DOCUMENT RESUME

ED 107 634 SP 009 259

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TITLE Effects of Fixatives and Buffers upon the Morphology of Heart and Skeletal Muscle Mitochondria from Exhausted Rats.
NOTE 14p.

EDRS PRICE MF-$0.76 HC-$1.58 PLUS POSTAGE
DESCRIPTORS *Biochemistry; *Chemical Reactions; *Fatigue (Biology); Metabolism; Physiology; *Rats
IDENTIFIERS *Mitochondria

ABSTRACT

This study describes the effects of several fixatives and buffers on the morphology of mitochondria from resting and exhausted rats. Rats were run to exhaustion and adjacent portions from the left ventricle or from the soleus were treated with the following fixation procedures: (a) glutaraldehyde buffered with cacodylate, S-collidine, or phosphate and postfixed in osmium tetroxide; or (b) osmium tetroxide (as a primary fixative) buffered with cacodylate, veronal acetate, S-collidine, or phosphate or veronal acetate-Ringers with varying concentrations of Ca^{2+} ions (dicalcium ion with a plus-one charge). Mitochondria disruption was absent in all tissues prepared in buffered glutaraldehyde solutions. Mitochondria swelling, following exhaustive exercise, occurred in all of the buffered osmium solutions which did not contain Ca^{2+}. It has been found that the degree of mitochondria swelling following exhaustive exercise was inversely related to the concentration of Ca^{2+}. In this study, the presence of Ca^{2+} in fixatives for muscles from resting or only moderately exercised rats produced mitochondria swelling. (Figures describing fixative mixtures and their effects upon mitochondria are included.) (Author/JS)

EFFECTS OF FIXATIVES AND BUFFERS UPON THE MORPHOLOGY OF HEART AND SKELETAL MUSCLE MITOCHONDRIA FROM EXHAUSTED RATS

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Swelling and disruption of heart and skeletal muscle mitochondria, following exhaustive exercise, has been shown to be the result of certain in vitro fixation procedures. That these same fixation procedures did not produce morphological changes in mito. from resting animals suggests that exhaustive exerise causes mito. to become more susceptible to disruption in certain fixation media. The purpose of this study was to describe the effects of several fixatives and buffers on the morphology of mito. from resting and exhausted rats. Rats were run to exhaustion and adjacent portions from the left ventricle or from the soleus were treated with the following fixation procedures: Glutaraldehyde buffered with cacodylate, S-collidine, or phosphate and post-fixed in osmium tetroxide; or osmium tetroxide (as a primary fixative) buffered with cacodylate, veronal acetate, S-collidine, phosphate or veronal acetate-Ringers with varying concentrations of Ca^{2+} ions. Mito. disruption was absent in all tissues prepared in buffered glutaraldehyde solutions. Mito. swelling, following exhaustive exercise, occurred in all of the buffered osmium solutions which did not contain Ca^{2+}. It has been found that the degree of mito. swelling following exhaustive exercise was inversely related to the concentration of Ca^{2+}. Surprisingly, the presence of Ca^{2+} in fixatives for muscles from resting or only moderatly exercised rats produced mito. swelling.

In the past, several laboratories have reported that mitochondrial swelling and, in some cases, disruption of cristae have occurred in heart and/or skeletal muscle following exhaustive exercise.

I have attempted to study this phenomenon by running both trained and untrained rats to exhaustion at a wide range of intensity and duration combinations. I found that the mitochondrial swelling which had been reported earlier was the result of in vitro fixation artifact. While at rest the tissues appeared similar and well fixed, a striking contrast occurred between methods of fixation applied to adjacent sections of muscle from exhausted rats. While no mitochondrial swelling occurred in muscles fixed with cacodylate buffered glutaraldehyde, mitochondrial disruption, following exhaustive exercise, was always found in veronal acetate buffered-Os fixed tissues.

The conclusion that the mitochondrial disruption reported earlier did not occur in vivo, has been confirmed biochemically by Brooks, et al and also by Terjung, et al. Bowers et al in 1974 have published electron micorgraphs which also agree with my findings. However, these investigators did find, as I have, that mitochondria from muscles of exhausted animals are susceptible to an interaction with certain fixatives and an in vitro, artifactual swelling may occur. Terjung, et al, had also published a micrograph of intact mitochondria but reported that "other buffers and fixatives resulted in unsatisfactory preservation."

It seemed that further study of the interaction of fixatives and buffers with mitochondria from both resting and exhausted animals

might be useful in explaining the artifactual swelling and, more importantly, might be a tool for studying exhaustion associated changes in mitochondria.

