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The Effect of Deuterium Ion Concentration on the Properties of Sarcoplasmic Reticulum

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Summary. 99.8% Deuterium oxide, as obtained commercially, has been shown to contain a contaminant which strongly inhibits calcium transport and binding by sarcoplasmic reticulum (S.R.) and the associated ATPase activity. The contaminant is removed by distillation of deuterium oxide. Calcium binding by S.R. is maximal at pH 6.5 whereas calcium transport (in the presence of oxalate) is maximal at a pH of 7.2 to 7.5. In the presence of deuterium oxide, these maxima are shifted to a pD of 7.2 and a pD of 7.5 to 8.0, respectively. The maximum binding and transport rates are not affected by the change from aqueous to deuterium oxide medium. The same phenomena are observed with the ATPase activity. In the presence of oxalate, calcium/magnesium ATPase is maximal at pH 7.2 and pD 8.0. The maximum rate is unchanged, however.

At pH 7.2 or higher, the amount of calcium which may be bound by S.R. remains constant with time. At lower pH, calcium initially bound is slowly displaced from the membrane with time. It has been reported that deuterium oxide inhibits excitation-contraction coupling. The results presented here indicate that S.R. is probably not the site of deuterium oxide inhibition, and raise the possibility that the measured inhibition is due to an impurity in the deuterium oxide.

In skeletal muscle, the sarcoplasmic reticulum membranes (S.R.) have the ability to release enough calcium ion into the myoplasm to bring about myofibrillar contraction, probably in response to the muscle action potential. S.R. also contain an active pump, manifest as a Ca/Mg-ATPase, which can lower calcium concentration in the cell to 10^{-7} M, at which level relaxation occurs [5, 21]. It has been postulated that deuterium oxide inhibits excitation-contraction coupling at a site as yet unknown [11]. We have examined, therefore, the possibility that S.R. is the site of deuterium oxide inhibition, using a rat muscle S.R. preparation isolated by continuous sucrose density gradient centrifugation. To our knowledge, this is the first report on the behavior of S.R. in deuterium oxide.

It has been suggested that intracellular pH of rat skeletal muscle may be a function of membrane potential [4]. The behavior of S.R. as a function

of pH may, therefore, be of physiological importance. On the basis of experiments demonstrating that sudden changes in pH can cause release of calcium from S.R. membranes, Schwartz and his co-workers have suggested that hydrogen ion concentration may participate in excitation-contraction coupling [15, 16].

Experimental Procedures

All counting was done in a Beckman LSC 250 counter. Sucrose used was the enzyme grade from Schwarz-Mann, Orangeburg, New York. ATP was obtained from P-L Biochemicals, Milwaukee, Wisconsin and ^{45}Ca from Amersham-Searle, Arlington Heights, Illinois. Sodium deuteroxide was obtained from K and K Laboratories, California, and deuterium chloride from Sigma. Deuterium oxide was obtained in four separate batches from Sigma between May and November, 1972. In addition, one batch of deuterium oxide was obtained from Diaprep, Inc., Atlanta, Georgia.

Isolation of Sarcoplasmic Reticulum Fraction

The isolation was as previously reported [10]. All incubations were done at 37 °C. Unless stated otherwise in the text, ATP was used as the Tris salt, prepared by passage of ATP through an AG 50 (H^+) ion exchange column, followed by adjustment to the desired pH with Tris base. Protein was determined by the biuret [7] or Folin method [13] and phosphate as previously described [9]. Mg-ATPase was measured in a solution containing 0.15 mg protein, 20 mM Tris, 3 mM $MgCl_2$, 0.2 mM EGTA and 4 mM ATP at pH 7.2. Total volume of incubation was 2 ml and length of incubation was 10 min.

The calcium binding assay (calcium uptake in the absence of oxalate) was measured in a solution containing 16 mM histidine, 4 mM ATP, 5 mM $MgCl_2$, 100 mM KCl, and 0.05 mM $^{45}CaCl_2$ at the appropriate pH. Reactions were terminated using the millipore filtration method of Martonosi and Feretos [14]. Phosphate and ^{45}Ca were determined in the filtrate. Individual blanks were run for every reaction to correct for phosphate background and changes in ^{45}Ca counting efficiency. Calcium transport was measured in a solution containing 3.5 mM $MgCl_2$, 100 mM KCl, 20 mM NaCl, 4 mM ATP, 3 mM potassium oxalate and 0.1 mM $^{45}CaCl_2$ at the appropriate pH (pD).

