A Critical Evaluation of Quantitative Histo- and Cytochemical Microscopic Techniques

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THE DEVELOPMENT OF MODERN TECHNIQUES for histo- and cytochemical investigations has placed new tools of wonderful keenness in the hands of medical and biological scientists. The understandable enthusiasm for these fine instruments has, in some instances, been overextended to the point where they have been applied to problems for which they are not fitted, or the results obtained have been interpreted incorrectly through a lack of understanding of their range of reliability. This has happened particularly in the case of microscopic techniques in contrast with the quantitative chemical methods of colorimetry, titrimetry, gasometric analysis, etc., which yield clearly defined histo- and cytochemical data that are much less apt to be misinterpreted. It is our purpose to point out the utility and the limitations of general procedures and approaches that are being increasingly used and misused, in connection with certain of the histo- and cytochemical techniques. These techniques comprise some of the most serviceable and most elegant yet devised, but the keener the tool and the finer the dissection possible, the easier it is to cut one's fingers.

Errors from Preparation of Material

Fixation. Since it is a goal of histo- and cytochemistry to localize and quantitate biologically significant substances and activities, the preparative treatment of the material to be investigated must be considered with respect to its effect on both the localization and quantitation. A very effective way to hold the cellular sample in its original state with regard to these factors is to freeze the material suddenly at a very low temperature immediately upon removal from its source; this can be readily accomplished by immersing the sample in isopentane cooled almost to its freezing temperature (−160° C) by liquid nitrogen. In contrast, the use of fixing solutions cannot as a rule be expected to yield material that has escaped morphological dislocations or concentration changes of chemical constituents. Possibly certain morphological studies might be accurately carried out by subjecting fresh-frozen tissue sections to gaseous fixatives, such as osmic or formaldehyde vapors, while the sections are held at subfreezing temperatures. The use of fixing solutions in studies claiming to reveal true localizations and to yield quantitative data must be considered critically in any specific instance. Although the necessity for fixation in many cases with present techniques is recognized, it would be preferable to avoid fixation whenever possible, and efforts should be directed to improving techniques for observations on living material.

Dehydration and embedding. That artifacts and change with respect to cellular topography and chemical composition result from the use of dehydrating liquids on fixed material is probably too well known to merit discussion. The vacuum dehydration of deep-frozen tissue will avoid many of these artifacts and changes.

Unfortunately, the thin-sectioning necessary for the proper observation of morphology requires the use of an embedding medium. The use of any such medium will obviate to some degree the advantages of the freezing-drying preparation, since it involves the introduction of a liquid phase that may cause dislocation and loss of certain constituents. This danger recurs in subsequent removal of the medium from the cut sections with a solvent. The difficulty may be minimal, as in the case of protein structures when paraffin and xylol are employed, or maximal, as in the case of fat structures when these agents are used. The problem could be avoided if a technique were developed that permitted good sectioning, at a suitable thinness, of fresh-frozen tissue, followed by dehydration of the still-frozen sections. Microtoming with a glass knife, which is particularly suited to thin-sectioning (*1*), in a subzero room or in a cryostat cabinet containing dehydration equipment, might accomplish this end, and an investigation of this possibility is now under way.

Separation of cellular particulates. The segregation of morphological constituents in cells by centrifugation is being used widely to obtain material for studies relating chemical composition and function to cytological architecture. The separation methods involve the mechanical disruption of cells, followed by differential centrifugations and washings in either aqueous saline or citric acid solutions, or in nonaqueous liquids (e.g., benzene–carbon tetrachloride mixtures). Accordingly, the extraction effect of the medium, as well as its chemical influence on the structural components, must be determined. Control experiments designed to test for such effects should be carried out to determine the reliability of any given procedure. Then, in addition, the possibility of removal of morphologically associated activators and inhibitors of particular chemical reactions during the treatments must also be considered, and appropriate tests, such as recombination of various fractions, should be employed to establish whether these effects are operative.

