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A Procedure for Determining the Relative Volume of Mitochondria in Hepatic Cells[1]

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About forty years ago Cowdry's monograph on mitochondria appeared evaluating the work of previous investigators, indicating synonymy and, in a sense, bringing the first exploratory phase of the study of these cellular constituents to a close (1). Reasonably specific methods for their demonstration had been devised, and it was possible to have some confidence in the conclusions that the so-called mitochondria of different kinds of cells, despite dissimilarities in size or shape, were comparable cellular parts.

The extreme variability of mitochondria, which had been at the root of much of the early confusion in terminology, became even more impressive when the observations were brought together. Variations in mitochondria were described for the same type of cell at different stages in the life cycle of an organism, and in the same type of cell under different physiological conditions. Eventually the Lewises, studying living cells from tissue cultures, described changes in mitochondrial shape and size in the same cell from moment to moment (2). While the variability would obviously require further description from more types of cells, it suggested that it was not so much the size or shape of the mitochondria as the materials of which they were composed which must be known for understanding their activity. Following Cowdry's summation of the early work, there were two main lines of mitochondrial studies. One line continued the description of mitochondria as seen in cells of various types, and the other pursued the goal of defining the mitochondrial substance in the chemical sense.

Histochemical methods carried out on vitally stained or fixed and stained cells had severe limitations. It was not until the technique of homogenates had been developed that a great deal of progress could be made. Studies on suspensions, however, quickly led to the conclusion that mitochondria are the loci of enzymes, particularly

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those associated with oxidation. Lists of enzymes found in normal and cancerous cell mitochondria emphasize the central role they have in cellular metabolism (3). It remains, of course, to bring the observations on modifications in mitochondrial form together with those on mitochondrial content. Early work along these lines, like that of Noel on rodent liver cells (4), was limited by lack of knowledge concerning the role of mitochondria in cellular metabolism. In more recent times a new interest in this phase has developed, and some pilot studies, like those of Harman and Fiegelson (5), are beginning to appear.

To bring form and function of mitochondria together would be much simpler if mitochondria had been found to contain but one or a few physiologically active components. The fact is that they have been found to contain diverse materials, and that these materials have a variety of potential contributions to make to metabolism. To discover whether a change in form is correlated with some change in activity will require a most detailed analysis. There are many unanswered questions. Do some of the described shifts in mitochondrial form involve changes in number to compensate for changes in size? Are changes in either size, shape, or number correlated with changes in the relative abundance of compounds present in mitochondria? Before these questions can be answered, the volumetric quantity of mitochondrial substance must be determined. The significance of mitochondrial differences in normal and cancerous, young and old, or other types of physiologically different cells depends upon these answers.

How can mitochondrial volume be determined? Cowdry (6) worked out a method, which although precise, has apparently proved too difficult and time-consuming to be used in conjunction with physiological observations. Their method was based upon constructing three dimensional models of cells which had been studied in very thin serial sections. It has the further disadvantage of being useful only when preserved, stained material can be studied. A determination of the number of mitochondria occurring in a definite area of a microscopic field has been used on vitally stained cells (7). This is useful as a relative statement of mitochondrial volume only insofar as the cells which are compared have mitochondria of the same size. A more definite method of estimating mitochondrial numbers in relation to the cytoplasmic volume was described by Thurlow (8), who used a cross-ruled insert in the ocular and counted the mitochondria in definite areas of sections of known thickness. This type of estimate was found to be relatively valid, with an experimental error of 1.3% or less.

This, and all methods based upon direct observation, have a neces-
sary limitation, in that they depend upon the recognition of mito-
chondria. Variations in size, shape and abundance in different cells
make the problem of recognition more difficult. Despite the develop-
ment of special staining techniques, none is perfectly specific, so no
single criterion for their identification is available. It is doubtful if
all cytoplasmic particulates that have been described as mitochondria
actually belong in the same category, and it remains to be shown that
the mitochondrial population of a single cell are all identical in der-
ivation and function. Experience, here, appears to be the best guide.
In our study comparisons with the liver cell mitochondria described
by Noel in the white mouse have been most helpful (4). His observa-
tions have checked very closely with ours, and aid in giving confi-
dence in our identification of mitochondrial material.

