

Different Effects of Thiopental in Severe Hypoxia, Total Ischemia, and Low-flow Ischemia in Rat Heart Muscle

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The effect of thiopental ($100 \text{ mg} \cdot \text{l}^{-1}$) during total ischemia, low-flow ischemia, and severe hypoxia with maintained flow was investigated in the isolated perfused rat heart. During total ischemia the rate of decline of tissue creatine phosphate and adenosine triphosphate was no different in thiopental-treated and untreated hearts. The development of ultrastructural damage during total ischemia, the release of creatine kinase on reperfusion, and the exacerbation of ultrastructural damage after reperfusion were unaffected by thiopental. When thiopental was added to the perfusate during hypoxia and during low-flow ischemia at a normal pH (7.4), creatine kinase release during reoxygenation and during reperfusion was significantly less ($P < 0.005$ and $P < 0.05$, respectively) than in the untreated groups. After low-flow ischemia at a low pH (6.5), creatine kinase release was no different in thiopental-treated and untreated hearts. Thus, thiopental afforded protection of the myocardium in hypoxia and low-flow ischemia at pH 7.4 but not in total ischemia and low-flow ischemia at pH 6.5. The data are consistent with the hypothesis that during total ischemia and low-flow ischemia at pH 6.5, acidosis favors the entry of thiopental into the cell, causing inhibition of mitochondrial function and reduction of ATP production. During hypoxic perfusion and low-flow ischemia at pH 7.4, when the decrease in pH is less, the cardiodepressant effect of thiopental may offset any deleterious effect of the drug on intracellular organelles such as mitochondria. (Key words: Anesthetics, intravenous: thiopental. Heart: hypoxia; ischemia; metabolism. Hypoxia: heart. Metabolism: heart.)

THE ABILITY of barbiturates to protect the brain from the consequences of ischemia is controversial.¹⁻⁷ As in brain, variable effects of barbiturates on ischemic damage have been reported in the heart. Barbiturates have been shown to have a protective effect⁸ or an adverse effect.⁹ Barbiturates are known to reduce the contractility of

heart muscle, inhibit calcium fluxes, inhibit mitochondrial function, decrease arterial blood pressure, and exert effects on cell membranes.¹⁰ In a previous study we found that large doses of barbiturates reduced the loss of enzymes during hypoxia and on reoxygenation of the isolated rat heart.¹¹ We have now compared the effects of barbiturates during severe hypoxia, total ischemia, and low-flow ischemia in rat heart muscle.

Methods and Materials

PERFUSION TECHNIQUE

Wistar rats of 200–250 g were anesthetized with diethyl ether. The rats were heparinized and their hearts quickly removed and perfused at 37° C by the Langendorff technique.¹² Three perfusion fluid reservoirs were connected to the aortic cannula by means of a specially designed stopcock, allowing alternate use of the reservoirs. The temperature of the perfusate was monitored continuously with the use of a needle thermocouple inserted just above the aortic cannula. The heart was kept in a temperature-controlled chamber in order that myocardial temperature could be maintained at 37° C irrespective of coronary flow. The standard perfusate had the following composition ($\text{mmol} \cdot \text{l}^{-1}$): NaCl, 124.0; KCl, 4.7; CaCl_2 , 1.3; MgCl_2 , 1.0; NaHCO_3 , 24.0; Na_2HPO_4 , 0.5; glucose, 11.0. After equilibration with 95% O_2 /5% CO_2 , the pH of the perfusate was 7.4.

EXPERIMENTAL GROUPS

Total Ischemia. After 15 min of control perfusion with standard perfusate at a constant pressure of 75 mmHg, two groups of hearts ($n = 8$ in each group) were made totally ischemic (no flow) for 90 min and then reperfused for 30 min at a pressure of 75 mmHg. In one group, thiopental ($100 \text{ mg} \cdot \text{l}^{-1} = 0.4 \text{ mmol} \cdot \text{l}^{-1}$) was added to the standard perfusate during the last 5 min before the period of ischemia.

Hypoxic Perfusion. After the initial 15-min perfusion period, the hearts were perfused for 40 min with hypoxic medium. The standard perfusate was equilibrated with 95% N_2 /5% CO_2 and contained mannitol ($11 \text{ mmol} \cdot \text{l}^{-1}$) instead of glucose. The P_{O_2} of the hypoxic perfusate was less than 5 mmHg, whereas with

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oxygenated perfusate the P_{O_2} was greater than 600 mmHg. Subsequently, the hearts were reoxygenated for 30 min. Thiopental ($100 \text{ mg} \cdot \text{l}^{-1}$) was added to the hypoxic perfusate in the intervention group ($n = 8$ for the untreated hypoxic group and $n = 8$ for the thiopental-treated hypoxic group). Perfusion pressure was 75 mmHg throughout the experiment.

