THE INTRACELLULAR TRANSPORT AND DISTRIBUTION OF CYSTEAMINE PHOSPHATE DERIVATIVES

SAMUEL B. HOROWITZ, I. ROBERT FENICHEL, BRUCE HOFFMAN, GEORGE KOLLMANN, *and* BERNARD SHAPIRO

From the Laboratory of Cellular Biophysics, Albert Einstein Medical Center Research Laboratories and the Radiation Research Laboratory, Department of Nuclear Medicine, Division of Radiology, Albert Einstein Medical Center, Northern Division, Philadelphia, Pennsylvania 19141

ABSTRACT Radioautography and extractive techniques were used to analyze the transport of cysteamine phosphate and its derivatives in salamander oocytes. The quantitative relations among the processes involved — membrane permeation, enzymatic dephosphorylation, binding through mixed disulfide formation, and cytoplasmic diffusion — were elucidated. Within the detection limits, all of the intracellular material is present as dephosphorylated derivatives. Cytoplasmic diffusion is effectively slowed by binding (the "chromatographic" effect) and makes an appreciable contribution to cellular flux rates. As a consequence, one can observe by radioautography a cortical diffusion ring which spreads inward as a function of influx time, while also increasing in peak density because of the finite membrane permeability. Good agreement was found between the transport parameters determined by radioautography and those from influx data for the whole oocyte. The ratio of nuclear to cytoplasmic concentrations of the cysteamine phosphate derivatives at equilibrium is about 0.4. The nuclear membrane is, however, a negligible barrier to transport, and the asymmetry appears to arise primarily from the quantity and sulfhydryl content of the binding proteins in the two compartments.

INTRODUCTION

In the present paper we describe the use of quantitative, low temperature radioautography and whole cell extractive techniques in the study of the intracellular transport of a radioprotective agent. This study was undertaken for a twofold purpose: to obtain specific knowledge of the intracellular distribution of a radioprotective agent, both during kinetic processes and at flux equilibrium, and to examine intracellular transport mechanisms in which specific site interaction (binding) between solute and cellular macromolecules is significant — in this case, cysteamine-protein mixed disulfide formation.

In these experiments amphibian oocytes were incubated in a solution of cysteamine phosphate-^{35}S and the metabolic changes and transport of the solute were studied. The amphibian oocyte was used for its large size and regular shape which greatly mitigate resolution problems associated with radioautography. Amphibian oocytes appear to be quite typical in a number of membrane properties (7), and results derived in the use of these cells may be expected to have general applicability. In the present study, the oocytes of the plethodontid salamander *Desmognathus o. ochrophaeus* were used.

MATERIALS

Oocytes

Mature ovarian oocytes were isolated from *Desmognathus o. ochrophaeus* collected in early June in streams of the Pine Creek drainage, Tioga County, Pa. Salamanders were kept sexually isolated at 12°C in large preparation dishes. They were fed weekly on vestigial-winged *Drosophila*, and their dishes cleaned and water replaced semiweekly with spring water. Under these conditions, no spontaneous ovulation took place during June, July, or August and no mortality occurred in the colony.

The reasons for using the oocytes of the plethodontid salamanders in these studies, rather than those of the frogs *Rana* or *Xenopus* more traditionally employed, are reviewed by Horowitz and Fenichel (8). Suffice it to say here, that the oocytes of *Desmognathus* are larger than those of frogs and are devoid of pigment granules.

Variation in weight of mature oocytes in the population of *Desmognathus* used in this study was greater than one finds in frogs. The mean oocyte size and its standard error (SE) among individual female salamanders utilized was 11.51 ± 3.73 mg. A typical animal yielded oocytes with an average weight and standard deviation (SD) of 11.85 ± 1.53 mg. The ratio of dry to wet weight obtained by drying oocytes for 24 hr at 105°C was 0.615 ± 0.017 (SD). The smallest animal used yielded oocytes weighing 6.33 ± 0.20 mg (SD), with a dry weight ratio of 0.599 ± 0.032 (SD). The average radius of the oocytes in these two groups was 0.1348 and 0.1094 cm, respectively, so that a large difference in weight reflected only a 25% difference in linear dimensions.

The oocytes were removed from decapitated salamanders which had been pinned to a cork surface and immersed in cold Ringer's solution. The right and left ovaries were removed; each contained 5–15 oocytes. Under a dissection microscope and using iridectomy scissors, the ovaries were carefully slit to form a sheet and the individual oocytes isolated with a small amount of adherent ovarian epithelium. The follicular envelopes were trimmed flush, except for a small tab of epithelium to permit transfer. Adherent small oocytes were carefully removed, or when this was not possible, crushed with forceps.

Solutions

The Ringer's solution used throughout consisted of 24.0 mM glucose; 92.7 mM NaCl; 2.5 mM KCl; 1.0 mM $CaCl_2$; 1.2 mM $MgSO_4$; 17.3 mM $NaHCO_3$; 2.0 mM NaH_2PO_4; 1.2 mM Na_2HPO_4. The pH was 7.2–7.3.

Sodium hydrogen cysteamine phosphate-^{35}S (H_2N-CH_2-CH_2-^{35}S-PO_3HNa) was synthesized according to the method of Akerfeldt (1) which yielded a chromatographically pure product with a specific activity of 10–15 mCi/mmole.

Radioautographic Plates

Kodak NTB emulsion-coated plates were made and used as described by Horowitz and Fenichel (1968).

METHODS

In the experimental procedure, a number of oocytes are incubated in a solution of cysteamine phosphate-^{35}S in Ringer's solution. Oocytes and aliquots of incubation fluid are removed at various times for one or more of the following analyses: (*a*) extraction and liquid scintillation counting to determine total ^{35}S activity; (*b*) paper radiochromatography to identify the extracellular and intracellular ^{35}S-containing metabolites; (*c*) low temperature radioautography of oocytes to determine the intracellular distribution of ^{35}S-containing substances; and (*d*) determination of oocyte Na^+ and K^+ content as a control on the toxic effect of mercaptoethylamine and self-irradiation.

