A Method for Estimating Volume-Surface Ratios

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WITH THE CONTINUED DEVELOPMENT of cytochemical and histochemical techniques and their increasing use in research involving problems in pathology and microscopic anatomy, there is increasing need for more accurate and speedier methods of analyzing the quantitative morphological characteristics of cells and tissues. In 1943 Chalkley (*2*) proposed a new method for estimating volume percentages of morphologic components in fixed preparations, or under proper conditions, *in vivo*. This method depends on the fact that if points are thrown at random on a block of tissue the percentage of points lying in any morphologic component will, as the number of points is increased, approach the percentage of the tissue block accounted for by the individual component. Tests in several different laboratories (*1, 5, 6*) have confirmed the original expectation that the method would prove expeditious and accurate.

In the present paper an attempt is made to extend this method to obtain estimates of the volume-surface ratio of morphologic components such as cells or nuclei. There are good reasons *a priori* for believing that changes in this ratio may be correlated with different physiological states, but until a method of measurement is available no confirmation of this expectation is possible. The method to be proposed takes as its point of departure Crofton's remarkable results (*3*) showing that if an indefinitely long line be repeatedly placed at random over a plane containing a closed figure, the average length of the chord intersected by the figure will be π area/perimeter, no matter what the shape of the figure, so long as it is not reentrant.

This result requires modifications in several respects before it can be used for the purpose at hand: a) it must be made applicable to lines of finite length; b) it must substitute some simple procedure for the laborious and inaccurate method of measuring chord length; c) it must be extended to cover reentrant figures; d) it must be extended to provide an estimate not merely of the area-perimeter ratio in the focal plane under observation but of the volume-surface ratio in three dimensions of which the observed focal plane is a two-dimensional sample.[1]

[1] A related problem has been considered by Wicksell (*8, 9*). A large number of spherical or ellipsoidal corpuscles of different sizes are distributed in a body, split in two by a plane section. Wicksell expresses the distribution of the diameters of the corpuscles in the body in terms of the distribution of the measured diameters in the plane section.

The first three of these modifications are accomplished by a single simple device. If we use a line of finite length, say r, and count the number of times the two end points fall in the interior of a plane figure, denoted by h for hits, and the number of times the line intersects the perimeter of the figure, denoted by c for cuts, then in a very large number of throws $rh/c = \pi$ area/perimeter for all closed figures, including reentrant ones (Fig. 1). The fourth modification is provided by the mathematical result that, when a line of length r is placed at random in three-dimensional space containing a closed figure, in a very large number of throws $rh/c = 4$ volume/surface.

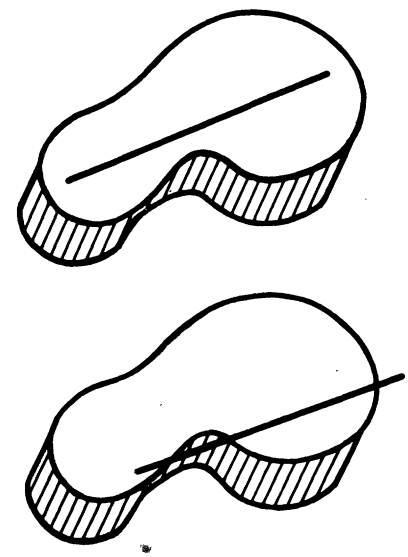

FIG. 1. Schematic representation of a randomly thrown line intersecting a closed three-dimensional figure. In the upper sketch the reading is two hits, no cuts; in the lower, 1 hit, 3 cuts.

This result satisfies condition *d*, since placing a line at random in three dimensions can be shown to be formally equivalent to placing a plane at random in three dimensions and placing the line at random on the resulting two-dimensional plane section.

If the space contains a series of figures of different volumes and surfaces, $rh/c = 4$ sum of volumes/sum of surfaces. As in two dimensions, this result applies whatever the shape of the figure, but covers reentrant as well as nonreentrant figures. Mathematical proofs are provided in a more complete discussion of this problem to be published elsewhere. It follows from this result that if a method can be found by which a

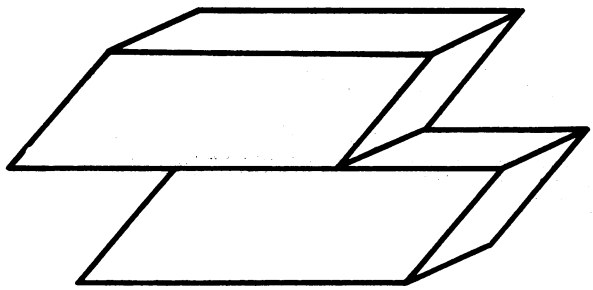

FIG. 2. Reentrant solid.

line of length r can be placed at random in a tissue and the hits and cuts on some specified morphological component counted, $\frac{1}{4}\ rh/c$ will yield an estimate of the average volume per unit of surface for that component.

