SOME OBSERVATIONS ON THE PHOTOMETRIC ESTIMATION OF MITOCHONDRIAL VOLUME*

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INTRODUCTION

It is now well established that various cytoplasmic granules are bounded by semi-permeable membranes (see, *e.g.*[1-5]). The importance of such semi-permeable membranes is indicated by the well-known observations (see, *e.g.*[6-9]) that the enzymic activity of cytoplasmic granules varies with the tonicity of the medium. These findings may be interpreted to mean that the membranes affect the accessibility of the enzymes, substrates, and cofactors. Conceivably, in normal cellular metabolism, such membranes aid in maintaining the optimal steady-state level of cofactors, intermediates, or products at particular sites. Indeed, one suspects that they may play a general role in regulating the rate of various intracellular enzyme systems and in directing the metabolic fate of particular substrates. A precise evaluation of these roles would be greatly aided by a knowledge of the permeability of the membranes.

Of the several methods available for the investigation of mitochondrial permeability, the photometric method seems the most convenient. Introduced in the classical studies of cell permeability[10,11], it greatly facilitated the investigation of the kinetics of exchange processes. The technique was later extended to the study of the osmotic behavior and permeability of isolated subcellular particles: pigment and yolk granules of the sea urchin egg[1], rat-heart sarcosomes[2], and rat-liver mitochondria[12,3]. The photometric method is particularly advantageous since: a) it does not demand complex instrumentation; b) it permits the application of well understood osmotic principles in experiments of simple design; and c) most significantly, it permits the investigator to deal with the high rates of exchange which result from the large surface area of mitochondria.

Although photometric methods have been widely used in studies of mitochondria (see, *e.g.*[1-3, 12-23]) and important data of a qualitative or semiquantitative nature obtained, the full potentialities of the technique have not been realized, and, in some instances the data have been difficult to interpret. These deficiencies stem from our incomplete knowledge of: the quantitative dependence of the optical density of the suspensions on the volume of the particles and on other variables; the limitations of the technique; and the difficulties one is likely to encounter. The present paper will be concerned primarily with these problems.

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METHODS

Many details of the experimental procedure have been described earlier[3]. In principle, livers of starved male rats (250–350 g) of the Sprague-Dawley strain were homogenized in a medium consisting of 0.25 *M* sucrose, 0.02 *M* potassium phosphate and 0.02 *M* sodium ethylenediamine tetraacetate (versene) at pH 7.5. After removing readily-sedimentable fractions by centrifugation at 900 *g* for 15 min, the mitochondria were isolated by centrifuging at 8500 *g* for 10 min. The resultant pellet was resuspended in a medium appropriate to the experiments. The suspensions were maintained during the experiments at 20 ± 1°C.

In a typical experiment, 10 ml of an experimental solution were added rapidly to 0.5 ml of suspension. Special care was necessary to avoid mixing artifacts in dealing with very viscous solutions; in these cases, mixing was aided by stirring rapidly with a glass rod. Samples which did not appear homogeneous, either because of the presence of air bubbles or because of incomplete mixing, were discarded. At suitable times, the optical density of the experimental suspension was read at 520 mμ or some other specified wavelength in a Coleman Junior Spectrophotometer. Further details of the individual experiments will be found in the legends of the corresponding figures.

It was frequently essential to measure the refractive index of solutions. This was done with white light at 18–22°C with a Zeiss immersion refractometer.

EXPERIMENTAL

The dependence of optical density on osmotic pressure

In a previous paper[3] it was shown that mitochondria obey osmotic law (eqn. (1))

$$V = K\frac{1}{c} + b \tag{1}$$

although leakage or lysis probably occurs in very dilute solutions. From measurements of mitochondrial diameters from photomicrographs the volume (V) was calculated and the constants K and b evaluated. In this experiment K was found to have a value of 0.032 osmolal μ^3 while b, the so-called osmotic dead space, was found to be 0.085 μ^3. The reproducibility of the constants was not established.

