Virchows Arch. A Path. Anat. and Histol. 379, 219–228 (1978)

Virchows Archiv A

An Effective Morphometric Method for Electron Microscopic Studies on Papillary Muscles

G. Mall, H. Reinhard, K. Kayser and J.A. Rossner

Pathologisches Institut der Universität Heidelberg (Direktor: Prof. Dr. W. Doerr)

Summary. Morphometry was performed on the left ventricular posterior papillary muscles of seven Wistar rats. The volume densities of myocardial cells, interstitial space, myocardial nuclei, sarcoplasm, mitochondria, myofibrils, ground substance and *T* tubules, and the surface densities of myocardial cells, mitochondrial membranes and *T* tubules, were calculated. Though only 1 ultrathin section per animal was evaluated the low standard errors of the means indicate that the method described here will be adequate in most experimental studies. Due to the anisotropy of the surfaces within myocardial cells, the papillary muscles were cut at an angle of 32.4° to their longitudinal axis. This angle is derived from an equation published by Whitehouse (1974). The procedure to correct the loss of cristal membrane images from oblique sectioning is discussed.

Key words: Morphometry – Myocardial fibres – Papillary muscles.

Introduction

Heart muscle tissue is composed of geometrically anisotropic tissue elements which do not have random orientation in space. The measurement and calculation of volume densities by point counting following Delesse's principle, is not influenced by the anisotropy (Weibel, 1969). On the other hand surface density S_V (surface area/volume) depends not only upon the profile border length per unit area of the test plane, B_A (length/area), but also upon the surface orientation in space and the relative orientation of the test planes (constant A):

$$AS_V = B_A \tag{1}$$

In the case of randomly oriented surfaces the value of A is $\frac{\pi}{4}$ (Saltykov, 1958;

Send offprint requests to: Dr. med. Gerhard Mall, Pathologisches Institut der Universität Heidelberg, Im Neuenheimer Feld 220/221, Postfach 104340, D-6900 Heidelberg 1, Federal Republic of Germany

0340-1227/78/0379/0219/$02.00

Whitehouse, 1974). The methods for calculating surface densities of heart muscle tissue components can be characterized as follows:

1. Random Orientation of the Section Planes in Relation to the Muscle Fibre Axis

Laguens (1971), Anversa et al. (1971), Reith and Fuchs (1973), Herbener et al. (1973), and Tate and Herbener (1976) removed heart muscle tissue samples and embedded them without regard to their fibre axis orientation. Therefore in this system it may be assumed that every tissue element which is to be cut is equally likely to be in any possible orientation in space, independent of its relationship to the fibre axis. Under these conditions $A=\frac{\pi}{4}$ may be correct.

However, this procedure has the disadvantage that if we consider a single, randomly cut, ultrathin section, we see in most cases that the anisotropic surfaces contained in this section are not adequately characterized by $A=\frac{\pi}{4}$. This value can only be determined from the average of a large number of sections. Thus B_A is influenced not only by the biological variability of surface densities in a tissue but also by an artificial variability of A. Therefore, the standard deviation of a B_A value tends to be greater than the variability caused exclusively by the biological variation. Even statistical tests presupposing normally distributed values are problematical.

2. Constant Angles between Section Planes and Muscle Fibre Axis

McCallister and Page (1973), Datta and Silver (1975) and Smith and Page (1976) took measurements at constant section angles. In cross sections the surface densities of T tubules, sarcolemma, sarcoplasmic reticulum, and outer mitochondrial membranes were evaluated and calculated from the well known relation $S_V=B_A$ (Sitte, 1967; $A=1$) presupposing that all the surfaces were normal to the section plane. Sections containing nuclear profiles or intercalated discs were excluded from the analysis. The inner mitochondrial membranes together with cristae were measured in longitudinal sections and calculated by $\frac{\pi}{4}S_V=B_A$.