Basic to the mathematical result is a particular definition of randomness. Other definitions, which lead to different results, are possible and cannot be excluded on purely mathematical grounds. Crofton's original result was criticized by Edgeworth (*4*) for this very reason. Although the definition used by Crofton and by us is the same as that used by Buffon in his solution of the needle problem (*7*), which solution has been empirically verified, a test of the method is nevertheless necessary.

For that purpose the following trial was conducted. Strips of balsa wood were sawed into geometrical figures of known shape. The figures we shall consider here are a cube $\frac{1}{2} \times \frac{1}{2} \times \frac{1}{2}$ in. and a reentrant figure found by taking two parallelepipeds $\frac{3}{4} \times \frac{1}{4} \times \frac{1}{4}$ in. and glueing one on top of the other to yield a figure of the shape shown in Fig. 2. The figures were dropped into a cardboard container filled with melted paraffin and talc and stirred. After cooling, the mixture was sliced into parallel sections, and all the resulting blocks were laid level, adjoining each other. Fig. 3 shows the resulting sections for the $\frac{1}{2} \times \frac{1}{2} \times \frac{1}{2}$-in. cubes. A needle was thrown onto the blocks 2000 times, and the number of hits and cuts made by the needle on the sections of the wooden blocks that appeared in the slices was recorded at each throw. After each 100 throws the sections were reshuffled. Fig. 4 shows the result for the cubes, Fig. 5 for the reentrant figure. In both cases the estimate of volume-surface ratio obtained differs from the known figure by less than 5%. This agreement appears satisfactory.

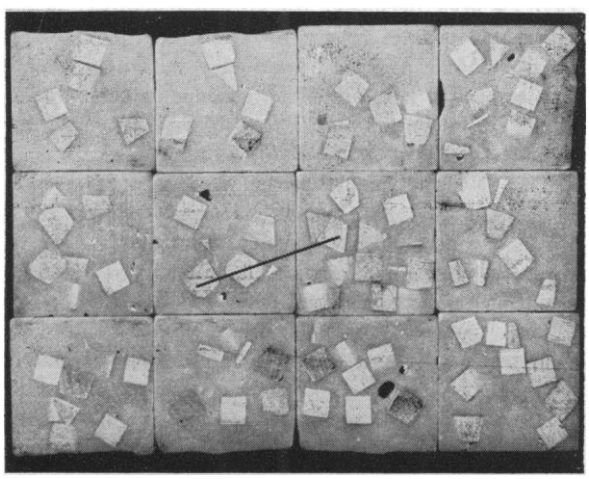

FIG. 3. Twelve parallel sections of $\frac{1}{2}$-in. cubes embedded in paraffin. The reading for the particular position of the needle is 2 hits and 4 cuts.

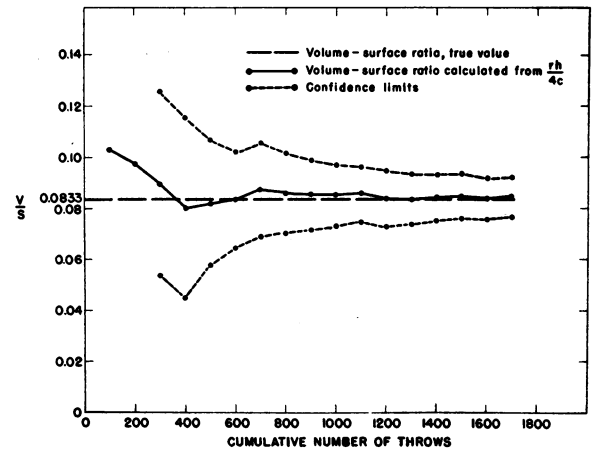

FIG. 4. Cumulative estimates from successive throws of volume-surface ratio for $\frac{1}{2}$-in. cubes.

