The Effects of Substrates on Rat Atria Depressed with Bicarbonate-Free Medium, Citrate, or Low Calcium[1] (34815)

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Cardiac contractility is depressed by citrate (1, 2), bicarbonate-free medium (3–5), and low extracellular calcium (6, 7). Biochemical studies involving homogenates of various tissues indicate that citrate is a potent inhibitor of phosphofructokinase (8–10). Bicarbonate-free medium also inhibits the phosphofructokinase activity of diaphragm muscle in the rat (11, 12). It is well established that phosphofructokinase plays an important role in the regulation of glycolysis in the cell (13–16), and that the operation of the glycolytic pathway is important for a fraction of the contractile activity of the myocardium (17, 18).

Exogenous substrates have served as useful tools for the study of the mechanism of cardiac-depressant agents. By the use of certain substrates the inhalation anesthetic halothane was found to inhibit either glucose uptake or an early stage in its metabolism (19, 20). The present experiment is an attempt to investigate the mechanism of other cardiac depressants by the use of exogenous substrates; in particular, this study attempts to elucidate the role of phosphofructokinase in the mechanism of action of bicarbonate-free medium and citrate. Experiments with low calcium were performed to determine whether citrate depression was due to chelation of extracellular calcium.

Methods. Male Sprague-Dawley rats, weighing 180–200 g, which had *ad libitum* access to food and water, were employed. Atria were removed from decapitated rats and suspended in 50 ml of modified Krebs–Ringer bicarbonate glucose medium (19, 21) of the following composition (m*M*): NaCl 120; KCl 4.8; $CaCl_2$ 1.22; $MgSO_4 \cdot 7H_2O$ 1.33; KH_2PO_4 1.2; $NaHCO_3$ 25.3; glucose 5.55. The medium was aerated with 95% O_2 and 5% CO_2 to maintain a pH of 7.4 at 30°. A constant resting tension of 750 mg was maintained throughout the experiment. The developed tension was recorded with a Statham strain gage, and the atria were electrically stimulated at a rate of 200 pulses/min. An equilibration period of 60 min was allowed before readings were taken. The experimental values of contractility (peak tension) were compared with control values obtained at zero time (after equilibration) and expressed as percentage of change in developed tension.

In the experiments with bicarbonate-free medium, the procedures were conducted by means of techniques previously described by Ko *et al.* (4). The bicarbonate-free medium was prepared by replacing the sodium bicarbonate from the Krebs–Ringer bicarbonate glucose medium with an equivalent concentration of sodium chloride and bubbling with 100% O_2. The pH of the bicarbonate-free medium was initially adjusted with dilute sodium hydroxide to 7.4 just prior to the experimental procedure. Electrodes, placed in the tissue bath to monitor pH, demonstrated no significant change from 7.4 throughout the course of the bicarbonate-free experiments. In the experiments with low-calcium medium, the medium was prepared by omitting a ½ or ¾ amount of calcium chloride from the Krebs–Ringer bicarbonate glucose medium. The sodium pyruvate, sodium acetate and L(+) lactic acid (grade L-I) employed in this

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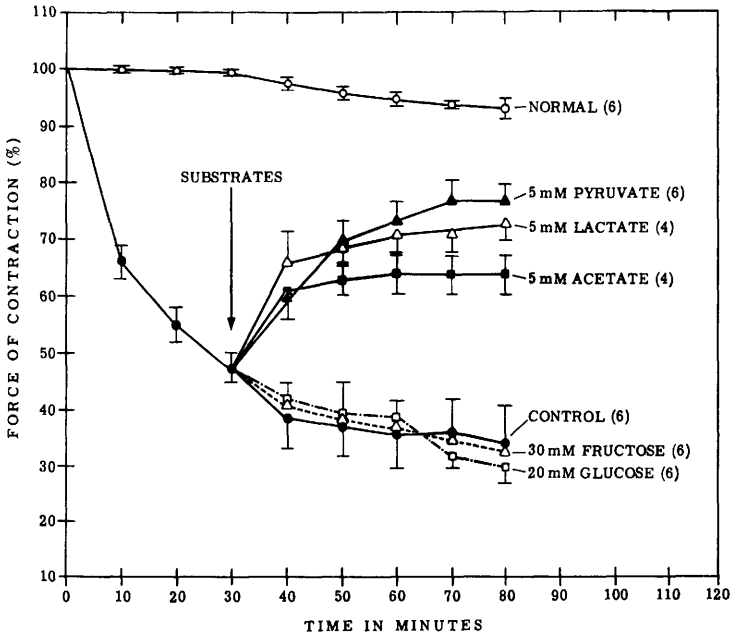

