BBA 75827

SUCROSE ANTAGONISM OF DRUG AND TEMPERATURE EFFECTS ON CARDIAC SARCOPLASMIC RETICULUM

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(Received July 15th, 1971)

SUMMARY

Sucrose, but not fructose, was found to antagonize the effects of exposing cardiac sarcoplasmic reticulum to a number of different drugs. The effect antagonized was the inhibition of ATP-dependent Ca^{2+} uptake. The drugs studied were quinidine, propanolol, chlorpromazine and chloroform. It was also discovered that sucrose, but again not fructose, could antagonize the inhibition of calcium uptake which follows the exposure of the sarcoplasmic reticulum to a temperature of 37°. This latter investigation was undertaken because there is evidence that 37° and the types of drugs studied produce similar alterations in the structure of biological membranes. Since sucrose was found not to influence the binding of one drug, chlorpromazine, to the sarcoplasmic reticulum, another mechanism for the action of sucrose had to be considered. This is discussed.

INTRODUCTION

In the course of studies with isolated myocardial sarcoplasmic reticulum, we accidentally observed that sucrose could antagonize propanolol inhibition of ATP-dependent Ca^{2+} uptake. A search of the literature revealed that although sucrose had previously been found[1-2] to antagonize interactions between biological membranes and such substances as anesthetics, antihistamines and phenothiazines; the mechanism of this effect had apparently not been the subject of any sort of intensive investigation. Since the red blood cell has been used in many of these studies it has been necessary to substitute, on an equal osmotic basis, sucrose for salt; and hence it has been difficult to decide whether sucrose antagonizes these drugs or salt potentiates them. Since the sarcoplasmic reticulum is quite permeable to molecules such as sucrose[3], this difficulty does not arise; and it should be possible, using this preparation, to decide between these alternative interpretations. The membrane function of the sarcoplasmic reticulum which we have studied is ATP-dependent Ca^{2+} uptake. This was particularly pertinent since the types of drugs

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

used in the studies with red blood cells inhibit the Ca^{2+} uptake[4-7] by the sarcoplasmic reticulum.

The sarcoplasmic reticulum has also been exposed to 37° to determine if this temperature produces effects similar to those of the drugs. By "similar" we here mean: Does exposure to this temperature inhibit calcium uptake and is this inhibition subject to modification by sucrose or other sugars? This particular type of investigation was undertaken because there is biophysical evidence that drugs with local anesthetic actions tend to fluidize[8] biological membranes in a manner which may not be very different from that produced by exposing membranes to a temperature of 37° (refs. 9–10). A limited investigation of the specificity of the action of the sugars has also been undertaken in order to more clearly decide if the drugs and exposure to 37° act on the membrane in a like manner.

METHODS

Fragmented sarcoplasmic reticulum was isolated by a modification of the method of CARSTEN[11] and BRIGGS *et al.*[12]. Mongrel dogs (12–20 kg) of either sex were anesthetized with pentobarbitol (30 mg/kg, intravenously), and intubated with an endotracheal tube. The animals were ventilated with 100% oxygen for several minutes; after which their hearts were quickly removed, washed free of blood with saline, and allowed to cool for 10 to 15 min in cold saline, 1–10°. All subsequent procedures were carried out in a cold room. The ventricles of the heart were dissected free of remaining portions of muscle and trimmed of fat, connective tissue, and remnants of valvular tissue. The ventricles (50–100 g) were then cut into small pieces weighed and homogenized in 4 vol. of 0.3 M sucrose and 10 mM imidazole at pH 7.0 (extraction solution) for 40 sec with a Servall Omnimixer. The homogenate was centrifuged at $1020 \times g_{max}$ for 15 min.

The fragmented sarcoplasmic reticulum was isolated by the following procedure. The supernatant fraction from the first centrifugation was strained through several layers of cheesecloth and centrifuged at $13200 \times g_{max}$ for 25 min. The supernatant fraction from this spin was centrifuged at $90000 \times g_{max}$ for 70 min in the ultracentrifuge. The pellet formed by this centrifugation consists of two distinct types of material. At the bottom of the pellet, there is some gelatin-like substance. Care was taken to exclude this when the pellet was resuspended in 1.3 ml of 0.6 M KCl per g heart to remove contaminant proteins[13]. The resuspended pellets were centrifuged at $198000 \times g_{max}$ in the ultracentrifuge for 30 min. The resultant pellets were combined and suspended in 1 ml of extraction solution per 10 g of muscle. The protein content of this final suspension was determined by the method of LOWRY *et al.*[14]. All studies on the fragmented sarcoplasmic reticulum were carried out on the day of isolation.

