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MEMBRANE POTENTIALS IN PINCHED-OFF PRESYNAPTIC NERVE TERMINALS MONITORED WITH A FLUORESCENT PROBE: EVIDENCE THAT SYNAPTOSOMES HAVE POTASSIUM DIFFUSION POTENTIALS

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SUMMARY

1. Some physiological properties of tissue fractions from rat brain homogenates have been examined. Of the three fractions studied (presynaptic nerve terminals, mitochondria and fragmented membranes), only the nerve terminals (synaptosomes) have the ability to accumulate ^{42}K from physiological salt solutions.

2. The ability to accumulate and retain K is lost if synaptosomes are exposed to very hypotonic solutions. The K uptake and total K content is reduced by ouabain and by inhibitors of glycolysis and oxidative phosphorylation.

3. These results suggest that synaptosomes in physiological saline accumulate K against a concentration gradient, and may have K diffusion potentials across their surface membranes. The voltage-sensitive fluorescent probe, 3,3′-dipentyl 2,2′-oxacarbocyanine (CC_5), was used to test this possibility.

4. In the squid axon, the fluorescent emission of CC_5 is directly proportional to membrane potential; depolarization causes an increase in fluorescence.

5. The fluorescence of synaptosomes ('synaptosome fluorescence') treated with CC_5 is increased when $[K]_o$ is increased or $[K]_i$ is reduced; replacement of external Na by Li or choline has little effect on the synaptosome fluorescence. In quantitative terms, synaptosome fluorescence is proportional to log $([K]_o + 0{\cdot}05\,[Na]_o)$. Rb is about as effective as K in enhancing synaptosome fluorescence; Cs is about 1/4 as effective. The effect of increased $[K]_o$ is reversible.

6. The fluorescence data provide corroborative evidence that there is

normally a large K gradient ($[K]_o \ll [K]_i$) across the synaptosome surface membrane. The data suggest the $[K]_i$ may be in excess of 100 mM.

7. Replacement of Cl^- by methylsulphate did not significantly affect the relationship between synaptosome fluorescence and $[K]_o$, nor did removal of external Ca.

8. The fluorescence of CC_5-treated mitochondria, membrane fragments, or lysed synaptosomes is unaffected by changes in the K concentration of the medium.

9. Veratridine and gramicidin D, both of which enhance Na permeability (P_{Na}) in some intact tissues, increase synaptosome fluorescence when added to the standard medium. The increment is greatly reduced or abolished when external Na is replaced by choline.

10. If synaptosomes are first Na-loaded (by pre-treatment with cyanide + iodoacetate), and then placed in a choline medium, addition of gramicidin D significantly *decreases* fluorescence. This effect could be explained if, with $[Na]_o \ll [Na]_i$, the increase in P_{Na} causes the synaptosomes to hyperpolarize.

11. The veratridine-induced increase in synaptosome fluorescence was prevented by 3×10^{-7} M tetrodotoxin, which also blocks the depolarizing effect of veratridine in intact neurones.

12. The main conclusion is that synaptosomes may retain resting membrane potentials and the ability to increase Na permeability.

INTRODUCTION

Presynaptic nerve ending (synaptosome) fractions prepared from brain homogenates (cf. Whittaker, 1965; DeRobertis, 1967) retain many of the metabolic (Bradford, 1969; Bradford & Thomas, 1969), osmotic (Marchbanks, 1967; Keen & White, 1970, 1971) and alkali metal ion transport (Ling & Abdel-Latif, 1968; Bradford, 1969; Escueta & Appel, 1969; Blaustein & Wiesmann, 1970) properties of more intact tissue preparations. These observations suggest that the synaptosome surface membrane may reseal when the ending is pinched-off during homogenization.

The finding that synaptosomes are much more permeable to K than to Na (Keen & White, 1971), and can accumulate K (Bradford, 1969; Escueta & Appel, 1969), may indicate that synaptosomes have a surface membrane potential which approximates a K diffusion potential as is the case for most intact neurones. Unfortunately, the small size of the presynaptic endings (about 0·5 μm diameter) precludes direct measurement of synaptosome membrane potentials by conventional micro-electrode techniques. Recently, however, certain fluorescent dyes have been found to accurately indicate membrane potential changes in squid giant axons

(Davila, Salzberg, Cohen & Waggoner, 1973). Davila *et al.* suggested that these dyes might be useful for the 'monitoring of membrane potential in systems where electrodes cannot be used'. The efficacy of this approach has already been demonstrated in the measurement of membrane potentials in human red blood cells (Hoffman & Laris, 1974).

In the present study, the fluorochrome, 3,3'-dipentyl-2,2'-oxacarbocyanine, has been used to estimate membrane potential changes in synaptosomes as a consequence of altering the external/internal K concentration ratio ($[K]_o/[K]_i$) or the Na/K permeability ratio (P_{Na}/P_K). The results provide further evidence that synaptosomes can accumulate and retain K, and that their membranes are normally K-selective. Synaptosome preparations may therefore be particularly suitable for investigating other aspects of presynaptic terminal physiology.

A preliminary report of some of these findings has been communicated to the Society for Neuroscience (Goldring & Blaustein, 1973).

METHODS

Solutions. The composition of some representative solutions used in these experiments is shown in Table 1. In many instances, mixtures of these solutions (e.g. Na + 5K and 137 mM-K saline) were used to obtain intermediate concentrations of alkali metal ions (see Results). Details regarding the addition of drugs will be given below. All solutions for these experiments were prepared with de-ionized glass-distilled water.

Preparation of presynaptic nerve terminals. Synaptosomes were prepared from whole rat brain by the differential centrifugation and sucrose gradient centrifugation procedures of Gray & Whittaker (1962). The fraction located at the 0·8–1·2 M sucrose interface of the sucrose density gradient is not a pure preparation of presynaptic nerve endings, but for simplicity it will be referred to as the 'synaptosome' preparation, in conformity with numerous other authors. The purity of the preparations has not been evaluated in the present study; however, the data of Michaelson & Whittaker (1963) indicate that about 70 % of the nitrogen recovered from the region between 0·8 and 1·2 M sucrose is associated with 'nerve-ending particles' (see Table 2). In most experiments, the brains from four 150–200 g rats were homogenized in 0·32 M sucrose; the synaptosome fraction obtained from the 0·8–1·2 M sucrose interface at the end of the 2 hr sucrose density gradient centrifugation contained about 65–100 mg protein suspended in a total of 20–22 ml. of approximately 1 M sucrose solution.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as a standard.

In several instances (see Results) the material from the 0·32–0·8 M sucrose interface (predominantly membrane fragments) and from the pellet at the bottom of the sucrose gradient (predominantly free mitochondria) were also examined for physiological activity. The former could be used directly, but the pellet had to be re-suspended (in 1·2 M sucrose) before additional procedures could be carried out. Subsequent treatment of these suspensions was identical to that used for the synaptosome fraction.

In three early experiments the morphologic characteristics of the particles in

each of the three sucrose gradient fractions was examined by electron microscopy. In agreement with the results of Gray & Whittaker (1962), we observed that the 0·32–0·8 M interface contained mostly membrane fragments and myelin figures; the pellet contained mitochondria, primarily. Membrane-enclosed particles about 0·5 μm in diameter (the synaptosomes) filled with 500 A vesicles, and usually containing one or two small mitochondria, were the most frequent particles at the 0·8–1·2 M interface; similar bodies were also observed in the other two fractions of the gradient, but they were much less common in these layers.

TABLE 1. Composition of representative solutions

Solution*	NaCl	Na_2SO_4	KCl	Choline Cl	$CaCl_2$
	(all in m-moles/litre)†				
Na + 5 K	132	0	5	0	1·2
K-free Na	137	0	0	0	1·2
Ca-free Na + 5 K	132	0	5	0	0
137 mM-K saline	0	0	137	0	1·2
Choline + 5 K	0	0	5	132	1·2
Na + 5 K (10 mM sulphate)	117	10	5	0	0

* Li + 5K, Rb + 5K and Cs + 5 K were similar in composition to the Na + 5 K, except that all of the Na was replaced by Li, Rb or Cs, respectively.

† In addition to the components listed, all solutions contained (in m-mole/l.): $MgCl_2$, 1·3; NaH_2PO_4, 1·2; glucose, 10; and tris hydroxymethylaminomethane base, 20. The solutions were titrated to pH 7·4 (at 30° C) with maleic acid.

Experimental procedures. In order to return the synaptosomes to a more physiological environment, the suspension from the sucrose gradient was diluted with a total of 12–15 volumes of Ca-free Na + 5K (Table 1) at 2–3° C, by progressive addition of small aliquots of the salt solution over a 15 min period. The diluted suspension was then evenly apportioned into twenty-four or thirty-six centrifuge tubes, and spun at 9000 *g* for 5 min at 3° C. The supernatant solution was decanted and discarded, and the pellet was re-suspended in saline (usually 0·5 or 1·0 ml. of Na + 5 K, but see Results for details regarding individual experiments). The re-suspended synaptosomes were then 'pre-incubated' for 12–15 min at 30° C in order to permit them to re-accumulate K and extrude Na (see Results), and come to a steady state. Additional aliquots of solutions were then added to the tubes; for the ^{42}K uptake experiments these solutions contained radioactive tracers (^{42}K and ^{35}S). The ionic content of these solutions (see Table 1) was often different from that of the pre-incubation medium in that, for example, the mixed medium might have an elevated K concentration. Details of incubation conditions, including the compositions of the mixed media and the timing of drug additions, will be given below.

