*Aust. J. Biol. Sci.*, 1976, **29**, 459–65

# Effect of Heptane Treatment on the Response of Sarcoplasmic Reticulum Preparations to Phosphate

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*Abstract*

The calcium-stimulated (extra) ATPase and calcium uptake activities of sarcoplasmic reticulum (SR) preparations treated with aqueous heptane mixtures were compared with those of untreated SR, and with those of SR treated with aqueous ether.

Both treatments altered the kinetic behaviour of the extra ATPase, the Lineweaver–Burk plot being changed from its normal non-linear shape to a straight line. Kinetic constants, $V_{max}$, $K_m$ for ATP and $K_i$ for phosphate, were measured. The extra ATPase activity of heptane-treated SR was inhibited by phosphate as was that of ether-treated SR, to a lesser extent. The magnitude of this inhibition by phosphate was found to be considerably less than the degree of stimulation of the extra ATPase activity of untreated SR caused by phosphate through its calcium-precipitating action.

The steady-state concentrations of the phosphoryl–enzyme intermediates were measured and together with the $K_m$ and $K_i$ values they indicate that the binding of ATP to heptane-treated SR is weaker than it is to untreated SR, and that phosphate is an efficient competitor for the binding sites.

## Introduction

The stimulatory effect of oxalate on the calcium uptake and calcium-stimulated (extra) ATPase activities of sarcoplasmic reticulum (SR) preparations is well known (Hasselbach and Makinose 1961; Makinose and Hasselbach 1965; Weber 1971). Although not studied as extensively, orthophosphate ($P_i$) appears to have an effect similar to that of oxalate (Martonosi and Feretos 1964; Ebashi and Endo 1968). This is due to the precipitation of the accumulated calcium as calcium phosphate within the vesicles and the consequent maintenance of a $Ca^{2+}$ concentration gradient favouring uptake.

On the other hand, phosphate, as a product of the ATPase reaction, might be expected to inhibit these activities. Although some indirect evidence exists for such an inhibitory effect (Hasselbach *et al.* 1974), it has not been clearly demonstrated probably because of the overwhelming stimulatory effect caused by calcium precipitation.

There are many reported treatments of SR preparations which reduce or destroy its calcium uptake activity while leaving the extra ATPase activity largely intact. For example, in a previous paper from this laboratory the effects of aging and addition of detergents on the kinetic behaviour of the extra ATPase activity were studied (Horgan 1974). Other treatments which destroy calcium uptake activity include extraction with aqueous diethyl ether (Inesi *et al.* 1967), and digestion with proteolytic enzymes (Ikemoto *et al.* 1968; Inesi and Asai 1968) or phospholipases

(Meissner and Fleischer 1972). However, apart from the addition of calcium-precipitating agents such as oxalate and phosphate, there is only one reported treatment which increases the calcium uptake activity of SR. This is the aqueous heptane treatment of Drabikowski *et al.* (1972).

In the present study the kinetic behaviour of heptane-treated SR is compared with that of untreated SR and that of ether-treated SR. Phosphate is shown to be a competitive inhibitor of both ether- and heptane-treated SR, the degree of inhibition being much greater in the case of heptane-treated SR. This effect is examined and a mechanism for the inhibition is proposed.

## Materials and Methods

SR preparations were made from the psoas muscles of New Zealand White rabbits essentially by the method of Martonosi *et al.* (1968). Dithiothreitol (1 mM) was present at all stages of the preparation (Van der Kloot 1969).

Extra ATPase activities in the presence and absence of phosphate were measured by a coupled-enzyme spectrophotometric method as described previously (Horgan 1974).

Calcium uptake activities in the presence and absence of phosphate were measured by the $^{45}Ca$ method of Martonosi and Feretos (1964) using Millipore filters with an average pore diameter of 0·22 $\mu$m. In addition calcium uptake and extra ATPase activities in the presence of phosphate were measured by the spectrophotometric method of Horgan *et al.* (1972).

Phosphoryl–enzyme intermediate concentrations were measured using $\gamma$-labelled [$^{32}P$]ATP according to the method of Martonosi (1969).

Treatments of SR preparations with heptane and ether were carried out at room temperature. Ether (10%, v/v) and heptane (20%, v/v) were added to SR preparations (protein concentration 5 mg/ml) and the samples were mixed by repeated inversion on a rotary mixer (12 rev/min) for 20 min. The samples were then centrifuged at 55 000 *g* for 30 min and the pellets resuspended in preparative KCl buffer (pH 7·3).

