Ischemic contracture begins when anaerobic glycolysis stops: a ^{31}P-NMR study of isolated rat hearts

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KINGSLEY, PETER B., EDWARD Y. SAKO, MAN QIU YANG, STEVAN D. ZIMMER, KAMIL UGURBIL, JOHN E. FOKER, AND ARTHUR H. L. FROM. *Ischemic contracture begins when anaerobic glycolysis stops: a ^{31}P-NMR study of isolated rat hearts.* Am. J. Physiol. 261 (Heart Circ. Physiol. 30): H469–H478, 1991.—The relationships among myocardial ATP, intracellular pH, and ischemic contracture in Langendorff-perfused rat hearts were investigated by ^{31}P nuclear magnetic resonance spectroscopy during total global normothermic ischemia while the left ventricular pressure was recorded continuously via an intraventricular balloon. Glucose-perfused hearts ($n = 63$) were divided into five groups based on the time of onset of contracture (TOC), and three other groups of hearts were treated to vary the ischemic glycogen availability. ATP levels, which showed no evidence of accelerated ATP depletion during contracture, were significant and variable at TOC. Intracellular pH initially declined and then leveled off at TOC, with lower final pH in hearts with later TOC. We conclude that contracture began when anaerobic glycolysis (and thus glycolytic ATP synthesis) stopped. These results, though consistent with the concept that ischemic contracture in normal hearts results from rigor bond formation due to low ATP levels at the myofibrils, suggest that TOC is more closely related to glycolytic ATP production than to total cellular ATP content, thus providing evidence of some degree of subcellular compartmentation or metabolite channeling. In glycolytically inhibited hearts, the quite early contracture may have a Ca^{2+} component.

rigor bonds; glycogen; intracellular pH; adenosine triphosphate; iodoacetate; metabolite channeling

WHEN A PERFUSED HEART is subjected to total (no flow) global ischemia, the peak systolic left ventricular pressure (LVP) falls rapidly, and contractile activity soon ceases. After a time, contracture (an increase in the resting LVP) begins (8, 15, 19, 21, 28, 37), and a similar contracture can occur in hypoxic or cyanide-treated myocardium (1, 2) and in metabolically inhibited myocytes (17, 27). The cause of ischemic contracture has been controversial, but current evidence (17–19, 23, 28, 39) favors rigor (ATP deficient) myosin cross-bridge binding to actin (5) rather than Ca^{2+}-dependent ATP-hydrolyzing cross-bridge cycling (15, 21) as the mechanism underlying the rise in resting tension.

It has been difficult to reconcile the rigor theory of contracture with the total myocardial ATP concentration ([ATP]) at the time of onset of contracture (TOC) (6, 8, 19, 21, 22, 38, 42), which, on the basis of skinned fiber studies, appears to be too high to allow rigor bonds to form (41). Although the total ATP content may be relatively high at TOC, it is possible that delivery of ATP to the myofibrils from the total intracellular pool may not be as efficient as ATP generation in the environs of the myofibrils by glycolysis (6, 18) or creatine kinase (CK) (41). If this is the case, then once the latter mechanisms for local ATP generation cease in the ischemic myocardium, only a modest reduction in the size of the total cytosolic ATP pool may be sufficient to induce rigor bond formation.

TOC can be delayed by interventions that decrease the rate of ATP utilization, such as oligomycin (17) or low perfusate Ca^{2+} (19, 21, 22), and accelerated by treatments that increase ATP utilization or inhibit ATP synthesis, including iodoacetate (IA) (19, 21, 28) and glycogen depletion (19, 30). It is unclear, however, whether these effects result from changes in the total ATP pool size or from altered glycolytic ATP production.

One index of glycolytic ATP production during total ischemia is the rate and magnitude of the fall of intracellular pH. During ischemia the intracellular pH initially declines steadily before reaching a plateau value (3, 4, 12, 22, 28), and lower plateau values of pH are associated with higher preischemic glycogen levels (3, 12, 30, 43). These observations suggest that the onset of the pH plateau phase is associated with the cessation of glycolytic production of lactic acid. The relationship between the onset of the pH plateau and the onset of contracture, however, has not been studied.

The relationships among [ATP], glycolytic ATP production, and ischemic contracture can be determined using ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR) to measure intracellular pH and [ATP] while continuously recording LVP in the isovolumic heart. The increasing use of ^{31}P-NMR in studies of myocardial ischemia (1, 3, 4, 12, 28, 30, 37, 38, 43, 44) is due largely to the ability to make relatively rapid (0.5–2 min), repetitive measurements of high energy phosphates [ATP and phosphocreatine (PCr)] and intracellular pH in the same heart nondestructively.

We have undertaken ^{31}P-NMR studies of glucose-perfused rat hearts subjected to total global normothermic ischemia to answer the following questions: *1*) What is the relationship between the rate and magnitude of

the ischemia-induced pH fall and TOC? *2*) Can ATP be detected by ^{31}P-NMR at TOC and if so, how much ATP is present at TOC? *3*) Is the rate of ATP decline altered when TOC is reached? Our results suggest that no-flow ischemic contracture occurs in concert with cessation of glycolytic ATP synthesis and that TOC is not associated with a specific cellular ATP content. The latter observation suggests that when there is no aerobic ATP production, glycolytically generated ATP is more efficiently delivered to the myofilaments than is ATP, which must diffuse to that region from the larger cytosolic pool.

MATERIALS AND METHODS

General methods. The perfusion system and conditions of ischemia have been reported in detail in a previous study of postischemic ATP synthesis kinetics (37) and will be described only briefly. Isovolumic rat hearts were perfused in the Langendorff mode with a modified phosphate-free Krebs-Henseleit buffer containing 1.8 mM $CaCl_2$ and 11 mM glucose. An intraventricular balloon was used to monitor LVP, and left ventricular end-diastolic pressure was set at 4–8 mmHg by adjusting the balloon volume. The balloon contained a 100 mM phenylphosphonate solution that was used as a concentration reference standard for ^{31}P-NMR spectra. The natural heart rate was ~300 beats/min.

After a period of stabilization of mechanical function during which the magnet was shimmed and a fully relaxed ^{31}P-NMR spectrum [146.1 MHz, 40 free induction decays (FIDs), 90° pulses, 15-s repetition time, 1,024 data points, 64-ms acquisition time] was recorded, perfusion was stopped, and the heart was subjected to 18 min of total global normothermic (37°C) ischemia. During this time ^{31}P-NMR spectra were recorded every 2 min (12 FIDs, 90° pulses, 10-s repetition time); a similar spectrum was recorded immediately before ischemia (but after the fully relaxed spectrum) to compare preischemic and ischemic metabolite levels and pH. Chemical shifts were referenced to 85% H_3PO_4 at 0.0 parts per million (ppm) using PCr at −2.50 ppm as an internal reference. FIDs were multiplied by an exponential function producing 20–30 Hz line broadening before Fourier transformation.