Binding and transport assays were run for 4 min and samples taken at 0.5, 1, 2, and 4 min and calcium bound and ATP hydrolyzed determined on these samples. Initial volume of incubation was 4 ml. For the binding assay, 0.2 ml (approximately 0.15 mg protein) of stock S.R. was used, and for the transport assay, 0.05 ml (approximately 0.04 mg protein) was used.

A lower concentration of $MgCl_2$ is used in the transport assay to lower the [calcium] [oxalate] product. Under the conditions used, [Ca] [oxalate] product is 23.1×10^{-9} M^2 at pH 7.2 and 254.9×10^{-9} M^2 at pH 6.5 [12]. These solutions are therefore supersaturated with calcium oxalate (solubility product is 2×10^{-9} M^2), but are metastable. No removal of calcium from solution occurs in the absence of protein, or in the presence of protein and the absence of ATP ([Ca] [oxalate] $= 300 \times 10^{-9}$ M^2 under these conditions).

Over the pH (pD) range 5 to 7.2, histidine (16 mM) buffer was used, and over the pH (pD) range 7.2 to 9 Tris (16 mM) buffer was used. Final adjustments of pH (pD) were made with HCl (DCl) or NaOH (NaOD). Protein concentration was so adjusted that calcium uptake did not exceed 75% of the calcium present in the transport assay. Owing to the precipitation of calcium oxalate within the S.R. vesicles, much greater uptakes of calcium occur with this assay.

Deuterium oxide was distilled from EDTA before use. Measurement of pD was performed by use of a pH meter, employing the relationship [6]:

$$pD = pH \text{ reading} + 0.4.$$

S.R. was added to deuterium oxide media either as a water suspension (0.05 ml in a 4 ml reaction medium for transport assay, and 0.2 ml in a 4 ml reaction medium for binding assay) or after being precipitated by centrifugation and resuspended in deuterium oxide.

Results

Effect of Undistilled Deuterium Oxide on S.R.

All samples of deuterium oxide (99.8%) as received almost totally inhibited the calcium transport and binding and ATPase functions of S.R., as shown by the data in Table 1. Much of this inhibitory activity disappeared after D_2O had been distilled from EDTA. The impurity present in the D_2O also interfered with the phosphate assay procedure [9] in such a way as to yield a high blank color value, perhaps indicating the presence of a heavy metal phosphate.

Effect of pH and pD on Calcium Binding by S.R.

Fig. 1 illustrates that calcium binding occurs to a greater extent at pH 6.5 than pH 7.2. At pH 7.2 the amount of calcium bound to the membrane at any one time is constant over the assay period, whereas at pH 6.5 there is a progressive release of calcium from the S.R. The fall in the amount of calcium bound is significant at the $p < 0.001$ level between any two

Table 1. Effect of undistilled deuterium oxide on S.R. functions

Experiment	Time (min)	ATPase (μmole P_i/mg prot.)		Calcium uptake (nmole/mg prot.)	
		H_2O	D_2O	H_2O	D_2O
Calcium binding pH or pD 6.5	0.5	0.7±0.2	0	165±46	0±0
	1	3.0±0.7	0	165±23	0±22
	2	5.5±0.5	0	152±17	0±3
	4	9.1±0.8	0	123±9	0±7
Calcium transport pH or pD 7.2	0.5	2.7±1.3	0	1470±470	190±100
	1	6.9±2.8	0	3010±500	540±260
	2	14.4±3.1	0±3.2	5610±720	1150±520
	4	26.3±5.8	0±5.1	9030±950	2310±690

Values shown ±SE. Number of paired preparations studied = 3. Time is duration of incubation. H_2O indicates incubation was performed in 100% water and D_2O in 95% deuterium oxide.

