Biochemical Pharmacology, Vol. 23, pp. 2369–2376. Pergamon Press, 1974. Printed in Great Britain.

EFFECTS OF ETHANOL ON CALCIUM BINDING AND CALCIUM UPTAKE BY CARDIAC MICROSOMES*

MARK H. SWARTZ, DORIS I. REPKE, ARNOLD M. KATZ† and EMANUEL RUBIN‡

Division of Cardiology, Department of Medicine and Pathology,
The Mount Sinai School of Medicine of the City University of New York, Fifth Avenue and 100th Street, New York, N.Y. 10029, U.S.A.

(*Received* 2 *October* 1973; *accepted* 18 *January* 1974)

Abstract—Calcium uptake and Ca binding by cardiac microsomes enriched in fragmented sarcoplasmic reticulum were inhibited by ethanol. After 5 min of incubation, half-maximal inhibition of the former was seen at approximately 1·3 M ethanol, that of the latter was seen at approximately 1·9 M ethanol. Neither Ca uptake nor Ca binding was stimulated at lower concentrations of ethanol. Prolonged exposure of the microsomes to ethanol increased the extent of inhibition of both Ca uptake and Ca binding. These inhibitory effects were almost completely reversed when microsomes were washed after exposure to ethanol. Inclusion of 0·12 M Na^+ instead of 0·12 M K^+ increased the inhibitory effect of ethanol on Ca uptake, but did not significantly change the sensitivity of Ca binding to ethanol. These inhibitory effects of ethanol were seen at concentrations that generally stabilize membrane structures and are associated with an anesthetic action. Even though the concentrations of ethanol needed to inhibit Ca uptake and Ca binding exceeded those found in the blood of chronic alcoholic patients, the time dependence of these effects raises the possibility that prolonged exposure to concentrations of ethanol might contribute to the myocardial weakness of chronic alcoholics by interfering with the retention of Ca^{2+} within the cardiac sarcoplasmic reticulum.

MUSCLE weakness has long been known to occur in chronic alcoholics,[1] and a well defined syndrome of muscle disease may result from prolonged alcohol abuse.[2] Similarly, impairment of cardiac function occurs in some chronic alcoholics.[3–7] These disorders are independent of nutritional factors because muscle damage[8] and disordered myocardial function have been produced by giving ethanol with adequate diets.[9] When administered acutely, ethanol causes impaired myocardial contractility in intact dogs,[10–13] the reported concentration range at which this negative inotropic effect was seen ranged between 15 and 65 mM. Similar findings have been reported for the isolated rat heart.[14,15] In one report, the maximum rate of left ventricular pressure rise was found not to be depressed in the isolated canine ventricle exposed to as much as 200 mM ethanol, although the data for end-diastolic volume included in this paper show a sizable rise in end-diastolic pressure at much lower ethanol concentrations.[16] There is general agreement that acute, as well as chronic, exposure to ethanol can impair the contractile performance of the heart.

* Supported by a Grant-in-Aid from the New York Heart Association, United States Public Health Service Grants HL-13191, AA 287 and AA 316, Contract No. NIH-NHLI-72-2973-M, and the Jack Martin Fund.

† Philip J. and Harriet L. Goodhart Professor of Medicine (Cardiology).

‡ Irene Heinz and John LaPorte Given Professor and Chairman of the Department of Pathology.

The sarcoplasmic reticulum of the heart is one of the systems which could be deleteriously affected by ethanol. This membrane system participates in the mediation of excitation–contraction coupling within the myocardium, presumably by releasing Ca^{2+} to the cardiac contractile proteins in response to a signal originating from the action potential. Calcium binding and calcium uptake, which represent two kinetically dissimilar mechanisms by which Ca^{2+} can be removed from solution by the membranes of the sarcoplasmic reticulum, can be examined in preparations of cardiac microsomes. Ca binding is a rapid but limited saturable mechanism by which Ca^{2+} becomes associated with cardiac microsomes, whereas Ca uptake is a slower but more extensive transport of Ca^{2+} into the interior of the microsomal vesicles, where the cation is trapped by a precipitating agent such as oxalate, which forms an insoluble complex with Ca^{2+}.[17] Since interaction of ethanol with hepatic microsomes[18] and an inhibition of certain microsomal enzyme systems in the liver[19] have been demonstrated, we studied actions of ethanol *in vitro* on Ca uptake and Ca binding by canine cardiac microsomes.