Three groups of hearts were also subjected to different preischemic treatments (5 hearts/group) to alter ischemic glycolytic ATP synthesis and TOC by adjusting the amount of myocardial glycogen available at the onset of ischemia or by inhibiting glycolysis. One group was perfused with 10 mM pyruvate + 11 mM glucose (PG hearts) for ~45 min to increase glycogen content relative to glucose-perfused hearts and delay TOC (44). A second group (depleted hearts) was perfused with substrate-free buffer for ~45 min to deplete glycogen (1, 19, 26, 30). A small amount of pyruvate (0.2–1 mM) was then added to maintain cardiac function without allowing glycogen synthesis. In both groups the carbon substrate was changed to 11 mM glucose ~2 min before ischemia so that the substrate available to the heart during ischemia was 11 mM glucose. A third group (IA hearts) was treated with IA (0.15 mM for 15 min, then 0.025 mM continuously) after glycogen depletion to prevent glycolytic ATP synthesis and lactic acid accumulation during ischemia. This level of IA inhibits glyceraldehyde 3-phosphate dehydrogenase completely with only slight inhibition of myocardial function (26). This method was chosen for complete glycolytic inhibition to avoid phosphate depletion due to the buildup of phosphorylated metabolites that occurs with 2-deoxyglucose (2-DG) (4, 20, 30, 34) or with IA in the presence of glucose or glycogen (26, 28, 34). Such phosphate depletion would lower the preischemic ATP and PCr levels (4, 20, 30, 34) and decrease ischemic accumulation of P_i (28, 34), which can affect the Ca^{2+} vs. force relationship (24). For these three groups, ^{31}P-NMR spectra during ischemia were collected using 70° pulses, 2,048 data points, and a 128-ms acquisition time. Eight FIDs were collected each 30 s for 2–6 min, followed by 16 FIDs each 1 min for the duration of the ischemic period (30 min for PG hearts, 18 min for depleted and IA hearts). In addition, four 30-s spectra were collected before ischemia to establish preischemic baseline data and to estimate errors in ^{31}P-NMR measurements.

LVP was monitored continuously via the intraventricular balloon. The onset of contracture was defined as an increase of at least 2 mmHg above the resting ischemic pressure with a rate of increase of at least 2 mmHg/min. TOC was recorded to the nearest 0.05 min in IA hearts and to the nearest 0.1 min in all other groups.

Determination of intracellular pH. Measurement of intracellular pH by ^{31}P-NMR spectroscopy is now well established (1, 3, 4, 12, 20, 28–30, 34, 43). Although the precise value of the pH may be in doubt because of ionic conditions used for calibration curves, the relative pH and, therefore, changes in pH can be determined accurately and repetitively by observing the chemical shift of the P_i resonance, using the PCr resonance as an internal standard. The pH was calculated from the formula pH = 6.72 + log [(chemical shift − 3.27)/(5.69 − chemical shift)], using the chemical shift of P_i in parts per million downfield from PCr (4); this equation is similar to the one calculated from the generalized formulas suggested recently (29). Chemical shifts were estimated to the nearest 0.01 ppm by interpolation with a Lorentzian fit for each resonance.

Determination of ATP content. Although the use of ^{31}P-NMR spectroscopy has several advantages over chemical analysis of tissue samples for measuring myocardial [ATP], care must be taken to avoid errors resulting from saturation and from overlapping resonances. [ATP] was determined in two ways: from the integrated intensity (area) or the curve-fit area of the ATP_β resonance, which is not near any other known resonance, and from the height of the ATP_γ resonance. The use of the ATP_γ resonance produced smoother ATP depletion curves during ischemia in individual hearts, probably because the ATP_γ had a narrower line width than the ATP_β peak (and, therefore, a better signal-to-noise ratio) and because of problems with baseline curvature near the ATP_β resonance (see Fig. 2, for example). The peak height is proportional to the integrated area if the line width does not change. Any line width changes during ischemia should be small and similar in all hearts so that comparisons of relative [ATP] at different times within each group and between groups should be valid even if

the absolute amounts are slightly inaccurate. Care was taken to position the spectrometer frequency the same distance from the ATP_γ resonance in every heart so that summing spectra of hearts in each group would not affect the apparent line width. Furthermore, although the position of the ATP_γ resonance is slightly pH dependent, all hearts in each group had a similar pH at each time point, and therefore this contribution to the overall line width in the summed spectra was minimal.

One possible complication with the use of the ATP_γ resonance is the presence of the resonance from the ADP_β phosphate group just upfield from the ATP_γ resonance (7). Under control conditions the level of free myocardial ADP is too low to be observed in the ^{31}P-NMR spectrum, and the areas of the ATP_γ and ATP_β peaks are equal within experimental error (1). Under severely ischemic conditions, however, there may be a significant contribution to the ATP_γ resonance from ADP. An upfield shoulder was, in fact, clearly visible beside the ATP_γ resonance of the ^{31}P-NMR spectra obtained during ischemia (see Fig. 2, middle spectrum). The use of peak heights rather than areas minimizes this interference from ADP because of the chemical shift difference between the ATP_γ and ADP_β peaks.

Errors in [ATP] measurements could also arise from saturation of some NMR resonances by pulsing too quickly. Because the spin-lattice relaxation times (T_1) of the ATP_β and ATP_γ resonances are ~1 s or less in normal rat hearts (data not shown), the use of a delay time of 10 s between pulses ensured that the ATP_β and ATP_γ resonances were fully relaxed in all the ischemic spectra even if the T_1 doubled during ischemia. Resonances with T_1 possibly longer than 2 s, such as PCr and the phenylphosphonate reference, may have been partially saturated. In the three groups of hearts treated to vary the preischemic glycogen levels, the faster pulse repetition rate (3.75 s with 70° pulses) allowed almost full relaxation of the ATP resonances and improved the signal-to-noise ratio for a given time of data accumulation. The use of a 15-s repetition time for a 10-min spectrum before ischemia allowed full relaxation of the phenylphosphonate resonance, permitting calculation of preischemic [ATP].