Even this method has some disadvantages:

a) The positioning of the sections with respect to the fibre axes is technically laborious.

b) The geometric model of surfaces running parallel to the fibre axis may be too simple in the case of outer mitochondrial membranes and T tubules.

c) Because of the periodically arranged intercalated discs some test areas may contain only intercalated discs which have to be excluded from the analysis.

Hence, Weibel (1972) recommends the use of oblique sections for measuring sarcoplasmic reticulum in skeletal muscle cells.

The aim of this investigation is to establish a morphometric method which is reliable in experimental pathology and efficient. We modified method 2, avoiding the disadvantages discussed above.

Materials and Methods

a) Choice of Sections

Papillary muscles were analyzed because of the well known orientation of their muscle fibres.

b) Geometrical Model of Surfaces

In performing an experimental study it is not necessary to know the exact value of A, if we suppose A is not influenced by the experimental conditions. In this case the systematic error would be the same in both the control and the experimental group. McCallister and Page (1973) assumed the outer mitochondrial membranes were parallel to the fibre axis. Apart from the fact that only a part of interfibrillar mitochondria is characterized adequately by this assumption it is obvious that mitochondrial shape changes under experimental conditions, for example: mitochondrial swelling and rounding off. In a recently published study (Mall et al., 1977) we assumed two mitochondrial surface classes, one parallel to the fibre axis and the other isotropic. From cross sections of left ventricular rabbit papillary muscles we derived a value of $0.86\ S_V = B_A$ whereas in longitudinal sections a figure of $0.73\ S_V = B_A$ was obtained. However, this method demands analysis of the relative frequencies of the two surface classes. Whitehouse (1974) published a formula for calculating A which is applicable to surfaces derivable from isotropic surfaces by linear expansion:

$$A(\alpha) = \frac{E(\varepsilon \sin \alpha)}{\frac{b}{a} + \frac{\sin^{-1}\varepsilon}{\varepsilon}} \tag{2}$$

where a = semi-major axis of a prolate spheroid,
b = semi-minor axis of a prolate spheroid,
ε = eccentricity ($\varepsilon = (a^2 - b^2)^{1/2}/a$),
α = angle between semi-minor axis and test plane,
E = complete elliptic integral.

For surfaces running parallel to an axis we derive $b/a = 0$, as the spheroid tends to become a cylinder ($\varepsilon = 1$). From (2) follows:

$$A(\alpha) = \frac{E(\sin \alpha)}{\frac{\pi}{2}}. \tag{3}$$

In cross sections the solution of (3) is the upper limit ($\alpha = 0°$, $A = 1$), in longitudinal sections the lower limit ($\alpha = 90°$, $A = \frac{2}{\pi}$). $\frac{\pi}{4}$, the constant for isotropic surfaces, is between these limits. Therefore, we can derive α from (3) under the condition $A \to \frac{\pi}{4}$:

