Evidence for Mediated Protein Uptake by Amphibian Oocyte Nuclei

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ABSTRACT The objective of this investigation was to determine whether there is mediated transport of endogenous proteins across the nuclear envelope. For this purpose, we studied the nuclear uptake of a 148,000-dalton *Rana* oocyte polypeptide (RN1) and compared its actual uptake rate with the rate that would be expected if RN1 crossed the envelope by simple diffusion through the nuclear pores. Nuclear uptake was studied in two ways: first, oocytes were incubated in L-[^{3}H]leucine for 1 h and, at various intervals after labeling, the amount of ^{3}H-RN1 present in the nucleoplasm was determined. Second, L-[^{3}H]leucine-labeled nuclear extracts, containing RN1, were microinjected into the cytoplasm of nonlabeled cells, and the proportion of ^{3}H-RN1 that subsequently entered the nucleus was measured. It was found that RN1 can readily penetrate the nuclear envelope; for example, after 6 h, ~36% of the newly synthesized RN1 and 17% of the injected RN1 had entered the nucleus. The diffusion rate through pores having a radius of 45 Å was calculated for several possible molecular configurations of RN1. Using axial ratios of 34, 7.5, 2, and 1, the estimated times required to reach 63% of diffusion equilibrium are 757, 468, 6,940 h, and infinity, respectively. Even assuming an axial ratio of 7.5 (the most diffusive configuration) and an equilibrium distribution of 45, simple diffusion through the pores could account for only ~1/20 the observed nuclear uptake of RN1. This and other comparisons indicate that some form of mediated transport is involved in the nucleocytoplasmic exchange of this polypeptide.

In a previous study performed on *Xenopus* oocytes, it was found that the nuclear uptake rates of specific endogenous polypeptides, ranging in molecular weight from 70,000 to 110,000, were not altered by mechanical disruption of the nuclear envelope, a procedure that significantly increases nuclear permeability (6). It was concluded that passage across the envelope is not necessarily a rate-limiting step for the translocation of endogenous proteins. Since the migration of exogenous macromolecules within this molecular weight range is restricted by the envelope (7, 14), it would appear that selective mechanisms exist for regulating the intracellular distribution of certain endogenous proteins.

Two possible mechanisms have been suggested (6). First, specific endogenous proteins could be transported across the envelope. If the transport rates were sufficiently rapid, or if transport occurred along structural elements, disruption of the envelope would have no apparent effect on nuclear uptake. Second, endogenous molecules might penetrate the envelope by simply diffusing through the pores, in which case the nuclear uptake rates would be regulated by nucleoplasmic or cytoplasmic processes, such as selective binding. For example, if reasonable assumptions are made for the functional size of the pores and nuclear binding affinity, even the largest *Xenopus* polypeptide that was studied (110,000 daltons) could, theoretically, diffuse into the nucleoplasm rapidly enough to account for the observed accumulation rates.

One line of evidence that would support the hypothesis that proteins are transported would be identifying a polypeptide that readily enters the nucleus but is too large to diffuse through the pores. Our efforts to identify such a molecule centered around a 148,000-dalton polypeptide (to be referred to as RN1), which is one of the major proteins in *Rana* oocyte nuclei (10). The nuclear uptake kinetics of RN1 were studied by determining the nucleocytoplasmic distribution of tritiated RN1 after (*a*) labeling whole oocytes with L-[^{3}H]leucine, or (*b*) microinjecting ^{3}H-labeled nuclear extracts into the cytoplasm of unlabeled cells. Furthermore, since the functional size of the *Rana* nuclear pores is known (14), it is possible to calculate, with a reasonable degree of accuracy, the diffusion rate of RN1 across the envelope. Overall, the results suggest that RN1 enters the nucleus at a greater rate than can be explained by simple diffusion.

MATERIALS AND METHODS

Rana pipiens were obtained from Nasco Corp. (Ft. Atkinson, WI) and stored at 4°C. Ovaries were dissected from pithed frogs, and mature oocytes, ~1,600 μm

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diam, were manually defolliculated in amphibian Ringer's solution. All of the procedures except the preparation of nuclear extracts (see below) were performed at room temperature (20–22°C).

