Application of Quantitative Stereology to the Evaluation of Enzyme-altered Foci in Rat Liver

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

The mathematical science of quantitative stereology has established relationships for the quantitation of elements in three-dimensional space from observations on two-dimensional planes. This report describes the utilization and importance of such mathematical relationships for the quantitative analysis of focal hepatic lesions in terms relative to the volume of the liver. Three examples are utilized to demonstrate the utility of such calculations in the three-dimensional quantitation of hepatic focal lesions. The first is that of a computer-simulated experiment based on defined hypothetical situations. The simulations demonstrate the applicability of the computations described in this report to the evaluation of two-dimensional data from typical animal experiments. The other two examples are taken from actual experiments and involve the transplantation of hepatic cell populations into the liver of suitably prepared hosts and the quantitation of altered foci produced by initiation with diethylnitrosamine-partial hepatectomy followed by promotion with phenobarbital. The quantitation of altered foci by means of a two-dimensional analysis (simple enumeration of focal intersections/area of tissue section) is proportional to the quantitation of foci per volume of liver provided that the mean diameter of the foci for each treatment is sufficiently uniform, as exemplified in the text by the transplantation experiment. When such mean diameters are unequal as in the diethylnitrosamine-phenobarbital experiment described herein, quantitation from three-dimensional analysis gives significantly different results as compared with enumeration of focal intersections on two-dimensional areas. These studies clearly demonstrate that the frequency and size of foci intersections viewed on two-dimensional tissue sections do not necessarily reflect the number or size of foci in the three-dimensional tissue. Only by quantitating the number and size of the foci in relation to the three-dimensional volume of the tissue can one determine the validity of the proportionality of data from two-dimensional measurements to the total number of foci per volume of tissue. Such a conclusion has important implications for quantitative studies on hepatocarcinogenesis as well as for the enumeration of premalignant lesions which occur during the natural history of carcinogenesis in any solid tissue.

INTRODUCTION

The quantitative enumeration and evaluation of microscopic, space-occupying premalignant lesions have been conducted in several solid organs of the rodent including mammary gland (29), pancreas (15), and liver (8). It is now apparent that the biological applications of the mathematical science of quantitative stereology (4, 26, 27) are numerous and go beyond the initial efforts. The methods are especially applicable to the problems involved in the quantitation of discrete lesions in solid tissues, especially liver. In 1848, the French geologist, Delesse (5), reported that the ratio of the sum of the areas of the cross-sections of elements within a solid structure to the total area of the random cross-section examined is equivalent to the ratio of the volume of that component to the total volume of the solid. This fundamental and useful relationship is essential to much of the analysis of focal elements presented in this report. To our knowledge, the earliest effort to solve the fundamental problems of particle enumeration and, in a solid mass, the particle size distribution was conducted in the field of anatomy by the mathematician Wicksell (28) in 1925. Wicksell dealt with the problem of quantitating the numbers and sizes of the secondary follicles or germinal centers in the lymphatic tissue of the spleen by methods less laborious than serially sectioning the spleen in toto. Thereafter, metallographers, biologists, and geologists entered the field of quantitative stereology as documented by DeHoff and Rhines (4), Elias (6), Underwood (26), and Weibel (27).

Statistically exact and mathematically rigorous relationships have been established for the quantitation of elements in three-dimensional space from observations on two-dimensional planes through such spatial structures (3, 9, 20). The statistically exact relationships for spheres in space reported by Fullman (9) have been used in our present analysis for the calculation of the total number of focal elements in three-dimensional space as well as for the mean diameter and hence the mean volume of such foci. Following the lead of Wicksell, the estimation of the size distribution of particles dispersed in space has been used in many fields of science by a variety of methods (12, 21, 22, 24, 26, 27).

The size distribution of focal elements in three-dimensional hepatic space and hence, by summation, the total number of enzyme-altered foci in livers of rats treated with DEN (2) were reported in 1972 by Scherer et al. (23). The formulations expressed in their computations of the number of foci in three-dimensional hepatic space were original, since these authors made no reference to the known body of information in the field of stereology. This important advance in the quantitative analysis of focal lesions in terms of three-dimensional liver space has since been utilized by several investigators including Pitot et al. (17) and Kunz et al. (13).

The purpose of the present report is to extend the analysis of focal hepatic lesions in terms of the three-dimensional space of the liver by utilizing the information and methodology established in quantitative stereology. In order to illustrate the tech-
H. A. Campbell et al.

In this paper, an intersection of a focus is defined as the profile of that focus on a plane intersecting the focus.

and its advantages, we discuss in some detail 3 specific examples of data, one taken from computer simulations and the other 2 taken from animal experimental data.

