition of the ionophore, then there will be a net movement of ion to achieve equilibrium. Fig. 2(*a*) shows that addition of the ionophore causes only a 2 mV change in the indicated potential. Therefore even though K^+ and Rb^+ are cycling across the plasma membrane driven by the $(Na^+ + K^+)$-dependent-ATPase (Fig. 1), the native Rb^+ permeability of the membrane is sufficient for the ion to remain close to thermodynamic equilibrium.

In Fig. 2(*a*) the effect of a simultaneous addition of valinomycin and 60 mM-KCl is shown. The rapid and complete collapse of the Rb^+ gradient indicates that there is no potential-independent association of Rb^+ within the synaptosome.

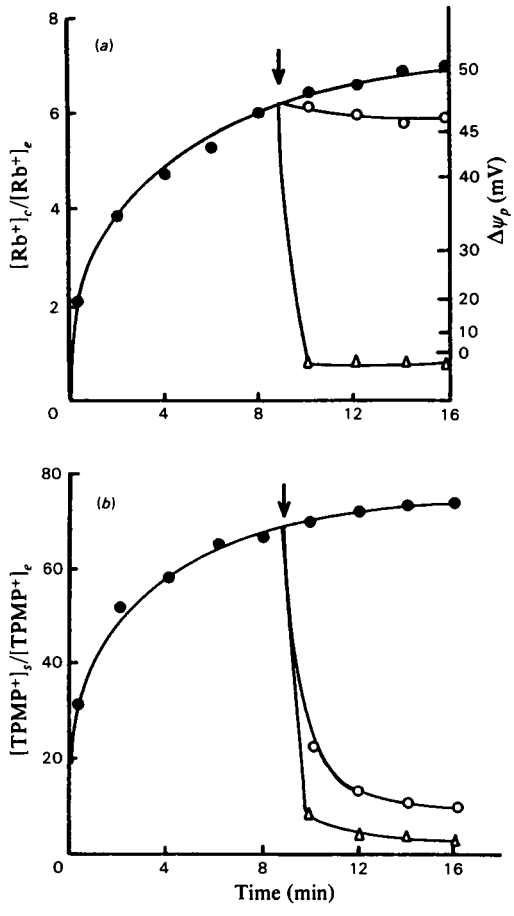

Fig. 2. *The accumulation of Rb^+ and $TPMP^+$ by synaptosomes: the influence of valinomycin*

Synaptosomes (1.5mg of protein/ml of incubation) were incubated at 30°C as described in the Experimental section. At 9min the following additions were made: ○, 5μM-valinomycin; △, 5μM-valinomycin plus 60mM-KCl; ●, control. (*a*) Accumulation ratio for Rb^+; (*b*) accumulation ratio for $TPMP^+$.

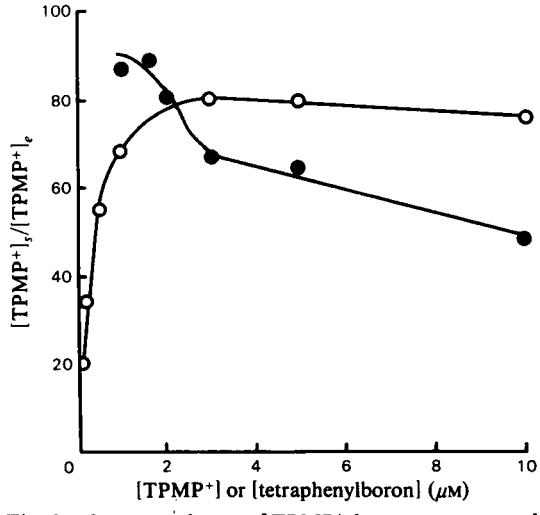

Fig. 3. *The accumulation of $TPMP^+$ by synaptosomes: the effect of varying the concentrations of $TPMP^+$ and tetraphenylboron*

Synaptosomes (1.5mg of protein/ml of incubation) were incubated for 12min as described in the Experimental section, except that the concentrations of $TPMP^+$ and tetraphenylboron were varied as indicated. Symbols: ○, 1μM-$TPMP^+$, tetraphenylboron varied; ●, 3μM-tetraphenylboron, $TPMP^+$ varied.

The accumulation of $TPMP^+$ by synaptosomes

The accumulation of $TPMP^+$ by synaptosomes is depicted in Fig. 3. Optimal accumulation of the cation requires the presence of tetraphenylboron, and when $TPMP^+$ is less than 1.5μM and tetraphenylboron is 3μM the accumulation ratio exceeds 80.