(Figure 1) This figure describes the series of fixative and buffer combinations which were examined. Two primary fixatives, glutaraldehyde, a peptide cross-linking agent, were used. Tissues which were fixed in glutaraldehyde were always post-fixed in osmium. The various combinations of buffers with fixatives may be noted for both soleus and heart muscle. In each fixation the glutaraldehyde and osmium concentrations were held constant, 2 1/2% and 1% respectively. Several more common buffers were used. Veronal acetate with Ringers solution has been utilized to increase the osmolarity to an approximation of tissue tonicity, 340 mOsmols. The osmolarity of the other osmium fixatives were all hypo-osmotic, ca. 250 mOsm. Glutaraldehyde fixatives were all hyperosmotic, ca. 500 mOsm. The pH of each fixative was carefully adjusted to 7.38 to 7.42. (Figure 2) Although there were slight variations in the appearance of tissues prepared, the general effects are reported here. Regardless of the buffer, all of the osmium fixations following exhaustive exercise were characterized by mitochondrial swelling and loss of matrix material except when Ringer's was added to veronal acetate buffered osmium. (Figure 3) Exhaustive exercise did <u>not</u> result in mitochondrial disruption when Ringers was used. Note that the fixation of resting tissue had deteriorated slightly, however.

At this time it appeared that the fixation artifact was, then, the result of the use of hypotonic fixatives. However, one component of the Ringers, Ca^{2+}, deserved closer attention. Many studies have reported the interaction of Ca^{2+} and mitochondria, including the

identification of high and low affinity binding sites, reaction with phospholipids and proteins (components of mitochondrial membranes) and Ca^{2+} accumulation as matrix granules. Mitochondria have also recently been studied, especially in heart muscle, as an active regulator of Ca^{2+} concentration in the contractile cycle.

Wood and Luft (in 1965) in a classic study of the effects of various buffers upon fixation reported a specific ion effect which was greater than the effects of osmolarity alone; Ca^{2+} wasone of the ions which they noted. Terjung et al had added Ca^{2+} to the osmium fixation in which they found no swelling after exhaustive exercise.

Another series of experiments was planned. In this study adjacent sections of both soleus and heart muscle were prepared with four differently buffered osmium fixatives, veronal acetate, veronal acetate w/4mM Ca^{2+}, and veronal acetate with Ringers. The Ringers either did or did not contain 1mM Ca^{2+}. Ringers without Ca^{2+} was prepared by substituting Na^{+} to maintain iso-osmolarity. This is a composite of micrographs of adjacent sections of osmium fixed heart muscle from an exhausted rat. (A one micron line is in the margin.) The upper left hand corner is of tissue fixed in veronal acetate buffered osmium; in the upper right, veronal acetate modified by adding 4mM Ca^{2+}; in the lower left, veronal acetate with Ringers containing 1mM Ca^{2+}; and in the lower right veronal acetate with modified Ringers without Ca^{2+}. The mitochondria are disrupted, electron lucid and the cristae are absent in the veronal buffered osmium. The addition of 4mM Ca^{2+} to this fixative has prevented loss of cristae. Ringers with Ca^{2+} is characterized by good fixation and more matrix material. Removal of the Ca^{2+} from Ringers resulted in the apparent loss of cristae, but a moderal level of matrix material remained. It is obvious that the presence of Ca^{2+}

is an important factor in preventing fixation artifact. Both 4 mM and 1 mM Ca^{2+} concentrations were compared. Less disruption was noted with the greater divalent ion concentration.

However, a paradox existed. Ca^{2+} seemed to be preventing swelling in in vitro fixation, yet in vitro studies of tissue homogenates have described Ca^{2+} as a swelling agent. A very surprising result occurred when the tissues from a resting rat were fixed in the same ways. Mitochondrial swelling occurred in those fixatives which did contain Ca^{2+} but not in those which did not. These same fixations were used on tissues from a rat which was only exercised for a duration about half of that normally required to produce exhaustion. The presence of Ca^{2+} again seemed to be related to increased swelling but in a manner opposite that in exhausted tissue. The results of this series of Ca^{2+} concentration variations are summarized in the next slide (Figure 4). There were slight differences between heart and soleus muscle. This should not be viewed with surprise. Mitochondria vary greatly between species, organs, and even location in organs. It is likely that the response to exercise and/or fixation of these mitochondria should be different. (Figure 5) Note the reversal of the effect of Ca^{2+} between exhaustion and resting states, with both isotonic fixatives. (Figure 6) The same reversal of effect was observed in hypotonic fixatives. It appears that Ca^{2+} ions affect mitochondrial morphology more than osmolarity.

