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# ON THE MECHANISM BY WHICH VERATRIDINE CAUSES A CALCIUM-INDEPENDENT RELEASE OF γ-AMINOBUTYRIC ACID FROM BRAIN SLICES

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**1** The mechanisms by which veratridine increases the release of γ-aminobutyric acid (GABA) from brain slices have been studied.

**2** Exposure of superfused cerebro-cortical, nigral or cerebellar slices to veratridine (5 μM) or KCl (50 mM) caused large increases in the efflux of [$^3$H]-GABA.

**3** Reduction of the external Ca concentration $[Ca]_o$ to zero had strikingly different effects on the veratridine and K-evoked release of [$^3$H]-GABA. The K-evoked release from all three areas was greatly reduced in Ca-free medium, but the veratridine-evoked release from cerebeller slices was not affected, and the release of [$^3$H]-GABA from cortical and nigral slices was increased three fold. The potentiation of the veratridine evoked release of GABA which occurred in Ca-free medium was not due to the reduction in divalent ions, because it still occurred in medium in which the Ca was replaced by an equivalent amount of Mg.

**4** The veratridine-evoked release of [$^{14}$C]-glycine from slices of spinal cord was also significantly increased in Ca-free medium. In contrast, the release of cortical [$^3$H]-noradrenaline and [$^{14}$C]-acetylcholine caused by the alkaloid was greatly diminished in Ca-free medium.

**5** The veratridine but not the K-evoked release of [$^3$H]-GABA was abolished when the external Na concentration $[Na]_o$ was reduced to zero and by tetrodotoxin (TTX) (0.2 μM). Cl-free medium did not affect the veratridine-evoked release of [$^3$H]-GABA or its potentiation by Ca-free medium.

**6** Exposure of the tissue to depolarizing concentrations of external K ($[K]_o$ = 120 mM) did not abolish the veratridine evoked release of [$^3$H]-GABA or its potentiation by Ca-free medium.

**7** Pre-incubation of cortical slices with L-2,4, diaminobutyric acid (DABA), or substitution of Na in the superfusion medium with Li, did not affect the veratridine-evoked release of [$^3$H]-GABA, indicating that the alkaloid does not stimulate GABA efflux by a carrier-mediated transport process.

**8** Exposure of the tissue to ruthenium red (10 μM) increased the veratridine evoked release of [$^3$H]-GABA in both normal and in Ca-free medium but almost abolished the K-evoked release.

**9** It is suggested that veratridine causes GABA release by increasing the permeability of the nerve terminals to Na. In normal medium, the resulting influx of $Ca^{2+}$ ions through voltage-dependent $Ca^{2+}$ channels may be involved in triggering the release of GABA. However, a major part of the GABA efflux appears to be triggered by the release of $Ca^{2+}$ ions from intraterminal mitochondria, which results from the increase in$[Na]_i$. Since $Ca^{2+}$ ions antagonize the action of veratridine, the potentiation of the drug-evoked release of GABA that occurs in Ca-free medium, might be due to the absence of the antagonistic $Ca^{2+}$ ions. The resulting greater increase in Na entry and $[Ca]_i$ caused by Ca release from intracellular stores, must presumably more than balance the contribution normally made by any influx of extracellular $Ca^{2+}$.

## Introduction

The alkaloid, veratridine, depolarizes excitable membranes by causing a selective increase in resting sodium permeability (for reviews, see Ulbricht, 1969; Narahashi, 1974). The mechanism by which veratridine produces this effect is unknown. However, because of its potent depolarizing action, veratridine has been used in the study of transmitter release (Blaustein, Johnson & Needleman, 1972; Blaustein, 1975; Redburn, Shelton & Cotman, 1976; Abita, Chicheportiche, Schweitz & Lazdunski, 1977) for which purpose, it may have certain advantages over potassium. For example, it might be considered

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that depolarization by an influx of Na ions is more physiological than by simply reducing the K diffusion potential. More importantly, veratridine may release γ-aminobutyric acid (GABA) selectively from neurones and be relatively ineffective in causing glial release (Minchin, 1975; Neal & Bowery, 1979). However, a complication in the case of veratridine is that the role of external Ca in transmitter release induced by the alkaloid is sometimes uncertain.

The release of noradrenaline from synaptosomes was found to be increased by veratridine and was associated with an increased influx of Ca (Blaustein *et al.*, 1972). The release of acetylcholine (ACh) from brain slices (Grewaal & Quastel, 1973) and of dopamine from synaptosomes (Patrick & Barchas, 1976) were both found to be Ca-dependent. However, Benjamin & Quastel (1972) noted that increases in the release of GABA, glutamate, aspartate and glycine from brain slices exposed to protoveratrine were enhanced in Ca-deficient medium. More recently, it has been found that the veratridine-evoked release of radiolabelled GABA from cortical slices was increased almost three fold in Ca-free medium (Neal, 1979) and similar effects of Ca-free medium on the alkaloid-evoked release of GABA from synaptosomes (Levi, Banay-Schwartz & Raiteri, 1978) and brain slices have been described (Szerb, 1979).

Since a potentiation of evoked transmitter release by calcium-free medium is in striking disagreement with the current calcium hypothesis of transmitter release, it seemed important to obtain further information on the mechanisms involved in the veratridine-evoked release of GABA. With this in mind, we have studied the mechanisms of the release process involved in the veratridine-evoked efflux of [$^3$H]-GABA from brain slices. For comparison, we have also examined the K-evoked release of [$^3$H]-GABA and the veratridine-evoked release of labelled noradrenaline and ACh. Some of these results have been published in a preliminary form (Neal, 1979; Cunningham & Neal, 1980).

## Methods

### *Incubation and superfusion procedures*

Male Wistar rats (weight 150–200 g) were killed by a blow on the head and the brains rapidly removed. Slices of cerebral cortex (approximately $3 \times 2 \times 0.3$ mm) were prepared with a mechanical tissue chopper. The slices were incubated at 25°C in Krebs bicarbonate Ringer containing [$^3$H]-GABA ($5 \times 10^{-8}$ M) for 30 min. The slices were then washed in fresh medium for 2 min and three or four slices were transferred to a small superfusion chamber (volume, 1 ml). The tissue was superfused at a rate of 1.2 ml/min and 2 min fractions were collected. The radioactivity in each fraction was determined by liquid scintillation counting after the addition of 10 ml phosphor (Aquasol, New England Nuclear). The counts were corrected for efficiency by the channels ratio method, and efflux curves were constructed by plotting the fractional rate coefficient against time (Bowery, Jones & Neal, 1976). The use of fractional rate coefficients in these experiments was for operational convenience only and is not intended to imply that GABA is necessarily released from a single pool of labelled transmitter.

