The RBC containing **1** were then incubated in buffered saline at 37°C, and fragility measurements were made at intervals. Typical results are shown in Fig. 1, which includes several fragility curves. In these measurements, the hemolysis is monitored as a function of decreasing salt concentration, which occurs linearly with time. The dashed curve is the control, a sample incubated with liposomes containing cholesterol but not **1**. The control showed no change in osmotic fragility after 80 minutes in saline at 37°C. Curve 1 was obtained for RBC exposed to a pulse of **1** (about 5 percent of the RBC cholesterol was exchanged for **1**) and then kept at 37°C in saline for 10 minutes; corresponding times in saline were 20 minutes for curve 2, 50 minutes for curve 3, 80 minutes for curve 4.

These observations show that incorporation of **1** into normal RBC membranes leads at first to a decrease in osmotic fragility (*19*); but after incubation for a time at 37°C an increase in fragility and hemolysis is achieved. That an "induction period" is observed before increased fragility may be rationalized in two ways. It may be that **1** is the precursor of the species that causes the hemolysis. That is, a rearrangement or decomposition product of **1** may be responsible for the increased fragility of the RBC, or the decomposition of **1** may initiate nonselective free-radical chain oxidation of unsaturated membrane lipids. Alternatively, the action of **1** may at first cause K^+ ions to leak out of the RBC faster than Na^+ ions leak in. This would cause the RBC to shrink initially and appear less fragile until most of the K^+ is gone (*20*).

In summary, we have found efficient formation of **1**, the product of attack of singlet oxygen upon cholesterol, after irradiation of RBC ghosts containing protoporphyrin. Incorporation of **1** into normal RBC membranes leads to increased osmotic fragility and eventual hemolysis of the RBC. Photohemolysis of RBC from EPP patients can be rationalized on the basis of these observations. We are performing several kinds of experiments to determine whether the photohemolysis mechanism that does operate involves singlet oxygen and **1** as important intermediates.

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Strychnine- and Pentylenetetrazol-Induced Changes of Excitability in Aplysia Neurons

Abstract. *In* Aplysia *neurons isolated from their synaptic input strychnine induces doublet discharges associated in voltage clamp with a decrease in the threshold for the inward current and a reduction and delayed onset of the outward current. Pentylenetetrazol causes oscillations and bursting behavior in normally silent cells together with an increased inactivation of the delayed outward current and induced or enhanced anomalous rectification.*

One experimental approach to the understanding of the neurophysiological basis of epileptic seizures has been the study of action of convulsant drugs (*1*). Unfortunately, there exists a myriad of convulsant agents with different, and possibly multiple, actions on nervous structures. Some of these drugs block inhibitory synaptic transmission, but these drugs constitute only a minority of the known convulsants. At the cellular level, focal epileptic activity is characterized primarily by high-frequency synchronous firing in association with oscillatory membrane potential changes (*1*). The experiments to be reported were undertaken to analyze the action of convulsant drugs responsible for repetitive firing at short intervals. Our hypothesis was that some convulsant may act by directly changing membrane properties related to excitability and stability.

The experiments were performed on neurons of the isolated abdominal ganglion of the marine mollusk *Aplysia californica*. Intracellular voltage recording and voltage and current clamping techniques were used to study the effects of two convulsants, strychnine and pentylenetetrazol (PTZ). The latter was chosen in comparison with strychnine because it does not seem to have a direct effect on synaptic transmission in *Aplysia*, while strychnine blocks acetylcholine, dopamine, and 5-hydroxytryptamine receptors in the ganglion (*2*). Previous investigations have demonstrated, furthermore, that both drugs produce high-frequency discharges with abnormally short interspike intervals and oscillatory membrane potential changes in the *Aplysia* neurons (*3*).

The soma of identified neurons (*4*) were penetrated with single or double microelectrodes (3 to 15 megohms). The drugs were added directly to the artificial seawater perfusate ($pH = 8.0$; 16°C). The effects studied generally took 5 to 8 minutes to become manifest, and all were readily reversible upon washing.