More indirect methods have also been developing. Schneider,

TREND OF PERCENTAGE OF MITOCHONDRIAL HITS WITH INCREASING NUMBERS OF OBSERVATIONS

NUMBER OF OBSERVATIONS
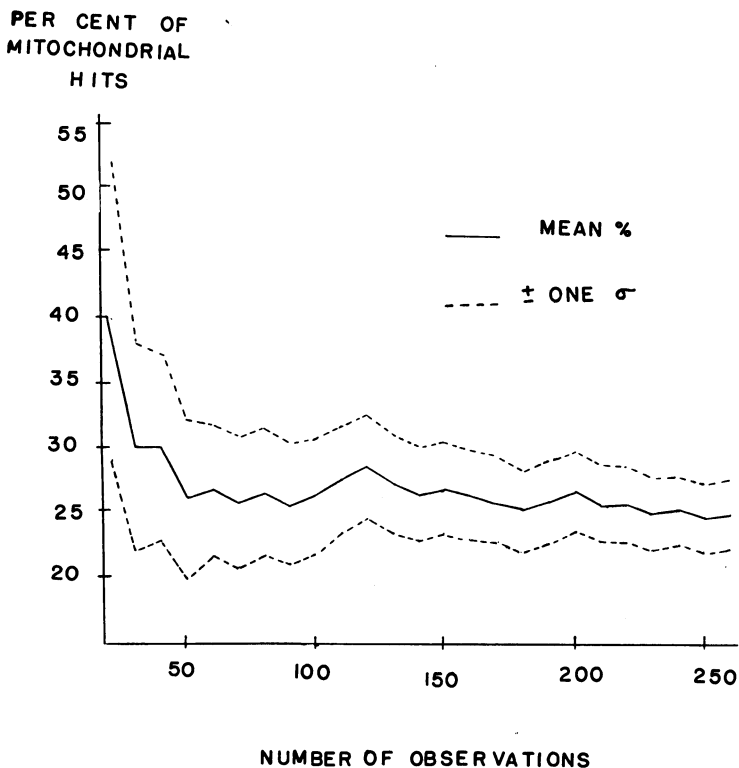
Hogeboom and their associates have compared the quantities of enzymes present in mitochondrial suspensions from normal and cancerous cells (9). Insofar as these fluctuations result from changes in mitochondrial volume they are significant. On the other hand, they may reveal differences in the quantity of an enzyme per unit of mitochondrial substance. A more definite attack upon the problem has appeared in the measurement of rhodanase activity (10). This method, as must any which depends upon activity in a mitochondrial suspension prepared from homogenates, is accurate only insofar as none of the mitochondrial material of the cell is lost in the preparation of the homogenate or in the concentration procedures which follow. Alternatively, it is relatively accurate insofar as the extent of mitochondrial loss is identical regardless of cell type or of the
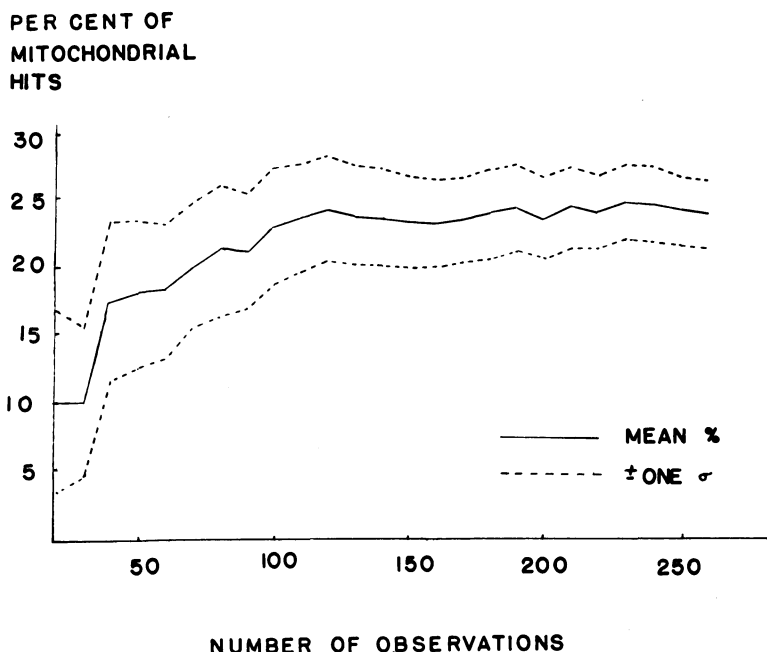
TREND OF PERCENTAGE OF MITOCHONDRIAL HITS WITH INCREASING NUMBERS OF OBSERVATIONS