Of course it is generally true of all the methods of

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preparing biological material that problems are posed by liquid treatments and cellular disruptions. Not only are extraction and separation of chemically significant components involved, but also the question of adsorption on interfaces, which either does not occur in the normal living cell but results when the organization of the cell is altered or disrupted by laboratory treatments, or vice versa. For example, does the mast cell granule stain metachromatically with toluidine blue because it contains heparin intrinsically, or because the substance is adsorbed on the granule surface from the cytoplasm as a result of the preparative treatment? Furthermore, does the fact that most of the heparin is present in the final supernatant after larger particles and microsomes are spun out (*2*) result from extraction of the heparin from particulate matter during the laboratory treatment, or because the compound is dispersed in the interparticulate phase *in vivo*? It is thus imperative to make interpretations sufficiently liberal to encompass all possibilities until more definite information is at hand.

It was pointed out by Potter (*3*) that a test can be made for contamination of separated particulates by other cellular material. Use is made of the principle that different particulates can be characterized by their content of specific substances or activities—e.g., desoxypentose nucleic acid for nuclei. Thus, the presence of desoxypentose nucleic acid in mitochondria would be taken to indicate contamination by nuclear or whole-cell material. In setting up biochemical criteria by which a particulate is to be characterized, it would contribute to reliability to establish by a method other than one involving centrifugal separations from homogenates that the biochemical properties in question are confined to the given particulate. This has been done in the case of desoxypentose nucleic acid in nuclei, since evidence for this localization has come not only from centrifugal separations but also from Feulgen studies and ultraviolet absorption observations. The possibility remains that chemical components other than the one or more tested may contaminate the particulate by some means, such as selective adsorption during the cellular homogenization, so that a morphologically pure fraction may still be biochemically impure with respect to the *in vivo* composition.

Errors in Chemical Staining Techniques

The use of staining reactions in histo- and cytochemistry contributes to the greatest volume of published work in the entire field. Both anatomists and pathologists in particular have been quick to appreciate the manifold advantages of endowing the ultimate biological structures with the significance of their chemical constitution and function. It might be of interest to set up some warning posts along the diverse paths that cross this area.

Specificity. The chemical specificity of any staining reaction must be clearly defined for it to be of service rather than disservice. That certain chemical groups are responsible for certain staining reactions is quite often beyond controversy. The difficulty arises when different chemical groups react with the same reagent —e.g., as in silver stains—or when the same reactive group occurs in different compounds—e.g., aldehyde visualized by Schiff's reagent. In such cases additional evidence must be presented to establish the claim that a stain has resulted from the occurrence of a particular compound or class of compounds. When this evidence is insufficient, no end of discussion is apt to develop concerning the relative merits of one possibility or another. It would be better to reserve judgment in these cases until positive conclusions can be justified. Some examples of the difficulties which develop when the chemical specificity of stains is open to variable interpretations may be found in a recent paper by Gomori (*4*).

Stoichiometry. It is obvious that the quantitation of a cellular substance *in situ* by a staining reaction requires that the reaction be a stoichiometrical one. The following consideration is given to the chances of achieving this end.

A staining reaction can be expressed by the following equation:

$$D + S \rightleftharpoons DS,$$

where D is the dye, S its substrate, and DS the stained substrate. It will be assumed that the stain is specific so that there is only one substrate for D. Applying the law of mass action one has:

$$\frac{(DS)}{(D)(S)} = K.$$

If K is very large the reaction can be written as follows:

$$D + S \rightarrow DS;$$

i.e., it is irreversible. In this case all of S will react with D to give DS, as long as D is present in excess. The excess D can be removed by washing without dissociating DS. Thus, the amount of DS, which is the quantity that is determined photometrically, will be directly proportional to the original amount of S. This would be the ideal case for a quantitative reaction.

If K is not very large, the following relation holds:

$$(DS) = K(D)(S).$$

It should be pointed out that this does not mean that (DS) is directly proportional to the initial concentration of D and the amount of S present in the section, since (D) and (S) are equilibrium, not initial, concentrations. The discussion will be simplified if it is assumed that (D), initial, is very great, so that the reaction with S will not appreciably decrease it—i.e., $(D) \simeq (D)_i$. We then have:

$$(DS) = K(D)_i(S) = K'(S).$$

Since $(S) = (S)_i - (DS)$, it follows:

$$(DS) = K'[(S)_i - (DS)], \text{ or}$$

$$(DS) = \frac{K'}{1+K'}(S)_i.$$

This equation shows that, under the conditions assumed in the derivation, (DS) is directly proportional

to $(S)_i$. It would then seem that a reversible reaction would be also satisfactory from a quantitative standpoint, if only the dye were present in a large excess. There is, however, one serious complication: the excess dye must be removed by washing, and even under standard conditions it will be difficult to attain a final (DS) that reflects the initial amount of S.