In Fig. 2 the accumulation ratios for Rb^+ and $TPMP^+$ are compared under identical conditions, the two isotopically-labelled substances being present in the same incubation. The far greater accumulation of $TPMP^+$ that is observed under initial incubation conditions could be due to two factors. First $TPMP^+$ might be additionally accumulated by an organelle within the cytosol possessing a high membrane potential, such as a mitochondrion, or secondly $TPMP^+$ within the cytosol might possess a very low activity coefficient due to binding, or to complexation with tetraphenylboron. To resolve the relative importance of these two factors, the effect of valinomycin was examined. As discussed above, by inducing a high electrical permeability to K^+ and Rb^+, valinomycin will depolarize any cytosolic organelle. Thus if the major accumulation of $TPMP^+$ was within these organelles, a considerable decrease in the overall accumulation ratio would be observed, and this is in fact the case (Fig. 2*b*). That the $TPMP^+$ responds not only to the potential across any internal organelles, but also to the plasma membrane potential, is indicated by the virtually complete efflux of the cation when 60mM-KCl and valinomycin are added together, thus additionally depolarizing the plasma membrane. Potential-independent complexation of $TPMP^+$ within the synaptosome is therefore slight. This is in contrast with the accumulation of dibenzyldimethylammonium by yeast cells in the presence of tetraphenylboron (Hoeberichts & Borst-Pauwels, 1975), where a complex appears to be formed between the cation and tetraphenylboron, which prevents the cation from responding to changes in potential. The reasons for this difference in behaviour are unclear.

In a variety of cells that have been examined by direct electrophysiological techniques the activity coefficient for K^+ in the cytosol appears to be close to that in free solution (Walker & Brown, 1977). If the same is true for Rb^+, then a comparison of the accumulation ratios for Rb^+ and $TPMP^+$ in the presence of valinomycin (when the gradients of the free cations will be equal) will enable the activity coefficient for $TPMP^+$ in the cytosol to be calculated relative to that in the medium. Under the conditions of Fig. 2, in the presence of valinomycin, the final accumulation ratio for $TPMP^+$ was 1.7 times that for Rb^+, indicating a relative activity coefficient of 0.6.

The obvious candidate for the intrasynaptosomal organelle responsible for the high accumulation of $TPMP^+$ is the mitochondrion. To confirm that $TPMP^+$ responds accurately to the membrane potential of brain mitochondria, the distribution of Rb^+ in the presence of valinomycin was compared with that of $TPMP^+$ in the presence of tetraphenylboron for isolated guinea-pig cerebral-cortical mitochondria. In Fig. 4 the membrane potential was determined at various K^+ concentrations. It is clear that a good fit between the two radioactive-isotope distributions is obtained if an activity coefficient of 0.4 is assumed for $TPMP^+$ in the mitochondrial matrix relative to the incubation medium. It should be noted that $TPMP^+$ and tetraphenylboron in low concentrations are without significant effect on mitochondrial respiratory control (Bakeeva *et al.*, 1970).

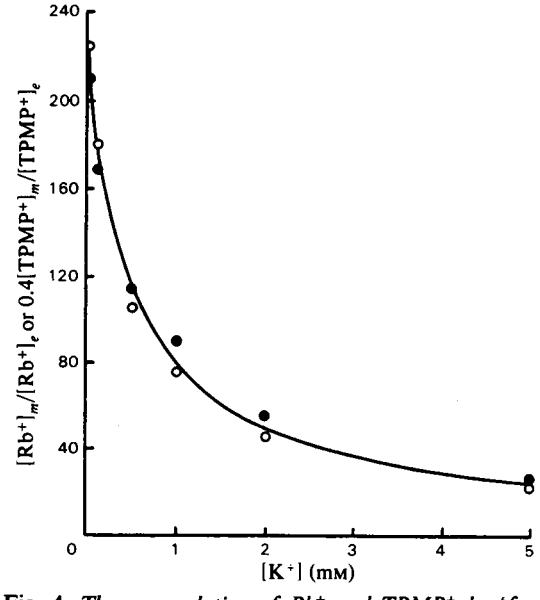

Fig. 4. *The accumulation of Rb^+ and $TPMP^+$ by 'free' mitochondria from cerebral cortex: the influence of K^+ in the presence of valinomycin*

Mitochondria (1 mg of protein/ml of incubation) were incubated for 3 min as described in the Experimental section, in a medium that contained various concentrations of KCl from 0 to 5 mM. Symbols: ○, Rb^+ accumulation ratio; ●, $TPMP^+$ accumulation ratio assuming an activity coefficient of 0.4 relative to the incubation.

It is possible that synaptic vesicles might contribute to the accumulation of $TPMP^+$ within the cytosol. However, if the membrane potential across the synaptic vesicle membrane is comparable with that across the chromaffin granule (i.e. less than 50 mV; Pollard *et al.*, 1976), any accumulation would not be detectable. In addition, as will be shown (see Fig. 8), accumulation of the cation by intrasynaptosomal organelles is prevented by specific mitochondrial inhibitors of proton extrusion.

The magnitude of the mitochondrial membrane potential and respiratory control in intact synaptosomes

The respiratory rate of the present preparation of synaptosomes incubated at 30°C in the presence of glucose is 4 nmol of O/min per mg of synaptosomal protein (Fig. 5). Contaminating 'free' mitochondria do not respire under these conditions (Bradford, 1969), and so the 5-fold stimulation of respiration induced by the addition of a proton translocator (Fig. 5*a*) can be ascribed to a loss of respiratory control by the internal mitochondria. The depolarization of the inner mitochondrial membrane induced by valinomycin (Fig. 2*b*) is associated with a similar stimulation of respiration (Fig. 5*b*). In contrast, addition of oligomycin to inhibit the ATP synthetase causes only a slight inhibition of the initial respiration (Fig. 5*d*), implying that under the initial conditions there is only a slow turnover of ATP within the synaptosome and that the internal mitochondria are close to State 4 (Chance & Williams, 1956).

