

# PHOTOMETRIC EVIDENCE FOR THE OSMOTIC BEHAVIOR OF RAT LIVER MICROSOMES

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## ABSTRACT

Electron microscope observations are consistent with the interpretation that the elements of the endoplasmic reticulum are osmotically active *in situ* as well as after isolation. More recently, it has been reported that microsomal suspensions equilibrate almost completely with added  $C^{14}$ -sucrose and that no osmotic behavior is evident from photometric data. These findings were considered at variance with the electron microscope data. However, equilibration with added label simply attests to a relatively high permeability, and, in addition, the photometric data need not be critical. Osmotic volume changes, measured photometrically, may be masked by concomitant events (*e.g.*, changes in the refractive index of the test solutions at varying osmotic pressures, breakdown of the particles, and agglutination). For these reasons the photometric experiments were repeated. In this work, the reciprocal of optical density of microsomal suspensions was found to vary linearly with the reciprocal of concentration of the medium at constant refractive index. These changes probably correspond to osmotic volume changes, since the effect was found to be (*a*) independent of substance used and (*b*) osmotically reversible. The transmission of the suspension was found to vary with the refractive index of the medium, the concentration of particles, and the wavelength of incident light, according to relationships that are similar to or identical with those obtained for mitochondrial suspensions.

## INTRODUCTION

The cell is subdivided into a number of compartments of characteristic composition and functional activity. Electron microscope studies have revealed that most of these compartments are bounded by membranes of varying complexity. At high resolution, the simplest of these membranes appears as a double layer, the unit membrane (1) and probably corresponds to a bimolecular lipid layer in combination with protein (1, 2). It would appear that the nuclear and mitochondrial membranes are more complex and are composed in part of two

unit membranes. Other elements, for example the endoplasmic vesicles, Golgi elements, and secretory granules, are in general bounded by single unit membranes.

The functional significance of these membranes is still not completely clear. In mitochondria (*e.g.*, 3, 4) and microsomes (5), they seem to play a vital role in the organization of their enzymes. In other cases, they play an obvious role in the segregation of material from other cell elements (see 6). Perhaps less obviously, in some

cases they act as semipermeable barriers, where they may play a more subtle role in regulating the accessibility to enzyme sites and in maintaining optimal levels of cofactors, intermediates, and products at particular sites. For an evaluation of this role, a precise knowledge of their permeability properties is necessary.

It would seem likely that a number of these systems are not freely permeable to relatively low molecular weight substances, since they respond to the osmotic pressure of the medium. In some cases this is evidenced, for example, by the release of their internal contents in dilute media (*e.g.*, catecholamine granules (7, 8), lysosomes (see 9), and pigment granules of sea urchin eggs (10)). In other cases, it is evidenced more rigorously by the fact that the particles follow osmotic law rather closely (*e.g.*, mitochondria (11-13)). From these considerations, it would appear probable that most of these intracellular membranes resemble functionally the cell membrane. In fact, studies of the permeability of mitochondria to non-electrolytes (11, 14) show that the permeability of these particles parallels closely that of cells. More recent studies suggest the presence of special transport mechanisms, analogous to those that have been encountered in the study of the cell membrane, in thymus nuclei (15), heart mitochondria (*e.g.*, 16), and muscle microsomes (17, 18).

The microsomal particles of isolated preparations seem to correspond structurally to elements of the endoplasmic reticulum (19, 20), from which they are thought to arise as a consequence of a fragmentation of the original cytoplasmic vesicles.

From electron microscope studies, there is some evidence that the vesicles of the endoplasmic reticulum respond to the osmotic pressure of the medium whether *in situ* (21) or isolated in the form of microsomal vesicles (20).

More recently, it has been claimed that sucrose-isolated microsomes do not respond to the osmotic pressure of the sucrose media, as evidenced by photometric measurements. In addition, the particles were found to equilibrate almost completely with added  $C^{14}$ -sucrose over a relatively long experimental period (22). In contrast to this equilibration, the particles were found not to be readily penetrated by  $C^{14}$ -carboxypolyglucose of high molecular weight.

These results have been thought to be at variance with those obtained by electron microscopy.

However, it should be recognized that the equilibration simply attests to a relatively high penetrability to added label and need not imply a lack of osmotic responses. In addition, the photometric data need not be critical. The transmission of incident light by a suspension can be altered by concomitant events such as agglutination, loss of high molecular weight components, breakdown of the particles, or differences in the refractive index at the different concentrations of the medium (see 12).

For these reasons, a systematic study of this question was undertaken. The reciprocal of the optical density of the suspensions was found to be directly proportional to the reciprocal of the osmotic pressure of the medium. These changes in transmission most probably correspond to osmotic volume changes, since (*a*) the effects are osmotically reversible and (*b*) they do not seem to depend on the nature of the solute.

The optical density of the microsomal suspensions was found also to depend on the refractive index of the medium, the concentration of the particles, and the wavelength of incident light, in a way somewhat similar to that of mitochondrial suspensions.