$$\frac{\pi}{4} = \frac{E(\sin \alpha)}{\frac{\pi}{2}}$$

$$\alpha \approx 57.6°.$$

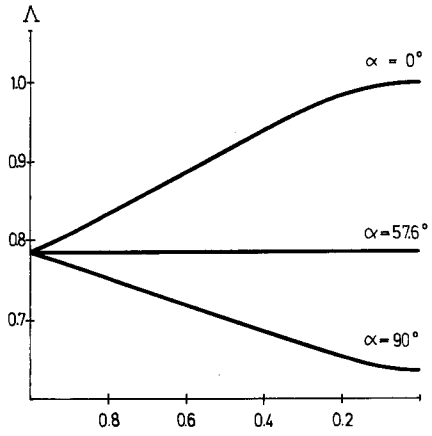

Fig. 1. If $\alpha = 57.6°$ Λ is independent on b/a

This implies that heart muscle surfaces cut at an angle of 32.4° to the fibre axis have to be calculated in both instance (isotropic and anisotropic) by the equation $\frac{\pi}{4} S_V = B_A$. Beyond this Figure 1 demonstrates the important fact that Λ is not affected by b/a if $\alpha = 57.6°$. We conclude, therefore, that not only isotropic surfaces and surfaces running parallel to an axis but also each possible anisotropic surface orientation derivable from isotropic surfaces by linear expansion may be calculated from $\frac{\pi}{4} S_V = B_A$ if $\alpha = 57.6°$. This general geometrical model characterizes all classes of surfaces which have the same orientation in space as a sphere (isotropy) or as any possible prolate spheroid (anisotropy) or as a cylinder (anisotropy). It is evident that this model is adequate for most surfaces contained in biological tissues. In heart muscle our model can be used for surfaces having a preferential orientation to the fibre axis such as outer mitochondrial membranes, sarcolemma, sarcoplasmic reticulum, and that small part of the T tubules running parallel to the fibre axis. Even for the mitochondrial cristae, which may have a random orientation in space, it is acceptable. However, in practise the model is not adequate for the sarcoplasmic reticulum because of the loss of membrane images, resulting from oblique sectioning. In this case the angle between fibre axis and section plane should be considerably greater than 32.4° (say 60°).

In general the model is not valid for T tubules, since most of their axes are randomly distributed in cross section planes of the muscle cells. From this, and from the assumption that the shape of T tubules may resemble cylinders (cf. Fawcett and McNutt, 1969) and from (3) we derive:

$$\Lambda(\vartheta) = \frac{4}{\pi^2} \int_0^{\frac{\pi}{2}} E\left(\sqrt{\frac{tg^2\alpha' + tg^2\vartheta}{1 + tg^2\alpha' + tg^2\vartheta}}\right) d\alpha', \tag{4}$$

where α' is the variable angle of T tubule axes to a standard section plane longitudinal to the fibre axis and ϑ is the angle between muscle fibre axis and the real section plane, in our case 32.4°. For $\vartheta = 90°$ (cross sections) we derive $\Lambda = \frac{2}{\pi}$, naturally the solution of (3) for longitudinally cut cylinders. From (4) and $\vartheta = 32.4°$ we derive $\Lambda = 0.805$. The difference from $\frac{\pi}{4}$ is smaller than 3 ‰, so we conclude that in practise even the surface of T tubules is reasonably calculated from $\Lambda = \frac{\pi}{4}$.

c) Longitudinal Periodicity

The periodicity of intercalated discs is not considered since we analyze oblique sections.

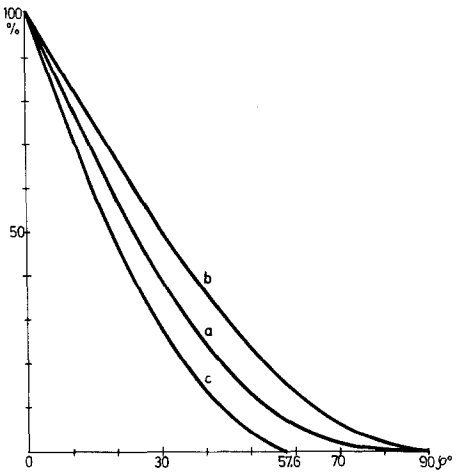

Fig. 2. Theoretical loss of membranes (%) depending on φ (Mall et al., 1977). Curve a: Isotropic surfaces, $\left(1-\frac{\sin 2\varphi+2\varphi}{\pi}\right)\cdot 100\,\%$. Curve b: Surfaces running parallel to an axis cut longitudinally to this axis, $(1-\sin\varphi)\cdot 100\,\%$ (cf. for derivation of (a) and (b) Mall et al., 1977). Curve c: Surfaces running parallel to an axis cut at an angle of 32.4° to this axis (graphical derivation, unpublished method)

The loss of cristal membrane images from oblique sectioning can be considered in the following way:

