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SOME ASPECTS OF THE APPLICATION OF QUANTITATIVE METHODS IN CARDIOLOGY

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Compared with liver tissue, myocardial tissue is difficult to investigate morphometrically. This is generally due to the non-homogeneous distribution and directional arrangement of some structural elements. Even histomorphometrically, differences can be observed for instance in myocyte interstitium in subepicardial, intramural and subendocardial heart muscle sections (Anversa et al. 1978). No differences in the number of organelles are found in the aforementioned myocardial layers and between ventricles and auricles. But structural clusters and arrangements are observed, wich are attributed to the occurrence and arrangement of contractile substance (of myofibrils).

If we examine the morphometric structural model proposed in Fig.1, we can see that the heart muscle cell can be used as a reference system for all organelles and intracellular structures. But then it must always be assumed that nearly all of the structures represented are not distributed homogeneously.

Mitochondria, the sources of energy of heart muscle cells, are situated beneath the sarcolemma in the interfibrillar and perinuclear areas, interfibrillar mitochondria accounting for the bulk of the mitochondria and thus representing an equivalent of the muscle cell's functional condition. For this reason one does not evaluate individual fractions of mitochondria in controls and in the experimental conditions.

Investigation conducted by us and designed to estimating the mitochondrial surface density with vertical and horizontal line grids revealid that mitochondria, unlike myofibrils, show no significant direction-dependent difference (Meyer et al. 1982). It can therefore be assumed that mitochondria in the

MORPHOMETRIC MODEL OF HEART MUSCLE CELL ULTRASTRUCTURE

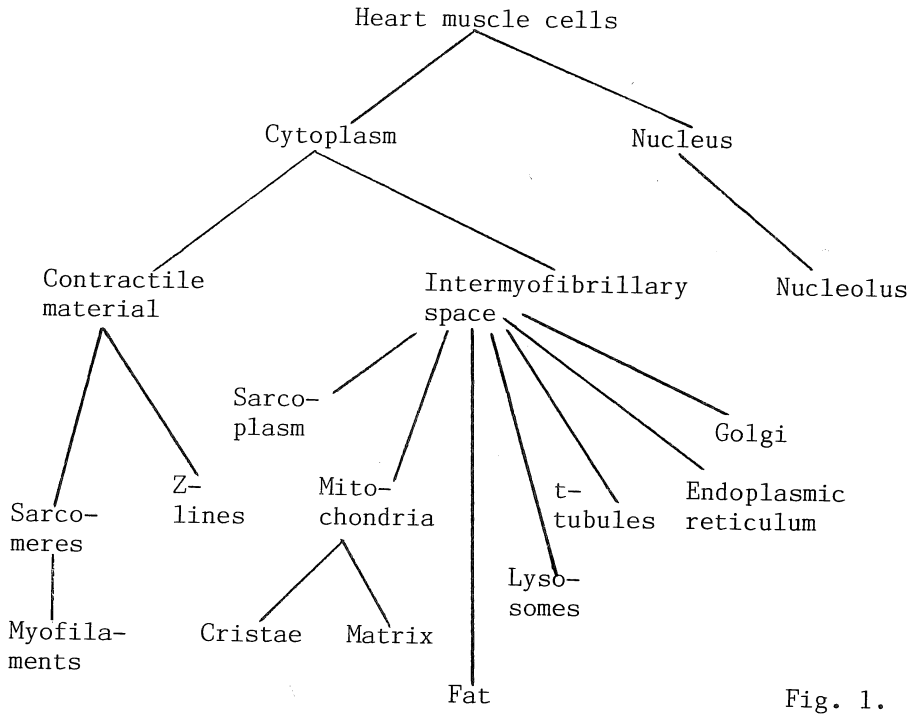

Fig. 1.

heart muscle cell are not arranged in a certain direction.

If we take the sarcoplasm as a reference system, we can say the following about the localized structures: the t-tubuli, which are found only in the vicinity of the Z strips, are not homogeneously distributed within the sarcoplasm. Under experimental conditions their changes are analogous to those of the contractile substance or of the Z strips. Decreases in relative total volume and in the number of Z strips are accompanied by decreases in these parameters in the t-tubuli.

The longitudinal system of the sarcoplasmic reticulum, which is responsible for the Ca++ intake, Ca++ binding capacity and hence for the myocardial contractility, is found in part in systematic arrangement in the form of diads and triads and in part in diffuse arrangement in the sarcoplasm.

The remaining cell organelles and intracellular structures are homogeneously distributed in the sarcoplasm. Some occur in the myocardium so rarely that a modified approach to the morphometric evaluation is called for.