It was further shown that an empirical relation (eqn. (2)) exists between the

$$\Phi = \kappa\frac{1}{c} + \beta \tag{2}$$

reciprocals of: optical density (1/O.D. = Φ), and solute concentration ($1/c$). The empirical constants κ and β, may be evaluated graphically (see, for example, Fig. 1). Eqns. (1) and (2) may be combined to give eqn. (3), which shows that the reciprocal

$$\Phi = \kappa\frac{V - b}{K} + \beta \tag{3}$$

of optical density is a linear function of mitochondrial volume and thus that one can calculate the average mitochondrial volume from the observed optical densities.

Eqn. (2), eqn. (3) and others to be described below must be applied with caution to mitochondria exposed to very hypotonic solutions. In such solutions the observed Φ frequently differs somewhat from that calculated by eqn. (2), particularly if exposure is prolonged. Since exposure to very hypotonic solutions is known to cause progressive damage to mitochondria[3], these deviations are probably due to some independent phenomenon such as leakage of internal solute of the mitochondria.

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In the course of continued experimentation, the dependence of κ and β on such variables as mitochondrial concentration, refractive index of the medium, and wavelength of incident light as well as their quantitative variation from experiment to experiment have been evaluated.

Mitochondrial concentration. It was previously shown (Fig. 4 of ref.[3]) that the linear relation between Φ and $1/c$ holds at any given mitochondrial concentration as expressed in eqn. (4). In this equation G_n is a variable dependent (eqn. (5)) on relative

$$\Phi = \frac{1}{G_n}\left[\kappa' \frac{1}{c} + \beta'\right] \qquad (4) \qquad\qquad G_n \equiv N/N_0 \qquad (5)$$

mitochondrial concentration (N/N_0). At low mitochondrial concentrations (O.D. $<$ 0.6) $G_n = N/N_0$ as required by Beer's law; in dense suspensions there are small deviations. Such deviations do not affect the validity of eqn. (4) which reduces at constant mitochondrial concentration to eqn. (2).

Wavelength. Most photometric measurements of the volume of mitochondria have been carried out at a wavelength of 520 mμ, although other wavelengths have been occasionally used[21]. Possibly the original choice[2] of wavelength may have been influenced by the small difference in absorption at this wavelength by oxidized and reduced cytochromes in mitochondria[24]. However, since it might prove useful to employ other wavelengths in certain experiments, it seemed worthwhile to investigate the dependence of optical density on the wavelength of the incident light.

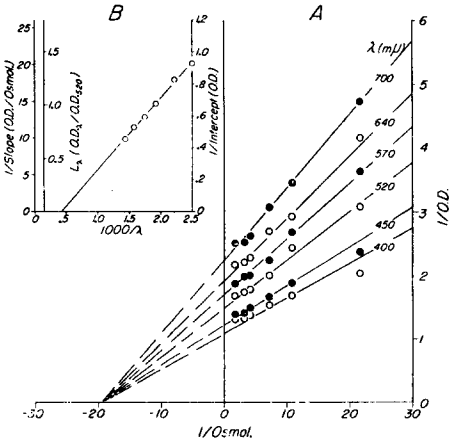

Fig. 1. The dependence of reciprocal optical density of mitochondrial suspensions on osmotic pressure and wavelength of incident light. The mitochondria were originally suspended in 0.32 molal sucrose, 0.01 *M* sodium versenate, pH 7.5. Potassium chloride solutions in 0.01 *M* sodium versenate, pH 7.5 were added to the suspension. Each point corresponds to the average of two independent determinations corresponding to an exposure of 20 and 60 sec each. See text for further details.

The results show (Fig. 1A) that similar linear relationships between reciprocal of optical density and reciprocal of concentration (eqn. (2)) obtain at all wavelengths. It will further be noted that all the lines of Fig. 1A converge at an imaginary point of the abcissa where $\Phi = 0$ and $\beta/\kappa = -1/c$. It follows (see Fig. 1B) that the wavelength affects both β and κ in equal degree and that one can calculate (eqn. (6)) the

$$\Phi_{520} = L_\lambda \Phi_\lambda \qquad (6)$$

reciprocal optical density at any wavelength (for example Φ_{520}) from the observed optical density at any other wavelength (Φ_λ). The parameter L_λ depends solely on wavelength and has been found from repeated experiments to be otherwise quite invariant.