In summary, I would like to extrapolate from my data and describe its possible importance and several of the experiments which would be necessary for disproof of the working hypothesis. (Figure 7) From the data presented here and in many other studies, I believe that the Ca^{2+} has reacted differentially with proteins within the mitochondrial

membrane. Many further studies are necessary to substantiate this hypothesis and complete this line of investigation. This reaction is either specific to Ca^{2+} or may be duplicated with other divalent cations. Ca^{2+} or other cations are either bound or not bound; autoradiography and perhaps microprobe techniques may be used to answer this. If the ions are bound they may play a physiological role or may merely be bound to exposed anionic sites. Perhaps biochemical techniques may be used to examine this question. Whether the ions react directly with mitochondria or merely affect other constituents, either endogenous or erogenous, fixative components, must also be examined. At some point down the road we may know whether the response we see is one of in vivo importance or only another in vitro occurrence. In vitro occurrences may help us to better understand conformational changes in mitochondria. In vivo occurrences may not only help our understanding but may provide a tool for modification of exhaustion.

Figure 1

Fixatives

Buffers	Osmium	Glutaraldehyde
Cacodylate	S	S H
Collidine	S	S
Millonig's Phosphate	S	S
Veronal	S H	
Veronal w/Ringers	S H	

Soleus - S

Heart - H

Figure 2

Various Buffers

	Glutaraldehyde (hypertonic)		Osmium (hypotonic)
Rest	Mito. intact Electron dense		Mito. intact Electron dense
Exhaustion	Mito. intact Electron dense	← 1 →	Mito. swelling Electron lucid

Figure 3

Various Buffers

	Glutaraldehyde (hypertonic)	Osmium (hypotonic)	Osmium in Veronal w/Ringer's (ca. isotonic?)
Rest	Mito. intact Electron dense	Mito. intact Electron dense	Mito. intact (slightly swollen) Less Electron dense
Exhaustion	Mito. intact ← 1 → Electron dense	Mito. swelling ← 2 → Electron lucid	Mito. intact Electron dense

Figure 4

Veronal-Osmium Fixation. Variable Ca^{2+}

	Veronal	Veronal w/Ringers	Veronal w/Ringers w/o Ca^{2+}	Veronal 4 mM Ca^{2+}
Exhausted	+	0	+0	0+
1/2 Exhausted	0	0+ (H) 0 (S)	0	+
Resting	0	0+	0	+ (H) +0 (S)

S - Soleus

H - Heart

\+ Swelling

0 No swelling

Figure 5

Veronal-Osmium Fixation: Variable Ca^{2+}

	Veronal	Veronal w/Ringers	Veronal w/Ringers w/o Ca^{2+}	Veronal 4 mM Ca^{2+}
Exhausted	+	0	+0	0+
1/2 Exhausted	0	0+ (H) 0 (S)	0	+
Resting	0	0+	0	+ (H) +0 (S)