Nuclear Uptake of Endogenous RN1: The nuclear uptake of RN1 was studied as follows. Oocytes were labeled for 1 h in Ringer's solution containing 500 μCi/ml of L-[^{3}H]leucine (sp act, 140 Ci/mmol; obtained from Amersham Corp., Arlington Heights, IL). The oocytes were then rinsed and maintained in Ringer's solution containing 0.47 mM cold leucine. There was no incorporation of L-[^{3}H]leucine into precipitable counts after the initial labeling period (data not included). Groups of 20 cells were manually enucleated at intervals ranging from 0 to 30 h after labeling. Immediately following enucleating, a procedure that requires about 30 s, the nuclei were fixed in EtOH and the envelopes were subsequently removed (5). The relative amount of RN1 in the nucleoplasm at each time interval was determined by running the ^{3}H-labeled nuclei with an equal number of ^{35}S-labeled nuclei, which served as internal standards, on two-dimensional gels. The groups of ^{35}S-labeled nuclei that were used in a given study were processed under identical conditions and presumably contained the same amount of labeled protein. Thus, the relative rate at which RN1 was taken up by the nucleus could be determined from the ^{3}H:^{35}S ratios obtained for extracted RN1 gel spots. To estimate the error inherent in this method, a control experiment was performed in which nuclear uptake was compared in four groups of oocytes, all of which were enucleated 6 h after labeling. It was found that the error in the nuclear uptake determinations was ±20%.

The procedures employed for two-dimensional gel analyses were identical to those described previously (6) except that a 7.5% SDS running gel was used rather than a 10% gel. The methods for preparing ^{35}S-labeled nuclei and measuring radioactivity in gel samples are also given in reference 6.

The following technique was used to determine the fraction of total ^{3}H-RN1 that was taken up by the nuclei at various times after labeling. Oocytes were labeled for 1 h and then incubated in cold leucine for 6, 12, or 24 h, as described above. At each time, labeled and unlabeled whole cells and isolated nuclei were fixed in alcohol and combined in two equivalent experimental groups. Group 1 contained 10 ^{3}H-labeled whole cells, 10 unlabeled nuclei, and 20 ^{35}S-labeled "standard" nuclei. Group 2 contained 10 unlabeled whole cells, 10 ^{3}H-labeled nuclei, and 20 ^{35}S-labeled nuclei. Both samples were sonicated in alcohol and centrifuged at 1,600 *g* for 5 min. The precipitates were dissolved in 120 μl of lysis buffer and run on two-dimensional gels. The fraction of ^{3}H-RN1 in the nucleus was then determined by dividing the ^{3}H:^{35}S ratio obtained for group 2 by that obtained for group 1.

Nuclear Uptake of Injected RN1: The exchange of RN1 across the envelope was also studied by injecting labeled nuclear extracts into the cytoplasm of unlabeled oocytes. Nuclear extracts were prepared using a modification of the procedure outlined by DeRobertis et al. (3). A group of ~200 oocytes was labeled for ~18 h in 2 ml of Ringer's solution containing 1 mCi of L-[^{3}H]leucine. After labeling, the nuclei were manually isolated and stored at 4°C in intracellular medium (5) containing 1.25% polyvinylpyrrolidone (PVP), mol wt 40,000. Approximately 150 nuclei, collected over a period of 45–50 min, were gently packed in a small conical tube by centrifuging at 400 *g* for 1 min. The supernatant PVP solution was removed and replaced by 25 μl of intracellular medium. The nuclei were then homogenized by drawing them through an Eppendorf pipette tip and extracted at 4°C for 90 min. Finally, the extracts were centrifuged in a Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA) at 100,000 *g* for 20 min and the supernatant was collected for injection. The amount of free L-[^{3}H]leucine in the extract was negligible.

