Electrolytes of Damaged Myocardial Mitochondria[1] (35086)

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Striking alterations in the structure of myocardial mitochondria appear after 40 to 60 min of ischemia or autolysis (1, 2). These changes include swelling, increased mitochondrial fragility, and the *de novo* appearance of prominent intramitochondrial dense bodies.

It would seem likely that alterations in mitochondrial electrolyte content would be associated with the structural changes observed in mitochondria isolated from myocardium damaged by ischemia or autolysis. On the one hand, mitochondria are membrane bound structures which are known to contain electrolytes (3), and on the other, these organelles are present in an intracellular milieu which shows marked changes in electrolyte concentration and distribution after lethal cellular injury (5). Furthermore, isolated myocardial mitochondria can accumulate cations such as Ca^{2+}, Mg^{2+}, and K^+ from the reaction medium in an energy dependent fashion (6, 14, 15). In the case of divalent cations, especially Ca^{2+}, the accumulation is known to be associated with the formation of dense intramitochondrial aggregates, which are superficially similar to the dense bodies noted in ischemia and autolysis (4).

Accordingly, we have investigated the distribution of electrolytes in mitochondria of control and damaged left ventricular myocardium. The results reported in this paper show that there is an apparent increase in the Ca^{2+} content and a decrease in Mg^{2+}, K^+, and nitrogen content of mitochondria isolated from tissues subjected to *in vitro* autolysis.

Materials and Methods. Hearts from six healthy mongrel dogs of either sex weighing between 11 and 18 kg were used. The dogs were allowed free access to Borden's dog chow and water but were fasted overnight before use. They were anesthetized with intravenous sodium pentobarbital (35 mg/kg of body wt).

Each heart was excised quickly through an incision in the left chest and was placed in a beaker surrounded by crushed ice and filled with 700 ml of 0.25 *M* sucrose at 0–2°. The heart was stirred to facilitate cooling. After 1 min, the left and right ventricles were incised and 1 min later, four samples of anterior superior septum, each weighing from 3.5 to 4.9 g were removed. Each piece was placed in a 2-oz jar containing a sponge soaked in isotonic sucrose. The jars, which had been heated to 37°, were tightly sealed and were immersed in a 37° water bath for periods of 60, 120, and 180 min. An additional portion of myocardium served as control.

Mitochondrial isolation procedure. Mitochondria were isolated using the general procedures and precautions described by Jennings *et al.* (7) except that 0.25 *M* sucrose was substituted for 0.18 *M* KCl. Briefly, myocardium was minced with iris scissors on the lip of a homogenizing tube and then digested in 1 ml of homogenizing solution/100 mg of tissue. The solution contained 0.25 *M* sucrose (Baker, A. R.), and 0.001 *M* EDTA with 0.5 mg of *B. subtilis* alkaline proteinase (Nagarse, Enzyme Development Corp.)/ml of solution. After 10 min of incubation, the

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tissue was homogenized with three strokes of a loosely fitting glass-impregnated Teflon homogenizer. The homogenate was diluted with an equal volume of homogenizing solution without Nagarse and centrifuged for 6 min at 225*g*. The supernatant was removed and passed through a stainless steel screen, and sedimented at 12,100*g* for 10 min. The supernatant was discarded and the pellet was washed with 5 ml of sucrose–EDTA and resedimented at 8700*g* for 5 min. This pellet was resuspended in sucrose–EDTA in 10% of the original volume of homogenizing fluid, and then centrifuged at 110*g*. Purity was checked by phase contrast microscopy and if tiny particles of undigested myocardium were present, a repeat centrifugation at 110*g* was performed.

In order to slow endogenous metabolism as much as possible and so allow a good estimate of the electrolyte concentration of the mitochondria *in situ,* the temperature during the isolation procedure was kept near 0°. The chance of ion translocation from the medium to the mitochondria during the isolation procedure was further decreased by using EDTA in the isolation fluid to chelate the small amount of Ca^{2+} known to be present in Nagarse. The sucrose used contained small amounts of Ca^{2+} which was chelated by the EDTA but no Na^+, K^+ or Mg^{2+} were present. These techniques were successful to the extent that significant quantities of electrolyte were found and control electrolyte values were consistent from dog to dog.

