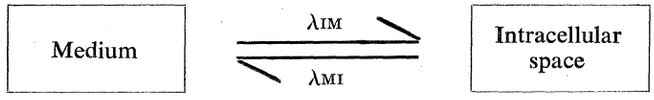
Table 1. Influence of temperature on kinetic parameters of sugar transport.



Incubation temperature (°C)	Medium pool size (μmole)	Steady state distribution ratio	Intracellular fluid pool (μmole)*	Fractional turnover rates per minute†		Net flux (μmole/min per 100 mg final wet weight)
				λ_{MI}	λ_{IM}	
37	4	2.5	0.263	0.105	0.0069	0.0276
25	4	4.0	.421	.037	.0039	.0156

* At 25°C intracellular space was found to be 52.6 percent of tissue weight. All calculations are based on 100 mg of tissue. † The rate constants are related by the equation $M \cdot \lambda_{IM} = ICS \cdot \lambda_{MI}$, where M is the medium concentration and ICS is the intracellular space.

ering the incubation temperature from 37° to 25°C, the renal tubule cells have a greater ability to concentrate α-MG. This paradox is explainable by a relatively greater inhibitory effect of temperature reduction on the sugar exit process than on the entry mechanism. These results differ from those obtained when the nonmetabolizable amino acid α-aminoisobutyric acid is affected by temperature reduction (*2*). A decrease from 37° to 27°C reduces the total amino acid flux by 60 percent; however, there is no difference in steady state gradients achieved since influx and efflux rate constants are reduced equally. In only one instance, that of cysteine transport, has a reduction of temperature resulted in an increased steady state gradient (*6*).

The phenomena reported here emphasize the importance of the efflux process in the establishment and maintenance of tissue concentration gradients. Our observations also underscore the need for analysis of tissue transport kinetics when perturbations are made in the incubation environment. This analytic requirement for amino acid and sugar uptake studies has been reviewed in depth previously (*7*). Our present knowledge of sugar transport in kidney cortex slices is derived mainly from the detailed reports of Kleinzeller and his associates (*3*, *8*) who have routinely employed a 25°C incubation temperature. Whether the latter observations reflect those at a physiological temperature remains to be determined.

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Calcium: Is It Required for Transmitter Secretion?

Abstract. *Ethanol multiplies miniature end-plate potential frequency independently of calcium ion concentrations and also multiplies calcium-dependent depolarization-evoked quantal release, to the same extent. This result implies a final common pathway, requiring little or no calcium, for both kinds of transmitter secretion. Chlorpromazine and hypertonicity act similarly to ethanol, but also depress depolarization-secretion coupling.*

Calcium ions, or divalent cations that can substitute for calcium, are essential to the process by which transmitter secretion by motor nerve terminals is accelerated by a presynaptic action potential or by presynaptic depolarization (*1*). A similar dependence on calcium has been demonstrated for a number of secretory systems, and it has been proposed that this reflects a fundamental similarity in the mechanism of such diverse secretory processes (*2*). However, it has been reported that raised osmotic pressure and black widow spider venom each cause an increase in the frequency of miniature end-plate potentials (MEPP's), which is the same in Ca^{2+}-free media as when Ca^{2+} is present (*3*). We have found that transmitter release by alcohols (*4*) is similarly independent of Ca^{2+}. Moreover, other "membrane stabilizers" (*5*) act in the same way as ethanol. The interaction of these agents and presynaptic depolarization indicates that the Ca^{2+}-independent release system shares at least its final step with the system by which transmitter is secreted in response to presynaptic depolarization. Thus, the actual transfer of transmitter quanta to the extracellular medium, as distinct from depolarization-secretion coupling, requires no Ca^{2+}.

Conventional methods were used for intracellular recording of MEPP's from end-plate regions of mouse diaphragms in vitro. To allow rapid changes of the bathing solution, we devised a superfusion system by which solution emerging from a nozzle of about 200 μm diameter flowed directly onto the surface of the muscle, keeping the solution there continuously mixed. The nozzle was connected by polyethylene tubes to reservoirs containing the various test solutions. With this system it was possible to change the solution in synaptic clefts within 5 seconds, as evidenced by changes of MEPP size when postsynaptic blocking agents or anticholinesterases were applied. To evoke transmitter release by nerve terminal depolarization, current was applied focally to end plates as described by Katz and Miledi (*6*). Using electrodes with tips of 25 to 50 μm diameter, we were able to obtain uniform polarization of the motor end plates, which are much more compact in the mouse than in the frog (*7*). Tetrodotoxin (Sankyo, 5×10^{-8} g/ml) was added to solutions to prevent action potential generation (*6*).