Although a strongly irreversible reaction would be ideal, a reversible reaction would be satisfactory if the dye were present in large excess, and if K were still relatively large. Other limiting factors, such as adsorption, diffusion, and imperfect accessibility of reagents to particular sites because of structural barriers, also contribute to the impossibility of satisfying the conditions for quantitation of a reversible reaction *in situ*. These factors can operate even in the case of an ideal irreversible reaction, and the aberrations arising from them are, for the most part, indeterminate. Hence, the chances for reliable quantitation by staining reactions are rigidly limited, although not necessarily eliminated, even when the reaction meets the requirements of specificity, and stoichiometry is obtained in the test tube.

Many of these difficulties are likewise encountered in purely morphological studies, in which the requirements for reliability are less rigorous. In this connection Gomori (*5*) and Novikoff (*6*) have discussed some of the errors involved in the special case of enzyme-staining.

Chemical composition. When a chemically complex substance, such as a protein, is to be determined by the measurement of one of its constituents, such as tyrosine, it is assumed that the constituent represents a constant fraction of the complex molecule. It cannot be assumed, however, that the composition of the complex substance in one cell or body fluid is identical with that to be found in other anatomical compartments in the same organism. Thus, the proportion of tyrosine in a serum protein cannot be taken to be the same as that in the protein of a liver cell nucleus unless experimental proof is supplied. In the case of tyrosine, its content in serum globulin has been reported as 6.2 per cent, in serum albumin 4.8 per cent, and in liver nucleoprotein 3.6 per cent (*7*). Of course all the tyrosine need not be in protein, and this too should be taken into account.

Another variable to be considered with respect to chemical composition is the effect of the degree of polymerization of certain dyes when bound to certain substances (*8–10*). This process will change the absorption spectrum of the dye, the magnitude of the change depending on the degree of polymerization. The latter will depend on the relative amount of the dye with respect to the amount and chemical nature of the substrate. The first factor will result in a wavelength shift that will vary with the substrate concentration, and thus large deviations from Beer's law will result. A metachromatic reaction would, therefore, not be suitable for photometric analysis in the usual manner—i.e., absorption measurements at a fixed wavelength. It is possible that some quantitative information might be obtained by measuring the degree of metachromasia—i.e., the magnitude of the shift of the absorption maximum, since this depends on the degree of polymerization, which in turn bears some relation to the substrate concentration.

Errors in Microspectrography

The physical errors occurring in the measurement of any kind of electromagnetic radiation used for analytical purposes can be grouped into two main classes: errors in the instruments and measuring technique used, and errors due to certain properties of the objects to be analyzed. When electromagnetic radiation is used for histo- and cytochemical analysis, the two groups of errors are considerably more difficult to master than in the case of absorption measurements on a liquid in a spectrophotometer. The theory and design of proper instrumentation for quantitative cytochemical analysis have been described for ultraviolet and visible light by Caspersson (*11, 12*) and Thorell (*13*), and for x-rays by Engström (*14*). A general treatment of optical methods used in biology appears in the *Transactions of the Faraday Society* (Sept. meeting [1950]). In the present survey only physical errors inherent in the biological object will be discussed.

Validity of the Lambert-Beer law. In biological systems such as parts of cells or tissues, there are areas having relatively high absorptions of radiation, which may be either natural or induced by staining. The first question that arises when radiation is passed through a sample for analytical purposes is whether the Lambert-Beer law holds. In other words, is the extinction of the radiation proportional to the product of concentration and thickness of the absorber? For many substances the Lambert-Beer law can be tested in macromodel systems. The unique conditions existing in thin slices of tissue having a high absorption make such macromodel tests less applicable. The test for the validity of the Lambert law can, for present purposes, be performed by measuring the absorptions by sections of different thicknesses of the *same homogeneous* tissue or cell.