#### METHODS

Microsomes were isolated from rat liver by a method similar to that used by a number of other investigators (*e.g.*, 23, 20). Male rats of the Holtzman strain, weighing approximately 200 to 250 grams, were fasted for approximately 16 hours and then killed by a blow on the head and cervical fracture of the spinal cord. The livers were removed and cut into large pieces which were washed in chilled 0.7 molal sucrose (about 4°C). The tissue pieces were then minced with scissors and homogenized in a motor-driven glass and Teflon homogenizer of the Potter-Elvehjem type. Eight to twelve livers were used, and each liver (~5 to 6 gm) was homogenized in about 20 cc of 0.7 molal sucrose, brought to pH 7.4 with 0.02 M tris [tris(hydroxymethyl)aminomethane] chloride buffer. The volume of the homogenate was brought to approximately 300 cc with the homogenization medium. Throughout these operations the preparation was maintained between 0° and 4°C, by keeping the vessels in an ice-saline bath.

Mitochondria, nuclei, and other large particles and debris were centrifuged out at 22,000 *g*, for 30 minutes, in a refrigerated LRA-1 Lourdes angle centrifuge operated at a chamber temperature of -2°C. The microsomes were then sedimented from the supernatant in a type 30 rotor of a Spinco model

L refrigerated preparatory ultracentrifuge operated at 30,000 RPM (78,000 *g*, average) for 1 hour. The supernatant of this last centrifugation was discarded by suction and the particles were suspended with a homogenizer of the Potter-Elvehjem type after mixing with approximately 20 to 30 cc of the isolation medium with a glass rod. Occasionally the particles were suspended with a glass rod without homogenization. The stock suspensions were stored at 0°C.

Observation of the preparation with the electron microscope, after fixation in 10 per cent buffered formalin, washing with ion-free water, and negative staining with phosphotungstic acid (see, *e.g.*, 24, 25), revealed a preparation of spherical or ellipsoid particles ranging in diameter from about 0.1 to 0.3  $\mu$ . In some favorable grids the vesicles appeared to be predominantly rough surfaced elements, in agreement with previous works (20, 26).

The photometric work was carried out with a Coleman junior spectrophotometer. The solutions were kept at a temperature between 23 and 26°C, depending on the experiment. Usually, the solutions were maintained within 1°C in each individual experiment (except for the experiment depicted in Fig. 3). Typically, 0.1, 0.2, or 0.5 cc of stock suspension was placed at the bottom of the vial, and 10 or 3 cc of test solution were added by means of a syringe provided with a rubber nozzle. In the reversal experiments, 8 or 25 cc of solutions were added to the 10 cc of the original suspension in the test solution. In these cases, the solutions were mixed with a glass rod or by gentle swirling of the suspensions. All solutions used were buffered in 0.02 M tris chloride at pH 7.4.

In the quantitative comparison of the effects of the different substances on optical density, speed was deemed important. In these experiments, for continuous recording, a Sanborn recording system was used, consisting of a low level preamplifier, model 150-1500, a power supply, model 150-400, and a single channel recorder, model 151-100. In these cases, 3 cc of test solution were added to 0.1 cc of stock suspension.

In a number of experiments it was found necessary to measure the refractive index of the medium. This was done with an American Optical Co. refractometer (TS Meter).

## RESULTS AND DISCUSSION

### *The Dependence of Optical Density on Osmotic Pressure*

It is generally recognized that the amount of light scattered by a suspension depends on the difference between the refractive index of the particles and that of the suspension medium (see 29, 30). In fact, osmotic volume changes of sus-

pended particles can be measured photometrically primarily because of the resultant alterations in internal refractive index. That this general relationship is valid has been repeatedly demonstrated for mitochondria (11, 12, 31) and erythrocytes (*e.g.*, 27).

In particles exhibiting osmotic behavior, the task of correlating volume changes with optical density changes is rather complex (see 12) if the refractive index of the medium is permitted to vary simultaneously with the osmotic pressure of the medium (and hence the volume of the particles). It is considerably simpler to carry out these tests while maintaining constant the refractive index of the medium.<sup>1</sup> It is possible to do so and simultaneously to vary osmotic pressure by the use of mixtures of two or more solutes. This technique has been described previously (see 12, legend to Fig. 2). Alternatively, solutions of very low refractive indices, such as inorganic salts, can be used.

In a few cases tested, microsomes were suspended in solutions containing potassium chloride and the optical density was found to decrease with increasing osmotic pressure. This effect was found to be irreversible, and it may correspond to lysis, aggregation, or rupture of the particles. Although other electrolyte solutions (*e.g.*, sodium sulfate and sodium citrate) do not behave in this fashion, they do reduce the optical density rather rapidly with time. For these reasons, in this study non-electrolyte solutions in tris buffer were used predominantly. Most of the experiments reported here made use of calculated concentrations of mannitol-raffinose mixtures, and the optical density of the suspension was measured 10 seconds after exposure to the solute (see discussion below).