The morphometry of cristal membrane surfaces is influenced by a systematic error resulting from the loss of obliquely cut membranes in electron microscopy (Loud, 1967). Reith (1977) pointed out the varied results published by several authors. We use a correction method similar to the method described by McCallister and Page (1973) (Mall et al., 1977) and our procedure is identical with that published recently by Reith (1977). We count the number of intersections between test lines and mitochondrial section profile border lines (I_{tot}), and also the number of intersections between test lines and the traces of inner mitochondrial envelope membranes (I_{app}). If all membranes contained in an ultrathin section led to membrane images, at every site of a mitochondrial profile border line inner mitochondrial membranes would be visible (Fig. 3). Therefore, we multiply the uncorrected cristal surface density by the factor I_{tot}/I_{app}. The reliability of this method was demonstrated recently (Mall et al., 1977). From I_{tot}/I_{app} in both longitudinal and cross sections we derived the datum that mitochondrial membranes tilted more than $\varphi = 25°$ to the direction of the electron beam are not visible in normal hearts under the conditions of our study. But if membranes were also visible between 25° and 30°, in other words, if $\varphi = 30°$, the uncorrected surface density would be one fifth greater even though a real difference does not exist. We therefore postulate that for experimental studies a correction is necessary, because φ may change under experimental conditions. But the loss of membranes does not only depend upon φ, but also upon their orientation in space. The object on which we wish to estimate the correction factor (the surface of heart muscle mitochondria) is anisotropic whereas the values for the object to be corrected (the mitochondrial cristae) may be approximately isotropic. If we infer two geometrical surface classes of outer mitochondrial envelope area as described above, one parallel to the fibre axis, the other randomly oriented in space, we can analyze the conditions of I_{tot}/I_{app} with respect to the surface orientation in space. Figure 2 illustrates the loss of membranes ($I_{tot}-I_{app}/I_{tot}\times 100\,\%$) depending upon φ. Curve (a) demonstrates the loss of isotropic membranes, curve (b) the loss of membranes running parallel to an axis cut parallel to this axis (longitudinal sections), and curve (c) the loss of membranes running parallel to an axis cut at an angle of 32.4° to this axis. Our correction factor I_{tot}/I_{app} is derived from membranes corresponding to a "mixture" of curves (a) and (c) whereas our objects to be corrected, the cristae, correspond to curve (a). From these theoretical considerations we derive the following conclusions:

1. Our correction factor tends to be underestimated. If the mitochondrial surface classes we discussed are in a 1:1 relationship the error would be about 10 %. But the real relationship seems to be a more favourable one (Mall et al., 1977).

2. Curves (a) and (c) are – in the relevant part of the curves – nearly parallel. Therefore, a change of φ under experimental conditions would not induce a significant change of the relative underestimation, i.e. the systematic error would be similar in both control and experimental groups. We conclude, therefore, that the procedure of correction described above is sufficient for the comparison between cristal surface densities of different groups of papillary muscles.