The incubation and superfusion medium usually contained amino-oxyacetic acid (AOAA) (0.1 mM) to prevent metabolism of GABA by γ-aminobutyrate aminotransferase (GABA-T). Under these conditions more than 95% of the released radioactivity was [$^3$H]-GABA, determined as described previously (Neal & Starr, 1973), and the amount of [$^3$H]-GABA remaining in the tissue after 40 min superfusion was 94–98% of that taken up. In some experiments, cortical slices were loaded with [$^3$H]-noradrenaline or [$^3$H]-choline instead of GABA. When slices were incubated with [$^3$H]-noradrenaline (0.6 μM), its breakdown was minimized by addition to the medium of disodium edetate (EDTA, 0.13 mM) and ascorbic acid (1.1 mM). In the experiments with [$^3$H]-choline, no inhibitor of acetylcholinesterase was used and it was assumed that as in similar experiments, the evoked release of radioactivity was due mainly to an increased release of [$^3$H]-ACh (Massey & Neal, 1979).

### *Solutions*

The 'normal' incubation and superfusion medium was Krebs bicarbonate Ringer solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.52, $MgSO_4$ 1.18, $KH_2PO_4$ 1.18, $NaHCO_3$ 25 and D-glucose 11. This solution was constantly gassed with a mixture of $CO_2$ (5%) in $O_2$ and had a pH of 7.4.

Modified solutions were prepared as follows:

*High K solutions:* KCL (25 mM, 50 mM or 120 mM) was added to the normal or modified media. This gave solutions containing a total K concentration of 30.9, 55.9 and 125.9 mM respectively.

*Cl-free solution:* NaCl was replaced with sodium isethionate and $CaCl_2$ was replaced with $(CH_3COO)_2Ca$.

*Na-free (Sucrose) solution:* NaCl and $NaHCO_3$ were replaced by Tris (tris-hydroxymethyl-aminomethane, 50 mM titrated to pH 7.4 with HCl) and sucrose (234 mM). This solution was gassed with $O_2$.

*Na-free (Li) solution:* NaCl and $NaHCO_3$ were replaced by Tris (50 mM) and LiCl (118 mM).

*Ca-free solutions:* $CaCl_2$ was omitted with or without replacement by $MgCl_2$ (2.52 mM).

*Materials*

Veratridine was obtained from Aldrich Chemicals. γ-[1-$^{14}$C]-aminobutyric acid (sp.act. = 50mCi/mmol), γ-[2,3-$^3$H(N)]-aminobutyric acid (sp.act. = 40 Ci/mmol) and (−)-[7,8-$^3$H(N)] noradrenaline (sp.act. = 40 Ci/mmol) were obtained from New England Nuclear chemicals, Gmbh. [$^3$H-methyl] choline (sp.act. = 8.4 Ci/mmol) and *cis*-3-amino[4,5(*n*)-$^3$H]cyclohexane carboxylic acid (sp.act. = 48 Ci/mmol) were obtained from the Radiochemical Centre, Amersham.

## Results

*Effect of* $[Ca]_o$ *on veratridine and K-evoked GABA release*

The influence of calcium ions on the effect of veratridine (5 μM for 4 min) and KCl (50 mM) on the release of [$^3$H]-GABA from cerebro-cortical slices is illustrated in Figure 1. In these experiments, two superfusions were performed simultaneously. In one, responses to veratridine and KCl were obtained in normal medium and then repeated in Ca-free medium (Figure 1a). In the other, responses were obtained first in Ca-free medium and then in normal medium (Figure 1b). Preliminary control experiments indicated that although reproducible evoked releases of [$^3$H]-GABA were produced by 50 mM KCl, a second exposure of the tissue to veratridine

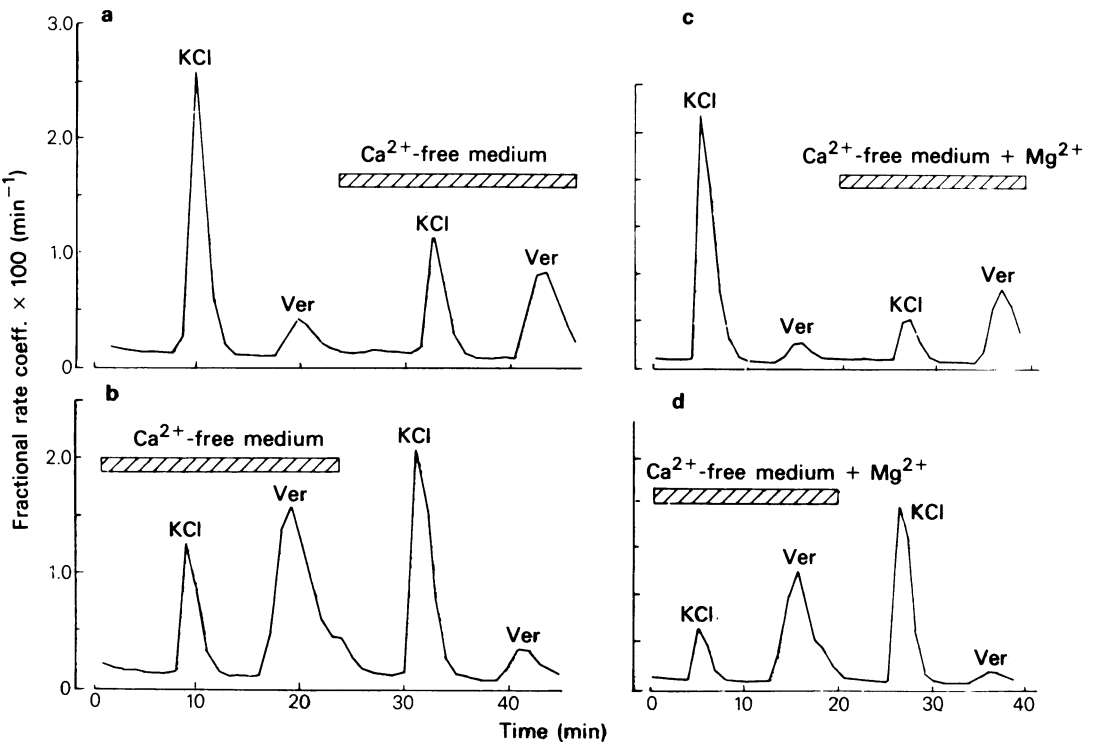


**Figure 1** Typical experiment showing [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA) release from superfused cortical slices. (a) Slices were exposed for 4 min to KCl (50 mM) and veratridine (5 μM) (Ver) in normal medium and then in Ca-free medium. (b) Experiment in which the drugs were applied first in calcium-free medium and then in normal medium. (c) and (d) As for (a) and (b) except that $MgCl_2$ (2.52 mM) was added to the Ca-free medium to keep the concentration of divalent ions constant. Each experiment was repeated at least 4 times with the same result. Slices for experiments shown in (a) and (b) were obtained from the same rat. Slices for experiments shown in (c) and (d) were from another rat.