When microsomal suspensions are placed in mannitol-raffinose solutions at constant refractive index, the optical density of the suspension increases with osmotic pressure (see Fig. 1 A). This relationship is similar to that encountered with erythrocytes (*e.g.*, 27) and mitochondrial suspensions (see 11, 12). Over a limited range of concentrations the relationship is approximately linear. However, the data are represented somewhat

<sup>1</sup>Where the optical density is not corrected for refractive index (*e.g.*, uncorrected data from the experiments carried out with mannitol, glucose, or sucrose), the effect of osmotic pressure is completely masked by the concomitant change in refractive index with concentration.

more faithfully when the reciprocal of the optical density is considered to vary linearly with the reciprocal of concentration, as shown in Fig. 1 B (see equation 2, below). Deviations usually occur below a concentration in the neighborhood of 0.30 osmolal, where the optical density is higher than predicted and does not change significantly with osmotic pressure. The exact concentration at which the relationship no longer applies varies from experiment to experiment and ranges from about 0.25 to 0.33 osmolal (e.g., compare Fig. 1

support the contention that microsomes follow osmotic law. In this respect, it is also encouraging to see the same general function describe the changes in optical density in relation to osmotic pressure in the case of microsomes as well as of erythrocytes and mitochondria. If we were to assume that these changes do represent osmotic volume changes, the relative osmotically active volume can be readily calculated from photometric data (see 12). Osmotic law (Boyle-Van't Hoff law) is defined by equation 1,

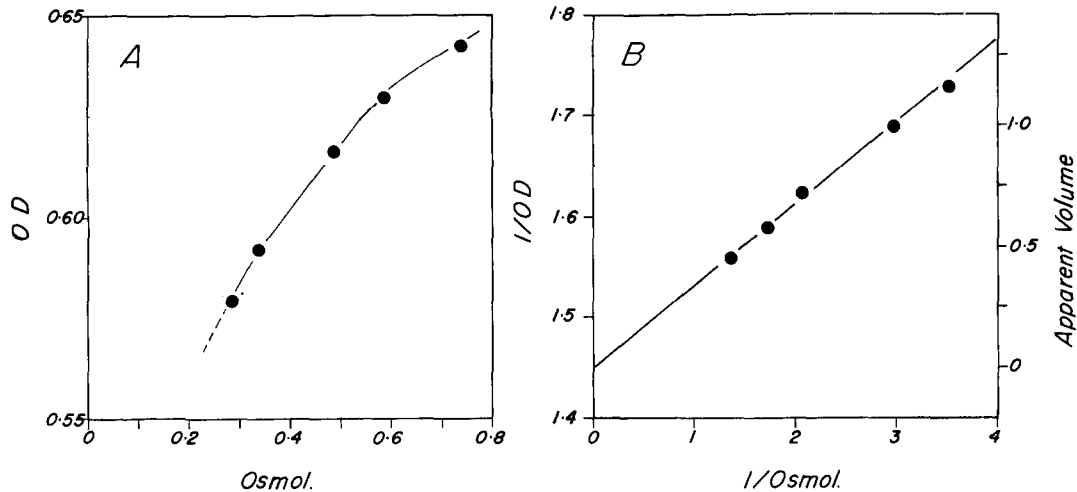


FIGURE 1 The dependence of the optical density of microsomal suspensions on the concentration of the medium. A mannitol solution or mannitol-raffinose mixtures at a  $\Delta r$  of 0.017 were used (see text). Each point corresponds to an average of 4 determinations carried out after 10 seconds of exposure. The standard error is smaller than the radius of the circles. The optical density was measured at 500  $\mu$  and the suspensions were maintained at 23°C.

A. Optical density (OD) as a function of the concentration of the medium (osmolal).

B. The reciprocal of the optical density (1/OD) as a function of the reciprocal of concentration (1/osmolal).

with Fig. 4).<sup>2</sup> A similar situation is evident in mitochondria at much lower concentrations. In the case of mitochondria, this effect was previously attributed to leakage of internal solute (11, 12). The extreme resistance of mitochondria to disruption by swelling is probably the result of the availability of the cristae in forming new surface membrane (see 11, 14, 28). An analogous mechanism is not likely to be present in microsomes.

These observed changes in optical density

<sup>2</sup> In this connection, it is interesting to note that experiments which failed to encounter an osmotic behavior in microsomes were carried out on preparations isolated in 0.30 M sucrose (22).

$$V - b = K \frac{1}{C} \quad (1)$$

where  $V$  is volume,  $b$  is the so-called osmotic dead space (thus  $V - b$  is the osmotically active volume),  $K$  a constant, and  $C$  the concentration of solute in the external medium. Equation 2 follows from the empirical relation found experimentally.

$$\phi - \beta = \kappa \frac{1}{C} \quad (2)$$

In this equation,  $\phi$  is the reciprocal of optical density, and  $\kappa$  and  $\beta$  empirical constants. The combination of these two equations results in

equation 3, which relates osmotically active volume ( $V - b$ ) with the reciprocal of optical density.

$$V - b = \frac{K(\phi - \beta)}{\kappa} \quad (3)$$

Many of the results of the experiments that follow have been expressed in terms of apparent relative osmotically active volume ( $V - b/V_0 - b$ ), where the osmotically active volume in 0.335 osmolal solutions ( $V_0 - b$ ) was arbitrarily taken as unity. The assumption of an osmotic volume change at this point in the discussion, is one of convenience, and the units may be regarded simply as relative units of optical density.

