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Acetylation of Synaptosomal Protein: Inhibition by Veratridine

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Abstract: Incubation of synaptosomes with [^{3}H]acetate results in rapid labeling of protein. Labeling is decreased in the presence of veratridine, and the effect of veratridine is blocked by tetrodotoxin. Most of the radioactivity can be removed by base or acid hydrolysis, and is probably incorporated as acetate; it is this fraction that is affected by the veratridine. The data suggest that veratridine stimulates deacetylation of synaptosomal protein. This raises the question whether acetylation-deacetylation is involved in membrane function. **Key Words:** Acetylation—Synaptosomes—Proteins—Veratridine—Tetrodotoxin. **Berl S. et al.** Acetylation of synaptosomal protein: Inhibition by veratridine. *J. Neurochem.* **40,** 176–183 (1983).

Isolated nerve ending particles prepared from brain (synaptosomes) take up, synthesize, and release putative neurotransmitters (Marchbanks, 1969; Blaustein et al., 1972; De Belleroche and Bradford, 1973; Levy et al., 1974). They contain specific mechanisms for the transport of Ca^{2+}, Na^+, and K^+ across their membranes (Lust and Robinson, 1970; Blaustein et al., 1972; Swanson et al., 1974; Blaustein, 1975) and ion flux studies show that sodium channels in synaptosomes have the same functional properties as those in intact nerve cells (Mathews et al., 1979; Krueger and Blaustein, 1980). Synaptosomes also retain a membrane potential (Blaustein and Goldring, 1975; Creveling et al., 1980) and bind neurotoxins to receptor sites associated with sodium channels (Abita et al., 1977; Catterall et al., 1979; 1981).

Actin, myosin, and tropomyosin have been identified in synaptosomal preparations (Puszkin et al., 1972; Blitz and Fine, 1974). Furthermore, actin (Wang and Mahler, 1976; Schwartz et al., 1977; Blomberg et al., 1977; Cohen et al., 1977; Mahler, 1977; Kelly and Cotman, 1978), myosin (Beach and Cotman, 1979), and the calcium regulator protein calmodulin (Grab et al., 1979; Lin et al., 1980; Wood et al., 1980) have been shown to be present in

synaptic membrane sites. The physiological activity of the contractile proteins in synaptic membranes has not yet been established. In part this is owing to the difficulty of formulating studies in which changes in this system could be related to function. It has been suggested that the actomyosin system present in nerve terminals functions in the release of transmitter materials at synaptic junctions (Poisner, 1970; Berl et al., 1973). Little direct evidence in support of such a function has been reported (Babitch et al., 1979).

Several of the proteins associated with this system—e.g., actin (Krans et al., 1965; Gaetjens and Barany, 1966), myosin (Offer, 1964), tropomyosin (Stone and Smillie, 1978; Mak et al., 1980), troponin C (Collins et al., 1977), and calmodulin (Watterson et al., 1980)—have a property in common; namely, their NH_2-terminal amino acid is blocked by the acetyl moiety covalently bound to the amino group. The functional significance of this terminal group is not known.

A number of years ago we observed that radiolabeled acetate can be incorporated into brain proteins, from which it can be readily released by acid hydrolysis and driven out upon drying in vacuum. This suggested to us that acetate may be

rapidly turning over in proteins as the acetyl moiety and might serve as a marker for synaptosomal activity. Therefore a study was undertaken to determine whether or not labile acetate incorporation can be demonstrated in synaptosomes and whether such incorporation would be affected by conditions that cause stimulated release of neurotransmitters.

The data reported in this paper support the latter notions. However, the results obtained to date provide no evidence for the participation of the contractile proteins in these processes.

MATERIALS AND METHODS

[^{3}H]Acetate (2 Ci/mmol) was obtained from New England Nuclear; veratridine and sodium fluoroacetate from Sigma Chemical Company; and tetrodotoxin (TTX) and D,L-fluorocitrate (barium salt) from Calbiochem.

Before use, 0.10 volume of 0.1 M NaOH was added to aliquots of the tritiated acetate, and the ethanol in which it was supplied was evaporated at 0°C with a gentle stream of N_2. The residue was dissolved in the same volume of H_2O. The veratridine was dissolved in 50% ethanol prior to use. The TTX was dissolved in 1.6 mM citrate buffer, pH 4.7. Control studies with amounts of ethanol, citrate buffer, or barium chloride equivalent to the amounts present experimentally showed that these substances were without effect on acetate incorporation.

The synaptosomes were prepared from the cerebral hemispheres of Sprague-Dawley rats by the flotation procedure of Booth and Clark (1978). Critical examination of this preparation has shown its purity to be approximately 85% or more (Deutsch et al., 1981).