METHODS

Microsomes were prepared from the hearts of dogs anesthetized with pentobarbital. The ventricles were washed in cold water and placed in crushed ice. Subsequent procedures were performed in the cold room (4°). The ventricular myocardium was excised, cut into 2 × 2 cm slices, weighed, placed in 5 vol. of 0·01 M $NaHCO_3$ solution and homogenized in a Waring blender equipped with a variable transformer. Homogenization at 120 V for 10 sec was repeated three times and the homogenate was centrifuged at 8000 *g* for 20 min. The supernatant was decanted through four layers of gauze and recentrifuged at 8000 *g* for 20 min. The supernatant was decanted into clean tubes and centrifuged at 37,000 *g* for 30 min. The pellets were suspended in 0·6 M KCl–20 mM Tris, pH 6·8, gently dispersed with a Potter–Elvehjem homogenizer and centrifuged at 37,000 *g* for 30 min. The final pellet was suspended in a small volume of 50 mM KCl–20 mM Tris HCl, gently homogenized in the Potter–Elvehjem homogenizer and stored on ice. The protein concentration was determined by the biuret method with bovine serum albumin as standard.

All studies were carried out within 6 hr after the removal of the heart. Calcium uptake and calcium binding were measured with ^{45}Ca-EGTA* buffers, as described previously.[20] Both were determined at microsomal concentrations of 0·2 mg/ml in solutions containing: $7{\cdot}2 \times 10^{-7}$ M Ca^{2+} (25 μM $CaCl_2$ and 88 μM EGTA); 0·12 M KCl; 40 mM histidine, pH 6·8; and 5 mM MgATP (Na^+-free) at 25°. Tris oxalate, 2·5 mM, was used in the measurement of calcium uptake. All reaction mixtures were incubated with MgATP for 5 min prior to addition of the Ca-EGTA buffer. The amount of calcium associated with the microsomes was determined by Millipore filtration as described earlier.[17]

To determine the reversibility of the effect of ethanol, washout experiments were performed as follows: 2 mg microsomal protein (initial concentration, 0·2 mg/ml) was added to a 15-ml centrifuge tube together with 2·5 ml of 0·48 M KCl–0·16 M histidine, pH 6·8, and 1·0 ml of 50 mM MgATP (Na^+-free). This constituted the control tube. The other tubes contained the same reaction mixture plus 1·06 M ethanol. After

* The abbreviations used are: EGTA, ethylene glycol bis-(β-aminoethyl) *N,N'*-tetraacetic acid; ATP, adenosine triphosphate.

30 min of incubation at 25°, all tubes, including the control, were centrifuged at 105,000 *g* for 20 min. Each pellet was resuspended in 1·0 ml of 50 mM KCl–20 mM Tris, pH 6·8, and homogenized gently in a Potter–Elvehjem homogenizer. After determination of protein concentration, studies of Ca binding and Ca uptake were carried out as described above.

All inorganic chemicals were reagent grade and obtained from the Mallinckrodt Chemical Works. Distilled water was deionized and redistilled in glass prior to use. Sodium-free ATP was prepared by passage of disodium ATP (Sigma Chemical Co.) through Dowex 50 in the H^+ form followed by neutralization with Tris and $MgCl_2$. Ethanol (95%) was redistilled and the constant boiling mixture was used in these studies.

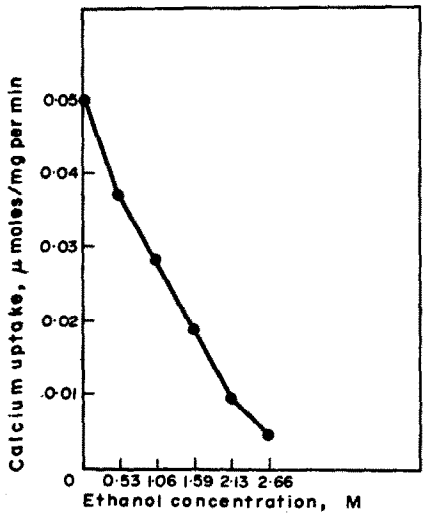

FIG. 1. Concentration dependence of ethanol-induced inhibition of Ca uptake by cardiac microsomes. Reactions were carried out with 0·2 mg/ml of cardiac microsomes in $7·2 \times 10^{-7}$ M Ca^{2+}, as described in Methods. Microsomes were incubated with ethanol for 5 min prior to addition of the ^{45}Ca-EGTA buffer.