S - Soleus

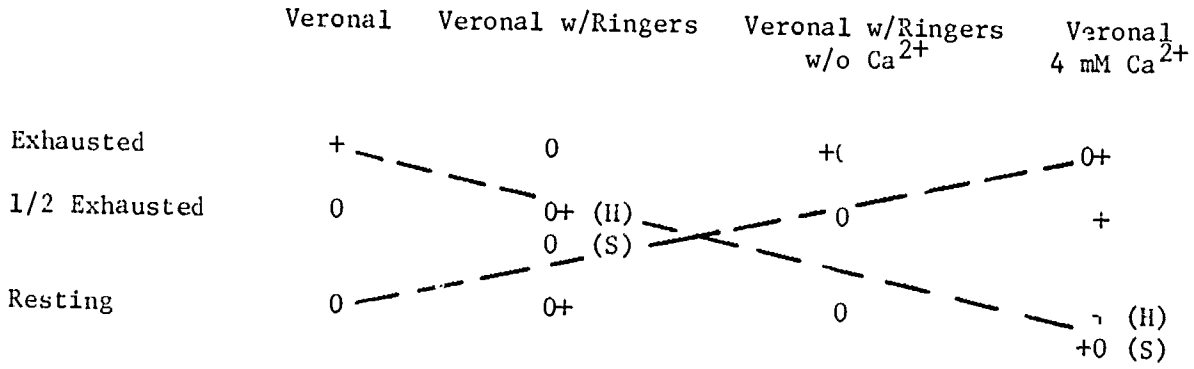
H - Heart

\+ Swelling

0 No swelling

Figure 6

Veronal-Osmium Fixation: Variable Ca^{2+}



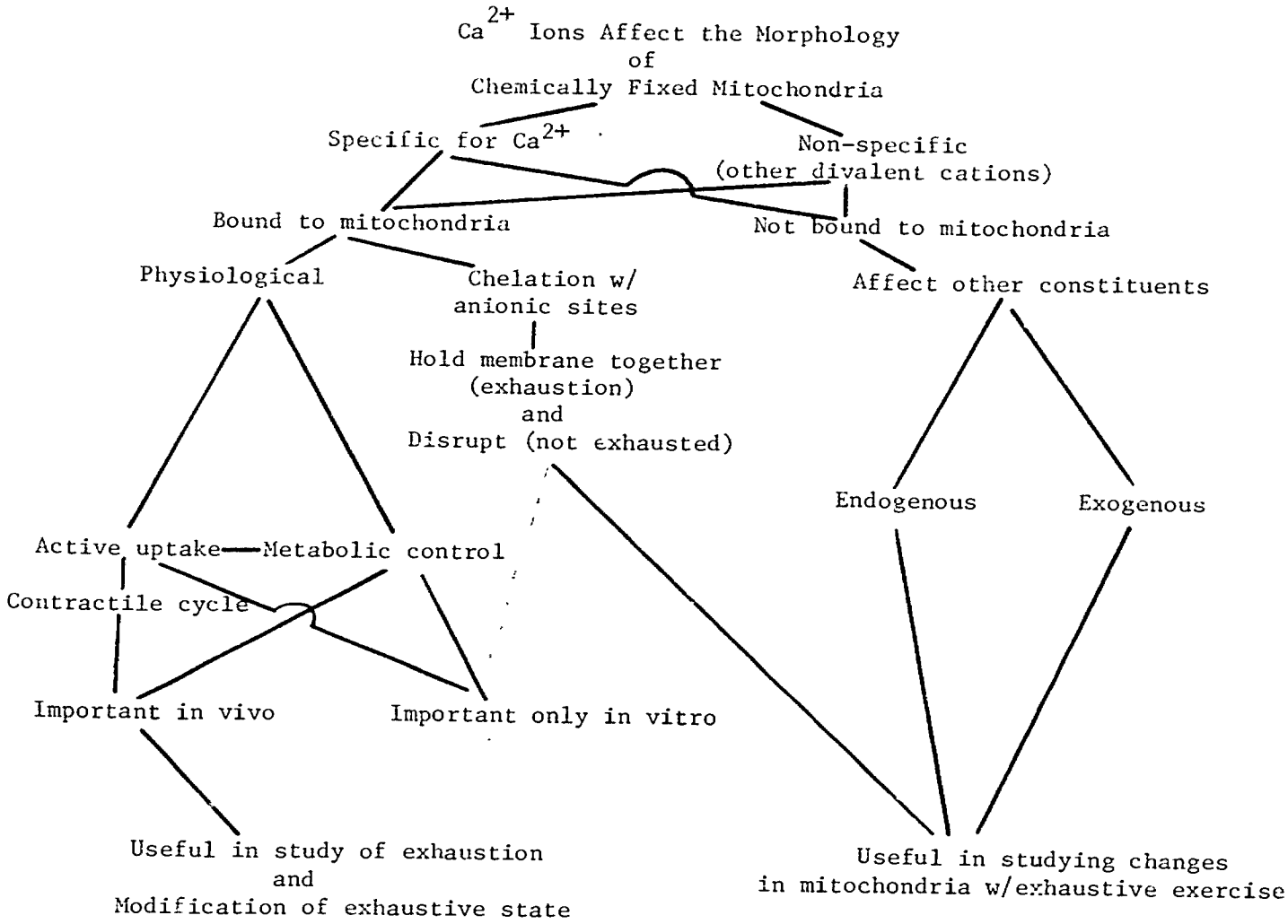
S - Soleus

H - Heart

\+ Swelling

0 No swelling

Figure 7


Ca^{2+} Ions Affect the Morphology of Chemically Fixed Mitochondria
Specific for Ca^{2+}
Non-specific (other divalent cations)
Bound to mitochondria
Not bound to mitochondria
Physiological
Chelation w/ anionic sites
Affect other constituents
Hold membrane together (exhaustion) and Disrupt (not exhausted)
Endogenous
Exogenous
Active uptake
Metabolic control
Contractile cycle
Important in vivo
Important only in vitro
Useful in study of exhaustion and Modification of exhaustive state
Useful in studying changes in mitochondria w/exhaustive exercise