MATERIALS AND METHODS

Computer-simulated Experiments

The data for the computer-simulated experiments were obtained by calculations for defined hypothetical situations. The purpose of these simulated experiments is to demonstrate the application of established relationships in quantitative stereology to the enumeration of hepatic lesions. This study is not intended to rigorously establish the accuracy of such relations since these have been well established by previous authors. It is recognized that, if a large number of parallel planes are randomly passed through the space under consideration, the density or spacing of the planes must be statistically uniform. Further, observations of our experimental data and distribution of the approximately circular focal intersections\(^4\) show no evidence of orientation or alignment of the foci. Thus, for simulated data, calculations have been based on the intersection of uniformly spaced parallel planes with spherical foci. By reducing the spacing between planes, it is possible to force the error in computation of foci to be as small as desired. It has been found that 1000 planes/cm (10 \(\mu\text{m}\) spacing) produce results of accuracy adequate for the simulated experiments reported herein.

Animal Experiments

Transplantation of Initiated Cell Populations. Young adult male F344 rats received a single injection of DEN (200 mg/kg) followed by AAF and PH. Liver cells from these donor rats were isolated 15 days or in some cases 6 months after PH and injected into host rats that had received a PH after a 7-day dietary administration of AAF (0.02%). The host animals were maintained on the AAF diet for an additional 7 days, shifted to basal diet, and killed 3 days later. Details of the orthotopic liver cell transplantation technique, histochemical staining for \(\gamma\)-glutamyl transpeptidase, and reading of the enzyme-altered focal intersections on the tissue section have been reported (14, 25).

DEN-Phenobarbital Regimen. Female CD (Sprague-Dawley-derived) rats were obtained from the Charles River Breeding Laboratory, Inc. (Wilmington, Mass.), and were subjected to a PH (11). DEN (100 mg/kg) was administered 24 hr later intragastrically (23). The rats received sodium phenobarbital (dose shown with the data) in the diet (27). All foci mentioned from experimental animal data will be understood to be enzyme-altered foci that are \(\gamma\)-glutamyl transpeptidase positive.

The values at each point in tables and charts represent the calculated number taken from the sum of all livers in animals under a given condition. By this method of calculation, statistical variation between the particles being enumerated (28). In our animal experiments, the microtome sections were 3 to 6 \(\mu\text{m}\) thick (about one-fourth of a hepatic cell diameter), and the smallest group of cells scored as a focus was of a minimum diameter of 50 \(\mu\text{m}\) as defined by the minimum diameter of Size Class 1. Thus, our tissue sections are thin with respect to even the smallest size class of foci, and a correction for counting in thick sections (2) is not necessary.

In the present experiments, we have made observations on the 2-dimensional tissue sections by marking measurements on photographs (10- to 15-fold magnification) of the original section (18) or by projection (25- to 35-fold magnification) of the images onto a screen for optical observations (14). The diameters of a circle estimated to have an area equal to the intersectional area of the focus is used as a measure of the size of the intersection of a focus in the plane of section.

The area of the tissue sections is determined by use of a planimeter, and the sum of the focal areas of intersection is calculated from the observed diameters of the intersections of the foci with the sectional plane.

Computations are made using relationships established by Fullman (9) for spheres in 3-dimensional space. The mean inverse of the diameters of the focal intersections \((\bar{d})\) is given by the equation:

\[
\bar{d} = \frac{1}{d_1} + \frac{1}{d_2} + \ldots + \frac{1}{d_n}/n
\]

where \(n\) is the number of focal intersections; \(d_1, d_2, \ldots, d_n\) are diameters of the focal intersections.\(^5\)

The mean diameter of the foci in the liver \((\bar{D})\) is given by:

\[
\bar{D} = \pi/2 \bar{d}
\]

and by definition the number of focal intersections per sq cm \((N_s)\) is given by:

\[
N_s = n/A
\]

where \(A\) is the area of the tissue section.

The number of foci per cu cm of liver \((N_v)\) is computed by the equation:

\[
N_v = N_s/\bar{D} = 2 \bar{d}N_s/\pi
\]

By use of the relationship reported by Delesse (5), the equivalence of the areal fraction and volume fraction, the volume fraction \((V_v)\) of the foci is obtained by the equation:

\[
V_v = A_s = [a_1 + a_2 + \ldots + a_n]/A
\]

where \(A_s\) is the areal fraction of the focal intersections on the tissue section area, and \(a_1, a_2, \ldots, a_n\) are the areas of the focal intersections.

The mean volume of the foci \((\bar{V})\) is given by:

\[
\bar{V} = V_v/N_v
\]

Estimates of Size Distribution. Methods for the estimation of the size distribution of particles in space have been based on the distribution of measurements on 2-dimensional random planes and include distribution of diameters, areas, volumes, chords, and points (4, 26, 27).