For these reasons, preischemic [ATP] was determined from the areas of the ATP_γ and phenylphosphonate resonances in the fully relaxed ^{31}P-NMR spectrum. Relative ATP levels during ischemia were determined from the integrated intensity and curve-fit area of the ATP_β resonance and the height of the ATP_γ resonance, in individual spectra and in summed spectra, and are reported as a percentage of the preischemic value; absolute levels can be determined from the values of 23–25 μmol/g dry wt found in glucose-perfused rat hearts at this work state (26). Similar preischemic ATP levels were found in the three treated groups: 20.9 ± 2.7 μmol/g dry wt (SD, $n = 5$) in PG hearts, 23.6 ± 6.9 μmol/g dry wt in depleted hearts, and 22.2 ± 3.8 μmol/g dry wt in IA hearts.

Statistics. Values are expressed as means ± SD and were analyzed for significant differences ($P < 0.05$) between groups by analysis of variance. When significant differences were found, individual groups were compared by Fisher's least significant difference test. The relationships between TOC and pH at TOC or [ATP] at TOC were also analyzed by linear regression.

RESULTS

Time course of contracture. When glucose-perfused rat hearts were subjected to total global ischemia as described above, the TOC varied between 4.9 min and 16.8 min, in the range of published values for rat hearts at 37°C (8, 19, 21, 22). The TOC values formed a continuum without clear groups of hearts clustered around certain values. For some analyses, however, such as summation of spectra, it was necessary to divide the hearts into groups. The 63 hearts in this study were divided into five groups on the basis of TOC, with 12–14 hearts in each group. The use of five groups allowed enough hearts in each group for statistical analysis and enough groups to observe trends in the data. Preischemic and ischemic LVP and heart rate data for these groups are reported in Table 1. Preischemic peak systolic LVP, end-diastolic pressure, heart rate, and developed rate-pressure product (RPP, the product of heart rate and developed pressure) were similar in all groups, as was the minimum ischemic LVP.

Resting LVPs recorded during ischemia for *groups 1–5* are plotted as a function of time in Fig. 1*A*. After the onset of contracture, the rate of increase in resting pressure and the maximum pressure achieved were similar in *groups 1–5*. For *groups 1–3*, the time from onset of contracture to maximum contracture pressure was ~5–7 min; comparisons among all of the five groups were not possible because many hearts in *groups 4* and *5* did not reach a plateau before 18 min of ischemia. This time

TABLE 1. *Preischemic and ischemic left ventricular pressure and heart rate data for five groups of glucose-perfused rat hearts*

	Total	*Group 1*	*Group 2*	*Group 3*	*Group 4*	*Group 5*
n	63	12	14	12	12	13
TOC, min	8.64±2.65	5.45±0.34	6.78±0.47	8.21±0.42	10.16±0.86	12.59±1.33
Preischemic peak LVP, mmHg	102±21	100±24	98±14	103±18	111±23	99±21
Preischemic EDP, mmHg	5±2	5±2	4±2	4±2	4±2	6±2
Preischemic HR, beats/min	286±22	294±14	293±12	290±24	275±26	280±24
Preischemic RPP, 10^3 mmHg/min	28±6	28±8	27±4	29±6	29±5	26±6
Minimum ischemic LVP, mmHg	0.1±1.8	0.3±2.0	0.6±1.3	0.1±1.7	1.2±1.6	0.4±2.1
Maximum contracture pressure, mmHg	50±12	55±12	52±12	51±7	ND	ND

All values are means ± SD; n = no. of hearts. TOC, time to onset of contracture; LVP, left ventricular pressure; EDP, end-diastolic pressure; HR, heart rate; RPP, developed rate-pressure product; ND, not determined because of incomplete data (not all hearts reached a maximum pressure plateau during the 18-min ischemic period).

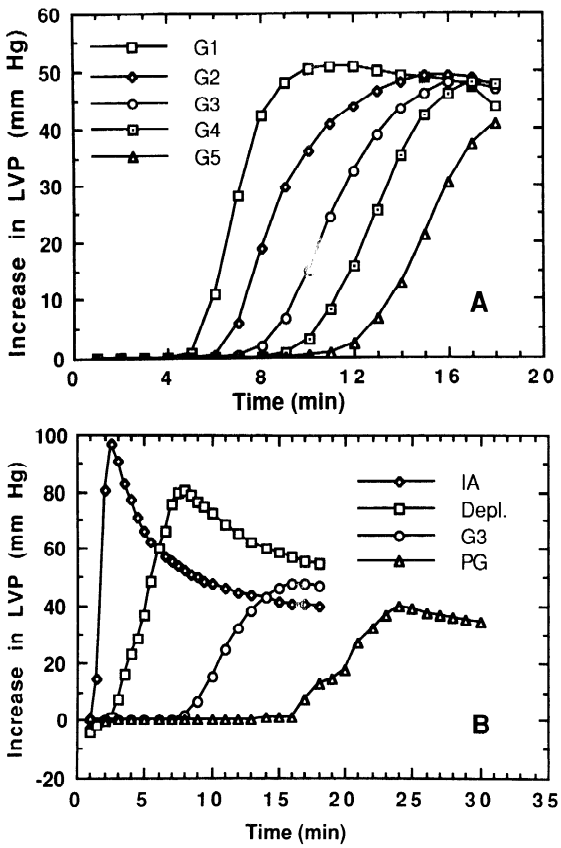

FIG. 1. Increase in left ventricular pressure (LVP) during 18 or 30 min of total global normothermic ischemia in Langendorff-perfused rat hearts. Error bars were omitted for clarity. *A*: *groups 1–5* (G1-G5) perfused with 11 mM glucose before ischemia. Sixty-three hearts were divided into 5 groups on the basis of TOC. Actual resting pressures varied from −3 to +5 mmHg at 3 min. SD values varied from 0 to 15.6 mmHg, with higher values corresponding to the steeper parts of the curves. *B*: 3 groups of hearts (IA, iodoacetate treated; depl., glycogen depleted; PG, pyruvate + glucose treated) treated to alter ischemic glycolysis and lactic acid production as described in text. Data from *group 3* are included to allow direct comparison.

span is similar to published values (8, 19), though times of 12–20 min or more have also been reported under apparently similar conditions (21, 22, 30). After the maximum resting pressure was attained, LVP began to decrease very slowly (Fig. 1*A*), as observed by others (8, 21, 30, 38), perhaps because of rupture of myocytes subjected to prolonged tension (21).