Incubation

All incubations were performed at 20°C. In a typical experiment 20–30 oocytes were transferred with Ringer's solution into a small vial and permitted to come to temperature equilibrium. The supernatant was carefully removed and 1 ml of Ringer's solution containing 1 mM cysteamine phosphate-^{35}S was added. The detection of metabolic products of cellular origin in the supernatant required that only a small volume of incubation fluid be used. There was therefore a decline in extracellular activity during the incubation. This decline amounted to about 40% in 3 hr and was small thereafter. Activity lost from the medium appeared quantitatively in the oocytes. The oocytes were shaken by hand continuously for the first 2000 sec of influx and, intermittently, three or four times an hour thereafter. It will be seen that the kinetic processes being studied are sufficiently slow that aqueous diffusion could not be rate limiting.

Incubation was terminated by removing the individual oocyte from the radioactive solution and rinsing it for 10 sec in ice-cold distilled water and blotting on filter paper. The duration of incubation is designated by t_I.

Extraction and Liquid Scintillation Counting for ^{35}S

Following the termination of incubation, the oocyte was placed in a 22 ml counting vial containing 1 ml of distilled water. Tube and contents were placed in boiling water for 20 min (under these conditions the oocytes ruptured) and then cooled to room temperature. 20 ml of Bray's solution was added to the tube, and counting was done in a Packard 3214 liquid scintillation spectrometer at 2°C. The standard error of the count was kept below 0.5%.

Radiochromatography

Descending chromatography was employed utilizing untreated strips of Whatman No. 1 paper. The solvent systems were ethanol:isopropanol: 1 N HCl (60:60:40), and ethanol: water:29% ammonia (60:30:10). The strips were run at 5°C for 40 hr. The paper strips were dried at room temperature and scanned for radioactive areas with an automatic strip scanner and Geiger-Müller flow counter. Planimetry of the areas under the peaks provided a measure of the relative amounts of ^{35}S in each peak.

RADIOAUTOGRAPHY

Technique

The radioautographic technique used for frog oocytes has been described in detail (7), as are its recent modifications and application to plethodontid oocytes (8). Briefly, the technique involves the following steps:

(*a*) Individual oocytes were frozen in a commercial mounting media, O.C.T. (Lab-Tek, Westmont, Ill.), in dichlorodifluoromethane cooled to −160°C with liquid nitrogen. The elapsed time from immersion in O.C.T. to the completion of freezing was about 10 sec.

(*b*) Sectioning and tissue-emulsion contact were carried out at −50°C in a Wedeen type cryostat (Refrigeration for Science, Inc., Oceanside, N.Y.). Sections were 16 μ in thickness and transferred to a Teflon sheet backed by a microscope slide. A radioautographic plate was placed, emulsion downward, on the sections to form a sandwich: slide, Teflon, sections, emulsion, slide.

(*c*) The sandwich was exposed at −73°C for 60–200 hr. Exposure was terminated by separating the sandwich at room temperature.

(*d*) The radioautographs were developed for 6 min in Kodak D19, followed by 10 sec in 1% acetic acid stop and 5 min in Kodak Rapid Fix.

(*e*) Grain densities were determined by grain counting at a magnification of 1250 × in either phase contrast or dark field illumination, using a Whipple-Hausser type eyepiece micrometer. The grains appeared essentially in a single plane of focus as bright refractile spots beneath the section. Grain densities (*g*) are expressed throughout as grains/1000 μ^2 per hr of radioautographic contact, and have had background density subtracted.

Resolution

The resolution of the radioautographs can be assessed by assuming that the concentration of ^{35}S at the surface of an oocyte, previously equilibrated with cysteamine phosphate-^{35}S and rinsed, changes as a step function, and by determining the grain density profile through the discontinuity. An example is given in Fig. 1. If we define resolution as the distance over which grain density falls by 50%, it is in this figure about 45 μ. This is a much greater distance than would be calculated by measuring from the edge of the source to the 50% grain density level (*cf.* 3, 11) but it is a conservative and probably more realistic estimate. By the latter criteria, resolution is about 7 μ. Our experience leads us to doubt that two planar sources, comparable to oocyte sections, having parallel edges 7 μ apart could be reliably distinguished.

Quantitation

For the purposes of this study, it was not considered necessary to construct calibration curves relating grain density to ^{35}S activity. We have assumed that by main-

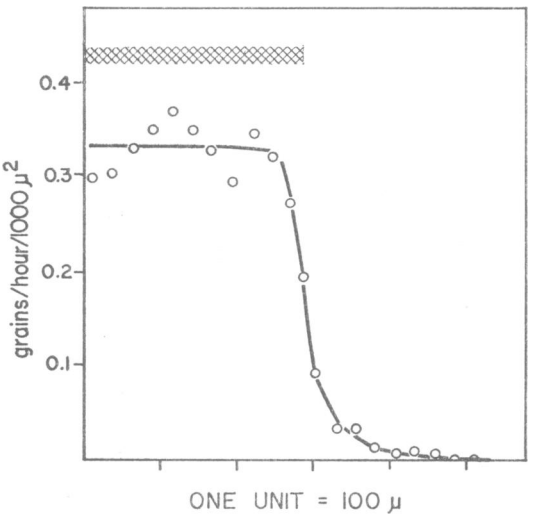


FIGURE 1 Grain density profile taken at the border of a 16 μ section of an oocyte equilibrated with cysteamine phosphate–^{35}S and briefly rinsed. The cross-hatched area shows the position of the overlying section.

taining grain densities at less than 200 grains/1000 μ^2, the multiple hit correction will be negligible (*cf.* 7), and grain density will be proportional to ^{35}S concentration in the overlying section. The validity of this assumption and the over-all reliability of treating grain density, normalized for time of radioautographic exposure, as proportional to the ^{35}S concentration was confirmed as follows. Profiles of grain density were taken along the radius of sections of oocytes which had been incubated in cysteamine phosphate–^{35}S (e.g. see Fig. 6) for various periods of time. From these profiles the total number of grains, G, that would be derived from the whole oocyte, per hour of radioautographic exposure, was estimated from the equation

$$G = 4\pi \sum g_i r_i^2 \delta r \qquad (1)$$

in which g_i is the local grain density in grains/μ^2 per hr, r_i the distance from the center of the section, and δr the measurement interval along the radius, in this case 50 μ. The summation is taken along the entire radius.