Oocytes were microinjected with ~100 nl of extract. During this procedure, care was taken to avoid contact with the nucleus. Groups of 20 nuclei were isolated 2, 6, and 12 h after injection. The nuclei were immediately fixed in alcohol and subsequently electrophoresed on two-dimensional gels. In addition, 2 μl of extract (a volume equivalent to 20 injections) was electrophoresed. All of the samples contained 20 ^{35}S-labeled "standard" nuclei. The proportion of injected RN1 that entered the nucleoplasm at each time point was determined by comparing the ^{3}H:^{35}S ratio for nuclear RN1 with that obtained for the extract.

Molecular Weight Controls: Calculations of the diffusion rate of RN1 are dependent on an accurate measurement of its molecular weight. Since the molecular weight data are based on gel analysis, it was necessary to determine whether RN1 migrates anomalously in SDS polyacrylamide gels. Anomalous results are commonly observed for proteins that contain >10% carbohydrate (16). Although it is not practical to measure the amount of carbohydrate, if any, in RN1, it is feasible to determine whether its migration is subject to deviations normally associated with a high carbohydrate content. Characteristically, the molecular weights obtained for glycoproteins are abnormally high when low acrylamide gel concentrations are used (i.e., when migration is limited by charge) and decrease with increasing gel concentrations (as molecular sieving becomes the limiting factor).

Possible variations in RN1 migration were studied using the general procedure outlined by Segrest and Jackson (16). Alcohol-fixed nuclei (nine per gel) were dissolved in sample buffer (11) and analyzed on one-dimensional SDS polyacrylamide gels made up with either 5, 7.5, or 10% running gel and 3% stacking gel (5). Myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, and ovalbumin were used as molecular weight standards.

Nucleic acids, when present in sufficient concentrations, can affect the separation of polypeptides in two-dimensional gels (11). To determine whether the migration pattern of RN1 was altered by nucleic acids, an experiment was performed in which isolated nuclei were incubated for 30 min at room temperature in a solution containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, and 25 μg/ml each of RNase and DNase 1. (Both enzymes were isolated from bovine pancreas and purchased from Sigma Chemical Co., St. Louis, MO.) The digest was then precipitated in alcohol and analyzed on a two-dimensional gel.

RESULTS

A typical two-dimensional gel obtained for *Rana* oocyte nuclei is shown in Fig. 1. RN1 migrates with an apparent isoelectric point of 4.7 and a molecular weight of 157,000. Neither value was effected by treating the nuclei with RNase and DNase prior to electrophoresis. Furthermore, adding 0.02% mecaptoacetic acid to the electrode buffer in the second dimension had no effect on the gel pattern.

When nuclei were analyzed on one-dimensional SDS gels containing 5, 7.5, or 10% running gel, the molecular weights recorded for RN1 were 147,000, 148,000, and 147,500, respectively. The consistency of these results indicates that even if RN1 is a glycoprotein, it does not contain a sufficient amount of carbohydrate to interfere with molecular weight determinations.

The 9,500-dalton difference in the molecular mass of RN1 that was observed when different gel systems were used might be due to the fact that only those samples analyzed on one-dimensional gels were boiled in an SDS solution. For some proteins this procedure is known to increase the amount of bound SDS (9), resulting in greater mobility and lower molecular weight readings. If this explanation is correct, the data obtained using one-dimensional gels should be more accurate; therefore, the molecular weight of RN1 was assumed to be 148,000.

Nuclear Uptake of Endogenous RN1

The nuclear uptake kinetics of RN1, as determined from double-labeling experiments, is shown in Fig. 2. The results of two separate studies demonstrate that over a 30-h period there is an appreciable uptake of endogenous RN1 into the nucleus.

Table I gives the percent of endogenous ^{3}H-RN1 incorporated by the nuclei 6, 12, and 24 h after labeling.

