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EFFECT OF HYPNORM, CHLORALOSANE AND PENTOBARBITAL ON THE ULTRASTRUCTURE OF THE INNER MEMBRANE OF RAT HEART MITOCHONDRIA

R. HERTSENS [a], W. JACOB [a] and A. VAN BOGAERT [b]

[a] *Department of Medicine, University of Antwerp, U.I.A., B-2610 Wilrijk, and* [b] *Foundation Group Comte de Launoit, Laboratory of Physiology, University of Antwerp, R.U.C.A., B-2020 Antwerp (Belgium)*

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Rat heart mitochondria were isolated from four groups of animals treated in a different way. The animals of the first group were killed after decapitation (D-group) without previous anaesthesia. The three other groups of animals were anaesthetised with different anaesthetics. The second group (N-group) was anaesthetised with nembutal (sodium pentobarbital), the third group with chloralosane (C-group) and the fourth group with hypnorm (H-group). From these three anaesthetics only nembutal is known to interact with mitochondria. After retrograde perfusion and excision of the heart, mitochondria were prepared from the ventricles by standard methods. After freeze-fracturing the mitochondrial suspension, the intramembrane particle dimension and density on both fracture faces of the inner mitochondrial membrane were measured. The intramembrane particle diameter on the P-face of the inner membrane of the N-group mitochondria was significantly different from D-, C- and H-group mitochondria. Also the density and diameter of the intramembrane particles on the mitochondrial inner membrane of D-group mitochondria compared to C- and H-group mitochondria were significantly different at the 95% level of confidence. Between C- and H-group mitochondria no differences of these parameters were observed. From these results it is clear that, depending on the pretreatment of the animals, a different substructure of the inner membrane of heart mitochondria is obtained.

Introduction

Isolation of mitochondria is necessary to study the energy metabolism of the heart. Criteria of functional intactness were developed and the influence of isolation techniques was examined [1,2]. Also the influence of the pretreatment of the animals was considered [3]. It was stressed that anaesthesia did not affect oxygen metabolism and regulation of the mitochondrion, nor did it change the structural integrity of the organels. However, from other experiments [4–7] it is known that anaesthetics do have an influence on the mitochondrial metabolism. The transport of pyruvate across the inner membrane is altered [6]. Even when no anaesthetics were used before killing the animals, changes in the integrity of rat heart mitochondria were proven [3,8]. From these investigations it became clear that the mitochondrion is an important index for the action of anaesthetics [5,6]. Several investigators have also shown that local as well as general anaesthetics interact with membranes. An excellent review on membrane action of anaesthetics is given by Seeman [9]. Concerning the membrane action of anaesthetics two hypothesises were developed. The first deals with the interaction of the anaesthetic with the membrane phospholipids. Studies were

done with several techniques including NMR [10–12], Raman scattering [13] and spin labeling studies [14,15]. The anaesthetic caused a loosening of the components of the membrane. There was a disordering or fluidization in the membrane which could be measured with a fluorescent hydrophobic probe, (ANS, 8-anilinonaphthalene-1-sulphonic acid). These experiments were mostly done on erythrocyte membranes [16–18]. The second hypothesis concerns the action of the anaesthetic through interaction with a protein. Early studies [19–22] reported the binding of anaesthetics with pure protein solutions. Later studies [10,11,23–30], used biological membranes where the action of the anaesthetic was measured on a specific protein such as mitochondrial F_1 ATPase [29], cytochrome *c* oxidase [10,11,28] and firefly luciferin and luciferase system [27,28]. Franks et al. [25,26] investigated the action of the anaesthetics with X-ray and neutron diffraction experiments. At clinical concentrations of anaesthetics they could not detect any change in the lipid bilayers. The interaction of the anaesthetic would occur with specialized regions of the membrane by interposition between the subunits of a multisubunit protein interfering with its function.

We have chosen three anaesthetics with a different chemical structure and a different mechanism of action.