usually resulted in a smaller response than to the first. For this reason, in the results summarized in Figure 2, the responses obtained in Ca-free medium were always obtained after the responses in normal medium. Thus, the results in Ca-free medium somewhat underestimate the potentiation caused by Ca-free conditions. However, the results show a striking difference in the calcium-dependence of the veratridine and K-evoked release of GABA. Thus, in Ca-free medium, the veratridine-induced release of GABA from cortical slices was almost three times greater than in control experiments. In contrast, the K-evoked release of GABA was reduced by approximately 50% in Ca-free medium.

The increase in the veratridine evoked release of [$^3$H]-GABA, which occurred in Ca-free medium, was not due to the reduction of divalent ions in the medium, because it also occurred in medium in which the Ca was replaced by equimolar Mg (Figure 1b,c).

The possibility that inhibition of GABA-T by the amino-oxyacetic acid present in the medium was resulting in a veratridine evoked release of [$^3$H]-GABA from pools not normally involved in the transmitter release process was tested in two ways: (1) The effect of veratridine and K on the release of [$^{14}$C]-GABA from cortical slices untreated with AOAA was studied and (2) the effect of drugs on the release of (±)-[$^3$H]-*cis*-3-aminocyclohexane carboxylic acid (ACHC) was examined. This conformationally restricted analogue of GABA is taken up selectively by the neuronal GABA transport process in brain slices and is not a substrate or inhibitor of GABA-T (Neal & Bowery, 1977). Cortical slices were incubated at 25°C for 30 min with either [$^3$H]-ACHC ($5 \times 10^{-8}$M) or [$^{14}$C]-GABA ($10^{-7}$M) in the absence of AOAA. The slices were then superfused in the absence of AOAA.

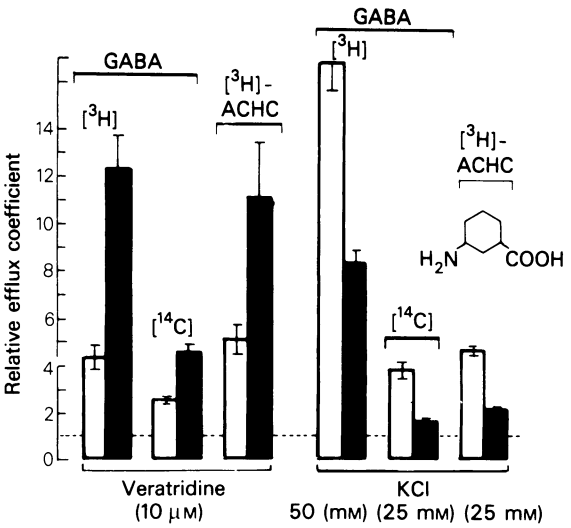


**Figure 2** Summary of results showing the effect of veratridine and KCl on the release from cortical slices of [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA), [$^{14}$C]-GABA and [$^3$H]-*cis*-3-aminocyclohexane carboxylic acid ([$^3$H]-ACHC). The results are expressed in terms of the relative efflux coefficient (peak release/resting release) and are the mean of 4 to 6 experiments; vertical lines show s.e. mean. The open columns show the evoked release in normal medium (controls) and the solid columns show the evoked release obtained in Ca-free medium. Amino-oxyacetic acid (AOAA) was present in the medium only in experiments with [$^3$H]-GABA. The smaller veratridine-evoked release of [$^{14}$C]-GABA was due to the absence of AOAA, since in its presence, the evoked release of [$^{14}$C]- and [$^3$H]-GABA was not significantly different.

**Table 1** Effect of veratridine and K on [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA) release from different brain areas

| | *Relative efflux coefficient* | | |
|---|---|---|---|
| | *Cerebral cortex* | *Cerebellum* | *Substantia nigra* |
| Veratridine | | | |
| 5 μM | 4.3 ± 0.58 | | |
| 5 μM, Ca-free medium | 12.3 ± 1.48* | | |
| 10 μM | | 6.5 ± 0.75 | 1.3 ± 0.07 |
| 10 μM, Ca-free medium | | 6.4 ± 0.90 | 4.0 ± 0.33* |
| KCl | | | |
| 25 mM | | 5.5 ± 1.10 | 24.1 ± 3.52 |
| 25 mM, Ca-free medium | | 2.1 ± 0.25§ | 5.9 ± 0.94* |
| 50 mM | 16.9 ± 1.24 | : | |
| 50 mM, Ca-free medium | 8.4 ± 0.41* | | |

The tissue slices were exposed to veratridine or KCl for 4 min. The results are expressed as the relative efflux coefficient (f.min$^{-1}$ at peak of response/f.min$^{-1}$ spontaneous resting release). Each result is the mean ± s.e. mean of 4 to 8 determinations. Calcium-free significantly different from control (Student's *t* test) at $^*P < 0.001$; §$P < 0.05$.

The results summarized in Figure 2 indicate that as with [$^3$H]-GABA, the veratridine-evoked release of both [$^{14}$C]-GABA and [$^3$H]-ACHC were greatly potentiated by the absence of Ca in the superfusion medium, whilst the K-evoked release of [$^{14}$C]-GABA and [$^3$H]-ACHC was more than halved in Ca-free medium.

*Other areas of the brain*

We have not systematically studied the effect of $[Ca]_o$ on the veratridine-induced GABA release in different areas of the brain. However, experiments with slices of cerebellum and substantia nigra, areas of the brain with relatively low and high levels of GABA respectively, suggest that there are important quantitative differences in the effect of $[Ca]_o$ on the veratridine-induced release of GABA from different areas of the brain. Thus, it can be seen from the results in Table 1, that in the case of cerebellar slices, whilst the absence of Ca did not actually reduce the veratridine-evoked release of GABA, the potentiation of GABA release seen with cortical slices was absent. In contrast, the absence of external Ca produced a striking increase in the veratridine-evoked release of [$^3$H]-GABA from slices of substantia nigra (Table 1). In this tissue, veratridine (10 μM) caused only a very small release of [$^3$H]-GABA in normal medium (approximately 1.2 to 1.5 times the resting release) but in Ca-free medium, the same concentration of drug caused a 3 to 6 fold increase in [$^3$H]-GABA efflux. On the other hand, the K-evoked release of GABA from both slices of cerebellum and substantia nigra showed a similar Ca-dependence to that seen with slices of cerebral cortex, although the K-evoked release from nigral slices appeared to be rather more sensitive to the absence of Ca in the medium (Table 1).