RESULTS

Inhibition of Ca uptake and Ca binding by ethanol—Concentration dependence. Calcium uptake was inhibited by ethanol, the extent of inhibition increasing at higher concentrations of ethanol (Fig. 1). Fifty per cent inhibition was seen at approximately 1·3 M ethanol. Calcium binding was less sensitive to ethanol than was calcium uptake, 50 per cent inhibition being seen at approximately 1·9 M ethanol (Fig. 2). Neither Ca uptake nor Ca binding was affected by ethanol concentrations below 0·1 M (Fig. 3).

Inhibition of Ca uptake and Ca binding by ethanol—Time dependence. The time dependence of the effect of ethanol on calcium uptake and calcium binding was assessed by varying the period of preincubation with 1·08 M ethanol prior to the addition of Ca^{2+}. As the preincubation time was increased from 0 to 60 min, the extent of inhibition of both calcium uptake and calcium binding increased (Fig. 4, Table 1). The control values, measured in the absence of ethanol, did not change significantly with increasing preincubation time (Fig. 4).

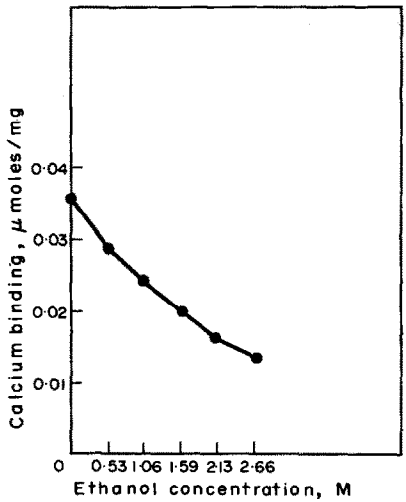

FIG. 2. Concentration dependence of ethanol-induced inhibition of Ca binding by cardiac microsomes. Reactions were carried out as in Fig. 1.

Reversibility of ethanol effects. Washing of the microsomes after exposure to ethanol greatly reduced the extent of inhibition, indicating that the deleterious effects of ethanol are reversible. In the case of Ca uptake, 30 min of exposure to 1·06 M ethanol caused 31 per cent inhibition of Ca uptake, whereas when measurements were made with microsomes exposed to this concentration of ethanol for 30 min and then washed, no inhibition was seen (Table 2). Similarly, in the case of Ca binding, reversibility of ethanol-induced inhibition was also seen in that exposure to 1·06 M ethanol followed by washing resulted in no significant inhibition, whereas Ca binding was inhibited by 36 per cent when ^{45}Ca-EGTA buffer was added directly to the microsomes after 30 min of exposure to 1·06 M ethanol (Table 2). The reversibility of

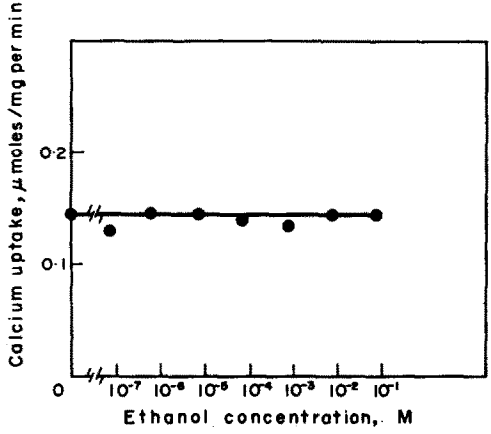

FIG. 3. Effects of low concentrations of ethanol on Ca uptake by cardiac microsomes. Reactions were carried out at $7{\cdot}2 \times 10^{-7}$ M Ca^{2+} with 0·015 mg/ml of microsomes. Microsomes were exposed to ethanol for 5 min in the presence of 5 mM MgATP prior to addition of the ^{45}Ca-ETGA buffer.