The method used herein for estimates of size distribution is that reported by Saltykov (21) and reviewed by Underwood (Ref. 26, pp. 24-27). We have not smoothed the data on focal intersection diameters. This is in contrast to Wickaell (28), who used data obtained from a continuous free-hand curve fitted to the frequency histogram of the intersection diameters, and also to Weibel (27, vol. I, page 174), who gives a rule-of-thumb graphic procedure for extrapolating to 0 the frequency histogram of the focal intersection diameters.

\[^4\] We have not smoothed the data on focal intersection diameters. This is in contrast to Wickaell (28), who used data obtained from a continuous free-hand curve fitted to the frequency histogram of the intersection diameters, and also to Weibel (27, vol. I, page 174), who gives a rule-of-thumb graphic procedure for extrapolating to 0 the frequency histogram of the focal intersection diameters.

\[^5\] We have not smoothed the data on focal intersection diameters. This is in contrast to Wickaell (28), who used data obtained from a continuous free-hand curve fitted to the frequency histogram of the intersection diameters, and also to Weibel (27, vol. I, page 174), who gives a rule-of-thumb graphic procedure for extrapolating to 0 the frequency histogram of the focal intersection diameters.

\[^6\] We have not smoothed the data on focal intersection diameters. This is in contrast to Wickaell (28), who used data obtained from a continuous free-hand curve fitted to the frequency histogram of the intersection diameters, and also to Weibel (27, vol. I, page 174), who gives a rule-of-thumb graphic procedure for extrapolating to 0 the frequency histogram of the focal intersection diameters.
123–126). In contrast to the original Saltykov method, the size classifications have been extended to include the larger foci observed in our experiments, and the smallest size class has been designated Size Class 1. The intersections of foci on the random-cut tissue sections are placed in size classifications by their intersectional diameters. The size classes are defined by a log scale of maximum diameters by the series: Size Class 1, antilog 1.8; Size Class 2, antilog 1.9; Size Class 3, antilog 2.0 . . . Size Class 25, antilog 4.2. The diameters are expressed in μm to avoid fractional values for the diameters and thus negative values in this log scale.

Using Saltykov’s equation and coefficients, the estimate of the number of foci per cu cm of liver in a specified size class is given by the equation:

\[ N_v, = \frac{1}{DK} \times (1.6461 N_v, - 0.4561 N_v, - 0.1162 N_v, - 0.0415 N_v, - 0.0003) \]

where \( N_v, \) is the estimate of the number of foci per cu cm of liver in Size Class \( K, \) \( DK \) is the diameter of the largest foci in Size Class \( K, \) \( N_v, \) is the number of focal intersections observed with diameters within the limits set for Size Class \( K, \) and \( N_v, + i \) to \( N_v, + 11 \) are the number of focal intersections with diameters within the limits set for size classes larger than \( K. \)

It is our present practice to use the number of foci computed by the statistically exact relation expressed in Equation D as the best estimate of the total number of foci rather than the summation of the estimates of the foci found in the size distribution computation, the latter being based on a smaller statistical base, i.e., the sum of estimates computed from the focal intersections after being distributed into several size classes. We then use the values found in the size distribution computation for distribution of the total foci computed by Equation D:

\[ N_v = (N_v, + 1)(N_v, + 2) \ldots N_v, \]

where \( N_v, \) is the estimated number of foci in the size class identified as \( i, \) and the other symbols are defined by Equations D and G.

The volume fraction of the foci in a particular size classification can now be calculated:

\[ V_v = N_v, V_r \]

where \( V_v, \) is the volume fraction of the foci in size class identified by \( i, \) \( N_v, \) is defined by Equation H, and \( V_r, \) is the mean volume for foci in size class identified as \( i. \) Multiplication of the volume fraction by 100 gives the percentage of the volume of the liver occupied by foci of Size Class \( i. \)

RESULTS

Computer-simulated Data. The computer simulation resulted in a series of values representing the diameters of the intersections of a plane with hypothetical particles in space, and these diameters were treated in the same way as those obtained from the tissue sections from the animal experiments. In most cases, for computer-simulated data, the agreement between the found and expected values is close (Table 1). In those cases where there is a significant difference between found values and theoretical values, the deviations are not greater than those expected from consideration of the convention of eliminating from the computation focal intersections with planes of a fraction of the uniform spacing. For spherical particles randomly distributed in space, the number of intersections with a random plane is directly proportional to the particle diameter. This relationship between focal intersections per sq cm of liver section and focal diameter is seen in the computer-simulated data identified by A, B, and C in Table 1.