Labeling of synaptosomes

The synaptosomal pellet was suspended in Krebs-Ringer phosphate fortified with 5.5 mM glucose (KRP) and oxygenated with 95% O_2–5% CO_2 prior to use (2.5–3 mg of synaptosomal protein/ml). The incubation temperature was 37°C, and the synaptosomes were kept at this temperature for 5–10 min before any additions were made. The final concentration of added [^{3}H]acetate was approximately 0.05 mM (100 μCi/ml) (unless otherwise stated); of veratridine 10–100 μM; and of TTX 2 μM, obtained by adding 0.01 ml of each stock solution per ml of synaptosomal suspension. Batrachotoxin (1 μM) was also added in some preliminary experiments.

Following incubation for varying periods of time (10s–15 min), 1-ml samples were immediately mixed with 0.1 ml of 4 M perchloric acid and kept in ice until centrifugation. The precipitates were exhaustively washed six times with 5 ml of 0.4 M perchloric acid and twice with organic solvents. The first three perchloric acid washes also contained 10 mM sodium acetate. In early experiments the protein was repeatedly washed with organic solvents by the procedure of Siekevitz (1952). In most of the experiments the protein was washed with acidified chloroform-methanol (3:2) and in later experiments also with ether. With either method, the last wash contained no radioactivity, nor were the results affected by either procedure. The protein was dissolved in 1 ml of 1 M NaOH at 60°C.

To determine how much of the radioactivity was incorporated in a hydrolyzable and acid-volatile state, probably as acetate, and how much was incorporated into the protein, probably as amino acids, portions of the protein (0.4 ml) were heated at 100°C in 1 M NaOH for 1–2 h. they were then acidified with 1 ml of 2M HCl and dried in vacuum at 60–65°C; the residue was dissolved in 1 ml 2 M HCl and again dried. Four milliliters of water was added, and the protein was dried again. The residue was dissolved in 1.2 ml of water and 1 ml was counted. A greater percentage of volatile radioactivity was obtained by acid hydrolysis with 2 M HCl for 3 h at 100°C (approximately 75–80% versus approximately 65%). This procedure was used in later experiments. Volatile radioactivity was calculated as the difference between total radioactivity and that remaining after hydrolysis.

Controls were run by adding the tritiated acetate to the synaptosomal preparation after the addition of the perchloric acid. Following the washing procedure the remaining radioactivity was approximately 10% or less of that usually found after 1 min of incubation. Following hydrolysis all of the radioactivity was acid-volatile.

Protein concentration was determined by the procedure of Lowry et al. (1951), with bovine serum albumin as a standard. Radioactivity was counted by liquid scintillation spectrometry in a Packard Tricarb instrument. Internal standards of ^3H_2O were used for the conversion of cpm to dpm.

RESULTS

Effect of veratridine and tetrodotoxin on labeling of protein

Veratridine, TTX, or both were added to the synaptosomal suspension 1–2 min prior to addition of the [^{3}H]acetate, and samples were taken at the indicated time periods (Fig. 1). The labeling of the protein was rapid and nonlinear beyond approximately 1.5 min. At 1 min after the addition of the [^{3}H]acetate the veratridine-treated synaptosomal protein contained approximately 17% less radioactivity than did the control protein, and this difference increased gradually with time largely because of continued labeling of the control samples. Further labeling of the protein was inhibited by veratridine (Fig. 1); TTX alone had no effect. The presence of TTX completely blocked the effect of veratridine, and therefore these values, as well as those with TTX alone, were averaged with the controls. As seen in Fig. 1, the major effect of veratridine occurred in the first 1–2 min.

Upon base hydrolysis and acid drying approximately two-thirds of the radioactivity was lost and one-third was not acid-volatile (Fig. 2). This latter fraction was apparently not affected by the presence of veratridine; it was the hydrolyzable and acid-volatile radioactivity that was inhibited by the veratridine from incorporation into the protein, as shown by the decreased amount found therein.