Nembutal is a frequently used anaesthetic in animal surgery. It has similar actions as amytal which is a potent inhibitor of the electron transport chain in mitochondria. The barbiturates have a high fat solubility and penetrate easily the cell membranes. It effects a general depression of the central nerve system with a respiration depression and a depression on the arteriolar musculature and the myocardium. It has no analgesic effect. Chloralose (chloralosane) has a chloral-hydrate derived structure. It excerts a longlasting depression on the central nerve system. Chloralose is broken down in the body to chloral and glucose. In the blood chloral is reduced to trichloroethanol which is taken up by the central nerve system and excerts the hypnotic effect. There is no action on blood pressure, no myocardial or respiratory effect. As third anaesthetic we have taken hypnorm which is a neuroleptanalgesic from the morphine type. After subcutaneous injection there is a blood pressure fall which results in a bradycardia. The anaesthetic also gives rise to a respiratory depression.

Considering the interaction of anaesthetics with proteins we have done a freeze-fracture investigation on the mitochondrial inner membrane. This technique has the advantage that membrane alterations can be detected in a simple way, especially in the mitochondrial inner membrane [31–33]. The aim of the present study was to determine possible alterations of the mitochondrial inner membrane ultrastructure which arise from anaesthetic action in the animal.

Morphological measurements were done to know if there was any correlation between particle densities and the overall size of the mitochondria.

Materials and Methods

Forty female Wistar rats (250–300 g body weight) were used in this study. Before use they were fed ad libitum.

Twenty animals in group D were decapitated without any previous anaesthesia. Group N consisted of ten animals which were injected intraperitoneally with nembutal (pentobarbital 80 mg/kg body weight, Abbott – 60 mg/ml stock solution). In group C, four animals were anaesthetised with chloralose (chloralosane 100 mg/kg body weight, BDH-Chemicals) injected in the tail vein. Six animals in group H were anaesthetised by subcutaneous injection of hypnorm (0.5 ml/kg body weight, Duphar).

Except for group D the hearts were stopped by heartwards perfusion with a thyrode solution containing 137 mM NaCl/0.5 mM $MgCl_2 \cdot 6H_2O$/0.36 mM $NaH_2PO_4 \cdot H_2O$/11.9 mM $NaHCO_3$/137 mM KCl/0.1 mM EDTA/3 mM saccharose adjusted to pH 7.4 with 0.1 M KOH.

The state of anaesthesia was checked on the degree of leg retrieval. After 10 min this reflex had totally disappeared. To perfuse the heart the abdominal aorta was cut free and a plastic cannula was inserted heartwards between the arteria femoralis and the arteria renalis. The perfusion was done at a pressure of 140 mmHg with ice-cold thyrode (4°C).

The high potassium concentration caused a quick stop of the heart contraction within 10 s.

After perfusion or after decapitation the hearts

were washed several times with homogenisation medium which had the same composition as the perfusion solution except for the saccharose concentration (250 mM instead of 3 mM). Mitochondria were prepared by standard methods [2]. The mitochondrial pellet was freeze-fractured by standard techniques ($-150°C$, 10^{-6} Torr) using a Balzers BAE 121 coating unit [34,35]. No cryoprotectants were used because of the danger to produce artefacts [36–40]. The fracture faces were shadowed with 30 Å Pt-C and replicated with a 200 Å carbon layer. The thickness was measured with a swinging quartz thickness meter. After floating off the replica's on cold bleach, they were cleaned on 6 M NaOH solution at 60°C, rinsed several times with water and caught on 300 mesh copper grids [41].

Pictures were taken with a Jeol 100B electron microscope at a magnification of 100000 times. The measurements were done on prints at a final magnification of 280000 times with a Videoplan equipment (Kontron).