*Effect of* $[Na]_o$ *on veratridine and K-evoked GABA release*

Cerebro-cortical slices were incubated with [$^3$H]-GABA in normal medium and then superfused with Tris/HCl buffered medium in which the NaCl was replaced with sucrose $[Na]_o = 0$. Under these conditions, exposure of the tissue to K still evoked a large release of [$^3$H]-GABA but the veratridine-evoked response was completely abolished (Figures 3 and 4). These results are similar to those obtained by Sandoval (1980) in experiments on the veratrine-evoked release of [$^3$H]-GABA from synaptosomes.

*Effect of* $[Cl]_o$ *on GABA release*

Cortical slices were incubated with [$^3$H]-GABA in normal medium and then superfused with Tris/HCl buffered medium in which NaCl was replaced with Na isethionate and $CaCl_2$ by Ca acetate. Superfusion of the tissue with Cl-free medium did not affect either

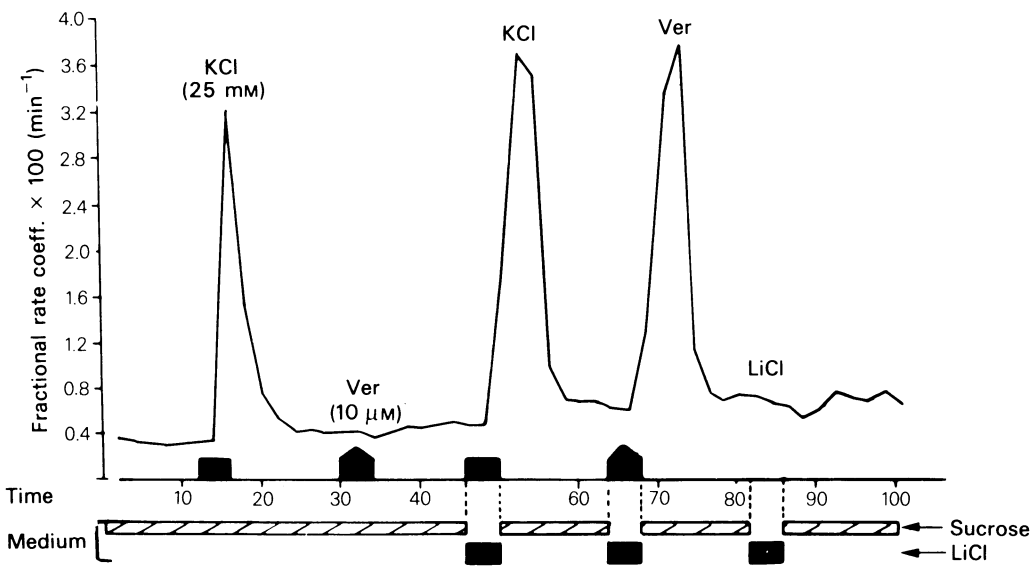


**Figure 3** Effect of Na-free medium on the veratridine (Ver)- and K-evoked release of [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA) from cortical slices. Slices were superfused as indicated with Na-free (sucrose) medium. This procedure abolished the effect of veratridine. However, when the tissue was exposed to veratridine dissolved in Na-free (Li) medium, the drug again caused a release of [$^3$H]-GABA. This experiment was repeated three times with the same result.

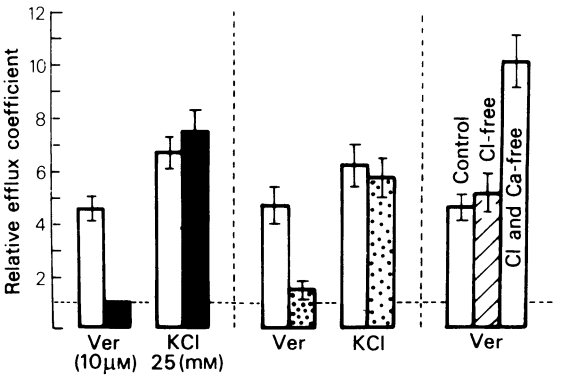


**Figure 4** Summary of results showing (a) effect of Na -free (sucrose) medium (solid columns) on the veratridine (Ver)- and K-evoked release of [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA) from cortical slices; (b) effect of tetrodotoxin (TTX, 0.2 μM, stippled column) and (c) effect of chloride-free medium. Open columns = controls. Results are expressed as the relative efflux co efficient and are the mean of 4 to 6 experiments; vertical lines show s.e. mean.

the veratridine-evoked release of [$^3$H]-GABA or the enhanced evoked release produced in Ca-free medium (Figure 4).

*Effect of tetrodotoxin (TTX) on GABA release*

Exposure of cortical slices, superfused in normal medium, to TTX (0.2 μM) almost abolished the veratridine-evoked release of [$^3$H]-GABA but had no significant effect on the release caused by KCl (Figure 4).

*Effect of* $[K]_o$ *on veratridine-evoked GABA release*

In an attempt to see whether depolarization *per se* was essential for veratridine to release GABA, cortical slices were loaded with [$^3$H]-GABA, and then superfused with medium containing a high concentration of KCl (120 mM), in addition to the normal amount of NaCl. The effect of KCl (120 mM) on GABA release in both the presence and absence of Ca is shown in Figure 5a,b. The large initial release of [$^3$H]-GABA, and the subsequent sustained release, were larger in the presence of Ca in the medium. When the brain slices were exposed to veratridine (10 μM) in the presence of KCl (120 mM), the alkaloid was still able to evoke an additional release of [$^3$H]-GABA and this release was greater in Ca-free medium (Figure 5a). The striking effect of Ca is even more obvious in Figure 5b which illustrates an experiment in which responses to veratridine were obtained before and after exposure of the cortical slices to KCl (120 mM). In Ca-free medium, veratridine evoked large increases in [$^3$H]-GABA release from