Figures 1, 2. The percentage of mitochondrial hits plotted as a function of the number of grid points counted, to show the trend of values obtained for a single cell from the intermediate zone. Unlike other determinations given in the report, the percentages are based upon total cell volume, including nucleus, rather than cytoplasmic volume only.

experimental procedures employed before measurement. Direct count-ing methods based on the study of homogenates have also been de-vised (11). Counts based on direct counting suffer from the same limitations as those based on enzyme activity in that any loss of mitochondria during the preparation of the homogenates will di-rectly affect the results. Such indirect methods, while admirable for comparative purposes, will be strengthened if checked by methods depending on direct observation of the original cells. All of the indirect methods based upon the study of homogenates have the distinct disadvantage that they cannot be used to compare two spe-cific cells, but rather must be used to compare two masses of tissue. In the case of hepatic tissue, where it is not possible to separate one type of cell from others, and where there is a great difference be-tween central and peripheral cells in the same lobule, this is a serious disadvantage.

The method of measurement described below has been devised to permit a reasonably objective statement of differences in quantity of mitochondria in cells. For comparisons it is believed to be sufficiently accurate to permit changes in percentage of cytoplasmic volume in the order of 2-3% to be detected, although for differences of a smaller order it may be found too time-consuming.

Mouse liver was fixed in a variety of standard mitochondrial fixa-tives, sectioned at 3 μ, and stained mith Altmann's stain, the Bensley-Cowdry modification, or iron haematoxylin. The stained slides were observed using standard oil immersion objectives. By means of a camera lucida, the image of the cells was superimposed on a target composed of a grid of points. A level of focus was chosen which brought a mitochondrium of unusual size or shape into sharp focus. A record was made of the number of grid points which were super-imposed on the images of mitochondria in focus, the number of grid points which were superimposed upon the nucleus, and the number of points superimposed on mitochondria-free cytoplasm. For the purposes of this paper, a record of a point which was superimposed on a mitochondrium is referred to as a mitochondrial hit, a point which was superimposed on the nucleus is termed a nuclear hit, and a point which was superimposed upon the mitochondria-free cyto-plasm is called a cytoplasmic hit. At regular intervals the level of focus was checked to make sure that it had not changed. The level of focus was changed whenever the whole grid had been counted. Another unusual signal mitochondrium was chosen for checking the level of focus, and the grid points recounted. This process was re-peated until sufficient data for comparative purposes had been obtained. It is evident that if this process is repeated with sufficient care the proportion of mitochondrial hits to total cytoplasmic hits

will approach the proportionate volumes of mitochondria and cyto-plasm. After the data were recorded the counts were transformed into percentage figures. The mitochondrial percentages were deter-mined by the formula:

$$\text{Mitochondrial Percentage} = \frac{\text{Mitochondrial hits}}{\text{Mitochondrial hits plus cytoplasmic hits}}$$

Nuclear hits were ignored, because areas sufficient to produce stastis-tically significant figures for comparisons of mitochondrial percent-ages were inadequate for determining nuclear volume with sufficient precision. The figures quoted, therefore, express the ratio of mito-chondrial volume to the total extranuclear cytoplasm.

To be reasonably certain of hits it was important to study the area to be quantitated before starting a count in order to locate the nu-clei, and sinusoids or other structures which were to be ignored. The use of a small piece of black paper cut into a blunt point and held below the mirror of the camera lucida increased the effectiveness of the procedure, since the cell showed more brightly and a target point could be concealed and revealed until it was certain that the correct decision had been made. In some cases counts were made on a series of cells; in other cases on the whole region covered by the target grid. In either case, only the portion of the grid which covered he-patic cells was counted. In all cases, once the counting of points was begun all significant grid points were recorded to ensure that no bias could creep in through inequities in portions of the cell or region being studied.

The number of points needed. The first question raised was the number of points needed to determine the abundance of mitochondria in a cell. For this question a series of hits were recorded from a single cell. A small target of 10 random (not gridded) points was used. This was placed at random, and the cell was focused at random by watching the calibrations on the fine adjustment of the scope rather than the cell. The points were recorded in clusters of 10, and a run-ning percentage of mitochondrial hits was computed including each new cluster of points. The mitochondrial percentage as obtained for a typical cell by this method is shown in Figures 1 and 2. At about 100 to 150 points the mitochondrial percentage found a level which re-mained relatively constant, variation remaining within plus or minus 1.5% during subsequent counting. It was concluded that for rough comparative purposes about 100 to 150 points were sufficient, but this was not found to be adequate for making statistically valid com-parisons.