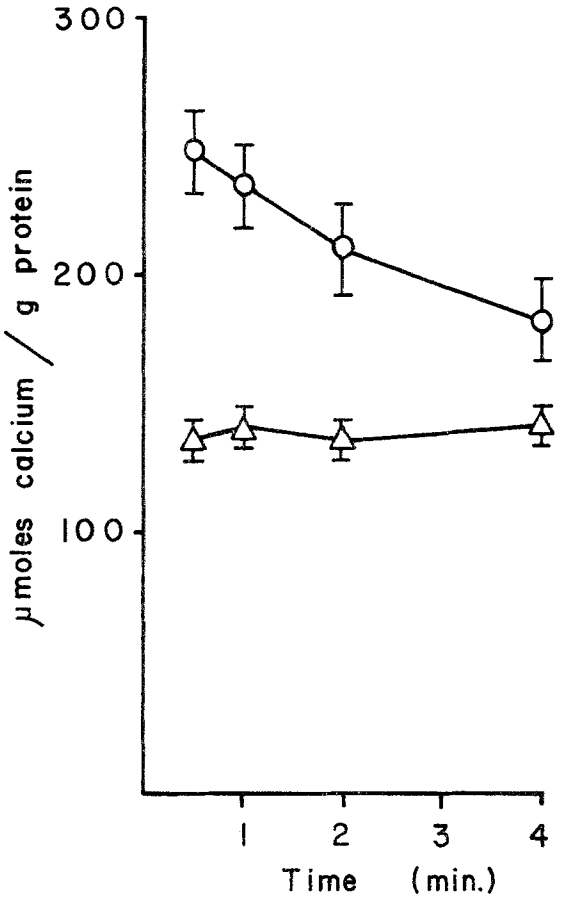

Fig. 1. The binding of calcium by S.R. Values shown ±SE. ○—○ pH 6.5 (Number of preparations studied = 33); △—△ pH 7.2 (Number of preparations studied = 26). At pH 6.5, the difference between any two consecutive time intervals is significant at the $p < 0.001$ level on a paired "t" test comparison

consecutive time intervals (Student's paired "t" test). The preincubation of S.R. at pH 6.5 in the absence of calcium does not alter the initial binding level and leak rate seen when reaction is initiated by the addition of calcium, implying that incubation at pH 6.5 does not damage the S.R. *per se*.

The pH dependence of calcium binding is shown in Fig. 2. Data were averages from three preparations, each preparation being studied throughout the pH range on one day to prevent differences due to aging. The dependency of calcium binding on pD is also shown in Fig. 2. The degree of binding under optimal conditions is unchanged, but maximum binding occurs at a pD of 7.2.

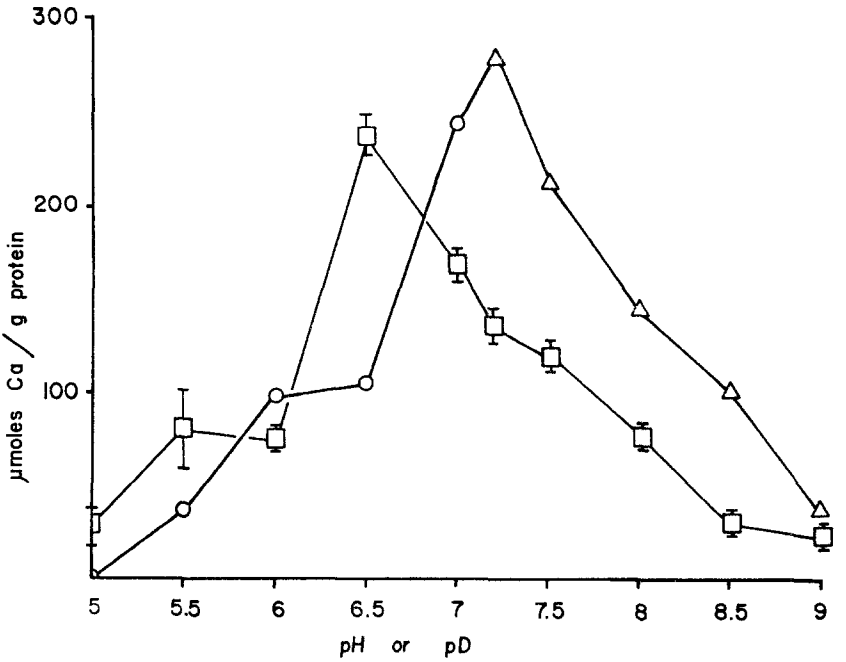

Fig. 2. Effect of pH and pD on degree of calcium binding. Points given ± SE. Number of preparations studied = 3. pD curve is a typical one of 3 run under slightly differing conditions. ○ Histidine buffer used; △ Tris buffer used. Binding averaged for 0.5 min and 4.0 min of incubation