Chemical analysis. Electrolytes were analyzed in quadruplicate by flame atomic absorption spectrophotometry in a model 82-526 Jarrell Ash Instrument using modifications of the technique of Willis (8) and Monder and Sells (9). Deionized water with a resistance of greater than 5×10^6 ohms was used throughout. One-ml aliquots of mitochondria were extracted for 1 hr in 50-ml Pyrex glass centrifuge tubes (10) which had been soaked for 1 month in dilute nitric acid before being used for extraction of electrolytes. The tubes were covered with sealed 5-cm glass funnels to prevent evaporation and volatilization of chloride and were heated in a boiling water bath for 1 hr to extract electrolytes from the mitochondria. After cooling, they were centrifuged at 1500 rpm (500*g*) for 10 min. A 3.0-ml aliquot of the extract was added to a 5-ml volumetric flask, diluted to 5.0 with enough $LaCl_3$ to bring the concentration of $LaCl_3$ to 10^4 ppm and enough 5 *N* HNO_3 to bring the final acid concentration to 1.0 *N*.

Standard curves for Ca^{2+}, Mg^{2+}, and K^+ were prepared using a standard solution containing these ions as well as PO_4 in a concentration equivalent to that found in heart muscle (11). This standard was diluted with $LaCl_3$ and HNO_3 in the same final concentrations used for the unknowns. Ca^{2+}, Mg^{2+} and K^+ absorbances were measured at concentrations of 0.5 to 2.0, 0.2 to 0.8, and 3.5 to 14.0 ppm, respectively. The relationship of absorbance to concentration was linear. An appropriate sucrose blank was employed.

Recovery experiments using lyophilized heart muscle with HNO_3 extraction and with the general methods described above, showed that we could recover 100% (± SE 2.6%) of added Ca^{2+} when its concentration was 0.4 μg of Ca^{2+} or more/ml of extract. Thus, a minimum of 120 mg of wet weight of normal dog left ventricle or 0.5 mg of myocardial mitochondrial nitrogen, must be used to insure accurate determination of Ca^{2+}. No difficulties were encountered with recovering Mg^{2+}, Na^+ and K^+ from heart muscle diluted to up to 5 times the volumes used for calcium. In each instance the final concentrations of $LaCl_3$ was 10,000 ppm and the HNO_3 concentration was 1 *N*. All the glassware and pipettes were washed with detergent, rinsed thoroughly with deionized water, and handled where necessary with polyethylene gloves to prevent finger print contamination. Nitrogen was determined by the micro-Kjeldahl technique (12).

Electron microscopy. Tiny aliquots of mitochondria isolated from control myocardium and from myocardium autolyzed for 120 and 180 min were spun through cold phosphate-buffered osmium tetroxide (pH 7.2) in clean polyethylene microcentrifuge tubes for 10 min at 15,000 rpm (10,000*g*) according to

