On the Kinetics of Acetylcholine at the Synapse

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Electrophysiologic, morphologic, and biochemical definitions of the synapse will be correlated spatially and temporally. Postsynaptic fatigue and facilitation follow oscillations of the free pool of acetylcholine, predicted by the kinetic theory and observed at the electric organ of *Torpedo marmorata*. The underlying thermodynamic instability exhibits properties of the Na^+—K^+-dependent hydrolysis of adenosine triphosphate and represents a necessary condition for synaptic memory.

«Le premier était de ne recevoir jamais aucune chose pour vraie, que je ne la connusse évidemment être telle.»
René Descartes (1636)

It is known that the vesicle hypothesis of synaptic transmission of signals, proposed by Katz [1] to unify the hypothesis of neurohumoral transmission [2], the theory of quantal components in miniature endplate potentials [3], and the observation of presynaptic vesicles [4] containing acetylcholine (AcCh) in sufficient amounts [5], has failed thus far to be verified by morphologic methods [6], where only pinocytosis could be proved under specific conditions [7]. Biochemically, too, Israël and Dunant discovered that, during periodic stimulation of the electric organ of *Torpedo marmorata*, the pool of $AcCh_{bound}$, inaccessible to the AcCh-esterases during homogenization and bound to the observed vesicular fraction, does not participate in the immediate hydrolysis and synthesis of the total acetylcholine, $AcCh_{tot}$, except for a decrease after prolonged stimulation [8–10]. Only the pool of 'free' acetylcholine, $AcCh_{free}(t) = AcCh_{tot}(t) - AcCh_{bound}$, depends on time t after onset of stimulation, $t \geq 0$, and correlates to the modifications of the amplitudes, $PSP(t)$, of the postsynaptic potentials. $AcCh_{free}$, though of unknown subcellular localization, proves to be kinetically independent from $AcCh_{bound}$, because their specific radioactivities due to [^{3}H]Ac or [^{14}C]Ch do not mix [8, 9]. Postsynaptic potentials may arise when vesicles do not contain AcCh [10], while they disappear completely when the pool of $AcCh_{free}$ in cholinergic neurones of *Aplysia* is specifically hydrolyzed [11].

From a cartesian point of view [12], the vesicle hypothesis is based on plausibility arguments, but not on evidence. Neurohumoral transmission (transport of AcCh across the synaptic cleft) has not been directly observed. Presynaptic release can therefore not be a priori derived from postsynaptic potentials. The total number of quantal components in miniature endplate potentials, which is to be compared with the number of presynaptic vesicles, is not measurable when they obey Poisson statistics. In the range of binomial statistics, values below 10 have been reported allowing only for eventual vesicular contribution to postsynaptic potentials [13]. While descriptively sufficient for many observations, the vesicle hypothesis does not follow by necessity [1].

The molecular membrane theory of Nachmansohn [14], on the other side, is based on necessary biochemical properties required for membrane excitations, in particular the localization at neuronal membranes of an enzymatic action faster than electrophysiologic excitation: the hydrolysis of AcCh by the enzyme AcCh-esterase (EC 3.1.1.7). Nachmansohn's theory would have predicted the observed accessability to AcCh-esterase of the pool of $AcCh_{free}$ responsible for the PSPs. It is, however, insufficient in its original form to correlate strictly with all physiologic and pharmacologic data. A unification with properties of receptors [15] has been proposed [16]; however, a decisive experimental test will be difficult; rigorously, it requires, among other things, measurement of the kinetics of AcCh processing at the membrane during excitation.

[1] "Plausibility estimates are *not* falsifiable. Neither, of course, are they verifiable." (Popper, K.: The Logic of Scientific Discovery, p. 191. London: Hutchinson 1972)

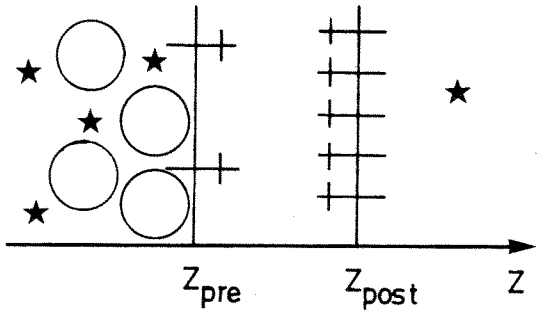

Fig. 1. The idealized synapse, defined by (1), depends on only one spatial coordinate z; this implies completely plain membranes at $z = z_{pre}$ and $z = z_{post}$ as well as homogeneity parallel to them. Therefore, the postsynaptic potential is constant along the postsynaptic membrane $z = z_{post}$ and is spatially correlated with the biochemical processes at this site. Approximately, the endplate of the electric organ of *Torpedo marmorata* depends only on z, including the AcCh-esterase activity (†), the choline acetylase activity (⋆), and the density of vesicles (○) (qualitatively according to [23, 24])