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As shown in Fig. 1B the magnitude of L_λ is approximately described by eqn. (7).

$$L_\lambda = 0.68\left[\frac{1000}{\lambda} - 0.4\right] \tag{7}$$

The deviations at certain wavelengths, though small, are significant and reproducible and thus eqn. (7) should be used only for approximate calculations of L_λ. According to this equation, the optical density should be zero at $1000/\lambda = 0.4$; this corresponds to a wavelength of 2.5μ. It is possibly of some significance that this is a distance 2–5 times as great as the diameter of mitochondria under these conditions.

Osmotic pressure and refractive index. It was earlier noted[3] that the optical density of mitochondrial suspensions is dependent on the refractive index of the medium. In the course of our work it became increasingly clear that a careful investigation of the dependence was essential.

A representative experiment from which we can examine the effect of refractive index on the optical density of a mitochondrial suspension is given in Fig. 2. The requisite solutions of controlled refractive index and osmotic pressure were obtained by preparing calculated mixtures of sugars and potassium chloride (see legend of Fig. 2). With these mixtures it was impossible to obtain solutions of low osmotic pressure and high refractive index and this area of the three-dimensional graph is therefore indicated by dashed lines.

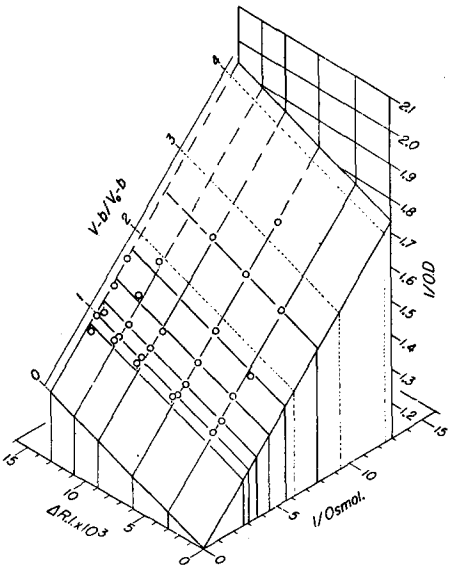

Fig. 2. The dependence of reciprocal of optical density on osmotic pressure and refractive index of the medium. Mitochondria were initially suspended in 0.32 molal sucrose, 0.01 *M* tris (trimethylol amino methane), pH 7.5. At zero time they were exposed to solutions of controlled osmotic pressure and refractive index. These solutions were prepared in 0.01 *M* tris (pH 7.5) and are mixtures of raffinose and potassium chloride (at $\Delta R = 0.012$, $1/c > 3.85$, the solutions are sucrose-raffinose mixtures). Readings were taken after 60-sec exposure. Solutions of requisite concentrations (c_T) and refractive index increment (ΔR) were calculated from the following considerations: For a solution of a single substance, the refractive index increment (ΔR_1) is simply $\Delta R_1 = a_1 c_1$. For a mixture of substances we may write $\Delta R = \Delta R_1 + \Delta R_2 + \ldots$, and $c_T = c_1 + c_2 + \ldots$. Thus for two substances $c_1 = (\Delta R - a_2 c_T)/(a_1 - a_2)$. For raffinose, sucrose and potassium chloride, the factor of proportionality, a, is 0.0690, 0.0467 and 0.0055 ΔR/osmolality respectively.

It will be noted that the experimental points can be closely approximated by the parallel lines shown on the surface of the three-dimensional graph. The lines of Fig. 2 and similar experiments are described by eqn. (8) which is a modification of eqn. (2) with slope κ and intercept $\beta = \beta_s(1 + \alpha\Delta R)$. In this equation β_s is the imag-

$$\Phi = \kappa\frac{1}{c} + \beta_s(1 + \alpha\Delta R) \tag{8}$$

inary point at $\Delta R = 0$ and $1/c = 0$. Repeated experiments indicate that the constant α is subject to minimal variation. While for precise calibrations it is necessary

to measure the magnitude of α, in most practical applications of eqn. (8) serious error will not result from assuming α to have a numerical magnitude equal to 17.