Table 1

Standard Error of Percentages Expressed As Percentages for Different Numbers of Observations

%	(p(1–p))	Number of observations								
		100	200	400	500	750	1000	2000	3000	5000
5	.0475	2.18	1.54	1.09	0.98	0.80	0.69	0.49	0.40	0.31
10	.09	3.00	2.12	1.50	1.34	1.10	0.95	0.67	0.55	0.35
15	.1275	3.56	2.51	1.78	1.59	1.30	1.13	0.80	0.65	0.50
20	.16	4.00	2.83	2.00	1.79	1.46	1.26	0.89	0.73	0.57
25	.1875	4.32	3.06	2.17	1.94	1.58	1.37	0.97	0.79	0.61
30	.21	4.58	3.24	2.29	2.05	1.67	1.45	1.02	0.84	0.65
35	.2275	4.77	3.37	2.39	2.13	1.75	1.51	1.07	0.87	0.67
40	.24	4.90	3.46	2.45	2.19	1.79	1.55	1.10	0.89	0.69
45	.2475	4.93	3.52	2.49	2.23	1.82	1.57	1.11	0.91	0.70
50	.25	5.00	3.54	2.50	2.24	1.82	1.58	1.12	0.92	0.71

The standard error of a percentage is calculated by the formula

$$\sigma_p = \sqrt{\frac{pq}{N}}$$

where p is the percent expressed as a decimal, q is 1-p, and N is the number of observations on which the percent is based. For this discussion each grid point recorded represents one observation. It is evident that standard errors can be calculated prior to experimentation where percentages are to be used. A table of the standard errors of percentages, prepared with a slide rule and subject to rule errors, is given (Table 1). It is evident that the standard error declines slowly with increasing numbers of observations, a factor which partially compensates for the fact that predetermination of the standard error is possible.

Where two percentages are to be compared, several methods are available to determine the statistical significance of differences between them. They may be compared with a Chi square test, or may be compared by computing the value of the difference between them divided by the standard error of the difference. Use of the latter method permits the formulation of a table giving the approximate differences between two percentages which is minimal to result in any desired probability of the differences not being due to random chance. This is possible because of the standard errors of the percentages themselves. The standard error of the difference between two percentages is given by the formula.

$$\sigma_{p1-p2} = \sqrt{\sigma_{p1}{}^2 + \sigma_{p2}{}^2}$$

Since the standard error of the percentages is a function of the numbers of observations made, and varies with the magnitude of the percentage, it is necessary to express the statistically significant differ-

Table 2

Minimum Percentage Differences to Produce a Probability of .001, at Different Percentage Levels and Different Numbers of Observations

Percent	100	200	400	500	750	1000	2000	3000	5000
5%	9.54%	6.75%	5.20%	4.33%	3.49%	3.02%	2.13%	1.74%	1.34%
10%	13.10%	9.27%	6.56%	5.89%	4.79%	4.26%	2.94%	2.39%	1.51%
15%	15.52%	11.00%	7.77%	6.68%	5.69%	4.92%	3.50%	2.85%	2.19%
20%	17.48%	12.36%	8.75%	8.20%	6.41%	5.54%	3.90%	3.20%	2.47%
25%	18.90%	13.40%	9.45%	8.40%	6.80%	6.00%	4.24%	3.59%	2.68%
30%	20.00%	14.10%	10.02%	8.96%	7.34%	6.45%	4.48%	3.65%	2.84%
35%	20.60%	14.70%	10.40%	9.34%	7.60%	6.59%	4.68%	3.80%	2.93%
40%	21.30%	15.10%	10.70%	9.60%	7.83%	6.78%	4.80%	3.90%	3.12%
45%	21.80%	15.32%	10.87%	9.75%	7.96%	6.88%	4.86%	3.96%	3.08%
50%	21.85%	15.45%	10.92%	9.76%	7.97%	6.92%	4.88%	4.05%	3.09%

Conversion   factors: for p of .005 x .833
for p of .01 x .755
for p of .05 x .536

ences as a function of the number of observations and the actual percentages. Table 2 gives a set of approximations of differences between percentages which are significant at the .001 level. To obtain predictions of the differences between percentages which are significant at other levels it is necessary to multiply the difference shown in the table with an appropriate factor. Appropriate factors are: probability of .005, .833; of .01, .755; of .05, .536. Table 2, also rule calculated, is subject to rule errors, and is approximate only. It is most nearly accurate when the two percentages being compared are equidistant from the quoted percentage values at the left.