Low-flow Ischemia. The hearts were electrically stimulated (Grass S88®) at a rate of $300 \text{ beats} \cdot \text{min}^{-1}$. After 15 min of perfusion with standard perfusate at constant pressure (75 mmHg), the hearts were perfused for 90 min at a constant flow, amounting to 5% of the flow at 10 min of control perfusion. In the intervention group of experiments, thiopental ($100 \text{ mg} \cdot \text{l}^{-1}$) was present in the perfusion fluid during the last 5 min of control perfusion and during the period of ischemia. Subsequently, the hearts were reperfused for 30 min at a pressure of 75 mmHg. During the period of low-flow ischemia, the standard perfusate was equilibrated with 95% $O_2/5\% \text{ CO}_2$ or 60% $O_2/40\% \text{ CO}_2$, resulting in a pH of 7.4 or 6.5, respectively ($n = 48$: untreated hearts at pH 7.4 [$n = 12$] and pH 6.5 [$n = 12$]; treated hearts at pH 7.4 [$n = 12$] and pH 6.5 [$n = 12$]).

ANALYTIC PROCEDURES

Coronary effluent was collected over 1 min and analyzed for creatine kinase (CK) activity at 25°C , with the use of a Vitatron Automatic Kinetic Enzyme System® (AKES) and a Boehringer® CK NAC-activated kit.^{13,14} Enzyme activities were expressed in international units (IU) $\text{min}^{-1} \cdot \text{g}^{-1}$ dry heart tissue. The effluent was collected after 10 min of control perfusion, at 10-min intervals during hypoxic perfusion and low-flow ischemia, and at 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 20, 25, and 30 min upon reperfusion or reoxygenation.

At the end of the control perfusion period and after 2.5, 5, 10, 20, 30, 60, and 90 min of total ischemia, hearts were freeze-clamped,¹⁵ extracted, and assayed for creatine phosphate (CP) and adenosine triphosphate (ATP) as previously described.¹⁶ Myocardial stores of CP and ATP were expressed in $\mu\text{mol} \cdot \text{g}^{-1}$ dry heart tissue.

ELECTRON MICROSCOPY

Electron microscopy was undertaken only on hearts subjected to control perfusion, total ischemia, or ischemia followed by reperfusion. Six groups of experiments were undertaken: control perfusion with and without thiopental, total ischemia with and without thiopental, and total ischemia with and without thiopental followed by reperfusion. Each group contained six hearts. Six pictures were obtained from each of three tissue blocks prepared for each heart. Thus, 108 images ($6 \times 3 \times 6$) were

analyzed for each group and a total of 648 images (108×6).

Control hearts were perfused for 135 min with standard perfusate with or without thiopental and then perfusion fixed with 4% glutaraldehyde prepared in $0.2 \text{ mol} \cdot \text{l}^{-1}$ sodium cacodylate buffer (pH 7.3). Thiopental was added to the perfusate after 10 min of control perfusion and remained in the perfusate for 95 min. Other hearts were perfusion-fixed after 90 min of total ischemia or after a subsequent 30 min of reperfusion. After 10 min of glutaraldehyde perfusion, the hearts were removed from the Langendorff apparatus and processed for electron microscopy as previously described.¹⁷

The percentage of mitochondria containing inclusion bodies¹⁸ was used as an index of myocardial cell damage in ischemic and reperfused left ventricle. Images were analyzed using the upper left hand corner of the specimen support grid as a reference system to prevent operator bias.¹⁹ Magnification was $\times 30,000$. From the images 19,062 control, 17,552 ischemic, and 16,944 ischemic reperfused mitochondrial profiles were examined for the presence or absence of mitochondrial inclusion bodies.

DATA ANALYSIS

The release of CK under different experimental conditions was analyzed as follows. The data for samples obtained at a given time from experiments in each group were shown not to be normally distributed. The median values were obtained and are shown in figures 1, 5 and 6. For clarity not all the points obtained at different times (see "Analytic Procedures") are shown. The area under the curve was calculated for each experiment on a HP1000 computer, and the median area was obtained for each group of experiments (see table 1). The significance of the differences between groups was analyzed by the Mann-Whitney test for two samples. $P < 0.05$ was considered to be a significant difference.