The effect of the orientation of the molecules on the absorption has been brought up as a cause of deviation from the Lambert-Beer law, especially when the amount of nucleic acid is determined at the absorption maximum of 260 mμ (*15*). The reason for the deviation is that light incident to the absorber will be absorbed differently, depending upon the planes of polarization in relation to the orientation of the molecules. It has recently been shown by Ruch (*16*) and Ruch and Thorell (*17*) that the dichroism of fibers prepared from a pure solution of desoxyribonucleic acid is very high; extinction values of over 3 have been recorded. If the nucleic acids were oriented to that degree in tissues, large deviations from the Lambert-Beer law would result when nonpolarized radiation is used for the absorption measurements. However, in structures where a strong orientation might be expected, such as in giant chromosomes from *Chiro-*

nomus or *Drosophila* and in muscle fibers, the actual ultraviolet dichroism observed is low, and the error is negligible when nonpolarized light is used for the absorption measurements.

Relative homogeneity of absorbing material. Other, greater errors occur in the absorption measurements. One of the most intriguing properties of the cell, or of biological tissue in general, is the inhomogeneous distribution of absorbing substance. When parallel x-rays are used for the estimation of cellular components, the inhomogeneities along the beam are superimposed, and thus only the inhomogeneities in the plane perpendicular to the beam have to be considered. When a microscopical system is used for micromeasurements of absorption, the situation is much more complicated because of the biconical shape of the beam. The actual depth of focus in a high-power optical system is of the order of magnitude of about 0.5 μ. Thus, inhomogeneities along the axis of the beam must be considered also, if histological sections of the usual thicknesses are used.

A simple estimate of the effect of the inhomogeneous distribution of an absorbing substance in one plane perpendicular to the beam gives the following result: The area under analysis is assumed to be unity, and the intensity of the incident radiation (I_o) is uniform over this area. If there is a homogeneous distribution of absorbing material of concentration c and thickness b, the intensity of the transmitted light (I) can be calculated from the Lambert-Beer law:

$$I = I_o e^{-kcb}, \text{ where } k \text{ is a constant.}$$

If, instead of being uniformly distributed, the absorbing material is concentrated in a fraction, $\frac{1}{q}$, of the total area, there will be an area $(1-\frac{1}{q})$ that is nonabsorbing. The following expression then gives the intensity of the transmitted light:

$$I_q = I_o(1-\frac{1}{q}+\frac{1}{q}e^{-kcqb}).$$

In practice the concentration of absorbing material is assumed to be proportional to the optical density. The magnitude of the error due to nonhomogeneity is therefore apparent if the ratio of the optical densities in the two cases (D and D_q) is calculated:

$$\frac{D}{D_q} = -\frac{0.434\,kcb}{\log\,(1-\frac{1}{q}+\frac{1}{q}e^{-kcqb})}$$

Fig. 1 gives the value of this ratio as a function of q for a number of different values of kcb. The ordinate tells how many times too small the concentration calculated from the absorption measurement is, compared to the true concentration. It is obvious that for any type of accurate absorption measurement the area under analysis must be quite homogeneous. One way to get greater accuracy is to carry out the measurements at a very high resolution and integrate them over the area being analyzed. In the determination of the amount of absorbing substance in a nucleus, for example, this procedure of arriving at a relatively correct value is exceedingly time-consuming. If a whole nucleus is measured, the errors depending on the inhomogeneous distribution become greater. In fact, all measurements of biological objects that are more or less inhomogeneous have a greater or smaller error that can be roughly estimated. The important point is that the measurements contain an inherent error that must be taken into account when interpreting the results.

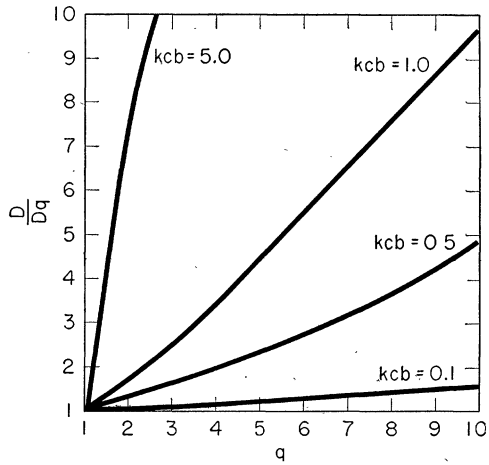

FIG. 1. Ratio of the optical density in a homogeneous optical field (D) to that in an inhomogeneous field (Dq) as a function of the degree of inhomogeneity, in terms of the number of times (q) reduction in the area containing the same amount of the radiation absorbing material.