The diameter of the intramembrane particles was measured as the width of the shadow cap perpendicular on the direction of shadowing. At least 400 particles were measured for each fracture face from 20 to 30 different fracture planes and that for each animal that was sacrified. The measurements were done in areas with no curvature so that errors from this side were excluded. The large number of different fracture planes together with double-blind measuring guaranteed the statistical demands.

After preparing the various mitochondrial suspensions they were taken up in equal volumes of homogenisation buffer (pH 7.4) and centrifuged for 1.5 min with an Eppendorf microcentrifuge. The mitochondrial pellet was then fixed for 1 h with 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) at 4°C, postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.4) at 4°C and then dehydrated in ethanol before being embedded in Epon. Thin sections of the various mitochondrial preparations were cut with a diamond knife and stained with 3% uranyl acetate for 1 min and 1% lead citrate for 30 s before examination in the electron microscope.

Pictures were taken at a magnification of 10000 times. The morphological measurements were done on prints at a final magnification of 28000 times. To determine the surface density of the inner mitochondrial membrane per unit mitochondrial volume the technique of Weibel and Bolender [42] was used.

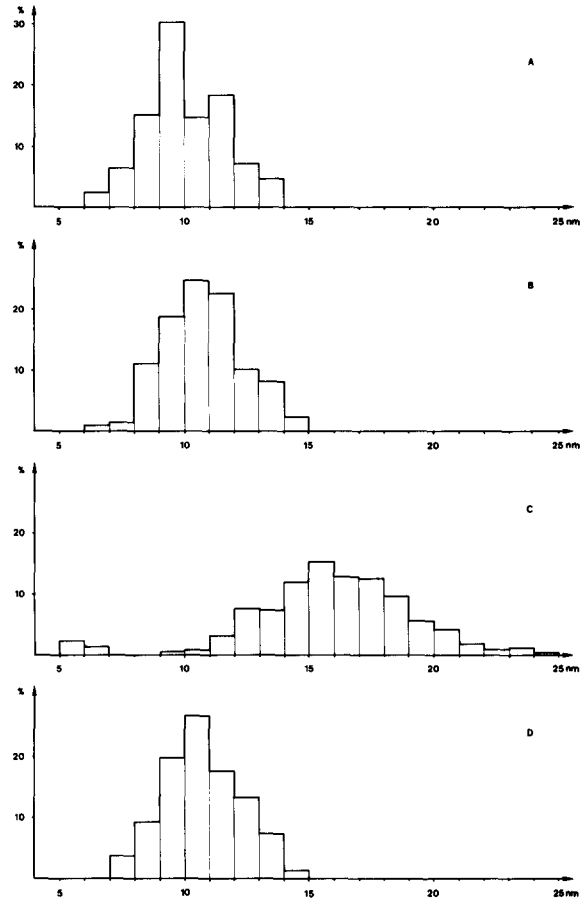

Fig. 1. Histogram of the distributions of the intramembrane particles on the P-face of the inner mitochondrial membrane. (A) A typical distribution after decapitation of the animal. 500 particles were measured. The whole distribution is shifted to low particle diameter (6 nm to 14 nm, with a mean value of 10 nm) due to the massive release of catecholamines in the blood. (B) A distribution after anaesthetising the animals with chloralosane (100 mg/kg). Again, 500 particles were measured. The distribution is also shifted to low particle diameter (6 nm to 15 nm, with a mean value of 10.79 nm). (C) A typical distribution after nembutal anaesthesia (80 mg/kg). The whole distribution is shifted to higher values (5 nm to 25 nm with a mean value of 15.8 nm). In this case, 498 particles were measured. (D) The distribution of the diameter of 644 particles after hypnorm anaesthesia (0.5 ml/kg). Again, the distribution is concentrated around 10 nm starting from 7 nm to 16 nm.

Results

Throughout this work the following terminology is used; the P-face represents the hydrophobic part of the inner mitochondrial membrane situated nearest to the matrix, the E-face is the other part of the hydrophobic side of the membrane pointing to the intermembrane space [43].