Over a concentration range of 0.01 to 1.0 m*M* strychnine blocked all excitatory and inhibitory postsynaptic potentials while it had only a slight effect on the inhibition of long duration (*2, 5*). Most neurons then fired in an irregular manner characterized by high-frequency bursts and doublets or triplets of spike appearance (Fig. 1A) (*6*). The following observations indicate that the short-interval doublets reflect a change in excitability: (i) they occurred in cells after axonal ligation blocked all synaptic inputs; (ii) they occurred in the white neurosecretory pacemaker cells (R-3 to R-13) which have little or no synaptic input (*4*); (iii) they occurred in silent cells after brief depolarizing current pulses and as anodal break phenomena; (iv) they persisted in higher strychnine concentrations (1 to 3 m*M*) when all excitatory synaptic transmission was blocked. On the other hand, PTZ (10 to 70 m*M*) induced doublets only in neurosecretory pacemaker cells.

The two drugs had pronounced, although different, effects on the action potentials of all cells studied. In the case of strychnine the most significant effect was an increase in spike duration up to three- to fivefold (Fig. 2A), correlated with a slowing of both the rising and decaying phases. In addition, spike overshoot and hyperpolarizing afterpotential were reduced by 5 to 20 percent, especially in higher concentrations (1 to 3 m*M*). In all cells PTZ reduced both spike overshoot and afterpotential, the reduction in the overshoot being significantly greater. Prolonged exposure to 70 m*M* PTZ generally resulted in a reduction of the spike amplitude to 10 to 20 mv.

Strychnine had virtually no effects on input resistance and caused slight reductions of the membrane potential (RMP), but PTZ had its most significant effect on these two parameters. Most cells depolarized by 10 to 15 mv or more following PTZ application. This depolarization was accompanied by high-frequency discharges and often led to the inactivation of the spike-generating mechanisms. On the other hand, a few cells, notably initially silent cells with high RMP (R-2), were unchanged or hyperpolarized and showed oscillations about their original RMP (Fig. 1B). These oscillations were often as large as 20 mv in amplitude, and, although they generally gave rise to a burst of spikes, they could occur in the absence of action potentials. Finally, cells which were depolarized by PTZ could be made to oscillate by the injection of hyperpolarizing current. All changes in RMP induced by PTZ and strychnine in different cells were in the direction opposite to E_{Cl} as determined by Cl^--dependent acetylcholine responses (*4, 5*), that is, both drugs do not change the RMP by increasing g_{Cl} (*7*).

As is shown in Fig. 1C for cell R-2, PTZ had two effects on membrane resistance: it increased the membrane resistance in the region of the resting potential (R_{RMP}) by an average of 41 percent in 11 cells, and it increased or introduced anomalous rectification (*8*).

Voltage clamping of *Aplysia* neurons demonstrates three kinds of membrane currents in response to a depolarizing (command) pulse. (i) There is a transient inward current (I_i) carried by both Na^+ and Ca^{2+} (*9*). The other two currents, which are presumably carried by K^+, are: (ii) a delayed outward current (I_o) which is inactivated in pacemaker cells when the command pulse lasts longer than 100 msec; (iii) after conditioning hyperpolarizing pulses of at least 400 msec, depolarization produces in some bursting cells a rapid transient outward current (I_A) which overlaps with I_i in time (*10*). In concentrations of 0.1 and 1.0 m*M*, strychnine reduced both I_i and I_o equally by 21 and 47 percent, respectively. Strychnine also increased the duration of I_i by 50 to 100 percent by increasing both its time to peak and its decay time. No shifts in the equilibrium potentials for the two currents were detected. These effects easily explain the strychnine action on the spike; the slow onset and decay of I_i and the slow onset of I_o account for the increased spike duration (*11*).

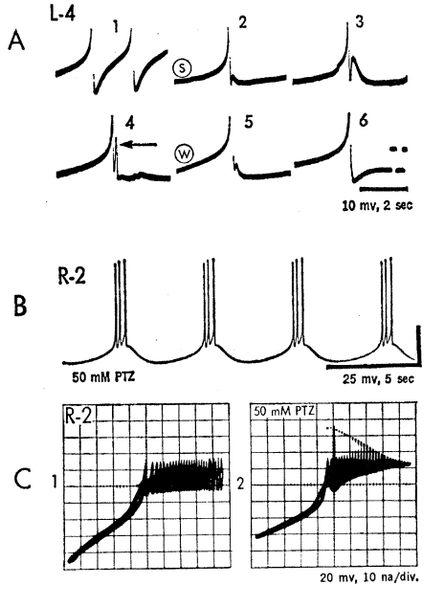

Fig. 1. (A) Initiation of doublet discharges in cell L-4 following the application of 1.0 m*M* strychnine (*S*). Progressive reduction of the hyperpolarizing afterpotential and increase of a depolarizing wave (frames 2 to 4) triggering a second spike after 11 minutes (arrow in 4); 5 and 6 illustrate reversibility after washing (*W*). Voltage calibration refers to pulses in frame 6. (B) Oscillation of the membrane potential and burst discharges in cell R-2, 8 minutes after 50 m*M* PTZ. (C) Pronounced anomalous rectification and changes in the delayed rectification in the current-voltage relationship of cell R-2 following the application of 50 m*M* PTZ. The current calibration refers to the horizontal axis; the injected current changed by 3 na/sec.