In order to take these special features of the myocardial cells into consideration it is necessary to determine additional parameters of cell organelles and cell components.

In the morphometric structural model of the myocardial cell (Fig. 1), the organelles and the intracellular components are represented in relation to the localization and to the corresponding reference system. Each higher stage of schematic representation can be used as a reference system for the structure beneath it. In this manner it is possible to determine for instance the surface density of cristae per unit volume of mitochondria, sarcoplasm and myocardial cell. Changes and structuring processes within the myocardial cell can be better observed and interpreted by determining the individual morphometric stereological parameters of the organelles and components in relation to different reference systems. In this respect, the determination of relations between different structures such as the mitochondria/myofibril relation or surface density of the outer membrane of mitochondria per unit volume of myofibril (a quotient which can be regarded as a ratio of ATP-binding and ATP-consuming structures (McCallister and Page 1973) and as a measure of myosin-ATP-ase activity (Smith and Page 1976) can also be helpful).

Statistical analysis of measured data and of morphometric stereological parameters is an essential part of the morphometric evalution process.

Even before setting up an experiment, one should try, if possible, to obtain information on the mean error to be expected and to take this into consideration when drawing up the plan for the experiment. In view of the fact that the distributions are known only in very exceptional cases, only approximate methods of experimental planning can be used.

The use of powerful EDP systems and main frame computers opens up the possibility of applying different approaches in calculating morphometric and statistical data.

Morphometric stereological parameters can be calculated for each individual random sample (microgram) and for each species, i.e. on the basis of the summed up measured data. The advantage of calculating the morphometric stereological parameters for each individual random sample is that one obtains an idea of the mean error of these parameters and thus of the necessary number of random samples (running calculation of the mean value according to Sitte). This is followed by an estimation of the mean values of the individual morphometric stereological parameters per species.

The calculation of morphometric stereological parameters based on the summed up measured values per species is called for, especially when evaluating cell organelles and intracellular structures which occur more rarely in myocardial cells (lysosomes, fat droplets, vacuoles) leading to 0 measured

values within a series of measurements. In extreme cases, such 0 measured values can also occur within an animal species. This varied approach to the calculation of morphometric stereological parameters can lead to differing results, especially in determining of quotients between structures occuring in myocardial cells (mitochondria/myofibril relation, etc.).

The group mean values of the morphometric stereological parameters can in turn be calculated either from the individual values of the random samples or from mean values of species. The most common method of calculating group mean values is by using mean values of species, the results obtained in this way being more accurate (Wassilew et al. 1984). The other method can be very useful in investigating cell cultures, where the cultivation of separate species is not possible, not common or not economical. If 0 values of morphometric stereological parameters occur per animal species, then they have to be taken into consideration in calculating group mean values of some parameters, such as the number of an object per unit area or unit volume, and neglected in other cases, such as mean cross-sectional area of objects. Another basic guideline should be the satisfaction of inequations of the type: Mean error (experimental animal, species) < mean (error experimental group).

This running inequation should be a general requirement for hierarchic levels of investigation developed step-bystep. To satisfy these inequations, a pilot study can be made prior to the actual experiment in order to be able to plan the necessary quantities in the groups to be statistically analyses. On problems of this kind see Gundersen (1980) and source literature mentioned by him.

As a rule, the following two questions figure prominently when comparing experimental groups:

1. Do certain groups differ with regard to certain features?
2. Do certain parameters show changes in time when the experimental groups are represented as "measuring points" in time?

The first question can be put with greater precision, i.e. can objects of different groups be distinguished on the basis of the parameters measured (classification)? If we start out from multi-dimensional sets of parameters, then questions of coherence arise in connection with the second question (dynamics of parameters, cell kinetics).

A very wide range of statistical methods is available for dealing with these problems. We should like briefly to consider the stastistical tests and to introduce a classifier,

which in our opinion is exceptionally well suited for dealing with biological questions.

The following two basic conditions must always be satisfied for carrying out statistical tests: firstly, the test must be adequate (i.e. the distributions must be of the type required for the test), and secondly, the number of test objects must be sufficient for the requirements, e.g. in order to be able to use approximative distributions.

Much has been written about the common t-test. Our experience shows that the normal distribution on which it is based renders this model unsuitable in many instances. Logarithmic normal distributions and mixed distributions, i.e. particularly multiple peak distributions, are more common. One is on the safe side if in such cases one chooses a test that is independent of distribution (Kolmogorov-Smirnov test, Wilcoxon test). But if there are clear indications that a certain distribution exists, e.g. exponential distribution in the case of survival times (Martin and coworkers 1984), then one would forfeit this information if tests independent of distribution are used.