Cation and water content of intact and lysed synaptosomes. In several experiments, samples of the synaptosome suspension from the sucrose gradient were diluted with 1·5 volumes of iced distilled water, to bring the sucrose concentration to about 0·4 M. The suspensions were then centrifuged at 20,000 *g* for 1 hr, at 3° C; the clear supernatant solutions were decanted and discarded. Some of the pellets were disrupted by resuspending them in 2 ml. 0·1 % (v/v) triton X-100; Na, K, Ca, Mg and protein concentrations were later measured in small aliquots of these suspensions. To determine the ionic content of the osmotically-sensitive compartment, some synaptosome pellets were rapidly resuspended in 1·95 ml. iced distilled water,

and immediately centrifuged at 20,000 *g* for 10 min at 3° C. The supernatant solutions were decanted, and 50 μl. 4 % triton X-100 was added; the pellets were resuspended in 2 ml 0·1 % triton X-100.

The cation content of these triton X-100-treated samples was determined on a Perkin–Elmer Model 303 atomic absorption spectrophotometer; Na and K were measured by flame emission, with 50 mM-CsCl present to minimize ionization. Ca and Mg were analysed by atomic absorption, with 70 mM-$LaCl_3$ (prepared from La_2O_3) present in the Ca samples to reduce flame suppression by phosphate.

Since synaptosomes are impermeable to sulphate (Keen & White, 1970; M. P. Blaustein, unpublished data), the difference between the total evaporative water loss and the sulphate space was used to measure the intrasynaptosomal ('occluded') water space. Lysed or intact synaptosome pellets (in tared centrifuge tubes) were suspended in 1·0 ml. 10 mM sulphate saline containing 0·8 μc $^{35}SO_4$ per μmole of sulphate, and incubated for 15 min at 30° C. The suspensions were centrifuged at 20,000 *g* for 5 min, at 3° C, and the supernatant solutions were decanted and saved. The walls of the centrifuge tubes were carefully wiped dry. The tubes were then weighed, dried for 24–48 hr in a vacuum desiccator, and re-weighed to determine the total evaporative water loss from the pellets. The dried pellets were digested in 2·0 ml. 1·0 N-NaOH at 60–70° C; 1·0 ml. was then transferred to a scintillation counting vial. Three drops of 30 % hydrogen peroxide (to help decolourize the sample), 1·5 ml. 0·67 N-HCl and 15 ml. Bray (1960) scintillation cocktail were added to each vial, and the ^{35}S counts were determined in a liquid scintillation counter (Packard Instrument Co.). The specific activity of ^{35}S in the supernatant solutions was measured by counting small aliquots which had been diluted, as above, with NaOH, HCl, H_2O_2 and Bray solution. All counts were corrected for quenching.

The protein content of the pellet digests was determined by the Lowry method.

K content of synaptosomes following incubation in salt solutions. The ability of synaptosomes to accumulate and retain K was tested in a few experiments by determining the effects of incubation with various drugs or metabolic poisons on the net K content of the synaptosomes. Immediately following incubation, the synaptosomes were centrifuged at 3° C for 5 min at 9000 *g*. The supernatant solution was decanted and discarded. The pellet (containing about 1·5–2 mg protein) was rinsed with 3–4 ml. iced-cold 0·32 M sucrose to remove the last few drops of incubation fluid, and was then suspended in 2 ml. 0·1 % triton X-100. The K content of these samples was determined by flame emission spectroscopy.

^{42}K uptake studies. The accumulation of ^{42}K by synaptosomes was determined by incubating synaptosomes at 30° C in 10 mM sulphate saline (Table 1) containing ^{42}K (1 μc/μmole K) and $^{35}SO_4$ (0·8 μc/μmole SO_4); in some instances (see below) ouabain or metabolic poisons were added to the medium. Uptake was terminated after centrifuging the synaptosome suspensions at 9000 *g* for 3 min at 3° C. The supernatant solutions were decanted and aliquots were saved for counting. The pellets were carefully rinsed with 8 ml. iced-cold 0·32 M sucrose to remove the remaining superficial 'extra-synaptosomal' fluid. They were then suspended in 1·3 ml. 1·0 N-NaOH and digested for 15 min at 60–70° C. A 1·0 ml. aliquot was placed in a counting vial, and the ^{42}K Cerenkov radiation (Haberer, 1965) was immediately counted in a liquid scintillation counter. The remainder of the digest was frozen and saved for protein analysis. Duplicate 50 μl. aliquots of the supernatant solutions were placed in counting vials; 1·0 ml. 1·0 N-NaOH was added, and the Cerenkov radiation in these samples was assayed. All counts were corrected for radioactivity decay and for quenching. About 2 weeks later (i.e. after decay of the ^{42}K), H_2O_2, HCl and Bray solution (see above) were added to each vial, and the samples were recounted in the liquid scintillation counter in order to determine the $^{35}SO_4$ content.

The $^{42}K/^{35}S$ ratio in the supernatant solutions was used to correct for 'extra-synaptosomal' ^{42}K trapped in the pellet.

Fluorescent dye studies. A Turner model 111 fluorometer was fitted with a motor to turn a small magnet just beneath the cuvette. This, in turn, rotated a small spin-bar in the bottom of the cuvette which kept suspensions well dispersed. The synaptosome suspension was illuminated with light at 450 nm and fluorescent emission was measured at 510 nm (Baird-Atomic type B-1 450 nm and 510 nm interference filters, with 10 nm half-widths, were used as the 1° and 2° filters, respectively). A Bausch and Lomb strip chart recorder was used to display the signal from the fluorometer.

A 5 μl. aliquot of fluorochrome (1·5 mg/ml. in ethanol) was added (at *d* in Fig. 4*A*) to 3·0 ml. of physiological saline in a 1 cm i.d. fluorometer cuvette, and the 'background' fluorescent intensity (i.e. due to dye, alone) was measured. The increment in fluorescence (= 'synaptosome fluorescence') upon addition of a 100 μl. aliquot of 'pre-incubated' synaptosomes (4–6 mg protein/ml.) was then recorded (at *s* in Fig. 4*A*1): addition of 0·6 mg of synaptosome protein approximately doubled the fluorescent intensity, when compared to dye alone. Synaptosome fluorescence in standard Na + 5 K medium (Table 1) was taken as the 'control' value, and was compared to synaptosome fluorescence in media containing various drugs or altered ion concentrations (see Results). In some experiments small aliquots (10–100 μl.) of drug or concentrated salt solutions were added to the synaptosome suspensions after a steady fluorescence had been attained: in other experiments, synaptosomes were pre-incubated in media which are known to alter intracellular ion concentrations. The increment (or decrement) in fluorescent intensity was then taken as a measure of the agent's action.

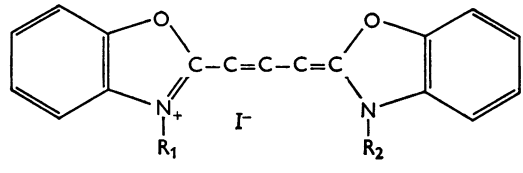

Fig. 1. Structural formula of 3,3′-dialkyl-2,2′-oxacarbocyanine dyes. The dipentyl analog, CC_5, with R_1 and R_2 both $-(CH_2)_4CH_3$, was used in all of the experiments in the present study.

At constant fluorochrome concentration, synaptosome fluorescence in both control and test media was directly proportional to synaptosome protein concentration. Consequently, for most experiments synaptosome fluorescence will be expressed in 'fluorescence units' per mg protein, where one fluorescence unit is defined as 1·0 μA of current output from the fluorometer photomultiplier.

A series of 3,3′-dialkyl-2,2′-oxacarbocyanine dyes (see Fig. 1), generously provided by Dr Alan S. Waggoner, was tested in these experiments. The dipentyl (CC_5) and dihexyl (CC_6) fluorochromes were found to give the largest external K-dependent fluorescence changes (presumably correlated with membrane potential; see below) when tested on rat brain synaptosomes. Furthermore, since CC_5 gave the most stable fluorescence, this dye was used for all the experiments to be reported below.

If the fluorochromes are to be used as indicators of membrane potential (V_M), it is particularly important to know the relationship between V_M and fluorescence. Although the molecular mechanisms responsible for the voltage-sensitive fluores-

cence changes are not completely understood (but see Sims, Wang, Waggoner & Hoffman, 1974), empirical observations in the squid giant axon (Fig. 2, and see Cohen, Salzberg, Davila, Ross, Landowne, Waggoner & Wang, 1974) show that the fluorescence of CC_5 changes linearly with voltage when the membrane is depolarized or hyperpolarized by as much as 100 mV from the resting potential; depolarization causes an increase in fluorescence. This linearity suggests that the dye, CC_5, may indeed be a useful membrane potential indicator.