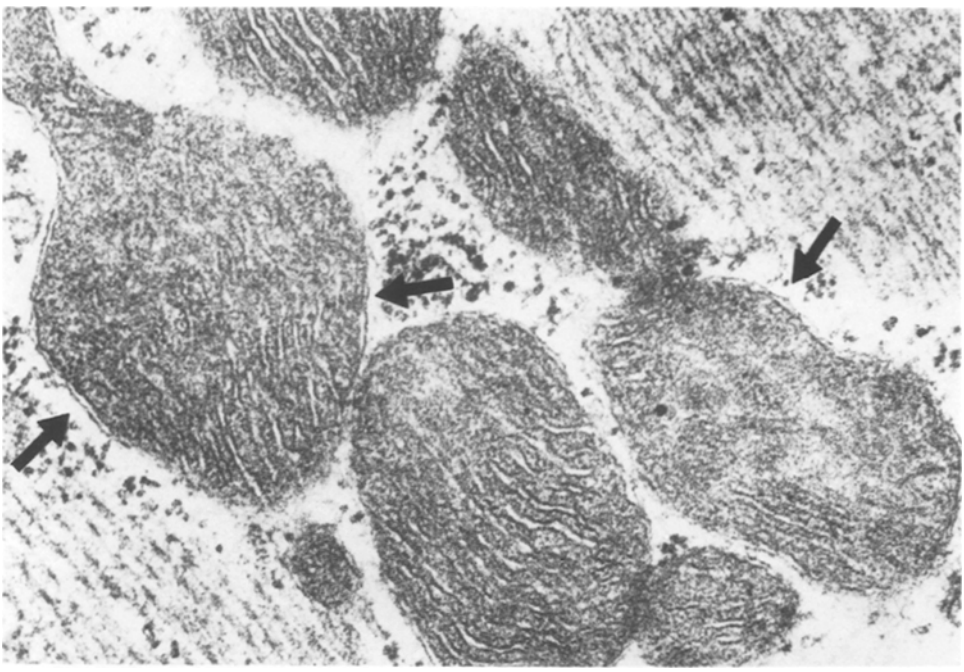

Fig. 3. Only a part of the inner mitochondrial envelope membranes is visible *(arrows)*

Experimental Studies

7 male Wistar rats weighing 146 ± 1.8 g were anaesthetized by intraperitoneal injection of 1 ml 10% chloralhydrate solution. The abdominal aorta was prepared, incised and catheterized retrogradely. Blood vessels were rinsed by a solution of Soerensen buffer containing PVP at a perfusion pressure of 80 mg Hg for 3 min. They were then fixed by perfusion by a solution of 3% glutaraldehyde containing Soerensen buffer and PVP for 12 min. After perfusion the left ventricular posterior papillary muscles were removed and imbedded in agar-agar on a filter paper. They were then cut at an angle of 32.4° to the longitudinal axis into 300 µm thick slices by a tissue slicer (Fig. 4). Three slices from the middle part of each muscle were postfixed in ice-cold OSO_4 for 4h, dehydrated in ethanol and embedded in araldite. The thin elliptical slices of heart muscle tissue in the araldite blocks can easily be cut parallel to the section plane of the slices, i.e. the ultrathin sections are cut at 32.4° to the papillary muscle axis. 1 tissue block per animal was chosen randomly for ultrathin section, being put on 300 mesh nickel grids. The test areas for morphometry were photographed on 7 × 7 film (AGFA-Gevaert 23D56) by a ZEISS EM 9, the films were developed by the reversal process and projected on screens with morphometric test systems. Volume densities were evaluated by point counting, surface densities by the coherent semicircular test grid of Merz (1967) excluding anisotropy of profile border lines. The morphometric data were evaluated at different magnifications.

Stage 1, primary magnification 1,300:1, 12 test areas per animal: Volume densities of myocardial cells, interstitial space, myocardial nuclei and sarcoplasm, surface density of myocardial cells.

Stage 2, primary magnification 6,200:1, 6 test areas per animal: Surface density of mitochondria, volume densities of mitochondria, myofibrils and ground substance.

Stage 3, primary magnification 10,000:1, 8 test areas per animal: Surface densities of inner mitochondrial membranes and cristae, and *T* tubules, volume density of *T* tubules.

Table 1 demonstrates the morphometric results.