The rate of Ca^{2+} uptake by fragmented sarcoplasmic reticulum was determined in the presence of oxalate using the Millipore filtration technique. Unless otherwise indicated, all incubations were carried out at 37°. The standard incubation media contained 100 mM KCl, 18 mM imidazole buffer at pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 1.8 mM potassium oxalate, and 0.18 mM $CaCl_2$ with 0.05 μC/ml $^{45}CaCl_2$. The final volume was 5 ml. The various agents studied were added to this standard incubation solution either alone (salt incubation) or in the presence of 300 mM sucrose

(sucrose incubation). Incubations in which chloroform was added were carried out in sealed reaction flasks. The reaction was started by the addition of fragmented sarcoplasmic reticulum suspension (0.1 ml) so that the final protein concentration was 0.075 or 0.15 mg/ml. In experiments involving preincubation the fragmented sarcoplasmic reticulum preparation was added to 4 ml of a solution containing 130 mM KCl, 2.24 mM potassium oxalate, 22.4 mM imidazole at pH 7.0, sugars when indicated, and 0.094 or 0.188 mg/ml fragmented sarcoplasmic reticulum protein. The reaction was started after the preincubation period by addition of 1 ml of a solution containing 25 mM $MgCl_2$, 25 mM ATP, 0.90 mM $CaCl_2$ with 0.25 μC/ml $^{45}CaCl_2$. Aliquots were taken at various time intervals and rapidly filtered by suction through Millipore filters of 0.45-μm pore diameters. Aliquots (0.5 ml) of filtrate were counted in a Beckman LS-100 liquid scintillation spectrometer in 10 ml of a medium containing TLZ fluoralloy (Beckman Inst.), Biosolv BBS-3 (Beckman Inst.), and toluene in a ratio of 1:2.5:10 giving a final concentration of 4.0 g/l PPO and 0.5 g/l POPOP. Since the uptake of $^{45}Ca^{2+}$ was linearly related to time at 0.5, 1.0 and 1.5 min, the comparison of uptake rates under various experimental conditions was made at 1 min.

The binding of [^{35}S]chlorpromazine (0.05 μC/ml) to fragmented sarcoplasmic reticulum membranes was determined in the same incubation media and under the same conditions used for measurement of Ca^{2+} uptake. Parallel Ca^{2+} uptake and chlorpromazine binding experiments were carried out on the same fragmented sarcoplasmic reticulum preparation. The incubation mixture was allowed to react for 2 min at 37° and then centrifuged in stainless steel tubes at 105000 $\times$ g for 45 min. The radioactivity was measured in the clear supernatant and used to calculate the extent of drug binding. Control experiments for determination of total radioactivity were carried out in the absence of protein under identical conditions of incubation and centrifugation.

All solutions were prepared with distilled, deionized water. The ATP was a product of P–L Biochemicals, Milwaukee, Wisc. Quinidine sulfate was obtained from Sigma Chemical Co., St. Louis, Mo., propanolol from Ayerst Lab., New York, N.Y. and chlorpromazine from Smith, Kline and French, Philadelphia, Pa. [^{35}S]-Chlorpromazine was obtained from Amersham-Searle Corporation, Arlington Heights, Ill.

RESULTS

Following the accidental discovery that sucrose could antagonize the effect of propanolol on Ca^{2+} uptake we undertook a series of experiments to further characterize this antagonism. Thus, the effect of 0.3 M sucrose on the action of propanolol was studied over a wide range of drug concentrations (Fig. 1). Although the antagonism was nearly complete at 0.6 and 0.9 mM and quite significant at 1.8 mM, it was not appreciable at 3.0 mM. In order to determine if the action of sucrose extends to substances other than propanolol we also studied its ability to antagonize the effects of quinidine and chloroform. The results are shown in Fig. 2 and 3. Although sucrose did protect against these inhibitors, the protection was not as marked as observed with propanolol.