G can be compared with the total activity (expressed as μmoles of S uptake) in sister oocytes incubated for comparable periods of time in the same tubes. The expectation is that in the absence of systematic errors these parameters will be linearly related; Fig. 2 shows this to be the case.

There are a number of significant sources of variation in grain density which must be kept in mind when working quantitatively. These include: (*a*) differences in emulsion thickness as a function of position on the radioautographic plate (see reference 13 for a discussion of this factor); (*b*) differences in emulsion sensitivity and emulsion dilution in different lots of radioautographic plates; and (*c*) differences in emulsion sensitivity as a function of the time and conditions of storage of the radioautographic plates. These factors complicate comparisons between grain

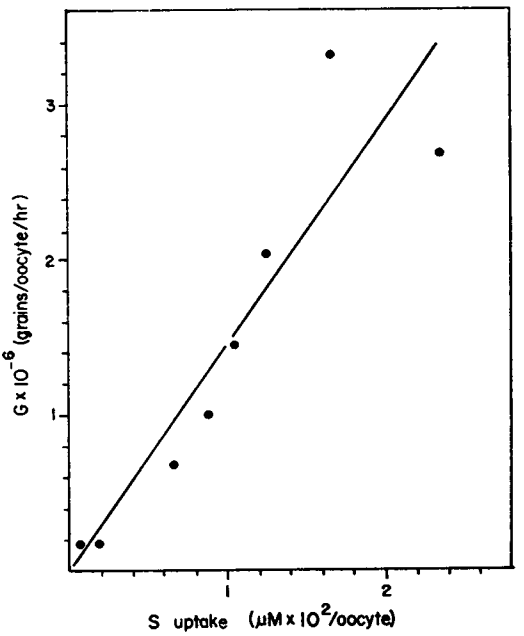


FIGURE 2 Relationship between the total grains derived from a whole oocyte per hour of autoradiographic exposure, G, and ^{35}S uptake of the oocyte. See text for procedure and additional explanation. Points experimental, line fitted.

densities on radioautographs made at different times. When quantitative comparisons are made herein, they are restricted to data which are comparable with respect to these variables. There are points to be made which do not require strictly quantitative grain density comparisons. For example in Fig. 6, *d* and *e* are derived from a different experiment than *a–c*, consequently there are apparent inconsistencies in peak grain densities between the two groups, in spite of the fact that there was good agreement in ^{35}S uptake as determined by whole oocyte analysis. In Fig. 7, where we are comparing peak grain densities, all of the data were derived from a single experiment, and from radioautographs that were kept constant with respect to the above mentioned variables.

Flame Photometry

Flame photometry was used to determine cellular Na^+ and K^+, as a control on the physiological state of the oocytes exposed over extended periods to cysteamine phosphate–^{35}S. Single oocytes were placed into carefully cleaned polypropylene containers with 5 ml of doubly distilled water. The containers were tightly sealed, boiled for 20 min, and cooled to room temperature. The ion content of the supernatant was determined on a Zeiss emission flame photometer using an oxy-hydrogen flame. Additional details of this procedure are provided by Century et al. (4).

In general both Na^+ and K^+ levels remained constant during the course of experiments. In the longest experiment controlled in this manner, an oocyte was taken at 0, 2.8, 6.9, 20.7, and 29.4 hr; Na^+ concentrations were 31.9 ± 1.4 (SD) and K^+

concentrations 52.1 ± 4.2 (SD) μEq/g oocyte; the small amount of variation was independent of incubation time.

RESULTS

Whole Oocyte Analysis

The time course of influx of ^{35}S derived from the cysteamine phosphate-^{35}S in the incubation fluid is shown in Fig. 3. The process is slow; its completion takes more than 10 hr. By 30 hr, the extracellular concentration of ^{35}S has fallen from 1.0 to 0.54 μmoles/ml Ringer's, and the intracellular concentration of ^{35}S was in excess of 2.4 μmoles/g oocyte or 6.0 μmoles/ml oocyte water, which is about 11.1 times the concentration in the bathing solution on a water basis.

The chemical identity of ^{35}S-containing material involved in the flux was established as follows. Whole oocytes were removed from the incubation fluid at 4.9 hr, rinsed in distilled water, and squashed and smeared out on a strip of filter paper for chromatography. The dried strips were chromatographed in either the acid or basic solvent systems as described in Methods. In both solvents about 90% of the activity remained at the origin and 8–10% moved as a single peak, in the acid medium at $R_f = 0.30$ and in the basic medium at $R_f = 0.72$.

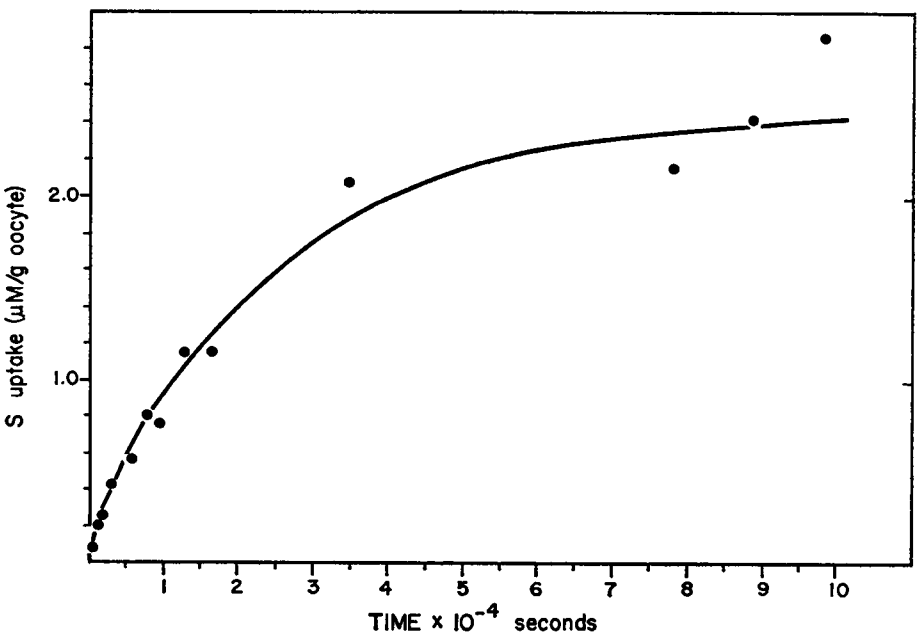


FIGURE 3 Concentration of cysteamine phosphate-derived S, determined by extraction and counting of ^{35}S, in oocytes as a function of influx time, t_I. Points experimental, line fitted.