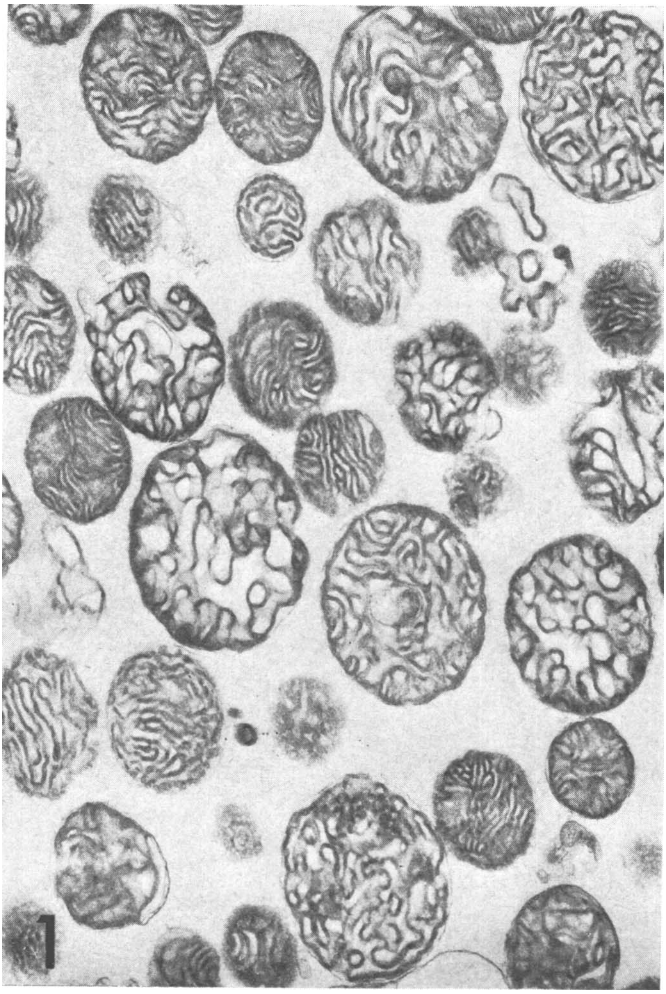

FIG. 1. Control; most of the mitochondria are intact. They show very fine outer membranes and cristae which are densely packed with varying degrees of contortion; 16,700×. Figs. 1 to 4 are of mitochondrial pellets isolated from either control or autolyzed dog left ventricular myocardium. Lead acetate stain.

techniques described elsewhere (7). In addition, pieces of intact myocardium from control tissue and from tissue autolyzed for 120 and 180 min were cut under osmium tetroxide into 1 mm^3 blocks and fixed at 4° for 1 hr. The fixed pellets of mitochondria were cut into 1 mm^3 blocks under 50% alcohol, and all the blocks were dehydrated through a graded series of alcohols and embedded in Epon 812. Thin sections were cut on a Porter-Blum MT-1 ultramicrotome using glass knives. Ultrathin sections were mounted on plain "Effa" copper grids, stained with lead and examined in a Philips EM 200 or a RCA EMU-3C electron microscope.

Results. Fine structure. The fine structural changes in the myocardium following autolysis at 37° for 60 to 180 min have been reported previously (2) and were not studied further in this experiment. Briefly, nuclear chromatin clumping and margination was evident following 30 to 60 min of autolysis, as was a decrease in glycogen granules. There were no striking changes in myofibrillar architecture. Prominent intramitochondrial granules first appeared at 60 min and in-

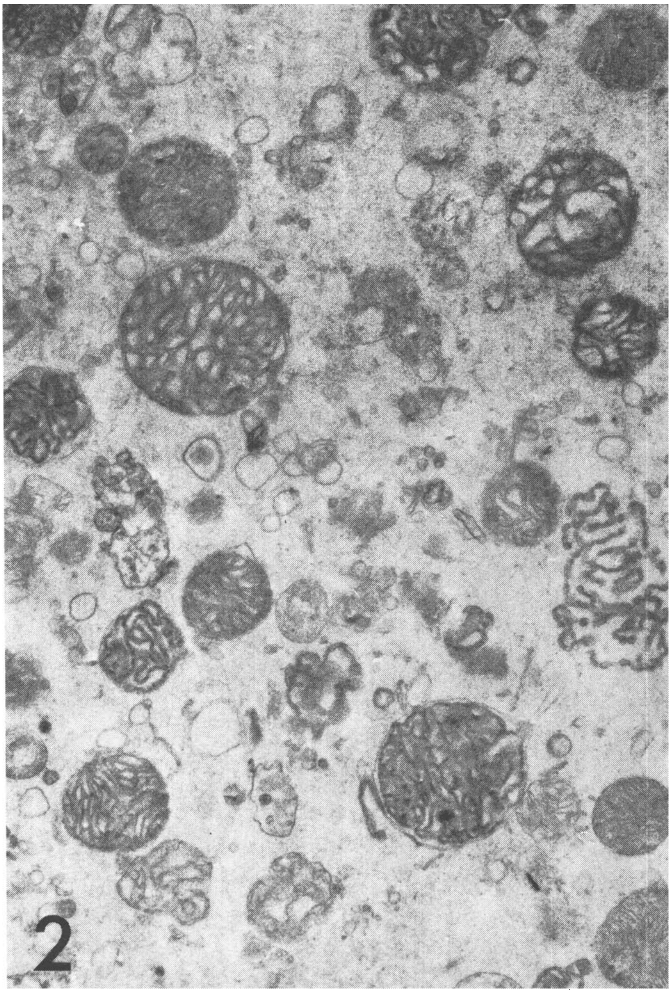

FIG. 2. 60 min of autolysis; numerous fragments considered to be of mitochondrial origin are present in this pellet. Some mitochondria are similar to control (see Fig. 1). Others have electron opacities; 12,600×.

creased in size and in number between 60 and 180 min.

Mitochondria of control myocardium isolated in sucrose showed a delicate outer membrane (Fig. 1). Most had densely packed, often wavy, cristae, with definite double membranes of uniform thickness. The matrix space dimensions and density were more variable. In general, the larger matrix spaces had lighter densities than the smaller ones. None of the mitochondrial sections examined contained any electron opaque granules of the type seen in autolyzed tissue.