Nuclear Uptake of Injected RN1

The first step in this experiment involved the preparation of a nuclear extract. Two-dimensional gel analysis demonstrated that many of the nuclear polypeptides, including RN1, are retained in these preparations (compare Figs. 1 and 3). Addi-

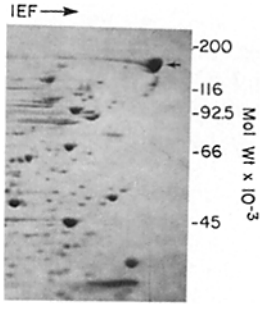

FIGURE 1 A two-dimensional gel, stained with Coomassie Blue, showing the distribution of acidic polypeptides in *Rana* nuclei. The pH range is ~6.3 (left) to 4.6 (right). RN1 is indicated by the arrow.

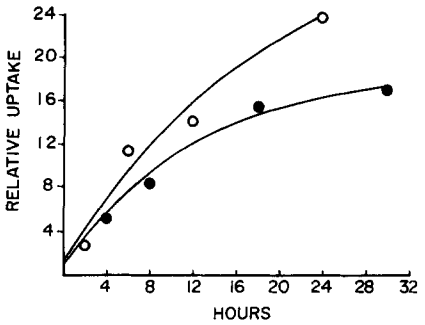

FIGURE 2 The relative nuclear uptake of endogenous RN1. The curves were derived by dividing the ^3H:^{35}S ratio for each time point by the ratio at 0 time, i.e., just after completion of the labeling period. Two independent experiments are shown. Assuming that the nucleus occupies 3% of the total cell volume, an equation for the uptake time can be derived: $1 - 0.03\ c_n/c_0 = e^{-t/t_c}$, where c_n is the concentration of RN1 in the nucleus at time t, c_0 is the cytoplasmic concentration at $t = 0$, and t_c is the characteristic uptake time. The parameters c_0 and t_c are unknown. Since c_0 is not known the equation may be reformulated as $\ln (R_\infty - R) = \ln R_\infty - t/t_c$, where R represents the ratios (data points) shown in this figure, and R_∞ is the ratio at infinite time. A plot of $\ln (R_\infty - R)$ vs. t has been curve-fitted by least squares to give the equilibrium value R_∞ and the slope $-t_c^{-1}$. The optimum values of t_c for experiments 1 (○) and 2 (●) are 18 and 13 h, respectively, for an average of 15 h.

The t_c values, however, are not very sensitive to the data points. R_∞ and t_c increase together. From the data in Table I, which are in percent of total amounts of RN1 taken up into the nucleus, it has been calculated that the maximum possible equilibrium R_∞ corresponds to t_c values of, at most, 50 h for both experiments. This was calculated from the most stringent conditions: all RN1 entering the nucleus remains, i.e., the highest R_∞ for the given data; the most positively deviant kinetic data points and the 20% standard deviation added to these points. The value of 50 h for t_c thus is the absolute upper limit compatible with both sets of data.

TABLE I

Nuclear Uptake of Endogenous RN1 (percent)

Experiment	6 h	12 h	24 h
1	32	—	—
2	39	—	—
3	—	41	51

The data are expressed as the ^3H:^{35}S ratio obtained for nuclear RN1 divided by the ratio obtained for whole cell RN1.

tional evidence that the migration pattern of RN1 was not altered is derived from the fact that extracted ^3H-RN1 co-migrates with ^{35}S-RN1 from nonextracted nuclei on two-dimensional gels.

The results that were obtained when labeled extracts were injected into the cytoplasm of unlabeled cells are given in Table II. The slower nuclear uptake of injected RN1 compared with endogenous RN1 (17% incorporation after 6 h vs. 36%) results, at least in part, because the injected polypeptides must diffuse from the injection site (the vegetal pole) to the nucleus before uptake can be initiated. It is difficult to calculate the exact effect of diffusion on the uptake rates, but for cells the size of *Rana* oocytes this could represent a delay of several hours (unpublished data).