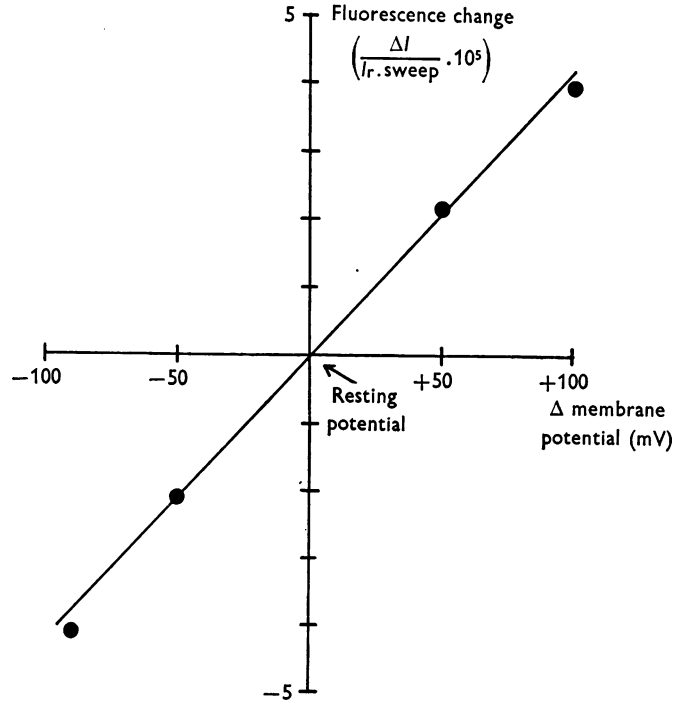

Fig. 2. Fluorescence of CC_5-treated squid axon graphed as a function of the membrane potential. The axon was incubated for 10 min in a 1:8 dilution (with sea water) of a saturated solution of CC_5 in sea water. The dye solution was then washed out with nitrogen-flushed sea water for 30 min. The axon was then voltage-clamped at its resting potential, and 2 msec hyperpolarizing and depolarizing voltage steps were applied. Incident light was 480 ± 15 nm; emission of wave-lengths longer than 515 nm was measured. For each voltage-clamp step, the fluorescence during multiple sweeps was summed on a computer-of-average-transients. The value plotted on the ordinate is the change in fluorescent intensity (ΔI) after 2 msec of depolarization or hyperpolarization, divided by the product of the resting fluorescence (I_r) and the number of sweeps. Temp. 13° C; axon diam.: 610 μm (unpublished experiment of L. B. Cohen, H. V. Davila and B. M. Salzberg, reproduced with their permission. For additional information cf. Davila *et al.* 1973).

RESULTS

^{42}K uptake and K content of synaptosomes

Data from several laboratories (Marchbanks, 1967; Bradford, 1969; Escueta & Appel, 1969) have established that synaptosomes can accumulate and retain K in an osmotically sensitive compartment. The K is presumably taken up via the Na-K exchange pump which is present in most animal cell membranes (cf. Baker *et al.* 1969), since the accumulation of K is ouabain-sensitive and is dependent upon intact metabolic pathways (Bradford, 1969; Escueta & Appel, 1969). These findings have been confirmed in our laboratory (M. P. Blaustein, unpublished data). In addition, in order to ascertain that the particles from brain homogenates which accumulate K are not simply contaminants of the synaptosome preparation, particles from all three layers of the sucrose density gradient (see Methods) have been tested for their ability to accumulate ^{42}K in the absence and presence of ouabain. The results indicate that the ouabain-sensitive fraction of the ^{42}K accumulation is associated primarily with the synaptosome fraction of the sucrose gradient (Table 2 and Fig. 3).

The synaptosome fraction from the sucrose gradient contains about 5 times as much K as Na (Table 3). When exposed to very hypotonic solutions, most of the Na and K (Marchbanks, 1967), but only a small fraction of the synaptosome Ca and Mg, is released from the particulate material (Table 3). These findings are consistent with the idea that the alkali metal ions are contained primarily in an osmotically sensitive compartment of the synaptosomes (Marchbanks, 1967), while a major fraction of the alkaline earth cations, especially Ca, may be stored within intracellular components such as mitochondria (cf. Tower, 1968) which are relatively insensitive to osmotic shock (Tedischi & Harris, 1955; Malamed & Rechnagel, 1959).

The data in Table 3 also indicate that the occluded water space (inaccessible to $^{35}SO_4$) is reduced by about 60 % when the synaptosomes are subjected to osmotic shock. From these data, the concentration of K in the osmotically sensitive compartment may be estimated by dividing the amount of K lost (243 μmole/g protein; Table 3, column 4, line 3) by the decrement in occluded water space (2·43 ml./g protein). This calculation suggests that the K concentration in the osmotically sensitive cytoplasmic compartment of synaptosomes may be as high as 100 mM.

The experimental results presented in Table 4 indicate that K uptake by synaptosomes requires an intact surface membrane, since the ^{42}K accumulation is markedly reduced, and the ouabain sensitivity is abolished following exposure to very hypotonic media. Further evidence that the

TABLE 2. Distribution in the sucrose gradient, of particles exhibiting ouabain-sensitive ^{42}K uptake

Sucrose gradient (and predominant particle morphology)	Incubation medium*	^{42}K accumulation		Protein in layer (mg)	Relative 'specific' distribution	
		Total	Ouabain-sensitive		Ouabain-sensitive ^{42}K Accumulation**	Synaptosomes‡
		(μmole K/g protein)				
0·32–0·8 M	Na + 5K	101·9 ± 5·4 (4)	46·1	43·02	0·84	0·38
Interface (membrane fragments)	Na + 5K + Ouabain	55·8 ± 2·3 (4)				
0·8–1·2 M	Na + 5K	127·4 ± 8·9 (3)	71·7	66·08	1·31	1·50
Interface (synaptosomes)	Na + 5K + Ouabain	55·7 ± 1·7 (3)				
Pellet (mitochondria)	Na + 5K	48·9 ± 3·2 (4)	29·6	28·62	0·54	0·57
	Na + 5K + Ouabain	19·3 ± 0·9 (4)				

* Samples were incubated for 10 min at 30° C in order to allow the synaptosome ^{42}K content to reach a steady level (Escueta & Appel, 1969; M. P. Blaustein, unpublished data). When used, the ouabain concentration was 1 mM.

† Mean ± S.E.; number of determinations is given in parentheses.

** Relative 'specific' distribution of ouabain-sensitive ^{42}K accumulation activity =

$$\frac{\text{Ouabain-sensitive } ^{42}K \text{ accumulation in layer}}{\text{Total ouabain-sensitive } ^{42}K \text{ accumulation}} \div \frac{\text{Protein in layer}}{\text{Total protein}}$$

‡ Calculated from Fig. 2 of Michaelson & Whittaker (1963) as:

$$\frac{\text{Fraction of recovered nitrogen associated with synaptosomes in layer}}{\text{Fraction of recovered nitrogen associated with synaptosomes in entire gradient}} \div \frac{\text{Protein in layer}}{\text{Total protein}}$$

K is accumulated in an osmotically sensitive compartment is provided by the observation (Table 4) that previously accumulated ^{42}K is lost much more rapidly from synaptosomes washed in hypotonic media ('post-lysed') than from synaptosomes washed in isotonic (Na + 5K) media ('post-washed').