Increase of MEPP frequency by ethanol was found to persist in the absence of Ca^{2+}. In preparations superfused with a Ca^{2+}-free solution—calculated (*8*) free $[Ca^{2+}] < 10^{-10}M$—containing 10 m*M* ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA) and 0.5 m*M*

Mg^{2+}, spontaneous MEPP frequencies fell rapidly. Within a few minutes neither muscle contraction nor increase of MEPP frequency could be elicited by focal depolarization. However, even after several hours, although muscle resting potentials were uniformly low (about 30 mv), spontaneous MEPP's at a low frequency (about 0.2 per second) could be found at many end plates, and application of solutions containing ethanol caused abrupt increases in frequency of the MEPP's, as well as an increased amplitude and prolonged time course. The MEPP's were recorded under such conditions in the presence of 0.8*M* and 1.2*M* ethanol (Fig. 1A). In this example the preparation was soaked more than 24 hours in Ca^{2+}-free solution. The typical effect of different concentrations of ethanol on frequency of MEPP's, at one end plate, is shown in Fig. 2. The MEPP frequency was related exponentially to ethanol concentration, the release rate quadrupling for each 0.40*M* increment in ethanol concentration. In each case the effects of ethanol on MEPP frequency were rapidly reversible; frequencies returned within a few seconds to control values after withdrawing the drug.

In a bathing solution containing the standard $[Ca^{2+}]$ of 2 m*M*, and 0 to 0.8*M* ethanol, the effect of ethanol on frequency was the same as in the absence of Ca^{2+}. Thus, at eight junctions tested in 2 m*M* Ca^{2+}, 0.8*M* ethanol accelerated MEPP's by a factor of 16.5 ± 2.9 (mean ± S.E.); at six end plates in Ca^{2+}-free solutions (10 m*M* EGTA) the factor was 15.9 ± 3.1. When Ca^{2+} was present, concentrations of ethanol above 0.8*M* caused muscle contraction, preventing accurate determination of the effects of higher ethanol concentrations on MEPP frequency.

Other experiments showed that raised $[Mg^{2+}]$ did not inhibit the response to ethanol and, in solutions with $[Mg^{2+}]$ less than $10^{-5}M$ and $[Ca^{2+}]$ less than $10^{-10}M$ [10 m*M* ethylenediaminetetraacetate (EDTA), 7.5 m*M* total Mg^{2+}, no added Ca^{2+}], the typical response to ethanol persisted (Fig. 1B).

Thus, the multiplication of MEPP frequency by ethanol, in contrast to presynaptic depolarization, appears not to require Ca^{2+} (or Mg^{2+} as a substitute). The possibility that intracellular Ca^{2+} or tightly bound membrane Ca^{2+} might mediate the action of ethanol seems to us remote. The rapid reduction of spontaneous MEPP frequency which occurs in Ca^{2+}-free solutions containing EGTA or EDTA indicates that the Ca^{2+} which normally maintains MEPP frequency is located at sites accessible to Ca^{2+} from the extracellular medium, directly or indirectly. If ethanol were to act by translocating Ca^{2+} from a sequestered pool to these sites, we would expect a gradual depletion of this Ca^{2+} pool with continuous or repeated application of ethanol in Ca^{2+}-free solution with added EGTA or EDTA, and a corresponding reduction of the response of MEPP frequency to ethanol. We have been unable to detect any such effect. The multiplication of frequency by ethanol persists unchanged for at least a day in such a solution and, with repeated applications of ethanol, there is no tachyphylaxis.

Table 1. Interaction of depolarizing pulses and ethanol, chlorpromazine (CPZ), or hypertonicity (added sucrose) on transmitter release (MEPP's/sec).