The effects of anesthetics and tranquilizers have been found to be associated

with expansion[15] and apparent liquification[8] of hydrophobic regions of the membrane. Since temperature has also been found to produce a phase change in membrane structure[10] which has been interpreted as liquification of structure, we carried out a series of experiments to determine if sucrose and other sugars can also modify the effects of temperature on the Ca^{2+} uptake system of the membrane. Fig. 4 shows the effect of 5 min of preincubation on Ca^{2+} uptake rate. While there was no measurable loss of rate at 17° and 27°, a 50 % loss of activity occurred at 37°. Fig. 5 shows the time course of loss of activity at 37°. This figure also shows that the presence of sucrose during preincubation largely antagonizes the loss of activity which normally occurs in 5 min at 37°. Figs. 6 and 7 show the ability of a number of sugars in various concentrations to protect the sarcoplasmic reticulum against the destructive effect of incubation at 37°. The monosaccharides glucose, fructose, and xylose are compared with sucrose in Fig. 6. Fructose is the only monosaccharide tested which did not demonstrate a protective action. The failure of fructose to protect offers evidence that protection is not simply an effect of increased osmolarity. The ketone group of the fructose is a major difference among these monosaccharides, and might be responsible for the difference in action. Fig. 7 shows that the disaccharides maltose and lactose protect against temperature denaturation as well as sucrose.

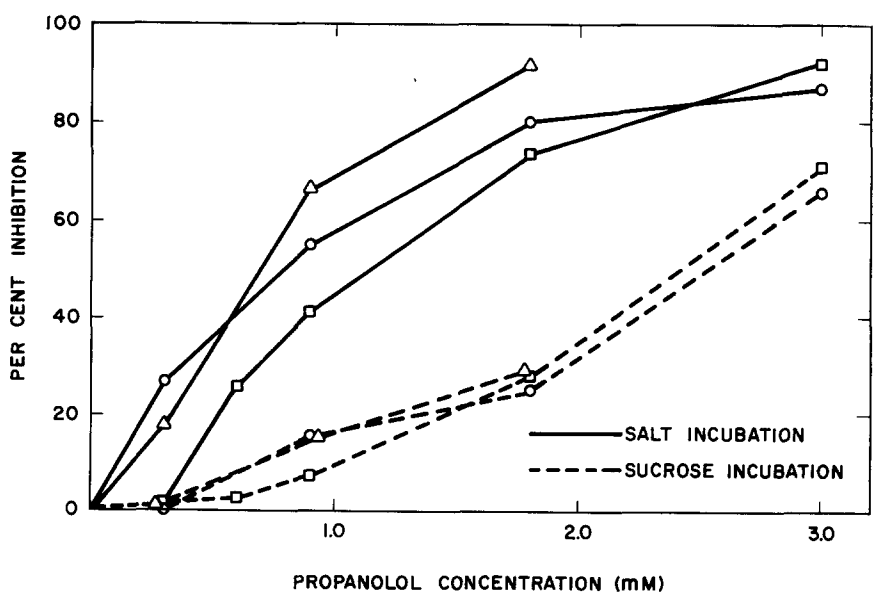

Fig. 1. Sucrose antagonism of propanolol inhibition of ATP-dependent Ca^{2+} uptake by cardiac sarcoplasmic reticulum. The salt incubation medium consisted of 100 mM KCl, 18 mM imidazole buffer (pH 7.0), 5 mM $MgCl_2$, 5 mM ATP, 1.8 mM oxalate. The sucrose incubation medium contained the same concentration of salts *plus* 300 mM sucrose. The temperature was 37°. Uptake rate was calculated from uptakes measured at 1 min, and ranged from 0.6–1.0 μmole/mg per min. Percent inhibition was calculated using zero drug concentration as control in the presence and absence of sucrose for the salt and sucrose experiment, respectively. Protein concentration was either 0.075 or 0.15 mg/ml. The symbols ○, △, and □ represent separate preparations of fragmented sarcoplasmic reticulum. Solid lines connecting these symbols are salt incubations, dashed lines are sucrose incubations.