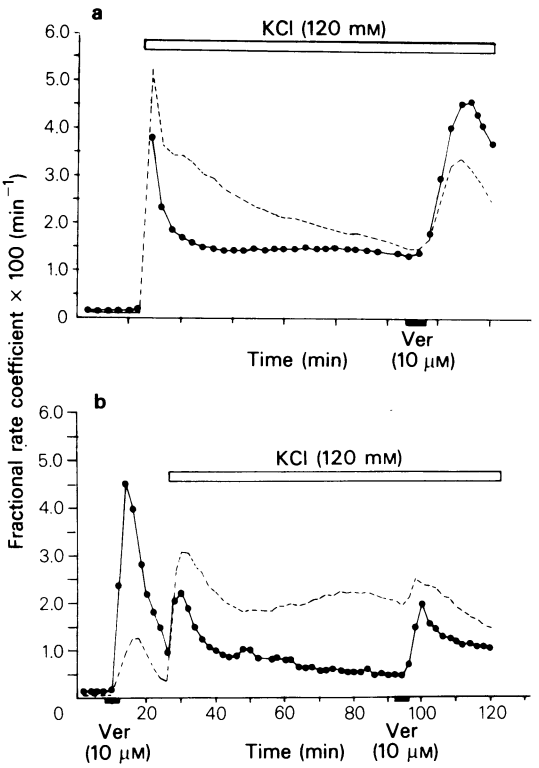


**Figure 5** Experiments showing (a) effect of KCl (120 mM) on the veratridine-evoked release of [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA) from cortical slices; (b) effect of veratridine (Ver) on [$^3$H]-GABA release before and after exposure of the tissue to KCl (120 mM). Dashed line = medium containing Ca; (●) Ca-free medium in both (a) and (b). Each of these experiments was repeated twice with the same results. The slices used for the two experiments in (a) were from one rat and the slices used for the two experiments shown in (b) were from another rat.

both normal and depolarized tissue. In contrast, in the presence of Ca, veratridine evoked relatively small increases in GABA release. The release of [$^3$H]-GABA by veratridine from K-depolarized slices was completely abolished by TTX (0.2 μM) or by the substitution of sucrose for the Na in the medium, indicating that in depolarized slices, veratridine was still able to open Na channels.

This experiment suggests that it may be the influx of Na *per se* and not the consequential depolarization that causes the release of GABA.

*Effect of L-2,4-diaminobutyric acid (DABA) and lithium (Li) on veratridine-evoked GABA release*

Since veratridine causes an increase in intracellular [Na], it seemed possible that veratridine might in-

crease the efflux of GABA by reversing the highly sodium-dependent transport process which normally accumulates the amino acid (Iversen & Neal, 1968). This was tested by preincubating cortical slices with a high concentration of DABA, an analogue of GABA which is an inhibitor of GABA transport (Harris, Hopkin & Neal, 1973) and is itself accumulated by nerve terminals in cortical slices (Weitsch-Dick, Jessell & Kelly, 1978). Therefore, its presence inside the cell would be expected to reduce any carrier mediated release of GABA.

A typical experiment is illustrated in Figure 6, which shows that loading the tissue with DABA did not reduce the veratridine-evoked release of [$^3$H]-GABA. This result with brain slices is identical to previously reported experiments using synaptosomes (Levi, *et al.*, 1978; Sandoval, 1980). In contrast, the release of [$^3$H]-GABA produced by exposing the DABA-loaded slices to non-radioactive GABA (1 mM) was reduced by over 70% compared with controls (Figure 6), presumably because of competition between GABA and DABA for the intracellular carrier site.

The possibility of a carrier-mediated efflux of GABA was further tested by studying the effect of replacing Na in the medium with Li. Li will support veratridine-evoked depolarization but not GABA transport (Iversen & Neal, 1968). Cortical slices were loaded with [$^3$H]-GABA and superfused in Na-free (sucrose) medium. Exposure of the tissue to veratridine dissolved in Na-free (Li) medium produced a large increase in the release of [$^3$H]-GABA (Figure 3) but no evoked release occurred if the drug was present in Na-free (sucrose) medium. The Li-medium itself had no effect on GABA release and did not significantly alter the K-evoked release of GABA (Figure 3).

*Effect of ruthenium red on veratridine and K-evoked GABA release*

The dye, ruthenium red, inhibits Ca uptake by mitochondria (Moore, 1971; Reed & Bygrave, 1974) and probably also the K-evoked Ca influx in synaptosomes (Tapia & Meza-Ruiz, 1977). This compound was used to inhibit the uptake of Ca by the intraterminal mitochondria, with a view to testing the possibility that veratridine might cause GABA release by

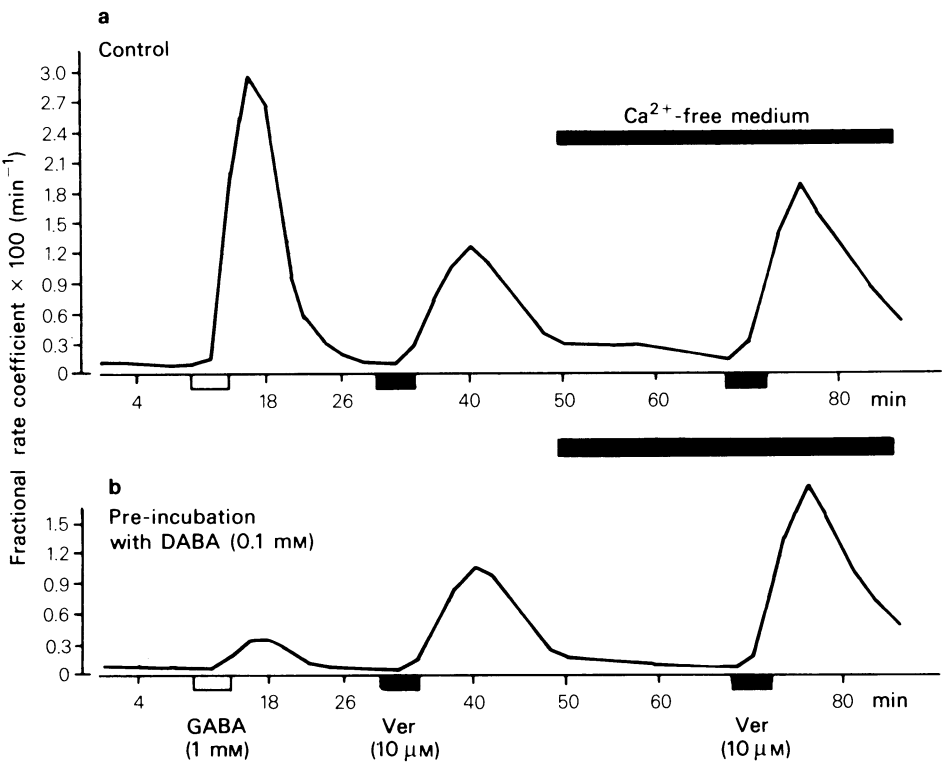


**Figure 6** Effect of veratridine (Ver) and γ-aminobutyric acid (GABA) on [$^3$H]-GABA release from cortical slices (a, control) and cortical slices preloaded with L-2,4-diaminobutyric acid (DABA) (b). This experiment was repeated three times with the same result.

increasing the concentration of free Ca ions in the cytosol.