Where there is one focus per cu cm of space and increasing diameter of foci, the linear relationship between the number of

Table 1

<table>
<thead>
<tr>
<th>Hepatic space (cu cm)</th>
<th>No. of foci</th>
<th>No. and diameter of foci (μm)</th>
<th>Total area of planes (sq cm)</th>
<th>2. Identifer for data</th>
<th>3. No of focal intersections</th>
<th>4. No of focal intersections/sq cm</th>
<th>5. No. of foci/cu cm of liver</th>
<th>6. Mean diameter of foci (μm)</th>
<th>7. Mean volume of foci (cu mm)</th>
<th>8. Volume % of foci in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4</td>
<td>4 (630)</td>
<td>4000</td>
<td>A</td>
<td>248</td>
<td>0.062</td>
<td>0.88</td>
<td>10</td>
<td>705</td>
<td>630</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4 (1259)</td>
<td>4000</td>
<td>B</td>
<td>496</td>
<td>0.124</td>
<td>0.89</td>
<td>1.0</td>
<td>1390</td>
<td>1259</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4 (2512)</td>
<td>4000</td>
<td>C</td>
<td>1000</td>
<td>0.250</td>
<td>0.98</td>
<td>1.0</td>
<td>2557</td>
<td>2512</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>2 (2512)</td>
<td>4000</td>
<td>D</td>
<td>1500</td>
<td>0.375</td>
<td>0.95</td>
<td>1.0</td>
<td>3963</td>
<td>3762</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>2 (5012)</td>
<td>4000</td>
<td>E</td>
<td>1999</td>
<td>0.500</td>
<td>0.95</td>
<td>1.0</td>
<td>5260</td>
<td>5009</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1 (5012)</td>
<td>4000</td>
<td>F</td>
<td>1999</td>
<td>0.500</td>
<td>0.95</td>
<td>1.0</td>
<td>5260</td>
<td>5009</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1 (10,000)</td>
<td>4000</td>
<td>G</td>
<td>1999</td>
<td>0.500</td>
<td>0.95</td>
<td>1.0</td>
<td>5260</td>
<td>5009</td>
</tr>
</tbody>
</table>

* The computed diameters of the intersections of foci defined in Section 1 with the test planes at 10 μm spacing were analyzed in the same way as were the diameters of the foci intersections observed on the tissue sections from the animal experiments.

** Numbers in parentheses, foci diameters (μm).
focal intersections and focal diameter is shown on Chart 1, open circles. This relationship, described by Wicksell (28), is expressed by Equation D.

For foci of uniform size dispersed in space, the pattern of number and size of focal intersections on a 2-dimensional plane is illustrated in Chart 2, A to C. The number of intersections is directly related to the diameter of the foci. The intersections of the foci occurring in sequentially smaller size classes have an identical pattern independent of the focal size. It is these relationships between focal diameter and the number and distribution of focal intersections for a system of spheres of the same size that form the basis of the computation of foci in a system of spheres of nonuniform size expressed numerically in Equation G. A theoretical treatment of these relationships has been published (21, 26). The application of the focal analysis procedures described in this report to the computer-simulated data demonstrates the utility of these procedures in analysis procedures described in this report to the computer-simulated data for situations defined and identified as A, B, C, D, and E in Table 1, Sections 1 and 2.

Part of the data from this experiment has been reported previously (14). Photomicrographs of tissue sections taken from the same tissue blocks used in this report have been published by Laishes and Rolfe (Ref. 14, Fig. 1). The results from calculations based on the application of statistically exact relationships in stereology (26) to observations of host liver sections are shown in Table 2. Under the conditions of this experiment, the number of focal intersections observed on the tissue sections varied from 2.1 intersections/sq cm up to 50.8 intersections/sq cm. These intersections varied considerably in diameter, as shown on Chart 3. It is recognized that the largest intersections represent the largest foci in the liver space, but the smaller intersections could occur from foci of the same diameter or from foci of larger diameter intersected at some distance from

\[
\text{H. A. Campbell et al.}
\]

\[
\text{CANCER RESEARCH VOL. 42}
\]

\[
\text{THE NUMBER OF FOCAL INTERSECTIONS OBSERVED FOR EACH SIZE CLASS}
\]

\[
\text{THE NUMBER OF FOCI COMPUTED FOR EACH SIZE CLASS}
\]

\[
\text{THE FOCAL VOLUME FRACTION COMPUTED FOR EACH SIZE CLASS}
\]

Chart 2. Computer-simulated data. The histograms show the distribution by group size classes of the focal intersections, number of foci, and focal volume fraction computed from the computer-simulated data for situations defined and identified as A, B, C, D, and E in Table 1, Sections 1 and 2.