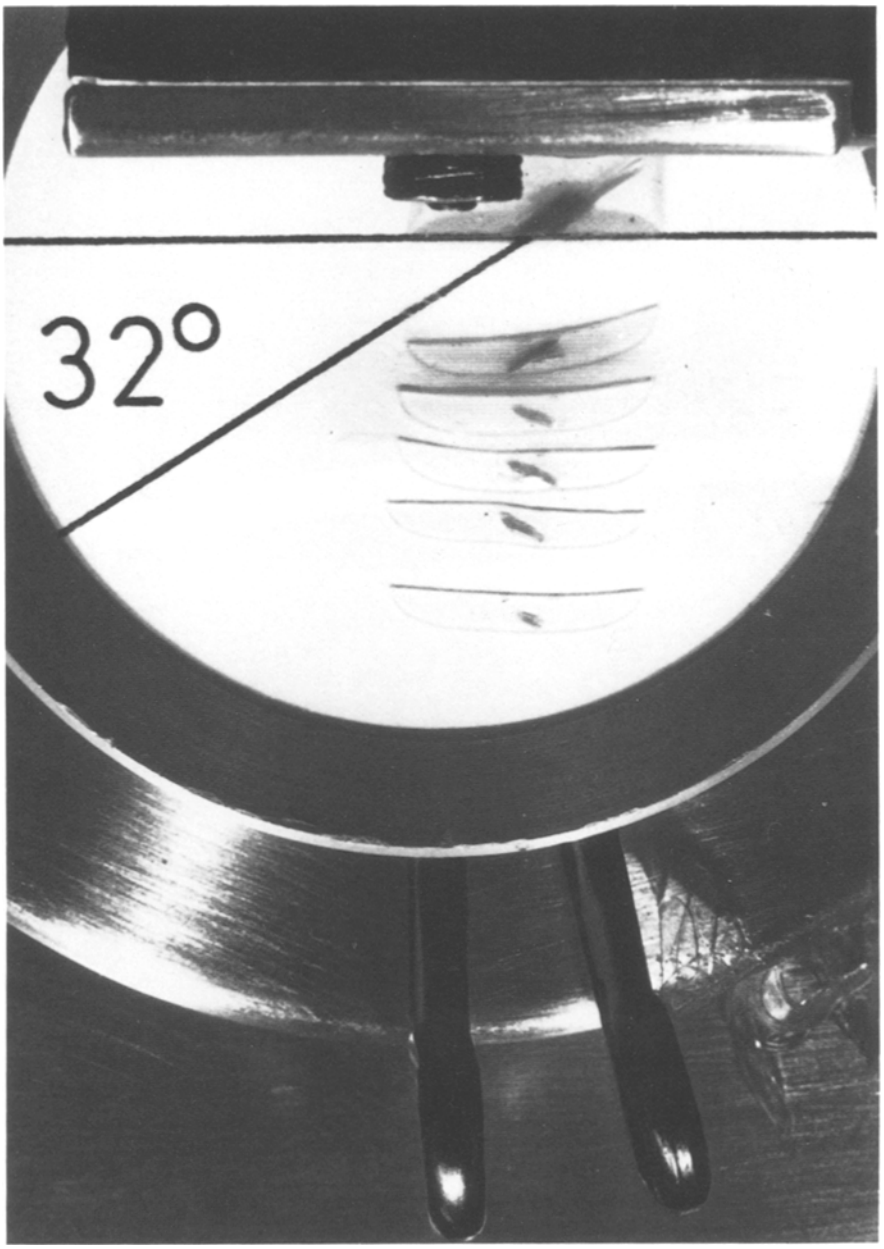

Fig. 4. Tissue sectioner (Sorvall, Newtown, Connecticut, USA) cutting a papillary muscle at an angle of 32.4° to the longitudinal axis

Table 1. Values of morphometric parameters in left ventricular papillary muscles

Component	Parameter	Reference volume	Symbol	Density/ml		Dim.
				Mean	S.E.	
Myocardial cells	Volume	Total tissue	VVMC	0.7972	0.0309	μ^3/μ^3
Interstitial space	Volume	Total tissue	VVEX	0.2128	0.0311	μ^3/μ^3
Myocardial nuclei	Volume	Total tissue	VVNC	0.0122	0.0013	μ^3/μ^3
Sarcoplasm	Volume	Total tissue	VVSP	0.7750	0.0301	μ^3/μ^3
Mitochondria	Volume	Sarcoplasm	VVMI	0.2529	0.0044	μ^3/μ^3
Myofibrils	Volume	Sarcoplasm	VVMY	0.6120	0.0061	μ^3/μ^3
Ground substance	Volume	Sarcoplasm	VVGP	0.1351	0.0098	μ^3/μ^3
T tubules	Volume	Sarcoplasm	VVTT	0.0073	0.0012	μ^3/μ^3
Myocardial cells	Surface	Myocardial cells	SVMC	0.3180	0.0149	μ^2/μ^3
Outer mitochondrial membranes	Surface	Mitochondria	SVOM	7.2614	0.2021	μ^2/μ^3
Inner mitochondrial membranes + cristae	Surface	Mitochondria	SVIM	38.22	0.86	μ^2/μ^3
T tubules	Surface	Sarcoplasm	SVTT	0.26	0.031	μ^2/μ^3
T tubules	Surface	*T* tubules	SVTB	36.05	2.25	μ^2/μ^3