When cortical slices were exposed to ruthenium red (10 μM), the K-evoked release of GABA was reduced to 21.2% of the control values (Figure 7). In contrast, the veratridine-evoked release of GABA was increased by over 50% in the presence of the dye (Figure 8a). Ruthenium red also further increased the larger veratridine-evoked release of GABA obtained in Ca-free medium (Figure 8b).

*Effect of* $[Ca^{2+}]_o$ *on veratridine-evoked release of* [$^{14}$C]-*glycine from spinal cord*

For comparison with GABA, the effect of veratridine on the efflux of [$^{14}$C]-glycine was studied, since glycine is believed to be a major inhibitory transmitter substance in the spinal cord (Krnjević, 1974). Slices of rat spinal cord were prepared as previously described (Neal, 1971) and incubated for 30 min with [$^{14}$C]-glycine (1 μM). The slices were then superfused in either normal medium (controls) or Ca-free medium. Veratridine (100 μM for 4 min) increased the efflux of [$^{14}$C]-glycine from both the control slices and those superfused in Ca-free medium. However, as with GABA, the evoked release in Ca-free medium (5.9 ± 0.52 times the spontaneous resting release) was significantly greater ($P < 0.05$ paired $t$ test, $n = 5$) than that occurring in control slices superfused in normal medium (3.8 ± 0.29 times the resting release).

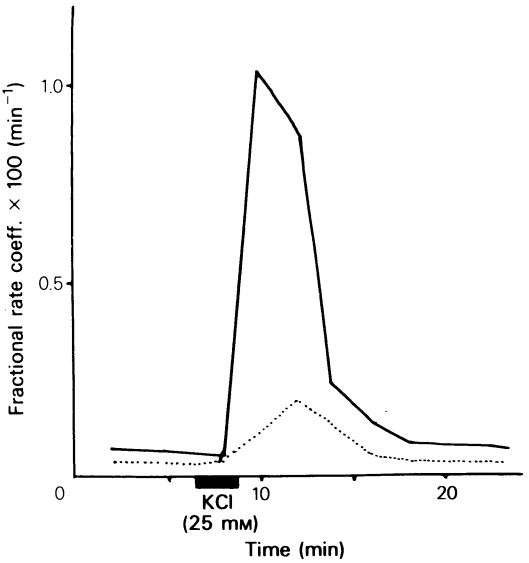


**Figure 7** Effect of ruthenium red (10 μM, dashed line) on the K-evoked release of [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA) from cortical slices. The K-evoked release of GABA was reduced to 21 ± 5.2% of the controls (mean ± s.e. mean of 4 experiments). Slices from the same animal were loaded with [$^3$H]-GABA in the presence of amino-oxyacetic acid and then superfused in medium (control) or medium containing ruthenium red. The medium containing high K, also contained ruthenium red if appropriate.

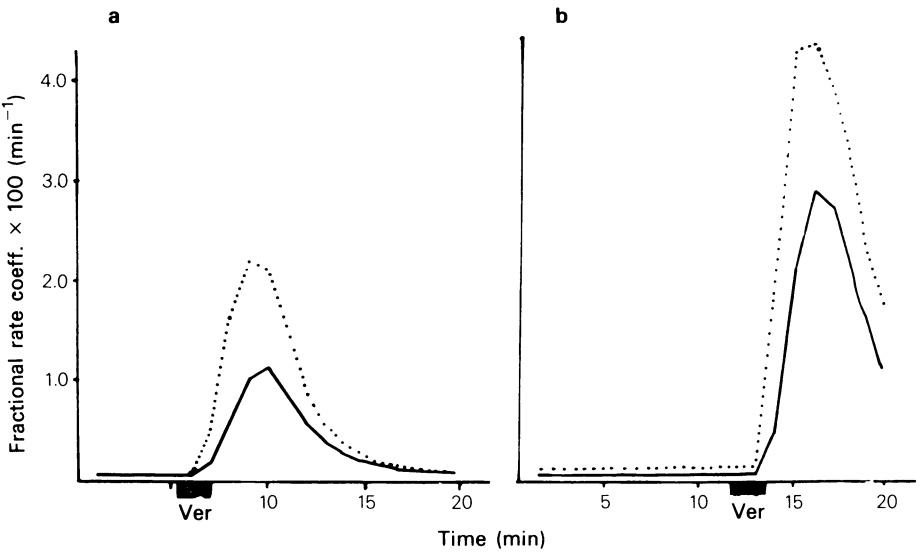


**Figure 8** Effect of ruthenium red (10 μM, dotted line) on the veratridine (Ver)-evoked release of [$^3$H]-γ-aminobutyric acid ([$^3$H]-GABA) from cortical slices (a) in normal medium ($[Ca]_o = 2.52$ mM); (b) in calcium-free ($[Ca]_o = 0$) medium. Solid line = control. The dye increased the veratridine-evoked release in normal medium by 59 ± 7.1% (mean ± s.e. mean of 9 experiments) and in Ca-free medium by 41 ± 7.0% (mean ± s.e. mean of 4 experiments) compared with controls. Slices for the experiments shown in (a) were from one rat; slices for the experiments shown in (b) were from another rat.

*Effect of* $[Ca]_o$ *on veratridine and K-evoked ACh release*

Both veratridine and KCl increased the release of radioactivity from cortical slices previously incubated in medium containing [$^3$H]-choline (Table 2). However, the evoked release of radioactivity was less sensitive than that of GABA to both these agents, and at a concentration of 100 μM, veratridine increased [$^3$H]-ACh release less than three fold (Table 2).

The effect of calcium on the veratridine and KCl-evoked release of [$^3$H]-ACh is summarized in Table 2. In contrast to GABA and ACHC, the veratridine-evoked release of [$^3$H]-ACh was almost abolished in calcium-free medium. Similarly, the release of [$^3$H]-ACh by KCl (50 mM) was also highly calcium dependent (Table 2).

**Table 2** Effect of $[Ca]_o$ on veratridine- and K-evoked release of [$^3$H]-acetylcholine ([$^3$H]-ACh) from cortical slices

| | *Relative efflux coefficient* |
|---|---|
| Veratridine | |
| 100 μM | 2.55 ± 0.22 |
| 100 μM, Ca-free medium | 1.35 ± 0.05* |
| KCl | |
| 50 mM | 2.56 ± 0.09 |
| 50 mM, Ca-free medium | 1.05 ± 0.007* |

Cortical slices were exposed to veratridine and KCl for 4 min. The results are expressed as the mean ± s.e. mean of 6 to 8 experiments. * Values significantly different from control, $P < 0.001$.