Table 2

<table>
<thead>
<tr>
<th>2. Observation basis</th>
<th>1. No. of viable cells injected(^a)</th>
<th>3. No. of tissue sections</th>
<th>4. Total sectional area (sq cm)</th>
<th>5. No. of focal intersections/sq cm</th>
<th>6. No. of foci/cu cm of liver</th>
<th>7. No. of foci/liver</th>
<th>8. Mean diameter of foci (μm)</th>
<th>9. Mean volume of foci (cu mm)</th>
<th>10. Volume % of foci in liver</th>
<th>11. No. of foci/1000 cells injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 days after PH (^b)</td>
<td>3</td>
<td>3.45</td>
<td>6</td>
<td>13.5</td>
<td>2.13</td>
<td>49</td>
<td>257</td>
<td>436</td>
<td>0.038</td>
<td>0.19</td>
</tr>
<tr>
<td>1 x 10^4</td>
<td>4</td>
<td>5.24</td>
<td>8</td>
<td>25.4</td>
<td>12.0</td>
<td>238</td>
<td>1031</td>
<td>505</td>
<td>0.058</td>
<td>1.39</td>
</tr>
<tr>
<td>3 x 10^4</td>
<td>3</td>
<td>4.33</td>
<td>10</td>
<td>16.4</td>
<td>20.1</td>
<td>457</td>
<td>2047</td>
<td>439</td>
<td>0.038</td>
<td>1.73</td>
</tr>
<tr>
<td>5 x 10^4</td>
<td>5</td>
<td>4.48</td>
<td>8</td>
<td>29.5</td>
<td>19.3</td>
<td>745</td>
<td>3519</td>
<td>528</td>
<td>0.062</td>
<td>5.96</td>
</tr>
<tr>
<td>7 x 10^4</td>
<td>2</td>
<td>3.58</td>
<td>10</td>
<td>9.38</td>
<td>42.5</td>
<td>1093</td>
<td>4713</td>
<td>417</td>
<td>0.032</td>
<td>3.53</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>2</td>
<td>3.29</td>
<td>4</td>
<td>9.43</td>
<td>30.8</td>
<td>963</td>
<td>3159</td>
<td>528</td>
<td>0.062</td>
<td>5.96</td>
</tr>
<tr>
<td>6 mos. after PH</td>
<td>1</td>
<td>5.02</td>
<td>4</td>
<td>6.4</td>
<td>1.90</td>
<td>59</td>
<td>296</td>
<td>474</td>
<td>0.040</td>
<td>0.24</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>1</td>
<td>2.82</td>
<td>2</td>
<td>4.2</td>
<td>14.5</td>
<td>340</td>
<td>959</td>
<td>425</td>
<td>0.034</td>
<td>1.16</td>
</tr>
<tr>
<td>3 x 10^6</td>
<td>1</td>
<td>3.25</td>
<td>2</td>
<td>4.4</td>
<td>32.4</td>
<td>719</td>
<td>2337</td>
<td>451</td>
<td>0.043</td>
<td>3.09</td>
</tr>
</tbody>
</table>

\(^a\) Host animals prepared according to the method described in Ref. 14 were killed 10 days after receiving intraportal injections of cells taken from donor rats given DEN and AAF and subjected to PH (14) at the times indicated.

\(^b\) Only one focus intersection was observed on the 6 tissue sections; thus, meaningful computations for this condition are precluded.
Quantitative Stereology during Hepatocarcinogenesis

the maximum diameter of the foci (i.e., acentric transections). The distribution of the focal intersections is shown for each group on Chart 4. The size distribution of the computed number of foci in the space of the liver shows a uniform pattern about a similar mean size for all groups. Although of similar mean diameter, the number of foci per cu cm of liver varied greatly for the different experimental conditions. The dose relationships between the number of cells injected, the number of foci per cu cm of liver, and the number of focal intersections observed per sq cm of tissue section are shown by the curves on Chart 5. In this experiment, the computed mean diameter of the foci for all of the treatment groups is quite similar [455 ± 39 (S.D.)] as shown in Table 2, Section 8; consequently, the foci per cu cm and the focal intersections per sq cm are proportional one to the other as in the relationship expressed by Equation D. Similarly, a curve representing the volume of the foci as a percentage of the volume of the liver is of similar shape (curve not shown). For this specific experiment where all experimental conditions resulted in foci of similar size as shown by the similar mean diameters, the parameters (foci per cu cm of liver, focal intersections per sq cm of tissue sections, volume of foci as percentage of volume of liver) show similar proportional dose relationships as would be expected.