Under State 4 conditions isolated mitochondria maintain a $\Delta\psi_m$ of about 150 mV (Mitchell & Moyle, 1969; Nicholls, 1974; Rottenberg, 1975), and a similar value has been obtained for 'free' guinea-pig cerebral-cortex mitochondria (Nicholls, 1978*a*). To quantify the potential for the internal synaptosomal mitochondria, eqn. (6) was invoked, and the 6-fold accumulation of Rb^+ and the 76-fold accumulation of $TPMP^+$ were substituted into the equation, employing the activity coefficients for $TPMP^+$ (0.6 for the cytosol and 0.4 for the matrix) deduced in the previous section. An accumulation ratio of free $TPMP^+$ of 300 was deduced across the mitochondrial inner membrane, corresponding to a membrane potential of 150 mV. In 28 experiments, the mean (± S.D.) $\Delta\psi_m$ attained was 148 ± 6 mV.

In a medium of high ionic strength, addition of sufficient proton translocator completely abolishes the mitochondrial membrane potential (Nicholls, 1974). In the experiment depicted in Fig. 6, 0.5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added where indicated. No effect was observed on the Rb^+ accumulation, which continued to indicate a $\Delta\psi_p$ of 47 mV, but the $TPMP^+$ accumulation ratio decreased from 76 to 10. This entire efflux can be ascribed to an altered distribution

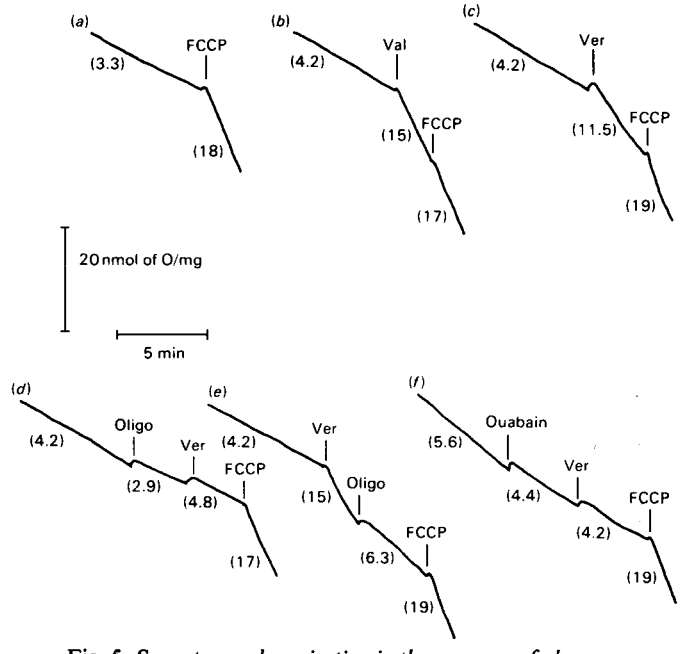

Fig. 5. *Synaptosomal respiration in the presence of glucose*
Synaptosomes (1.5 mg of protein/ml of incubation) were incubated in an oxygen electrode chamber as described in the Experimental section. After preincubating for 8 min, further additions were made as detailed in the Figure. Abbreviations used: FCCP, 0.5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Val, 4 μM-valinomycin; Ver, 100 μM-veratridine; Oligo, 4 μg of oligomycin/ml. Ouabain was present at a concentration of 1 mM. Values in parentheses represent the respiratory rates in nmol of O/min per mg of synaptosomal protein.

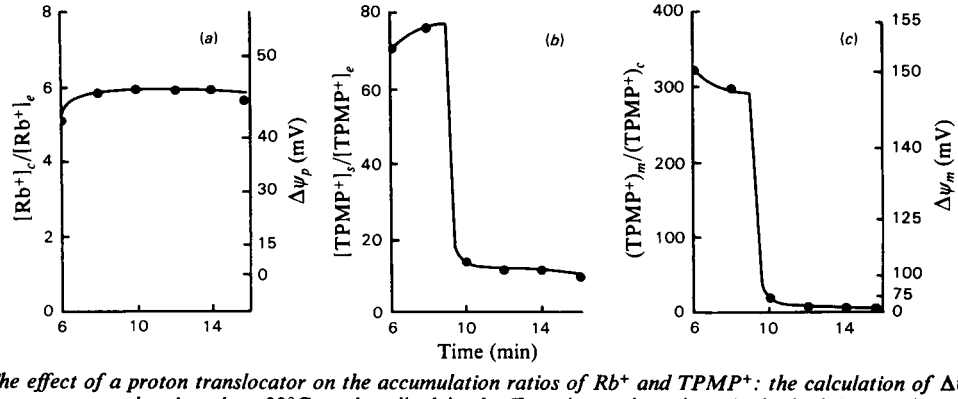

Fig. 6. *The effect of a proton translocator on the accumulation ratios of Rb^+ and $TPMP^+$: the calculation of $\Delta\psi_p$ and $\Delta\psi_m$*
Synaptosomes were incubated at 30°C as described in the Experimental section. At 9 min 0.5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added. Square brackets represent concentrations, rounded brackets represent activities.

across the inner membranes of the included mitochondria, the computed gradient decreasing from 300 to less than 20, implying a decrease in $\Delta\psi_m$ to below the limits of detection (i.e. less than 75mV).