Effect of pH and pD on Calcium Transport by S.R.

pH dependence for calcium transport was measured in a similar way to the experiment shown in Fig. 2, and with the same precautions. The most rapid uptake and greatest storage of calcium oxalate occured at pH 7.2 to 7.5. The pD curve was shifted by half a pD (pH) unit higher relative to the pH curve, but the maximum uptake remained the same.

Effect of pH and pD on ATPase Activity of S.R.

The dependency of ATPase activity on pH is shown in Fig. 3. The curves shown are typical of four or more preparations studied. Each S.R. was studied throughout the pH range in one day. There is sufficient contaminating calcium in the isolated S.R. to cause the ATPase activity in the presence of magnesium but in the absence of exogenous calcium to be appreciably higher in the absence of EGTA. In the presence of oxalate, the Ca/Mg-ATPase activity rises to a sharp maximum at pH 7.2. When oxalate is omitted, ATPase activity is essentially constant over the range pH 6 to 8.

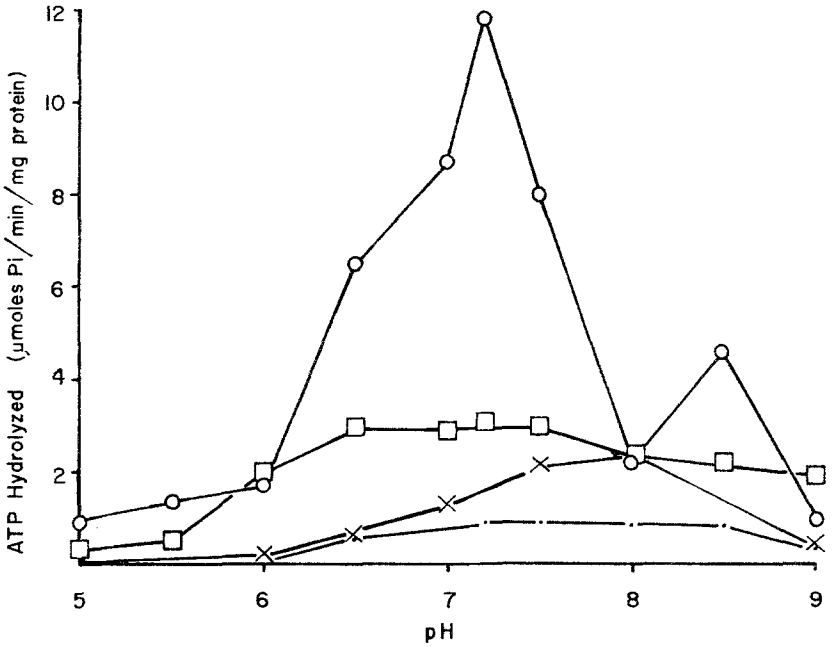

Fig. 3. Effect of pH on ATPase. ○—○ Ca/Mg-ATPase measured under transport assay conditions; □—□ Ca/Mg-ATPase measured under binding assay conditions; ×—× ATPase in absence of EGTA and exogenous calcium; ·—· ATPase in presence of 0.2 mM EGTA and absence of exogenous calcium