Effects of ischemia on intracellular pH. ^{31}P-NMR spectra from hearts in *group 3* obtained before ischemia, just before TOC (6–8 min of ischemia), and at 16–18 min of ischemia are shown in Fig. 2. Each spectrum resulted from adding the individual spectra for all 12 hearts in the group to enhance the signal-to-noise ratio and allow quantitation of low levels of ATP and PCr near the end of the ischemic period. Note the upfield shift (toward the right) of the P_i resonance in the ischemic spectra (bottom two spectra) compared with the preischemic spectrum (top spectrum), and the significant amount of ATP at TOC (middle spectrum).

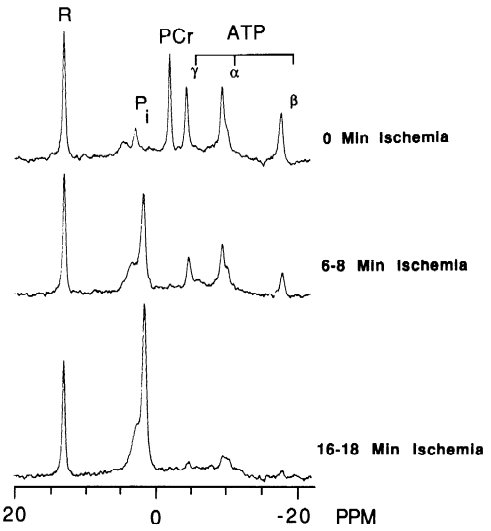

FIG. 2. ^{31}P-NMR spectra of hearts from *group 3* before and during ischemia. Each spectrum results from the addition of 12 individual spectra from 12 different hearts. For individual spectra 12 free-induction decays (FIDs) with 90° pulses and a repetition time of 10 s were recorded. R, phenylphosphonate reference in the left ventricular balloon; PCr, phosphocreatine; ATP-γ, α, and β, γ, α, and β phosphates of ATP. Chemical shifts are referenced to 85% H_3PO_4 at 0.0 ppm, using PCr at −2.50 ppm as an internal reference.

Preischemic and ischemic pH data are summarized in Table 2. The preischemic pH was 7.05 ± 0.04 and did not differ significantly among the five groups, but the final ischemic pH levels were different, presumably because of different preischemic glycogen contents. The intracellular pH measured by ^{31}P-NMR is plotted as a function of ischemic time in Fig. 3*A*. Error bars were omitted for clarity; SD ranged from 0.059 to 0.135 pH units for all points. The solid symbols mark the average time at which the onset of contracture occurred for each

TABLE 2. *Preischemic and ischemic pH and ATP data for five groups of glucose-perfused rat hearts*

	Total	*Group 1*	*Group 2*	*Group 3*	*Group 4*	*Group 5*
Preischemic pH	7.05±0.04	7.06±0.04	7.05±0.02	7.04±0.04	7.07±0.05	7.05±0.02
pH at TOC	6.13±0.17	6.35±0.10[a]	6.24±0.11[b]	6.10±0.07[c]	6.04±0.07[c]	5.93±0.09[d]
Final pH	6.08±0.18	6.28±0.11[a]	6.19±0.13[b]	6.04±0.07[c]	5.99±0.08[c]	5.87±0.08[d]
pH drop after TOC	0.06±0.04	0.06±0.04	0.05±0.03	0.06±0.04	0.05±0.05	0.06±0.04
%Preischemic [ATP] at TOC, %						
ATP_β areas						
Summed	41	43	42	41	33	36
Individual	41±18	42±26	47±17	40±12	39±16	38±12
ATP_γ height						
Summed	39.6	52.3	42.0	42.6	36.8	34.2
Individual	43.3±9.7	52.8±11.8[a]	45.5±5.7[b]	43.5±7.9[b]	40.3±5.7[b c]	34.6±6.0[b c]

All numbers are means ± SD. TOC, time to onset of contracture. In rows with superscripts, entries with a common superscript are not significantly different ($P > 0.05$).

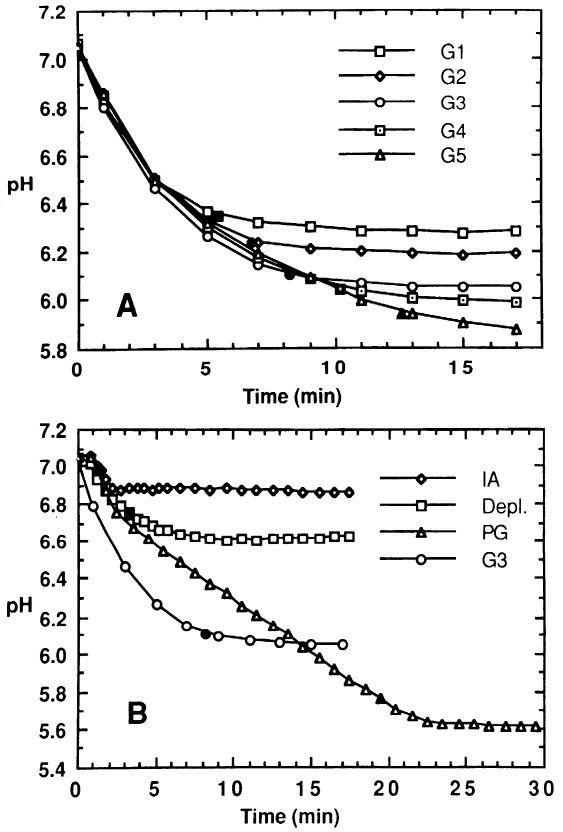

FIG. 3. Intracellular pH during ischemia in Langendorff-perfused rat hearts. Error bars were omitted for clarity. The pH was calculated from the chemical shift of the P_i resonance in the ^{31}P-NMR spectra as described in the text and represents an average over the entire volume of the heart. Solid symbols indicate time of onset of contracture (TOC). *A*: 5 groups of hearts perfused with 11 mM glucose before ischemia. SD during ischemia ranged from 0.059 to 0.135 pH units. *B*: 3 groups of hearts treated to alter ischemic glycolysis and lactic acid production as described in the text. Data from *group 3* are included to allow direct comparison.

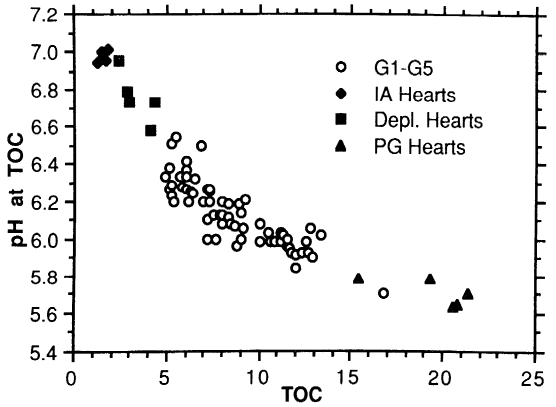

FIG. 4. Intracellular pH at TOC for individual hearts. The pH was measured using the chemical shift of the P_i resonance as described in the text and represents an average over the entire volume of the heart.