The maintenance of synaptosomal ATP and phosphocreatine

Under the initial conditions in a medium containing 0.5mM-P_i, the total ATP and phosphocreatine concentrations were each about 3nmol/mg of synaptosomal protein, and these values were maintained for the 20min duration of the experiment (Fig. 7*a*). These values closely correspond to those found for rat synaptosomes (Bradford, 1969).

The ability of any cell to maintain ATP-dependent functions when respiration is inhibited will depend on the capacity of anaerobic glycolysis to supply ATP (Fig. 1). As would be predicted, the simultaneous addition of iodoacetate to inhibit glycolysis and cyanide to inhibit respiration leads to a complete depletion of synaptosomal ATP (Bradford, 1969) and to depolarization of the plasma membrane (Blaustein & Goldring, 1975). With the present preparation, addition of sufficient rotenone to inhibit respiration leads to a partial decrease in ATP and phosphocreatine concentrations (Fig. 7*b*). However, it is not immediately apparent whether the residual ATP possesses sufficient thermodynamic potential to drive ATP-dependent events in the cytosol; for example some ATP could be associated with synaptic vesicles (Dowdall *et al.*, 1974). A more sensitive measure of the Gibbs free energy of the cytosolic ATP/(ADP+P_i) pool in the presence of rotenone is to determine the ability of the synaptosome to maintain a $\Delta\psi_m$. The mitochondrial ATP synthetase is reversible (Mitchell, 1976) and can catalyse a close thermodynamic equilibrium between $\Delta\mu_{H^+}$ and the ATP pool.

As shown in Fig. 8(*b*), addition of rotenone results in a decrease of less than 25mV in $\Delta\psi_m$. This implies that anaerobic glycolysis can maintain the cytosolic ATP/(ADP+P_i) pool at a Gibbs free energy that is only slightly lower than that achieved by oxidative phosphorylation, and that the ATP synthetase by reversing can maintain the mitochondrial inner membrane in an 'energized' state. This in turn suggests that the internal mitochondria can retain their putative role in the regulation of cytosolic free Ca^{2+} concentrations (Nicholls, 1978*b*) even during anoxia. As the ATP pool in the presence of rotenone is evidently capable of driving mitochondrial ion translocation, it is not surprising that plasma membrane transport does not seem to be significantly affected (Fig. 8*a*). A similar ability of anaerobic glycolysis to maintain $\Delta\psi_m$ has been shown for Ehrlich ascites cells (Åkerman, 1979).

If $\Delta\psi_m$ is maintained by reversal of the ATP synthetase, it would be predicted that the simultaneous addition of rotenone and oligomycin would lead to a complete collapse of $\Delta\psi_m$ (Fig. 1). This is confirmed in Fig. 8(*b*). As would be predicted, the addition of oligomycin in the absence of rotenone does not decrease the mitochondrial potential, but may even lead to a slight increase in $\Delta\psi_m$ (Fig. 8*b*). As this increase is not more than 2 or 3mV, this confirms the evidence from the oxygen electrode that the mitochondria within synaptosomes under control conditions are already very close to State 4.

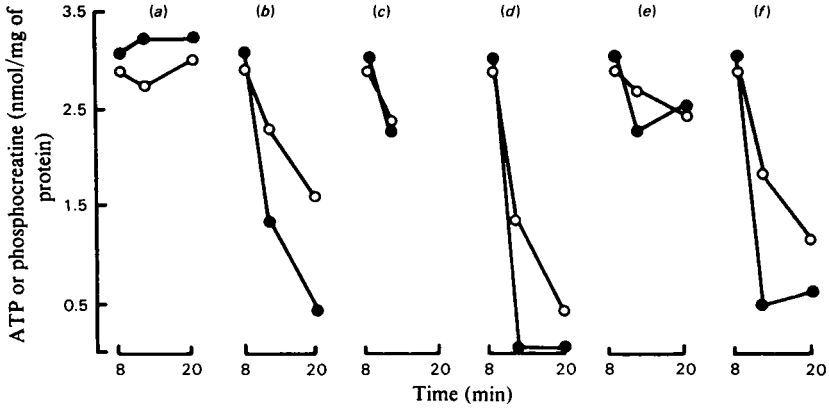

Fig. 7. *ATP and phosphocreatine concentrations within synaptosomes incubated in the presence of glucose*
Synaptosomes (1.5mg of protein/ml of incubation) were incubated in the basic medium, without radioisotopes, as described for the determination of membrane potentials. At defined times portions were quenched by the addition of $HClO_4$, and assayed as described in the Experimental section. The following additions were made at 9min: (*a*), control; (*b*), 4μM-rotenone; (*c*), 100μM-veratridine; (*d*), 4μM-rotenone plus 100μM-veratridine; (*e*), 1mM-ouabain plus 100μM-veratridine; (*f*), 1mM-ouabain plus 100μM-veratridine plus 4μM-rotenone. Symbols: ○, ATP; ●, phosphocreatine.