Statistical analysis of the values for tissue CP and ATP was performed with the use of the Mann-Whitney test for two samples. Student's *t* test was used to determine any differences between the groups of experiments in which mitochondrial inclusion bodies were counted. $P < 0.05$ was considered to be a significant difference.

Results

TOTAL ISCHEMIA

During the initial period of perfusion with standard perfusate, the hearts released negligible amounts of CK. Figure 1 shows CK release during the reperfusion

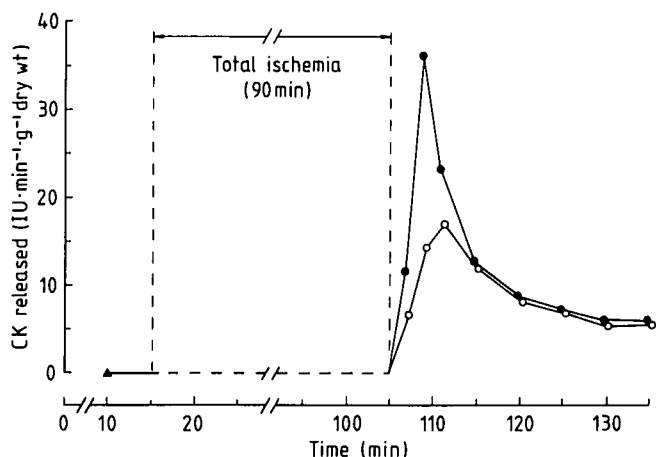


FIG. 1. Effect of total ischemia and subsequent reperfusion on the release of creatine kinase (CK) from isolated rat heart. CK release during control perfusion (\blacktriangle) and during reperfusion from untreated (\circ ; $n = 8$) and thiopental-treated (\bullet ; $n = 8$) hearts are shown. See "Methods" for explanation of thiopental treatment and statistical analysis. Each point is the median value of eight measurements. This method of analysis was chosen because of the skewness of the distribution of the data. For the sake of clarity, the figure does not contain all data that were used for statistical analysis of the release of CK (see table 1). The release of CK on reperfusion in the two groups was not significantly different.

period. Reperfusion after 90 min of total ischemia resulted in more release of CK from the thiopental-treated than from the untreated hearts, but this difference did not reach statistical significance (table 1). For the sake of clarity, figure 1 does not contain all data that were used for statistical analysis of the release of CK.

After 15 min of control perfusion, myocardial ATP content was not significantly different in treated and untreated hearts. CP content was lower ($P < 0.05$) in the hearts that had been perfused with thiopental-containing perfusate during the last 5 min of control perfusion (table 2). Total ischemia resulted in a rapid

reduction of myocardial CP content in both the treated and untreated hearts, reaching 12% of control after 90 min of ischemia. ATP decline was somewhat delayed, reaching 12% (treated) and 17% (untreated) of control after 90 min of ischemia. During ischemia, only small differences in CP and ATP content were found between treated and untreated hearts.

After 90 min of ischemia, hearts showed mitochondria containing inclusion bodies, irrespective of whether thiopental had been present or not (not shown). The cells appeared to be swollen and exhibited empty cytoplasmic spaces. The nucleus showed aggregation of the chromatin. Subsequent reperfusion for 30 min resulted in marked swelling of the cells and the mitochondria and disruption of the myofibrils in both groups of experiments (figs. 2 and 3). Figure 4 shows the percentage myocardial mitochondria containing inclusion bodies counted in untreated and thiopental-treated control, ischemic, and ischemic reperfused groups. The control groups showed virtually no mitochondria containing these inclusion bodies. The percentage of mitochondria containing inclusion bodies under untreated ischemic conditions was estimated to be $33.3 \pm 10.2\%$ (mean of six hearts \pm SD), which was seen to increase to $37.2 \pm 10.0\%$ upon subsequent reperfusion. This trend was not abolished by treatment with thiopental with values of 24.9 ± 6.9 and $29.1 \pm 5.0\%$ for ischemia and reperfusion, respectively. Although these values were lower than in the untreated groups, the differences were not significant.