The protein concentrations of the SR preparations were measured by the method of Lowry *et al.* (1951).

Chemicals used were obtained as follows: ATP, phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, and NADH from Boehringer, Mannheim; [$^{32}P$]ATP from Radiochemical Centre, Amersham.

## Results

Fig. 1 shows the double reciprocal Lineweaver–Burk plots of the extra ATPase activities of untreated SR, heptane-treated SR and ether-treated SR measured at pH 7·4. The untreated SR yielded a characteristic curved plot (Horgan 1974) whilst both the ether- and heptane-treated SR gave linear plots. The ether-treated SR had a much higher extra ATPase activity than the other SR preparations. These activities were measured in the absence of phosphate or other calcium-precipitating agents such as oxalate. Similar shaped curves were obtained at pH 6·4.

The calcium uptake activity in the absence of calcium-precipitating anions [sometimes called calcium binding (Katz and Repke 1973)] of heptane-treated SR varied but in all cases differed little from that of the untreated SR. Ether treatment, as was expected (Inesi *et al.* 1967), completely destroyed calcium uptake activity.

Table 1 shows typical results for the extra ATPase and calcium uptake activities of untreated and heptane-treated SR preparations measured in the presence of 50 mM phosphate and 5 mM ATP by the spectrophotometric method of Horgan *et al.* (1972). The extra ATPase and calcium uptake activities are greatly reduced by heptane treatment, especially at pH 6·4. The heptane-treated SR is, however, slightly more

efficient in the presence of phosphate than the normal SR, i.e. it uses less ATP to pump the same amount of calcium.

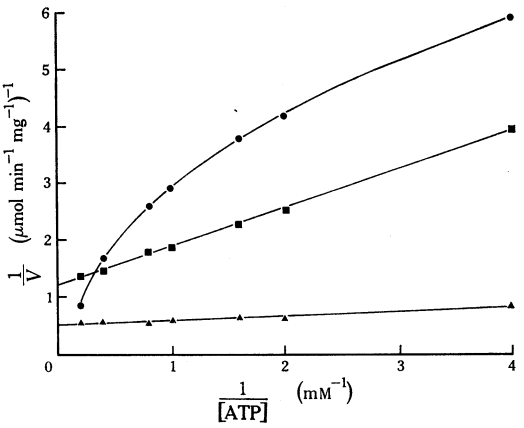


**Fig. 1.** Effect of substrate concentration on extra ATPase activities of SR preparations in the absence of phosphate, plotted in Lineweaver–Burk form. pH 7·4.
● Untreated SR.
■ Heptane-treated SR.
▲ Ether-treated SR.

The effect of aging on the extra ATPase and calcium uptake activities of untreated and heptane-treated SR measured in the presence of 50 mM phosphate and 5 mM ATP is shown in Table 2. The effect on heptane-treated SR varied considerably in magnitude and two examples illustrating the range of the effect are shown. In both examples the extra ATPase activity of untreated SR increased as it aged whilst the calcium uptake activity steadily decreased. On the other hand, the extra ATPase activity of heptane-treated SR increased rapidly, whilst the calcium uptake activity also increased in most cases. In some cases, however, as shown in the second example, the increase in extra ATPase activity was much greater whilst the calcium uptake activity was reduced to zero after 2 days.

**Table 1. SR activities in the presence of 50 mM phosphate[A]**

| | Extra ATPase activity [$\mu$mol min$^{-1}$(mg protein)$^{-1}$] | Ca uptake activity [$\mu$mol min$^{-1}$(mg protein)$^{-1}$] | Ca uptake activity / Extra ATPase activity[B] |
|---|---|---|---|
| pH 6·4 | | | |
| Untreated SR | 0·790 | 0·742 | 0·93 |
| Heptane-treated SR | 0·184 | 0·217 | 1·18 |
| pH 7·4 | | | |
| Untreated SR | 1·710 | 0·770 | 0·45 |
| Heptane-treated SR | 0·690 | 0·377 | 0·55 |

[A] 50 mM phosphate was used as this concentration was sufficient to give a large stimulation of the calcium uptake activity by untreated SR and to cause a distinct and easily measured inhibition of the ATPase activity of heptane-treated SR.
[B] In this type of assay the ratio is an average value for the entire reaction rather than a ratio of initial rates.