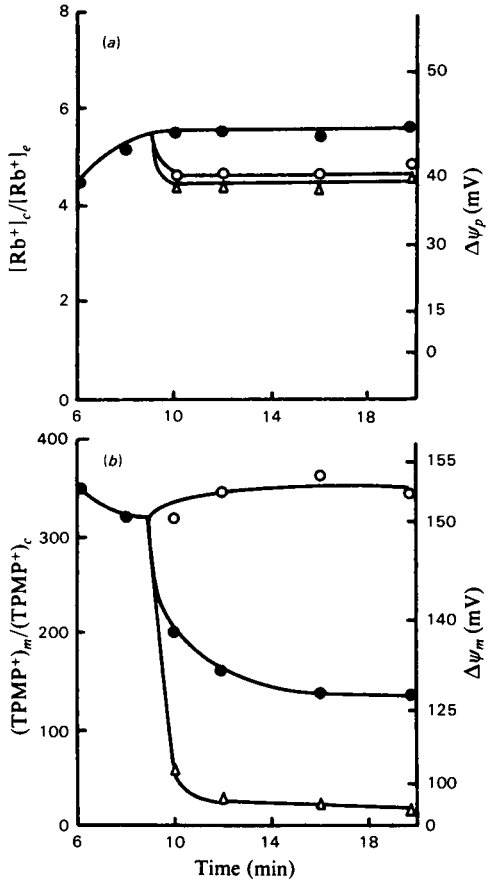

Fig. 8. $\Delta\psi_p$ *and* $\Delta\psi_m$*: the influence of rotenone and oligomycin*
Synaptosomes were incubated as described in the Experimental section. At 9min the following additions were made: ●, 4μM-rotenone; ○, 4μg of oligomycin/ml; △, 4μM-rotenone plus 4μg of oligomycin/ml.

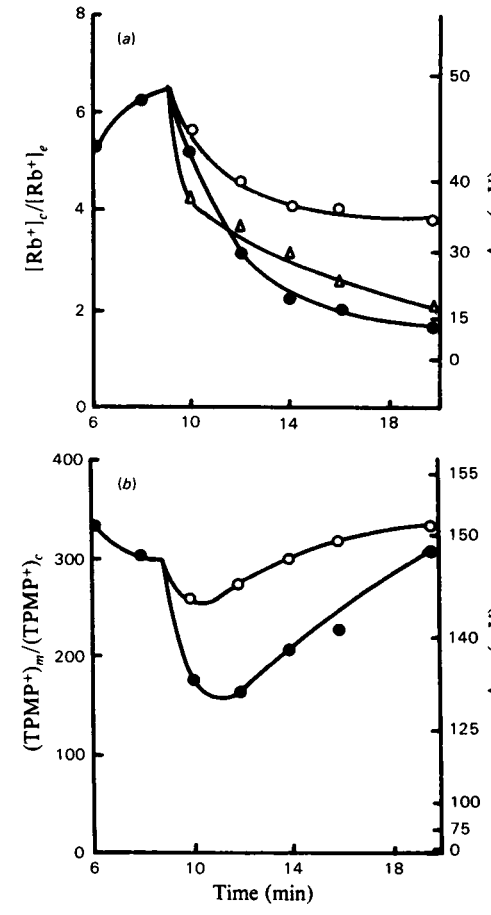

Fig. 9. $\Delta\psi_p$ *and* $\Delta\psi_m$*: the influence of ouabain and KCl*
Synaptosomes were incubated as described in the Experimental section. At 9min the following additions were made: ●, 60mM-KCl; ○, 1mM-ouabain; △, 1mM-ouabain plus 5μM-valinomycin.

The influence of effectors of the plasma membrane potential

Even under the initial conditions the cycling of ions across the plasma membrane driven by the $(Na^+ + K^+)$-dependent ATPase appears to utilize a significant proportion of the synaptosomal ATP supply. Thus respiration can be significantly inhibited not only by oligomycin (Fig. 5*d*), but also by ouabain (Fig. 5*f*). Addition of ouabain results in a slow decrease of the Rb^+ diffusion potential to 33mV (Fig. 9*a*). As would be predicted, ouabain does not significantly affect $\Delta\psi_m$ (Fig. 9*b*), and the efflux of $TPMP^+$ from the synaptosomes that occurs can therefore be accounted for by the decrease in $\Delta\psi_p$. The rate at which the plasma membrane depolarizes appears to be largely limited by the low Na^+ permeability of the membrane, rather than by the K^+ permeability, as addition of valinomycin to introduce a very high K^+ permeability leads to only a slight enhancement of the rate of depolarization (Fig. 9*a*).