An examination of the table shows that the number of observations required to demonstrate a statistically valid difference depends upon the magnitude of the difference being studied. For very large differences few observations are needed, but if small differences are to be detected a large number of observations is required. For work dealing with differences in the order of 2-3%, however, not so many observations are required that the method becomes excessively time-consuming.

Consistency of results. Regardless of the mathematical validity of comparisons between two sets of data of the type being used, it remains to be shown that observations can be made with sufficient consistency to place confidence in the percentages obtained. For this purpose the mitochondrial volume was determined in a series of cells on one day, and repeated the next day, without reference to the original data. The target grid was placed repeatedly over the cell until a minimum of 200 points had been recorded, except in a few instances where unavoidable interruptions prevented completion of a count. The results are tabulated in Table 3. Even with such a short series of observations it is evident that individual counts are quite

reproducible, and for a whole series of cells, the total percentages obtained are satisfactory for comparative work.

For determining the p values of percentages being compared where the number of observations on which each percentage is based is different, the standard error of the difference is computed differently. Except for the construction of the Table 2, the standard error of differences was routinely computed using the following formula:

$$\sigma_{p1-p2} = \sqrt{ p_{1+2} \ q_{1+2} \left(\frac{N_1 \quad N_2}{N_1 N_2} \right) }$$

where p_{1+2} represents the combined percentages obtained from the two sets of observations, expressed as a decimal; q_{1+2} equals $1-p_{1+2}$; N_1 is the number of observations on which the first percentage is based and N_2 the number of observations on which the second percentage is based. (p for pooled data)

While one investigator may be able to reproduce his counts, it is another matter for two investigators to agree in their counts. There are many places for error or differences in interpretation. To check this point, two different observers counted a short series of cells, using the same method as that mentioned above. The results of this series of counts are given in Table 4. It is evident that the two investigators differed to an extent which would invalidate comparisons between counts made by one and counts made by the other on experimental material. The probability that the differences in the pooled counts of the two observers could have occurred as the result of random chance was approximately .001. It is also evident that the two observers varied consistently, one recording more and the other fewer mitochondrial hits. When checked with the Kendall sum the probability that the two observers were not differing consistently was

Table 3

Comparison of Duplicate Determinations of Mitochondrial Volume on the Same Cells

	TRIAL I			TRIAL II		
	N	Mit. Vol.	S.E.	N	Mit. Vol.	S.E.
	147	36.8%	3.96%	151	35.2%	3.94%
	147	42.8%	4.08%	141	42.6%	4.18%
	215	46.2%	3.43%	240	42.6%	3.18%
	217	41.5%	3.34%	221	42.1%	3.33%
	233	33.0%	3.08%	222	32.0%	3.12%
	244	40.5%	3.12%	253	40.7%	3.08%
Totals	12.03	40.1%	1.41%	1228	39.2%	1.39%

Table 4

Comparison of Mitochondrial Volumes Reported By Two Independent
Observers for the Same Cells.

Cell No.	Observer 1			Observer 2		
	N	Mit. Vol.	S.E.	N	Mit. Vol.	S.E.
1	189	63.5%	3.50%	202	50.4%	3.51%
2	241	58.6%	3.13%	241	57.1%	3.19%
3	228	65.0%	3.00%	180	52.9%	3.73%
4	204	57.0%	3.47%	200	50.0%	3.54%
5	184	62.0%	3.54%	212	50.5%	3.42%
Totals	1046	60.8%	1.51%	1035	52.4%	1.55%

found to be between .01 and .02, giving evidence that they were consistently differing in their interpretation of levels of focus, or other factors of the kind. The two observers were not experienced in mitochondrial observations. It is probable that with experienced observers there would be greater agreement. The implication is, however, that where two investigators wish to use this method they should check carefully to see to what extent they agree in their counts, and wherever possible, all comparisons should be based on counts made by the same person. The implication is also evident that the mitochondrial volumes obtained by this method are approximations, which are useful in showing relative changes rather than absolute changes.