DISCUSSION

In general, molecules cross biological membranes either by simple diffusion or by mediated transport processes. Regarding

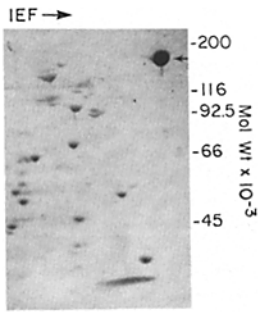

FIGURE 3 A two-dimensional gel of a nuclear extract. The migration of RN1 (arrow) is not affected by the extraction procedure. The gel was stained with Coomassie Blue.

TABLE II

Nuclear Uptake of Injected RN1 (percent)

Experiment	2 h	6 h	12 h
1	4	16	24
2	—	18	—

The data are expressed as the ^3H:^{35}S ratio of nuclear RN1 divided by the ^3H:^{35}S ratio obtained for RN1 in the injected extract. Thus, the results represent the percent of the total injected ^3H-RN1 taken up by the nuclei. 20 oocytes were analyzed at each time.

the nuclear envelope, it is known that proteins can enter the nucleoplasm by diffusing through the pores, and that the rates of diffusion are directly related to the size of the permeating molecules (2, 12). It is not known, however, whether mediated mechanisms also exist for the translocation of specific proteins across the envelope. Such mechanisms could be required for certain endogenous polypeptides that, due to their size or specific chemical properties, might not be able to diffuse through the pores at sufficient rates to maintain essential nuclear functions. In an effort to resolve this question, we studied the nuclear uptake kinetics of a 148,000-dalton *Rana* protein and subsequently determined whether the kinetic results could be accounted for by simple diffusion.

The data presented above demonstrate that RN1 rapidly accumulates within the nucleoplasm. For example, 6 h after labeling oocytes with L-[^{3}H]leucine, ~36% of the newly synthesized RN1 was localized within the nucleus. Extracted RN1 also concentrated in the nucleus following microinjection into the cytoplasm. A mathematical analysis of the diffusion rate of RN1 across the envelope is given in the Appendix. Even assuming the most diffusive configuration for RN1 and a nuclear binding coefficient of 45, it is estimated that in the absence of a mediated transport mechanism only ~1.7% of the newly synthesized RN1 would accumulate in the nucleus in 6 h. Other possible configurations would enter much more slowly, if at all. Thus, we have concluded that a transport process exists that facilitates the nucleocytoplasmic exchange of RN1 and, perhaps other nuclear proteins.

The mechanism by which transport occurs is not known. However, since extracted nuclear RN1 can re-enter the nucleoplasm following microinjection, it appears unlikely that the exchange of RN1 across the envelope is coupled to translation, as is the case for the passage of secretory proteins into the lumen of the endoplasmic reticulum (15).

The present results are consistent with the recent finding by Dingwall et al. (4) that nucleoplasmin, a 165,000-dalton pentamer, is selectively transported into the nucleus of *Xenopus* oocytes. These investigators demonstrated that the 12,000-dalton tail regions of the monomeric subunits of nucleoplasmin are required for transport. Whether similar "signal" regions are present in RN1 remains to be determined.

TABLE III

Stokes' Radii and Hindrance Factors for Spherical Proteins Passing through a Small Pore

Mol wt	a*	Steric factor $(r-a)^2/r^2$	K_1	Steric factor/K_1
	Å			
—	12.0	0.54	2.12	2.54×10^{-1}
—	23.0	0.24	6.32	3.80×10^{-2}
81,000	36.0	0.040	78.5	5.09×10^{-4}
90,000	37.5	0.028	128	2.19×10^{-4}
100,000	38.9	0.020	225	8.89×10^{-5}
110,000	40.3	0.011	737	1.48×10^{-5}
120,000	41.5	0.006	—	—
150,000	44.9	—	—	—
160,000	45.9	—	—	—

* Stokes' radii for globular proteins interpolated from known a. The hindrance factors are calculated based on a pore radius, r, of 45 Å (13, 14). The bulk diffusion constant decreases by ~30% through this size range.