In passing we should like to point to the possibilities offered by multi-dimensional analysis for discovering causal relations in the parameter proper (regression analysis, variance analysis, covariance analysis, ...). the process library published by Rasch, Herrendörfer, Bock and Busch is an excellent reference work for this purpose.

THE HIERARCHIC CLASSIFIER

If we wish to go beyond the statement "the two parameter groups vary significanly with regard to such and such parameters", then we can do this be changing from the test to classification. There has been substantial development in the field of classification through progress made in pattern recognition. Detailed information on this can be found in the book by Wysotzki, Klix and Sydow entitled "Recognition and classification processes" published in 1974 and the book by Kittler and Devijer entitled "Pattern recognition" published in 1982. Just as certain tests, many classifiers (e.g. Bayes classifiers) can be used effectively only if the distribution is known or if it can be estimated with sufficient accuracy. If both the distributions and the class of the objects are unknown, then cluster analysis methods have to be resorted to.

Here we shall assume that the class of the objects is known. The "hierarchic classifier" (Voss 1980) introduced in the following requires no previous knowledge on the distribu-

tions of the features. The classifier described here briefly is for separating two classes (detailed description in Hufnagl and Voss 1984).

The classifier consists of a number of systematic and simple rules. An example is as follows:

IF	M1	a	THEN	O
IF	M2	b	THEN	X
IF	M2	c	THEN	X
IF	M1	d	THEN	O
			ELSE	X

Thus the hierarchic classifier permits complex separating surfaces between populations, which are set up in stairlike arrangement. With each step we look for a lower threshold tij and upper threshold Tij for each feature "Mi" in such a manner that the rules Mi < tij or Mi > Tij result in the best possible separation (few errors) of objects of class "j". We use

$$Wij\ (c,t) = rj - d \sum_{k=j} tk$$

as an optimation function for a step and a given feature "i". In the equation "c" is the comparison operator (<or>), t the threshold parameter, rj the number of correctly separated objects of class "j", tk the number of incorrectly separated objects of class "k" and "d" is an optimation parameter.

Classification errors can be severely (d 10) or mildly (d 1) punished with the aid of d.

The threshold values mentioned above for a given feature "Mi" and a fixed class "j" can be found by assuming maximum values of Wij (<, t) or Wij (>, t). The empirical distribution functions of the parameters of the individual classes are used for this purpose. This maximum might have been reached by the comparison separator cij and the threshold value tij. Taking into consideration all features, we determine the optimum value W by

$$W = \max_i \max_j Wij\ (cij\ ,\ tij)$$

In this manner we obtain an optimum separation of class J with the feature I, using the comparison operator C and the threshold T. Thus, a classification step is characterized by a quadrouple E = 1,C,T,J). The objects separated in the preceding steps are neglected in the steps to follow (hierarchic principle).

After s classification steps, the quality triad (R,F,N) reads out; R=number of correctly classified objects, F=number of incorrectly classified objects, N=number of objects not yet

classified. This triad is an unequivocal function of the step number s and of dj in each step.

The classifier we have described has the following advantages:

1. Easy interpretation and high clarity
2. Good ability to separate surfaces between classes
3. Possibility of direct error estimation (i.e. no test random sample or bootstrap is required)

The structure and working principle of the classifier are described in depth in the works by Voss and Hufnagl (1979, 1984, 1984). Up till now the classifier has been used mainly for automatic analysis of micrographs (ct. for instance Martin and Voss 1984, Gottschalk and co-workers 1984).

This somewhat differentiated method of obtaining measured data, of calculating morphometric stereological parameters and of their statistical evaluation calls for the use of efficient and adaptable morphometry programs. The program system developed by us and described on various occasions (Wassilew, Frölich, 1980, 1982), which we called the "Program generator for drawing up user programs for processing morphometric and statistical data", meets these requirements. It generates user program according to the measured value parameters obtained and to the mathematical formulas contained in the program generator. The user programs contain only those formulas that are required for calculating any primary or secondary parameters. These user programs are thus generated, not programmed. Their generation costs only as much as the computer time it takes to generate them. They can be used over and over without having to be generated anew.

It is also possible, without any programming work and with little time expenditure, to instal new mathematical formulas and to take out those that are used only once. This gives the program system a multi-purpose character.

This means that the program system can accept and process measured data obtained with the aid of different measuring methods (direct measurement, point and intersection point counting methods, planimetry, automatic and semiautomatic measuring methods). These data as well as morphometric stereological parameters are accepted and printed out using common international symbols.

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