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Deuterium Oxide: Inhibition of Calcium Release in Muscle

Abstract. *Calcium release, measured as luminescence of the protein aequorin, was measured simultaneously with membrane potential and isometric tension in single muscle fibers of the barnacle* (Balanus nubilus). *Deuterium oxide inhibited calcium release and isometric tension but did not affect membrane potential, a result consistent with the postulate that deuterium oxide inhibits the coupling between excitation and contraction.*

Deuterium oxide (D_2O) retards numerous biochemical and biological reactions (*1*, *2*). Biological effects of D_2O are due mainly to the exchange of hydrogen by deuterium at specific sites. The deuterium bond, having a lower zero-point energy, is more stable than the hydrogen bond and thus requires a greater activation energy (*3*). Some biological effects may also be related to certain differences between the solvent properties of D_2O and H_2O. For example D_2O has a higher melting point, greater density, and lower ionic conductance than does H_2O (*2*).

When the effects of D_2O on different types of muscle were studied, contraction was markedly or completely inhibited. However, even in instances of complete inhibition, the excitatory impulse continued to be propagated. In contrast, D_2O added to muscle extracted with glycerol did not retard the contraction induced by adenosine triphosphate, a result suggesting that the contractile mechanism was not influenced by D_2O. Hence, Kaminer postulated that the main inhibitory effect was on the coupling between excitation and contraction (*4*). Hotta and Morales (*5*) confirmed the lack of effect of D_2O on the contractile proteins by showing that the adenosine triphosphatase of myosin B was not influenced at neutral *p*H. Goodall (*6*) interpreted his results for living muscle as being due to the effect of D_2O on the contractile process, whereas Svensmark (*7*) remained uncertain about the site of action of D_2O. In support of Kaminer's postulate, we report evidence that calcium release, a step in the coupling of excitation and contraction (*8*), is prevented by D_2O.

Methods were similar to those of Ashley and Ridgway (*9*), who demonstrated the release of calcium in muscle by the use of aequorin, a protein which luminesces in the presence of calcium (*10*). The lateral and ventral depressor muscles of the barnacle (*Balanus nubilus*) were used (*11*). A single muscle fiber was placed in a horizontal bath in Ringer solution (*12*) buffered by 5 m*M* *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) at *p*H 7.4. The experiments were done at 4°C.

After injection of 1 μl of an aequorin solution, the muscle fiber was impaled with a combination electrode for both stimulation and recording (*13*). Simultaneous measurements were made of the membrane potential, the "flash" of aequorin, and the isometric tension. After the control measurements were made, the solution was replaced with Ringer solution that contained 99.8 percent D_2O and was at the same temperature, had the same concentrations of salts, and had a *p*D equivalent to a *p*H of 7.4 (*p*D = *p*H reading + 0.4) (*14*). Recordings were then made at 5-minute intervals for 10 to 15 minutes. Figure 1 shows that the flash and the tension disappeared within 5 to 10 minutes in D_2O, while the membrane potential remained. This effect was reversed if Ringer solu-

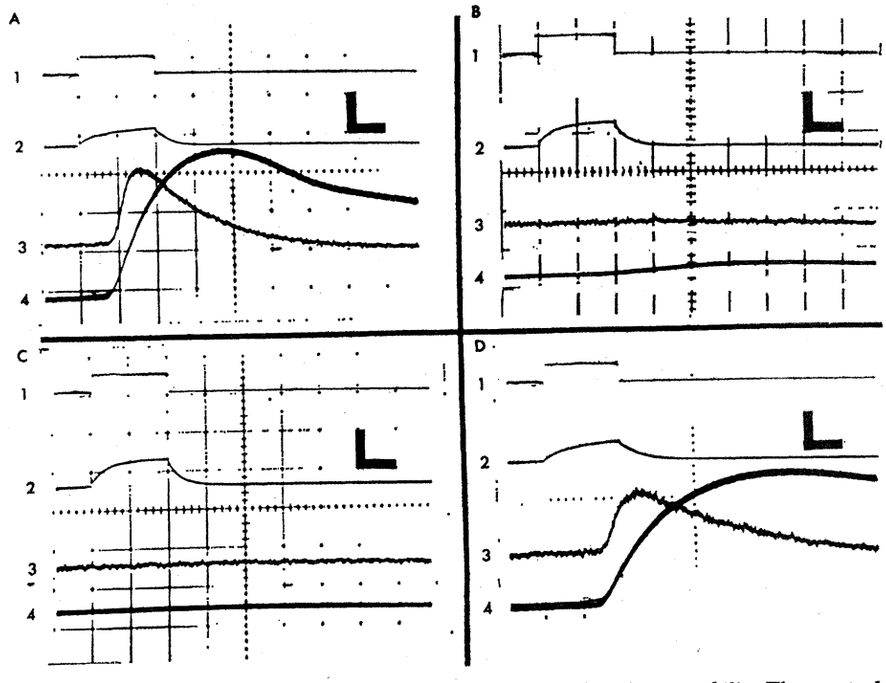

Fig. 1. Effects of 99.8 percent D_2O in a muscle fiber of *Balanus nubilis*. The control incubation (A) contained Ringer solution with H_2O. Records were made after Ringer solution with D_2O had been present for 5 minutes (B) and 10 minutes (C), and 10 minutes after the D_2O solution had been replaced with that containing H_2O (D). Traces are 1, the stimulus (45 μa, 200 msec); 2, membrane potential (20 mv/cm); 3, light emission of aequorin (1 μl of 0.1 to 0.5 m*M* aequorin was injected, and emission was measured with an RCA 6342A photomultiplier tube); and 4, isometric tension (0.5 g/cm), measured with a Grass FT03 transducer. The calibration bar equals 1 cm. After the preparation was in D_2O for 5 minutes (B) the flash disappeared; the contraction was minimal at this time and disappeared after 10 minutes in D_2O (C). There were variations in the amplitude and shape of the membrane potential. In this example, recovery is not complete; in others, recovery was complete.

tion with D_2O was replaced by that with H_2O.