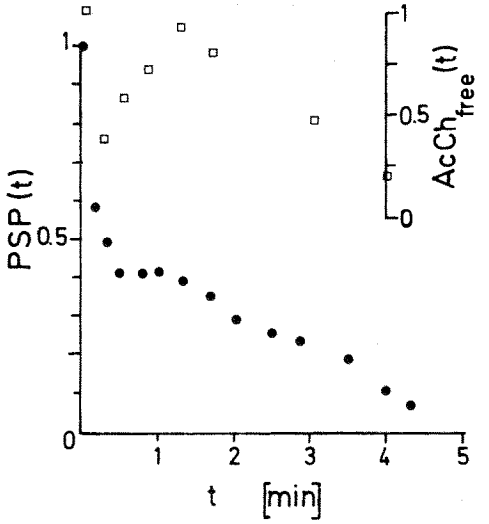

Fig. 2. The modifications of the amplitudes PSP(t) (●) of the postsynaptic potentials during periodic stimulation are temporally correlated with the hydrolysis and synthesis of the free pool of acetylcholine, $AcCh_{free}(t)$ (□), observable at the electric organ of *Torpedo marmorata* (from [9]). This example: stimulation frequency $\nu = 5/s$, temperature $T = 19\,°C$; normalized to the initial value at onset of stimulation, time $t = 0$. Oscillations with a period of 2 min [Eq. (4)] are predicted for these conditions from the temporal difference between the first minimum and maximum of $AcCh_{free}$ reproducibly observed [9]. After 4 min, i.e., 1200 stimuli, the kinetic pools are not preserved, and only rather exponential fatigue is obtained

Definition

A theory of 'the synapse' must correlate the morphologic synapse [17, 18] with its electrophysiologic definition [19, 20] as well as with its biochemical mechanisms [21, 22].

For this purpose, we define a one-dimensional synapse by

$$\{n_i(z, t)\} \tag{1}$$

which would allow this correlation spatially [23, 24] (Fig. 1).

The set $\{i\}$ may imply all molecular or ionic species i describing the morphologic, biochemical, and electrophysiologic observations. n_i is the respective mole number in a compartment at coordinate z.

No biological synapse fulfills definition (1), but endplates come close to it particularly in the electric organ [25] of *Torpedo marmorata*, which is poor in Schwann cells at the interface and rather homogeneous parallel to the membranes, morphologically as well as histochemically. Since it is homogeneous and rich in AcCh and related enzymes, this system may also permit quantification of the observables of the synapse (1). For example, at rest $t \leqq 0$, the metabolic pool $AcCh_{free}$ (to be denoted by $i = 1$) can be quantified (e.g., $n_1(t \leqq 0) = 760\,\text{nmol/g}$ of wet tissue) and defines the unity in Figure 2. Its temporal evolution $n_1(t)$ during periodic stimulation is measurable after homogenization at a time $t > 0$ (experimental variance $\Delta t \sim 1\,\text{s}$). The following analysis of the underlying kinetics may therefore average over several stimuli within Δt.

According to the spatial (Fig. 1) and temporal (Fig. 2) correlations, this analysis is also relevant to the kinetics of the modifications of the PSPs.

Kinetic Degrees of Freedom

A kinetic theory is, in principle, measurable directly, however, only in vitro and under specific conditions [26]. Its general formulation is

$$dn_i/dt = v_i(\{n_k\}); \quad i = 1, 2, 3, \ldots, K. \tag{2}$$

The rate v_i contains positive and negative terms describing formation and utilization of n_i. v_i is in general a nonlinear function of the variables n_k defining the system, e.g., the synapse (1). Spatial inhomogeneities are approximated by sufficiently small homogeneous compartments.