It has been reported previously that the relationship between the number of focal intersections per “standard liver section” and the number of cells injected is linear and that the curve passes through the origin when the cells are collected from donor rats 15 days after partial hepatectomy (14). The data from the same specimens obtained by the present methodology do not differ markedly from the earlier findings (Chart 5). However, we now have data on cells that were transplanted 6 months after treatment of the donor rats. In this case, the number of foci in the recipients was greatly reduced and was not proportional to the number of cells injected. The relationship can be expressed as a power equation \( y = 62 x^{2.3} \) or as a linear equation with an x-axis intercept of 0.875 million injected cells and slope of 328. The former seems preferable.

The reason for the decreased yield of foci 6 months after the treatment of the donors is not known at present. In this in

the maximum diameter of the foci (i.e., acentric transections). The distribution of the focal intersections is shown for each group on Chart 4. The size distribution of the computed number of foci in the space of the liver shows a uniform pattern about a similar mean size for all groups. Although of similar mean diameter, the number of foci per cu cm of liver varied greatly for the different experimental conditions. The dose relationships between the number of cells injected, the number of foci per cu cm of liver, and the number of focal intersections observed per sq cm of tissue section are shown by the curves on Chart 5. In this experiment, the computed mean diameter of the foci for all of the treatment groups is quite similar [455 ± 39 (S.D.)] as shown in Table 2, Section 8; consequently, the foci per cu cm and the focal intersections per sq cm are proportional one to the other as in the relationship expressed by Equation D. Similarly, a curve representing the volume of the foci as a percentage of the volume of the liver is of similar shape (curve not shown). For this specific experiment where all experimental conditions resulted in foci of similar size as shown by the similar mean diameters, the parameters (foci per cu cm of liver, focal intersections per sq cm of tissue sections, volume of foci as percentage of volume of liver) show similar proportional dose relationships as would be expected.

It has been reported previously that the relationship between the number of focal intersections per “standard liver section” and the number of cells injected is linear and that the curve passes through the origin when the cells are collected from donor rats 15 days after partial hepatectomy (14). The data from the same specimens obtained by the present methodology do not differ markedly from the earlier findings (Chart 5). However, we now have data on cells that were transplanted 6 months after treatment of the donor rats. In this case, the number of foci in the recipients was greatly reduced and was not proportional to the number of cells injected. The relationship can be expressed as a power equation \( y = 62 x^{2.3} \) or as a linear equation with an x-axis intercept of 0.875 million injected cells and slope of 328. The former seems preferable.

The reason for the decreased yield of foci 6 months after the treatment of the donors is not known at present. In this instance, the altered relationship between number of foci and number of cells injected may indicate a need for cooperativity between injected cells or a greater capacity of the host to suppress the growth of the cells collected at 6 months.

**DEN-Phenobarbital Regimen.** When animals are treated with DEN followed by phenobarbital in the diet (10, 18), the size of the foci as well as the number of foci increase with increasing doses of phenobarbital as shown in Table 3 (see Ref. 17 for examples of sections showing foci). In this experiment, unlike

<table>
<thead>
<tr>
<th>1. Dietary phenobarbital levels (%)</th>
<th>No. of rats</th>
<th>Mean liver wt (g)</th>
<th>No. of tissue sections</th>
<th>Total sectional area (sq cm)</th>
<th>2. Observation basis</th>
<th>3. No. of focal intersections/sq cm</th>
<th>4. No. of foci/cu cm of liver</th>
<th>5. No. of foci/cu cm of liver</th>
<th>6. Mean diameter of foci (µm)</th>
<th>7. Mean volume of foci (cu mm)</th>
<th>8. Volume % of foci in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13</td>
<td>10.9</td>
<td>19</td>
<td>49.6</td>
<td>1.63</td>
<td>55.7</td>
<td>587</td>
<td>293</td>
<td>0.023</td>
<td>0.13</td>
<td>8.0</td>
</tr>
<tr>
<td>0.000005</td>
<td>6</td>
<td>10.9</td>
<td>11</td>
<td>24.8</td>
<td>3.51</td>
<td>136</td>
<td>1480</td>
<td>258</td>
<td>0.014</td>
<td>0.19</td>
<td>50</td>
</tr>
<tr>
<td>0.01</td>
<td>7</td>
<td>11.2</td>
<td>12</td>
<td>32.0</td>
<td>8.10</td>
<td>174</td>
<td>1942</td>
<td>464</td>
<td>0.289</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.05</td>
<td>5</td>
<td>12.8</td>
<td>10</td>
<td>32.9</td>
<td>13.1</td>
<td>215</td>
<td>2743</td>
<td>607</td>
<td>0.272</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>14.8</td>
<td>12</td>
<td>38.2</td>
<td>10.1</td>
<td>158</td>
<td>2342</td>
<td>642</td>
<td>0.750</td>
<td>11.8</td>
<td>11.8</td>
</tr>
</tbody>
</table>