Experiment	Transmitter release (MEPP's/sec)		
	No pulse	Pulse	Difference
Control	1.0	16.4	15.4
Ethanol (400 m*M*)	5.0	74.9	69.9
Recontrol	1.4	15.7	14.3
Control	3.6	29.4	25.8
CPZ (20 μM)*	9.2	115	105.8
CPZ (20 μM)†	45.6	168	122.4
Recontrol	5.3	55.7	50.4
Control	0.8	23.3	22.5
Sucrose (100 m*M*)	9.1	55	45.9
Recontrol	1.2	21.2	20
Control	11.8	~25‡	~13
Sucrose (200 m*M*)	114	224	110
Recontrol	12.3	28.4	16.1

* One minute in CPZ. † Three minutes in CPZ. ‡ By interpolation.

Other substances that act like ethanol included alcohols such as propanol, butanol, and chloral hydrate, and also pentobarbital, ether, and chloroform. The last three were tested in the presence of ethanol, so that relatively small changes of frequency could be detected; each had a considerable postsynaptic blocking action that forced the use of concentrations with rather small presynaptic effects. In its presynaptic action butanol is nearly three times as effective as propanol, which is in turn nearly three times as effective as ethanol.

Seeman and his colleagues (*9*) found that the above agents cause increased binding of Ca^{2+} to erythrocyte membranes; in contrast, local anesthetics and chlorpromazine displace membrane Ca^{2+} (*10*). However, we find that chlor-

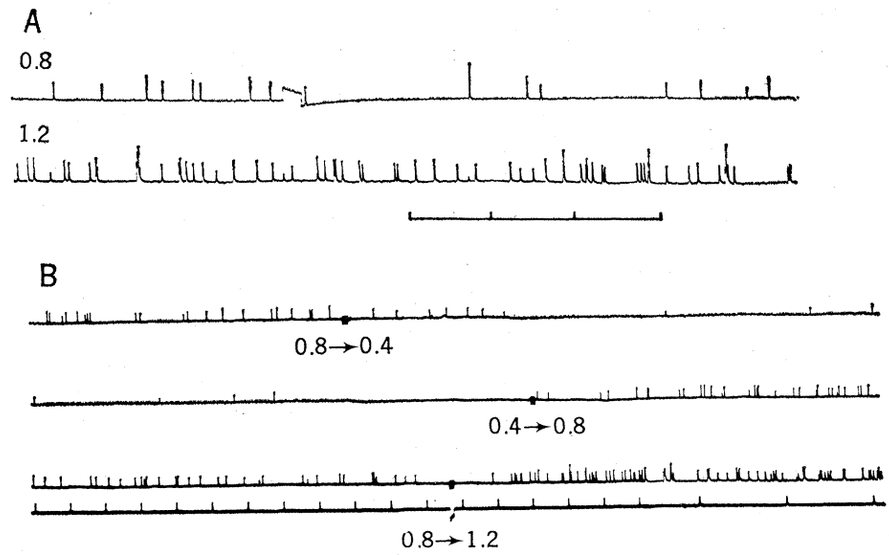

Fig. 1. MEPP's in Ca^{2+}-free solution plus ethanol. (A) After 24 hours soaked in Ca^{2+}-free solution containing 10 m*M* EGTA and 0.5 m*M* Mg^{2+} (otherwise standard solution). Upper trace, in same solution plus 0.8*M* ethanol. Lower trace 30 seconds after switching to 1.2*M* ethanol. Calibrating pulse on upper trace 2 mv. Time scale is seconds. (B) After 4 hours soaked and superfused with Ca^{2+}-free solution containing 7.5 m*M* EDTA (magnesium salt) and 2.5 m*M* EDTA (disodium salt). Upper trace, switch from 0.8 to 0.4*M* ethanol. Middle trace, switch from 0.4 to 0.8*M* ethanol. Lower trace, switch to 1.2*M* ethanol. Time scale is seconds. Note change of recording speed in 1.2*M* ethanol, only. Record continuous except 30 seconds omitted between upper and middle trace, and between middle and lower trace.