Each complete kinetic system possesses at least *one* stationary state $\{n_k^0\}$ defined by $v_i^0 = 0$. In the vicinity of that state the solutions are

$$n_i(t) = n_i^0 + \sum_{\lambda=1}^{K} n_i^\lambda \exp \omega^\lambda t;$$
$$\omega^\lambda = \omega_{real}^\lambda + \omega_{im}^\lambda \sqrt{-1}. \tag{3}$$

An instable mode $\omega_{real}^\lambda > 0$ leads to nonlinear solutions deviating from the exponential or sinusoidal

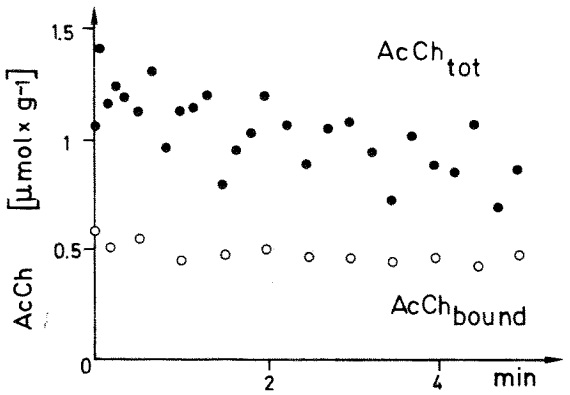

Fig. 3. Total acetylcholine (●) of the electric organ of *Torpedo marmorata* during sustained stimulation with frequency $\nu = 10/s$ (from [27]) is consistently characterized by oscillations with a period of 1 min. These prove the instability, upon stimulation, of the stationary kinetic state of this synapse, which, therefore, fulfills a necessary criterion for evolution. The compartment bound to the vesicular fraction, $AcCh_{bound}$ (○), is stable, and only the free pool $AcCh_{free} = AcCh_{tot} - AcCh_{bound}$ is responsible for the kinetics of this synapse

shape (3). The number, K, of independent variables (degrees of freedom), n_k, is equal to the number of eigenvalues, ω^λ. To determine K, the quasilinear approximation (3) is necessary due to the lack of any general theory for nonlinear differential equations. It will, however, turn out to approximate the observations. At rest $\nu = 0$, $t \leqq 0$, only a stationary state is observed. During stimulation, e.g., for $\nu = 5/s$ at $T = 19\,°C$, at least $K = 2$ eigenvalues

$$\omega^{1,2} \approx \pm \pi \sqrt{-1}/\text{min} \tag{4}$$

are necessary to fit the observations (see Fig. 2). The rapid decay from the initial condition $n_1(0)$ is due to a transient solution that will not be investigated here. The late decrease of $AcCh_{free}$ and $AcCh_{bound}$ [8–10] can also not be described, because the kinetic pools are not preserved in that state [9]. In the intermediate time interval, Eq. (4) predicts rather undamped stationary oscillations of a period of about 2 min for these experimental conditions.

Using a more rapid sampling technique, this has been verified [27] in good approximation (Fig. 3; for $\nu = 5/s$ see Fig. 5). The reproducibility of the fitted periods justifies the description by oscillations.

Biochemical oscillations are usually analyzed by sufficient molecular model reactions [28, 29] that are, by necessity, difficult to derive [2].

[2] Kinetic observations require defined experimental conditions $v_i'(n_j) = v_i(n_j; \{n_{k \neq j}\} = \text{constant})$. By consequence, a complete nonlinear system $v_i(\{n_k\}) = f_i + \sum_j g_{ij} n_j + \sum_j \sum_k h_{ijk} n_j n_k$ is reduced from third to second matrix order: $v_i'(n_j) = f_i' + \sum_j g_{ij}' n_j + \sum_j h_{ijj} n_j^2$; all f, g, h constant with respect to $\{n_k\}$. Therefore, in general, nonlinear oscillations cannot be completely analyzed in kinetic terms

Thermodynamic Instability

Thermodynamics may be more advantageous for a unique analysis of complex kinetic systems far from equilibrium. It provides rather general criteria of stability [30]. Fluctuations can also be described if the generalized thermodynamic potential is known [31]. For a stable stationary state, the second-order variation of the entropy, $\delta^2 S < 0$, can be used as a Lyapunoff function in order to formulate the sufficient criterion of stability

$$\mathrm{d}\delta^2 S/\mathrm{d}t = \frac{1}{T} \sum_j \delta w_j \cdot \delta A_j > 0 \tag{5}$$

with the rate w_j of normal coordinate ('reaction') j, and the corresponding affinity A_j for a kinetic system. This criterion is violated by cycles of autocatalytic properties, discussed by Eigen [32] in relation to the selection of molecular species in evolution; are there analogies to long-term modification of neural networks? Obviously, for the short-term modification of the synapse (Figs. 2 and 3), the stability criterion is violated, too.