The slope, κ, varies rather markedly (3 fold) from experiment to experiment even after suitable corrections are made for differences in mitochondrial concentration. The reasons for such variation are obscure but the most plausible explanation is probably the presence of variable amounts of osmotically inactive material (*e.g.* damaged mitochondria). We do know that a variety of treatments (*e.g.* washing mitochondria in nonelectrolyte medium, freeze-thawing, etc.) can reduce or destroy the optical response of mitochondria to variations of osmotic pressure.

It was necessary to see if the several relations between optical density, wavelength, osmotic pressure, refractive index and mitochondrial concentration, discovered in part individually, were correct in all possible combinations as expressed in eqn. (9). An experiment giving a diverse sample was therefore undertaken. The

$$\Phi_{\text{Standard}} = \frac{L_\lambda}{G_n}\left[\frac{\kappa' I}{c} + \beta_s'(1 + \alpha \Delta R)\right] \qquad (9)$$

results are summarized in Fig. 3 and Table I. Fig. 3 shows that the fit of the equation to the observed data is excellent. Table I shows, for the several conditions employed, the agreement with the weighted averages in statistical terms. It is apparent from Table I that any wavelength or mitochondrial concentration may be employed with equal reliability.

As a final check on the utility of eqn. (9) an experiment was performed (Fig. 4) in which mitochondria were exposed to varying concentrations of sucrose (curve 1), mannitol (curve 2) and potassium chloride (curve 3). Under these conditions the

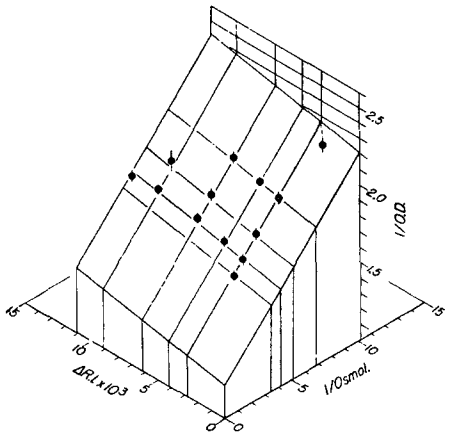

Fig. 3. The dependence of reciprocal of optical density on osmotic pressure and refractive index of the medium. The mitochondria were originally suspended in 0.30 molal sucrose, 0.02 *M* tris, pH 7.5. At zero time the mitochondria were exposed to raffinose–potassium chloride–tris solutions of known osmotic pressure and refractive index (see legend of Fig. 2) and the optical density read 20 and 60 sec later. Each point is in effect the weighted average of 32 separate measurements (4 mitochondrial concentrations, 4 wavelengths and 2 exposure times). Standard deviations are represented by vertical lines. See also Table I.

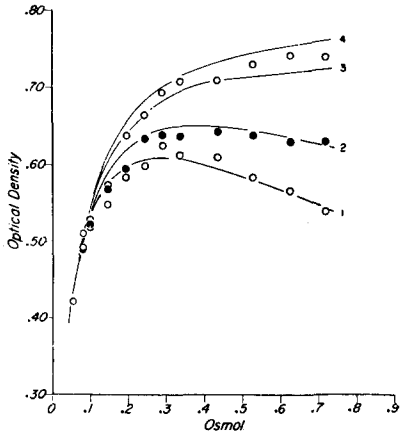

Fig. 4. The variation of optical density with osmotic pressure in solutions of a single nonpenetrant. The mitochondria were first suspended in 0.30 molal sucrose, 0.02 *M* tris, pH 7.5. They were then resuspended in sucrose (curve 1), mannitol (curve 2) or potassium chloride (curve 3) media (0.02 *M* tris, pH 7.5). Each circle represents the average optical density of three readings taken at 20 sec. The curves have been drawn from eqn. (8). The constants $\kappa = 0.060$, $\beta_s = 1.23$ and $\alpha = 13.9$ were obtained from a calibration curve (see text).