Depending upon the treatment of the animal, different results in particle size and distribution on the P- and E-face are observed. The histogrammic distributions of the IMP diameter are shown in Figs. 1 and 2. Fig. 1 represents the particles observed on the P-face scored in different dimension classes, whereas Fig. 2 represents those on the E-face. There is a remarkable influence of nembutal on the diameter and density of the intramembrane particles on the P-face of the inner membrane.

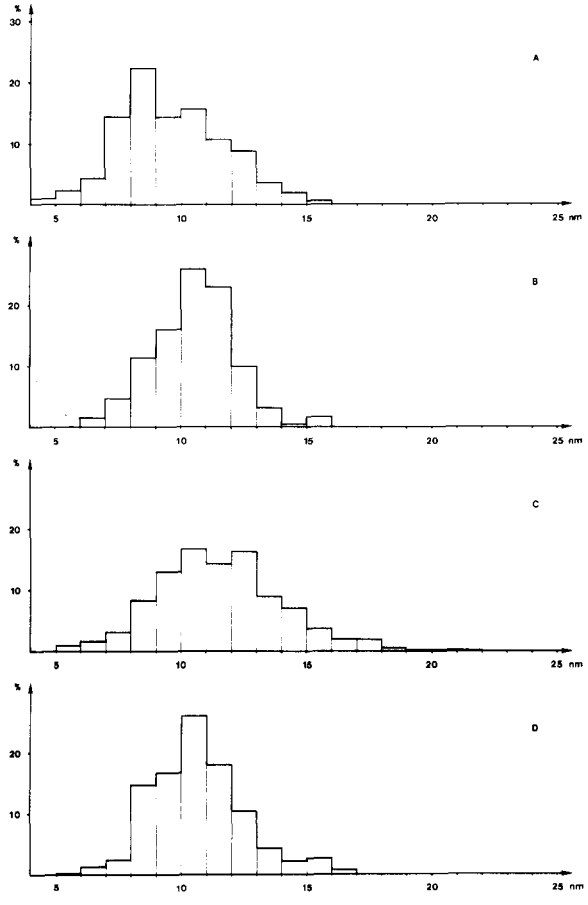

Fig. 2. Histogram of the distributions of the diameter of the intramembrane particles of the E-face of the inner mitochondrial membrane. (A) Decapitation, (B) chloralosane anaesthesia (100 mg/kg); (C) nembutal anaesthesia (80 mg/kg) and (D) hypnorm anaesthesia. All the particles are in the same range of diameter (4 nm to 19 nm, with mean values of 10.5 nm). Only after nembutal anaesthesia (C) larger particles are seen (up to 20 nm) which causes a mean value which is slightly bigger (11.5 nm). The distributions are plotted after counting 445 particles in (A), 407 in (B), 590 in (C) and 403 in (D).

The dimension of the intramembrane particles on the inner membrane of N-mitochondria is significantly greater than those from D- and C- and H-mitochondria.

On the E-face the difference in mean diameter and density is not so big although significant differences exist at the 95% level of confidence. The mean diameter and mean density of the intramembrane particles are presented in Table I.

Figs. 3 and 4 represent P- and E-faces of the inner mitochondrial membrane of N- and C-mitochondria. From these pictures one can easily see the differences in dimension and density of the intramembrane particles by the naked eye.

The surface density of the inner mitochondrial membrane per unit mitochondrial volume is significantly smaller after nembutal anaesthesia (17.43 μm^{-1}) compared to decapitation (23.22 μm^{-1}), ($P < 0.005$). Between the surface density after nembutal anaesthesia and the surface density's after chloralosane anaesthesia (21.17 μm^{-1}) or hypnorm anaesthesia (20.67 μm^{-1}) there is a significant difference at the 95% level of confidence. There is no significant difference between the surface densities after chloralosane and hypnorm anaesthesia.