HYPOXIC PERFUSION

Hypoxic glucose-free perfusion caused a small CK release (fig. 5). When thiopental was added to the hypoxic medium, the CK release was little changed. Reoxygenation of the hearts resulted in an exacerbation of the CK release. Enzyme release from the thiopental-treated hearts was less ($P < 0.005$) than from the un-

TABLE 1. Creatine Kinase (CK) Release from Isolated Rat Hearts during 30 Min of Normal Reperfusion after Total Ischemia, Hypoxic Perfusion, and Low-flow Ischemia, in the Presence and Absence of Thiopental ($100 \text{ mg} \cdot \text{l}^{-1}$)

Experimental Conditions	Median value*	n	Range	P
After 90 min of total ischemia				
Without thiopental	418	8	289-533	NS
With thiopental	491	8	334-670	
After 40 min of hypoxic perfusion				
Without thiopental	683	8	363-893	$P < 0.005$
With thiopental	280	8	113-429	
After 90 min of low-flow ischemia				
At pH 7.4 without thiopental	562	12	296-1131	$P < 0.05$
At pH 7.4 with thiopental	445	12	108-613	
At pH 6.5 without thiopental	166	12	75-335	NS
At pH 6.5 with thiopental	211	12	118-409	

* Median value (in arbitrary units) of the areas under the creatine kinase release curves during normal reperfusion are shown. See

"Methods" for explanation of thiopental treatment and statistical analysis. n = number of experiments; NS = not significantly different.

TABLE 2. Effect of Ischemia on Myocardial Stores of Creatine Phosphate (CP) and Adenosine Triphosphate (ATP)

Treatment	CP		ATP	
	- Thiopental	+ Thiopental	- Thiopental	+ Thiopental
Control perfusion (15 min)	34.1 29.8-43.1	26.2* 23.2-34.0	19.5 16.9-21.0	19.9 18.6-20.4
Total ischemia 2.5 min	6.8 5.2-10.1	5.0* 3.2-7.4	18.8 17.6-21.4	18.3 16.0-22.8
5 min	4.6 2.8-5.1	3.7 2.3-6.6	16.9 14.8-17.3	19.0 13.5-22.6
10 min	3.4 3.1-3.8	3.2 2.4-4.4	12.3 11.8-13.0	12.9 11.3-14.1
20 min	3.6 2.6-4.6	3.3 2.7-4.4	7.5 6.8-11.1	4.9* 4.0-8.2
30 min	3.2 1.9-4.7	3.4 2.5-4.7	4.7 3.7-5.6	4.9 3.8-5.6
60 min	3.9 2.5-4.4	2.9 2.3-3.3	3.0 2.7-4.0	2.3* 2.0-2.8
90 min	4.2 3.0-5.0	3.1* 2.3-4.0	3.4 2.3-5.1	2.3 1.7-2.7

After 15 min of control perfusion, the hearts were made totally ischemic for 90 min. When required, 100 mg · l⁻¹ thiopental was added during the last 5 min before ischemia. Median values and range are

presented. Values are given in μmol g⁻¹ dry heart tissue (n = 6).
* Significantly different from the untreated hearts (P < 0.05).

treated hearts (table 1). This result is in contrast to the results in total ischemia, where during reperfusion CK release from the thiopental-treated hearts compared with untreated hearts was not significantly different.

LOW-FLOW ISCHEMIA

During low-flow ischemia there was a small release of CK, which never exceeded a value of 1 IU · min⁻¹ · g⁻¹ dry heart tissue (fig. 6). Reperfusion at a constant pressure of 75 mmHg, after low-flow ischemia at pH 7.4, resulted in an exacerbation of the CK release. Enzyme release during reperfusion from the thiopental-treated hearts was less (P < 0.05) than from the untreated hearts (fig. 6, upper panel; table 1). When low-flow ischemia was imposed at pH 6.5, CK release during reperfusion from the treated hearts was not significantly different to CK release from untreated hearts (fig. 6, lower panel; table 1).

Discussion

Large doses of barbiturates in excess of those used for the induction of anesthesia have been reported to protect the brain against damage during a period of hypoxia or ischemia,¹⁻³ but this has not been a universal finding. Contradictory results may be attributed to differences between species or in techniques used for the induction of hypoxia or ischemia. Another important consideration is that the protection afforded by barbiturates has been shown only during hypoxia and incomplete ischemia^{4,5} but is controversial during total ischemia.^{1,6,7} We have studied the effect of these different conditions in the isolated heart.