Discussion

Though only one ultrathin section per animal was evaluated, the standard errors of the means of most of the variables are small. Thus, the proposed method is both appropriate and efficient for the recording of quantitative morphologic changes under experimental conditions (cf. Mall and Reinhard, 1977). Naturally the quantitative relations of a papillary muscle do not represent the average relationships for a whole chamber wall and absolute quantitative data can only be estimated. In comparison with the results of other authors for left ventricular chamber walls of rats (Table 2) the following can be stated:

Most of our results are compatible with the results of others, but there is a striking difference in the mitochondrial and myofibrillar volume densities. In rabbit papillary muscles we found similar values when our results are compared with the results of Anversa et al. (1971) who analyzed the left ventricular chamber wall (Mall et al., 1977). Winkler et al. (1977) did not find any differences between the chamber walls and papillary muscles of dog hearts. It is therefore not possible to refute the argument that our values are influenced by different methods of preparation or morphometric analysis. But the morphometry of volume densities is much less sensitive to errors than that of surface densities (Blouin et al., 1977) and the comparison of the results of several authors for myocardial and liver cells demonstrates the reliability of volume analysis. Therefore we think it is more likely that there is a genuine quantitative difference between left ventricular papillary muscles and chamber wall muscle, than that the effect is an artifact of different methodology. Perhaps this difference is only evident in small animals (rats or rabbits) and not in larger animals like dogs because of the higher mitochondrial volume density of the smaller animals.

Table 2. Comparison of our results with the results of other authors

Parameter	Papillary muscle	Left ventricular chamber wall							Dim.
		Datta (1975)	Laguens (1971)	McCallister (1973)	Page (1973)	Page (1973)	Reith (1973)	Smith (1976)	
VVMI	0.25	0.38	0.37	0.31 – 0.36	0.36	–	0.36	–	μ^3/μ^3
SVOM	7.2	4.6	–	8.3 – 8.8	–	–	5.3	–	μ^2/μ^3
SVIM	38[a]	66	–	36 –52[b]	–	54–61	37	55[c]	μ^2/μ^3
SVMC	0.32	–	–	0.28 – 0.36	0.30	–	0.1	–	μ^2/μ^3
VVTT	0.0073	–	–	0.006– 0.011	0.01	–	–	–	μ^3/μ^3
VVMY	0.61	–	–	0.49 – 0.52	0.48	–	0.42	–	μ^3/μ^3

[a] Low underestimation because the correction of the loss of cristal membrane images was performed on oblique sections (Fig. 2)

[b] Low overestimation because the correction was performed on longitudinal sections

[c] The morphometric procedure described leads to an artificial anisotropy of membrane surfaces. We derived that in this case rather than $\frac{\pi}{4} S_V = B_A$ $S_V = \frac{4 \sin \varphi}{\sin 2\varphi + 2\varphi} B_A$ should be used. The cristal surface area therefore might be calculated with an unrecognized overestimation of about 20% if we assume that $\varphi \approx 25°$

This investigation was supported by the "Sonderforschungsbereich 90" of the "Deutsche Forschungsgemeinschaft". The skillful assistance of Zlata Antoni, Harald Derks and Peter Rieger are gratefully acknowledged.