In the presence of D_2O, calcium is not released and there is consequently no contractile response. These results are similar to those of Ashley and Ridgway (*9*) when they used hypertonic sucrose.

Results were reproducible with some minor variations. In some instances, both the flash and tension were more intense for a brief period during the recovery period than in the control whereas in others recovery was not complete. Such an increase in tension during recovery had been found in frog atrial muscle (*4*). In a few experiments the D_2O did not completely eliminate either the tension or the light flash.

The possibility that D_2O inhibited the luminescent reaction of aequorin and calcium was considered. By visual means, the aequorin flash in preparations exposed to $CaCl_2$ in the presence of more than 80 percent D_2O was compared to that in H_2O; no difference was observed. The kinetics of the reaction in D_2O should be studied, as should the site and mechanism of D_2O action.

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Radioautography of the Optic Tectum of the Goldfish after Intraocular Injection of [^{3}H]Proline

Abstract. *Radioautography of the optic tectum of the goldfish, performed after injection of [^{3}H]proline into the contralateral eye, effectively resolves several distinct layers of retinal synapses. Silver grains are found unilaterally over nerve tracts containing efferent fibers from the tectum, a result that suggests intercellular migration of labeled molecules. The low background and high specific grain density obtained with [^{3}H]proline radioautography indicate the usefulness of this technique for the elucidation of neuroanatomical connections in the visual system.*

Newly synthesized proteins that are labeled with radioactive amino acid precursors in neuronal cell bodies are transported to nerve terminals by the process of axonal flow (*1*). The goldfish visual system has proved useful for studies of this process; this is true because the optic tracts are completely crossed, and the labeled proteins that are formed in retinal ganglion cells after intraocular injection of radioactive amino acids migrate to a distinct region of the contralateral optic tectum (COT) (*2, 3*). Radioactive protein appears in the COT, 4 to 6 hours after injection of [^{3}H]leucine into the eye; this time course represents rapid axonal transport of newly synthesized protein (*4*). In addition, labeled protein that appears in the ipsilateral optic tectum (IOT) and throughout the brain represents [^{3}H]leucine that has escaped into the circulation and has been taken up by the brain and incorporated into protein. The systemic background labeling thus limits the usefulness of radioautography as a method of identifying nerve endings to which the labeled proteins migrate or of detecting possible intercellular movement of labeled molecules.

We previously measured the relative efficacy of 18 amino acids (*3*) in selectively labeling proteins that are synthesized in the retinal ganglion cell of the goldfish and transported to the optic tectum. [^{3}H]Proline and [^{3}H]-asparagine, injected into the eye, were both more efficient than [^{3}H]leucine in labeling substances recovered in the COT fraction that was insoluble in trichloroacetic acid (TCA), and these amino acids were much less active than [^{3}H]leucine in labeling the brain systematically (that is, in labeling TCA-insoluble material in the IOT). While the ratio of the radioactivity in protein recovered from the COT to that in the IOT was less than 2 : 1 with [^{3}H]leucine, the ratio was from 17 : 1 to 70 : 1 with [^{3}H]proline, depending upon the specific activity of the proline. Studies in visual systems of the chick (*5*) and mouse (*6*) have confirmed the usefulness of labeled proline as a marker for the study of axonally transported proteins. Proline's effectiveness in part may be due to the fact that it is poorly incorporated into the brain from blood (*7*) and is metabolized into glutamic and aspartic acids, which do not become highly labeled because they are present in high concentrations relative to other amino acids (*8*). When COT protein that is synthesized after [^{3}H]proline injection is hydrolyzed in acid, more than 90 percent of the radioactivity can be accounted for as proline (*3*).

Three goldfish, weighing 8 to 10 g, were each injected in the right eye with 40 μc of L-[2,3-^{3}H]proline (45.7 c/mmole) in a volume of 5 μl. After 24 hours, brains were removed and fixed in Bouin's solution, dehydrated with butyl alcohol, and embedded in paraffin. Sections for radioautography were dipped in Kodak NTB3 emulsion (*9*), and were developed 6 to 13 days later and stained with hematoxylin-eosin. Other sections were treated with phosphotungstic acid–hematoxylin or Bodian's stain to facilitate classification of the tectal layers according to Leghissa (*10*). Cross sections through midtectal regions were selected for study, and mediolateral areas of similar thickness were used for counting grains. At least 7000 grains were counted per brain.

Regions typical of those counted are indicated in Fig. 1A. At low magnification (Fig. 1A), a dark band of silver grains restricted to the COT is readily identified, but few exposed grains are seen in the IOT, even at higher magnification (Fig. 1B). There are several distinct layers of grains in the COT (Fig. 1C), which receives fibers from the injected eye. The highest concentrations of grains are seen in a discrete area (S) medial to the marginal fiber (MF) layer, in the external plexiform (EP) layer containing retinal axons and nerve endings, in the internal plexiform (IP) layer, and in the periventricular (PV) layer. These areas appear to correspond to retinal synaptic regions