The R_f values for cysteamine phosphate in acid and basic media are 0.50 and 0.58, respectively; for cysteamine 0.65 and 0.72, respectively; and for cystamine 0.30 and 0.72, respectively. We conclude that about 10% of the intracellular activity is in the form of cystamine-^{35}S.

To determine the nature of the ^{35}S that did not move in the original chromatogram, the origin of the chromatogram was cut off, sewn to fresh paper, and saturated with 10 mM cysteine or glutathione and dried. This treatment has been shown to free thiols present as mixed disulfides with cellular proteins (14). The preparation was

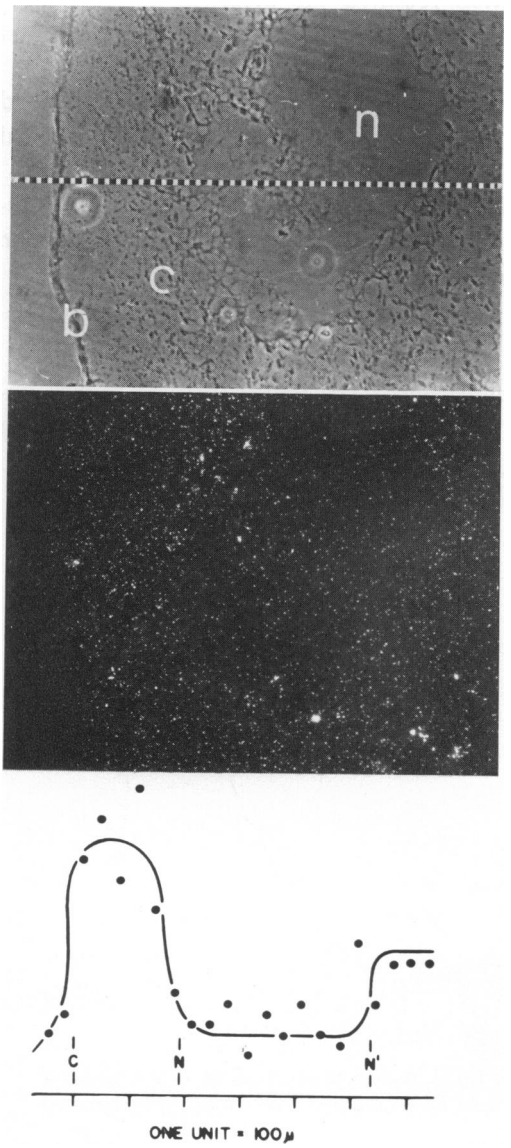


FIGURE 4 (Top) Phase contrast view of a radioautograph of an oocyte at equilibrium (t_I = 21.0 hr) showing the oocyte boundary, *b*; cytoplasm, *c*; and nucleus, *n*. (Middle) The same view in dark field illumination showing the distribution of silver grains in the various compartments. (Bottom) A grain density profile taken along the dotted line above, from outside the cell through the nucleus. Magnification: ×73.3.

chromatographed. Less than 2% of the activity remained at the origin, and a single peak was observed at R_f 0.65 and 0.72 in the acid and basic solvents, respectively.

Finally, samples of incubation fluid were removed for chromatography at 2.1, 4.9, 23.8, and 27.2 hr. In each case, a single peak characteristic of cysteamine phosphate was found. This was also the case in samples of fluid that were incubated in the absence of oocytes, whether or not the fluid had been in contact with oocytes.

Within the resolution of the chromatographic method, which is probably about 5% of the activity present in the oocyte, we draw the following conclusions:

(*a*) All of the intracellular ^{35}S was present as dephosphorylated derivatives of cysteamine phosphate. Dephosphorylation required the presence of cells; once having been dephosphorylated neither the free thiol nor its derivatives were found in measurable quantities outside of the cell.

(*b*) After dephosphorylation, 90% of the free thiol cysteamine reacts to form mixed disulfides with thiol groups of cellular protein while 10% forms the symmetrical disulfide cystamine.

For the remainder of this paper, we will use the following abbreviations for cysteamine phospate (MEAP) and its various derivatives: cysteamine (MEA), cystamine (MEAD), and the mixed cysteamine-protein disulfide (MEAR).

Radioautographic Analysis

General Features of the Radioautograph. The major features of a radioautograph of an oocyte section are seen in Fig. 4. The oocyte in this case had a long