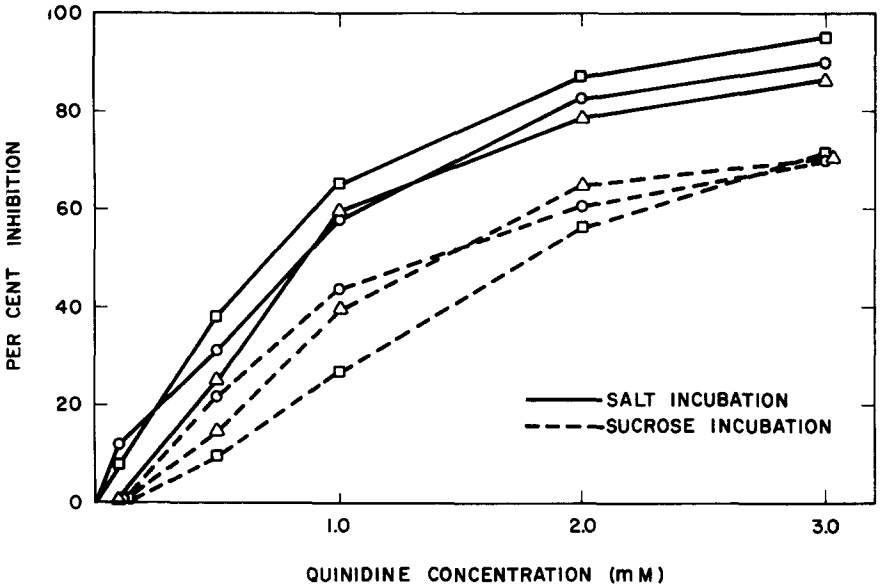

Fig. 2. Sucrose antagonism of quinidine inhibition of ATP-dependent Ca^{2+} uptake by cardiac sarcoplasmic reticulum. See legend to Fig. 1 for experimental conditions and explanation of symbols.

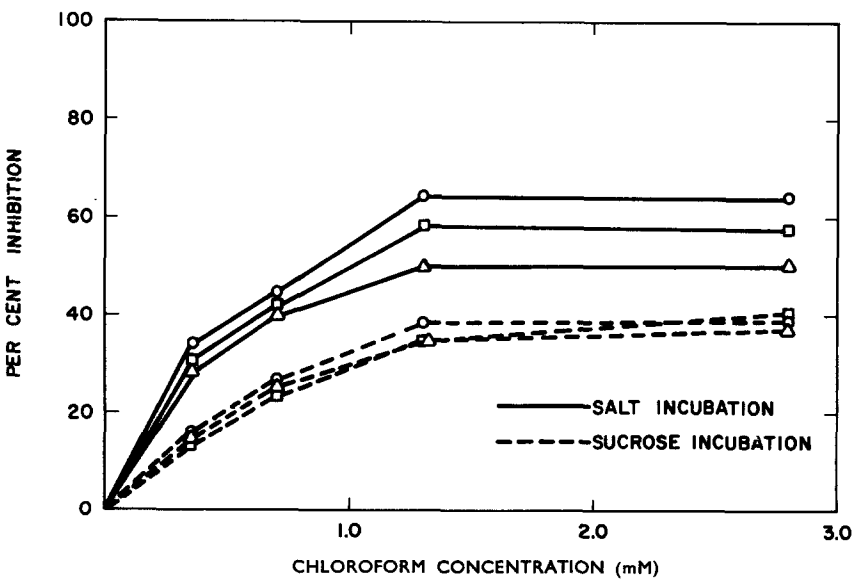

Fig. 3. Sucrose antagonism of chloroform inhibition of ATP-dependent Ca^{2+} uptake by cardiac sarcoplasmic reticulum. See legend to Fig. 1 for experimental conditions and explanation of symbols.

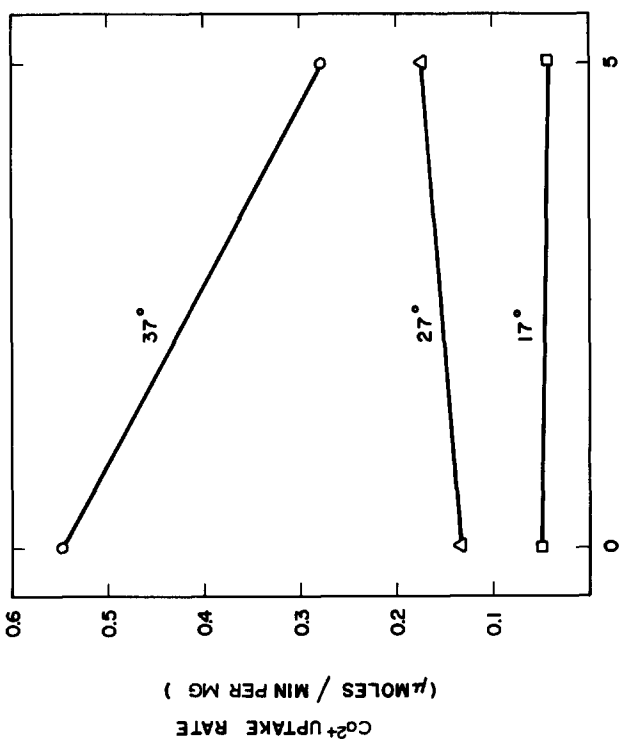

Fig. 4. The effect of preincubation temperature on rate of ATP-dependent Ca^{2+} uptake. Preincubations for 0 and 5 min were carried out in a salt medium the composition of which was 130 mM KCl, 22.4 mM imidazole buffer (pH 7.0), 2.24 mM potassium oxalate. Ca^{2+} uptake was initiated by the addition of ATP, Mg^{2+} and Ca^{2+}. For details see METHODS. Uptake rates were calculated from Ca^{2+} accumulated 2 min (17°), 1 min (27°), and 0.5 min (37°) after initiation of the reaction.