A photometric effect underlain by an osmotic event should be measurable over a wide range of concentration of microsomes, refractive index of the suspending medium, and wavelength of incident light. In addition, a knowledge of these factors is necessary for the effective use of the photometric technique. In the case of mitochondria, the dependence of optical density on the osmotic pressure of the medium is modified characteristically by these parameters. It would be of some interest to determine whether results with microsomal suspensions yield similar relationships.

It should be noted that changes in optical density do not necessarily correspond to volume changes. They may be the result of (a) loss of high molecular weight substance from the particles (lysis), (b) agglutination of the particles, (c) fragmentation of the particles, (d) changes in light absorption, and (e) other effects not clearly evaluated at this time (see 12 for a discussion of some of these factors). Changes in the transmission of microsomal suspensions which are probably not osmotic in nature have in fact been encountered in the presence of high molecular weight substances (Tedeschi and James, unpublished observations). Because of these factors, the interpretation of photometric observations requires a good deal of caution. Fortunately, in most cases, it is possible to differentiate between osmotic effects and effects stemming from other factors. In contrast to most other effects, osmotic phenomena are (a) osmotically reversible in a predictable manner and (b) independent of the substance used. Experiments were carried out to clarify these points.

### *Dependence of the Optical Density on the Concentration of Microsomes*

The linearity of the relationship between  $\phi$  and  $1/C$  holds at any given microsomal concentration. This is represented in Fig. 2. It may be noted that at any given reciprocal of osmolality, the  $\phi$  varies linearly with the reciprocal of the concentration of microsomes ( $1/N$ ). However, small deviations occur at high microsomal concentration. Apparently, deviations from Beer's law occur at the extreme of this range, although they are minor in nature. The slope of the lines and the intercepts on the  $\phi$  axis are equally affected by the concentration of microsomes. To take this new information into account, the results can be expressed by equation 4, a modification of equation 3.

$$\phi = \frac{1}{G_n} \left( \kappa' \frac{1}{C} + \beta' \right) \quad (4)$$

$G_n$  is constant only at constant microsomal concentration, and it is a function of particle concentration. In the range in which Beer's law applies,  $G_n$  corresponds to  $N/N_0$  (the relative concentration of particles). These relationships are identical with those observed for mitochondria (11, 12).

### *Dependence of the Optical Density on the Wavelength of the Incident Light*

The relationship between  $\phi$  and  $1/C$  is approximately linear, regardless of wavelength used. As shown in Fig. 3, the results are approximated by lines which converge at an imaginary point on the abscissa where  $\phi = 0$  and  $\beta/K = 1/C$  (in this case, approximately 12). Consequently, and analogously with the results shown in Fig. 2, the slope and the intercept at  $1/C = 0$  are equally affected, in this case by wavelength. The relationship between two equivalent measurements at two different wavelengths can be expressed as in equation 5,

$$\phi_1 = L_\lambda \phi_2 \quad (5)$$

where  $\phi_1$  and  $\phi_2$  are any  $\phi$  at a particular wavelength, and  $L_\lambda$  is a constant. It is interesting to note, however, that this relationship does not hold below 450 m $\mu$ . These deviations are real and have been confirmed by experiments spanning the range

from 400 to 450  $m\mu$ . Above these wavelengths, any optical density can be predicted after estimation of the constants  $L_\lambda$ ,  $\kappa$ , and  $\beta$ . The deviations at the lower wavelengths may perhaps be the result of the absorption peak encountered in this range (20).

#### *Dependence of Optical Density on the Refractive Index of the Medium*

We have seen that the optical density of a suspension of particles is a function of the difference between the refractive index of the particles and that of the medium. In the case of osmotically active particles (*e.g.*, erythrocytes, mitochondria), osmotic volume changes will alter the internal refractive index. This leads to a change in the amount of light scattered by the suspension. Altering the refractive index of the suspension medium

should also alter the optical density of the suspension.

Experiments were carried out to study systematically the effect of external refractive index on optical density. The purpose of the experiments was to clarify the relation between optical density and osmotic pressure with changes in the refractive index of the medium in order (a) to find out whether microsomes and other semipermeable systems behave analogously, (b) to evaluate whether the amount of light scattered by a suspension in different media at the same osmotic pressure but differing refractive index is quantitatively equivalent (*e.g.*, the experiments summarized in Table I), and (c) to provide information that would facilitate future experiments, where suspensions in media of different refractive indices would have to be used.