Comparison of the steady-state values for ^{42}K accumulation from Na + 5K (114 ± 8 μmole K/g protein, mean ± S.E. of data from four experiments – cf. Tables 2 and 4) with the net K content determined by flame analysis of synaptosomes incubated under identical conditions (193 ± 8 μmole/g protein: six experiments) suggests that not all of the synaptosome K is exchangeable. However, the magnitude of the ouabain-sensitive or metabolically dependent (i.e. reduced with 2 mM-CN + 2 mM-IAA) K

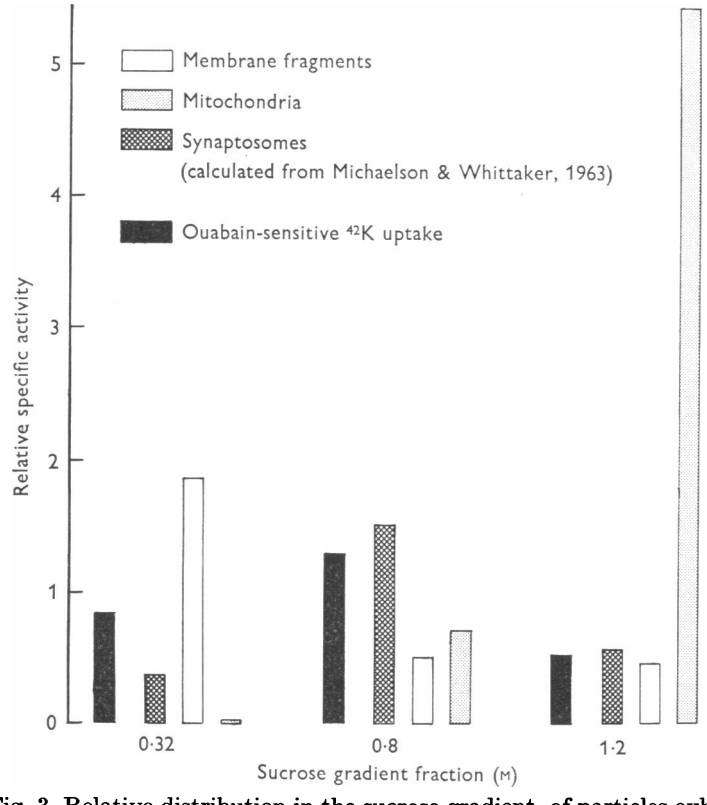

Fig. 3. Relative distribution in the sucrose gradient, of particles exhibiting ouabain-sensitive ^{42}K uptake (data from Table 2). Relative distributions of membrane fragments, synaptosomes and mitochondria were calculated (see Table 2) from Fig. 2 of Michaelson & Whittaker (1963). The 0·32 fraction refers to the 0·32–0·8 M sucrose interface, the 0·8 fraction, to the 0·8–1·2 M interface, and the 1·2 fraction, to the pellet (below the 1·2 M sucrose layer).

TABLE 3. Effect of osmotic shock on cation and water content of synaptosomes

	Protein (mg/pellet)	Na (μmole/g protein)†	K (μmole/g protein)†	Na/K	Ca (μmole/g protein)†	Mg (μmole/g protein)†	H_2O* (ml./g protein)†
Intact synaptosomes	1·45	61 ± 5	291 ± 4	0·210	10·9 ± 0·6	42·4 ± 1·5	4·05 ± 0·14
Lysed synaptosomes‡	1·16	14 ± 3	48 ± 3	0·288	9·7 ± 1·1	35·3 ± 3·7	1·62 ± 0·13
Lost in supernate due to lysis	0·29	47	243	0·193	1·2	7·1	2·43
Percentage lost into supernate	20 %	77 %	81 %		11 %	17 %	60 %

* 'Occluded water space' obtained with ^{35}S-labelled sulphate as extra-synaptosomal marker (see text).

† Values for lysed synaptosomes refer to protein content of intact synaptosomes. Each value is the mean of four determinations ± S.E.

‡ Synaptosome pellets (approx. 1·2 mg protein) were lysed in 1·95 ml. iced distilled water.

content determined by flame analysis (59 ± 7 μmole K/g protein; five experiments) is virtually identical to the value obtained from ^{42}K uptake experiments (60 ± 5 μmole K/g protein; four experiments). Thus the ouabain-sensitive and metabolically dependent fractions of the synaptosome K may be identical, and are likely associated with the activity of the Na pump (cf. Baker *et al.* 1969).

TABLE 4. ^{42}K accumulation by synaptosomes

Conditions*	Total†	^{42}K accumulation Ouabain-sensitive	% Decrease
	(μmoles K/g protein)		
Na + 5 K	115·4 ± 1·0	—	—
Na + 5 K + ouabain	43·7 ± 1·0	71·7	62
Na + 5 K, pre-lysed§	12·1 ± 1·0	—	—
Na + 5 K + ouabain, pre-lysed§	13·0 ± 0·2	(+0·9)	0
Na + 5 K, post-washed**	43·9 ± 0·6	—	—
Na + 5 K + ouabain, post-washed**	13·0 ± 1·1	30·9	70
Na + 5 K, post-lysed**	11·6 ± 0·3	—	—
Na + 5 K + ouabain, post-lysed**	4·7 ± 0·3	6·9	58

* Incubations with ^{42}K and $^{35}SO_4$ lasted 10 min, at 30° C.

† Mean of three determinations ± S.E.

§ These synaptosomes were equilibrated with Na + 5 K and centrifuged in the usual fashion. The pellets were then resuspended in 2 ml. Na + 5 K diluted 1:50 with H_2O, and immediately recentrifuged at 15,000 *g* for 5 min at 3° C. The lysed pellets were subsequently incubated in media containing $^{42}K + ^{35}SO_4$.

** After incubation (with $^{42}K + ^{35}SO_4$), centrifugation and rinsing (see Methods), these pellets were resuspended in 8 ml. iced, unlabelled 10 mM sulphate saline ('post-washed' samples) or 8 ml. of a 1:50 dilution of this saline with water ('post-lysed' samples). The suspensions were immediately centrifuged at 15,000 *g* for 5 min, at 3° C. The supernatant solutions were decanted and discarded, and the pellets were prepared in the usual fashion for counting.

These data may also provide information about the K concentration in the osmotically sensitive compartment (2·4 ml./g protein; see Table 3). Values of about 30 or 50 mM are obtained, depending, respectively, upon whether the ouabain-sensitive or the total exchangeable K values are taken for the calculation.

These calculated concentrations may be spuriously low estimates if the osmotically sensitive compartment also contains some non-exchangeable K or if a fraction of the terminals are very leaky to K but not to sulphate. Moreover, the fraction of the synaptosome volume presumed to be occupied by K (60 %; see Table 3) may be an over-estimate if K is excluded from the many small vesicles (synaptic vesicles) which occupy a significant fraction of the osmotically sensitive compartment (cf. Gray & Whittaker, 1962; Whittaker, Michaelson & Kirkland, 1964), and which may in many cases contain quaternary amine transmitters as their main cations.

Despite the uncertainties in the calculation of the intrasynaptosomal K concentration, the most straightforward explanation of these data is

that a significant fraction of the synaptosomes have re-sealed surface membranes and are able to accumulate K against a concentration gradient. Coupled with the observation that synaptosomes are about 19 times more permeable to K than to Na (Keen & White, 1971), the presence of a K concentration gradient suggests that pinched-off nerve terminals may have resting membrane potentials determined primarily by the K diffusion potential. In view of its efficacy as an indicator of membrane potential in the squid axon (see Methods), the fluorochrome, CC_5, was used as an indirect indicator of membrane potential changes in synaptosomes exposed to a variety of cations and drugs.

Fluorochrome dye studies: evidence that synaptosomes have membrane potentials

Effects of cations on synaptosome fluorescence. Original records of the photomultiplier output from a fluorescence experiment are illustrated in Fig. 4. In panel 4A1, it can be seen that, after the synaptosome fluorescence (in standard Na+5K) attained a steady level, addition of 50 μl. 1 M-NaCl (at *n*) had no effect on the fluorescence, while subsequent addition of an equal volume of 1 M-KCl (at *k*) significantly increased fluorescence. (It is worth reiterating here than an increased fluorescence of CC_5 is associated with depolarization in squid axons.) Similar additions of 1 M-KCl (at *k*) to suspensions containing mitochondria (from the sucrose gradient pellet) or fragmented membranes (from the 0·32–0·8 M sucrose gradient interface) induced only a small increase or no change in the fluorescence (Figs. 4*A*2 and 4*A*3, respectively). When synaptosomes were subjected to osmotic shock by suspension in distilled water (Fig. 4*A*4), the initial fluorescence was much greater than the fluorescence of synaptosomes suspended in Na+5K, and subsequent addition of 50 μl. 1 M-KCl did not affect the fluorescence.

Since addition of small volumes of concentrated salt solutions alters tonicity and ionic strength as well as ionic concentrations, this method was used only in preliminary experiments. Fig. 4*C* shows a series of records of synaptosome fluorescence in which the concentrations of Na ($[Na]_o$) and K($[K]_o$) in the medium were varied but the sum of $[Na]_o+[K]_o$ remained 137 mM. When synaptosome fluorescence is plotted as a function of log $[K]_o$, as in Fig. 5, an approximately linear relationship is observed at high $[K]_o$. The fact that complete replacement of $[Na]_o$ by choline (e.g. Figs. 7 and 9) does not increase synaptosome fluorescence indicates that the increased fluorescence in K-rich media (Figs. 4*C* and 5) is a consequence of increasing $[K]_o$ rather than reducing $[Na]_o$. It is unlikely that chloride played a significant role in this effect because a curve identical to that of Fig. 5 was obtained when nearly all of the external Cl was

replaced by the impermeant anion (Hutter & Noble, 1960), methylsulphate (see Fig. 4*B*).