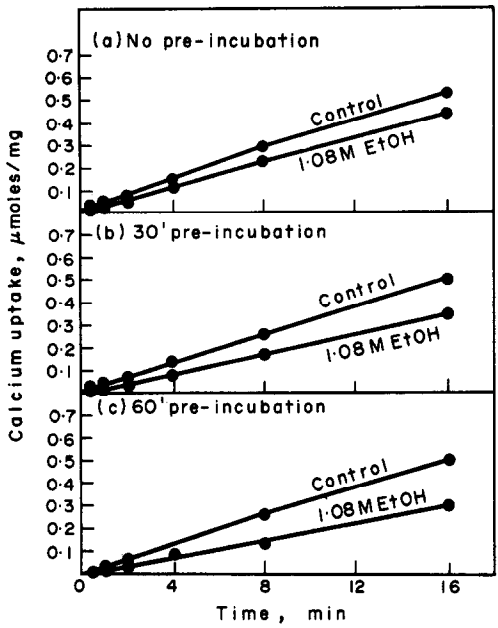

FIG. 4. Time dependence of ethanol-induced inhibition of Ca uptake by cardiac microsomes. Microsomes (0·2 mg/ml) were incubated in 1·08 M ethanol for 0 min (panel a), 30 min (panel b) and 60 min (panel c), after which MgATP was added to a final concentration of 5 mM. Other reaction conditions were as described in Methods. Five min after addition of MgATP, the reactions were started by the addition of the ^{45}Ca-EGTA buffer ($[Ca^{2+}] = 7{\cdot}2 \times 10^{-7}$).

these ethanol effects could not be attributed to selective recovery of "native" membranes after washing because the recovery of protein after washing was not reduced by ethanol treatment (Table 2).

Effects of Na^+ and K^+ on ethanol-induced inhibition of Ca uptake and Ca binding. The inhibitory effect of ethanol on Ca uptake was greater in the presence of Na^+ than in the presence of an equimolar concentration of K^+ (Table 3). As has been reported previously,[21] Ca uptake was less in the presence of 0·12 M NaCl than with

TABLE 1. TIME DEPENDENCE OF INHIBITION OF Ca UPTAKE AND Ca BINDING BY 1·08 M ETHANOL*

Preincubation time (min)	Control	1·08 M ethanol	% Inhibition
Ca uptake (μmoles/mg/min)			
0	0·034	0·030	12
30	0·033	0·022	33
60	0·034	0·019	44
Ca binding (μmoles/mg)			
0	0·028	0·020	29
30	0·028	0·015	46
60	0·030	0·013	56

* Microsomes were incubated with MgATP with and without ethanol (1·08 M) prior to addition of the ^{45}Ca-EGTA buffer. Protein concentration was 0·2 mg/ml for both Ca uptake and Ca binding, each of which was studied with a different preparation.

TABLE 2. REVERSIBILITY OF ETHANOL INHIBITION OF Ca UPTAKE AND Ca BINDING*

	Control	1·06 M ethanol	% Inhibition
Ca uptake (μmoles/mg/min)			
Not washed	0·088	0·060	32
Washed	0·074	0·080	none
Ca binding (μmoles/mg)			
Not washed	0·023	0·015	35
Washed	0·029	0·029	none
Recovery after washing (%)			
Control microsomes 72			
1·06 M ethanol-treated microsomes 70			

* Microsomes (0·2 mg/ml) were incubated with ethanol for 30 min and washed. Subsequent Ca binding and Ca uptake were carried out as described in Methods. The "Not washed" microsomes were also preincubated in ethanol for 30 min prior to the addition of ^{45}Ca-EGTA.

TABLE 3. $Na^+ + K^+$ EFFECTS ON ETHANOL-INDUCED INHIBITION OF Ca UPTAKE AND Ca BINDING*

	0·12 M KCl	0·12 M NaCl
Ca uptake (μmoles/mg/min)		
Control	0·019	0·010
1·06 M ethanol	0·014	0·006
% Inhibition	26	40
Ca binding (μmoles/mg)		
Control	0·036	0·034
1·06 M ethanol	0·025	0·022
% Inhibition	30	35

* The effects of 5 min of exposure to ethanol in 0·12 M KCl and 0·12 M NaCl were examined as described in Methods. Protein concentrations were 0·2 mg/ml for both Ca uptake and Ca binding.

TABLE 4. COMPARISON OF GLYCEROL AND ETHANOL EFFECTS ON Ca BINDING AND Ca UPTAKE*

	Ca binding (nmoles/mg)	% Inhibition
Control	0·0146	
0·5 M glycerol	0·0132	9·6
0·5 M ethanol	0·0112	23·3
1·0 M glycerol	0·0142	2·7
1·0 M ethanol	0·0082	43·8
1·5 M glycerol	0·0130	11·0
1·5 M ethanol	0·0057	61·0
	Ca uptake (μmoles/mg/min)	**% Inhibition**
Control	0·050	
1·0 M glycerol	0·036	28·0
1·0 M ethanol	0·016	68·0

* Microsomes (0·2 mg/ml) were incubated with either glycerol or ethanol for 30 min prior to addition of the ^{45}Ca-EGTA buffers.