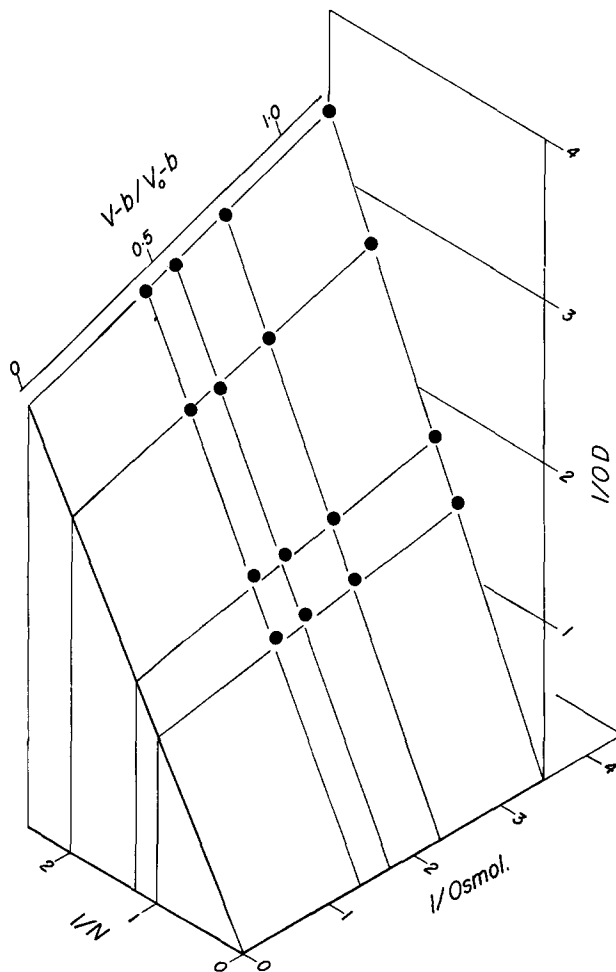


FIGURE 2 The dependence of the reciprocal of optical density ( $1/OD$ ) on the reciprocal of the concentration of the medium ( $1/osmol$ ) and the reciprocal of the relative concentration of the particles ( $1/N$ ). Each point represents an average of 4 determinations. The standard errors of the measurements are smaller than the radius of the circles. The solutions were at a  $\Delta r$  of 0.017 (see text). The measurements were carried out at 500  $m\mu$  after 10 seconds of exposure. The suspensions were maintained at 23°C.