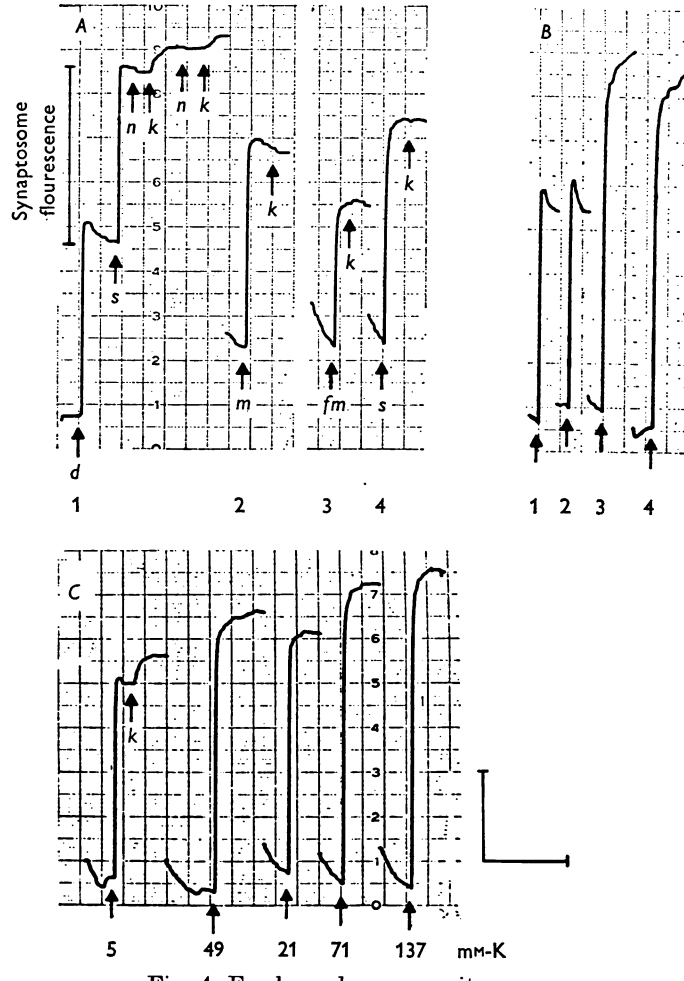

Fig. 4. For legend see opposite page.

The use of the impermeant anion may help to rule out the possibility that the fluorescence increase in K-rich media is a consequence of synaptosome swelling (due to net gain of salt and water). This problem deserves serious consideration because synaptosomes exposed to K-rich solutions containing a permeable anion are known to swell and to exhibit an increase in light-scattering (Keen & White, 1970, 1971; Kamino, Inouye & Inouye, 1973). However, it seems unlikely that light-scattering could account for the observations reported here. In the first place, use of interference filters with 10 nm half-widths permitted only a negligible fraction of the scattered incident light to pass the secondary filter. The magnitude of the changes in light intensity we observed were considerably greater than the changes in intensity reported in the aforementioned light-scattering studies. Furthermore, we were

unable to detect any changes in light output, as a consequence of change in $[K]_o$, when the fluorochrome was omitted from the medium. Finally, light-scattering at the emission wave-length could be eliminated as a source of the change in emission intensity, since the scattering should be identical in all directions.

Other alkali metal ions which are known to depolarize excitable cells (Shanes, 1958; Sjodin, 1959; Adrian & Slayman, 1966) also increase synaptosome fluorescence. As shown in Fig. 5, Rb, which is approximately

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Legend to Fig. 4

Fig. 4. Original records of fluorometer photomultiplier current output from three experiments.

A, the effect of increased $[KCl]_o$ on the fluorescence of synaptosomes (1), mitochondria (2), fragmented membranes (3) and lysed synaptosomes (4). In record *A*1, arrow *d* indicates the time of addition of 7·5 μgm of CC_5 to the fluorometer cuvette containing 3·0 ml. Na + 5 K. In all of the other records shown here and in subsequent Figures, the recorder was turned on after the addition of dye, so that this early portion of each record is omitted. Synaptosomes (and mitochondria and membrane fragments; see Methods) for these experiments were pre-incubated in Na + 5 K for 15–30 min at 30° C. At *s* in *A*1 and *A*4, a 100 μl. aliquot of the pre-incubated synaptosome suspension (0·58 mg protein/100 μl.) was added to the fluorometer cuvette. The cuvette contained 3·0 ml. distilled water + fluorochrome in *A*4. In *A*2, 100 μl. of mitochondrial (*m*) suspension (0·66 mg protein/100 μl.) and in *A*3, 50 μl. of fragmented membrane (*fm*) suspension (0·18 mg protein/50 μl.) was added to the cuvette containing 3·0 ml. Na + 5 K + fluorochrome. The increment in fluorescence due to the addition of synaptosomes, the 'synaptosome fluorescence' is labelled in record *A*1. In some instances, after the addition of the tissue suspension, as indicated by the arrows labelled *k* or *n*, the K or Na concentration of the medium was increased by addition of 50 μl. aliquots of 1 M-KCl or 1 M-NaCl, respectively. (Arrows labelled *k* in subsequent records refer to similar increments in the external KCl concentration).

B, effect on synaptosome fluorescence of replacing external chloride by methylsulphate. In record *B*1 (and in all subsequent records) the bottom (unlabelled) arrow indicates the time of addition of pre-incubated synaptosome suspension (0·44 mg protein/100 μl. in this experiment) to the cuvette. In *B*1 the cuvette contained 3·0 ml. Na + 5 K. The solution in *B*2 was similar to Na + 5 K, but with 137 mM chloride replaced by methylsulphate. The test solutions in *B*3 and *B*4 were, respectively, 137 mM-K (chloride) saline and 137 mM-K (methylsulphate) saline.

C, the effect of replacing external Na by K on synaptosome fluorescence. The cuvette contained 3·0 ml. Na + 5 K (left-hand record), or 137 mM-K saline (right-hand record), or mixtures of the two to give the K concentration shown below each record. At the time indicated by the unlabelled arrow in each record, 100 μl. of pre-incubated synaptosome suspension (0·55 mg protein/100 μl.) was added to the cuvette. At *k*, in the Na + 5 K record, 50 μl. 1 M-KCl was added to the cuvette. Calibrations for *A*, *B* and *C*: vertical bar = 20 μA; horizontal bar = 5 min. The temperature in the cuvette was 34° C.

as effective as K as a depolarizing agent, increases fluorescence to about the same extent as does K. About five times as much Cs as K is required to produce a given level of depolarization in frog skeletal muscle (cf. Sjodin, 1959); by comparison, as shown in Fig. 5, replacement of 132 mmoles/liter of Na by an equal amount of Cs gave the same increment in fluorescence as the replacement of only 25 m-mole/l. of Na by either K or Rb. Although not illustrated here, complete replacement of Na by Li, which does not depolarize excitable cells (Shanes, 1958), also had no influence on synaptosome fluorescence.

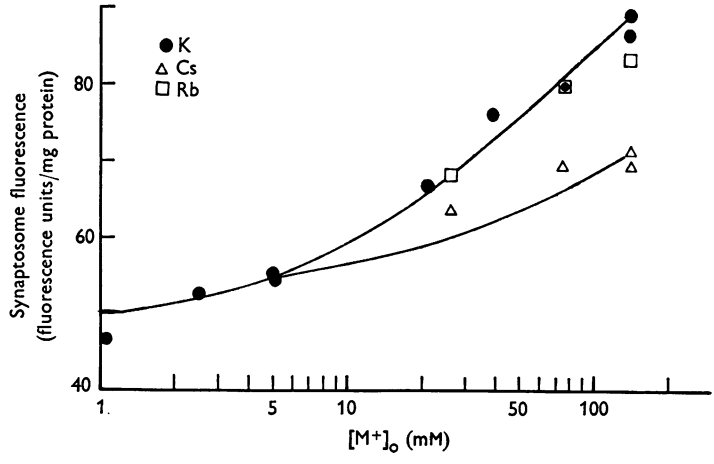


Fig. 5. Relationship between external cation concentration and synaptosome fluorescence. Synaptosomes were pre-incubated in Na + 5 K for 10–30 min at 30° C. Aliquots (100 μl., containing 0·65 mg protein) were then added to 3·0 ml. of test saline + dye in the fluorometer cuvette. The total alkali metal ion concentration in the test solution was always 137 mM. For the K curve (●), the sum of Na + K was 137 mM, and the K concentration is given on the abscissa; the fluorescence records from which the curve is plotted were comparable to those of Fig. 2*B*. For the Rb (□) and Cs (△) curves, 5 mM-K was present in all of the test solutions, and concentrations on the abscissa correspond to the sum of K + Rb or K + Cs, respectively. One 'fluorescence unit' is equal to an increment of 1 μA in the output of the fluorometer photomultiplier. The curve through the K and Rb points was calculated from the equation (see Discussion).