Range of extinction coefficients. In some papers dealing with absorption measurements of biological material, extinctions as high as 2 (i.e., 1 per cent transmission) have been reported. If the error in estimating the transmission is assumed to be constant, the error in concentration will vary depending on the exponential absorption of the light, provided the Lambert-Beer law holds.

The following formula has been derived by Ringbom (*18*):

$$\frac{\frac{100\ dc}{c}}{dI} = -\frac{43.4}{\rho I}.$$

This expression gives directly the relative analytical error caused by an absolute photometric error of 1 per cent, when I_o is set at a transmission of 100 on the galvanometer scale. The magnitude of this error as a function of the transmission is given in Fig. 2. The curve in Fig. 2 is obtained on the assumption that dI is 1 per cent over the entire transmission range. Since the value of dI may vary with the instrument used, and even with the same instrument if a switch is included to change the range of sensitivity, as in the Beckman spectrophotometer, the curve in Fig. 2 must be interpreted accordingly. For example, if dI at a given transmission value is 0.5 per cent, the error would be half that indicated in Fig. 2. The best results in absorption measurements will be obtained in the range of 20–60 per cent transmission, which corresponds to an extinction range of 0.7–0.2.

A discussion of photometric error has appeared in recent papers by Cole (*19*) and Robinson (*20*), and a subsequent note by Cole and Robinson (*21*) indicates areas of agreement and a correction of some overgeneralization in the paper of Cole (*19*).

Nonspecific losses of radiation. A factor that complicates many absorption measurements is the scattering of radiation. In the x-ray region the scattering is only a fraction of a per cent of the absorption and therefore completely negligible. In the visible and ultraviolet region, however, the increased scattering can be a highly complicating factor when quantitative measurements of biological material are desired. There are very great difficulties in estimating the radiation scattering in parts of cells, etc. (*22*). Attempts at applying Rayleigh corrections in the ultraviolet region, based on measurements at longer wavelengths, cannot be very successful, since the magnitude of the exponent of the wavelength cannot be predicted. It is also difficult to estimate the proportions of the inherently reflected and scattered radiation.

Thickness of radiation absorbing structure.[2] Finally, another complicating factor should be mentioned. Depending on the different methods used to prepare samples for microspectrography, the thickness of the absorbing layer may vary from point to point in the sample. In comparing amounts of substances in different parts of cells the thickness must be known. At present there is no easy method for a very exact determination of the thickness of ordinary histological sections. The usual technique of focusing with a microscope on the upper and lower surfaces may not be reliable, since the movement of the tube is not a linear function of the fine screw motion in many microscopes. For a high-power optical system, NA 1.30 and a total magnification of 1,000, the depth of focus is about 0.5 μ (*24*). In estimating the thickness of a section, the maximal error in this case would then be 1.0 μ. The error can be diminished by performing a great number of measurements and treating the results statistically.

Interference methods have also been proposed for measuring the thickness of microtome sections. For that purpose flat surfaces are required, and these are found only in the tissue-free areas of embedded sections. The thickness of the section at a tissue-free area, however, may not be the same as that at the point where the structure to be investigated is found. For structures lying near the edge of the section and adjacent to the tissue-free edge of the embedding medium, the method may be applicable. Furthermore, absorption measurements of x-rays, α-rays, or β-rays may be used in a tissue-free area of the section for the determination of thickness, but these methods will have the same limitations as the interferometric procedure.

Shadow-casting at oblique angles can be used for the determination of the thickness of a specimen close to the edge of a section, but not for structures near the center. For isolated cells or structures, as in blood smears and squash preparations of chromosomes, shadow-casting can be utilized for the determination of thickness. However, the necessity of performing the shadow-casting in high vacuum excludes the use of the method for living cells.

Thickness measurements may be avoided altogether if the results of absorption measurements are referred to dry weight per unit area, as determined by x-ray absorption.

This discussion has dealt only with physical errors in microspectrography, but one important source of error of a chemical nature should be pointed out, namely, the error resulting from the effect of certain radiations on cellular compounds to be analyzed (*25*, *26*). For example, ultraviolet absorption by solutions of pentosenucleic acid decreases with time because of the breakdown of the molecules by radiation. In any given case it would be necessary to establish that the time of exposure to the radiation during measurement was such as to confine the error to an allowable known range.