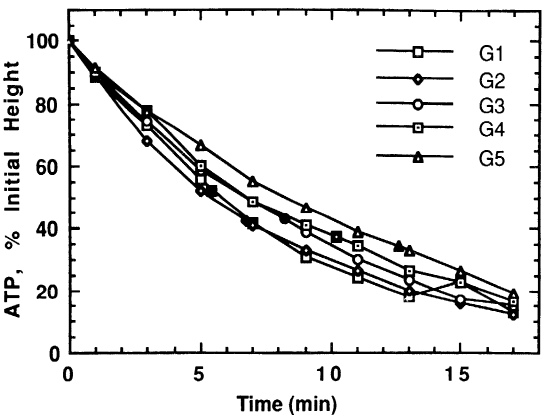

FIG. 5. ATP levels during ischemia as percent preischemic concentration for *groups 1–5*. Relative ATP levels were determined from the height of the ATP_γ resonance in ^{31}P-NMR spectra as described in text. Within each group, spectra at each time point were added together to increase the signal-to-noise ratio and allow quantitation of ATP at later time points. Solid symbols indicate TOC for each group.

group. In each group the pH initially fell and then stabilized. Similar results were observed when hearts were examined individually. The rate of decline of pH was similar in *groups 1–5* until the plateau phase was approached, despite presumed differences in preischemic glycogen levels.

It is clear from Fig. 3*A* and Table 2 that contracture did not occur at any specific value of intracellular pH. In fact, as the TOC increased, pH at TOC decreased ($r = -0.86$, $P < 0.001$; Fig. 4, Table 2). On the other hand, TOC correlated very well with the time of onset of the plateau in pH. In other words, pH fell until contracture began, after which time further change in pH was modest and similar in all groups (Table 2, Fig. 3*A*).

Effects of ischemia on ATP and PCr contents. Myocardial [ATP] and [PCr] during ischemia were measured from individual ^{31}P-NMR spectra and from summed spectra for each group as described in MATERIALS AND METHODS. ATP was visible in the ^{31}P-NMR spectra of all hearts at TOC, and in the summed spectra [ATP] could be quantitated throughout the entire ischemic period (see, for example, Fig. 2). During ischemia [ATP] decreased in a monophasic manner, with no plateau before contracture and no acceleration of the rate of ATP depletion after contracture started, reaching ~30–50% of preischemic levels at TOC (Fig. 5). Because of considerable scatter in [ATP] measured in individual hearts using the integrated intensity or curve-fit area of the ATP_β resonance, statistically significant differences in [ATP_β] were not found. Measurements of the heights of the ATP_γ resonances, however, showed significant differences between some groups at TOC (Table 2), and linear regression analysis revealed a significant correlation between TOC and [ATP] at TOC ($r = -0.58$), showing that TOC did not occur at a unique [ATP] ($P < 0.001$). Slight differences in the rate of [ATP] decline during ischemia were observed (Fig. 5), and at several times significant differences in [ATP] were found, especially in *group 1* vs. *group 5* hearts.

As expected, [PCr] dropped quickly during ischemia to a low residual level of ~10% of preischemic values, as has been reported by others (19, 33).

Effects of varying preischemic glycogen levels and glycolytic inhibition. The experiments presented above involved hearts perfused with only 11 mM glucose in the preischemic period to minimize artifacts due to different metabolic states, e.g., enzyme activation, and took advantage of the presumed natural variation in myocardial glycogen content and TOC in individual hearts. To investigate hearts with a wider TOC range, PG, depleted,

and IA hearts ($n = 5$ in each group) were given different preischemic treatments to alter glycolytic ATP synthesis during ischemia and thus vary the TOC over a wider range (see MATERIALS AND METHODS).

Results for these three groups are summarized in Table 3, and some data for these groups are compared with *groups 1–5* in Figs. 1*B*, 3*B*, and 4. ^{31}P-NMR spectra at TOC for these three groups of hearts are shown in Fig. 6. For each spectrum shown, spectra of the five individual hearts that were being collected at TOC have been added together to increase the signal-to-noise ratio. In all three groups the pH dropped until TOC and then reached a plateau (Fig. 3*B*). Interestingly, in all three groups little or no change in [P_i], [PCr], or pH was observed during the first 30 s of ischemia. The LVP dropped quickly during the first few seconds of ischemia, as expected. In most PG, depleted, and IA hearts, as well as in several hearts in *groups 1–5*, though, after ~30 s LVP stabilized briefly or increased slightly before resuming its downward trend. During this time (30–60 s of ischemia), the average P_i level increased and PCr declined by ~40%, while the pH actually increased slightly in PG, depleted, and IA hearts; in *groups 1–5* the time resolution (2 min vs. 30 s) was not sufficient to observe this effect. This inotropic response after metabolic inhibition was qualitatively similar to effects seen during hypoxia or cyanide treatment (1, 2) and demonstrates the sensitivity of myocardial function to intracellular pH.

TABLE 3. *Preischemic and ischemic LVP, pH, and ATP data for IA, depleted, and PG hearts*

	Treatment		
	IA	Depleted	PG
TOC, min	1.5±0.3	3.3±0.8	19.5±2.4
Preischemic LVP, mmHg	72±23	88±13	81±13
Preischemic RPP, mmHg/min	17±9	24±7	19±7
pH at TOC	6.97±0.03	6.76±0.14	5.71±0.07
Final pH	6.86±0.08	6.63±0.09	5.61±0.08
pH drop after TOC	0.11±0.05	0.13±0.07	0.10±0.05
%Preischemic [ATP] at TOC			
ATP_β area	59	66	10
ATP_α height	69	65	19
Maximum contracture pressure, mmHg	109±23	83±22	45±14

All values are means ± SD; $n = 5$ hearts for each group. IA, iodoacetate; PG, pyruvate + glucose, see Table 1 legend for other definitions. [ATP] was measured in the summed spectra.