In order to define the initial slope of the incorporation curve, 1-ml samples were incubated in separate tubes, the reaction being stopped by the addition of 0.1 ml of 4 M perchloric acid added at 10, 20, 30, and 60 s. As seen in Fig. 3, the initial rate of
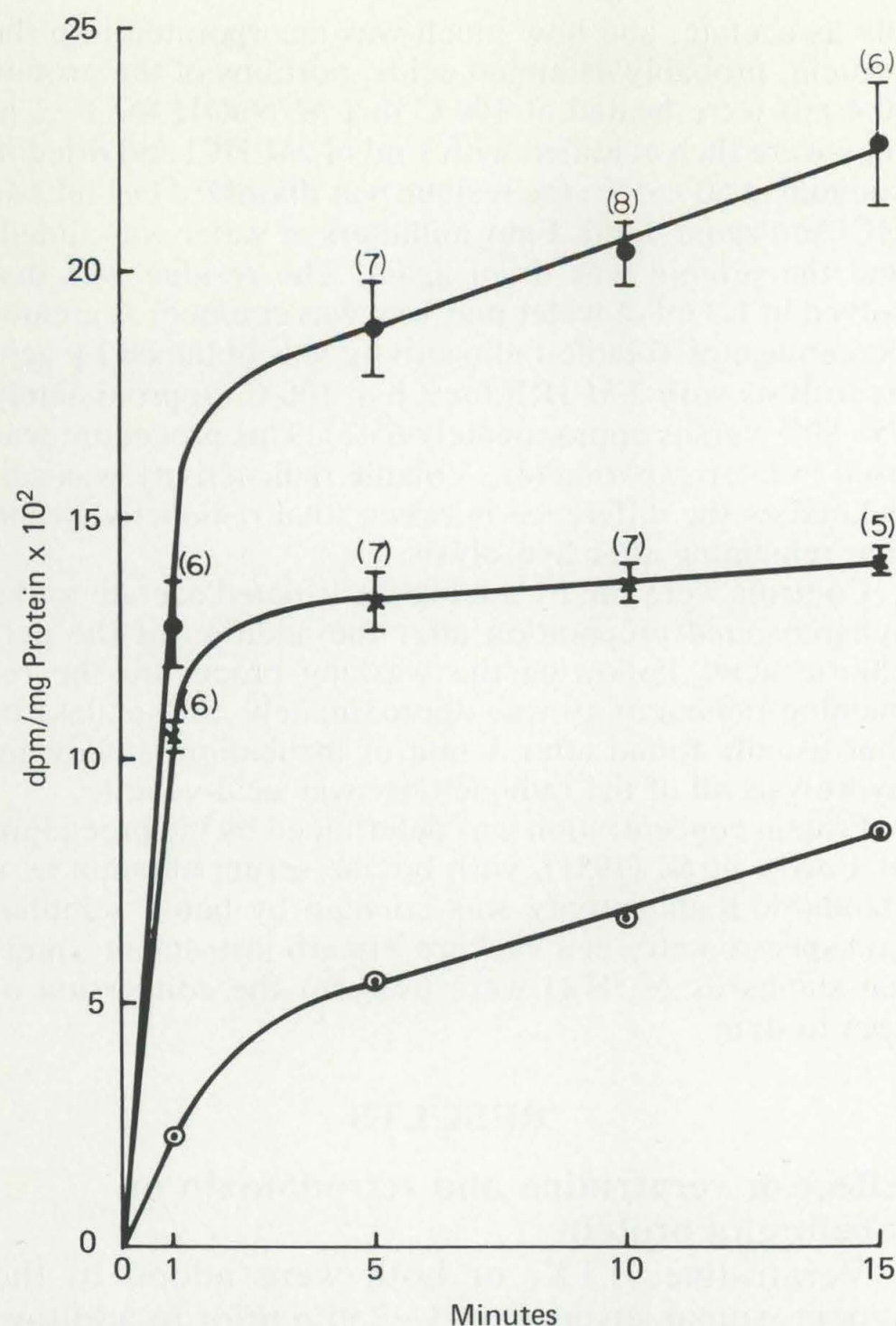
FIG. 1. Effect of veratridine on the labeling of synaptosomal protein with [³H]acetate. Suspensions of synaptosomes in KRP were preincubated for 10 min at 37°C. At 1-min intervals TTX, veratridine, and [³H]acetate (100 μCi/ml, 0.05 μmol) were added and 1-ml samples taken at the indicated times after addition of the [³H]acetate. (●) Averaged values obtained with no drug, with TTX (2 μM), and with TTX + veratridine (75–100 μM); (×) veratridine, 75–100 μM; (○) difference between the averaged and the veratridine values. The bars represent SEM; the numbers in parentheses represent the number of experimental values averaged.

FIG. 2. Effect of veratridine on the distribution of labile radioactivity in the synaptosomal protein. Experimental conditions are the same as in Fig. 1. Aliquots of the protein were base-hydrolyzed, acidified, and dried as described in the text. (—) Nonvolatile radioactivity; (---) volatile radioactivity; (●) no drug; (○) TTX; (×) veratridine; (△) TTX + veratridine.

acetate incorporation was greater than was estimated in Fig. 1. The effect of veratridine was evident even at 10 s after the addition of [³H]acetate.