The abnormal discharges induced by strychnine are dependent upon the calcium and sodium concentrations of the seawater (*12*). Reducing the Ca^{2+} concentration induces in the presence of strychnine long-lasting depolarizations similar to the action of cobalt plus strychnine (*12*). On the other hand, increasing the Ca^{2+} concentration eightfold or replacing Na^+ by tris(hydroxymethyl)aminomethane reduces but does not block completely the amplitudes of the depolarizing waves, thus preventing the initiation of a doublet. The above procedures also reduce the changes in the RMP due to strychnine. Strychnine interacts with the two ionic channels carrying the inward current, that is, Na^+ and Ca^{2+} (*9*); the reduction of the calcium spike by strychnine in sodium-free solution is comparable to the reduction of the sodium component of the spike in calcium-free solution.

Does strychnine act by interfering with a calcium system controlling Na^+, K^+ conductances? Although there are some similarities between the action of strychnine on *Aplysia* membranes and the effect of low calcium on squid and lobster axon (*13*), strychnine seems to have a more unique action. First, as is shown in the voltage clamp (Fig. 2A), strychnine resembles local anesthetics and barbiturates in reducing both inward and outward currents (*14*). This is even more true for the effect of PTZ on I_i and I_o (see below). Different from the effect of those substances but similar to the effect of low calcium, strychnine very often decreased the potential necessary to produce a maximum of I_i by 5 to 15 mv; in other words, it shifted the potential dependency of the Na^+ and Ca^{2+} conductances to lower voltages. This effect should be an im-

portant factor for the generation of oscillations underlying depolarizing waves and involves both Na^+ and Ca^{2+} (*15*). But the effect of strychnine differs from that expected for low calcium in that the threshold for the outward current is shifted to higher voltages (Fig. 2C). These shifts provide a satisfactory explanation for the occurrence of the doublets in most cells which we examined, and strychnine produces *I/V* (current/voltage) relationships similar to those in experiments in which sustained activity was induced in Ranvier nodes by lowering calcium concentration and adding TEA to the bathing medium (*16*). Strychnine did not specifically affect I_A in a manner that would explain the doublets.

Pentylenetetrazol also reduced I_i and I_o; the reduction for both currents averaged 24 percent with 30 m*M*, but while increasing the PTZ concentration to 70 m*M*, I_i was reduced by an additional 35 percent with no further change of I_o. These results explain the somewhat large reduction in spike amplitude produced by higher concentration, since, as in the case of strychnine, equal reductions in both conductances could have minimal effects on the spike amplitude (*17*). Pentylenetetrazol also increased the voltage necessary for the maximal inward current. All these effects favor a decrease in excitability and do not seem to be related to the described "convulsive" effects of this drug. Pentylenetetrazol dramatically increased K^+-inactivation during maintained depolarizing commands (Fig. 2B). The depolarization and the increase in membrane resistance, typical for the effect of PTZ, probably resulted from this increased potassium inactivation.

Our results demonstrate that two convulsant agents, one which interacts with postsynaptic receptors and one which apparently does not, both produce an epilepsy-like spike pattern in *Aplysia* neurons owing to several direct effects on membrane excitability. The different effects of strychnine and PTZ on different cell types in this ganglion are similar to their different effects on mammalian neurons, for example, motoneurons, pyramidal tract cells, interneurons, Purkinje cells, and so forth, and underline the fact that there is no "standard neuron" or membrane for the study of epileptic phenomena; this makes extrapolation and generalization extremely hazardous. Nevertheless, it is apparent that similar changes in cell membrane properties, for example in spinal cord interneurons (*18*), could be an important factor in the genesis of epilepsy.