The results of the present study show that thiopental did not protect heart muscle against the consequences



FIG. 2. Heart perfused for 15 min with standard perfusate, followed by 90 min of total ischemia and 30 min of reperfusion, without thiopental treatment. The mitochondria are swollen and contain inclusion bodies (arrows). The myofibrils (M) are disrupted, and the cells are swollen. The nucleus (N) shows aggregation of the chromatin. The bar represents 1 μm.

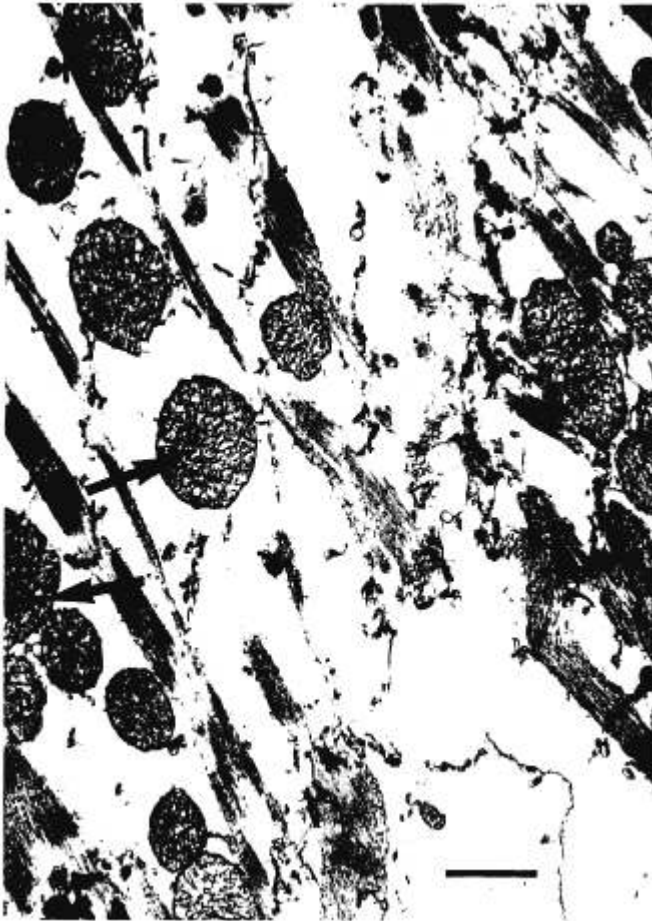


FIG. 3. Heart perfused for 15 min with standard perfusate, followed by 90 min of total ischemia and 30 min of reperfusion, with thiopental treatment. See "Methods" for explanation of thiopental treatment. The mitochondria are swollen and contain inclusion bodies (arrows). The myofibrils (M) are disrupted, and the cells are swollen. The bar represents 1 μ m.

of total ischemia. There were no significant differences between thiopental-treated and untreated hearts in the rate of decline of myocardial CP and ATP content and development of ultrastructural damage during ischemia. During reperfusion there was no difference in the release of CK and the exacerbation of ultrastructural damage between the two groups of experiments. The number of mitochondria containing inclusion bodies was used to quantitate ultrastructural damage.

When thiopental was added during hypoxic perfusion, CK release during reoxygenation was significantly less than in the untreated group, which confirms the results of our previous investigation.¹¹ When thiopental was present during low-flow ischemia at a normal pH (7.4), there was a significant reduction in CK release during reperfusion, compared with the untreated group. After low-flow ischemia at a low pH (6.5), however, CK release was no different in treated and untreated hearts. A