*Effect of* $[Ca]_o$ *on veratridine and K-evoked noradrenaline release*

The release of radioactivity from cortical slices previously loaded with [$^3$H]-noradrenaline was greatly increased by exposure of the tissue to veratridine (10 μM for 4 min). Veratridine increased the efflux of radioactivity more than four fold (Table 3) and this effect was Ca-dependent since a second exposure of the tissue to veratridine following a change to Ca-free medium increased the efflux of radioactivity by only 2.2 times the resting release. The significantly smaller evoked release of radioactivity seen in Ca-free medium was not due simply to a diminished second response to the drug, since an identical result was obtained when the first application of veratridine was made in Ca-free medium followed by a second (control) exposure of the tissue to veratridine in normal medium (Table 3). KCl (25 mM) also produced a calcium-dependent increase in the release of radioactivity (Table 3).

**Table 3** Effect of $[Ca]_o$ on veratridine- and K-evoked release of [$^3$H]-noradrenaline from cortical slices

| | *Relative efflux coefficient* | |
|---|---|---|
| Veratridine | | |
| 10 μM | 4.16 ± 0.38 (8) | (a) |
| 10 μM, Ca-free medium | 2.2 ± 0.29* (8) | (a) |
| 10 μM | 5.73 ± 0.067 (3) | (b) |
| 10 μM, Ca-free medium | 2.2 ± 0.46* (3) | (b) |
| KCl | | |
| 25 mM | 7.13 ± 0.99 (9) | |
| 25 mM, Ca-free medium | 2.11 ± 0.56* (9) | |

In each experiment the tissue either was exposed to veratridine first in normal medium and then in Ca-free medium (a) or was exposed to veratridine first in Ca-free medium and then in normal medium (b). The incubation and superfusion media contained EDTA (0.13 mM) and ascorbic acid (1.1 mM) to reduce chemical breakdown of noradrenaline, but [$^3$H]-noradrenaline in the superfusate was not separated from $^3$H-metabolites.

Results are expressed as the mean ± s.e. mean of the number of experiments indicated in parentheses. * Values significantly different from control ($P < 0.002$).

## Discussion

The present study shows that the veratridine-induced release of [$^3$H]-GABA from rat cerebrocortical, nigral and cerebellar slices is not dependent on the presence of extracellular calcium. Indeed, the evoked release of GABA from cortical and nigral slices was potentiated by the absence of calcium ions in the medium bathing the tissue.

These results confirm our previous findings (Neal, 1979; Neal & Bowery, 1979) and are in agreement with several other studies in which the release of both endogenous and labelled GABA by veratridine alkaloids has been found to be either unaffected or increased in Ca-free medium (Benjamin & Quastel, 1972; Levi *et al.*, 1978; Minchin, 1979; Szerb, 1979).

In contrast to veratridine, K evoked a release of [$^3$H]-GABA from cortical slices which was greatly reduced in Ca-free medium, a result which has been reported many times previously (see Krnjević, 1974 for references).

It is apparent, therefore, that the mechanism underlying the K-evoked release of [$^3$H]-GABA requires extracellular Ca, whilst that underlying the veratridine-evoked response does not. We do not know whether the veratridine-evoked release of other labelled amino acid transmitters from brain tissues is also independent of extracellular Ca, but this seems likely for two reasons: (a) the veratridine-evoked release of [$^{14}$C]-glycine from slices of spinal cord was significantly increased in Ca-free medium and (b) Benjamin & Quastel (1972) reported that the protoveratrine-evoked release of endogenous

GABA, aspartate, glycine and glutamate from brain slices was facilitated by Ca-deficient medium.

In contrast to GABA and glycine, the veratridine-evoked release of labelled noradrenaline and ACh from cortical slices was clearly Ca-dependent, a result which is in agreement with previous reports (Blaustein *et al.*, 1972; Grewaal & Quastel, 1973; Patrick & Barchas, 1976).

It has generally been accepted that the release of transmitters by veratridine involves an influx of Ca following the drug-induced increase in Na permeability of the nerve terminal membrane. This mechanism is supported by the work of Li & White (1977), who found that veratridine caused a tetrodotoxin-sensitive influx of labelled Na into synaptosomes, and by Blaustein (1975) who reported that the alkaloid caused an influx of Ca into synaptosomes. Furthermore, Blaustein *et al.* (1972) found that the release of noradrenaline from synaptosomes, produced by veratridine, was associated with an increased Ca influx and these results were taken as being consistent with the calcium hypothesis of transmitter release.

The present study of the veratridine-evoked release of noradrenaline and ACh is also consistent with the calcium hypothesis of transmitter release, but the results with [$^3$H]-GABA are apparently not. However, as will be argued later, the most likely explanation for the present results involves the release of bound Ca within the GABAergic nerve terminals, and so the release of GABA by veratridine may, after all, be consistent with the calcium hypothesis of transmitter release.

What then is the explanation for the apparent independence from $[Ca]_o$ of the veratridine-evoked release of GABA from brain slices, and why is the release actually greater in Ca-free medium?

The possibility that the striking difference produced by Ca-free medium on the veratridine-evoked release of GABA, and noradrenaline and ACh, might be due to release of the amino acid from non-neuronal compartments is very unlikely for the following reasons: (a) In thin cortical slices, [$^3$H]-GABA has been shown by radioautography to be accumulated predominantly in nerve terminals (Iversen & Bloom, 1972). (b) Veratridine at the concentration and application time used in the present experiments has little or no effect on the release of GABA from glial cells (Neal & Bowery, 1979). (c) An increased release of [$^3$H]-GABA evoked from synaptosomes by veratridine has been demonstrated in Ca-free medium (Levi *et al.*, 1978).

It is unlikely that metabolic degradation of the [$^3$H]-GABA is important, since in most of the experiments, AOAA was included in both the incubation and superfusion medium to inhibit GABA-T. The presence of AOAA itself could not be responsible for the potentiation of the veratridine-evoked release of GABA observed in Ca-free medium, because the same effect was found when [$^{14}$C]-GABA was used in the absence of AOAA.

Furthermore, the veratridine-evoked release of [$^3$H]-ACHC, a GABA analogue which is accumulated by cortical neurones but not metabolized (Neal & Bowery, 1977), was also enhanced in Ca-free medium.