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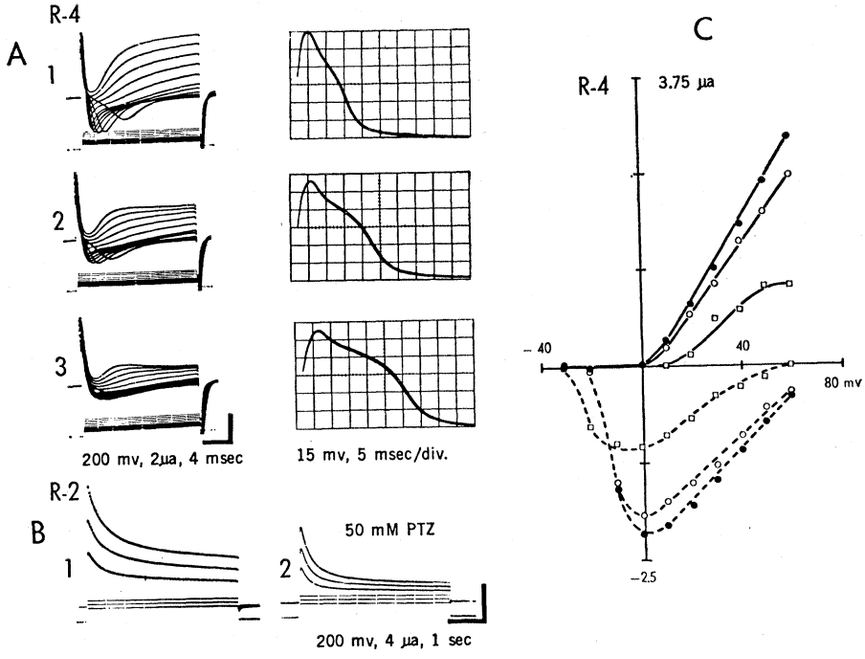

Fig. 2. (A) Changes in spike duration and amplitude of cell R-4 related to different concentrations of strychnine (right column). The left column shows families of membrane currents associated with step depolarizations (lower records) of voltage clamps during the same time. Reduction of both the transient inward and the outward current (latter shown upward). Holding potential $Vh = -40$ mv, command pulses (Vc) = -10 to $+70$ mv. (1) Control; (2) 0.1 m*M* strychnine; (3) 1.0 m*M* strychnine. (B) Enhancement of the inactivation of the delayed rectification outward current in R-2 after application of 50 m*M* PTZ in (2). $Vh = -40$ mv; $Vc = +30$, $+50$, and $+70$ mv. (C) Current-voltage relationship of the voltage clamp experiment shown in (A). The application of strychnine caused a reduction of the inward current (broken lines) and shifted its maxima on the voltage axis to the left. At the same time the outward current (solid lines) is also reduced but shifted to the right. ●, Control; ○, 0.1 m*M* strychnine; □, 1.0 m*M* strychnine.

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Detection of Inborn Errors of Metabolism: Galactosemia

Abstract. *Radioautography of cultured, human, galactosemic and nongalactosemic cells shows that, in the presence of 0.05*M D-*galactono-γ-lactone, the former incorporate much less galactose in acid-insoluble form than the latter. Presumably the lactone inhibits incorporation of the labeled galactose into pathways which do not require galactose-1-phosphate uridylyltransferase activity. Definite differences between the galactosemic and nongalactosemic condition can be demonstrated with as few as 100 to 1000 cells. This approach may be useful in facilitating prenatal detection of several kinds of inborn errors of metabolism.*

The detection of galactosemic and similar biochemical defects in utero requires growth in vitro of cells biopsied from the amniotic fluid of a patient. Obtaining a sufficient number of growing cells may require several weeks during which the patient may undergo emotional stress, and the difficulty of terminating a defective pregnancy may be increased. If a highly reliable radioautographic procedure could be devised, the number of cells needed might be materially decreased with consequent decrease in time required for cell growth and improvement in the usefulness of the diagnostic procedure.

Galactosemia was selected as a model system for study. There are four known pathways for the metabolism of galactose (Fig. 1).

1) Reduction of galactose to galactitol. This reaction occurs in several tissues and the product, galactitol, accumulates in urine and in various tissues, especially lens, in patients deficient in galactokinase or galactose-1-phosphate uridylyltransferase activity (*1*). The galactitol does not appear to be further metabolized.