A second method for depolarizing the synaptosomal plasma membrane is to increase the K^+ concentration in the medium. Addition of 60mM-KCl results in a gradual lowering of the Rb^+ diffusion potential to less than 15mV (Fig. 9*a*). In contrast with a published report (Bradford *et al.*, 1973) no stimulation of respiration was observed on adding 60mM-K^+. As the permeability of the plasma membrane to $TPMP^+$ appears to be greater than to Rb^+, the presumably instantaneous depolarization of the plasma membrane induced by the addition of

K^+ results in a more rapid efflux of $TPMP^+$ compared with Rb^+. This results in a transient overestimation of $\Delta\psi_p$, and hence to an equivalent transient under-estimate of $\Delta\psi_m$, which accounts for the time-dependency of the apparent $\Delta\psi_m$ depicted in Fig. 9(*b*).

The third technique for depolarizing the plasma membrane involves the addition of the alkaloid veratridine, which stabilizes the high-conductance conformation of the potential-dependent Na^+ ionophore in the plasma membrane (Ohta *et al.*, 1973), and hence induces a long-lasting depolarization of the plasma membrane (Blaustein & Goldring, 1975). If the rate of Na^+ cycling across the plasma membrane is limited by the activity of the Na^+ ionophore under initial conditions, then it would be predicted that increasing the activity of this pathway would increase the rate of ion cycling. This in turn would increase the cytosolic demand for ATP, and finally increase the respiratory rate of the included mitochondria (Fig. 1). In Fig. 5(*c*) it is shown that the increased respiration induced by veratridine is very substantial. In eight determinations, a mean increase of 7.4nmol of O/min per mg of synaptosomal protein was found on addition of 100μM-veratridine.

If the mitochondrial P/O ratio is 3 under these conditions, the increased ATP turnover indicated by the respiratory response is 22nmol of ATP/min per mg of protein, and should be well within the capacity of the $(Na^+ + K^+)$-dependent ATPase, which in rat brain synaptosomes at 37°C has an activity of 130nmol of ATP/min per mg of protein (Ata *et al.*, 1970). It is clear that the internal mitochondria possess sufficient respiratory capacity to maintain cytosolic ATP concentrations, as respiratory rates in the presence of veratridine are significantly less than those following the addition of a proton translocator (Fig. 5*c*), and as 3min after addition of the alkaloid the synaptosome retains high concentrations of both ATP and phosphocreatine (Fig. 7*c*).

If the $(Na^+ + K^+)$-dependent ATPase translocates $3Na^+$ and $2K^+$ ions for each ATP hydrolysed (Thomas, 1972), then the addition of veratridine increases the rate of cycling of Na^+ and K^+ by respectively 66 and 44nmol of ion/min per mg of protein. The enhanced rate of K^+ (and presumably Rb^+) cycling will increase any thermodynamic disequilibrium of the cation across the plasma membrane, and will lead to an overestimate of $\Delta\psi_p$ if this is based on the Rb^+ diffusion potential. In Fig. 10(*a*), the effect of veratridine on the Rb^+ diffusion potential is depicted. There is a 10mV decrease in diffusion potential on adding the alkaloid alone, a 25mV decrease when rotenone and veratridine are added together, and a rapid complete depolarization when veratridine and ouabain are added together. In the presence of rotenone, as will be shown below, ATP supply limits the rate of veratridine-stimulated

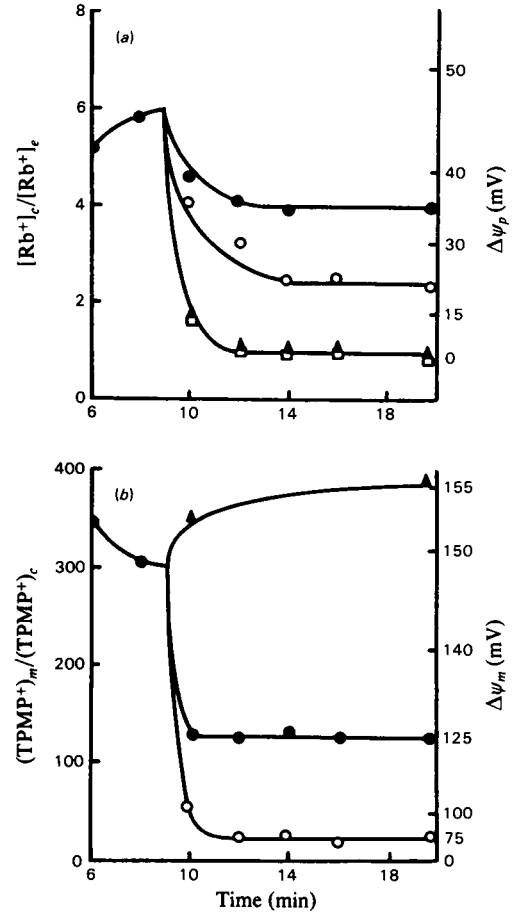

Fig. 10. $\Delta\psi_p$ *and* $\Delta\psi_m$*: the influence of veratridine*
Synaptosomes were incubated as described in the Experimental section. At 9min the following additions were made: ●, 100μM-veratridine; ▲, 100μM-veratridine plus 1mM-ouabain; ○, 100μM-veratridine plus 4μM-rotenone; □, 100μM-veratridine plus 4μM-valinomycin together with either 4μM-rotenone or 1mM-ouabain.