Discussion

Among the three chosen anaesthetics, pentobarbital is frequently used to anaesthetise laboratory animals prior to isolation of different tissues and organs. Therefore, it is essential to know whether and to what extend it does influence the functional integrity of the organ or the subcellular fraction studied. We have studied the influence of the anaesthetics used, on heart mitochondria. As is known, mitochondria are an important index for action of anaesthetics [3,5,6]. Using the heart, we could easily follow the action of the anaesthetic on the contractility and blood pressure [44–46].

At levels far in excess of those required to

TABLE I

MEAN DIAMETER AND PARTICLE DENSITY OF THE INTRAMEMBRANE PARTICLES ON THE E- AND P-FACE OF THE INNER MEMBRANE OF HEART MITOCHONDRIA AFTER DIFFERENT PRETREATMENTS OF THE ANIMAL

The diameter of the particles was measured perpendicular on the direction of shadowing. From each animal 20 to 30 different fracture planes were examined so that the values in this table are means of 2000 particles. The mean density was obtained after counting the number of particles in 400 squares of 1 μm^2 with respect to the magnification of the electron microscopical picture. The measurements were done double-blind. The figures are presented as means ± S.D.

Treatment	P-face		E-face	
	Diameter (nm)	Density (μm^{-2})	Diameter (nm)	Density (μm^{-2})
Decapitated (D)	10.0 ± 1.5	3360 ± 20	9.7 ± 2.1	1890 ± 20
Chloralosane (C)	10.8 ± 1.6	2520 ± 20	10.5 ± 1.6	2053 ± 20
Nembutal (N)	15.8 ± 3.2	2370 ± 20	11.5 ± 2.5	1070 ± 50
Hypnorm (H)	10.7 ± 1.6	2660 ± 20	10.5 ± 1.8	1870 ± 20

produce clinical anaesthesia, Pumphrey [47,48] showed polarographically that barbiturates inhibit oxidation of NAD-linked substrates almost completely. However, from our results it is clear that even at levels of clinical anaesthesia there is an influence on the mitochondrion.

In a previous study [49] we have shown that the diameter of the intramembrane particles on the P-face of the inner membrane of heart mitochondria varies in a concentration-dependent manner in respect to the amytal concentration. With pentobarbital as anaesthetic the intramembrane particle diameter enlarges on the P-face as it is seen in Table I and Fig. 1. This enlargement together with the lowering of the particle density is in agreement with the inhibition of the electron transport in vivo and in vitro with 1.8 mM amytal after chloralosane anaesthesia [50].

Since the histogram of the distribution of the intramembrane particles on the P-face also shows particles with a large diameter, we must conclude on purely morphological considerations that pentobarbital anaesthesia affects the electron transport in rat heart mitochondria in the same way. This is in agreement with other studies [4–7]. On the other hand, no such influence of pentobarbital is seen when P/O ratio or respiratory control are measured [3]. The different experimental conditions most probably explain this discrepancy. Indeed, polarographic measurements are performed on mitochondrial suspension after more or less long equilibration periods in reaction media during which membrane protein rearrangements can take place. In this study, however, the mitochondrial suspensions were at no time exposed to metabolisable substrates, nor were they allowed to warm up. On the contrary, due to the extreme rapidity of cryofixation, intramembrane protein rearrangements are excluded. Because of the standardized isolation technique only the different way of anaesthesia could have influenced the intramembrane protein organization. Since the barbituratic effect is concentration dependent [49], it is conceivable that at clinical concentrations the alterations are too small to be detected by macroscopic P/O-ratio determination. Alternatively, due to the modest interference of pentobarbital with the electron transport [47,48] the organisational changes of the inner mitochondrial membrane may be reversed under incubation conditions of P/O-ratio measurements. Since the commonly used isolation techniques were developed to guarantee membrane integrity by carefully adjusting ionic environment and osmotic pressure, the morphometric characterisation after freeze-fracturing, truely reflects the momentanous metabolic state of the mitochondrion.