2) Oxidation of galactose to galactono-γ-lactone. Galactose dehydrogenase has been reported to be present in rat liver (*2*). However, its significance in the metabolism of galactose has been questioned (*3*, *4*). Hydrolysis of the lactone would lead to galactonic acid which has been identified in large amounts in the urines of galactosemic patients (*5*). Srivastava and Beutler (*4*) suggest that the substrate for such a dehydrogenation might be galactose-6-phosphate.

3) Phosphorylation of galactose to galactose-1-phosphate and its conversion to uridine diphosphogalactose (UDPGal) by reaction with uridine triphosphate (UTP). Isselbacher first described UDPGal pyrophosphorylase and suggested that it might be involved in the metabolism of galactose by galactosemic patients (*6*). Recently, it has been suggested that this enzyme and uridine diphosphoglucose (UDPG) pyrophosphorylase are the same (*7*).

4) Reaction of galactose-1-phosphate with UDPG to form UDPGal and glucose-1-phosphate. (The enzyme galactose-1-phosphate uridylyltransferase is absent or greatly reduced in activity in galactosemia.) This route is probably the major route for galactose metabolism in normal cells. Further metabolism of UDPGal would lead the galactose backbone into the usual routes of glucose metabolism.

Galactose can be utilized by galactosemic cells since they incorporate [1-^{14}C]galactose into trichloroacetic acid–precipitable cell material at about one-half the rate of normal cells (*8*). Normal cells may use the same route or routes, but in addition they can utilize the transferase route. During short incubations normal cells produce $^{14}CO_2$ from [1-^{14}C]galactose, whereas galactosemic cells do not (*9*). The $^{14}CO_2$ produced by normal cells must come from the transferase pathway, probably through the pentose phosphate shunt. Galactosemic fibroblasts do produce $^{14}CO_2$ from [l-^{14}C]galactose, but this does not begin until after about 4 days of incubation (*10*). Since galactosemic cells display little lag in the conversion of [1-^{14}C]galactose into trichloroacetic acid–insoluble cell material (*8*), this $^{14}CO_2$ could come from indirect or secondary slowly acting pathways, from an increase in activity of the pyrophosphorylase, or from an increase in the transferase; the increase in the activity of the transferase seems unlikely.

The dehydrogenase route might be a major route for galactose metabolism in galactosemic cells from the following considerations: (i) Galactitol produced by reductase does not appear to be further metabolized in vivo (*1*).

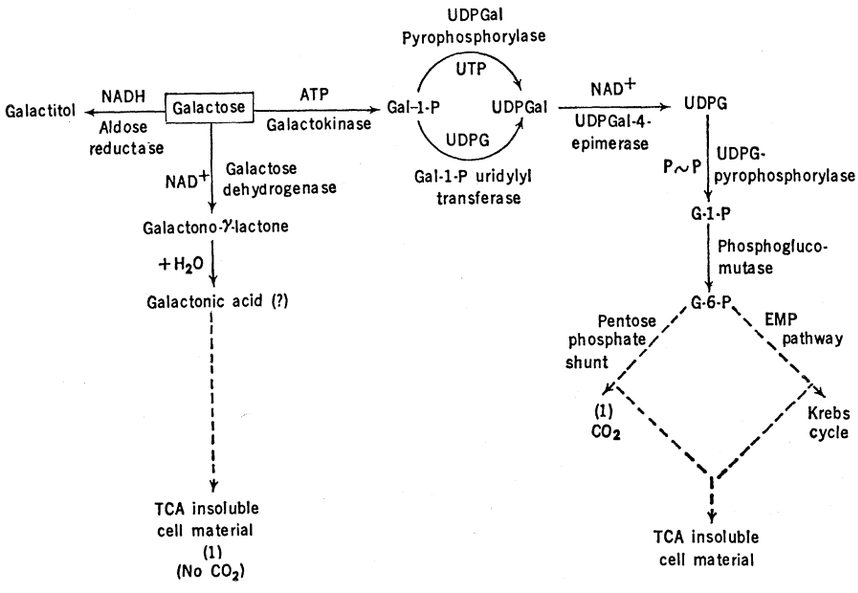

Fig. 1. Proposed scheme for galactose metabolism in cultured human cells. Abbreviations: UDPGal, uridine diphosphogalactose; UTP, uridine triphosphate; UDPG, uridine diphosphoglucose; Gal-1-P, galactose-1-phosphate; NADH, reduced form of nicotinamide adenine dinucleotide (NAD^+); ATP, adenosine triphosphate; EMP, Embden-Myerhof-Parnas pathway; and TCA, trichloroacetic acid. See (*14*).