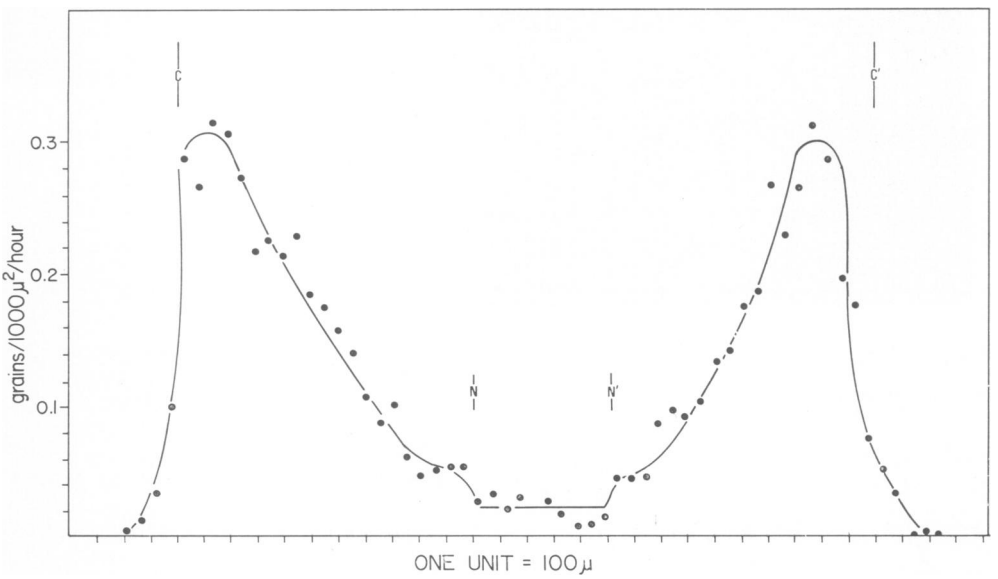


FIGURE 5 A grain density profile through the animal hemisphere of an oocyte at t_I = 7.5 hr. *C* and *C'* are oocyte boundaries, *N* and *N'* are nuclear boundaries. For additional explanation, see text.

incubation (t_I = 21.0 hr) and was sectioned so that the nucleus appears strongly eccentric and close to the cell surface. At the top is a phase contrast view including portions of the cell boundary, cytoplasm, and nucleus. Below is the same field in dark field illumination; the silver grains appear as bright spots. At the bottom is a grain density profile taken along the dotted line from outside the cell through the nucleus.

Fig. 4 shows the grain density edge at the cell surface (used in Fig. 1 to establish the resolution of the method), the high grain density of the cytoplasm, and the lower grain density in the nucleus. At extended influx times (t_I > 20 hr) grain density

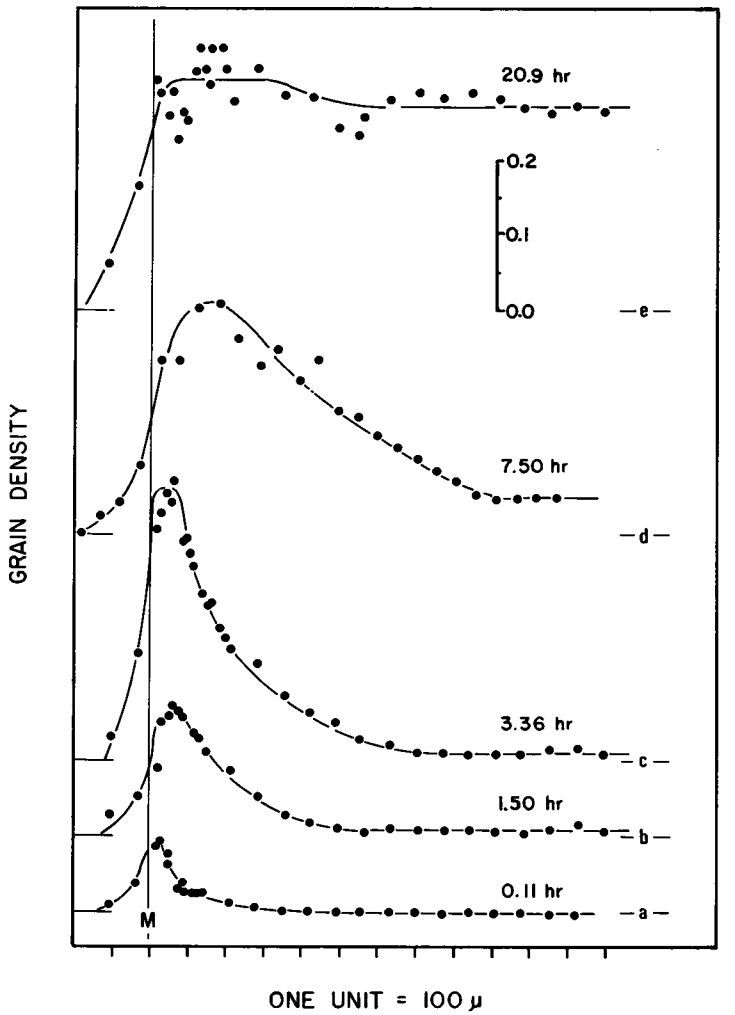


FIGURE 6 Radial grain density profiles through the cytoplasm of oocytes at t_I from 0.11 to 20.9 hr. The vertical scale is in grains/1000 μ^2 per hr. The horizontal lines labeled *a–e* indicate the base line background grain densities. The position of the cell surface is indicated by the line *M*.

in the cytoplasm is more or less uniform (e.g. Fig. 6 *e*) while at shorter t_I, this is not the case. Fig. 5 is a grain density profile through an oocyte incubated for 7.5 hr and sectioned latitudinally in the animal hemisphere. The activity is nonuniform; a diffusional ring exists, grain density being highest in the cell cortex and decreasing radially to the cell center. Furthermore, as seen in Fig. 4, there is discontinuity at the nuclear border, the nucleus having a lower concentration of ^{35}S than the adjacent cytoplasm.

Cytoplasm. A diffusional ring is characteristic of oocytes incubated for short times. Fig. 6 *a–e* are radial profiles for various t_I. The diffusional ring both broadens and increases in peak density with increasing t_I. Width and height are essentially uniform around the perimeter of a given section, hence, significant regional variation in permeability is not indicated.

Fig. 7 shows the peak cortical grain density as a function of t_I for a series of grain density profiles from oocytes of a single experiment. After a rapid early rise, the increment is linear with respect to time.

We know from other experiments that peak cortical grain density asymptotes between 4.6 and 7.5 hr, and is constant after 7.5 hr.