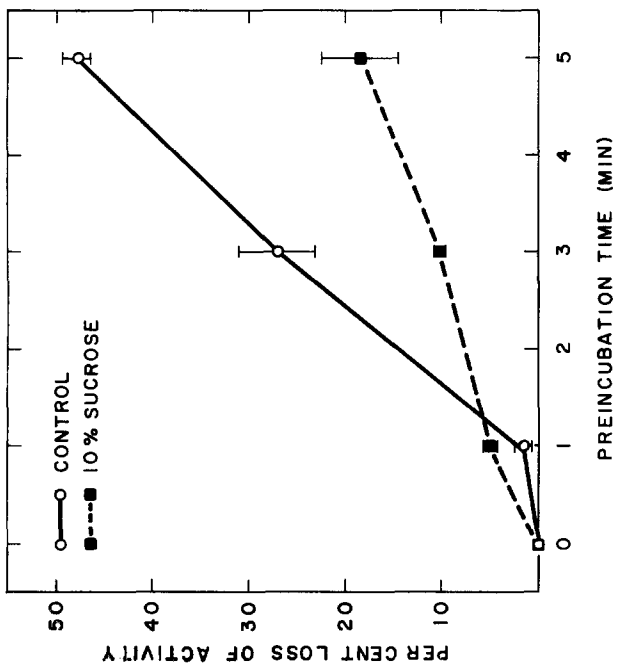

Fig. 5. The effect of preincubation time on rate of Ca^{2+} uptake. The composition of the salt solution was the same as in Fig. 4. Sucrose was added to a concentration of 300 mM in the sucrose *plus* salt experiment. Temperature, 37°, protein concentration, 0.15 mg/ml. Loss of activity was determined by comparison with the zero min preincubation rate. Control rates were obtained in the salt and salt sucrose experiments, respectively. The vertical bars present standard errors of the mean. The percent loss of activity in sucrose was significantly different ($P < 0.02$) from that in salt after incubation for 3 and 5 min. Three preparations were studied.

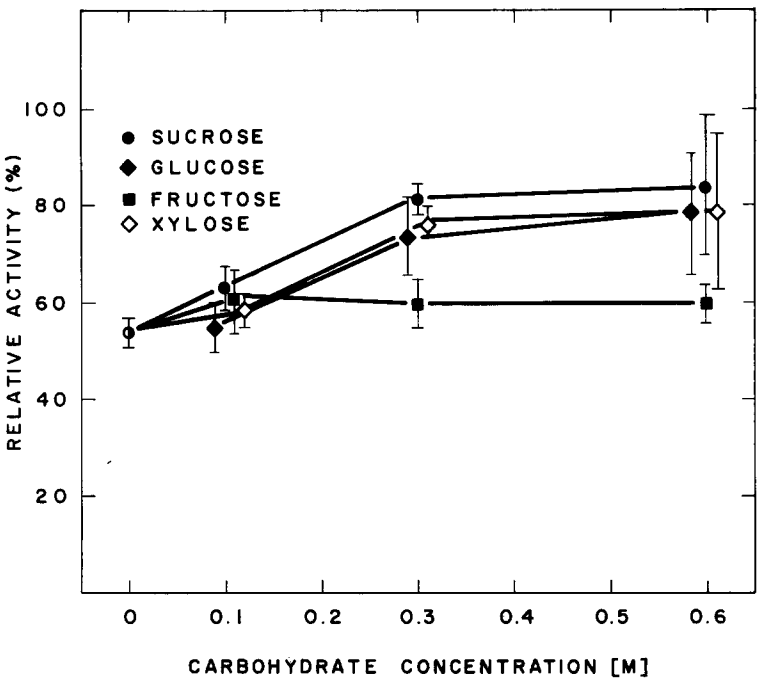

Fig. 6. The effect of monosaccharides on the deterioration of Ca^{2+} uptake rate by preincubation of sarcoplasmic reticulum for 5 min at 37°. The conditions during preincubation are the same as in Fig. 4. Three preparations were studied. Relative activity compares Ca^{2+} uptake rate after 5 min of preincubation in the indicated salt *plus* sugar solution with the rate of Ca^{2+} uptake in the same medium without preincubation.