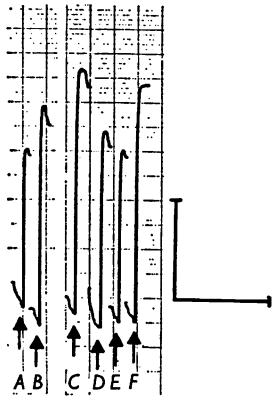
$$\text{Synaptosome fluorescence} = 31{\cdot}5 \log ([K]_o + [Rb]_o + 0{\cdot}05\,[Na]_o) + 22.$$

The lower curve was calculated from the equation:

$$\text{Synaptosome fluorescence} = 31{\cdot}5 \log ([K]_o + 0{\cdot}25\,[Cs]_o + 0{\cdot}05\,[Na]_o) + 22.$$

The implication from the foregoing experiments is that synaptosome fluorescence may be a function of a K diffusion potential (i.e. a function of $\log [K]_o/[K]_i$). If this is the case, agents which are known to decrease $[K]_i$ should also increase synaptosome fluorescence above control values.

Accordingly, Fig. 6 illustrates the observation that synaptosomes pre-incubated in the presence of CN + IAA, or ouabain, or in nominally K-free media, all exhibited increased fluorescence when compared to synaptosomes from the control Na + 5K medium. The small increment seen following K-free pre-incubation may indicate that a considerable amount of K leaked into the medium and was pumped back into the synaptosomes during the pre-incubation.



	Treatment	Fluorescence units per mg protein.
A	Control	64
B	Ouabain	83
C	IAA-CN	94
D	K-free	76
E	Control	70
F	137 K	97

Fig. 6. Effect of metabolic poisons, ouabain and K-free media on synaptosome fluorescence. Synaptosomes were pre-incubated for 10–20 min at 30° C in: Na + 5 K (for records *A*, *E* and *F*), Na + 5 K + 10^{-3} M ouabain (record *B*), Na + 5 K + 2 mM-NaCN + 2 mM-Na iodoacetate (record *C*) or K-free Na (for record *D*). Aliquots of 100 μl. were then transferred to the fluorometer cuvette containing 3·0 ml. Na + 5 K (137 mM-K saline, in the case of record *F*) + 7·5 μg of CC_5, to obtain the fluorescence records shown in the Figure. The column at the right indicates the increment in photomultiplier current output (1 fluorescence unit equals 1 μA) per mg protein for each of the records shown at the left. Calibrations: vertical bar = 20 μA; horizontal bar = 5 min.

It seemed possible that the fluorescence changes observed in these experiments could have been the result of a change in membrane surface area or the consequence of irreversible changes in the synaptosomes caused either by the K-rich media, or by the fluorochrome itself. Several experiments were made in an effort to eliminate these possibilities. For example, if K-rich media stimulate Ca uptake and Ca-dependent transmitter release (Blaustein, 1975) by an exocytic process (e.g. Heuser & Reese, 1973), the increased synaptosome fluorescence might simply be the result of the increase in neurilemma surface area (cf. Simms *et al.* 1974). However, omission of Ca from the bathing medium, a manoeuvre which greatly inhibits synaptosome transmitter release (Blaustein, 1975), had no effect on the K-evoked increase in synaptosome fluorescence.

The effects of exposure to K-rich media were apparently quite reversible. For example, synaptosomes were exposed to a 70 mM-K (+ 67 mM-Na) medium for 1–2 min, and then returned to a 7 mM-K environment (by

diluting the medium with Na + 5K). The fluorescence increment of these pre-treated terminals, in response to a subsequent increase in $[K]_o$, was indistinguishable from the response of synaptosomes maintained in Na + 5K throughout the pre-treatment period.

The fluorochrome also apparently had no significant deleterious effect on some other physiological properties of the terminals. In one experiment, the effect of CC_5 on net K content was determined. Synaptosomes were incubated in Na + 5 K or in K-free Na + 10^{-3} M ouabain for 10 min at 30° C. In the absence of dye, the K content was 194 ± 3 μmole/g protein in the absence of ouabain, and 56 ± 4 μmole/g protein with ouabain present; with CC_5 added to the incubation medium to give a final concentration of 12 μg/g synaptosome protein, the respective K values (μmole/g protein), without and with ouabain, were 173 ± 7 and 61 ± 6 (each value is the mean of three determinations ± S.E.). In another type of experiment, the K-stimulated ^{45}Ca uptake (cf. Blaustein, 1975) also was not significantly affected by CC_5.

Effects of gramicidin D and veratridine on synaptosome fluorescence. The application of agents known to depolarize cells by altering the permeability of membranes to ions should provide a test for the hypothesis that the synaptosome membrane is normally relatively K-permeable. Gramicidin is an ionophore which is known to make biological membranes (e.g. electroplax; Podleski & Changeaux, 1969), including synaptosome membranes (Keen & White, 1971), indiscriminately permeable to Na and K. Fig. 7*A*1 shows that the addition of 10 μl. of a 3 mg/ml. ethanol solution of gramicidin D (ethanol, alone, had no effect) markedly increased the fluorescence of synaptosomes suspended in Na + 5K. When choline replaced Na in the medium, gramicidin D caused little change or a slight decrease in fluorescence (Fig. 7*A*2).

These are the results to be expected if the action of gramicidin D is due primarily to its increase of Na permeability (cf. Podleski & Changeaux, 1969). In the presence of a large inwardly directed Na concentration gradient and an outwardly directed K concentration gradient, if both P_{Na} and P_K (the Na and K permeabilities, respectively) are relatively large, the membrane potential should lie between the Na and K equilibrium potentials. In the absence of external Na, increasing P_{Na} should cause little change in membrane potential, or even slight hyperpolarization (see Fig. 7*A*2), because $[Na]_i > [Na]_o$.

As shown in Fig. 8, gramicidin D increased the fluorescence of synaptosomes in the standard medium to about the value observed in 137 mM-K saline; however, gramicidin D had little effect on the fluorescence of synaptosomes in 137 mM-K saline. Podleski & Changeaux (1969) found that gramicidin A depolarized electroplax to about 0 mV. If their observations are applicable to the present study, the data in Fig. 8 may indicate that synaptosome fluorescence in 137 mM-K saline is approximately equivalent to a synaptosome membrane potential of 0 mV.

When synaptosomes are depleted of K and loaded with Na, by pre-treatment with CN + IAA, synaptosome fluorescence should be increased due to the dissipation of the K gradient (see Figs. 6 and 7 *B*2). Furthermore, the increment in fluorescent emission (if this reflects a membrane potential change) due to gramicidin D, should be reduced or abolished,

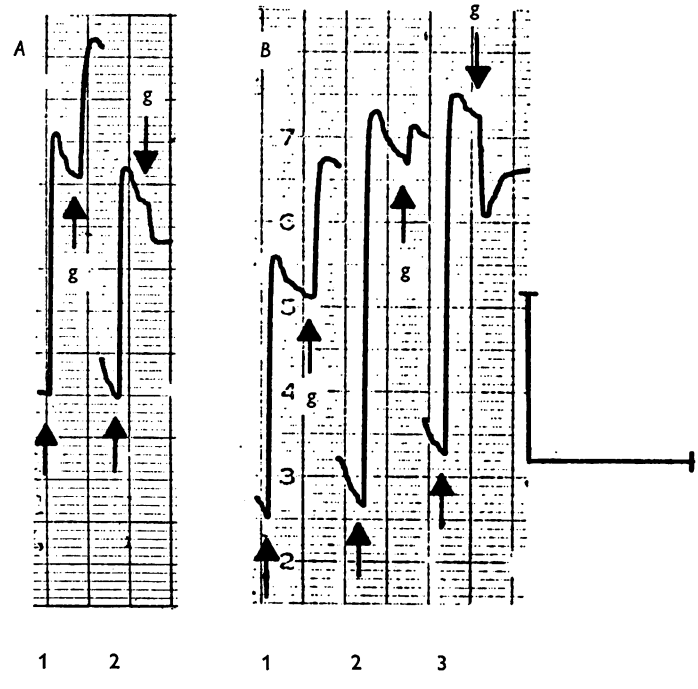


Fig. 7. The effect of gramicidin D on synaptosome fluorescence. Synaptosomes were preincubated in Na + 5 K (records *A*1, *A*2 and *B*1) or Na + 5 K + 2 mM-Na CN + 2 mM-IAA (records *B*2 and *B*3) for 15–30 min at 30° C. 100 μl. aliquots were then transferred to the fluorometer cuvette containing 7·5 μg CC_5 in 3·0 ml. Na + 5 K (records *A*1, *B*1 and *B*2) or choline + 5K (records *A*2 and *B*3). Gramicidin D (10 μl. of a 3 mg/ml. solution in ethanol) was added to each suspension at arrow g. The protein concentrations in the pre-incubation suspensions were: 0·46 mg/100 μl. (records *A*1 and *A*2), 0·50 mg/100 μl. (record *B*1), and 0·47 mg/100 μl. (records *B*2 and *B*3). *A* and *B* are from different experiments. Calibrations: vertical bar = 20 μA; horizontal bar = 5 min.

since the $[Na]_o/[Na]_i$ gradient is decreased and changes in P_{Na} should have little effect (Fig. 7 *B*2). However, if previously poisoned synaptosomes are suspended in Na-free (choline) media, an outwardly directed Na concentration gradient (and no K gradient) will be present. In this case, increasing P_{Na} with gramicidin D should cause a significant hyperpolarization

(cf. Podleski & Changeaux, 1969), which may account for the large decrease of fluorescence seen in Fig. 7*B*3.