Nucleus. As shown in Figs. 4 and 5, the nuclear activity is less than that in the adjacent cytoplasm. The nuclear/cytoplasmic distribution taken as the ratio of nuclear (g_n) to cytoplasmic (g_c) grain densities at the longest times studied were

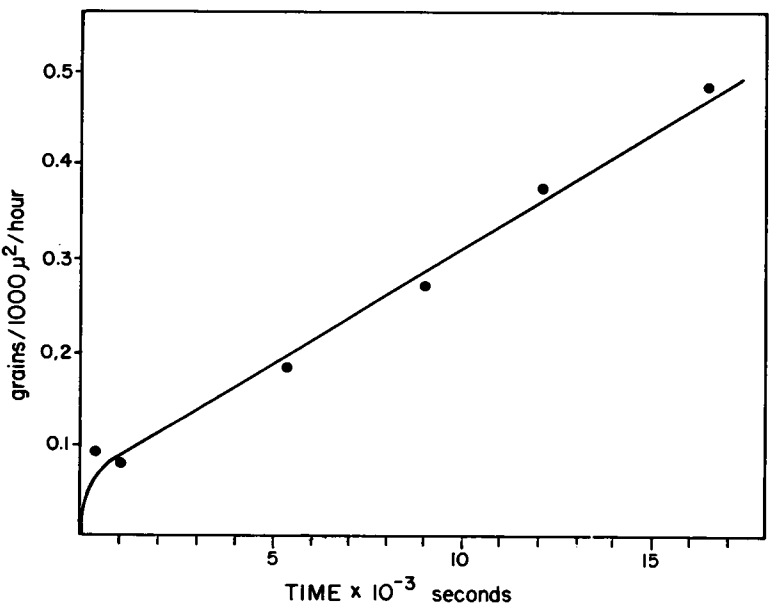


FIGURE 7 The peak cortical grain density in radioautographs of oocytes exposed to cysteamine phosphate-^{35}S as a function of influx time, t_I.

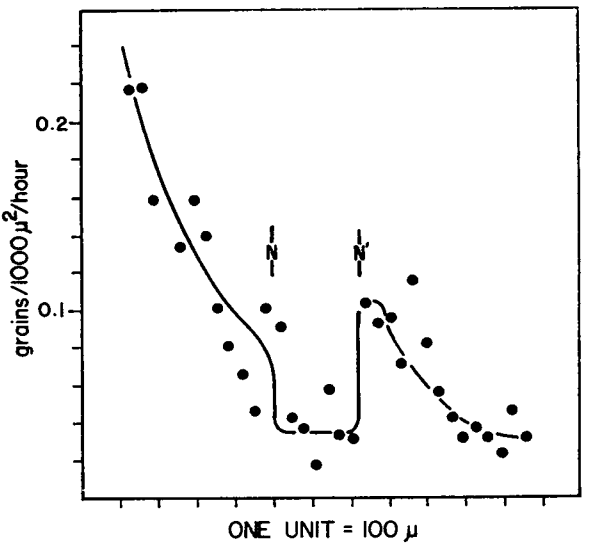


FIGURE 8 A grain density profile through cytoplasm and nucleus located on a portion of a diffusional gradient at t_I = 4.6 hr. N and N' indicate the nuclear boundaries.

at t_I = 20.9 hr, 0.42 ± 0.18 (SD; 6 nuclear, 19 cytoplasmic determinations) and at t_I = 29.6 hr, 0.40 ± 0.16 (SD; 6 nuclear, 25 cytoplasmic determinations). As no significant change took place in g_n/g_c during the final 9 hr of incubation, 0.41 may be taken as the equilibrium ratio.

We have no evidence to suggest that the entry of tracer into the nucleus is delayed by the nuclear membrane, implying that g_n/g_c is entirely due to differences in the composition of the nucleus and cytoplasm. Evidence against an appreciable transport barrier at the nuclear surface is seen in Fig. 8 which is a profile through a fortuitous section in which the oocyte nucleus is located on a portion of a cytoplasmic diffusion gradient. If an appreciable transport barrier existed at the nuclear surface, we would expect to find that the nuclear grain density was less than 0.41, the equilibrium value of g_n/g_c, of the immediately adjacent cytoplasm. The actual ratio in Fig. 8 is not less than 0.43.

DISCUSSION

The inability to detect MEAP in the oocyte, and the fact that dephosphorylation takes place only within the oocyte, imply that dephosphorylation is limited by the rate of MEAP permeation. This is supported by radioautographs taken at short influx times in that the restriction of activity to a thin cortical layer would seem to mean that dephosphorylation is so rapid as to be complete before the MEAP can diffuse an appreciable distance inward from the cell border. As MEA itself is not present at detectable levels, we surmise that after its production MEA rapidly oxidizes to MEAD or forms the mixed disulfide, MEAR. The latter we refer to as

binding. We will assume that the rate of diffusion of MEAR is insignificant compared to that of MEA or MEAD and, further, that MEAD is the principal diffusing species which carries the thiol group into the interior of the cell.

The mathematical analysis of influx into a body involving a surface permeation process, binding, and diffusion is extremely complicated. It may be simplified by making an appropriate assumption about the relative rates of binding and diffusion. In the oocyte, the presence of a diffusional ring indicates that the appropriate approximation is to assume that binding is rapid relative to diffusion. We can also assume that binding is reversible, as suggested by the ease with which MEA is displaced from cellular protein by other sulfhydryl compounds during chromatography. In these circumstances, intracellular transport is kinetically equivalent to pure diffusion (5, 6) with an effective diffusion coefficient in cytoplasm, D_c', equal to the true cytoplasmic coefficient, D_c, divided by the ratio (R) of total to free material, that is

$$D_c' = D_c/R \tag{2}$$

so that $D_c' = 0.10\, D_c$.

The expression for uptake into a sphere by a combined diffusion-permeation flux is

$$\frac{M(t)}{M(\infty)} = 1 - \sum_{n=1}^{\infty} \frac{6L^2\, e^{-\beta_n^2 D_c' t/a^2}}{\beta_n^2[\beta_n^2 + L(L-1)]} \tag{3}$$

(Crank, 1956) where $M(t)/M(\infty)$ is the fractional uptake at time t. L is given by

$$L = \frac{aP}{D_c'} \tag{4}$$

where a is the cell radius and P is the rate constant for the first-order surface process, the permeability of MEAP. The β_n's are the roots of $\beta_n \cot \beta_n + L - 1 = 0$.