The extra ATPase activities of both heptane- and ether-treated SR were shown to be competitively inhibited by phosphate. The inhibition was much greater in the case of the heptane-treated SR and was also much larger at pH 6·4 than at pH 7·4. Table 3 shows the kinetic parameters measured at pH 6·4 for the two types of SR

preparations. The extra ATPase of ether-treated SR is approximately three times as active as that of heptane-treated SR whilst its $K_m$ for ATP was approximately one-third that for heptane-treated SR. The $K_i$ for phosphate with ether-treated SR was approximately three times that for heptane-treated SR. When heptane-treated SR was aged two of these parameters changed. $V_{max}$ increased steadily over a period of

**Table 2. Effect of aging on SR activities in the presence of 50 mM phosphate, pH 6·4**
Activities are measured as $\mu$mol min$^{-1}$ (mg protein)$^{-1}$

| Example | Time of aging (days) | Untreated SR | | Heptane-treated SR | |
|---|---|---|---|---|---|
| | | Extra ATPase activity | Ca uptake activity[A] | Extra ATPase activity | Ca uptake activity[A] |
| 1 | 0 | 0·680 | 0·715 | 0·163 | 0·142 |
| | 1 | 0·840 | 0·600 | 0·320 | 0·230 |
| | 2 | 0·830 | 0·540 | 0·575 | 0·342 |
| 2 | 0 | 0·780 | 0·740 | 0·184 | 0·217 |
| | 1 | 0·870 | 0·673 | 0·990 | 0·435 |
| | 2 | 0·990 | 0·520 | 2·4 | 0 [B] |

[A] Measured spectrophotometrically. [B] No measurable activity.

3 days to approximately three times its original value while $K_i$ for phosphate increased steadily over the same period to 140 mM. $K_m$ for ATP remained almost constant over this period.

**Table 3. Kinetic parameters of the extra ATPase activity of treated SR preparations**
Values expressed are means ± s.e.m. of five preparations, pH 6·4

| | $V_{max}$ [A] [$\mu$mol min$^{-1}$ (mg protein)$^{-1}$] | $K_m$ [B] (mM) | $K_i$ [C] (mM) |
|---|---|---|---|
| Ether-treated SR | 1·40 ± 0·21 | 0·10 ± 0·01 | 49·8 ± 4·4 |
| Heptane-treated SR | 0·51 ± 0·07 | 0·34 ± 0·06 | 16·4 ± 1·8 |

[A] Measured both in the presence and in the absence of $P_i$.
[B] ATP as substrate, measured in the absence of $P_i$.
[C] Value measured for competitive inhibition by 25 mM and 50 mM orthophosphate of extra ATPase activity.

Table 4 shows the steady-state concentrations of the phosphoryl–enzyme intermediates in the extra ATPase reactions catalysed by the various SR preparations. Ether treatment increased the extra ATPase activity but had little or no effect on the phosphoryl–enzyme concentration. Phosphate had no significant effect on the phosphoryl–enzyme concentration of either untreated or ether-treated SR. However the steady-state level of phosphoryl–enzyme in the reactions catalysed by heptane-treated SR was approximately half that for untreated SR and this level was halved again in the presence of phosphate. The extra ATPase activity of heptane-treated SR was greater than that of untreated SR in the absence of phosphate, as the substrate concentration was only 0·25 mM (see Fig. 1). When 1 mM ATP was used the extra ATPase activities of the untreated and heptane-treated SR preparations were very similar in the absence of phosphate but the phosphoryl–enzyme level of the reaction catalysed by heptane-treated SR was still only half that of the control.

## Discussion

Previous work (Yamamoto and Tonomura 1967; Horgan 1974) has shown that the Lineweaver–Burk plot of extra ATPase activity of untreated SR with varying ATP concentration is non-linear. Fig. 1 shows that both heptane and ether treatments result in linear plots. Heptane-treated SR is less active than untreated SR at high substrate concentrations and more active at low substrate concentrations but is several-fold less active than the ether-treated SR at all substrate concentrations. The effect of ether treatment on the Lineweaver–Burk plot is similar to that previously reported for Triton X-100 and aging (Horgan 1974). These treatments also cause a loss of calcium uptake activity and this, together with the change in shape of the Lineweaver–Burk plot, can be followed in a progressive manner. The point at which the Lineweaver–Burk plot becomes linear has been associated with complete loss of calcium uptake activity (D. J. Horgan, unpublished data). Therefore it is of interest that heptane-treated SR gave a linear plot and yet still had an efficient calcium uptake activity. The heptane treatment, unlike the other treatments, did not produce a marked increase in the extra ATPase activity.