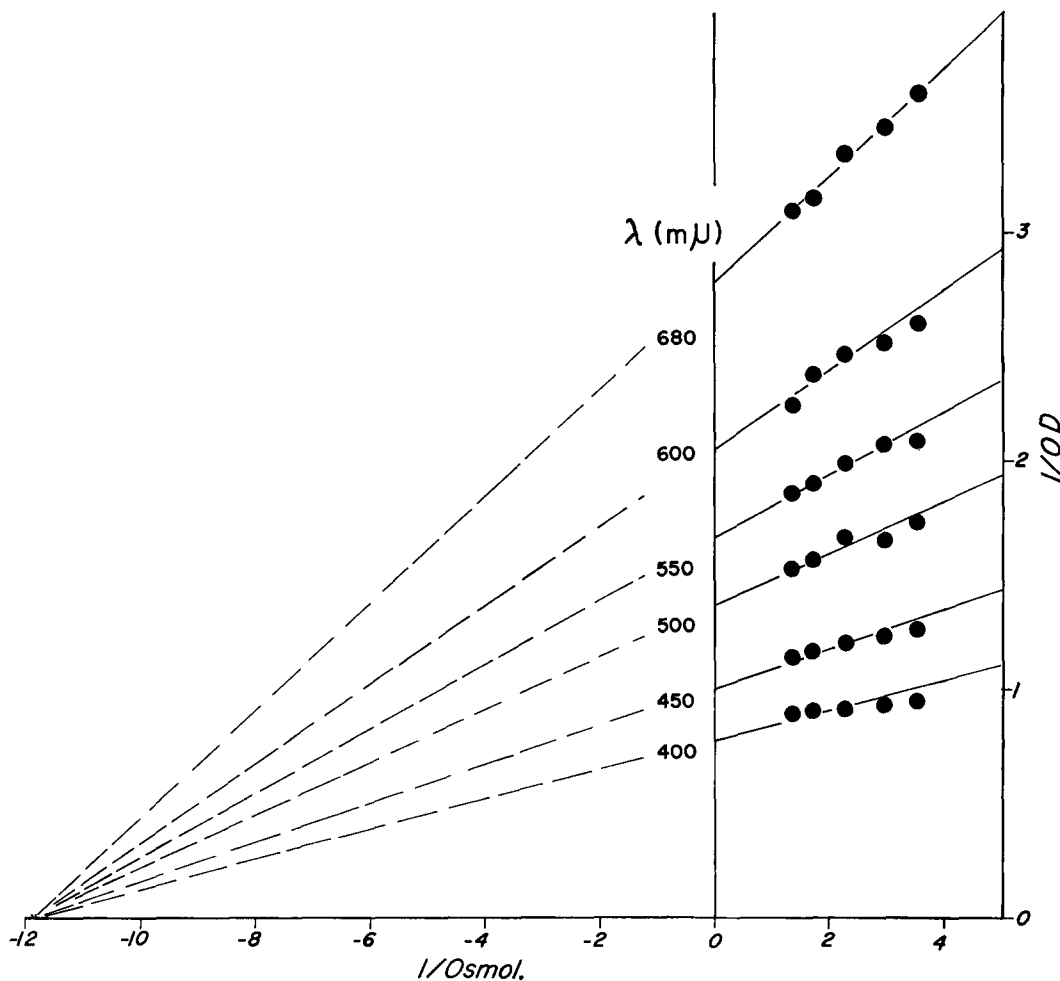


FIGURE 3 The dependence of the reciprocal of optical density ( $1/OD$ ) on the reciprocal of concentration ( $1/osmol$ ) at various wavelengths of incident light. Each point represents an average of 4 determinations. The standard errors of the measurements are smaller than the radius of the circles. The solutions were at a  $\Delta r$  of 0.017 (see text). The temperature of the suspensions was maintained between 20 and 25°C.

The use of mannitol-raffinose mixtures necessarily limits the range of refractive index that can be tested. With these mixtures, it is not possible to obtain solutions of low refractive index and high osmotic pressure. In addition, the limited solubility of raffinose does not permit the preparation of some solutions of high refractive indices and low osmotic pressures. Therefore, it was not possible to study the complete range of concentrations and refractive indices.

Within the range studied, a systematic variation in the refractive index is reflected in the optical density. The experimental data can be closely approximated by parallel straight lines,

each representing the reciprocal of optical density as a function of the reciprocal of concentration at a different refractive index (expressed as  $\Delta r$ , the difference between the refractive index of the solutions and that of water). An increase in the refractive index results in a systematic increase in the reciprocal of optical density (see Fig. 4).

This relation can be expressed in the form of equation 6,

$$\phi = \frac{\kappa}{C} + \beta_s(1 + \alpha \Delta r) \quad (6)$$

where  $\beta_s$  is the imaginary  $\phi$  where  $1/C = 0$ ,  $\Delta r = 0$  (in this case 1.03), and  $\alpha$  is a constant (in

this case 17.5). In this experiment,  $\kappa$  is 0.110. This relationship is the same as that describing the behavior of mitochondrial suspensions, as previously shown (12). In fact, the constant  $\alpha$  is approximately the same.

The demonstration that the same relationship holds at the different refractive indices for the two

rigorously tested. Sucrose, glucose, and mannitol-raffinose were chosen for this test because their effects are somewhat slower than those of other substances used.

Selection of an exposure time is somewhat problematic. After exposure to a solution which differs in osmotic pressure from the original suspen-

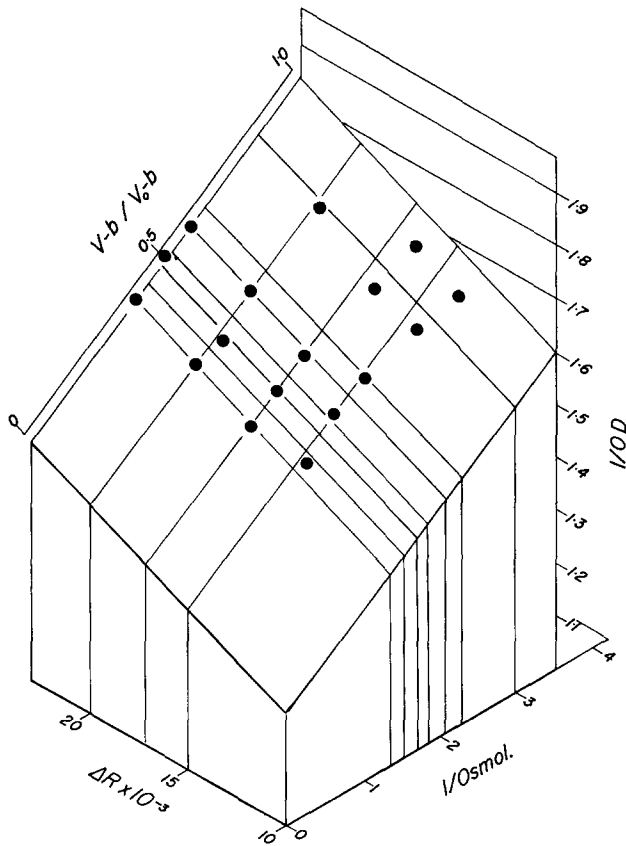


FIGURE 4 The dependence of the reciprocal of optical density ( $1/OD$ ) on the reciprocal of concentration of the medium ( $1/osmol$ ) at various refractive indices of the suspending medium, expressed as the difference between the refractive index of the solution and water ( $\Delta r$ ) (see text and 12, Fig. 2). The suspensions were mixed with the mannitol-raffinose mixture of the proper refractive index, and the optical density was measured after 10 seconds of exposure at  $500 m\mu$ . The suspensions were maintained at  $25^\circ C$ .

systems is encouraging. However, it would be more meaningful if it were possible to show that the same optical density or the same apparent osmotically active volume results from the same osmotic pressure, regardless of the substance used.