0·12 M KCl (Table 3). Ca binding, on the other hand, is not significantly influenced by replacement of K^+ by Na^+,[21] nor was the extent of inhibition by ethanol significantly affected by the nature of the cation (Table 3).

Nonspecific osmotic effects of ethanol. In view of the high ethanol concentrations used in this study, it was necessary to exclude the possibility that inhibition of Ca binding and Ca uptake was due solely to hyperosmolarity. When the effects of glycerol were compared with those of ethanol, slight inhibition of Ca binding and a modest inhibition of the Ca uptake were seen, though these were much less than the inhibitory effects of ethanol (Table 4).

DISCUSSION

Ethanol is one of the large class of chemical compounds that can induce general anesthesia. These agents share the general property of stabilizing membrane functions, e.g. protecting erythrocytes against osmotic hemolysis at low concentrations and disrupting cell membranes at higher concentrations.[22] In the case of ethanol, the stabilizing effects are seen at concentrations above 0·1 M; for example, half-maximal antihemolysis of human erythrocytes occurs at 0·22 M ethanol,[23] a level only slightly less than that which blocks conduction in the frog sciatic nerve at 22°.[23] The concentration of ethanol required for a disrupting effect is appreciably higher, as evidenced by the finding that the electrical resistance of bimolecular (black) lipid membranes falls significantly at ethanol concentrations greater than 1 M.[24] This decrease in electrical resistance, which in the case of higher alcohols occurs at concentrations similar to those that disorient spin-labeled lipid bilayers,[22] may reflect disruption of hydrophobic bonds, although changes due to an increase in the dielectric constant near charged groups, or the replacement of water of hydration with polar groups, cannot be excluded.[24] The finding that ethanol at concentrations below 1 M, i.e. in the range where membrane stabilization occurs, impairs both Ca uptake and Ca binding (Figs. 1,2) suggests that these effects do not result simply from a lipid-disordering effect of ethanol.

The effects of ethanol on Ca uptake and Ca binding by cardiac microsomes are dependent on both concentration and duration of exposure. There is no evidence for a "stabilizing" action of this compound that is manifest as an enhancement of the Ca transport system. These findings with an enriched preparation of cardiac sarcoplasmic reticulum contrast with the effects of general anesthetics on human erythrocyte ghosts, in which Ca binding is increased.[22] In the case of human erythrocytes, Ca binding has been reported to increase as ethanol concentration was increased from $<0·1$ M in the presence of 0·8 mM free Ca^{2+}.[25] This stimulation of Ca binding was attributed to enhanced Ca^{2+} affinity of the erythrocyte membrane rather than to an increase in the number of sites accessible to Ca^{2+}.[25] Thus, the responses of the high affinity Ca binding and Ca uptake systems of the heart's sarcoplasmic reticulum differ from those of the lower affinity Ca binding system of the erythrocyte membrane.

Ethanol has been noted previously to be a weak inhibitor of Ca binding by skeletal muscle microsomes.[26] Inhibition of Ca uptake[27] and Ca binding[28] have also been observed with ether-treated skeletal muscle microsomes. Moreover, aromatic alcohols, notably thymol,[26,29,30] inhibit Ca binding by skeletal microsomes, and inhibition

of Ca uptake by skeletal muscle microsomes by n-alcohols has been found to increase with increasing number of carbon atoms.[31]

It is premature to attribute the negative inotropic actions of ethanol to its inhibitory actions on the transport of Ca^{2+} by the sarcoplasmic reticulum. While a plausible hypothesis can be made to link inhibition of Ca^{2+} transport into intracellular stores to a reduction in the Ca^{2+} released at the onset of systole,[32] the sensitivities of both Ca uptake and Ca binding by cardiac microsomes to ethanol *in vitro* are low, especially when K^+ is the major cation. For example, exposure of the cardiac microsomes to 100 mM ethanol, the lower level of the lethal range of blood ethanol concentration in man, has little effect on these activities. The finding that prolonged exposure of cardiac microsomes to ethanol increases their sensitivity to the inhibitory effects provides some basis for the speculation that the negative inotropic action of ethanol may be linked to the reversible effects on the sarcoplasmic reticulum that are documented in the present study.