FIG. 1. Effect of substrates on atria in bicarbonate-free medium. In this and subsequent figures zero time represents the period after a 60-min equilibration of the atria in the normal medium containing 25 m*M* sodium bicarbonate and 5.5 m*M* glucose. At zero time, in this figure, the normal medium was replaced by one in which the sodium bicarbonate was replaced by equimolar sodium chloride and the pH adjusted to 7.4. Substrates were added to the bicarbonate-free medium at the 30-min period. Control represents those atria incubated in the bicarbonate-free medium for 80 min without addition of substrate.

study were obtained from Sigma Chemical Co.

The lactic acid was neutralized to pH 7.4 by sodium hydroxide in the experiments with bicarbonate-free medium.

Results. Effects of substrates on atria depressed with bicarbonate-free medium. The behavior of atria in bicarbonate-free medium was determined to provide data with which the responses to substrates of the depressed atria could be compared. The rat atria were exposed to bicarbonate-free medium immediately after the 60-min equilibration period in Krebs–Ringer bicarbonate glucose medium. The contractility of rat atria was markedly depressed in the bicarbonate-free medium, despite the fact that the medium contained 5.5 m*M* glucose (Fig. 1). After 30 min of incubation in the bicarbonate-free medium, substrates were added to the depressed atria. Pyruvate, lactate, and acetate partially restored the contractility of atria depressed with bicarbonate-free medium, but fructose and additional glucose were without effect (Fig. 1). Since fructose and glucose can serve as a source of fuel for the contractile process in rat atria (20), these studies indicate a defect in the utilization of these substrates prior to their conversion to pyruvate and are consistent with data obtained by Shaw and Stadie in diaphragm muscle in which phosphofructokinase was inhibited by bicarbonate-free medium (11, 12).

Effects of substrates on atria depressed with citrate. After a 60-min equilibration period in the normal Krebs–Ringer bicarbonate glucose medium, addition of sodium citrate produced dose-dependent decreases in the force of contraction (Fig. 2). After 2 hr of exposure to citrate, the medium was changed to the normal medium. This resulted in complete restoration of the contractility to the control levels.

Substrates were added to the bathing medium 30 min after the atria were depressed approximately 50% with 1.5 m*M* sodium ci-