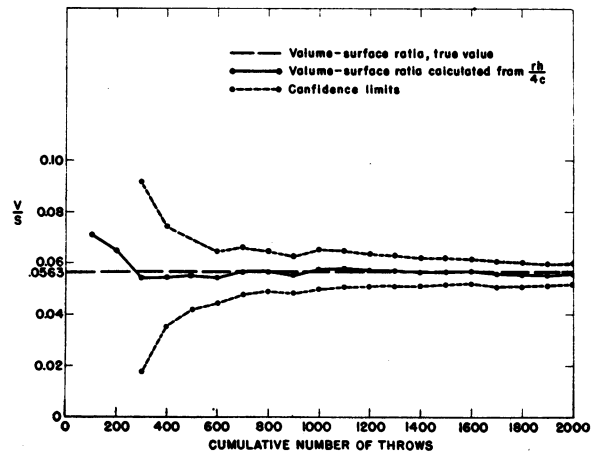

FIG. 5. Cumulative estimate from successive throws of volume-surface ratio for the reentrant figure.

The 95% confidence limits shown in Figs. 3 and 4 have been calculated from standard deviations computed from groups of 100 throws. They provide the basis for deciding when a sufficient number of throws has been made. Thus, after 2000 throws we can conclude, even in the absence of knowledge of the correct answer, that the correct value lies within 8% of the observed value with a probability of 0.95. After 1400 throws the correct value lies within 10% of the ob-

served value, and after 900 throws within 15%. Thus, by calculating error limits it is possible to decide at any point when sufficient accuracy for the purpose at hand has been attained.

To determine the amount of subjectivity involved in reading hits and cuts, two different observers independently recorded hits and cuts for the same 2000 throws on the reentrant figure. In all doubtful cases they recorded separately their first and second choices. In no case do the differences exceed 6% (Table 1).

TABLE 1

ESTIMATES OF A TRUE RATIO OF 0.0563 INDEPENDENTLY OBTAINED BY TWO OBSERVERS FROM THE SAME 2000 THROWS

Observer	Estimated $\frac{V}{S}$ based on	
	Use of first choice	Use of second choice in doubtful cases
A	0.0584	0.0572
B	0.0557	0.0550

In applying the present method to microscopic sections, the major problem is in achieving randomness. When the morphologic components under study are not oriented, or when they are approximately spherical, the technique used by Chalkley for achieving randomness (*2*) should be satisfactory. For this purpose a reticle with a line of suitable length engraved or otherwise inscribed thereon is substituted for the five pointers used in the method referred to. The manipulation is identical but cuts as well as hits are observed and recorded. Of course, the length of the visual image of the line in the focal plane must be measured for the particular magnification used, and treated as the contant r in the calculations. When the components are oriented and are not approximately spherical, however, parallel sections cut through the tissue will not provide random sections. In such cases special precautions will be needed to insure randomness.

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TECHNICAL PAPERS

The Enzymatic Hydrolysis of Chloramphenicol (Chloromycetin)[1]

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With the development of the new antibiotic chloramphenicol, (chloromycetin) (*1, 3, 6*) and identification as D(-) threo-1-*p*-nitrophenyl-2-dichloracetamido-1, 3-propanediol (*2, 4, 5*), experiments were undertaken to determine if this compound could be inactivated by enzymatic hydrolysis of the amide linkage present in the compound. This linkage appears to contribute substantially to the bacteriostatic properties of chloramphenicol.

In the initial series of experiments with *E. coli*, *B. mycoides*, *P. vulgaris*, and *B. subtilis*, it was found that chloramphenicol could be hydrolyzed when introduced into actively growing broth cultures of these organisms. The decrease in bacteriostatic potency of the filtrates from these cultures was followed by means of a microbiological assay method for chloramphenicol using *Shigella sonnei* (*6*).

The organisms tested showed considerable variation in ability to hydrolyze the antibiotic. *P. vulgaris* and *B. subtilis* proved to be the best source of the enzyme. Cultures of these organisms possessed approximately twice the enzymatic activity of *B. mycoides* cultures and about three times the activity exhibited by *E. coli* cultures.

The results obtained, using actively growing cultures, did not indicate whether the enzyme destroying the antibiotic was an extracellular or endocellular enzyme. When filtrates from 24-hr broth cultures of the organisms were incubated for 24 hr at 37° C with chloramphenicol, no hydrolysis of the antibiotic was observed. However, filtrates from cultures 2–4 weeks of age actively destroyed chloramphenicol and a fairly active preparation of the enzyme could be obtained by concentration of the filtrate. The enzyme is therefore liberated into the culture medium. The enzyme could also be detected in suspensions of washed bacterial cells and in the autolyzates prepared from the washed cells.

[1] Trademark Parke, Davis & Company.

The enzyme involved in the hydrolysis of chloramphenicol has been tentatively designated as enzyme A to distinguish it from the other enzymes which might be involved in the enzymatic destruction of the antibiotic through attacks on other groups present in the molecule. The action of the enzyme is to hydrolyze the amide linkage of chloramphenicol and thus liberate the corresponding basic amine and dichloroacetic acid, which have been isolated and identified by chemical means from the enzymatic digests of chloramphenicol.