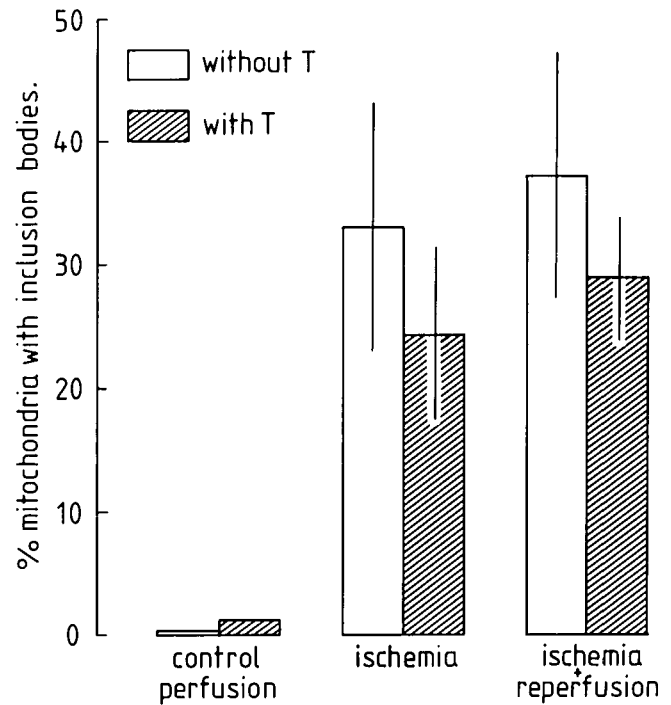


FIG. 4. Percentage (mean \pm SD) myocardial mitochondria containing inclusion bodies counted in untreated and thiopental-treated control perfused, ischemic and reperfused hearts. The following numbers of mitochondria were examined for the presence or absence of inclusion bodies: 19,062 from control perfused, 17,552 from ischemic, and 16,944 from reperfused hearts. See "Methods" for explanation of thiopental treatment. There were no significant differences between treated and untreated hearts in each of the three experimental groups. T = thiopental.

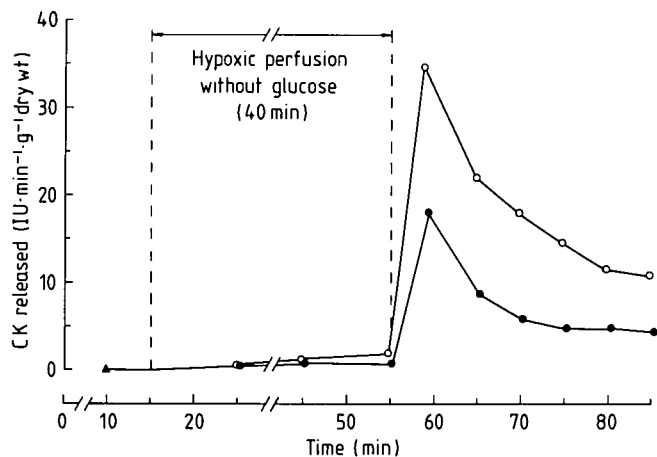


FIG. 5. Effect of hypoxic perfusion without glucose and subsequent reoxygenation on the release of creatine kinase (CK) from isolated rat heart. CK release during control perfusion (\blacktriangle), and during hypoxic perfusion and reoxygenation from untreated (\circ ; $n = 8$) and thiopental-treated (\bullet ; $n = 8$) hearts are shown. See "Methods" for explanation of thiopental treatment and statistical analysis. Each point is the median value of eight measurements. The release of CK was less in thiopental-treated hearts ($P < 0.005$) (see table 1).

similar pattern of response to hypoxia, incomplete ischemia, and total ischemia has been reported in brain.^{1,4-7}

In the present study, thiopental was used in a concentration of $100 \text{ mg} \cdot \text{l}^{-1}$, which is grossly in excess of the dose used for induction of anesthesia. High doses ($50\text{--}500 \text{ mg} \cdot \text{l}^{-1}$ and $30\text{--}120 \text{ mg} \cdot \text{kg}^{-1}$) have been used in several studies on protection by barbiturates of ischemic and hypoxic brain and heart.^{1,6,8,11} In a previous study on rabbit ventricular myocardium, it was shown that $100 \text{ mg} \cdot \text{l}^{-1}$ thiopental reduced developed tension to less than 20%.¹⁰

Marked differences in the release of enzymes are known to exist in hearts reperfused after ischemia in comparison with reoxygenation after hypoxia.²⁰ Our results (figs. 1 and 5) are compatible with these previous reports. Acidosis of moderate severity is known to be protective to the hypoxic myocardium²¹ partly as a result of a cardioplegic effect. Our results are compatible with these observations in that the release of CK on reperfusion after low-flow ischemia was reduced by an acidosis (fig. 6). For these reasons, comparisons in the present study have been made between results obtained in the presence or absence of thiopental under different experimental conditions, namely, total ischemia with reperfusion, hypoxia with reoxygenation, and low-flow ischemia at different pH with normal reperfusion. A detailed comparison has not been made between results obtained under different experimental conditions.