A comparison of $M(t)/M(\infty)$ from equation 3 with the value determined experimentally by extractive analysis (Fig. 3) can be made using independently derived parameters for P, D_c', and a.

An estimate of P of 3.3×10^{-6} cm/sec can be obtained from Fig. 7 if we assume that the peak cortical concentration is determined solely by the rate of permeation.[1]

D_c for MEAD in oocyte cytoplasm can be estimated from the approximation

$$D_c \approx D_c^w\, (m_{H_2O}^{-1/2}/m_{MEAD}^{-1/2}) \tag{5}$$

where D_c^w is the diffusion coefficient of water in oocyte cytoplasm, and m_{H_2O} and m_{MEAD} are the molecular weights of water and cystamine, respectively. Fenichel

[1] P as derived is an apparent rather than a true permeability coefficient. This is because the surface of the oocyte is microvillous, the true surface being greater than the surface of the simple sphere assumed. In young *R. pipiens* oocytes, the surface area is about 35 times that of a smooth sphere (9), but it is unlikely that the ratio in the mature oocyte is as high.

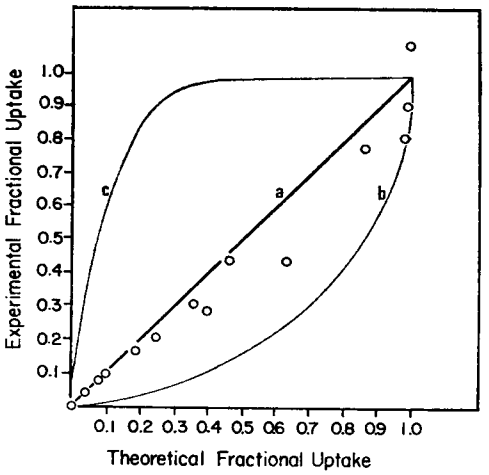


FIGURE 9 Comparison of the theoretical fractional uptake of ^{35}S from equation 3 using the parameters $P = 3.3 \times 10^{-6}$ cm/sec, $D_c' = 1.5 \times 10^{-7}$ cm²/sec, with the experimentally determined fractional uptake derived from extractive analysis, using $M_\infty = 24.3 \times 10^{-3}$ μmole/oocyte. *a* indicates the line of perfect coincidence of experimentally and theoretically derived values. See text for additional details, derivation of parameters, and the meaning of theoretical curves *b* and *c*.

and Horowitz (6) estimated D_c^w to be about 0.25 the self-diffusion coefficient of water in free solution (15), or at 20°C about 4.7×10^{-6} cm²/sec. Then $D_c \approx 1.5 \times 10^{-6}$ cm²/sec and, from equation 2, $D_c' \approx 1.5 \times 10^{-7}$ cm²/sec.

The value of *a* for each oocyte is calculated from the oocyte weight assuming it is a perfect sphere and has a specific gravity of 1.15. The average value of L is 2.4.

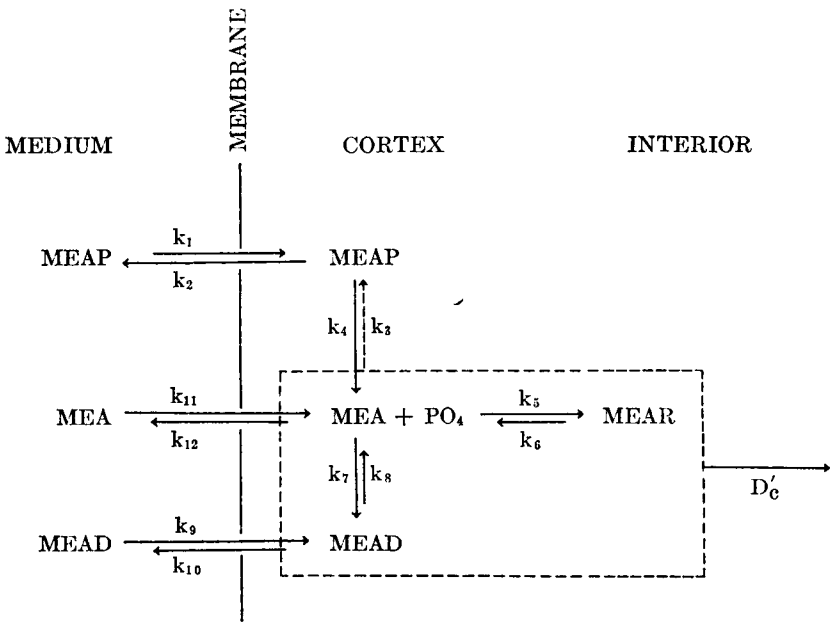
The fractional uptake of tracer predicted by equation 3 and that determined directly are compared in Fig. 9. Line *a* is theoretical and corresponds to a perfect coincidence of derived and measured values; the points indicate the actual coincidence observed. The coincidence is quite good at the lower values (short influx times), but there is an overestimation by about 14% of the rate of approach to equilibrium at longer times. The cause of this deviation is not known but it may arise from either R or D_c not being constant everywhere within the oocyte, but rather varying from the cortex to the center. This explanation seems likely, as the particulate contents of the cytoplasm are not subject to mixing and are laid down cortically so that the material at the center of the oocyte is older, and presumably somewhat different, than that at the periphery (9, 16).

Curves are presented in Fig. 9 for two additional theoretical cases which help underscore the significance of both permeability and binding in determining the influx. Curve *b* results if the membrane permeability to MEAP is assumed to be appreciably greater than 3.3×10^{-6} cm/sec, so that D_c' is the sole determinant of influx. Curve *c* results if binding is assumed to be absent ($R = 1$) and the determinant of influx is membrane permeability. Clearly, neither of these cases prevails.