The optimum conditions for the enzymatic hydrolysis of chloramphenicol have been found to be pH 7.5 and a temperature of 37.5 to 40.0° C. The rate of enzymatic hydrolysis increased with substrate concentration up to 2 mg per ml. This is approximately the maximum solubility of chloramphenicol under the test conditions.

One unit of enzyme activity has been arbitrarily chosen as that amount of the enzyme which will hydrolyze 1 μg of chloramphenicol in 1 hr at pH 7.5 and 37.5° C.

Initial studies on the properties of this enzyme involved in the hydrolysis of chloramphenicol indicate that it is probably very similar to other proteolytic enzymes that have been isolated from bacterial cells. Moreover, this enzyme is neither a true papain nor a trypsin, since highly active preparations of these latter enzymes do not hydrolyze chloramphenicol to any great extent. The enzyme appears, however, to be activated by cysteine and other reducing agents, so that it is probably more closely related to the papain group than to the trypsin group of enzymes. Crystalline preparations of pepsin and chymotrypsin were found to be inactive on chloramphenicol.

Further studies on the distribution and properties of this enzyme are in progress and will be reported later.

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Absorption Spectrum of Beta Carotene in Liquid Solution at the Temperature of Liquid Nitrogen[1]

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In a recent paper in another journal (*1*), we describe solutions of salts fluid at the temperature of liquid nitrogen. One of the solvents employed consists of one part in ten of *n*-propyl alcohol and the remainder is equally divided between the liquids propane and propene.

[1] This document is based on work performed under Contract Number W-7405 eng 26 for the Atomic Energy Project at Oak Ridge National Laboratory.

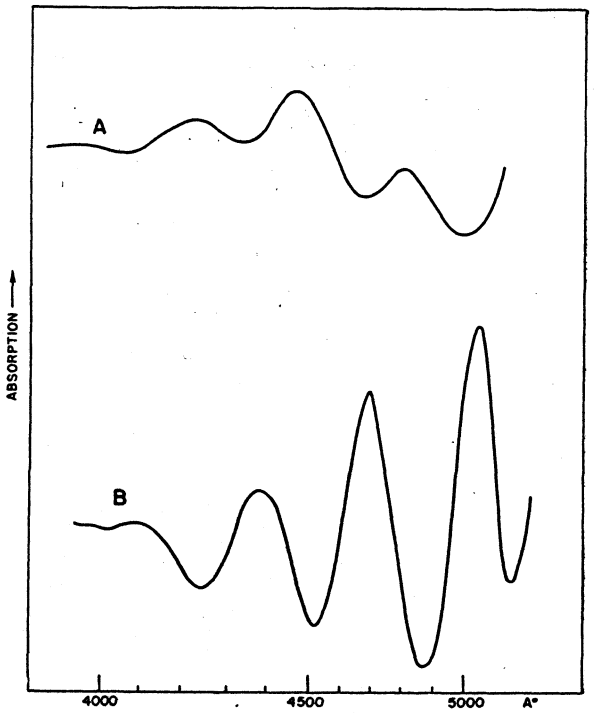

FIG. 1. Absorption spectra of carotene (90% alpha and 10% beta). A—In heptane at room temperature; B—In equal volume mixture of propane and propene at −196° C.

To obtain as refined a spectrum of a solute as possible, it is recognized that solvents such as alcohol and ether are to be avoided, since they possess permanent electric dipole moments, and they would be expected to distort the solute molecules. For this reason, hydrocarbons such as hexane frequently serve as solvents.

Our mixed solvent without alcohol, namely, a solvent consisting only of liquid propene and liquid propane in equal volumes, proved to dissolve carotene rather rapidly at about −50° C and maintain it in solution at −196° C in sufficient concentration to give an absorption spectrum in a path length of 25 cm.

The spectrum at −196° C, as may be seen from the traces, differs markedly from that of carotene at room temperature dissolved in heptane. The number of absorption peaks seems to be the same, but their relative intensities differ greatly. They are much sharpened and are in general displaced toward long wavelengths at the low temperature.

On lowering the temperature of the solution in propane-propene from −80° C to −196° C, changes consistent with these took place.

The solute consisted of 90% beta carotene and 10% alpha carotene.

We are much indebted to Dr. Martin Kuna, of our Biology Division, not only for some biologically important substances but also for a discussion of great value to us on their chemical and spectroscopic properties.

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