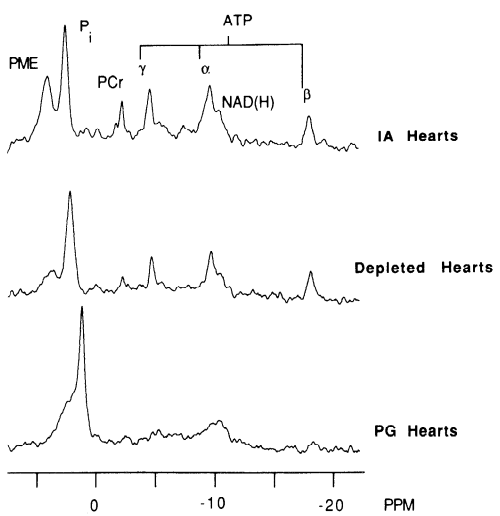

FIG. 6. ^{31}P-NMR spectra of 3 groups of hearts at TOC. For each spectrum shown, spectra from the 5 individual hearts in the group were summed to improve the signal-to-noise ratio. Individual spectra were collected as described in the text using 70° pulses, with 8 FIDs being collected in 30 s (IA and depleted hearts) or 16 FIDs in 1 min (PG hearts). Peak assignments are as in Fig. 2, with 2 additions: PME, phosphomonoesters (mostly fructose 1,6-bisphosphate in IA hearts); NAD(H), the diphosphodiester phosphates of NAD^+, NADH, $NADP^+$, and NADPH.

In PG hearts the TOC of 19.5 min was longer than in *group 5* (Fig. 4, Table 1). Intracellular pH leveled off at pH ~5.6, significantly lower than in *group 5* hearts (Fig. 3). The slightly greater drop in measured pH after TOC (Table 3) may be due to the fact that the pH is one full pH unit below the pK of the P_i whose resonance is used to measure pH, so that a small change in apparent chemical shift corresponds to a large change in the calculated pH. [ATP] at TOC was lower than in any other group (Table 3, Fig. 6).

In depleted hearts the TOC of 3.3 min was shorter than in *group 1* hearts. The pH reached a plateau at pH ~6.6, higher than in *group 1* hearts (Fig. 3), while [ATP] at TOC was significantly greater than in PG hearts (Fig. 6, Table 3) and slightly greater than in *groups 1–5*.

In IA hearts the TOC of 1.5 min was even faster than in depleted hearts, and both pH at TOC and final pH were higher. Contracture occurred so quickly, in fact, that there was significant PCr in the ^{31}P-NMR spectra of IA hearts at TOC (Fig. 6), and in some cases a small amount of developed pressure was still present at TOC. In addition to the rapid onset of contracture, the rate of pressure development during contracture and the maximum contracture pressure achieved were also much greater than in other groups; a similar though weaker effect was seen in depleted hearts (Fig. 1*B*, Table 3). After reaching a maximum of ~100 mmHg, the contracture pressure decreased to ~50 mmHg, similar to other groups. These observations on contracture pressure in IA-treated hearts are similar to those reported elsewhere (21, 28).

DISCUSSION

Choice of contracture model. Myocardial contracture occurs under a variety of conditions of energy deprivation, including total ischemia, low flow ischemia, hypoxia, and metabolic inhibition by cyanide plus either IA or 2-DG. Studies of contracture have been performed with different species and with a variety of experimental systems and experimental conditions. Because the relative importance of various factors that may influence contracture (e.g., the rate of ATP depletion and accumulation of lactate, H^+, and P_i) will not be the same in all these systems, quantitative comparisons between studies must be done with caution. We chose the perfused heart subjected to total global ischemia for our contracture studies because this model most closely resembles the "stone heart" occasionally seen during human cardiac surgery (23).

Identification of the metabolic events underlying ischemic contracture is limited by transmural heteroge-

neity in myocardial metabolism. Techniques such as spatially nonlocalized NMR (which makes measurements of average transmural $[Ca^{2+}]_i$, high-energy phosphate levels, and pH in both ventricles) or indo-1 and aequorin measurements (which determine $[Ca^{2+}]_i$ in the epicardium) may not adequately represent changes occurring in the subendocardium. Thus, if contracture begins in the subendocardium in the perfused small animal heart, then global measurements of phosphorylated compounds or epicardial measurements of $[Ca^{2+}]_i$ may not permit precise correlation of biochemical events with the onset of ischemic contracture.

Intracellular pH during ischemia. The major source of H^+ in ischemic myocardium is glycolytic production of lactic acid coupled to ATP hydrolysis and resynthesis, although small amounts are also generated by net ATP hydrolysis and removed by PCr hydrolysis (13). [At pH < 6.5, though, ATP hydrolysis may actually remove H^+ (43).] Consequently, the fall in pH after the onset of ischemia has been attributed to the persistence of anaerobic glycogenolysis and glycolysis (3, 4, 12, 43). In support of this view, glycogen levels have been shown to decline during ischemia while lactic acid levels increased (3, 17, 19, 33, 42, 43), and close correlations have been noted during ischemia between glycogen breakdown and lactate accumulation (3, 33) and between lactate accumulation and pH (43). A secondary effect in the early stages of energy deprivation is the possible increase in pH caused by PCr hydrolysis (1, 13), as seen in most PG, depleted, and IA hearts.

Because the rate of decrease in pH (preceding the plateau onset) in *groups 1–5* was similar until TOC (Fig. 3*A*), we conclude that the rate of glycogen metabolism to lactate was independent of the total quantity of utilizable glycogen under our experimental conditions. If phosphorylase was the rate-limiting enzyme, then the concentration of metabolizable glycogen sites appears to have been much greater than the Michaelis constant (K_m) of phosphorylase, so that glycogen utilization was limited by enzyme activity, not by glycogen concentration. Alternatively, a glycolytic enzyme may have been rate limiting. In either case the velocity of the rate-limiting step appears to have been similar in all hearts.

Other metabolic variables such as myosin isozymes, mitochondrial H^+-ATPase inhibitor activity (35), and mitochondrial H^+ leakiness could also affect the rates of ATP depletion and glycogenolysis, and the delayed TOC in hearts from hypothyroid rats (8) may be due to both increased glycogen levels and decreased metabolic rate. Such metabolic differences may be responsible, in part, for the slightly different rates of ATP loss in *groups 1–5* (Fig. 5), which had similar preischemic RPP (Table 1). A lower average metabolic rate may have not only contributed to higher preischemic glycogen stores in *group 5* hearts but also led to a slower rate of ischemic ATP utilization. Finally, although the rate of glycolysis may be modified by intracellular pH and lactate ions (36), intracellular acidification was not the primary mechanism for the ultimate cessation of anaerobic glycolysis. This follows from the fact that the time-dependent decrease in pH stopped at a variety of different pH values ranging from 5.7 to 6.4 in *groups 1–5*, above 6.7 in some depleted hearts, and ~5.6 in PG hearts (Figs. 3 and 4, Tables 2 and 3), and a similar range of final ischemic pH has been observed by others (3, 4, 12, 30, 43).