Veratridine is also known to depolarize excitable cells (Straub, 1956; Ulbricht, 1969), but by a different mechanism: it increases conductance by apparently holding Na channels (of cells with an Na-action potential) in an open configuration and by blocking inactivation of the Na conductance (Ohta, Narahashi & Keeler, 1973). When synaptosomes are suspended in Na+5K containing 75 μM veratridine, synaptosome fluorescence is significantly increased, relative to control values; this increase is

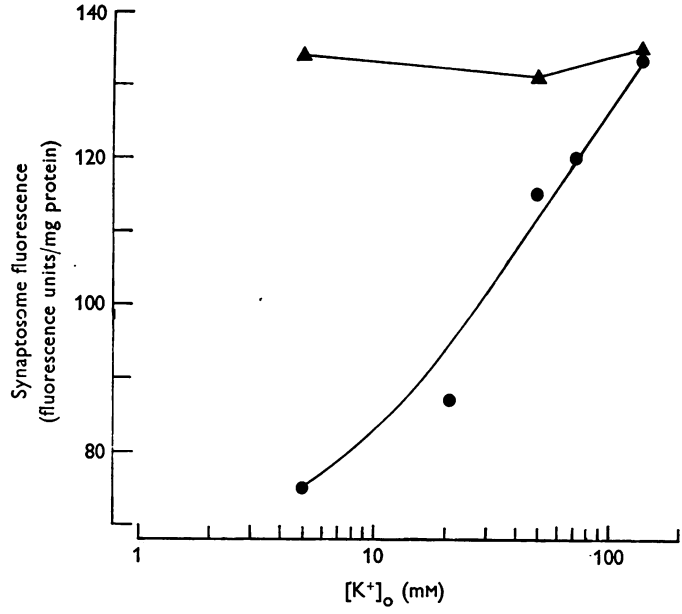


Fig. 8. Effect of gramicidin D on the relationship between synaptosome fluorescence and log $[K]_o$. Synaptosomes were pre-incubated in Na+5 K for 15–30 min at 30° C. Aliquots of 100 μl. (0·52 mg protein/100 μl.) were then transferred to the fluorometer cuvette containing 7·5 μg CC_5 in 3·0 ml. test saline. The test solutions were Na+5 K, 137 mM-K saline, or mixtures of the two, as indicated by the K concentration on the abscissa. In some instances (▲), 30 μg gramicidin D was also present in the cuvette. The lower curve was calculated from the equation:

$$\text{Synaptosome fluorescence} = 55 \log ([K]_o + 0{\cdot}05\,[Na]_o) + 17$$

The upper curve was drawn to connect the experimental points. ●, Control.

greatly reduced if 3×10^{-7} M tetrodotoxin is also present in the medium (Fig. 9*A*). Furthermore, the increment in synaptosome fluorescence due to veratridine is reduced as $[Na]_o$ is progressively replaced by K or choline; little or no increment is observed in Na-free media (137 mM-K, Fig. 10, or choline+5K, Fig. 9*B*). The effect of veratridine on the synaptosome

fluorescence *vs.* log $[K]_o$ curve (Fig. 10) could be accounted for if veratridine increased the P_{Na}/P_K ratio about fivefold (see Discussion). These observations on the veratridine-induced increase in synaptosome fluorescence parallel the effects of veratridine on the membrane potential of excitable cells in which the veratridine-induced depolarization is blocked by tetrodotoxin and does not occur in the absence of external Na (Ohta *et al.* 1973).

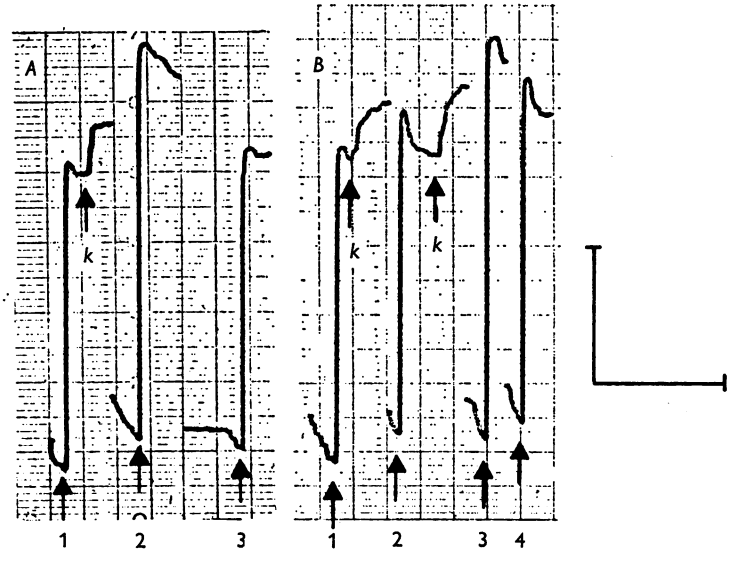


Fig. 9 *A*, effect of veratridine and tetrodotoxin on synaptosome fluorescence. Synaptosomes were pre-incubated in Na + 5 K for 15 min at 30° C. Aliquots of 100 μl. (0·58 mg protein/100 μl.) were then transferred to the fluorometer cuvette containing 7·5 μg of CC_5 in 3·0 ml. Na + 5 K (record 1) or Na + 5 K + $7{\cdot}5 \times 10^{-5}$ M veratridine without (record 2) or with (record 3) 3×10^{-7} M tetrodotoxin. In record 1, 50 μl. 1 M-KCl solution was added to the cuvette at *k*.

B, effect of choline on veratridine-induced fluorescence increase. Synaptosomes in all records were pre-incubated in Na + 5 K at 30° C for 15–30 min. 100 μl. aliquots (0·63 mg protein/100 μl.) were transferred to the fluorometer cuvette containing 3·0 ml. Na + 5 K (records 1 and 3) or choline + 5 K (records 2 and 4). In records 3 and 4, $7{\cdot}5 \times 10^{-5}$ M veratridine was present in the medium. At *k* in records 1 and 2, 50 μl. 1 M-KCl solution was added to the cuvette. Calibrations: vertical bar = 20 μA; horizontal bar = 5 min.

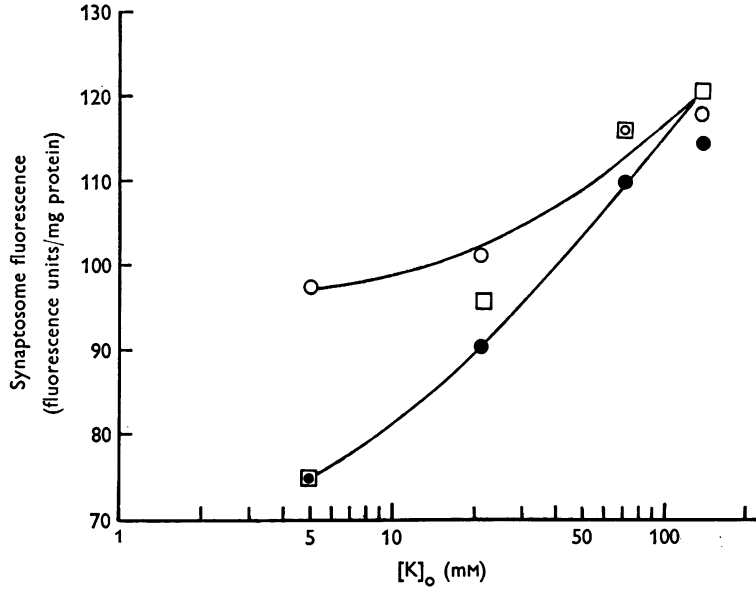


Fig. 10. Effect of veratridine and tetrodotoxin on the relationship between $[K]_o$ and synaptosome fluorescence. Data are from the same experiment as that of Fig. 9*A*. The procedure was identical to that given in the caption to Fig. 9*A*, except that the test solutions in the cuvette also included mixtures of Na + 5 K and 137 mM-K saline: the K concentrations are shown on the abscissa. The concentration of veratridine in the test solutions (○, ⊡) was $7{\cdot}5 \times 10^{-5}$ M; the tetrodotoxin concentration used (□) was 3×10^{-7} M. The controls (●) contained neither veratridine nor tetrodotoxin. The curves were calculated from eqn. (2*b*) (see Discussion):

$$\text{Synaptosome fluorescence} = 43 \log ([K]_o + \alpha[Na]_o) + 29$$

where α has a value of 0·05 for the lower curve, and 0·25 for the upper curve.