References

Anversa, P., Vitali Mazza, L., Visioli, O., Marchetti, G.: Experimental cardiac hypertrophy: a quantitative ultrastructural study in the compensatory stage. J. Mol. Cell. Cardiol. **3**, 213–228 (1971)

Blouin, A., Bolender, R.P., Weibel, E.R.: Distribution of organelles and membranes between hepatocytes and nonheptatocytes in the rat liver parenchyma. A stereological study. J. Cell. Biol. **72**, 441–445 (1977)

Datta, B.N., Silver, M.D.: Cardiomegaly in chronic anemia in rats. Lab. Invest. **32**, 503–514 (1975)

Fawcett, D.W., McNutt, N.S.: The ultrastructure of the cat myocardium. I. Ventricular papillary muscle. J. Cell. Biol. **42**, 1–44 (1969)

Herbener, G.H., Swigart, R.H., Lang, C.A.: Morphometric comparison of the mitochondrial populations of normal and hypertrophic hearts. Lab. Invest. **28**, 96–103 (1973)

Laguens, R.: Morphometric study of myocardial mitochondria in the rat. J. Cell. Biol. **48**, 673–676 (1971)

Loud, A.V.: Quantitative estimation of the loss of membrane images resulting from oblique sectioning of biological membranes. In: Proceedings 25th Anniversary Meeting of Electron Microscopy Society of America, C.J. Arceneaux, editor. Baton Rouge: Claitor's Book Store 1967

Mall, G., Reinhard, H.: Morphometrische Untersuchungen am Rattenherzen nach hochdosierter Gabe von Cortisol. Verh. Dtsch. Ges. Path. **61**, 445 (1977)

Mall, G., Kayser, K., Roßner, J.A.: The loss of membrane images from oblique sectioning of biological membranes and the availability of morphometric principles – demonstrated by the examination of heart muscle mitochondria. Mikroskopie **33**, 246–254 (1977)

McCallister, L.P., Page, E.: Effects of thyroxin on ultrastructure of rat myocardial cells: A stereological study. J. Ultrastructure Res. **42**, 136–155 (1973)

Merz, W.A.: Die Streckenmessung an gerichteten Strukturen im Mikroskop und ihre Anwendung zur Bestimmung von Oberflächen-Volumen-Relationen im Knochengewebe. Mikroskopie **22**, 132–142 (1967)

Page, E.: Morphometry of mitochondrial membranes and size distribution in rat heart. J. Cell. Biol. **59**, 256a (1973)

Page, E., McCallister, L.P.: Quantitative electron microscopic description of heart muscle cells. Am. J. Cardiol. **31**, 172–181 (1973)

Saltykov, S.A.: Stereometric metallography, 2nd Ed. Moscow: State Publishing House for Metals Sciences 1958

Sitte, H.: Morphometrische Untersuchungen an Zellen. In: Symposion über quantitative Methoden in der Morphologie, 8. Anat. Kongr. Wiesbaden 1965 (E.R. Weibel und H. Elias (ed.)), S. 167. Berlin-Heidelberg-New York: Springer 1967

Smith, H.E., Page, E.: Morphometry of rat mitochondrial subcompartiments and membranes: application to myocardial cell atrophy after hypophysectomy. J. Ultrastruct. Res. **55**, 31–41 (1976)

Tate, E.L., Herbener, G.H.: A morphometric study of the density of mitochondrial cristae in heart and liver of aging mice. J. Gerontol **31**, 129–134 (1976)

Reith, A.: Probleme bei der Bestimmung von Oberflächendichten (OD) von Biomembranen. Mikroskopie **33**, 80–87 (1977)

Reith, A., Fuchs, S.: The heart muscle of the rat under influence of triiod thyronine and riboflavin deficiency with special reference to mitochondria. Lab. Invest. **29**, 229–235 (1973)

Weibel, E.R.: Stereological principles for morphometry in electron microscopic cytology. Int. Rev. Cytol. **26**, 235–302 (1969)

Weibel, E.R.: A stereological method for estimating volume and surface of sarcoplasmic reticulum. J. Microsc. **95**, 229–242 (1972)

Whitehouse, W.J.: A stereological method for calculating internal surface areas in structures which have become anisotropic as the result of linear expansions or contractions. J. Microsc. **101**, 169–176 (1974)

Winkler, B., Schaper, J., Thiedemann, K.U.: Hypertrophy due to chronic volume overloading in the dog heart. A morphometric study. Basic Res. Cardiol. **72**, 222–227 (1977)

Received April 28, 1978