The mitochondrial pellets isolated from myocardium autolyzed for 60 min showed numerous fragments, many of which were highly suggestive of parts of cristae from disrupted mitochondria, others being less readily identifiable (Fig. 2). Numerous whole mitochondria were seen. These had retained their outer membranes. A few had one or more electron opaque dense bodies which were indistinguishable from those seen in tissue sections. After 120 and 180 min of autolysis (Figs. 3 and 4), isolated mitochondrial pellets contained mitochondria which were markedly swollen and showed disruption of the normal arrangement of the cristae. The

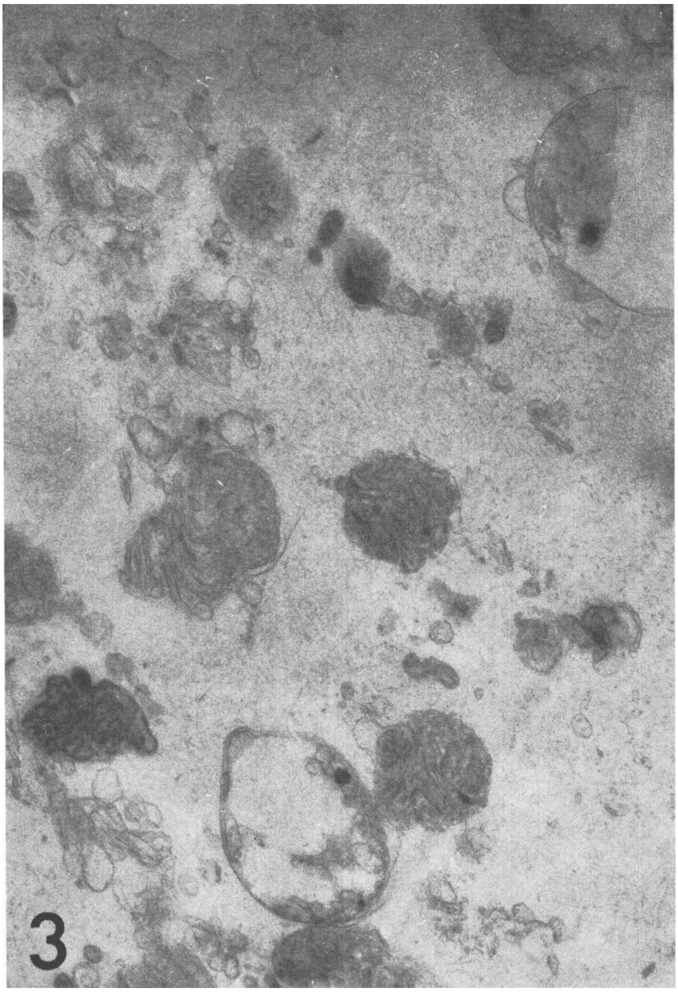

FIG. 3. 120 min of autolysis; most of the mitochondria are swollen with disruption of the normal cristae pattern. Electron opaque granules are present and are seemingly associated with the cristae; 19,200×.

outer membranes, however, were largely retained. Sections of most of the whole mitochondria contained one or more electron opaque dense bodies which appeared to be associated with the cristae. These bodies were globular in form, measured up to 300 mμ in diameter and had indistinct outlines. In addition to whole mitochondria, these pellets contained numerous smaller fragments, many of which were indisputably of mitochondrial origin. There was also a finely fibrillar ground substance which stained indistinctly with lead.

Electrolyte changes. The Ca^{2+}, Mg^{2+}, K^+, and nitrogen content of the isolated mitochondria are listed in Table I. The Mg^{2+} and K^+ content were much higher than the calcium content which was only 6.09 ± 0.73 mmoles/100 g of mitochondrial N. These values are in the range found by other authors in rat liver mitochondria isolated in sucrose (3).