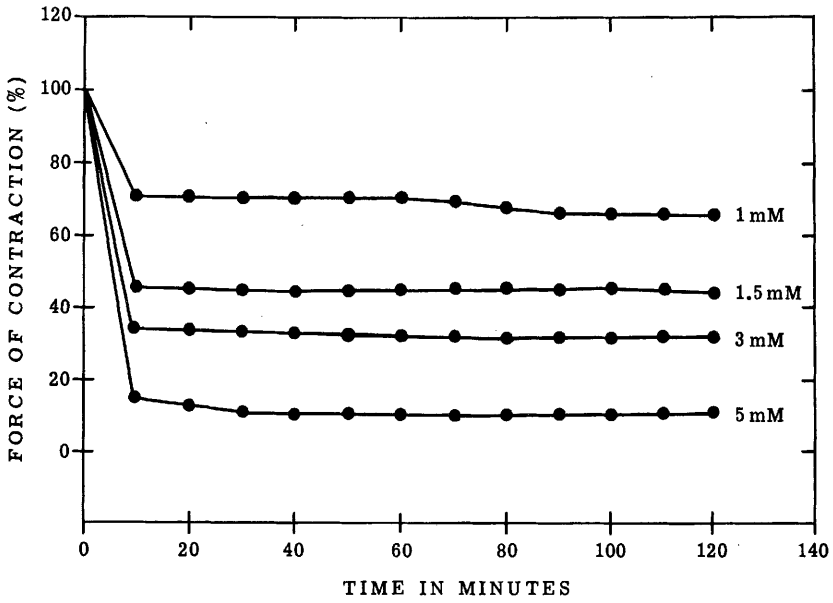

FIG. 2. Effect of citrate (1–5 m*M*) on atria in normal medium. After 2 hr of exposure to citrate the medium was changed to the normal medium without citrate.

trate. This concentration of citrate was chosen for the substrate study because it produced about the same degree of depression as that seen with bicarbonate-free medium. It is evident from Fig. 3 that pyruvate and lactic acid partially restored the depressed contractility of the atria and that fructose was without effect, similar to the effect of these sub-

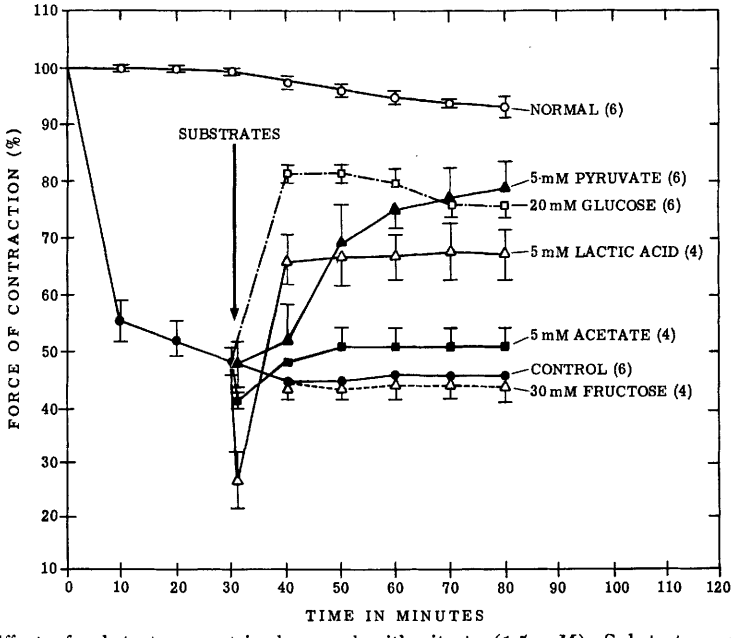

FIG. 3. Effect of substrates on atria depressed with citrate (1.5 m*M*). Substrates were added to citrate-depressed atria at the 30-min period. Control represents atria incubated with citrate for 80 min without addition of substrate.

strates in the experiments with bicarbonate-free medium. Depression by citrate, however, differs from that by bicarbonate-free medium in that acetate was without appreciable effect in citrate-depressed atria. Also, additional glucose, which had no effect in bicarbonate-free medium, produced a marked positive inotropic action in citrate-depressed atria.

A transient depression of the contractility of atria immediately after the addition of lactic acid is due to a temporary changing of pH in the medium by the lactic acid (19). An initial rapid drop in pH was followed by a gradual return to a stable value in about 5 min. This drop in pH was responsible for the decrease in contractility, since similar initial changes in both pH and contractility occurred after addition of hydrochloric acid to normal atria.

Effects of substrates on atria depressed with low calcium. It is well established that calcium ions play an important role for the contractile activity of the myocardium, and that calcium ions are chelated by citrate. Experiments were, therefore, performed to determine whether the depression of contractile activity of rat atria by citrate is due to the effect of low ionized calcium in the medium, caused by chelating of calcium ions by citrate. After a 1-hr equilibration period, the normal medium was replaced with low-calcium medium. The stability of the developed tension of rat atria suspended in the low-calcium medium is shown in Fig. 4. It is evident from the figure that the removal of half the calcium from the normal medium resulted in an approximately 50% decrease in atrial contractility similar to that seen with 1.5 m*M* citrate or bicarbonate-free medium. After 30 min of incubation of atria in this low-calcium medium, addition of pyruvate, lactic acid, acetate, or fructose was without effect on the contractility except for the initial transient depression seen with lactic acid and previously discussed.