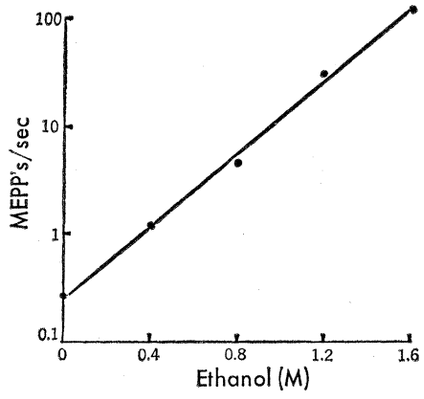

Fig. 2. The effect of ethanol on MEPP frequency. All bathing solutions were Ca^{2+}-free and contained 10 m*M* EGTA and 0.5 m*M* Mg^{2+}.

promazine also increases quantal transmitter release in the presence or absence of Ca^{2+}. Indeed, the high potency of chlorpromazine (20 μM increases release about twentyfold) indicates that the action of these drugs on release of transmitter closely parallels their stabilization of erythrocyte membranes, and is independent of any effect on calcium binding. Chlorpromazine, however, differs from ethanol in that its full effect takes several minutes rather than a few seconds to develop, and on withdrawal of the drug some acceleration of MEPP frequency remains for at least 10 minutes.

The relation between release rate and drug concentration for these agents is exponential, as is the relation between MEPP frequency and osmotic pressure, and (over a large range) that between transmitter release and presynaptic depolarization (*11*). It is therefore possible to distinguish between the alternative possibilities: (i) a Ca^{2+}-independent release system distinct from that concerned with liberation of transmitter by nerve impulse or (ii) one release system, which can be activated by nerve terminal depolarization, with Ca^{2+} an essential cofactor, and also by agents which do not require Ca^{2+} for their action. If the first alternative held, then the effect of alcohol and other agents on release would add arithmetically to the effect of nerve terminal depolarization. For example, if a given depolarization were to raise MEPP frequency from 1 to 10 per second, and a given amount of ethanol also raise it from 1 to 10 per second, applying the two together would cause a frequency of 19 per second. On the other hand, if there is a single mechanism which is activated independently by the two modes of stimulation, there should result a frequency of 100 per second.

Table 1 shows the effects of depolarization pulses (2 m*M* Ca^{2+} present) with and without added ethanol (400 m*M*), chlorpromazine (20 μM), and raised osmotic pressure (100 and 200 m*M* sucrose). The pulses were different in each experiment. The recontrol determinations were made about 1 minute after switching back to control solution from test solution. The hypothesis of independent release systems is contradicted (Table 1). The increase in MEPP's per second by a pulse was greater in the presence of the other activating agent. For ethanol the multiplication of frequency by the pulse was identical in the presence or absence of the drug. Thus, ethanol multiplies the transmitter release, which is secondary to depolarization, by the same factor as it multiplies spontaneous release. With the other agents the situation is slightly more complicated. Although the release evoked by depolarization was in each case increased, the multiplications were less than that of spontaneous release. This relation implies an action the same as that of ethanol, but, in addition, it implies an inhibitory effect on the acceleration of release by depolarization. The latter effect may reflect depression of a change of Ca^{2+} permeability of the presynaptic membrane in response to depolarization (*1*, *2*).

In general, we have found that MEPP frequency (F) can be expressed as follows:

$$\log F = \phi\,(V_m) + a_1 + a_2\,\Delta\pi + a_3\,[\text{drug}_1] + a_4\,[\text{drug}_2] \ldots$$

where a_1, a_2, a_3, and so on are constants. The function of the presynaptic membrane potential, $\phi\,(V_m)$, is flat for hyperpolarization and linearly ascending for at least moderate depolarization. It is a function also of Ca^{2+} and Mg^{2+} concentrations and can be influenced by osmotic pressure gradient ($\Delta\pi$), chlorpromazine, and perhaps other drugs. The exponential relation between F and the magnitude of stimulus by any of the agents that affect frequency suggests that there is an energy barrier, limiting release of quanta, which can be reduced in a graded fashion by any of these agents. The Ca^{2+} may act directly at this level or earlier in a series of steps. However, Ca^{2+} is by no means essential to the final step or steps by which transmitter is released. The role of Ca^{2+} is restricted to the mechanism by which presynaptic polarization is coupled to the release process. It follows that a common calcium dependence of many secretory processes need not reflect basically similar mechanisms. Instead, it would appear that although calcium ions commonly play a role in coupling physiological stimuli to secretion, there is no reason to suppose that this must always be the case. This conclusion is reinforced by the recent finding that stimulation of adrenocorticotropic hormone release by a number of agents is not associated with increased influx of calcium into cells of the adenohypophysis (*12*).

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