Effect of veratridine on prelabeled proteins

The synaptosomal suspension in separate aliquots was prelabeled by incubation for 20 min in the presence of [³H]acetate. At 1-min intervals, TTX, veratridine, or both were then added to the separate flasks and 1-ml samples taken at 1, 5, 10, and 15 min. The synaptosomal protein was labeled by the [³H]acetate, but further labeling was again completely inhibited by the addition of veratridine, that in the control samples continuing to rise at a low rate (Fig. 4A). At 1 min the veratridine-treated synaptosomal protein contained approximately 15% less radioactivity than the control without veratridine. The decreased percentage of label in the
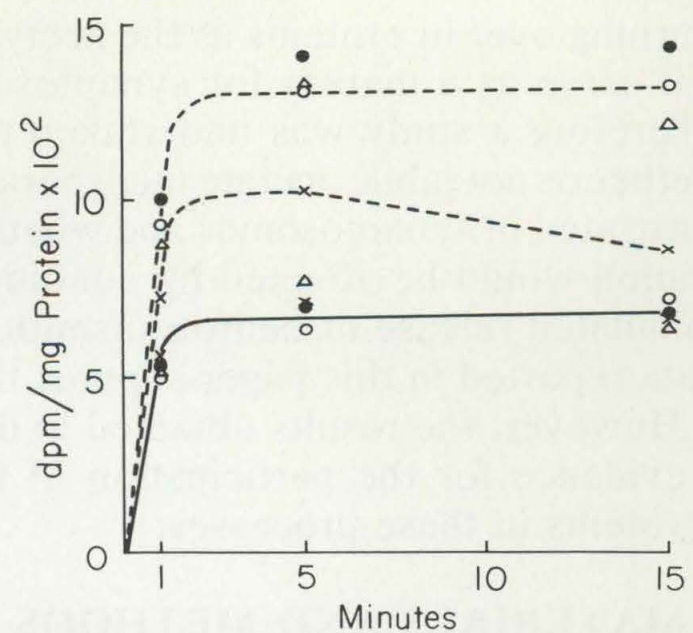
veratridine-treated samples was due to an absolute loss of radioactivity, as well as an increased labeling of the control sample. It appeared that in the presence of veratridine a portion of the radioactive acetate previously attached to protein was removed. The presence of TTX blocked the effect of the veratridine, and the labeling of the protein was even greater than that in the control samples (Fig. 4A). In Fig. 4B, the loss of radioactivity after base hydolysis and drying under acidic conditions is depicted. The same general effects of veratridine and TTX were observed under these conditions as previously. The loss of radioactivity due to incubation with veratridine was in the volatile fraction, following hydrolysis. It should be noted that the same difference curves between control and veratridine-treated samples were obtained before and after hydrolysis (compare Fig. 4A and 4B). Extrapolation back to zero time (point of addition of veratridine) suggests that the effect of veratridine was extremely rapid, with no time lag.

In another set of experiments the release of label from the protein caused by the presence of veratridine was examined by the addition of the veratridine to the synaptosomal suspension in the same flask after previous labeling with [³H]acetate (Fig. 5). Samples were taken at 1, 2, and 5 min. Veratridine (100 μM) was added, and samples were taken at 6, 7, and 10 min. Addition of the veratridine caused a rapid loss in the amount of acid-hydrolyzable, volatile radioactivity from the protein; it had no effect on the nonvolatile fraction. Similar results were obtained with batrachotoxin (1 μM), another neurotoxin whose action is similar to that of veratridine, but is much more potent (data not shown).

Acetate chase

Another demonstration of the labile nature of a part of the incorporated acetate is shown in Fig. 6.
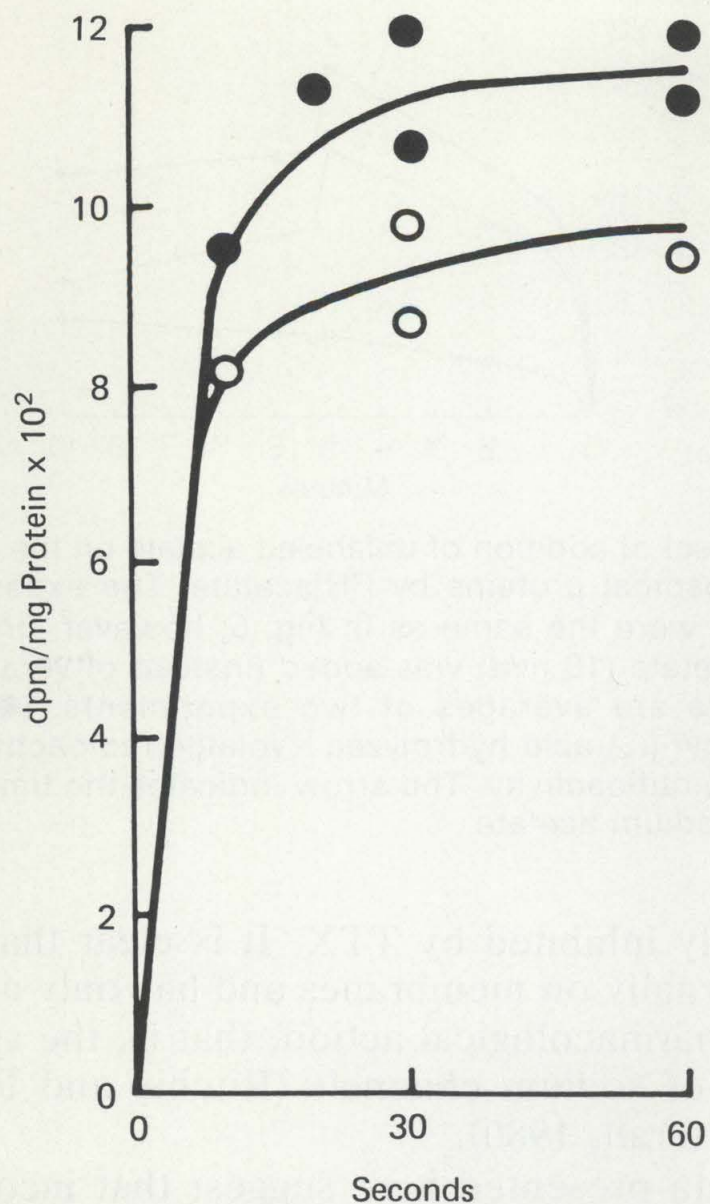
FIG. 3. Initial rate of labeling of synaptosomal protein with [³H]acetate and the effect of veratridine. One-milliliter aliquots of the synaptosomal suspension were preincubated for 10 min. Veratridine (100 μM) was added to one set of tubes and after 1 min [³H]acetate was added (100 μCi, 0.05 μmol) to both sets. The reaction was stopped after 10, 20, 30, and 60 s by the addition of 0.1 ml of 4 M perchloric acid. The results from two experiments are shown. (●) Without veratridine; (○) with veratridine.