From (5) follows a *necessary* criterion for instability: at least one coordinate $j = 0$ has to obey

$$\delta w_0 \delta A_0 < 0. \tag{6}$$

Consider the minimum of only one irreversible reaction $X + \sum_\alpha Y_\alpha \xrightarrow{w_0} \sum_\beta Z_\beta$ and of one reactant X determining the fluctuations, $\delta n_X \neq 0$. Then

$$(\delta w_0/\delta n_X)(\delta A_0/\delta n_X) < 0.$$

Choosing $\delta w_0/\delta n_X > 0$, we define the simplest irreversible reaction where the flux w_0 monotonically increases with the mole number of a reactant. The instability must then be due to the variation of the corresponding thermodynamic force: $\delta A_0/\delta n_X < 0$.

The meaning of a thermodynamic force is not clear in nonequilibrium systems generally. Attempts for a definition [31] require the knowledge of the kinetic system, which we are inversely interested in deriving. Therefore we approximate A_0 by

$$RT \log (n_X \prod_\alpha n_{Y_\alpha} / \prod_\beta n_{Z_\beta})$$

as in equilibrium [3], with gas constant R and the product operator $\prod$. For instability, it follows that the mole numbers of at least two reaction products $Z_{1,2}$ have to be proportional to n_X. The mole number

[3] Flux w_j and thermodynamic force A_j of reaction j are defined by $\delta n_i = \sum_j \nu_{ji} \delta w_j$; $A_j = -\sum_i \nu_{ji} \mu_i$; with chemical potential $\mu_i = \mu_i^0 + RT \log a_i$, activity $a_i \sim n_i$ and stoichiometric coefficient $\nu_{ji} > 0$ of reaction product ($\nu_{ji} < 0$ of reactant) i

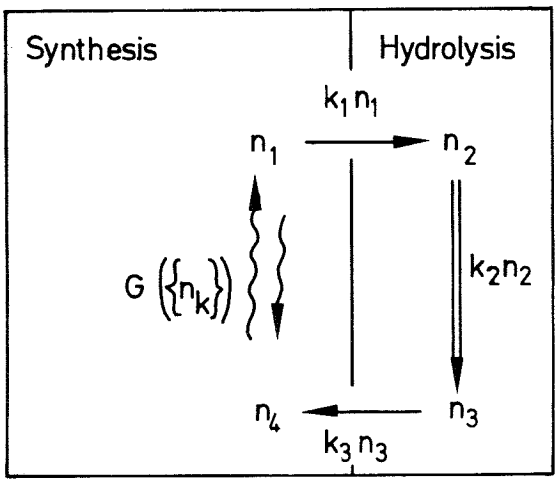

Fig. 4. The two functional compartments and the necessary cycle of $AcCh_{free} = n_1$. Due to the rapid hydrolysis (rate $k_2 n_2$, averaged over several stimuli) in a small volume (compare Fig. 1), the transport 'release' ($k_1 n_1$) and 'uptake' ($k_3 n_3$) is only passive, i.e., from high toward low concentrations. Oscillations of n_1 must be caused by at least one instable reaction coupled to the rate-limiting nonlinear synthesis $G(\{n_k\})$. The hydrolysis rate is correlated to the postsynaptic potentials spatially (Fig. 1) and may therefore contribute to the temporal correlation (Fig. 2) between $n_1(t)$ and the synaptic modifications PSP(t)

n_X then increases in a de facto autocatalytic way:

$$dn_X/dt = +k_X n_X - F(\{n_k\}), \quad k_X > 0. \tag{7}$$

We note that autocatalysis $X \to 2X$ is only a special case of, but not a requirement for, instability and evolution of kinetic systems. It is necessary to supplement a nonlinear function $F(\{n_k\})$ to avoid 'explosion' of the system. This instable reaction has to be coupled to a rate-determining process in the cycle of $AcCh_{free}$ that will now be derived. Identification of species X and reaction (7) will be discussed afterwards.

The Necessary Cycle of Acetylcholine

At least, $n_1 = AcCh_{free}$ and one of its hydrolytic products, choline (Ch) or acetate (Ac), have to be included in the set $\{n_i(t)\}$ defining the synapse (1). During periodic stimulation, at least two functional compartments of AcCh, a synthesizing and a hydrolyzing one, are manifest in the observations (Fig. 2). We need not specify their localization, but we may consider mainly presynaptic synthesis and postsynaptic hydrolysis according to Figure 1. A decisive test of this hypothesis of neurohumoral transmission, however, would require a quantitative histochemical analysis of these enzymes. In conclusion, the set $\{n_i(t)\}$ contains at least 4 members, 2 in each compartment, connected by at least 4 reaction steps (Fig. 4).