Sources of error. The method of determining mitochondrial volume described here has certain inherent sources of error, all of which tend to result in an overestimation rather than an underestimation of the mitochondrial volume. It is almost impossible to determine whether a mitochondrium is partly in focus or immediately below the level of focus. The focal plane of the lens is not a perfect plane, so that each target point represents a certain depth rather than a mathematical point. Furthermore, the target point has its own dimensions, and it is evident that the larger the target point the more frequently a hit will be recorded, despite attempts to count only hits in which half or more of the point is superimposed on a mitochondrium.

The most serious deficiency of the method is encountered in cells having extremely small mitochondria. Experience has shown that the method cannot be used with any degree of accuracy unless the target points, as seen in the camera lucida, are no larger than the diameter of the mitochondrial image. In the case of extremely delicate, thread-like mitochondria the target points must be so small that they are very difficult to see. In the case of the mouse liver, the peripheral cells in a lobule have plump, large mitochondria. Here the method works very well. As one follows a plate of cells toward the central vein the mitochondria become smaller, at first appearing

as slender rods, and finally appearing as extremely fine threads or granules in the cells adjacent to the central vein. In the central region of the lobule the method is not applicable.

After considerable experimentation with various target grids and counts made with them, it appears that the spacing of points on the target grid is not an important factor. The dimensions of the points, on the other hand, are important, as a larger grid point results in recording a larger number of hits. Eventually target grids were prepared with a typewriter, the period being a reproducible spot with a relatively constant size. The figures quoted in the paper are based on counts made with a target grid made up of 26 rows of periods, with 26 periods in each row, prepared with elite type. The distance between the rows was half the distance between normal lines of type. The first targets were made on shiny white paper. It was found later that the visibility of the areas being studied was increased if the paper was not glaringly white.

Another potential source of error is the technique used to prepare the material for mitochondrial counts. To what extent does the fixative or staining method used affect the results obtained? To check the consistency of results obtained when different fixatives were used, a series of determinations was made on hepatic cells from the same mouse after fixation in a number of different fixatives. All of the areas studied were stained with iron haematoxylin. To minimize the effects of variability within the liver used, five areas were selected for study following each fixative. The areas chosen were taken from different lobules, or alternatively, at some distance apart in the same lobule. Except for two of the counts made on Regaud-fixed tissue, only areas at the periphery of a lobule were used.

Table 5 summarizes the results obtained. The total percentages obtained by pooling the data from the five areas studied for each fixative appear to be relatively constant, with somewhat less than 6% separating the highest and lowest percentages recorded. The highest percentages were found after Schridde's fixative, the lowest after Carleton's fixative. Table 6 shows the p values obtained by comparing the mitochondrial percentages obtained after the different fixatives. The relatively small differences appear somewhat more significant here. The approximately 3% difference between the mitochondrial volume obtained after Schridde's and Altmann's fixatives could be expected by random chance only about 8 times in a hundred, while the nearly 4% difference observed between Schridde's and Hoyer's fixatives could be expected only about 3 times in a hundred. It seems clear that it would be unwise to do comparative work on cells which had been fixed with different fixatives. The low percent-

age figure recorded for Regaud's fixation was the direct result of the inclusion of two areas which were not at the periphery of the lobule. The low percentage found after Carleton's fixative was, in our subjective judgment, the result of imperfect mitochondrial fixation, which may be borne out by the greater discrepancies found between different areas. The Altmann fixation also resulted in areas with somewhat greater variability than had been found in the chrome-formol or chrome-sublimate fixatives. It was again a subjective judgment that this was related to unequal penetration of the fixative throughout the block of tissue fixed.

Not only the fixative used, but the stain used may have an influence on mitochondrial counts. To determine whether the stain influenced

Table 5

Comparison of Mitochondrial Volume of Hepatic Cells From the Same Mouse After Fixation With Different Fixatives and Staining in Hematoxylin.