ion cycling, whereas in the presence of ouabain all such cycling is inhibited. It therefore appears that the actual depolarization induced by veratridine is more or less complete, but that the Rb^+ diffusion potential overestimates $\Delta\psi_p$ to an extent proportional to the rate of K^+ (and Rb^+) cycling across the membrane. It is clear from comparisons of the Rb^+ diffusion potential on addition of ouabain alone (Fig. 9*a*), ouabain plus valinomycin (Fig. 9*a*) or ouabain plus veratridine (Fig. 10*a*) that Na^+ permeability, rather than $K^+(Rb^+)$ permeability, is normally rate limiting. The addition of valinomycin will ensure that the

Rb^+ diffusion potential will be equal to, rather than an overestimate of, $\Delta\psi_p$. When the ionophore is added simultaneously with veratridine and either ouabain or rotenone (Fig. 10*a*), a very rapid and complete depolarization is observed.

If the veratridine-induced stimulation of respiration is due only to enhanced ion cycling across the plasma membrane, then it should be inhibited by the addition of either oligomycin or ouabain as is the case for the basal respiration (Figs. 5*d* and 5*f*). This is in fact observed (Figs. 5*d*–5*f*), and demonstrates that the changes in respiratory rates are not secondary consequences of an altered cytosolic ionic composition on adding veratridine. In this context it should be noted that the final rate of uncontrolled respiration after addition of a proton translocator is essentially the same in each of the traces of Fig. 5, even though the cytosolic Rb^+ (and hence K^+) concentrations vary considerably according to the incubation conditions (Fig. 10*a*). It therefore appears that the dependency on K^+ concentration that is observed for the rate of uncontrolled pyruvate respiration by isolated brain mitochondria (Nicklas *et al.*, 1971; Lai & Clark, 1976) is less apparent in intact synaptosomes incubated under the present conditions.

The uncertainty in the estimation of $\Delta\psi_p$ in the presence of veratridine induces an equivalent error in the calculation of $\Delta\psi_m$ (eqn. 6). If the Rb^+ diffusion potential is taken to represent $\Delta\psi_p$, then the mitochondrial potential apparently decreases by 23 mV (Fig. 10*b*). Although to some extent a decrease in $\Delta\psi_m$ would be predicted to accompany the increase in respiration associated with the addition of veratridine (Fig. 5*c*), the decrease in ATP and phosphocreatine is only slight (Fig. 7*c*). This implies that the actual mitochondrial membrane potential changes by less than 23 mV.

In the presence of ouabain and veratridine, the Rb^+ diffusion potential should accurately reflect $\Delta\psi_p$, and the mitochondrial potential calculated on this basis shows no decrease (Fig. 10*b*), and may even slightly increase. Under these conditions there is no significant change in the concentration of either ATP or phosphocreatine (Fig. 7*e*). This, together with the respiratory and potential evidence, confirms that veratridine has no effects on synaptosomal bioenergetics that are not a direct consequence of the increased ATP demand by the $(Na^+ + K^+)$-dependent ATPase.

Although anaerobic glycolysis is able to satisfy the cytoplasmic demand for ATP under initial conditions, it does not follow that the same is true in the presence of veratridine. When rotenone and veratridine are added simultaneously (Fig. 7*d*) the phosphocreatine is rapidly depleted, while the total synaptosomal ATP is greatly decreased. This decrease is much more pronounced than with veratridine or rotenone alone (Figs. 7*b* and 7*c*) and indicates that anaerobic glycolysis is unable to keep up with the enhanced demand for ATP by the $(Na^+ + K^+)$-dependent ATPase.

In the presence of rotenone, the high mitochondrial membrane potential was maintained by a reversal of the mitochondrial ATP synthetase driven by cytosolic ATP (Fig. 1). Evidently, if the cytosolic ATP is depleted then this reversal will not occur, and this is shown in Fig. 10(*b*), where no detectable mitochondrial potential is maintained under these conditions. It thus appears that synaptosomes possess sufficient glycolytic capacity to maintain basal transport functions during anoxia, but not to cope with the increased energy demand concomitant with persistent depolarization of the plasma membrane.

Discussion

Heterogeneity of the preparation

In any study of generalized synaptosomal functions, it is important to appreciate the limitations that are imposed by the inherent heterogeneity of the preparation. First, all synaptosomal preparations, including the present one (Nicholls, 1978*a*) are contaminated to some extent with 'free' mitochondria not bounded by a plasma membrane (Whittaker, 1969; Booth & Clark, 1978). These mitochondria will not respire in the presence of glucose (Bradford, 1969), and by virtue of the high Ca^{2+} concentration to which they are exposed will not develop a significant membrane potential. However, if the incubation medium contains a mitochondrial substrate such as glutamate or succinate then the contaminating mitochondria can respire and even exhibit respiratory control (in the absence of Ca^{2+}) on addition of ADP (Verity, 1972; Booth & Clark, 1978). It is important to distinguish between these studies and the present one, where the respiratory stimulations reflect cytosolic effects on internal mitochondria.