While our studies do not permit a complete characterization of the concentrations and equilibria that exist among all the various molecular species derived from MEAP and found in the medium and the cytoplasm and nucleus of the oocyte, a picture consistent with the data available can be drawn. At flux equilibrium, the cellular content of MEAP derivatives is about 2.4 μmole/g oocyte, or 4 times the

MEAP concentration in the medium; at least four molecular species, MEAP, MEAR, MEA, and MEAD coexist at this time. Of these, we have detected only MEAP in the incubation medium, and only MEAR and MEAD in the cell. The explanation for these results appears to lie in the resolution of the chromatographic method, which we estimate to be about 5% of the activity on the chromatogram, and in the rapid hydrolysis of MEAP and the transmembrane asymmetries expected for the various molecular species due to the oocyte Donnan equilibrium. Morrill and Watson (10) have reported the resting potential of *Rana pipiens* oocytes to be −63 mv with respect to Ringer's solution. If a similar potential exists in *Desmognathus* oocytes, we would expect the three permeable MEAP derivatives to distribute between the medium and cell as a function of their net charge. At pH 7.2, MEA and MEAD have one and two positive charges respectively, while MEAP has a negative charge. The expected Gibbs-Donnan ratios, r, will be 10 and 100 for MEA and MEAD, respectively, and 0.1 for MEAP. As a consequence of these ratios, the concentration of MEAD outside the cell and MEAP inside the cell are below the chromatographic resolution. The concentration of MEA, even within the cell, appears to be too low to be detected.

The postulated metabolic and translational changes taking place in the cytoplasm when the oocyte is bathed in MEAP can be schematically summarized as follows:



in which $k_2 = (S/V)P \approx 8.1 \times 10^{-5}$ sec^{-1} where S/V is the cell surface-to-volume ratio; $k_1/k_2 \approx 0.1$; $k_3/k_4 \ll 1$; $k_5/k_6 = K_a \gg 1$; $k_7/k_8 = K_b \gg 1$; $K_a/K_b \approx 9$; k_9/k_{10}

$\approx$ 100; $k_{11}/k_{12} \approx 10$; k_{13}/k_{14} may equal k_7/k_8 . Diffusion from the site of hydrolysis involves the system MEAR $\leftrightarrows$ MEA $\rightleftarrows$ MEAD and has an apparent coefficient, D_c', of 1.7×10^{-7} cm²/sec.

Akerfeldt (2) first demonstrated MEAP cleavage by an enzyme that was intracellular in distribution. Neumann (12) has reported that MEAP cleavage, k_4 , is catalyzed in vitro by alkaline but not acid phosphatase, the rate of reaction being dependent on the concentration of $MgCl_2$.

The finding that the nuclear membrane does not limit the rate of tracer entry into the nucleus was expected. Horowitz and Fenichel (7, 8) showed that the rate at which exogeneous solutes, specifically glycerol-^{3}H and ^{22}Na, enter the nucleus is established not at the nuclear but at the cortical membrane. In the present case, binding in the cytoplasm appreciably slows transport below even the rate established by the cortical membrane, and this factor tends to further reduce the transport significance of the nuclear membrane. On the other hand, the very slow rate at which tracer reaches the nucleus through the cortical membrane—cytoplasm route permits a more rigorous test than was previously possible of the notion that the nucleus has transport pathways to the extracellular compartment which short-circuit the cytoplasm. Of the dozens of oocytes examined, at values of t_I from 1 min to 30 hr, the nucleus invariably exhibited a lower grain density than the adjacent cytoplasm. Since MEAP, as a function of its charge, size, and binding potential, is likely to be among the slowest moving of those solutes whose transport is dependent on ordinary diffusional and permeation mechanisms, we conclude that if endoplasmic connections between the nuclear membrane and the extracellular compartment exist, they do not serve the role of facilitating solute transport. A role in the transport of macromolecules, of course, is not excluded.

The absolute concentrations of MEAP and its derivatives in both nucleus and cytoplasm can, in principle, be directly determined by the application of recently described microdissection techniques (4) and chromatography. Factors that can be anticipated to lead to appreciable differences between nuclear and cytoplasmic concentrations of these substances include: a potential difference between nucleus and cytoplasm (10), a difference in the water content of cytoplasm and nucleus (4), as well as differences in the specific proteins found in the two compartments. Nuclear dry weight in *Desmognathus* oocytes is only 0.33 of that of cytoplasm, and since protein binding occurs to such a great degree, one would expect a lower concentration in the nucleus; but in view of the above considerations, attempts to quantitatively account for the observed value of about 0.4 seem premature.

MEA and MEAP are of pharmacological interest because of their radioprotective properties (14). The slow effective rate of diffusion in the oocyte and the capability of visualizing the intracellular location of the agents appear to make it feasible to perform experiments directed towards localizing the intracellular site of radioprotection. In particular, it appears possible to selectively protect the cell membrane and/or the cortical cytoplasm, keeping the nucleus free of the agents while

irradiating the oocyte. The feasibility of such experiments, of course, is likely to decrease as cell size decreases. For example, if the oocyte were 50 μ in radius rather than over 1200 μ, diffusional equilibrium would be 50% complete in appreciably less than 10 sec, and the nonequilibrium distribution of the MEA derivatives among the intracellular organelles would be impossible to establish.

The permeability coefficient of MEAP in the oocyte is sufficiently low as to suggest that an appreciable time lag, even in small cells, may occur between exposure to MEAP and full radiological protection. As pointed out above, because of the microvillous nature of the oocyte surface, the true permeability of the oocyte membrane may be less than 0.1 of the apparent value. Taking 10^{-6} cm/sec as a conservative upper estimate of MEAP permeability in cortical membranes, a spherical cell of 10 μ radius would have a half-time of exchange in excess of 500 sec. In this regard, it may be relevant to note that Shapiro et al. (14) found radioprotection, following intraperitoneal injection of MEAP into mice, was not maximal until times in excess of 10 min.

We wish to thank Mr. Theodore Century for his help during this investigation.

This study was supported in part by National Science Foundation Grant GB 8032, National Institutes of Health Grant GM 14886, and U.S. Army Medical Research and Development Command, Department of the Army, Contract No. DADA-17-67-C-7165.

Received for publication 2 March 1970 and in revised form 8 May 1970.

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