Fixative		N	Mit. Vol.	S.E.
Regaud*		463	43.2%	2.30%
		438	41.9%	2.36%
		455	32.5%	2.19%
		243	30.2%	2.94%
		249	41.2%	3.11%
	Total	1848	38.8%	1.13%
Schridde		210	45.1%	3.44%
		203	46.4%	3.50%
		224	41.4%	3.30%
		208	45.2%	3.43%
		226	41.2%	3.26%
	Total	1071	43.8%	1.51%
Altmann		251	44.7%	3.12%
		290	42.4%	2.90%
		216	41.6%	3.33%
		242	38.4%	3.11%
		234	37.6%	3.14%
	Total	1233	40.9%	1.39%
Carleton		285	38.8%	2.84%
		261	37.6%	3.00%
		220	34.6%	3.21%
		273	39.2%	2.95%
		227	37.0%	3.21%
	Total	1269	37.9%	1.36%
Hoyer		299	38.9%	2.82%
		296	40.0%	2.84%
		372	39.4%	2.53%
		407	41.6%	2.45%
		350	41.0%	2.64%
	Total	1724	40.1%	1.18%

*Two of the areas recorded are not from the periphery of the lobule.

Table 6

Probability of Significant Differences When Different Fixatives Are Compared

	Regaud	Schridde	Altmann	Carleton
Schridde	.004			
Altmann	.121	.084		
Carleton	.308	.002	.063	
Hoyer	.218	.026	.334	.011

the results, sections which had been prepared from tissues fixed with Carleton's and Hoyer's fixative were stained with iron haematoxylin and fuchsin. As in the comparison of fixatives, all of the tissues used came from the same mouse liver, and five peripheral areas were selected for study for each stain and fixative. The results of this comparison are given in Table 7. It is interesting to note that fuchsin-stained areas varied after Carleton's fixation as they had in haematoxylin-stained material. The areas studied after Hoyer's fixation were relatively constant after fuchsin staining. It was an unexpected result to find that the mitochondrial volume recorded after fuchsin staining is consistently greater than that after haematoxylin staining. It had been supposed that fuchsin was a more selective stain, and might, therefore, give more dependable results. Actually, the haematoxylin-stained material was easier to count, as the margins of the mitochondria were more easily recognized, and the greater transparency of the fuchsin-mitochondria made it somewhat more difficult to recognize the portion of a mitochondrium in sharp focus. Differences in interpretation, however, may not account for all of the differences recorded. Mitochondria of the same liver appeared to be somewhat more plump in the fuchsin-stained slides. This was especially noticeable in the cells near the periphery of the lobules, from which the data were collected, but seemed to be no less true of the mitochondria from regions nearer the center of the lobule. The p value of the differences between haematoxylin and fuchsin-stained cells following Carleton's fixation was .0011; and following Hoyer's fixation was .0001. The results show, certainly, that any comparisons

Table 7
Comparison of Mitochondrial Volume in Peripheral Hepatic Cells After Staining With Haematoxylin or Fuchsin

	Haematoxylin			Fuchsin		
	N	Mit. Vol.	S.E.	N	Mit. Vol.	S.E.
Carleton	295	38.8%	2.84%	252	45.2%	3.12%
	261	37.6%	3.00%	206	38.9%	3.39%
	220	34.6%	3.21%	214	46.2%	3.48%
	275	39.2%	2.95%	305	42.4%	2.82%
	227	37.0%	3.21%	225	49.2%	3.33%
Total	1268	37.9%	1.36%	1192	44.8%	1.44%
Hoyer	299	38.9%	2.82%	335	48.4%	2.73%
	296	40.0%	2.84%	334	50.0%	2.68%
	372	39.4%	2.53%	339	46.4%	2.70%
	407	41.6%	2.45%	380	44.6%	2.55%
	350	41.0%	2.64%	309	43.5%	2.82%
Total	1724	40.1%	1.18%	1709	46.6%	1.20%

of experimental material should be made with cells that were stained in the same manner. A second conclusion reached was that greater confidence could be placed in the study of haematoxylin-stained material for determining mitochondrial percentage, assuming that the

cells have been destained sufficiently to permit the recognition of mitochondrial outlines despite any ergastoplasm present.

SUMMARY

A procedure for determining the relative volume of mitochondrial substance in a cell or region is reported. The method can be used to determine the relative volume of mitochondrial substance and to make comparisons between cells of a given type in experimental material. Some of the statistical factors are discussed, and the method is examined with respect to the consistency of the results obtained and the major sources of error inherent in the method. It is found to be sufficiently delicate to detect changes in the order of 2-3%, and to make identical fixation and staining methods necessary in the preparation of material for study.

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