#### *Independence of the Nature of the Medium*

Any purely osmotic effect should occur independently of the solute used, provided its permeability is relatively low. The same relationship holds for a number of substances, for example, sodium citrate-raffinose mixtures and sodium sulfate alone. However, in these cases, after the initial drop, the optical density decreases rather rapidly with time and the question cannot be

sion medium, equilibrium, in relation to initial water movement, is likely to be reached rapidly. The initial decrease in optical density after exposure to dilute solutions of sucrose, raffinose, and mannitol seems to be essentially over within the mixing period (less than half a second). However, this initial decline is followed by a slower one which probably corresponds to the effect of the penetration of the solute. In the experiments described in the previous section, a 10-second exposure to the experimental medium was used for convenience. Although this procedure is suitable for the experiments carried out in the initial characterization of the system, it probably includes in the measurement the effect of pene-



tration of some of the solute. Although this effect is probably small, it is significant. The results obtained at shorter exposure times ( $\frac{1}{2}$  second or 1 second) most closely approximate the apparent volumes predicted from osmotic law. Accordingly, for the experiments to be described in this section, the transmission of the suspension was measured

TABLE I  
*Independence of the Effect from the Nature of the Solute*

The effects were measured at 500 m $\mu$  and the suspension was maintained at 24°C. The relative osmotically active volumes were calculated by using equation 6 after determining the constants  $\alpha$ ,  $\beta_s$ , and  $\kappa$  with mannitol-raffinose calibration curves (see text). The results reported under experiment 1 represent the average of 4 determinations, those under experiment 2 the average of 8 determinations. The deviations reported are standard errors.

Experiment	Substance	$V - b/V_0 - b$	
		Estimated	Theoretical
1	Sucrose	0.75 $\pm$ 0.06	0.77
	Glucose	0.77 $\pm$ 0.06	0.77
	Sucrose	0.57 $\pm$ 0.06	0.57
	Glucose	0.60 $\pm$ 0.03	0.57
	Sucrose	0.41 $\pm$ 0.10	0.45
	Glucose	0.45 $\pm$ 0.05	0.45
2	Sucrose	0.70 $\pm$ 0.06	0.77
	Glucose	0.70 $\pm$ 0.01	0.77
	Sucrose	0.59 $\pm$ 0.03	0.57
	Glucose	0.58 $\pm$ 0.08	0.57
	Sucrose	0.37 $\pm$ 0.07	0.45
	Glucose	0.45 $\pm$ 0.02	0.45

with time with a rapid recording system (see Methods).

The optical densities recorded after 1 second (experiment 1, Table I) or  $\frac{1}{2}$  second (experiment 2, Table I) were used in the analysis of the data of this experiment. These were the earliest recordings consistently free of mixing artifacts in each experiment.

The same apparent volume is evident for the three cases. This is shown in Table I. The values predicted from osmotic law (theoretical  $V - b/V_0 - b$ ) are compared with the relative osmotically active volume (apparent  $V - b/V_0 - b$ ) obtained experimentally for sucrose or glucose. The neces-

sary constants ( $\kappa$ ,  $\beta_s$ ,  $\alpha$ ) for the calculations were obtained by determining the effect of osmotic pressure and refractive index independently by means of raffinose-mannitol solutions. The apparent relative osmotically active volumes ( $V - b/V_0 - b$ ) were calculated by means of equation 6. In effect, the optical densities corrected for refractive index were compared for sucrose, glucose, and mannitol-raffinose (since the latter was used to determine the constants) solutions, each at three different concentrations.

It is clear that the effect, as far as tested, is independent of the substance used. Therefore, it is likely to represent an osmotic event. As we have seen earlier, osmotic effects should be osmotically reversible. Osmotic reversibility could thus constitute an additional criterion for differentiating between an osmotic effect and an effect brought about by other phenomena.

### *Osmotic Reversal*

Changes in optical density which correspond to osmotic volume changes should be reversible osmotically in a predictable fashion. For example, an osmotically induced low optical density should be reversed by the addition of a calculated amount of solute. On the other hand, an osmotically induced high optical density should be predictably reversed by dilution of the solute. In either case, the final optical density should be the same as, or very close to, that of other suspensions of corresponding microsomal concentration and refractive index, which were exposed to a single osmotic pressure of the medium (represented in these experiments by the raffinose-mannitol calibration suspensions). Since the final microsomal concentration and refractive index in these experiments are not the same as the initial values, the results have been expressed as apparent relative osmotically active volume ( $V - b/V_0 - b$ ) as discussed above (see equation 3 and its discussion). As we have seen, these values are found readily by calculation after obtaining the necessary constants from calibration suspensions of microsomal concentration, refractive index of the medium, and wavelength of incident light identical with those of the experimental determinations.