APPENDIX

The diffusion rate of molecules through large pores depends on the cross-sectional area of the pore and, roughly, on the square root of the molecular weight. When the dimensions of the two approach each other, hindrance factors must be considered. Movement through the pore is restricted by a steric hindrance factor and a wall drag factor. Usually these effects are viewed as decreasing the effective cross-sectional area, A_{eff}. For pure diffusion of a rigid sphere through a cylindrical pore with no bulk solvent flow, the effective cross-section is given by

$$A_{\text{eff}} = A_0(r-a)^2/K_1r^2, \quad (1)$$

where A_0 is the unrestricted cross-sectional area, a is the radius of the sphere, r is the radius of the pore, and K_1 is a drag coefficient. The corrective factor for steric hindrance reflects the condition that the sphere must enter the pore without striking the edge so that only the area defined by the distance $r-a$ is available. The drag coefficient is a complex function of a/r and the radial distance of the sphere's center from the pore's central axis. By assuming axisymmetric flow, Haberman and Sayre (8) greatly simplified the calculations of K_1 with only small errors. Paine and Scherr (13) have provided numerical values through a large range of a/r. Paine et al. (14) determined the nuclear pore radius of *Rana* to be ~45 Å. Using the above information, hindrance factors for various size molecules have been calculated and are presented in Table III.

The characteristic times (t_c, inverse of the rate constant) for diffusion through the *Rana* pore were experimentally determined by Paine et al. (14) for dextrans of various sizes. These times increase dramatically with size: for radii of 12, 23, and 36 Å, t_c = 0.13, 3.9, and 347 h, respectively. The diffusional rate for the largest dextran is decreased by ~2,000 times relative to free bulk flow due to steric hindrance and drag. For a 95,000-dalton protein, Ra_0 = 38 Å, diffusion is slowed by 6,200, and at 110,000 daltons, Ra_0 = 40 Å, diffusion is slowed 68,000-fold. For these larger ranges of a/r, the drag on the sphere is more important than the steric factor. Unfortunately, the drag coefficient cannot be calculated for $a/r > 0.9$. Obviously, the trend is to greatly decrease the permeation rate at sizes well below the pore size, and this decrease is very strongly dependent upon a/r.

The *Rana* RN1 protein has a molecular weight of ~150,000 which gives a Stokes' radius of 45 Å, assuming the molecule to be spherical. Thus, one would not expect RN1 to be able to traverse the *Rana* pore. However, it is necessary to consider the possibility that there might be an error in the value used for the pore size or for the size of RN1. Furthermore, RN1 might not be spherical.

If the protein is spherical, it could be 30% smaller, or the pore could be 30% larger, and the diffusion rate would still be at least an order of magnitude slower than that observed for 36-Å dextran. This is much too slow to give the data reported here.

If RN1 were a prolate ellipsoid, it would still diffuse very slowly through the pore. Although one dimension will be shortened, the orientation factor for entering the pore is much smaller than for a comparable sphere. Defining b as the short radius, and $1/2\ l$, the long radius, the orientation factor R_0 for accessing a pore of radius r is $\pi(r-b)^2/2\pi l^2$, $b \leq r$. We have calculated the diffusion times for two representative prolate ellipsoids having the same volume as a sphere with a 45-Å radius; the values used for the short radii were 23 and 36 Å. The corresponding lengths are 345 and 141 Å, respectively. The orientation factor has a broad maximum in this range: R_0 is 0.0020 in either case for a pore having a 45-Å radius. The longer hypothetical protein would have a diffusion time of 468 h, that is, at least 120 times slower than the 23-Å dextran sphere with a steric factor of 0.24. The 36-Å ellipsoid will be 20 times slower to diffuse than the 36-Å dextran sphere, i.e., t_c would be at least 6,940 h. The diffusion time estimate is not very dependent on the choice of pore size since that quantity was derived from Eq. 1 using the experimental diffusion times given above. Thus, for a hypothetical pore size of 50 Å, the 23-Å ellipsoid will still take 372 h to pass through.