The nitrogen data is given as milligrams of mitochondrial nitrogen per gram of left ventricular wet weight. It shows that less mitochondrial nitrogen was isolated from each unit quantity of autolyzed tissue, regardless of the duration of autolysis, than from con-

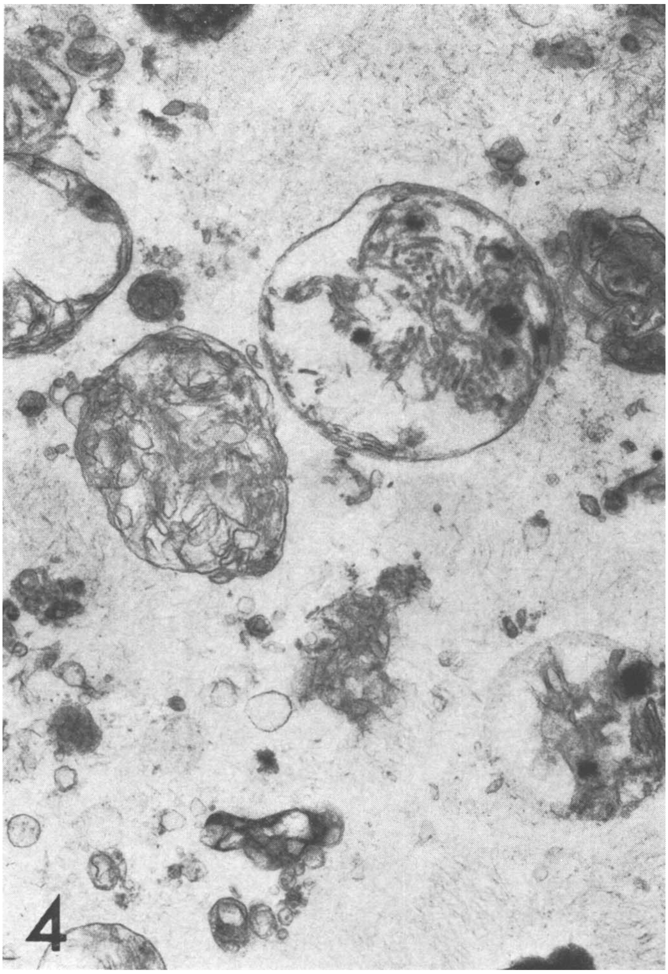

FIG. 4. 180 min of autolysis; similar to Fig. 3, except that electron opaque, intramitochondrial granules are more numerous; 19,200×.

trol tissue. This has been shown previously to be due to an increase in mitochondrial fragility (*2*) and is reflected in the fact that significantly more mitochondrial debris was present in micrographs of specimens of 2- and 3-hr autolysis (Figs. 3 and 4) than was present in preparations of control mitochondria.

The Mg^{2+} and K^+ content of the mitochondrial fraction isolated from the tissue after 2 and 3 hr of autolysis was significantly decreased from control levels. The decreased Mg^{2+} concentration also was significant after 1 hr of autolysis. This decrease is based on the mitochondrial nitrogen, a substance which shows a much greater proportionate decrease at 1 hr than the decrease in either the Mg^{2+} or K^+ concentration.

On the other hand, the Ca^{2+} concentration was significantly increased in mitochondria isolated from tissue after autolysis for either 1 or 2 hr. The increase is of the order of 100 and 150%, respectively, (Fig. 5) but Ca^{2+} is not significantly increased in mitochondria isolated after 3 hr of autolysis. The order of magnitude of these increases is relatively small in absolute terms but may be large in the sense that there is very little Ca^{2+} in control mitochondria and only 0.42 mmoles of Ca^{2+} in 100 g of dry dog left ventricular

TABLE I. Myocardium Mitochondrial Electrolyte Content.

		Duration of autolysis (min)			
		0	60	120	180
(mmole/100 g of mitochondrial N_2)[a]	Ca	6.09 ± 0.73[b]	12.28 ± 1.55[c]	15.62 ± 2.60[d]	8.60 ± 2.79
	Mg	17.37 ± 0.74	13.23 ± 1.78[c]	11.02 ± 1.66[d]	8.64 ± 1.07[e]
	K	99.22 ± 6.38	72.31 ±10.80	31.53 ± 6.31[d]	25.01 ± 4.81[e]
(mg/g of wet wt)	N_2	0.9314± 0.0974	0.5019± 0.0645[d]	0.2673± 0.0274[e]	0.3673± 0.0274[f]

[a] Standard isolation procedures used throughout.

[b] Values shown are mean ± standard error.

[c] Significantly different from control (0 min) by paired *t* test [$p < 0.05$]; [d] [$p < 0.01$]; [e] [$p < 0.001$]; [f] [$p < 0.0005$].

tissue to serve as a source of Ca^{2+} in the mitochondria (13). It is of interest that following 2 hr of autolysis, mitochondria showed a 75% decrease in nitrogen and a 156% increase in Ca^{2+}.