FIG. 4. Prelabeling of synaptosomal protein with [³H]acetate and the effect of veratridine. Suspensions of synaptosomes in KRP were preincubated for 5 min at 37°C, [³H]Acetate (100 μCi/ml, 0.05 μmol) was added and incubation continued for 20 min. Then at 1-min intervals TTX and veratridine were added and 1-ml samples were taken at indicated times after addition of the veratridine. **(A)** Total radioactivity in the protein; **(B)** radioactivity removed from the protein by base hydrolysis and drying under acidic conditions. (●) No drug; (×) veratridine; (○) average of TTX and TTX + veratridine; (◉) no drug - veratridine. The bars represent SEM.

After labeling the synaptosomal suspension for 5 min, unlabeled sodium acetate (10 mM) was added and samples taken at 15 s, 1 min, and 5 min. A rapid exchange and dilution of the acid-hydrolyzable and volatile radioactivity was observed. Again there was no effect on the nonvolatile portion of the radioactivity.

Effect of temperature

Aliquots of the synaptosomal suspension were incubated at 0 or 37°C. After 10 min of preincubation veratridine was added, and 1 min later the labeled acetate was added and samples were taken at 1, 2, and 5 min. At 1 min after addition of the labeled acetate the incorporation of the radioactivity into the protein was approximately 50% less at 0 than at 37°C (Table 1). The percent inhibition decreased with time, apparently because the incorporation of the labeled acetate approached its peak at approximately 1 min at 37°C, whereas at 0°C the incorporation of radiolabeled acetate continued over the entire experimental time period and had not yet peaked at 5 min. At 0°C the effect of veratridine was not evident at 1 min, although the char-
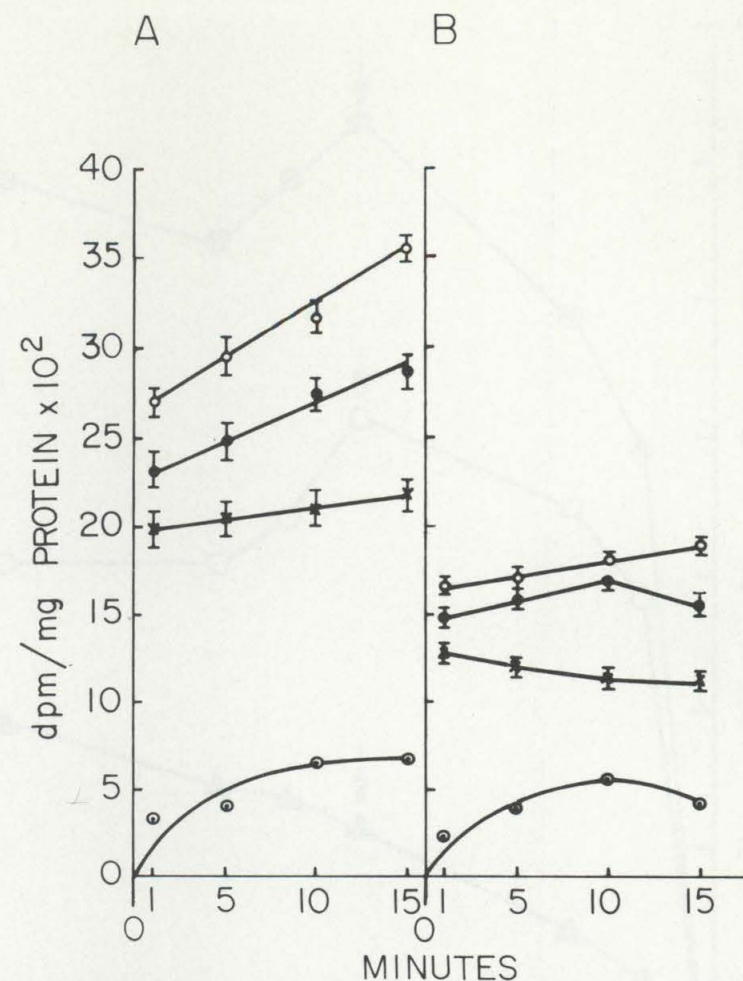
acteristic inhibition of labeled acetate incorporation was evident at 37°C (Table 1).