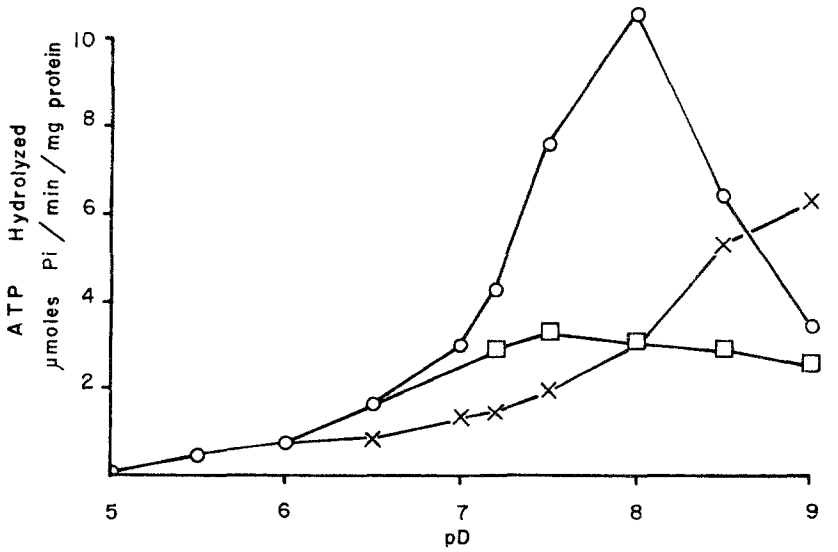

Fig. 4. Effect of pD on ATPase. ○—○ Ca/Mg-ATPase measured under transport assay conditions; □—□ Ca/Mg ATPase measured under binding assay conditions; ×—× ATPase measured in absence of EGTA and exogenous calcium

A similar result was obtained with the effect of pD on ATPase activity (Fig. 4 compared to Fig. 3). The initial pD of incubation has been used for the Figures. On average, the pD dropped by 0.2 during the course of a 4-min incubation.

For these experiments, S.R. suspended in aqueous media were used. Experiments in which S.R. were centrifuged out and resuspended in aqueous or D_2O containing media yielded similar results.

Discussion

Effect of Undistilled Deuterium Oxide on S.R.

It has long been known that deuterium oxide can retard muscle contraction [1, 3, 20]. It has been reported that deuterium oxide inhibited the calcium release without affecting membrane potential [11]. Kaminer and Kimura postulate on this basis that deuterium oxide inhibits excitation-contraction coupling, and so we have examined the possibility that the site of inhibition is the S.R. Our results show that 99.8% deuterium oxide from the bottle will almost totally inhibit calcium binding and transport functions and ATPase activities on S.R. However, to a large extent, the inhibition was due to an impurity in the deuterium oxide, removable by distillation, which gave a color reaction in the colorimetric method used to determined orthophosphate. These results clearly indicate the need for investigators to purify deuterium oxide before its use in biological experiments. Only rarely have investigators indicated that redistilled deuterium oxide was used (e.g., Ref. [8]). No mention is made by Kaminer and Kimura of having used distilled deuterium oxide, leaving the question as to whether their results are attributable to deuterium oxide or to a contaminant in it.

Release of Calcium from S.R. in the Binding Assay

Schwartz [18] has shown that in the absence of oxalate, maximum binding of calcium occurs within a few seconds. The first time interval at which we measure calcium binding is 30 sec after exposure of the S.R. to calcium. At pH 7.2 and above once equilibrium has been reached, the binding remains constant. Below pH 7.2, however, there is a progressive leakage of calcium from the membrane. The function or mechanism of this behavior is not known, but is highly characteristic of the S.R. The leaking occurred without any diminution in ATPase activity. Schwartz and Besch have observed a similar phenomenon using double beam spectrophotometry [2, 18] at shorter times than are possible by the methodology used here. It

has been reported that monolayers of phosphatidylinositol and diacetylphosphoric acid bound calcium in a pH-dependent manner, and that a leaking of the initially-bound calcium occurred between pH 3 and 6.5 [17]. The authors excluded the possibility that acid-catalyzed hydrolysis led to the calcium leakage, and concluded that H^+ slowly displaced Ca^{2+} from binding sites. This implies that the leaking phenomenon is a fundamental property of the phospholipid structure.

Effect of pH and pD on S.R.

The pH maximum for calcium binding and calcium transport differ by about a pH unit. This is in approximate agreement with results previously obtained with rabbit muscle S.R. [19].

Purified deuterium oxide had inhibitory effects on S.R. which were a function of the pD of the medium. In general, the pD dependency curves for calcium binding and transport, and ATPase activities were shifted towards lower D^+ concentrations (higher pD) relative to the corresponding pH dependency curves.

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