DISCUSSION

The synaptosome preparation was first introduced as a means of studying the chemical and morphological characteristics of vertebrate presynaptic nerve endings (DeRobertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz & Salganicoff, 1962; Gray & Whittaker, 1962). However, a number of recent studies have provided evidence that this preparation also retains many functional properties usually associated with intact cells (see the review by Rodriguez de Lores Arnaiz & DeRobertis, 1972). These observations suggest that during the preparative homogenization process, the pinched-off presynaptic terminals reseal and retain mitochondria, synaptic vesicles, soluble cytoplasmic enzymes, and even small inorganic ions. The data reported above provide additional evidence of

functional integrity as indicated by the ability of synaptosomes to accumulate and retain K against a concentration gradient. Taken together with the evidence that synaptosomes are much more permeable to K than to Na (Keen & White, 1971), these observations indicate that synaptosomes may have resting membrane potentials which approximate the K diffusion potential, as is the case in most resting nerve cells. When the voltage-sensitive fluorochrome, CC_5, was added to synaptosome suspensions, the fluorescent intensity changes which resulted from the addition of depolarizing agents and/or changes in ion concentrations were consistent with this hypothesis.

As noted in the Methods section (see Fig. 2), there is evidence that, at least for squid axons, the fluorescence of CC_5 is directly proportional to membrane potential (V_m). It may therefore be of interest to consider some of the synaptosome fluorescence data in terms of the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$V_M = 2{\cdot}3\frac{RT}{F}\log\frac{P_K[K]_o + P_{Na}[Na]_o}{P_K[K]_i + P_{Na}[Na]_i}, \tag{1}$$

or, with $\alpha = P_{Na}/P_K$:

$$V_M = 2{\cdot}3\frac{RT}{F}\log\frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i}. \tag{1a}$$

Assuming that synaptosome fluorescence is proportional to V_M, the analogous expression for fluorescence is:

$$\text{Synaptosome fluorescence} = A\log\frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i} + B, \tag{2}$$

where A is a scaling factor which may, in part, be a function of the fluorochrome concentration (at constant protein concentration) and, in part, a function of the number of 'intact' synaptosomes per mg total protein in the suspension. B is the synaptosome fluorescence when the log term is zero.

According to eqn (2), with little change in the normal $P_{Na}:P_K$ ratio (about 0·05:1), a significant decrease of $[K]_i$ should increase synaptosome fluorescence – as was observed after treatment with CN + IAA, ouabain, or K-free media (Fig. 6).

For conditions in which the internal cation concentrations remain constant, eqn. (2) reduces to:

$$\text{Synaptosome fluorescence} = A\log([K]_o + \alpha[Na]_o) + B', \tag{2a}$$

where $$B' = B - A\log([K]_i + \alpha[Na]_i).$$

The chloride terms have been omitted from the foregoing equations and will be disregarded in the subsequent discussion. The main reason for employing this simplification is that in our experience (see Results), complete replacement of Cl by the presumably impermeant methylsulfate ion did not significantly alter the relationship between fluorescence and log $[K]_o$ (cf. Fig. 4). This may indicate that the $P_K:P_{Cl}$ permeability ratio for the synaptosomes in these experiments was somewhat higher than that calculated by Keen & White (1971), 1:0·6.

The curves for synaptosome fluorescence versus log $[K]_o$ in Figs. 5, 8 and 10 have been calculated from eqn. (2*a*). In these and four other experiments, the equation provided a good fit when α varied between 0·03 and 0·05, which suggests

that $P_K \simeq 20\, P_{Na}$. This value is very close to the result obtained by Keen & White (1971) with light-scattering methods: $P_K = 19\, P_{Na}$.

Equations analogous to (2*a*) can be used to determine the relative permeabilities of other cations (cf. Hille, 1972):

$$\text{Synaptosome fluorescence} = A \log ([K]_o + \alpha[Na]_o + \alpha'[M^+]_o) + B' \qquad (2b)$$

where M^+ is any other cation, and α' is the permeability ratio, $P_M : P_K$. This expression has been used to evaluate the relative permeabilities of Rb and Cs. The results (Fig. 5) suggest that $P_K \simeq P_{Rb} \simeq 4P_{Cs}$ for synaptosomes, values which are reasonably close to those of other excitable membranes (cf. Shanes, 1958; Sjodin, 1959; Adrian & Slayman, 1966).

The effects of gramicidin D and veratridine may also be evaluated in terms of eqn. (2*a*). Both of these agents should increase α, and this may explain why they greatly increase synaptosome fluorescence when added to Na + 5 K, but not to Na-free media.

Eqn. (2) may be useful in helping to explain the effects of altered $[Na]_o$ and $[Na]_i$ on the response to gramicidin D. As already noted, with the normal cation gradient present ($[K]_o \ll [K]_i$ and $[Na]_o \gg [Na]_i$), a large increase in α should cause a depolarization and a concomitant increase in synaptosome fluorescence (Fig. 7*A* 1 and 7*B* 1); but a small hyperpolarization should result if Na_o is replaced by the relatively impermeant cation, choline (Fig. 7*A* 2). However, when the cation gradients are reduced ($[K]_o \leqslant [K]_i$ and $[Na]_o \geqslant [Na]_i$), as a consequence of cyanide-IAA poisoning, the increment in synaptosome fluorescence, due to the gramicidin-induced increase in α should be reduced (Fig. 7*B* 2) or abolished (depending upon how close the cation concentration ratios, $[K]_o/[K]_i$ and $[Na]_o/[Na]_i$, are to unity). If, under these circumstances ($[Na]_i \gg [K]_i$), external Na is replaced by choline, an increase in α should result in a *decrease* of fluorescence (Fig. 7*A* 3).

Although the foregoing considerations do not provide absolute values for the synaptosome membrane potentials, sufficient data are available to permit certain approximations. If it is assumed that the cation concentration ratios, $[K]_o/[K]_i$ and $[Na]_o/[Na]_i$ fall approximately to a value of 1 when synaptosomes are pre-incubated with cyanide + iodoacetate or with ouabain, the fluorescence of these synaptosomes should correspond to a membrane potential of about 0 mV. In the four experiments for which these data are available (e.g., see the CN + IAA record of Fig. 6), the synaptosome fluorescence of CN + IAA-poisoned synaptosomes corresponds to $[K]_o$'s (on the fluorescence *vs.* log $[K]_o$ curves of unpoisoned synaptosomes from the same experiments) of between 100 and 150 mM. Somewhat lower values of synaptosome fluorescence, and therefore, of the extrapolated [K]'s (between 50 and 70 mM, in three experiments) were obtained with ouabain alone (e.g. see Fig. 6*B*). One possible explanation is that the increase in $[K]_o$ in the incubation medium (due to leak from the synaptosomes) may have partially counteracted the action of ouabain. In one experiment, in which synaptosomes were pre-incubated with ouabain in K-free Na, the synaptosome fluorescence of these synaptosomes corresponded to a $[K]_o$ of 100 mM on the fluorescence *versus* log $[K]_o$ curve for unpoisoned synaptosomes.

Additional support for the validity of this approximation to 0 mV comes from the experiments of Podleski & Changeaux (1969), who observed that gramicidin A (which apparently acts in a manner similar to gramicidin D) depolarizes electroplax to approximately 0 mV. As seen in Fig. 8, gramicidin D increases synaptosome fluorescence to about the value obtained when $[K]_o$ is 130–140 mM, perhaps indicating that this value of synaptosome fluorescence corresponds to a membrane potential of 0 mV.

Assuming that in K-rich media both Na terms ($P_{Na}[Na]_o$ and $P_{Na}[Na]_i$) in equation 1 are sufficiently small and can be ignored, the $[K]_i$ of the unpoisoned synaptosomes in the aforementioned experiments should also be about 100–150 mM. As noted earlier (p. 596), net K concentration data also indicate that $[K]_i$ may be of the order of 100 mM. These $[K]_i$ values may then be inserted into eqn. (1) to determine the synaptosome 'resting potential'. By ignoring the small $P_{Na}[Na]_i$ term, and by using a value of 0·05 for the P_{Na}/P_K ratio (cf. Figs. 5, 8 and 10, and Keen & White, 1971) and a value of 25 mV for RT/F, potentials of about -55 to -60 mV are obtained for synaptosomes in Na + 5 K. These calculated potentials are surprisingly close to the resting potentials of mammalian central neurons measured with microelectrodes (e.g. Phillips, 1956; Li, 1959).

The data described in the present communication indicate that synaptosomes may be a very useful preparation for studying some of the physiological and pharmacological properties of vertebrate presynaptic nerve endings – properties which cannot presently be examined in more standard preparations by currently available techniques. One example of this utility stems from the observation that veratridine (which specifically affects Na conductance inactivation) increases synaptosome fluorescence (i.e. presumably depolarizes synaptosomes) and that this effect is blocked by tetrodotoxin. This suggests that the nerve terminal membrane contains Na channels and that action potentials conducted down nerve fibres may propagate directly into the terminals (cf. Katz & Miledi, 1965), rather than depolarizing the terminals by electrotonic spread from adjacent membrane. Further examples of how the synaptosome preparation may be used to obtain information about the physiology of presynaptic terminals will be described in the subsequent articles (Blaustein, 1975; Blaustein & Oborn, 1975).

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