A second form of heterogeneity that is inherent in any synaptosomal preparation is that the number of mitochondria per synaptosome is variable, while some completely lack internal mitochondria (Whittaker, 1969). This does not affect the validity of the membrane potential calculations, as these are purely dependent on the total cytosolic and internal matrix volumes in the incubation (eqn. 6).

Yet another degree of heterogeneity is due to the presence of distinct sub-populations of synaptosomes containing different neurotransmitters. As the properties examined in the present paper are averaged over the entire incubation, it is impossible to describe accurately those bioenergetic effects that are restricted to any one class of synaptosomes. This, however, should not apply to the generalized functions discussed here.

Validity of the membrane-potential determinations

All three indirect techniques that have been utilized to estimate membrane potentials in isolated cells suffer from limitations and differ in the sensitivity with which they respond to plasma membrane and mitochondrial membrane potentials. Cyanine dyes are relatively insensitive to high potentials (Burckhardt, 1977) and the possibility of a mitochondrial contribution to the fluorescent response is generally not considered (Blaustein & Goldring, 1975; Laris *et al.*, 1976). The differential spectrum of safranine responds linearly up to 200mV (Åkerman & Wikström, 1976) and can thus report on the mitochondrial potential, although quantification is difficult (Åkerman, 1979). The accumulation of $TPMP^+$ responds logarithmically to the sum of the mitochondrial and plasma membrane potentials (Hoek *et al.*, 1979; eqn. 4 of the present paper) and can thus detect small variations in either potential, although a second indicator is required to resolve them. Although the isotope technique allows a measure of quantification, its response time is somewhat limited, and activity coefficients must be estimated for the different compartments. Nevertheless if the intention, as in the present paper, is not to determine a definitive and precise value for the plasma membrane potential, but rather to be able to provide an overall description of the bioenergetic pathways within the synaptosome, then it is apparent that the isotope technique is useful.

General conclusions

The brain is a highly aerobic tissue that is dependent almost entirely on glycolysis (Bachelard, 1978), and this is reflected in the behaviour of the isolated synaptosome. The respiratory rates we observe in the presence of glucose are compatible with the published values (Bradford, 1969; Whittaker, 1969; Booth & Clark, 1978) and if the included mitochondria account for about 12% of the synaptosomal protein (Nicholls, 1978*a*), they correspond to a rate of mitochondrial respiration that varies from 24nmol of O/min per mg of mitochondrial protein in the presence of oligomycin (Fig. 5*d*) to 150nmol of O/min per mg of mitochondrial protein in the presence of a proton translocator. These rates are compatible with those published for isolated synaptosomal mitochondria oxidizing pyruvate and malate (Lai & Clark, 1976; Nicholls, 1978*a*).

One advantage of the synaptosome as a model system in which to study cellular bioenergetics is that the extra-mitochondrial ATP demand can be varied by altering the rate of ion cycling at the plasma membrane. In this way the consequences of a 'physiological' transition away from State 4 can be readily investigated. It is significant that a 200% increase in respiration on addition of veratridine (Fig. 5*c*) is caused by only a slight decrease in synaptosomal ATP and phosphocreatine concentrations. As discussed in the Results section, the apparent 23mV decrease in mitochondrial membrane potential is probably exaggerated due to problems in accurately determining the plasma membrane potential under conditions of increased Na^+ permeability. Thus it appears that as for isolated mitochondria (Nicholls & Bernson, 1977) a slight decrease in proton electrochemical potential (and hence in membrane potential) is sufficient to produce a considerable increase in respiration.

When respiration is inhibited by addition of rotenone, ATP can still be generated by anaerobic glycolysis, and the cytosolic ATP pool can still maintain the mitochondrial membrane potential by reversal of the ATP synthase (Fig. 8). It is significant that tumour cells also appear to maintain the mitochondrial potential during anaerobiosis (Åkerman, 1979). It is also important to note that addition of a respiratory inhibitor such as CN^- cannot automatically be assumed to deplete cytosolic ATP to the extent that ATP-dependent processes cease to function.

Anaerobic glycolysis is, however, incapable of maintaining ATP when the ATP turnover at the plasma membrane is enhanced by addition of veratridine. Thus phosphocreatine becomes undetectable, and ATP decreases to an extremely low concentration (Fig. 7*d*). Most compellingly, the mitochondria are no longer capable of maintaining an appreciable membrane potential (Fig. 10*b*).

In conclusion, the present paper demonstrates the central bioenergetic role played by the two ion-translocating ATPases located respectively in the plasma membrane and inner mitochondrial membrane and linked through the cytoplasmic ATP pool (Fig. 1). It is clear that the close interrelationship between these systems should be considered in future studies of synaptosomal bioenergetics.

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