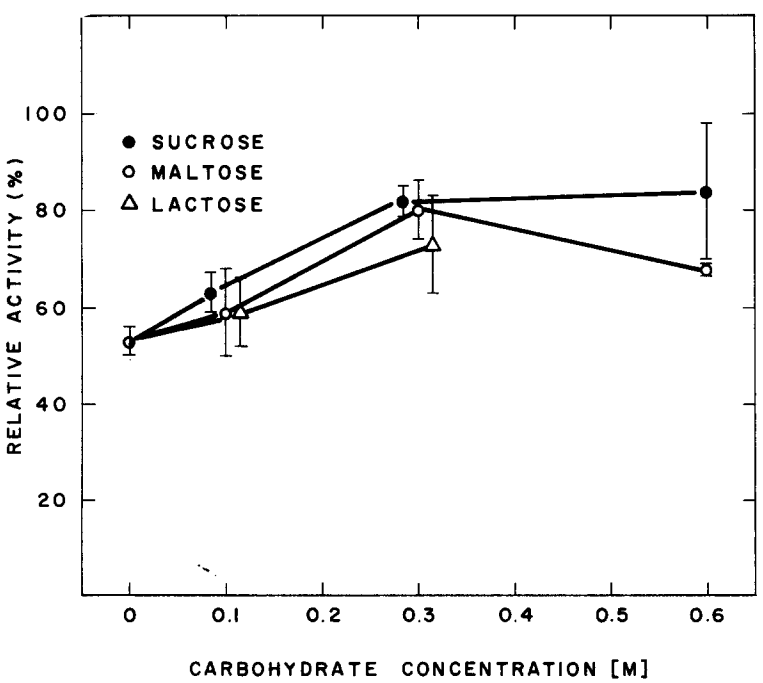

Fig. 7. The effect of disaccharides on the deterioration of Ca^{2+} uptake rate by preincubation of sarcoplasmic reticulum for 5 min at 37°. Experimental conditions are the same as in Fig. 6.

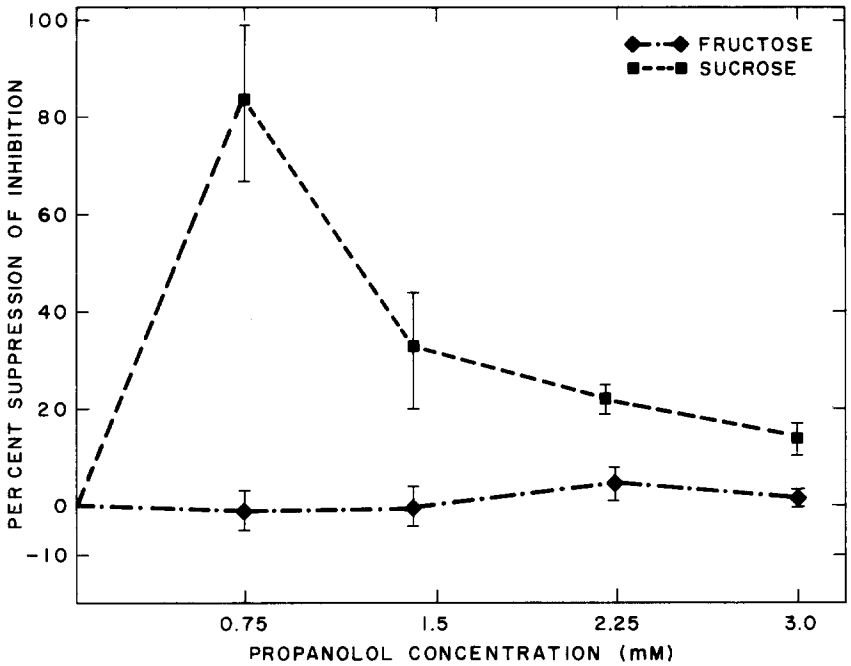

Fig. 8. The effect of fructose and sucrose on propanolol inhibition of Ca^{2+} uptake by cardiac sarcoplasmic reticulum. See legend to Fig. 1 for experimental conditions. Three preparations were studied. Vertical bars represent standard errors of the mean. Fructose suppression of inhibition was not significantly different from zero. Sucrose suppression was significantly different ($P < 0.05$) at all concentrations of propanolol.