The curvature in the pH vs. time plots (Fig. 3*A*) is probably due to three factors. First, pooling of data from several hearts with slightly different time courses will tend to blur any changes or transitions. Second, there is an increased myocardial buffering capacity at lower pH, which produces a nonlinear relationship between lactic acid production and pH (43). Third, glycolysis is inhibited by both H^+ and lactate (36).

Intracellular pH and TOC. The plateauing of ischemic pH values after the initial fall (Fig. 3) is thought to reflect the depletion of utilizable glycogen (3, 4, 12). In support of this hypothesis, preischemic depletion of myocardial glycogen by substrate-free perfusion decreased the ischemic acidosis (12, 30, 43), and increasing preischemic glycogen content by treatment with insulin resulted in a greater fall in pH (3). Our own data in hearts with decreased or increased glycogen content agree with these earlier studies (Fig. 3*B*), suggesting that most of the variability we observed in the magnitude of the fall in pH and the time of onset of the pH plateau was due to variation in preischemic myocardial glycogen stores. The small pH drop (~0.2 pH units) in IA hearts (Fig. 3*B*) was probably due to nonglycolytic H^+ production, presumably from ATP hydrolysis (13) and perhaps other unidentified sources, and a similar pH drop in the other groups probably came from these nonglycolytic H^+ sources. Some variability within each group may also have been caused by different intracellular buffering capacity and Na^+-H^+ exchange rates or by slight variations in experimental conditions such as temperature. It seems unlikely, however, that these small effects could account for the wide range of TOC and pH at TOC among the *groups 1–5* and in PG and depleted hearts.

In all groups studied, TOC occurred at the beginning of the pH plateau phase. The slight drop in pH after TOC (Fig. 3, Tables 2 and 3) was probably due to two factors. First, ATP continued to be hydrolyzed slowly, and ATP hydrolysis can release H^+ (13). The slightly greater drop in pH after TOC observed in IA hearts and depleted hearts may have been due, in part, to their relatively high [ATP] at TOC (Table 3, Fig. 6). In addition, the elevated pH at TOC in these two groups resulted in more H^+ release per ATP hydrolyzed than at lower pH, as well as a decreased buffering capacity (43). Second, contracture may have begun when glycogenolysis and glycolysis ceased in one region of the myocardium (e.g., left ventricle or subendocardium of the left ventricle), while other regions (right ventricle or subepicardium of the left ventricle) may have had a small amount of glycogen left. Continued lactic acid production in these latter regions for a few minutes during the development of contracture would result in a slight decrease in the average myocardial pH measured by ^{31}P-NMR. This explanation is supported by transmural and regional differences in the metabolism of 2-DG (40) and glycogen (9) in rat hearts.

The observation that TOC occurred at the onset of the pH plateau phase implies that contracture began in concert with cessation of significant glycolytic produc-

tion of ATP. (Although glycolytic NADH production also ceased at this time, we know of no mechanism by which this could induce contracture.) This temporal relationship between the pH plateau and TOC has not, to our knowledge, been previously reported, though Figs. 4 and 6 in Ref. 28 appear to show a similar trend, and a similar relationship between lactate production and contracture has been reported in isolated myocytes (18). In contrast, in a study using a rat heart model similar to ours, glycogen decreased from 120 μmol glucose equivalents/g dry wt to ~25% of the initial level at TOC (5–8 min) and then continued to decline slowly, reaching a value of ~5% of the initial level at 18–20 min (19). We have no explanation for the significant glycogenolysis and glycolysis reported to occur after TOC in light of the very modest fall in pH that we observed (Fig. 3 and Tables 1 and 2), though transmural or regional metabolic differences may be responsible for this apparent discrepancy.

Any direct causal link between a specific pH value and TOC is contradicted by our data (Tables 1 and 2 and Figs. 3 and 4). In agreement with our results, contracture can occur during hypoxia despite little change (1) or no change in pH (22). In the case of hypoxic hearts in which perfusion is present, the efflux of lactic acid appears adequate to reduce lactic acid accumulation and decrease the consequent fall of pH (33).

ATP and TOC. Our observation of a monophasic decline in [ATP] throughout the ischemic period, with no evidence of a plateau in [ATP] before contracture or an acceleration of ATP depletion after TOC, is consistent with other recent reports (4, 28, 42) and contradicts the apparent biphasic fall of [ATP] reported in some studies (19, 21). The steady ATP decline does not support the hypotheses that ATP depletion accelerates during contracture because of either rigor bond formation (19) or Ca^{2+}-stimulated actomyosin ATPase activity. Our data indicate that the onset of contracture is not associated with a unique [ATP] (Fig. 6 and Tables 2 and 3), although in all groups ATP was significantly depleted at TOC (Tables 2 and 3). The observation that TOC is associated with significant but variable [ATP] is consistent with previous observations (6, 38, 42) and argues against the suggestion that contracture begins when the total cellular ATP pool falls below some critical level (17, 19, 28).

Mechanism of ischemic contracture in control hearts. In light of *1*) the Ca^{2+}-desensitizing effect of low intracellular pH (11), *2*) a rise in $[Ca^{2+}]_i$ preceding the onset of contracture in perfused rabbit (32) and ferret hearts (25, 28), and *3*) scattered light intensity fluctuation data demonstrating that Ca^{2+}-activated cross-bridge activity was low at TOC in perfused rat and rabbit hearts (39), increased $[Ca^{2+}]_i$ is unlikely to have been the precipitating event for ischemic contracture in glycolytically competent hearts. We therefore conclude that the onset of contracture was due to formation of rigor bonds that persisted in the absence of sufficient ATP to dissociate the myosin cross bridges from actin filaments. While elevated $[Ca^{2+}]_i$ was probably not a direct factor in the onset of contracture, elevated $[Ca^{2+}]_i$ may have increased the rate of ATP depletion (perhaps by stimulating mitochondrial or sarcoplasmic reticulum ATP consumption), thus accelerating contracture (27).

Rigor bonds, by definition, consist of myosin cross-bridge binding to actin in the absence of ATP, which is necessary for the release of cross bridges from actin. Without a cycle of cross-bridge binding and release, how can contracture pressure develop by rigor bond formation? There is evidence that rigor complexes can remove the troponin inhibition of myosin-actin interaction even in the absence of Ca^{2+} (5, 14). Thus when [ATP] is sufficient to allow the coexistence of significant numbers of myosin cross bridges with ATP and without ATP (~1–10 μM), cross bridges could cycle slowly between the ATP-deficient (rigor) state and an ATP-bound state, allowing pressure to develop gradually, independent of Ca^{2+}. As [ATP] falls through this range in ischemic muscle, myosin ATPase activity and cross-bridge cycling could increase, thus slowly creating tension even in the absence of elevated $[Ca^{2+}]_i$ (or at low pH, which decreases the Ca^{2+} sensitivity of myofibrils), and then decrease as [ATP] declines even further.