The action of chloralosane and hypnorm on the heart is more or less the same. So the intramembrane particles should have equal dimensions, which is seen on both P- and E-face. There is a small rise in heart rate [51] which can be the

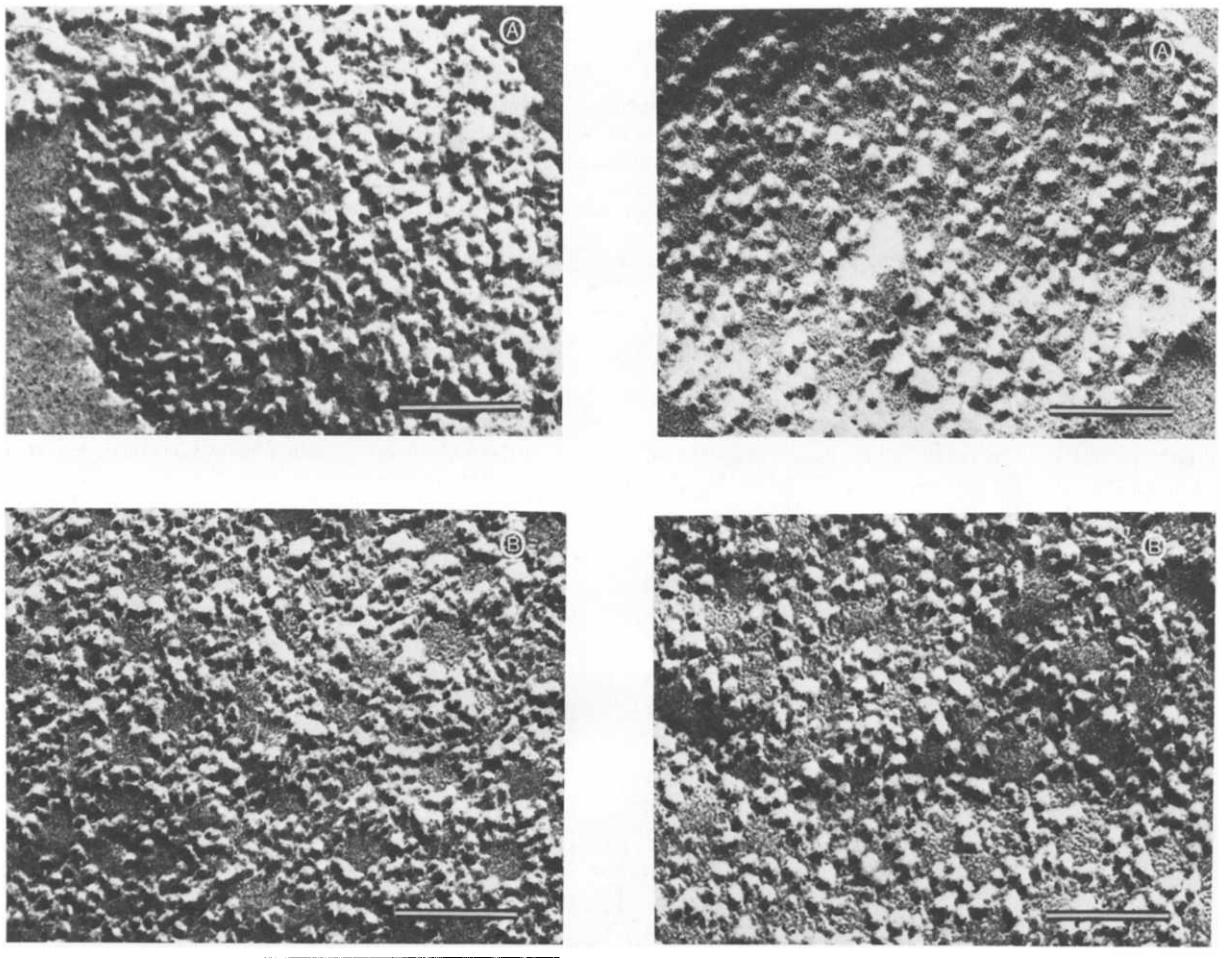

Fig. 3. Freeze-fracture faces of mitochondrial inner membrane: (A) the P-face after nembutal anaesthesia (80 mg/kg); (B) the P-face after chloralosane anaesthesia (100 mg/kg). After isolation, freezing (at −210°C in nitrogen sluch) and freeze-fracturing (−150°C, 10^{-6} torr) pictures were taken with a Jeol 100 B. Note the larger particles after nembutal anaesthesia (A) (Bar 0.1 μm). (Left).

Fig. 4. Freeze-fracture faces of mitochondrial inner membrane: (A) the E-face after nembutal anaesthesia (80 mg/kg), (B) the E-face after chloralosane anaesthesia (100 mg/kg). After isolation of the mitochondria, they were quickly frozen in nitrogen sluch at −210°C. No cryoprotectants were used. All particles are more or less of the same size (bar 0.1 μm). (Right-hand column).

result of the release of noradrenalin in the blood by manipulation of the animal [3,52]. This is confirmed by the decapitation experiment. It is well documented [3] that this way of killing provokes a massive release of catecholamines in the blood stream. The effect of catecholamines on the cardiac metabolism is well known [53,55]. The energy metabolism is highly stimulated. Even so earlier rexperiments in our laboratory [50,56] indicate that the injection of noradrenalin (50 μg/kg) yields small diameters of intramembrane particles on the P-face of the inner mitochondrial membrane. Consequently, the small diameter of the intramembrane particles on the P-face of the inner membrane of D-, C- and H-group mitochondria is due to the release of catecholamines in the blood which in turn stimulates the energy metabolism. Why the diameter of the intramembrane particles on the E-face of the inner membrane stays in the same range is not yet clear.

The freeze-fracture technique gives also an idea of the particle density in the fracture plane of the