There are several explanations for the variable effects of barbiturates on the ischemic and hypoxic myocardium. Barbiturates have a dose-dependent cardiodepressant effect, decrease arterial blood pressure and cardiac output, reduce the slow calcium current, block the uptake of calcium by the sarcoplasmic reticulum, and inhibit mitochondrial function reducing ATP production.^{10,22-24} The overall result in any experiment will depend on the interplay of these factors. For example, if blood pressure decreases, the perfusion of ischemic muscle may be reduced and necrosis hastened.⁹ In the isolated heart, protection during ischemia was only observed at very high concentrations of pentobarbital ($500 \text{ mg} \cdot \text{l}^{-1}$) and may be largely a consequence of cardioplegia.⁸ In the present experiments, a cardiodepressant effect might have been expected to protect the myocardium. This was observed after a period of hypoxia and low-flow ischemia at pH 7.4 but not after total ischemia and low-flow ischemia at pH 6.5.

Our results may be explained as follows. Thiopental is presumed to have two major effects. The first is to reduce contraction and thus to have a cardiodepressant effect. The second is to enter the cell, inhibit mitochondrial function, and limit the production of high-energy phosphates.²⁴ The latter may explain the reduced CP content of hearts after control perfusion in the presence

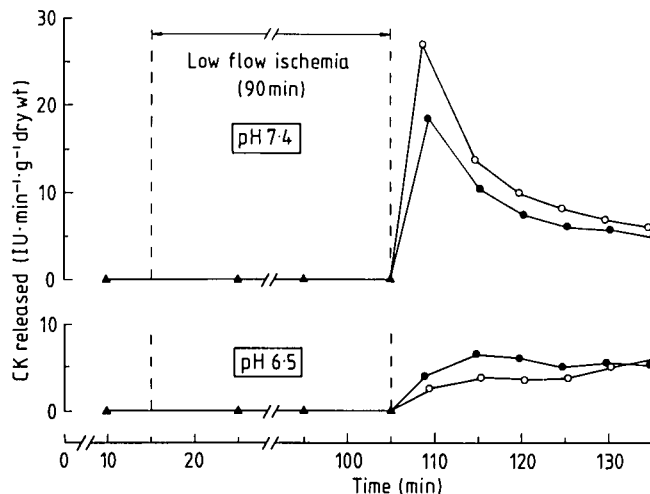


FIG. 6. Effect of low-flow ischemia and subsequent reperfusion on the release of creatine kinase (CK) from isolated rat heart; pH of the perfusate during ischemia was 7.4 (upper panel) or 6.5 (lower panel). CK release during control perfusion and ischemia (▲) and during reperfusion from untreated (○; $n = 12$ at pH 7.4 and $n = 12$ at pH 6.5) and thiopental-treated (●; $n = 12$ at pH 7.4 and $n = 12$ at pH 6.5) hearts are shown. See "Methods" for explanation of thiopental treatment and statistical analysis. Each point is the median value of 12 measurements. At pH 6.5, CK release was not significantly different between the two groups. At pH 7.4, CK release was less in thiopental-treated hearts ($P < 0.05$) (see table 1).

of thiopental. The ability of thiopental to affect mitochondrial function is dependent on the entry of the drug into the myocardial cell. This process is known to be dependent on pH.²⁵ In total myocardial ischemia, pH decreases to low values rapidly. Such conditions would favor the influx of thiopental into the cell. During hypoxia with continued coronary flow the decrease in pH is less, and under those conditions the cardiodepressant effect of thiopental may counteract any smaller effect on mitochondrial function. Thus, we observed no protection in total ischemia but did observe protection in hypoxia.

This hypothesis was tested further by studies on low-flow ischemia. In these experiments, the coronary flow rate and the heart rate were kept constant. Under low-flow conditions, the decrease in extracellular pH is less than during total ischemia²⁶ and protection was found. But if pH was reduced further by the introduction of a respiratory acidosis, the protective effect was abolished. The result is compatible with the idea that the acidosis favored the intracellular movement of thiopental and greater inhibition of mitochondrial function.

Barbiturates are known to have many effects on the heart. The overall consequence in a pathologic state will depend on the balance of these effects. In intact animals or humans, a decrease in blood pressure due to a direct negative inotropic effect on the heart and vasodilatation

would be expected to be harmful. When a decrease in perfusion pressure is avoided, the effect of barbiturates may depend on the balance between the advantages of a cardiodepressant action and the disadvantages of inhibition of metabolic pathways. It would seem that the concentrations of barbiturates that afford protection of the myocardium will not be achieved in intact humans and that the potential benefits to the myocardium of barbiturates during cardiac arrest or cardiopulmonary bypass are small.

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