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TABLE I

RELIABILITY OF MEASUREMENTS ($\Phi/\bar{\Phi}$) AT VARIOUS WAVELENGTHS (λ) AND MITOCHONDRIAL CONCENTRATIONS (N)

The figures given are the mean and standard deviations of all measurements made at specified wavelength and relative mitochondrial concentration (N/N_0); statistically, $\Phi/\bar{\Phi} = 1.000 \pm 0.020$.

λ	N/N_0			
	1	5/6	4/6	1/2
400	1.017 ± 0.021	1.015 ± 0.016	1.001 ± 0.012	0.978 ± 0.016
520	1.005 ± 0.012	0.998 ± 0.022	0.998 ± 0.015	0.995 ± 0.021
600	0.990 ± 0.016	0.991 ± 0.024	0.994 ± 0.015	1.015 ± 0.017
700	0.989 ± 0.015	0.987 ± 0.039	1.007 ± 0.020	1.011 ± 0.041

osmotic pressure of a solution depends solely on the concentration of solute while the refractive index depends on both its concentration and nature. As the fit of the data about the calculated curves shows, the equation has reasonable predictive value (the variation of the data (standard deviation) from the calculated values is $\pm$ 2.3%). It is particularly gratifying that the equation accounts for the asymptotic rise in optical density seen in curve 3 and the slight decrease in optical density seen in curves 1 and 2 at high concentrations. Curve 4 is the ideal curve at $\Delta R = 0$. The extent of deviations of optical density due to systematic variation in refractive index can be seen by comparing curves 1, 2 and 3 with curve 4.

Application of photometric data to the study of permeability. The use of photometric measurements of mitochondrial volume in the study of permeability depends on the fact that mitochondria exposed to solutions containing penetrating substances must, in accordance with osmotic law, undergo volume changes. For as the substance penetrates, the osmotic pressure of the interior will increase and water will inevitably follow. From the kinetics of the volume changes it is possible in theory to calculate the permeability constants. For a useful discussion of the problem see JACOBS[25].

In certain cases it may prove useful to calculate the amount of penetrant in the mitochondria at any given time. If we are dealing with a substance which passes the membrane slowly in comparison to water, the calculation is simple. In such cases, water follows the penetrant across the membrane essentially instantaneously and the system may be considered to be at osmotic equilibrium at all times[25]. We can solve for the desired quantity (S) by use of eqns. (10, 11) in which c_0 is initial concentration, V_0 is the initial volume and V_t is the volume at time, t.

$$S = c_0 (V_t - V_0) \qquad (10) \qquad\qquad S = \frac{c_0 K}{\kappa} [\Phi_t - \Phi_0] \qquad (11)$$

A possible source of error in the estimation of the transient volume of mitochondria from photometric data might conceivably arise from an alteration of the refractive index of the interior due to the presence of the penetrant. This possibility is readily tested. If mitochondria are exposed to solutions containing both non-penetrant and penetrant, the volume of the mitochondria at equilibrium should be

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dependent solely on the concentration of non-penetrant and should be independent of the concentration of the penetrant. In such an experiment, alterations of the internal refractive index of the mitochondria by the penetrant would appear as a systematic deviation of the apparent volume as estimated from photometric data. A graphical solution of eqn. (8) for purposes of calibration is shown in Fig. 5A. It will be noted in Fig. 5B that no such systematic deviation of apparent volume occurs. It follows that any increment of refractive index in the interior due to the introduction of penetrant is negligible in the range tested (ΔR = 0–0.0046). This must be considered to be a fortunate circumstance since it simplifies the calibration.