Effect of inhibitors

The effect of the metabolic inhibitors fluoroacetate and fluorocitrate on the incorporation of labeled acetate into the synaptosomal protein was measured (Table 2). The additional effect of veratridine was also determined. Fluoroacetate at 10 mM inhibited the incorporation of the tritiated acetate by 31–34%. Fluorocitrate at 1 mM inhibited the incorporation of the tritiated acetate by 34–43%. The presence of veratridine was not additive but consistently increased the inhibition caused by the fluoroacetate and fluorocitrate to a small extent. The inhibitory effects were somewhat higher in the base-hydrolyzed, acid-volatile fraction than in the total incorporated radioactivity. Fluoroacetate at 1 mM concentration had a slight effect in inhibiting the incorporation of the labeled acetate (data not shown).

Effect of acetate concentration

Rates of incorporation were measured at 30 s with increasing amounts of tritiated acetate (Table 3). The incorporation of total radioactivity, as well as the acid-hydrolyzable volatile radioactivity, was approximately proportional to the amount of ace-
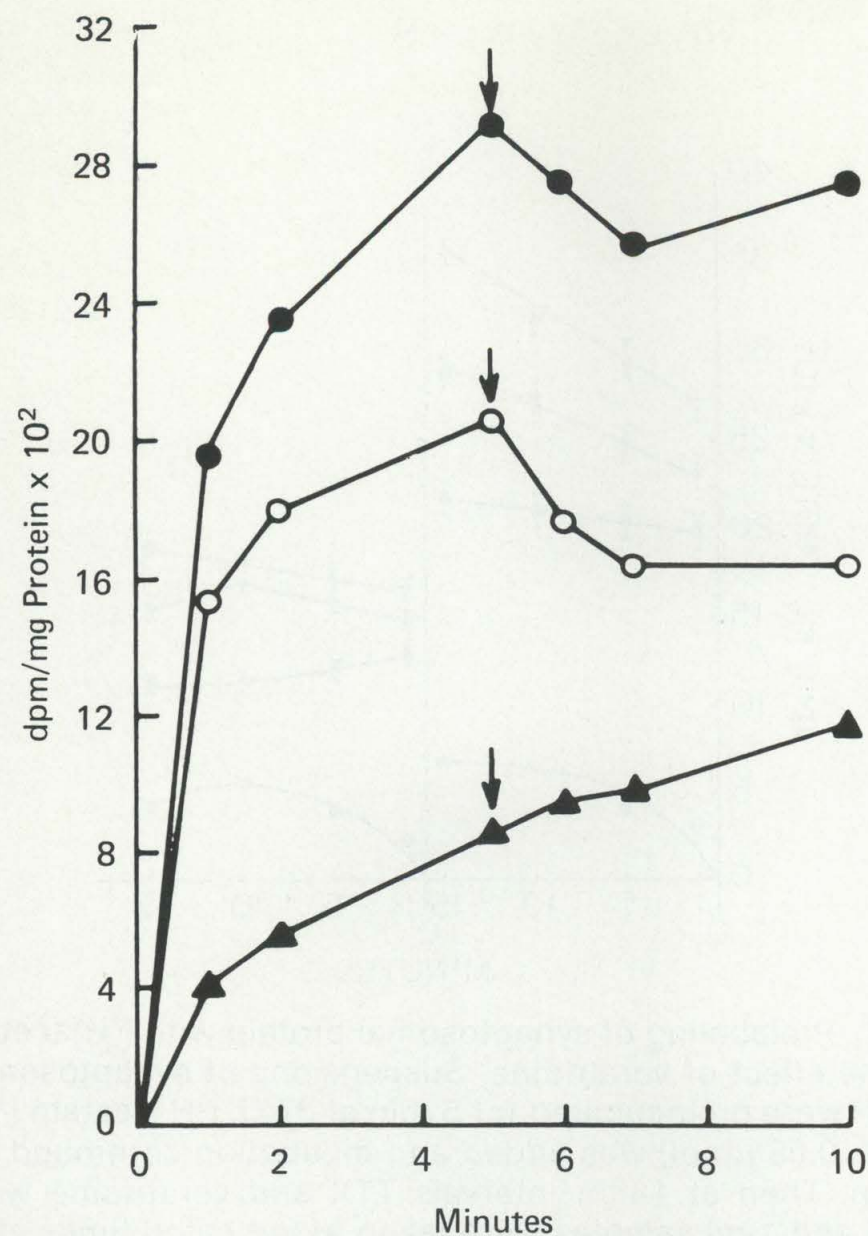
FIG. 5. The effect of the addition of veratridine to a synaptosomal suspension labeled with [³H]acetate. The synaptosomal suspension was preincubated for 10 min. [³H]Acetate was added (100 μCi, 0.05 μmol/ml) and 1-ml samples were taken at 1, 2, and 5 min. Veratridine was added (100 μM) and 1-ml samples taken at 6, 7, and 10 min. The 1-ml samples were immediately mixed with 0.1 ml 4 M perchloric acid. Aliquots of the proteins were counted, acid-hydrolyzed, and dried as described in Materials and Methods. The results are averages of two experiments. (●) Total radioactivity; (○) acid hydrolyzed - volatile radioactivity; ▲, nonvolatile radioactivity. Arrows indicate time of addition of veratridine.

tate added. The veratridine effect was evident at the three concentrations of acetate used.