Mechanism of ischemic contracture in hearts with glycolysis inhibited. The presence of slight contractile activity at TOC in some IA hearts, along with significant PCr in the ^{31}P-NMR spectra (Fig. 6), could be explained by myocardial heterogeneity: some cells (e.g., subendocardium) could have begun a rigor contracture while other cells (e.g., subepicardium) still had some contractile activity. This hypothesis, however, does not explain the contracture pattern observed in the limited glycolysis groups (this is most evident in the IA group), where there was a rapid and marked rise in contracture pressure, which then fell toward the lower pressure found in the other groups, a pattern that has been reported by others in IA-treated hearts (21, 28) and 2-DG-treated hearts (30). We believe that the high, early contracture pressures noted in the limited glycolysis groups may reflect an initial $[Ca^{2+}]_i$-mediated phase of contracture that then was converted to rigor contracture as cross-bridge ATP availability further decreased. This hypothesis is supported by the lack of a Ca^{2+}-desensitizing drop in pH. The idea that a Ca^{2+}-initiated contracture can be maintained by Ca^{2+}-independent rigor bonds has been suggested previously in myocytes (10). Thus, during ischemia associated with limited glycolytic H^+ production such as was the case in our IA and depleted groups, the initial phase of ischemic contracture may have been Ca^{2+}-dependent, with the active contracture relaxing later into a rigor contracture.

This interpretation contrasts with the report of Koretsune and Marban (28) that $[Ca^{2+}]_i$ does not increase until after TOC in IA-treated ferret hearts at 30°C. Differences in animals (ferret vs. rat) and experimental conditions (30 vs. 37°C) could contribute to this apparent discrepancy, but a more important factor is likely to be the low time resolution (2.5 min) of their Ca^{2+} measurements, which used 5-fluoro-1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (5F-BAPTA) and ^{19}F-NMR. Their measured $[Ca^{2+}]_i$ increased from baseline levels of ~0.3 μM to a stable level of ~1.8 μM in consecutive spectra, with no intermediate concentrations (their Fig. 7). Because the $[Ca^{2+}]_i$ could not have in-

creased instantaneously between these two spectra, Ca^{2+} levels may have begun to increase during the earlier spectrum, with the increase masked by the relatively low signal-to-noise ratio. Furthermore, because they did not deplete glycogen before IA treatment, phosphorylated metabolites accumulated during the ensuing ischemic period (compare their Fig. 5 with our Fig. 6), causing extremely rapid ATP depletion to nearly undetectable levels at TOC. Under these conditions, both ATP depletion and elevation of $[Ca^{2+}]_i$ may have contributed to the onset of contracture. If this is so, then the $[Ca^{2+}]_i$-mediated component of contracture could have caused the LVP vs. time relationship that we (Fig. 1*B*) and others (21) have observed in IA-treated hearts.

The correlation between pH and TOC in the IA group, where the pH fall was modest, glycolysis was minimal, and contracture began soon after the onset of ischemia, may have been merely fortuitous. Alternatively, under ischemic conditions sarcolemmal (16) or sarcoplasmic reticulum function (21) may be quite dependent on glycolytically generated ATP, and alteration of the function of these organelles could result in rapid elevation of $[Ca^{2+}]_i$ and contracture. This latter reasoning can also be applied to the depleted hearts, which contracted somewhat later and probably had some early glycolytic activity.

Is the ATP content too high to permit ischemic rigor contracture? Rigor bond formation can be suppressed by as little as ~30 μM ATP (41), and isolated myocytes can lose up to 90% of their ATP without undergoing contracture (18). The occurrence of ischemic contracture in the presence of significant cellular stores of ATP (8, 19, 21, 22, 30, 38, 42) (see also Tables 2 and 3 and Figs. 2 and 6), which are adequate to support reasonably normal systolic and diastolic function in postischemic hearts (33, 37, 44) and in adequately oxygenated hearts whose PCr and ATP have been markedly depleted by 2-DG (20, 30), suggests partial cytosolic compartmentation or channeling of metabolites: ATP produced from glycolysis or CK may be delivered to the myofibrils more efficiently than is ATP from the intracellular pool (6, 18, 19). In support of this idea, certain glycolytic enzymes have been shown to be bound to the contractile apparatus in skeletal muscle and thus near the cross bridges (31). Furthermore, an isozyme of CK is associated with myofibrils, and in chemically skinned cardiac muscle the buffer ATP concentration required to prevent rigor-like bonds was decreased by more than an order of magnitude in the presence of the PCr-CK system (41).

Another possible explanation for the onset of rigor bond contracture in the presence of relatively high tissue [ATP] is the suggestion that individual myocardial cells undergo contracture when their ATP is depleted (17, 18). The degree of contracture in this case would reflect the percent of myocytes that have lost all their ATP, and ATP observed in ^{31}P-NMR spectra would be in the cells that have not undergone contracture. It is difficult to explain with this model, though, why contracture tension reached a maximum when tissue [ATP] in *group 1* hearts was still 30% of preischemic values and failed to increase as [ATP] declined further, to ~12% (Figs. 1 and 5).

Conclusions. We make the following conclusions about the no-flow globally ischemic myocardium. *1*) Anaerobic glycolysis is associated with a pH decline whose initial rate is independent of the final pH achieved (and presumably independent of the initial glycogen content). *2*) Ischemic contracture begins when the pH plateaus, at which time ATP synthesis via glycogenolysis and anaerobic glycolysis has presumably ceased. *3*) Variation in preischemic glycogen stores does not affect the relationship between the onset of the pH plateau and TOC, although glycogen depletion shortens TOC. *4*) ATP declines in a monophasic manner, with no acceleration in ATP loss after TOC. *5*) The onset of ischemic contracture is normally a result of rigor bonds formed because of ATP deficiency at the myofibrils. After severe glycogen depletion or complete glycolytic inhibition, however, the onset of contracture may have an active Ca^{2+}-dependent component. *6*) ATP delivery to (and/or ADP removal from) the cross-bridge microenvironment may be supported better by the PCr-CK system and glycolysis than by diffusion from the cytosolic ATP pool. Thus metabolite channeling via glycolytic reactions may delay the onset of ischemic contracture.

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