Because the observed specific radioactivity of $AcCh_{free}$ is preserved during some minutes of stimulation (e.g., 4 min for the example in Fig. 2), the four-membered set is closed in the sense that

$$\sum_{i=1}^{4} n_i(t) = \text{constant}. \tag{8}$$

It follows the necessary cycle of acetylcholine (Fig. 4):

$$\begin{aligned} dn_1/dt &= -k_1 n_1 + G(\{n_k\}), \\ dn_2/dt &= -k_2 n_2 + k_1 n_1, \\ dn_3/dt &= -k_3 n_3 + k_2 n_2, \\ dn_4/dt &= -G(\{n_k\}) + k_3 n_3. \end{aligned} \tag{9}$$

The nonlinear synthesis rate $G(\{n_k\})$ has to account for the observed deviations from the sinusoidal shape (3) at low stimulation frequencies [27], and to ensure coupling to the unstable reaction (7). To make the theory applicable to the experiment, further specification of the nonlinear functions F and G is inevitable. Without conclusive data on the substructure of rate-determining processes, we try the mathematically simplest possibility, a quadratic coupling of $AcCh_{free}$ to X:

$$F(\{n_k\}) = G(\{n_k\}) = k_{X1} n_X n_1. \tag{10}$$

We note that we have phenomenologically introduced two *necessary* parameters: k_X to account for instability and k_{X1} for nonlinearity.

With specification (10), Eq. (7) and the first equation in (9) represent an autonomous cycle between $AcCh_{free}$ and X of the type discussed by Lotka and Volterra [33] with the following properties:

1) It possesses the necessary number of kinetic degrees of freedom, $K = 2$, Eq. (4), corresponding to n_1 and n_X.

2) It violates stability condition (5) giving rise to weak instability $\omega^{\lambda}_{real} = 0$.

3) It therefore already possesses completely sustained oscillations in the quasilinear vicinity of its stationary state, i.e., Eq. (3) holds with $\omega^{1,2} = \pm 2\pi\sqrt{-1}/\tau$ as in Eq. (4).

4) The period τ of the oscillations does not depend on their amplitudes in the quasilinear, and only weakly in the nonlinear neighborhood of the stationary state, i.e., at the same period τ rather different amplitudes should appear.

5) Nonlinear deviations from the sinusoidal shape occur only during longer oscillation periods.

6) The phenomenologic parameters k_1 and k_X give rise to a dispersion that, in the quasilinear range, obeys

$$\tau = 2\pi/\sqrt{k_1 k_X}. \tag{11}$$

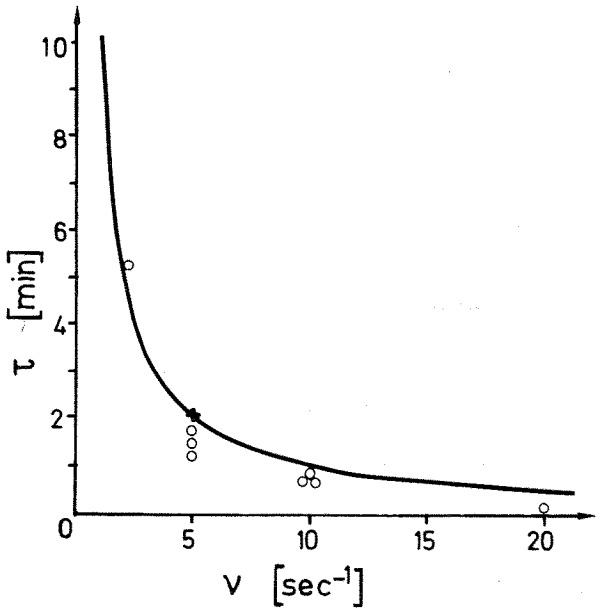

Fig. 5. For small stimulation frequency ν, the minimally necessary kinetic system of Eqs. (7), (9), (10) (Fig. 4) leads to a hyperbolic dependence of the oscillation period τ on ν [the cross + is predicted from Eq. (4), Fig. 2, the line from Eq. (12)]. Each circle (o) represents one observed oscillation of $AcCh_{free}$ such as is shown in Fig. 3 (from [27])