Effect of Ca²⁺ and K⁺

The presence or absence of calcium (2 mM Ca²⁺ or 1 mM EGTA) did not have a significant effect on the action of veratridine. High concentrations of K⁺ (100 mM) also had no effect on the labeling of the synaptosomal protein by acetate (data not shown). Therefore the inhibition of incorporation of acetate does not appear to be related to depolarization of the membrane.

DISCUSSION

It is well established that veratridine has a major effect on sodium channels, causing them to open and thereby stimulating the entrance of Na⁺ and Ca²⁺ and the release of transmitter materials from presynaptic sites (Ulbricht, 1969; Blaustein et al., 1972; De Belleroche and Bradford, 1973; Narahashi, 1974; Abita et al., 1977). These effects are
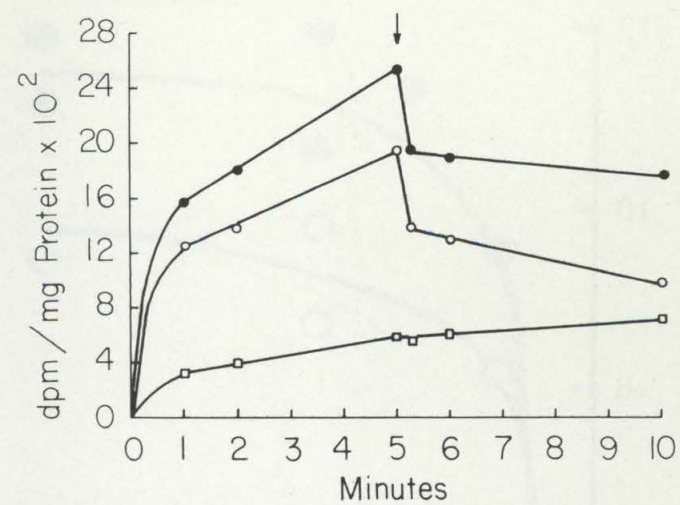

FIG. 6. Effect of addition of unlabeled acetate on the labeling of synaptosomal proteins by [³H]acetate. The experimental conditions were the same as in Fig. 5; however, unlabeled sodium acetate (10 mM) was added (instead of veratridine). The results are averages of two experiments. (●) Total radioactivity; (○) acid hydrolyzed - volatile radioactivity; (□) nonvolatile radioactivity. The arrow indicates the time of addition of sodium acetate.

completely inhibited by TTX. It is clear that TTX acts externally on membranes and has only a single known pharmacological action, that is, the specific blocking of sodium channels (Ritchie and Rogart, 1977; Catterall, 1980).

The data presented here suggest that incorporation of acetate into protein, probably as the acetyl group, is affected by veratridine. It is not proved, (although it might logically be expected) that this incorporation is in the NH₂-terminal amino acid, since a number of proteins are known in which the

TABLE 1. *Effect of temperature on the incorporation of* [³H]acetate into synaptosomal protein

	Minutes		
	1	2	5
Total radioactivity			
0°C[a]	50	36	20
Veratridine at 0°C[b]	0	0	14
Veratridine at 37°C[a]	22	25	31
Acid-hydrolyzed, volatile radioactivity			
0°C[a]	49	35	10
Veratridine at 0°C[b]	0	7	14
Veratridine at 37°C[a]	28	—	36
Acid-hydrolyzed, nonvolatile radioactivity			
0°C[a]	48	38	36
Veratridine at 0°C[b]	0	0	15
Veratridine at 37°[a]	0	—	14

Synaptosomes were incubated at 0 or 37°C. After 10 min of preincubation, veratridine (100 μM, final concentration) was added. One minute later [³H]acetate (100 μCi, 0.047 μmol acetate/ml) was added and samples taken at 1, 2, and 5 min and mixed with 0.1 ml of 4 M perchloric acid. See Materials and Methods for further details. Values are percentage of inhibition and are averages of two experiments.

[a] Compared with controls run at 37°C.

[b] Compared with the 0°C values.