There is an attitude prevalent in some circles that, although spectrophotometric measurements on stained tissue admittedly cannot give data regarding absolute quantities of chemical constituents in given cases, they still may offer reliable "semiquantitative" or relative values. This would be acceptable if the order of magnitude of the error were established by objective means and proved to fall within justifiable limits.

From the foregoing it is apparent that uncertainties exist in both the attempts at localization and quantitation of chemical constituents in cells and tissues. Errors arise from the artificial conditions imposed by fixation, dehydration, and embedding and staining procedures, as well as from those attending the techniques used for the separation of cellular

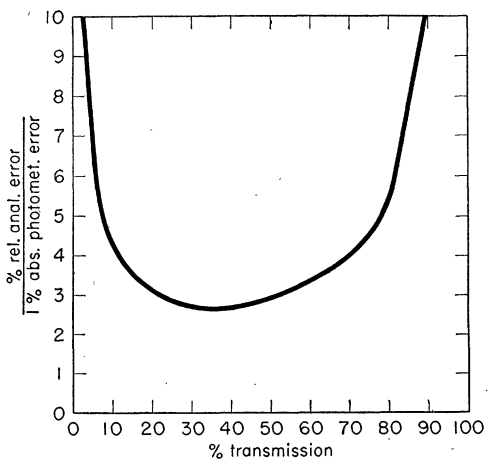

FIG. 2. Relative analytical error for an absolute photometric error of 1 per cent as a function of the percentage of transmission.

[2] After this manuscript was submitted for publication a note by Glimstedt and Hakansson (*23*) appeared on the mechanical measurement of thickness in histological sections.

particulates, but within limits these errors can be minimized. The questions of specificity, stoichiometry, and variable chemical composition as applied to staining reactions must also be considered with respect to the errors of quantitation *in situ* that may result from these factors.

With reference to errors inherent in microspectrography as practiced in histo- and cytochemistry, the validity of the Lambert-Beer law does not appear to be threatened by the degree of orientation of the molecules in most biological materials. However, the relative inhomogeneity in the distribution of absorbing substances introduces an error that can be significant. Reliable measurements are possible only in a well-defined extinction range, and nonspecific losses of radiation in some cases can be serious in the visible and ultraviolet region. The determination of the thickness of a cellular sample for analytical purposes likewise presents difficulties, and the chemical effects of radiation used for absorption measurements cannot be ignored.

From these considerations, it appears unwise to accept, too literally, the localizations of chemical substances in finer cellular structures as indicated by staining reactions. Furthermore, little reliability can be expected from attempts at quantitation *in situ* by means of microspectrography of stained structures in much biological material. Reliable localizations of chemical substances can be obtained in unstained material by microspectrography in the ultraviolet and x-ray regions. However, the errors of inhomogeneous distribution of the radiation-absorbing substance, nonspecific radiation losses, and chemical effects can gravely interfere with quantitative work in the ultraviolet. X-ray absorption, as it has been employed, is affected less by these factors, and its use can yield reliable quantitative data, as well as true morphological patterns.

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Technical Papers

Oxygenation of Blood by Isolated Lung

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Recent progress in intracardiac surgery has necessitated the diversion of blood from that side of the heart, usually the left, involved in the operative field. This has been accomplished by the use of mechanical pumps (*1*), which have short-circuited blood from the area. However, future advances in major intracardiac operative procedures—i.e., repair of septal defects—may well involve the diversion of blood from all chambers of the heart (except that to the coronary vessels). Although most of the technical difficulties governing the artificial propulsion of blood have been overcome, that of insuring adequate oxygenation persists.

Previously proposed methods of oxygenating blood *in vitro* in an extracorporeal blood circuit involving either mechanical mixing of blood with oxygen (*2*) or intravenous introduction of chemical oxidants (*3*) have proved unsatisfactory.

Although older isolated heart-lung preparations as a rule operated far below physiological efficiency, pulmonary oxygenation was well maintained. It occurred to us, therefore, that an isolated donor lung, functionally attached to a mechanical propulsive unit, would successfully oxygenate venous blood pumped through it without the disadvantage of the foaming or hemolysis of red blood cells associated with the use of artificial oxygenators.

To verify this point, the heart and lungs of a cat were removed following death by exsanguination. No attempt was made to maintain sterility of the operative field. As previously described by one of us (*4*), the pulmonary artery and veins of one side were

[1] The authors wish to express their appreciation to A. S. Buchberg for his technical aid.