inner mitochondrial membrane. Migration of proteins from one or from both sides of the membrane to the hydrophobic part of the membrane or vice-versa, can alter the particle density [57]. Since chloralosane and hypnorm have no, or very little influence [51] on the heart or on the mitochondrial metabolism the particle density and the surface density of the inner mitochondrial membrane may be considered as reference values, to evaluate changes induced by anaesthetics having a definite influence on heart metabolism, e.g., nembutal. We are aware of the fact that the best control for these experiments should be killing the animals by potassium injection inducing a quick stop of the heart. However, we do not see how this can be done practically. If one tries to kill the animals in this way without any anaesthetic given, the animals will be stressed in such a way that one cannot speak of a control either by the massive release of catecholamines. The particle density after nembutal anaesthesia decreases significantly ($2370/\mu m^2$ versus $2590/\mu m^2$). The fact that both particle density and surface density decrease after nembutal anaesthesia implicates that there is a loss of proteineous material at least out of the fracture plane. Indeed, membrane proteins can migrate vertically in the membrane [57]. Whether these intramembrane particles (proteins?) stay in the membrane as surface proteins or leave the membrane is not known at the moment. Also an association phenomenon of smaller particles has to be considered as a possible explanation for the lowered practicle density.

Increase of the surface density per unit mitochondrial volume, together with the increase of the intramembrane particle density in the case of decapitation, could be explained as the reverse phenomenon. Again, it is not known whether these particles migrate from the surface of the membrane to the centre or that the extra material is imported in the membrane from matrix or intermembrane space or originate from dissociation of larger clusters.

In conclusion, depending on the pretreatment of the animal, the ultrastructure of the inner membrane of heart mitochondria changes.

Moreover, it also follows that pentobarbital anaesthesia, although it antagonizes the stress reaction, is not the best choice for anaesthetising purposes in experiments designed to study mitochondrial metabolism.

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