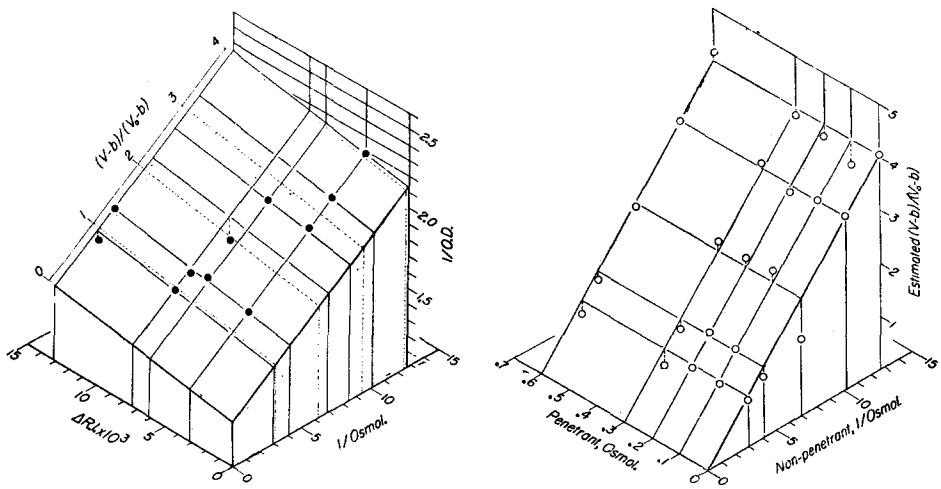

Fig. 5. The effect of the presence of a penetrant on relative optical density (expressed as apparent volume). A mitochondrial suspension in 0.3 molal sucrose, 0.02 *M* tris, pH 7.5 was resuspended in a medium containing a non-penetrant (raffinose, potassium chloride, 0.02 *M* tris, pH 7.5) and varying concentrations of a penetrant (propylene glycol). Readings were taken after an equilibration of 2 min. Each point represents an average of no less than four determinations (see text).

The data of Fig. 5 further suggest that mixing solutions of fast penetrants and non-penetrants might be a convenient technique for controlling the effective osmotic pressure and refractive index of solutions. This would be particularly useful for preparing solutions of high refractive index and low effective osmotic pressure (see discussion of Fig. 2 above). Experiments carried out in this fashion do show the utility of this technique and the reliability of eqn. (8) in the range not covered in Fig. 2.

Necessary validations. Although it is clear from the data of the foregoing sections that changes in mitochondrial volume produce predictable changes in optical density of a mitochondrial suspension, it is by no means certain that all changes in optical density are due to changes in volume. Indeed a number of cases have been encountered[3,26] in which changes in optical density almost certainly do not correspond to volume changes. Moreover, a number of processes are known to occur in mitochondria or in other subcellular granules which might produce changes in optical density. These include (a) a hemolysis-like leakage of high molecular weight substances and a consequent decrease in internal refractive index (*e.g.*[27,8,9]), (b) fragmentation of mitochondria[28], (c) a change in the absorption spectrum of substances within

mitochondria (*e.g.*[24]), (d) agglutination of the suspension (*e.g.*[29]). Agglutination occurs under a number of conditions particularly at an acid pH and high ionic strength. Such changes can usually be prevented or reversed by adequate mixing. Occasionally, however, relatively fast agglutinations are encountered, not readily controlled by mixing, which are accompanied by a sharp increase in optical density with time. It is not as yet clear that these changes in optical density are caused exclusively by the agglutination.

It is thus essential to have some independent way of demonstrating that volume changes have, in fact, occurred. This can be done in a number of more or less painful ways: (e) by estimating the volume from mitochondrial diameters as measured directly or from photomicrographs[3]; (f) by estimating the content of water in the centrifugal pellet[14,30,31,21]; (g) by demonstrating by analytical procedures that exchanges of osmotically active material and hence osmotic swelling are quantitatively correlated with the observed optical density changes[32]; and (h) by demonstrating that the effect is reversible by osmotic means alone[2,3].

It may be noted that none of the effects enumerated above (a–d) can be reversed by simple osmotic means; such reversal thus constitutes sufficiently rigorous proof that an observed optical density change is due to a change in volume. Where desirable, the degree of reversal can be varied; the resultant optical density should then be quantitatively predictable from osmotic law.