Fig. 5 A represents an experiment in which an apparent swelling was reversed osmotically. The microsomes were initially exposed to a low concentration of raffinose. After 20 seconds of exposure, a concentrated solution of glucose was

added. The apparent swelling appears fully or almost fully reversible (closed circles). The reversal volume predicted from osmotic law is represented by the lower dashed line, the initial theoretical volume by the upper dashed line. An equivalent volume of the original raffinose solution was added to the control suspensions (open circles).

ments, exposure to mannitol or mannitol-raffinose rather than sucrose). In this experiment, the effect of concentrated mannitol is reversed by dilution (closed circles). A control, where the suspension is diluted by the addition of the original concentration of mannitol, is represented by the open circles. In this figure, the dashed lines repre-

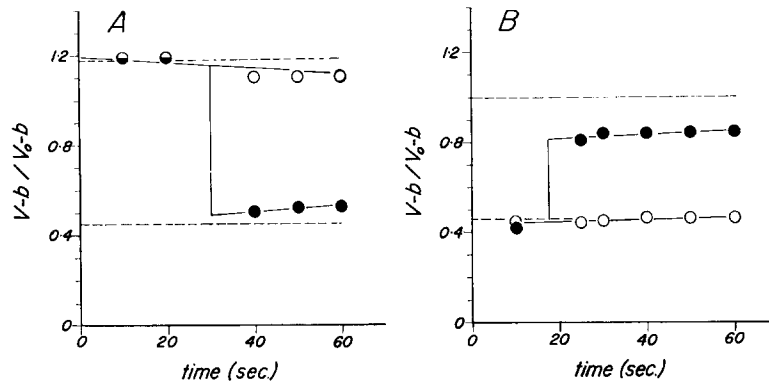


FIGURE 5 Osmotic reversal of the photometric changes. The calibration determinations were carried out at the refractive index, microsomal concentration, and wavelength of incident light of the experimental suspensions after 10 seconds of exposure. The apparent relative osmotically active volume  $(V - b/V_0 - b)$  was calculated using equation 3 (see text).

A. Reversal of an apparent swelling. At zero time, 10 cc of 0.284 osmolal raffinose-tris were added to the stock suspension. For reversal, 8 cc of 1.396 osmolal glucose-tris were added (closed circles). For the control, 8 cc of 0.284 osmolal raffinose-tris were added (open circles). Each point represents an average of 8 determinations for the reversals and of 7 for the controls. The standard error of each point is smaller than the radius of the circles. The temperature of the suspensions was maintained at 20°C. The measurements were carried out at 600  $m\mu$  before dilutions and at 500  $m\mu$  after dilution.

B. Reversal of apparent shrinkage. At zero time, 10 cc of 0.736 osmolal mannitol-tris were added. For reversal, 25 cc of 0.125 osmolal raffinose-tris were added (closed circles). In the controls, 25 cc of 0.736 mannitol-tris were added (open circles). Each point represents 8 determinations in the experimental curve and 6 in the control curve. The standard error of each point is smaller than the radius of the circles. The temperature of the suspensions was maintained at 25°C. The measurements were carried out at 700  $m\mu$  before dilution and at 500  $m\mu$  after dilution.

Fig. 5 B represents an experiment in which the microsomes were exposed to hypertonic conditions by exposure to a concentrated mannitol solution. After 10 seconds, the concentration was diluted by the addition of 25 cc of a dilute raffinose solution. Since our suspension medium is hypertonic (0.736 osmolal sucrose-tris), every experiment or calibration in this study is probably a version of this experiment. The fact that transmission changes can be measured in experiments using these suspensions argues for a reversal. However, it is possible that non-osmotic effects could result from changes introduced during the resuspension (*e.g.*, change in temperature, dilution of the suspension, and, for most of these experi-

ent volumes predicted by osmotic law; the lower line represents the initial volume, and the upper line represents the final volume. The deviation from the predicted reversal volume is significant. It is possible that this deviation reflects a small non-osmotic effect of mannitol. On the other hand, it might be the result of the exposure of a small fraction of the particles to the very dilute reversal solution (0.125 osmolal raffinose-tris) before mixing is complete. We have seen (see discussion of the dependence of optical density on osmotic pressure) that below a concentration ranging from 0.25 to 0.33 osmolal the values of  $\phi$  obtained are lower than predicted. This effect

might be the result of the leakage of internal solute. This question was not pursued further.

## CONCLUSIONS

The data presented in these experiments are consistent with the interpretation that microsomes behave osmotically. This concept is supported by the facts that (a) the same relationship describes optical density in relation to osmotic pressure regardless of the number of particles in the suspension, the wavelength of incident light, and the refractive index of the medium, (b) the function describing optical density in terms of osmotic

pressure is modified by these parameters in a way analogous to that found for other osmotic systems (e.g., erythrocytes and mitochondria), (c) where tested under valid conditions the effect was found to be independent of the nature of the solute, and (d) the photometric changes were found to be completely or largely reversible by osmotic means.

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