Comparison with the Experiment

The reported stationary oscillations of $AcCh_{free}$ [27] are compatible with properties 1) to 5) of our minimal description. To test property 6), we investigate the observable influence of the stimulation frequency ν on the oscillation period (11). We assume that the kinetic coefficients k are analytical functions of the stimulation frequency ν, i.e., that there exists a Taylor expansion $k(\nu)=k(0)+\nu\left.\frac{\delta k}{\delta \nu}\right|_0+O(\nu^2)$ with a linear range for small ν. At rest $\nu=0$, hydrolysis of $AcCh_{free}$ (Fig. 4) is negligible [34], and no instability is observed [9]. Hence, $k_1(\nu)\geqq 0$, $k_X(\nu)\geqq 0$ for $\nu\geqq 0$.
It follows that the period (11) obeys a hyperbolic law for small stimulation frequencies:

$$\tau(\nu)=\nu^{-1}\cdot 2\pi\Big/\sqrt{\left.\frac{\delta k_X}{\delta \nu}\right|_0\cdot\left.\frac{\delta k_1}{\delta \nu}\right|_0}\,. \qquad (12)$$

The constant factor can be determined from the predicted period $\tau\approx 2$ min at $\nu=5$/s (Fig. 2). As shown in Fig. 5, the derived law (12) is verified reasonably well in the experiment. The minimally necessary kinetic system (7), (9), (10) is therefore sufficient for the description of these data on AcCh oscillations.

Does Na^+—K^+-ATPase (EC 3.6.1.3) Cause Synaptic Modifyability?

Kinetic coefficients that are proportional to the stimulation frequency are initiated by each stimulus in the same way. The corresponding molecular processes must therefore be localized either at or sufficiently close to the excitable membrane. As $k_1\sim\nu$, $k_X\sim\nu$ is descriptively sufficient, the *membrane localization* of the corresponding molecular processes can be predicted.
Concerning k_1, membrane localization is histochemically confirmed (compare Fig. 4 with Fig. 1); $k_1\sim\nu$ may be caused simply by the same passive diffusion constant for $AcCh_{free}$ during each stimulus.
Concerning k_X, the de facto autocatalytical reaction (7) should also be 1) membrane-localized, 2) initiated by each stimulus in the same way, and, in addition, 3) be coupled to AcCh synthesis, see (9).
Na^+—K^+-ATPase activity seems to be the only candidate not excluded by these criteria [35]. Therefore, adenosine triphosphate (ATP) may represent the unknown species X and should in this case oscillate temporally correlated to $AcCh_{free}$; in the perfusion fluid, such a temporal correlation of ATP has already been reported [36]. In addition, to generate the instability Eq. (7), Na^+—K^+-ATPase activity may be inhibited de facto autocatalytically

$$\delta(-dn_X/dt)/\delta n_X=-k_X$$

by each stimulus in the same way ($k_X\sim\nu$). A qualitatively similar reversal of Na^+—K^+-ATPase activity due to high-sodium, potassium-free medium has already been reported for resealed erythrocyte ghosts [37].

Is Presynaptic Kinetically Independent from Postsynaptic Acetylcholine?

Superimposed "rapid" oscillations of period 4–5 s have been reported recently [38]. Faster by an order of magnitude, these either are caused by a mechanism independent from the "slow" oscillations (Figs. 2–5), a hypothesis which has no histochemical support so far; or by a similar mechanism, but in independent compartments. The presynaptic axon terminal and the postsynaptic electroplax represent the only known histochemical possibility. Due to its larger synthesizing activity (Fig. 1), $z\leqq z_{pre}$ should correspond then to the "rapid", and $z\geqq z_{post}$ to the "slow" compartments.

This is a testable prediction, falsified, when *Torpedo* synaptosomes [39] produce the slow, and verified, if they produce the rapid oscillations alone. Confirmed

by a postsynaptic origin of the slow kinetics of ATP [40], it does explain the fact that only the slow, but not the rapid kinetics of the free pool of acetylcholine determines the time course of the physiological modifications of membrane conductivity [38] and postsynaptic potentials (Fig. 2) upon presynaptic stimulation of the electric organ of *Torpedo marmorata*.

The stimulus for this work arose out of discussions about the theory of evolution set up by Manfred Eigen, and its completion owes much to the support of Otto Creutzfeldt, Jean Scherrer, and Yves Dunant. The work was begun in cooperation with my friend Maurice Israël, to whom this paper is dedicated.

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