There are two convenient methods employing the principle of osmotic reversal. The first is basically an equilibrium measurement; it rests on the fact that osmotic theory demands that the volume at equilibrium depend solely on the concentration of non-permeating substances within the mitochondria and in the medium. It is this sort of equilibrium measurement which was employed in our previous study[3] where, following exposure of mitochondria to hypotonic solutions or to solutions of penetrating substances, sufficient quantity of the non-penetrant sucrose was added to the suspension to bring its concentration to 0.3 molal and restore the mitochondria to their initial volume. This principle is also implicity illustrated in Fig. 5 which shows that the equilibrium volume depends solely on the concentration of non-penetrant.

In dealing with processes which are slow compared to the rate of water movement, it may be either inconvenient or inadvisable to use equilibrium measurements. For such purposes an instantaneous or transient reversal is better. If mitochondria are exposed to a medium of given concentration (c_e) reaching at any time (t) the apparent osmotically active volume ($V_t - b$), then the desired concentration (c_d) which must be added to change the volume of the mitochondria to a desired volume ($V_d - b$) is according to osmotic law simply: $c_d = (V_t - b)\, c_e/(V_d - b)$. The application of such a technique is illustrated in Fig. 6. In this experiment, the mitochondria were exposed at zero time to 0.3 osmolal erythritol (curve 1). After 8 minutes, a calculated amount of potassium chloride was added (curve 2). The mitochondria immediately returned to the volume theoretically predicted (dashed line).

It may be noted that the use of the "instantaneous" reversal technique is not confined to the case of penetrating substances as is the equilibrium measurement, but can be applied to any sort of slow or discontinuous volume change. Thus, for example, if mitochondria were to undergo any metabolic change that altered the concentration of the osmotically active solute within the mitochondria, volume changes would occur and these could be identified as such by the "instantaneous" reversal technique.

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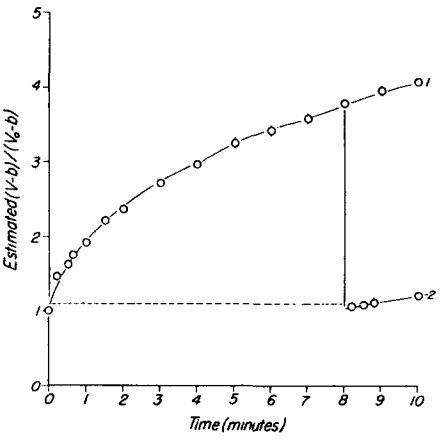

Fig. 6. Osmotic reversal. Mitochondria originally suspended in 0.3 molal sucrose, 0.02 *M* tris, pH 7.5 are resuspended at zero time in 0.3 molal erythritol (0.02 *M* tris, pH 7.5). Curve 1: Relative volume of control. Curve 2: Instantaneous reversal by the addition of 0.651 molal potassium chloride after 8 min exposure. Five determinations were carried out per each curve. The relative volumes were estimated by calibration (see text). The broken line represents the volume predicted by osmotic law. The vertical lines at each circle represent standard errors, where larger than the diameter of the circle.

DISCUSSION

The data presented above have provided us with an empirical equation (eqn. (9)) by which the relative volume of mitochondria can be calculated from photometric measurements. This equation appears to be reliable over a wide range of experimental conditions.

It should be emphasized that the photometric calibration is a measure of the osmotically active volume $[(V-b)/(V_0-b) = c_0/c]$ and gives no information about the absolute magnitude of V, V_0 or b. For many purposes, for example the calculation of relative permeability constants, it is sufficient to know this relative volume. To translate the relative permeability constants to absolute constants one must have estimates of both volume and surface area. As yet, relatively little is known about the magnitude or variability of these quantities.

For purposes of calibration, it is essential to measure the optical densities of mitochondrial suspensions exposed to a minimum of two and preferably many different solutions of known osmotic pressures and refractive index. It should be emphasized that the work of collecting and interpreting photometric data is greatly simplified if the experiments are so designed that the refractive index of the experimental and calibrating solutions is held constant (see legend of Fig. 2).