Since fructose was unique in its inability to protect the sarcoplasmic reticulum from the effects of temperature, we examined its ability to protect the sarcoplasmic reticulum from the inhibitory action of propanolol. Fig. 8 compares the effect of sucrose and fructose on the propanolol inhibition of Ca^{2+} uptake. Fructose did not provide any protection while sucrose was highly antagonistic. The relative abilities of fructose and sucrose to protect against temperature and the drug propanolol are thus the same. The ability of fructose to antagonize other drugs was not tested.

Although it seemed unlikely, the possibility that sucrose might inhibit the binding of the drugs to the membrane needed to be eliminated. Chlorpromazine was chosen for this study since it was readily available as an isotopically labeled compound. Fig. 9 provides the results of a study in which the effects of sucrose on chlorpromazine binding and on Ca^{2+} uptake rate were compared under identical experimental conditions. The extent of chlorpromazine binding to cardiac sarcoplasmic reticulum preparations determined here is very similar to that measured by BALZER *et al.*[16] on skeletal muscle sarcoplasmic reticulum preparations. Although sucrose clearly antagonized the chlorpromazine inhibition of Ca^{2+} uptake, it was completely without effect on the binding of the drug to the membrane. It seems, therefore, that the action of sucrose must be involved more with the consequences of drug binding than with binding *per se*.

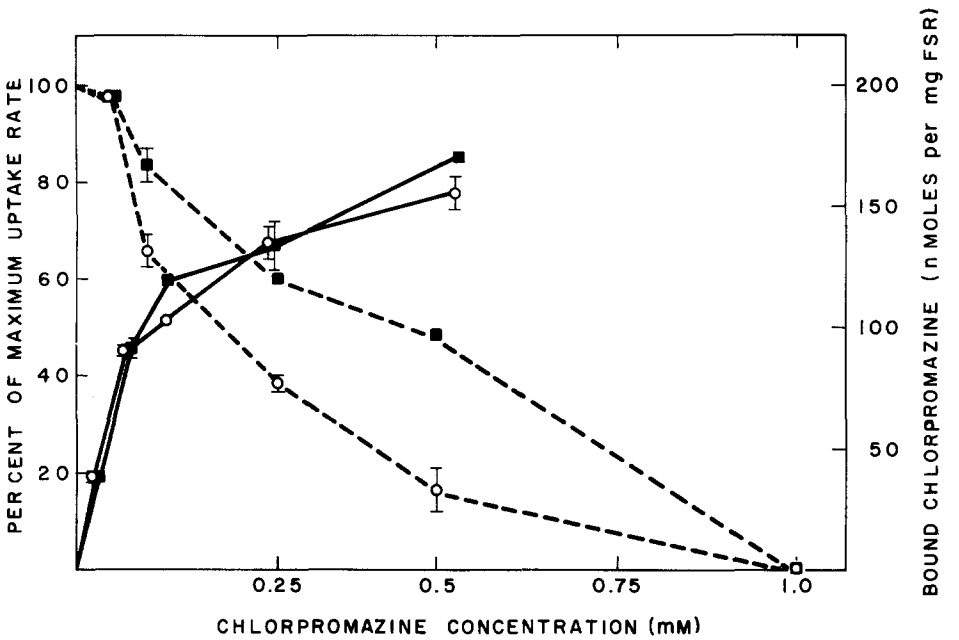

Fig. 9. Effect of sucrose on chlorpromazine binding and inhibition of Ca^{2+} uptake. Conditions for salt and sucrose incubation as in Fig. 1. Chlorpromazine binding was measured as outlined in METHODS. Symbols represent the mean and range of two experiments on separate fragmented sarcoplasmic reticulum preparations. Percent maximum uptake rate in salt incubation (○---○), and in sucrose incubation (■---■). Chlorpromazine binding in salt incubation (○—○) and in sucrose incubation (■—■).

DISCUSSION

The results presented above indicate that sucrose antagonizes the effect of temperature as well as a number of drugs on Ca^{2+} uptake by the cardiac sarcoplasmic reticulum. Fructose, in contrast, was unable to antagonize either type of inhibition. This similarity in chemical specificity suggests that the drugs and the temperature of 37° may influence membrane structure in a similar manner. This conclusion has also been reached recently by JOHNSON AND INESI[17] who found that temperature induced denaturation and pharmacological intervention produce parallel alterations in the permeability of the sarcoplasmic reticulum to Ca^{2+}.