Discussion. The results of these experiments show that the magnesium, potassium, and nitrogen contents of the mitochondria from myocardium are decreased, while the calcium content is increased after 2 hr of autolysis (Table I). The calcium level after 3 hr of autolysis was less than that observed at 2 hr and this latter increase was not significant. In these experiments, we are assuming that the average nitrogen content per individual mitochondrion remains constant during autolysis, thus allowing comparison of electrolyte contents of control and injured mitochondria. The isolation procedure is designed to isolate mitochondria or large mitochondrial fragments. Electron micrographs of the pellets demonstrate that this in fact is what has been isolated and that after 1 hr of autolysis in particular, there is a relatively large population of structurally intact mitochondria. Thus, our tentative conclusions about electrolyte changes seem correct, because the nitrogen value at 1 hr appears to be an accurate reference base against which we may assess the electrolyte changes in mitochondria of damaged vs control tissue.

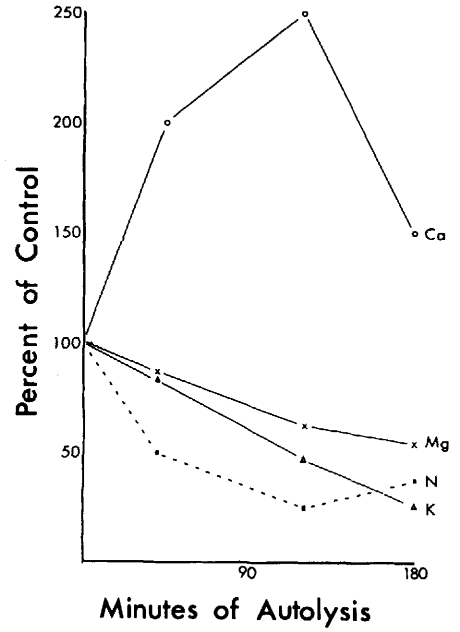

FIG. 5. Effect of autolysis on dog myocardial mitochondrial electrolyte and nitrogen content. Mitochondria were isolated in 0.25 *M* sucrose.

On the other hand, mitochondrial nitrogen may not be a good reference measure of the mitochondrial mass in tissue autolyzed for 120 min or longer. This is because the increased fragmentation of mitochondria may have resulted in a disproportionate change in the amount of nitrogen present per mitochondrion. For example, if there is nitrogen in the matrix space, and if the nitrogen of this space is held in it by the membranes, one would have good reason to suspect that mitochondria isolated from tissue subjected to two or more hours of autolysis in which there is a large proportion of fragments of cristae would have a different nitrogen content per unit than control mitochondria.

A measure of mitochondrial mass, which is partially independent of the mitochondrial nitrogen is mitochondrial phospholipid. This substance is the principal component of mitochondrial membranes and might represent a more stable point of reference for comparison of concentrations of various mitochondrial constituents as a function of time. Preliminary experiments with this reference base confirm the increase in mitochondrial calcium content after 2 hr of autolysis.

One of the original aims of these experiments was to relate the development of the intramitochondrial electron opaque dense bodies to the calcium and magnesium content of the mitochondria. We hypothesized that the location and structural characteristics of the granules might be associated with the accumulation of divalent cations. The results of the present experiment support but do not establish this hypothesis. The results also suggest that if the hypothesis is true, Mg^{2+} is unlikely to be the ion accumulated into the dense bodies, since mitochondrial Mg^{2+} decreases rather than increases at the time the dense bodies appear. On the other hand, the data suggest that the dense bodies may contain a significant amount of calcium. If calcium is present, it probably was accumulated by an active metabolic process, perhaps equivalent to that observed *in vitro* (4, 6).

Summary. The concentrations of Mg^{2+}, K^+ and nitrogen in mitochondria isolated from dog myocardium after 2 and 3 hr of autolysis at 37° were significantly decreased from the control while the mitochondrial Ca^{2+} concentration showed a small apparent increase after 1 and 2 but not 3 hr of autolysis. Intramitochondrial dense bodies were found in the mitochondria isolated from damaged myocardium at all time intervals, but an increasing proportion of large mitochondrial fragments were isolated from the damaged tissue as the duration of autolysis increased, suggesting that the decreased nitrogen yield per unit quantity of heart was the result of increased mitochondrial fragility. These experiments do not finally establish whether the mitochondrial granules contain significant quantities of Ca^{2+}, but do suggest that Mg^{2+} probably is not a significant constituent of the granules.

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