Although this empirical treatment is completely satisfactory for practical purposes, it would, of course, be far better to have a detailed theoretical basis for these equations. At the present time this seems difficult, except in a broad sense. The optical density of a suspension of particles seems to be due in large part to a scatter of the incident light by the particles. Although we have not investigated this question in detail, we do know that the scatter measured at a 90° angle, 520 mμ in a Coleman Universal Spectrophotometer under a variety of conditions of osmotic pressure and concentration of mitochondria is directly proportional to observed optical density, as is the case with small isotropic particles (*e.g.*[33]). It is also known that the scatter by a suspension of particles depends on the refractive index of the particles and of the medium (*e.g.*[34]).

It is therefore not surprising to find that the optical density of a mitochondrial suspension does depend on the refractive index of the medium. However, we have

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not established the exact nature of this dependence. The empirical equation described above was chosen from several which fit the data reasonably well.

The linear relations between the reciprocal of optical density and mitochondrial volume as expressed in eqn. (3) or the reciprocal of osmotic pressure as expressed in eqns. (2), (4), (8) and (9) are of considerably greater interest. In an osmotic system such as mitochondria, the refractile material inside, on which the optical density depends, will undergo dilution when the mitochondria swell. In principle then, the refractive index and hence the optical density will be inversely dependent on volume; it is thus obvious that the reciprocal optical density should be directly dependent on volume as has been found to be the case empirically. Moreover, if mitochondria are exposed to extremely hypertonic solutions the refractile material becomes confined to a minimum volume, b, and the reciprocal optical density approaches a minimum, β. Thus the empirical equation corresponds, at least roughly, to reasonable theoretical expectations. Much additional work would be required to establish a more sophisticated theoretical basis.

SUMMARY

1. The dependence of the optical density of a mitochondrial suspension on mitochondrial concentration, osmotic pressure, the refractive index of the medium (Fig. 2), and the wavelength of incident light (Fig. 1) have been examined.

2. Empirical equations which describe the observed relation have been deduced and subjected to empirical verification (*e.g.* eqn. (8)).

3. The measurements necessary for using these equations for calculating mitochondrial volume from photometric data are described.

4. The simplifications resulting from keeping constant the refractive index of experimental and calibrating solutions are pointed out.

5. The application of photometric techniques to a biological problem, the study of permeability, has been discussed.

6. It is pointed out that a number of changes can occur in mitochondria producing changes in optical density which might be spuriously interpreted as volume changes. Techniques for testing whether an observed optical density change is in fact due to osmotic phenomena are described.

7. A rough theoretical basis for the empirical equations is discussed.

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THE INTRACELLULAR AMINO ACIDS OF *STAPHYLOCOCCUS AUREUS*: RELEASE AND ANALYSIS

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The investigations of GALE[1] and TAYLOR[2] showed that cells of a number of gram-positive bacteria (14 species) and yeasts (3 species) contain certain free amino acids, which were detected and estimated by enzymic methods after liberation by treatment of washed cells with boiling water or detergent substances. *Staphylococcus aureus* was shown to contain free glutamic acid and lysine; washed suspensions of cells of this organism were able to establish concentration gradients of these amino acids across the cell surface, and further results showed that a number of other amino acids could be concentrated within the cells (GALE[3]). Chromatographic investigations of the free internal amino acids have been made with *Corynebacterium diptheriae*[4], *Neurospora crassa*[5,6], *Aspergillus nidulans*[7] and *Saccharomyces cerivisiae*[8].

No evidence was found in early studies for the existence of free amino acids within cells of gram-negative bacteria; however MANDELSTAM[9] demonstrated chromatographically the presence of certain free amino acids within cells of *Escherichia coli*, and accumulation of radioactive amino acids in cells of this organism has been observed[10,11]. Loss of internal amino acids from cells of *E. coli* on washing with media of low osmotic pressure was observed by BRITTEN[12] and osmotic sensitivity of this type may have been responsible for the failure of earlier attempts to detect free amino acids in cells of gram-negative organisms.

The objects of the work described here were to determine the conditions under which the free internal amino acids of *S. aureus* are released, and to investigate the nature of the amino acids present in the internal pool.

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