TABLE 2. *Effect of inhibitors on the incorporation of*
[³H]acetate into synaptosomal protein

		Minutes	
Inhibitor	1	2	5
Total radioactivity			
Veratridine	25.0	29.1	31.0
Fluoroacetate	31.3	33.7	33.6
Fluoroacetate + veratridine	42.5	45.8	48.7
Fluorocitrate	33.5	33.0	43.4
Fluorocitrate + veratridine	40.6	45.3	46.1
Based-hydrolyzed, acid-volatile radioactivity			
Veratridine	30.2	37.0	31.0
Fluoroacetate	36.0	42.5	43.0
Fluoroacetate + veratridine	49.6	50.5	51.2
Fluorocitrate	42.2	39.6	45.0
Fluorocitrate + veratridine	45.3	51.0	49.1

Synaptosomes in Krebs-Ringer phosphate were preincubated for 5 min at 37°C. Fluoroacetate (10 mM) or fluorocitrate (1 mM) was added and incubation continued for 15 min prior to addition of veratridine (100 μM), followed in 2 min by [³H]-acetate (100 μCi, 0.047 μmol/ml). One-milliliter samples were taken at 1, 2, and 5 min and mixed with 0.1 ml 4 M perchloric acid. See Materials and Methods for further details.
Values are percentage inhibition and are averages of three experiments.

NH$_2$-terminal amino acid is blocked by the acetyl moiety. However, the acetate may also be bound in an acetyl-O-linkage or in glycoproteins. We are now attempting to identify the proteins into which the acetate is incorporated.

It has been demonstrated that actin acetylation probably occurs as a posttranslational process and that under certain conditions a small amount of nonacetylated actin may exist in brain preparations (Palmer and Saborio, 1978; Rubenstein and Deuch-

TABLE 3. *Incorporation of [³H]acetate into*
synaptosomal protein with increasing concentration
of [³H]acetate

[³H]Acetate (mM)	Total dpm/mg protein	
	− Veratridine	+ Veratradine
0.036	10,800	9035
0.072	18,860	17,610
0.144	36,730	33,370
Acid-hydrolyzable and volatile, dpm/mg protein		
0.036	7760	6400
0.072	14,770	13,385
0.144	27,380	24,770

One-milliliter aliquots of synaptosomal suspension were preincubated for 10 min at 37°C. [³H]Acetate (2.4 Ci/mmol) was added and the reaction stopped after 30 s by the addition of 0.1 ml 4 M perchloric acid. Veratridine (100 μM) was added to one set of tubes 2 min prior to the addition of the [³H]-acetate. Other details are given in Materials and Methods. Values are those of a representative experiment.

ler, 1979; Garrels and Hunter, 1979; Palmer et al., 1980). In the present study, hydrolyzable, acid-volatile radioactivity was found associated with crude synaptosomal actin; however, the radioactivity did not concentrate as the actin was carried through several steps of purification. These findings suggest that actin is probably not the major protein into which the acetate is incorporated.

The identification of the protein or proteins into which the acetate is incorporated is rendered difficult by the small amount of [³H]acetate fixed in the protein. We estimate on the basis of the added acetate that 1−2 pmol/mg protein is bound. If the synaptosomes contain acetate in concentrations usually found in tissue (Knowles et al., 1974; Tyce et al., 1981), then the amount fixed could be 3−6 pmol/mg protein. This amount is of the same order of magnitude as that found for the binding of TTX to synaptosomal membranes (Abita et al., 1977) and of TTX or saxitoxin to other conducting membranes (Ritchie and Rogart, 1977). The binding of these toxins is related to the density of the Na$^+$ channels in the membranes.

The inhibition of incorporation of acetate at 0°C and in the presence of the inhibitors fluoroacetate and fluorocitrate indicates that the incorporation is probably enzymatic. The toxicity of fluoroacetate is usually attributed to its conversion to fluorocitrate, and to the reversible inhibition of aconitase in the Krebs citric acid cycle (Peters, 1972). However, Goldberg et al. (1966) have shown that fluoroacetate at a dose of 100 mg/kg in mice did not lower ATP levels or affect high-energy phosphate stores in brain even though it produced convulsions at 20 and 40 min. Gonda and Quastel (1966) showed that fluoroacetate (1 mM) did not inhibit O$_2$ utilization, ^{14}CO$_2$ production from glucose, or ATP formation by rat brain cortex slices. Paradoxically, it did inhibit metabolism of acetate and its conversion to amino acids (Gonda and Quastel, 1966; Clarke et al., 1970). Since fluoroacetate can be converted to fluoroacetyl CoA, it is possible that its toxicity may be due in part to fluoroacetylation of synaptosomal protein. In addition, Kun et al. (1977) have shown that fluorocitrate is bound to protein associated with the citrate transport system in mitochondrial membranes. Further work is required to determine more specifically whether or not these inhibitors affect membrane function, e.g., uptake or release of transmitter substances or ionic flux, by binding to membrane proteins.

The chase experiments with unlabeled acetate support the idea that the acetylation process is rapid and reversible. The results of the experiments in which the veratridine was added to the acetylated protein agree with the interpretation that in the presence of veratridine deacetylation of a portion of the protein occurs. The data suggest that the veratridine stimulates deacetylation rather than inhibits

acetylation of protein and thus establishes a new steady state in the ratio of acetylation to deacetylation. It is conceivable that deacetylation and acetylation of proteins can sufficiently change their conformation or polarity to permit movement of protein from less polar to more polar regions of the membrane and vice versa, and thus affect opening and closing of ionic channels and/or transmitter release.

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