These results differ from those seen with citrate in that pyruvate and lactic acid were effective in increasing the contractility of citrate-depressed atria and glucose was more effective in atria depressed by citrate rather than by low calcium. Thus, depression of

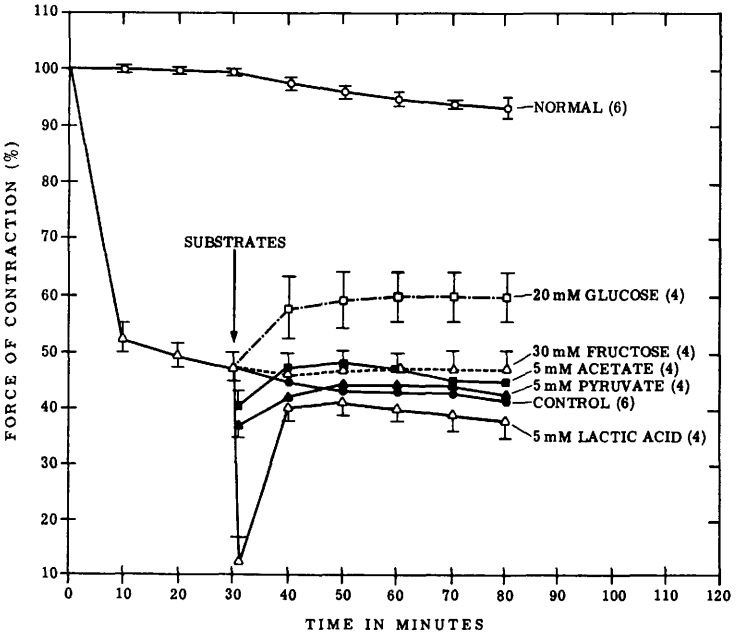

FIG. 4. Effect of substrates on atria in low-calcium media (½ amount of calcium). Substrates were added to low-calcium-depressed atria at the 30-min period. Control represents atria incubated with ½ calcium medium for 80 min without addition of substrate.

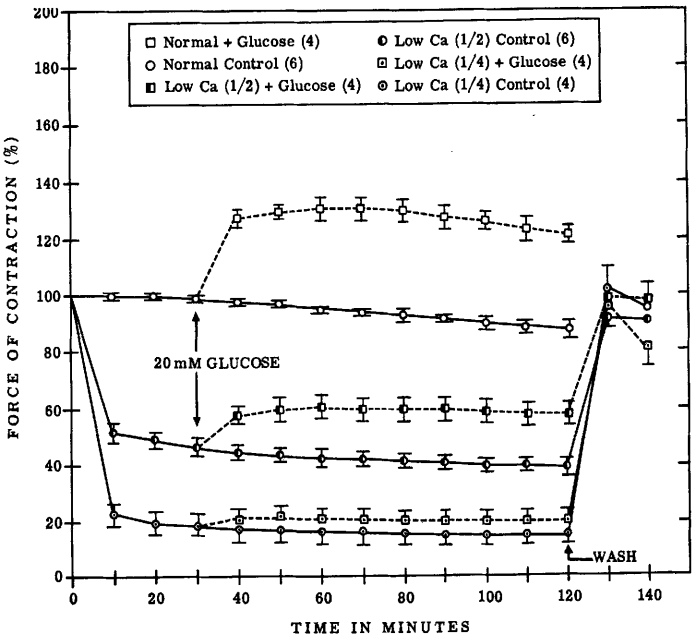

FIG. 5. Effect of additional glucose on normal atria, or atria in low-calcium medium (½ or ¼ calcium). Glucose (20 m*M*) added at 30-min period. Medium changed to the normal medium at the 120-min period.

atria by citrate must involve something other than a simple lowering of extracellular calcium ion activity by chelation.