The possibility that K and veratridine stimulate the release of [$^3$H]-GABA from different pools within the nerve terminal is also unlikely, since De Belleroche & Bradford (1977) found that both these agents caused GABA release from the same, cytoplasmic, pool of synaptosomes.

Veratridine depolarizes excitable membranes by a selective increase in resting sodium permeability (see Ulbricht, 1969; Narahashi, 1974); and in the present study, the veratridine-evoked release of GABA was completely abolished both by sodium-free medium and by tetrodotoxin, indicating that an influx of $Na^+$ into the nerve terminals through voltage sensitive Na-channels was necessary for GABA release. Furthermore, the degree of GABA release was highly dependent on both the concentration of veratridine, and on the time for which the tissue was exposed to the drug (Neal & Bowery, 1979), suggesting that the amount of GABA released was related to the increase in $[Na]_i$.

The potentiation of the veratridine-evoked release of GABA in Ca-free medium might be due to a greater influx of Na under these conditions. This could occur for at least two reasons: firstly, Catterall (1975) has shown that the action of veratridine on voltage-sensitive Na channels in neuroblastoma cells is inhibited in a competitive manner by divalent cations. Thus, in Ca-free medium, an ion antagonistic to the action of veratridine is absent and so the drug would be expected to cause a greater influx of Na than in normal medium. Furthermore, since Ca is a more potent inhibitor of veratridine than Mg, a similar enhancement of Na influx would be expected in medium in which the Ca was replaced by Mg. We have not measured Na fluxes in the present experiments, but the results of Catterall (1975) clearly show that Na uptake by neuroblastoma cells was considerably greater in medium in which Ca was replaced by Mg.

Secondly, medium low in Ca has been shown to reduce the depolarization required to open voltage-sensitive Na channels and delays their inactivation (Frankenhaeuser & Hogkin, 1957; Hille, 1968). Thus, reducing $[Ca]_o$ without increasing $[Mg]_o$ shifts the activation curve of Na channels and can produce an artifactual depolarization and release of transmitter. For example, Matthews & Wickelgren (1977), who did not keep constant the divalent ions concentration found that low $[Ca]_o$ increased the frequency of frog muscle miniature endplate potentials (m.e.p.ps). In contrast, Madden & Van der Kloot

(1978) found that if the divalent ions concentration was kept constant, the m.e.p.p. frequency was a monotonically increasing function of $[Ca]_o$.

Since the potentiation of the veratridine-evoked release of GABA from cortical slices occurred not only in Ca-free medium, but also in medium where the Ca was replaced with equimolar Mg, it would seem that shifts of the Na channel activation curve are not important in the present experiments. Thus, the enhanced effectiveness of veratridine in causing GABA release in Ca-free medium is probably due to the absence of the antagonistic ion.

It may, then, be reasonable to assume that in cortical slices a greater influx of Na into the nerve terminals occurs in Ca-free medium, and that this results in a larger depolarization than that occurring in normal medium. The question which arises is whether it is the influx of Na or the consequential depolarization which causes GABA release. Since veratridine evoked the release of GABA from slices depolarized by a high concentration of K (120 mM), it is probable that it is the influx of Na which causes GABA release. However, we cannot exclude the possibility that the hyperosmotic, high-K solutions may have caused cell shrinkage and an increase in $[K]_i$. If so, $E_m$ might still have been negative and the influx of Na caused by veratridine might have produced further depolarization of the brain slices.

It has been suggested (Martin, 1976) that increased $[Na]_i$ might reverse the Na-dependent transport process for GABA. However, this plausible hypothesis is not compatible with the present study. Thus, preloading the tissue with L-DABA, a relatively potent inhibitor of the GABA transport process (Harris *et al.*, 1973), did not affect the veratridine-evoked release of [$^3$H]-GABA, although the release caused by exposing the slices to a high concentration of GABA was greatly reduced. Furthermore, the veratridine-evoked release of GABA was unaffected by medium in which the Na was replaced by Li. Since Li will enter through Na channels (Richelson, 1977) and cause depolarization but will not support the GABA transport process (Iversen & Neal, 1968), these results provide further evidence that the veratridine-evoked release of GABA does not involve reversal of the GABA transport process.

Although release processes generally appear to require the presence of extracellular Ca, there have been reports that secretion from the adrenal medulla (Lastowecka & Trifaro, 1974), neuropituitary (Douglas, Ishida & Poisner, 1965; Russel, Warberg & Thorn, 1974) and endocrine pancreas (Lowe, Richardson, Taylor & Donatsch, 1976) can occur in the absence of extracellular Ca. Also, high frequency stimulation of motonerves caused ACh release in the absence of extracellular Ca (Erulkar & Rahamimoff, 1978). In these studies, it has been suggested that the mobilization of bound intracellular Ca may be involved in the release process and it is possible that a similar mechanism underlies the veratridine-evoked release of GABA from brain slices. Veratridine increases $[Na]_i$ and this could increase the concentration of free $Ca^{2+}$ ions by several mechanisms (Baker, 1972). For example, Na has been shown to induce a rapid efflux of Ca from mitochondria of brain and certain other tissues (Crompton, Moser, Lüdi & Carafoli, 1978). It is not known whether a similar Na-activated release of Ca from mitochondria occurs in intact tissue, but the present study in which ruthenium red, an inhibitor of mitochondrial Ca uptake, was found to potentiate the veratridine-evoked release of GABA, even in Ca-free medium, supports the suggestion that the release of $Ca^{2+}$ from intracellular stores is important. Veratridine was found to cause GABA release from brain slices in sodium-free (Li) medium, and since Li also causes Ca-release, at least from heart mitochondria (Crompton, Copano & Carafoli, 1976), this result is also compatible with the idea that the veratridine-evoked release of GABA involves the mobilization of intracellular Ca stores.

Although the mitochondria are probably the most important store of intracellular Ca, it is possible that Ca ions may also originate from sites on the internal surface of the membrane (Oschman, Hall, Peters & Wall, 1974), soluble molecules and macromolecules (Baker, 1976).

It is likely that in normal solution, $[Ca]_i$ in nerve terminals increases both because it enters from outside and because $[Na]_i$ releases it from internal stores. In Ca-free solutions, the first effect will be reduced but the second will be increased. The fact that Ca-free solutions increase the veratridine-evoked release of GABA but decrease the release of ACh may be because the balance between the two effects of Ca-free medium is different in GABAergic and cholinergic terminals. This may be due to a variety of factors and it might be significant that synaptosomes which accumulate [$^3$H]-GABA, i.e. GABAergic terminals, have a higher density of mitochondria than those which do not (Iversen & Bloom, 1972).

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