**Table 4. Extra ATPase activities and associated phosphoryl–enzyme intermediate levels of SR preparations, using 0·25 mM [$^{32}$P]ATP as substrate**

| | Extra ATPase activity [$\mu$mol min$^{-1}$ (mg protein)$^{-1}$] | | Phosphoryl–enzyme level [A] [nmol (mg protein)$^{-1}$] | |
|---|---|---|---|---|
| | No $P_i$ | 50 mM $P_i$ | No $P_i$ | 50 mM $P_i$ |
| Untreated SR | 0·246 | 0·390 | 2·05 | 1·99 |
| Ether-treated SR | 1·56 | 1·27 | 1·95 | 2·06 |
| Heptane-treated SR | 0·370 | 0·157 | 1·09 | 0·58 |

[A] Steady-state concentration.

Compared to the extra ATPase activity of untreated SR which is stimulated by phosphate, the extra ATPase activity of heptane-treated SR in the presence of phosphate is low (Table 1). The $Ca/P_i$ ratio, i.e. the number of moles of calcium pumped per mole of ATP hydrolysed, of heptane-treated SR is, however, higher than that of untreated SR. If net calcium uptake by the SR equals calcium pumped in minus calcium diffusing out then the heptane treatment would appear to be affecting only the rate at which the calcium is pumped into the SR.

This hypothesis is supported by the results of experiments, not shown here, which show that whilst phosphate increases the calcium accumulating ability of heptane-treated SR, the increase is considerably less than that for untreated SR.

During the aging of untreated SR there is a gradual loss of calcium uptake activity which is associated with an increase in the activity of the extra ATPase (Table 2). This effect also occurs with heptane-treated SR but appears to be more rapid. The situation is complicated by a second effect with heptane-treated SR, i.e. the reduction in inhibition of the extra ATPase by phosphate as the SR ages ($K_i$ increases). This increase in the activity of the extra ATPase stimulates the rate of calcium uptake. Hence with heptane-treated SR the measured rate of calcium uptake during aging is the net result of two opposing effects, i.e. decrease in activity by increase in the permeability of the membrane, and increase in activity due to the increase in pumping

activity. Depending on the relative rates of these two effects the net result can be either an increase in calcium uptake activity over the 2-day aging period as shown in the first example, or, as in the second example where presumably the increase in the permeability of the membrane is more rapid, a faster increase in extra ATPase activity together with a complete loss of calcium uptake activity in the second day.

Phosphate has a stronger inhibitory effect on the extra ATPase activity of heptane-treated SR than on that of ether-treated SR (Table 3). If the ether treatment simply destroys the calcium uptake activity of the SR without altering the extra ATPase activity as has been claimed (Inesi *et al.* 1967), then these results constitute the first direct demonstration of an inhibition by phosphate of the SR extra ATPase activity. The relative degrees of inhibition produced at pH 7·4 and pH 6·4 indicate that the monobasic form of phosphate ($HP_2O_4^-$) is the inhibitory form. Because of its competitive nature the inhibition by phosphate of the extra ATPase activity is masked by the stimulatory effect at high substrate concentrations. For example, with 5 mM ATP as substrate, 50 mM phosphate will cause an inhibition of 5% or less whilst with intact SR this phosphate level will stimulate the extra ATPase (by means of its calcium-precipitating action) by up to 100%.

The results in Table 4 have been interpreted assuming that the reaction scheme proposed by Martonosi *et al.* (1974) for SR-catalysed extra ATPase reactions is substantially correct. In this scheme it is the breakdown of the phosphoryl–enzyme intermediate which is the rate limiting step. Therefore, as the extra ATPase activity of the heptane-treated SR is either slightly greater than (0·25 mM ATP) or equal to (1 mM ATP) that of the untreated SR, the low level of phosphoryl–enzyme intermediate in the reactions catalysed by heptane-treated SR must be due to a lower rate of formation of the intermediate. This is supported by the $K_m$ values in Table 3 which indicate a weaker binding of ATP to the heptane-treated SR. Phosphate, which, according to the $K_i$ values in Table 3, binds more strongly to the heptane-treated SR, presumably competes with ATP for binding sites on the heptane-treated SR and thus further lowers the phosphoryl–enzyme intermediate concentration.

## Acknowledgments

I am grateful to Mrs Sue Thompson for skilled technical assistance. This work was supported in part by the Australian Meat Research Committee.

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Manuscript received 21 June 1976