REFERENCES

1. G. T. PERKOFF, *A. Rev. Med.* **22,** 125 (1971).
2. H. FAHLGREN, R. HED and C. LUNDMARK, *Acta med. scand.* **158,** 405 (1957).
3. W. EVANS, *Am. Heart J.* **61,** 556 (1961).
4. G. E. BURCH and T. D. GILES, *Am. J. Med.* **50,** 141 (1971).
5. T. J. REGAN, G. E. LEVINSON and H. A. OLDEWURTEL, *J. clin. Invest.* **48,** 397 (1969).
6. T. J. REGAN, *Circulation,* **44,** 957 (1971).
7. D. H. SPODNICK, V. M. PIGGOTT and R. CHIRIFE, *New Engl. J. Med.* **287,** 677 (1972).
8. S. K. SONG and E. RUBIN, *Science, N.Y.* **175,** 327 (1972).
9. D. S. MIERZWIAK, K. WILDENTHAL and J. H. MITCHELL, *Clin. Res.* **15,** 215 (1967).
10. I. U. DEGERLI and W. R. WEBB, *Surg. Forum* **14,** 252 (1963).
11. T. J. REGAN, G. T. KOROXENISIS, C. B. MOSCHOS, H. A. OLDEWURTEL, P. H. LEHAN and H. K. HELLEMS, *J. clin. Invest.* **45,** 270 (1966).
12. L. C. MENDOZA, K. HELBERG, A. RICKART, G. TILLICH and R. J. BING, *J. clin. Pharmac.* **11,** 165 (1971).
13. J. NAKANO and J. M. KESSINGER, *Eur. J. Pharmac.* **17,** 195 (1972).
14. A. L. GIMENO, M. F. GIMENO and J. L. WEBB, *Am. J. Physiol.* **203,** 194 (1962).
15. A. LOCHNER, R. COWLEY and A. J. BRINK, *Am. Heart J.* **78,** 770 (1969).
16. W. R. WEBB, D. N. GUPTA, W. A. COOK, W. L. SUGG, F. A. BASHOUR and M. O. UNAL, *Dis. Chest* **52,** 602 (1967).
17. D. I. REPKE and A. M. KATZ, *J. molec. cell. Cardiol.* **4,** 401 (1972).
18. E. RUBIN, C. S. LIEBER, A. P. ALVARES, W. LEVIN and R. KUNTZMAN, *Biochem. Pharmac.* **20,** 229 (1971).
19. E. RUBIN, H. GANG, P. MISRA and C. S. LIEBER, *Am. J. Med.* **49,** 801 (1970).
20. A. M. KATZ, D. I. REPKE, J. E. UPSHAW and M. A. POLASCIK, *Biochim. biophys. Acta* **205,** 473 (1970).
21. A. M. KATZ and D. I. REPKE, *Circulat. Res.* **21,** 767 (1967).
22. P. SEEMAN, *Pharmac. Rev.* **24,** 583 (1972).
23. S. ROTH and P. SEEMAN, *Nature New Biol.* **231,** 284 (1971).
24. J. GUTKNECHT and D. C. TOSTESON, *J. gen. Physiol.* **55,** 359 (1970).
25. P. SEEMAN, M. CHAU, M. GOLDBERG, T. SAUKS and L. SAX, *Biochim. biophys. Acta* **225,** 185 (1971).
26. S. EBASHI, F. OOSAWA, T. SEKINE and Y. TONOMURA (Eds.), *Molecular Biology of Muscular Contraction,* p. 197. Igaku Shoin, Tokyo (1965).
27. W. FIEHN and W. HASSELBACH, *Eur. J. Biochem.* **9,** 574 (1969).
28. G. INESI, J. J. GOODMAN and S. WATANABE, *J. biol. Chem.* **242,** 4637 (1967).
29. J. GERGELY, G. KALDOR and F. N. BRIGGS, *Biochim. biophys. Acta* **34,** 218 (1959).
30. Y. OGAWA, *J. Biochem., Tokyo* **67,** 667 (1970).
31. M. KONDO and M. KASAI, *Biochim. biophys. Acta* **311,** 391 (1973).
32. A. M. KATZ and D. I. REPKE, *Am. J. Cardiol.* **31,** 193 (1973).