*The animals were subjected to PH followed by intragastric DEN (10 mg/kg) 24 hr later. Phenobarbital administration was begun 1 week later and continued for 6 to 8 months (18).*
the foregoing transplantation experiments, the size distribution of foci varies from group to group with respect to the mean diameter as well as the pattern of distribution about the mean as shown on Chart 6. The progressive increase in focus diameter with increasing dose of phenobarbital and the differences in scatter of the diameters of the foci about the mean (Chart 6) are in contrast with the rather uniform distribution patterns and means found for the various groups in the experiment involving the injection of cells from treated donors (Chart 4). Thus, for the DEN-phenobarbital experiment, the curve representing the relationship of the number of foci per cu cm of liver to dose and the curve representing the relationship of the number of focal intersections of foci per sq cm of tissue section to dose are quite different in shape (Chart 7). For the group receiving 0.05% phenobarbital, the number of foci per cu cm of liver is 4 times that of the group receiving no phenobarbital, while the number of intersections of the foci per sq cm of tissue section is 9 times that of the group receiving no phenobarbital. Since the relationship between foci intersections per sq cm and foci per cu cm is a function of mean diameter (Equation D), it may be recognized that the differences in shape of these curves are a consequence of the differences in the mean diameters of the foci in the various groups, which varied from 258 to 642 μm in this experiment (see Table 3).

While the dependent ordinates of Charts 5 and 7 are the same, the abscissas are different due to the nature of the experiments. This is of no significance to the present report, since virtually any parameter in relation to the number of focal intersections per sq cm of tissue section and the foci calculated per cu cm of liver would show proportionality between the values in Chart 5 and the disproportionality in Chart 7.

## DISCUSSION

The enumeration of foci by use of the statistically exact relationships established in the discipline of quantitative stereology (26) has the advantage of being limited only by the capacity of the data to represent the sample statistically. Number of focal intersections per sq cm of liver section, number of foci per cu cm of liver, mean diameter of foci, and mean volume of foci are all readily calculated without complications brought about by grouping focal intersections into size classes as required by available size distribution methods. For many experiments, the number of foci per cu cm of liver, the mean diameter of the foci, and the volume of the foci relative to the liver volume are adequate to be the basis for interpretation of the experimental results (16, 18).

In some experimental systems such as the investigation of the effect of promoters on the development of foci, information on the size distribution of foci per cu cm of liver provides a perception of the situation in the 3-dimensional space of the liver not provided by the mean values. Scherer et al. (23) have described in detail a method for calculation of the size distribution of foci and hence by summation the number of foci per cu cm of liver. They defined the size classes in such a way that each subsequent larger class of foci has an intersection area twice that of the preceding class. In the Saltykov method for computing the size distribution, the size classes are defined by a logarithmic scale of the diameters. Saltykov (21) and others have noted that small dispersed particles often fall into classes appropriate. More important for our use is the observation that the enzyme-altered foci in liver may be the result of clonal development (7) and thus could appropriately be represented by volume-related size classes. We have identified the size classes starting with Size Class 1, foci diameters of 50 to 63 μm. This is a size similar to the smallest size class (Size Class 1) described by Scherer et al. (23), which has about 5 cells on the focal intersection observed on the liver section. Because of differences in size class definitions, our Size Classes Nos. 2 to 25 are not the same as those of Scherer et al. These authors used the mean diameter of the size class in their equation rather than the maximum diameter in the size class used by Saltykov (21). The use of the mean diameter by Scherer et al. rather than the maximum diameter for the class results in the estimates of the number of foci per cu cm in the size class being 17% greater than if the estimates were based on the use

### Chart 6: DEN-phenobarbital regimen. The histograms show the distribution by group size classes of the focal intersections observed, the number of foci computed, and the focal volume fraction computed for the group of animals treated according to the DEN-phenobarbital regimen at the percentage levels of dietary phenobarbital shown.