Effects of additional glucose on atria depressed with low calcium. In view of the smaller effect of additional glucose on atria depressed by ½ calcium rather than by citrate, we decided to try still lower calcium to see whether the glucose effects could be reduced further. Figure 5 shows the effects of 20 m*M* glucose on atria in normal, ½ calcium, and ¼ calcium medium. The positive inotropic action of glucose is seen to be a function of the extracellular calcium concentration. This may be due simply to an inability of the actomyocin filaments to respond to added glucose rather than to any specific block of glucose metabolism by low calcium. This point needs further clarification.

Discussion. Cardiac metabolism has been well studied in recent years, and it has been demonstrated that glucose, pyruvate, lactate, acetate, and fructose can be metabolized by the myocardium for the purpose of sustaining the contractile process (2, 3, 19, 20, 22–29).

In the present investigation the contractile activity of rat atria was markedly depressed by bicarbonate-free medium. This depression was partially overcome by pyruvate, lactate, and acetate, but not by fructose or additional glucose. These results are consistent with the biochemical data of Shaw and Stadie on rat diaphragm which indicate that the Embden–Meyerhof pathway is inhibited at the phosphofructokinase step by a bicarbonate-free medium (11, 12). It can be interpreted that the depression of atrial contractility by bicarbonate-free medium is due to the blockade of phosphofructokinase enzyme activity in the bicarbonate-free medium. Thus, the exogeneous substrates pyruvate, lactate, and acetate could be utilized for the contractile activity, but fructose and additional glucose could not be metabolized. The results are also in agreement with a number of studies from rat ventricles concerning a lack of phosphofructokinase activity in bicarbonate-free medium. In this medium, glucose is relatively ineffective in maintaining the contractile activity of rat ventricle strips (3, 22). Rice and Berman demonstrated (23, 24) that the oxidation of glucose by heart strips

incubated in bicarbonate-free medium is lower than the oxidation of pyruvate or acetate. In contrast, they have observed that in medium containing bicarbonate, glucose maintains contractile activity (3), and Hood and Saunders have reported that glucose is rapidly oxidized in this medium (25). The results are also in agreement with the previous reports that the phosphofructokinase reaction is an important regulatory step in glycolysis (13–16), and that the operation of the Emden–Meyerhof pathway is important for a fraction of the contractile activity of the myocardium (17, 18).

The mechanism of the cardiac-depressant action of citrate can be discussed from two possible viewpoints: (1) chelation of extracellular calcium, (2) inhibition of phosphofructokinase activity.

Chelation of extracellular calcium was mentioned by Webb (1) as a possible mechanism for the depressant action of citrate on rabbit atria. This is a reasonable postulate because calcium is known to be chelated by citrate (30) and low extracellular calcium is known to depress the heart (6, 7). If this were the only mechanism involved, one would expect the atria, depressed either by citrate or low calcium, to behave in a similar fashion on addition of metabolizable substrates. Such was not the case, however. Pyruvate and lactic acid, which produced marked increases in cardiac contractility in atria depressed by citrate (Fig. 3), had no positive inotropic effect on atria depressed to the same extent by low extracellular calcium (Fig. 4). Also, 20 m*M* glucose produced a greater positive inotropic action in atria depressed by citrate rather than by low calcium. It seems apparent, then, that the negative inotropic action of citrate is not mediated entirely by virtue of its ability to reduce extracellular calcium ion activity.