The easiest way to account for the above phenomena would be to find a common mechanism for the action of the drugs and temperature. One possible mechanism involves the tendency of both types of treatments to produce membrane effects which are related to a disordering of lipoprotein structure which is associated with an increase in hydrophobic interaction following exposure to temperature or drugs. This tendency is often referred to as fluidizing or melting of a portion of the membrane structure. Evidence for such an action by drugs has been presented by HUBBELL AND MCCONNELL[8] who found that tetracaine enhances the solubility of the spin label TEMPO in membranes. These authors suggest that this increase in solubility "is due to a local disordering or 'melting' of the hydrophobic regions of the membranes, which increases the 'fluid' volume available to

TEMPO". A similar conclusion has been reached by METCALFE *et al.*[18] using NMR relaxation methods. The component of the membrane, lipid, protein or both, involved in this fluidization by local anesthetics is currently unknown[8]. The lipids of the membrane, however, are well known to engage in hydrophobic interactions[19], and the drugs tested in this study have been shown to bind to lipids of the sarcoplasmic reticulum[16, 20]. Temperatures in the range of 37° apparently also increase the fluidity of membranes. For example, CHAPMAN *et al.*[21] report that the ^2H_2O NMR spectrum is markedly sharpened as the temperature is increased to 30–40°. This sharpening is interpreted as indicating a change in membrane structure from crystalline to liquid crystalline. The same type of temperature induced structural change has been inferred by REINERT AND STEIN[10] using a scanning differential calorimeter. It is of interest that the temperature range, 30–40°, where the phase transition sets in corresponds to the temperature at which we found loss of Ca^{2+} transport activity. There was no loss of activity in 5 min at 17 and 27°.

If 37° and the types of drugs studied fluidize the membrane as discussed above, then the consequent loss of Ca^{2+} uptake activity could almost be predicted. A number of laboratories[22, 23] have shown that removal of lipid from the membranes of the sarcoplasmic reticulum can lead to a loss of both Ca^{2+} uptake activity and ATPase activity. The interpretation which has been put on these results is that lipid is required to provide the conformation necessary for the protein to operate as an ATPase and presumably to participate in Ca^{2+} transport. With a decrease in lipid–protein interaction which could be caused either by 37° or by drugs, the lipid fraction of the membrane might easily not confer the necessary protein conformations and Ca^{2+} transport activity would be lost as observed in our studies.

If the drugs and 37° decrease Ca^{2+} uptake by fluidizing the membrane and rendering the ATPase site inactive, then how do sucrose and the sugars other than fructose antagonize this loss of activity? The studies with ^{35}S-labeled chlorpromazine clearly indicate that sucrose does not influence the binding of the drug to the membrane. It appears, therefore, that sucrose probably does not interfere with the effect of the drugs or 37° on membrane lipid. It has been speculated in previous studies that the effects of sucrose on drug–membrane interaction are not related to the sucrose molecule but rather to the decrease in ionic strength that accompanies the substitution of sucrose for salt[24]. The present studies, which did not involve such a substitution indicate that sucrose, *per se*, can be responsible for the loss of drug effect. Since a loss of protein conformation following alteration in lipid structure is central to our explanation for the loss of activity, it is necessary to examine how the sugars might protect against this conformational change. One possibility that we would like to suggest is the following: The ATPase site which is involved in Ca^{2+} transport must be hydrophilic in nature if the substrate, ATP, is to approach this site. A conformation change at this site might, therefore, ultimately involve hydrogen bonding between protein and water. It is this latter reaction against which sucrose might protect. MACRITCHIE AND ALEXANDER[25] have indeed shown that sucrose can decrease the hydrogen bonding between water and protein and suggest that sucrose does this by competing with the protein for water. The disparity in the effects of fructose and sucrose on the parameters studied is intriguing but difficult to explain. On the basis of the above proposition one might infer that fructose does not participate in hydrogen bonding with water molecules as well as sucrose or pro-

teins. In this regard TAYLOR AND ROWLINSON[26] have shown a stability difference in the packing of water molecules in association with different sugars although they did not study fructose *per se*.

ACKNOWLEDGMENTS

This investigation was supported by a research grant from the Public Health Service No. HE 06782, by Public Health Service Training Grant No. GM 01404 to R. J. S. and E. W. G., and by a Public Health Service Career Award No. 1K6 HE 5290 to F. N. B.

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