<table>
<thead>
<tr>
<th>Sodium Phenobarbital in the Diet (%)</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Foci per cu cm of Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>Number of Focal Intersections per sq cm of Tissue Section</td>
<td>0.05</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**THE NUMBER OF FOCAL INTERSECTIONS OBSERVED IN EACH SIZE CLASS**

**THE NUMBER OF FOCI COMPUTED FOR EACH SIZE CLASS**

**THE FOCAL VOLUME FRACTION COMPUTED FOR EACH SIZE CLASS**

**Chart 7: DEN-phenobarbital regimen. The relationship is shown between the number of foci computed and the number of focal intersections observed with the dose of phenobarbital.**

---

H. A. Campbell et al.
of the maximum diameter of the size class. A comparison of the methods for estimation of size distribution of dispersed particles by Aaron et al. (1) showed that the methods of Scheil (22), Schwartz (24), Saltykov (20), and Johnson (12) have approximately the same precision and are in good agreement with one another. Our choice of the Saltykov method is based on the ease of computations of this method and a preference for the size classes of this method, the foci of each larger size class having twice the volume of the preceding class. The use of the size distribution method to estimate only the relative proportion of the foci in the size class, rather than the absolute number of foci, makes our analysis of foci comparatively insensitive to possible errors in the absolute values found in the size distribution method.

The number of cells comprising the foci can be estimated from the volume of the foci if an independent measurement of cell size is made. Alternatively, if it is established that the cell size of the foci is the same as the cell size of the normal tissue, then the proportion of cells exhibiting the abnormal phenotype is directly shown by the relative proportion of focal volume to the total tissue volume.

Such a relationship is also important if other parameters such as autoradiographic labeling or immunohistochemical properties distinctive for certain cells within each focus are to be measured within the cells of individual foci. In particular, Pugh and Goldfarb (19) have demonstrated that enzyme-altered foci exhibiting different phenotypes as determined histochemically show different levels of thymidine labeling of their nuclei. The foci resulting from injected cells (Chart 5) are predominantly of only one phenotype and thus presumably exhibit similar labeling indices among all such foci (14).

For the DEN-phenobarbital regimen, the number and size of the foci generally increase with increase in the dose of phenobarbital, as shown in Chart 7 (10, 17). The number of focal intersections observed per sq cm of tissue section has frequently been used by investigators to express their results. Enumeration of foci intersections per sq cm of section as a representation of results is limited by the fact that both the number of foci and the size of foci affect the number of intersections of the foci in the plane of section. Although limited in defining the number and size of the foci in the liver, the expression of results as the number of focal intersections per sq cm and the size of such intersections has the advantage of requiring little or no mathematical manipulation of the original observations. The marked increase in volume of enzyme-altered cells as a percentage of the liver space and the increase in the computed focal mean diameter with the increase in phenobarbital are markedly apparent when data are expressed per cu cm as in Table 3. It is recognized that the measurements of number of foci per cu cm of liver and the volume of foci as percentage of the liver have the characteristics of frequency or concentration measurements. Thus, if the foci increase in size with a concurrent increase in volume and weight of the liver, the number of foci per cu cm of liver will be decreased even though the number of foci in the liver as a whole is not decreased (Table 3). The small decrease in the computed number of foci in the liver at the 0.1% dose of phenobarbital as compared with the 0.05% dose may be due to experimental variation rather than a real decrease in the total number of foci at the highest phenobarbital level.

Of major importance is the demonstration in Chart 7 that in this particular instance the number of focal intersections observed in 2 dimensions is very different from the number of foci computed in 3 dimensions. The 2 curves differ markedly in the pattern of each relationship to the level of phenobarbital in the diet. Relative to the values observed with 0.0005% dietary phenobarbital, the foci per cu cm at 0.01 and 0.05% phenobarbital are 128 and 158%, respectively, while the increases in focal intersections per sq cm are 231 and 373%, respectively. This is in contrast to the data in Chart 5 where the foci from the various treatment groups have a similar mean diameter so that the number of foci per cu cm and the number of focal intersections per sq cm do not significantly differ relative to each other. This finding was mathematically predictable as was the marked relative difference in the number of foci calculated per unit liver substance and the intersections per unit area when the mean diameter of the lesions differed. In summary, these studies clearly demonstrate that the frequency and size of focal intersections seen on 2-dimensional tissue sections may not be a direct quantitative measure of the number or size of foci in the 3-dimensional tissue space. Only by quantitating the number and size of the foci in 3 dimensions can it be decided whether the 2-dimensional data actually reflect the relationship of number of foci to various independent variables.

ACKNOWLEDGMENTS
The authors wish to express their gratitude to Tom Goldsworthy and Sue Moran for their help in drawing and measuring the foci in sections from DEN-phenobarbital-treated rats; to Jane Weeks and Mary Foltz-Erbs for cutting and staining the sections used in these studies; to P. B. Rolfe, B’Ann True, and Mark Buckley for their technical assistance with the cell transplantation experiments; and to Dr. I. Riegel for assistance in the preparation of the manuscript.

REFERENCES


Application of Quantitative Stereology to the Evaluation of Enzyme-altered Foci in Rat Liver


**Updated version**

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/42/2/465

---

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.