Citrate has been shown to be a powerful inhibitor of phosphofructokinase in homogenates of brain, liver (8), skeletal (9), and cardiac muscle (10). In order to have such an effect in the intact auricle preparation, citrate would have to cross the cell membrane and be taken into the cell cytoplasm to the site where the enzyme is found. Such would not be the case with the broken cell homogenate preparations cited above. There is some question whether citrate could cross cell membranes since it is a tricarboxylic acid in a highly charged state at pH 7.4. Masuoka *et al.* (2) have shown that citrate could not serve as a suitable substrate for restoration of contractile activity of rat ventricle strips depressed by substrate-free medium. The limiting membrane for citrate uptake and utilization, however, could be the mitochondrial rather than the plasma membrane. If such were the case, citrate could gain access to the extramitochondrial phosphofructokinase. Figure 3 presents some evidence which is compatible with a citrate inhibition of phosphofructokinase. Pyruvate and lactic acid, not metabolized via phosphofructokinase, produced marked positive inotropic effects in citrate-depressed atria (these same substrates produce little or no effect on atria in normal or low-calcium media). Also, fructose, metabolized via the phosphofructokinase step is without effect on the citrate-depressed atria. Acetate, however, which is not metabolized via phosphofructokinase and is effective in bicarbonate-free treated atria, produces little or no effect on citrate-depressed atria. It is possible that citrate inhibits the conversion of acetate to acetyl coenzyme A, although no information is available to us on this point.

Of great interest is the positive-inotropic action of 20 m*M* glucose on citrate-depressed atria. If citrate were inhibiting phosphofructokinase, one would expect no effect from added glucose just as added fructose (30 m*M*) produced no effect and just as added glucose had no effect on atria in bicarbonate-free medium (Fig. 1), another inhibitor of phosphofructokinase. One may speculate that glucose can cause a positive inotropic action without the necessity of being metabolized via the phosphofructokinase step. If such were the case then this "extra" effect of glucose would have to be blocked in bicarbonate-free medium where added glucose has no effect. Actually, there is a growing body of evidence to suggest that some early step or steps in the metabolism of glucose is responsible for a portion of the positive-inotropic action of glucose (17, 31). Further speculation on the

mechanism of the negative inotropic action of citrate seems unwarranted until more experiments are done.

Summary. Atria, incubated in bicarbonate-free medium containing 5.5 m*M* glucose at pH 7.4, demonstrated a progressive decline in contractility not seen in bicarbonate-containing normal medium. Addition of 5 m*M* pyruvate, lactate, or acetate to atria depressed by bicarbonate-free medium produced a marked increase in contractility. Such an effect was not seen by addition of glucose (30 m*M*) or fructose (20 m*M*) indicating a block in the Embden–Meyerhof pathway produced by the bicarbonate-free medium at some point between fructose and pyruvate and most probably at the phosphofructokinase step.

Addition of citrate to atria incubated in the normal bicarbonate-containing medium produced dose-dependent decreases in contractility. The effects of substrates on citrate-depressed atria were in some respects similar to their effects on atria depressed by bicarbonate-free medium. Pyruvate (5 m*M*) and lactic acid (5 m*M*) increased the force of contraction of citrate-depressed atria whereas fructose (30 m*M*) was without effect. These results are consistent with an inhibitory effect of citrate at some point in the Embden–Meyerhof pathway such as the phosphofructokinase step. Addition of acetate (5 m*M*) to citrate-depressed atria produced little, if any, effect. Additional glucose (20 m*M*) produced a marked positive inotropic effect in citrate-depressed atria. This is not in accord with a citrate-induced block of phosphofructokinase unless one postulates an effect of glucose on contractility which is not dependent on its metabolism via the phosphofructokinase step. The mechanism of the depressant action of citrate cannot be solely attributed to a lowering of ionized medium calcium by chelation with citrate since the action of substrates on atria depressed by low calcium was quite different from their action on citrate-depressed atria. Pyruvate and lactic acid, which produced marked increases in contractility of citrate-depressed atria, were without effect on atria depressed by low calcium. Addition of glucose had less effect on atrial contraction when added to the low-calcium medium than when added to the citrate medium. Indeed, the marked positive inotropic action of 20 m*M* additional glucose seen in normal or citrate-depressed atria was virtually abolished in atria depressed by removal of ¾ of the calcium from the medium.

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