Extending the 150,000-dalton protein to a long, thin rod slows passage of the protein through the pore. For a cylindrical rod of radius b = 12 Å, the length, l, would be 844 Å (the volume of a cylinder = $\pi b^2 l$). The same formula for the orientation factor, R_0, as was applied to a prolate ellipsoid is appropriate, and substitution gives R_0 = 0.00076 for this rod in the *Rana* pore. A lower limit for the drag factor for a cylinder moving axisymmetrically inside another cylinder is given by $2\pi\eta l/\ln(r/b)$, which can be derived from a simple shell integration of Newton's definition of viscosity (1). This estimate of the drag factor is a minimal one since it does not account for all the contributions to drag that Haberman and Sayre (8) included in their calculations. No such calculations exist for a cylinder within a pore. To obtain a minimum estimate of the diffusion time for this rod, we can compare it with one of the spheres for which a characteristic time is known. The drag on an unimpeded sphere is $6\pi\eta R$ where R is its radius. Comparing the drag on the rod with that of an unimpeded sphere with a radius of 12 Å gives a ratio of 17:7. The entry rate of a 12-Å dextran particle is 0.13 h (14) and the steric factor/K_1, is 0.25 (Table II). Thus, the characteristic passage time for the long, thin rod is expected to be at least 0.13 h × 0.25 × 17.7 ÷ 0.00076 = 757 h. Extending the molecule even longer actually increases passage time, as can easily be verified from the definition of R_0.

With respect to the possible molecular configurations that were considered, a minimum diffusion time was calculated for a 23-Å prolate ellipsoid. By assuming different values for orientation, steric, and drag factors, it is estimated that this minimum time (468 h) is within 10% of the theoretical minimum that could be achieved by a 150,000-dalton polypeptide diffusing through a 45-Å pore.

The final question to be considered is whether the observed nuclear uptake rates can be explained by simple diffusion of RN1 or by diffusion plus binding in the nucleus. Taking $X_{n,c}$ as the ratio of protein concentration within the nucleus to that in the cytoplasm, $K_{n,c}$ as the partition coefficient between the nucleus and cytoplasm (i.e., the concentration ratio at infinite time), and t_c as the characteristic time for entering the nucleus, the following rate equation can be easily derived (14):

$$1 - X_{n,c}/K_{n,c} = e^{-t/t_c}. \quad (2)$$

For simple diffusion with no binding, and not taking water activity into account, $K_{n,c}$ = 1. For the most diffusible form of RN1 that can be envisioned, the 23-Å prolate ellipsoid, the concentration ratio between nucleus and cytoplasm is calculated to be 0.013 at 6 h. Since the nucleus occupies only ~3% of the volume, only 0.038% of the total amount of protein would be found in the nucleus at 6 h, obviously far less than the 36% actually found. However, RN1 is probably bound in the nucleus. Similar proteins (N1 and N2) accumulate in the nucleus of *Xenopus* oocytes ~120-fold (3). Our results indicate less affinity in the *Rana* system. The uptake kinetics in Fig. 2 yield a characteristic time, t_c, of about 15 h with an upper limit of 50 h (see figure legend). From Table I and Eq. 2, the partition coefficient, $K_{n,c}$, is calculated as 45 with an upper limit of 100. For simple diffusion and a partition coefficient of 45, the calculated percent uptakes at 6, 12, and 24 h are only 1.7, 3.4, and 6.5%, respectively, an order of magnitude less than the observed uptake. For $K_{n,c}$ = 100, uptake is estimated as 3.8, 7.3, and 13.4%, respectively, still considerably less than the observed. To account for the results obtained in Table I by simple diffusion, the partition coefficient implied from Eq. 2 would have to be ~1,000. This is incompatible with the actual partition coefficients computed from all the data and also with the kinetics of nuclear permeation in Fig. 2. Only a t_c of 15–50 h and a $K_{n,c}$ of 45–100 are consistent with all the data.

Clearly, even proposing the most extreme case, it is highly unlikely that the 150,000-dalton protein, RN1, enters the nucleus by simple diffusion through nuclear pores.

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