tervened. The nucleolar cycle in embryos with small nucleoli appeared much the same, except that the nucleoli were generally smaller and tended to fuse much less rapidly.

If these nucleoli were really immature because of the rapid cell division of cleavage, stopping cell division should allow them to mature into large prominent nucleoli. Fluorodeoxyuridine (FUdR), a compound that stops DNA synthesis by inhibiting thymidylate synthetase (*6*), was added to eggs in the presence of uridine at five times the FUdR concentration. Even at $10^{-2}M$, FUdR had no effect on cleavage until the 16-cell stage was reached. At this stage cell division was stopped by $10^{-2}$ to $10^{-3}M$ FUdR. As the concentration was lowered below $10^{-3}M$, cell division proceeded for longer periods beyond the 16-cell stage before stopping. At all concentrations of inhibitor, addition of thymidine at five times the FUdR concentration completely prevented the inhibition of cell division.

The nucleoli in cells treated with FUdR increased in size and density for about 2 hours after cell division stopped (Fig. 3, e and f). In many cases the fusion of the initial nucleolar blobs was inhibited, and eight to ten large nucleoli were formed. In some cases fusion was normal in FUdR, and a few large nucleoli resulted when cell division stopped. These results support the conclusion that the apparently atypical, multiple nucleoli are initial stages in the normal development of nucleoli that appear in all cells because the nucleoli do not have time to develop beyond these stages in the rapidly dividing cells of early embryos.

Our biochemical and cytological data indicate that rRNA synthesis is not repressed but only obscured by DNA-like RNA synthesis during cleavage of sea urchin embryos and that the "atypical" morphology of the nucleoli, usually attributed to decreased rRNA synthesis, is due to rapid cell division. We have reexamined the data from embryos (*1*) of other animals in which the results suggest that rRNA synthesis is repressed and find that the evidence available is entirely consistent with these new interpretations.

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## p-Nitrophenyl Phosphate Hydrolysis and Calcium Ion Transport in Fragmented Sarcoplasmic Reticulum

Abstract. *The calcium ion pump of fragmented sarcoplasmic reticulum can be coupled to hydrolysis of* p*-nitrophenyl phosphate, in the absence of added adenosine triphosphate. Comparison of the activities obtained with the two substrates suggests an analogous mechanism of transport. Independent of the substrate, a 2:1 ratio between calcium ion transport and substrate hydrolysis is displayed by the system, and an identical amount of work is required for ion transport against a given gradient. A phosphate ester appears necessary for substrate utilization in the pump mechanism, whereas the structure of the substrate determines the rates of activity and the affinity of the system for calcium ion.*

Vesicular fragments of sarcoplasmic membrane (SR) obtained from skeletal muscle homogenates rapidly accumulate $Ca^{2+}$ in the presence of adenosine triphosphate (ATP). Simultaneous with $Ca^{2+}$ uptake, a burst of ATP hydrolysis is also catalyzed by SR (*1*). Kinetic analysis of these activities indicates that, while a small amount of $Ca^{2+}$ may bind to SR, most of the accumulated $Ca^{2+}$ is transported inside the vesicles, forming a concentration gradient (*2, 3*). The energy required by this pump is met by ATP hydrolysis (*4*). I now report experiments in which $Ca^{2+}$ transport was coupled to hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP). *p*-Nitrophenyl phosphatase activity has been observed in other membranes (*5*), but no direct relation to ion pumps was demonstrated.

Sarcoplasmic membrane was prepared by differential centrifugation of homogenized skeletal muscle (rabbit) (*1*). Sucrose (10 percent) was added to the homogenization and resuspension medium. To eliminate contaminations of myosin, the final SR suspensions were exposed for 1 hour to 0.6*M* KCl, recentrifuged, and resuspended. Accumulation of $Ca^{2+}$ was measured by incubating SR in the presence of $^{45}CaCl_2$ in the reaction mixtures as required. After incubation, SR was removed by rapid filtration (Millipore HA0,45 $\mu$m); the residual radioactivity in the filtrate was determined by scintillation counting. In the experiments on *p*-NPP or ATP hydrolysis, incubations were interrupted by the addition of trichloroacetic acid (5 percent). After the denatured protein was removed by centrifugation, inorganic phosphate was determined by the method of Fiske and Subbarow (*6*). *p*-Nitrophenol was determined by direct spectrophotometric reading (400 nm) after addition of NaOH to the supernatants.

Curves of the rate of $Ca^{2+}$ accumulation by SR, in the presence of *p*-NPP or ATP, are shown in Fig. 1. In spite of slower rates of uptake, relatively large amounts of $Ca^{2+}$ are accumulated in the presence of *p*-NPP. In fact, the

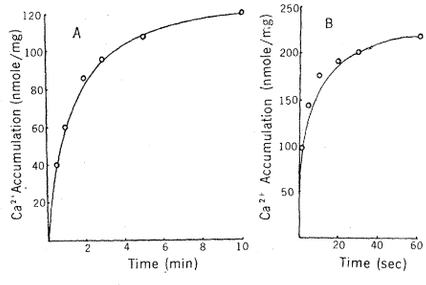


Fig. 1. Time curves of $Ca^{2+}$ accumulation (nanomoles per milligram of protein) by SR. The reaction mixtures consisted of 16 m*M* tris-maleate (*p*H 6.8), 80 m*M* KCl, 0.1 m*M* EGTA, 0.12 m*M* $CaCl_2$, 10 m*M* $MgCl_2$; and either 10 m*M* *p*-NPP (A) or 5 m*M* ATP (B); and either 0.35 mg (A) or 0.175 mg (B) of SR protein per milliliter.

steady state levels obtained with *p*-NPP are approximately half of those obtained with ATP. These levels are sensitive to changes in the $Ca^{2+}$, $Mg^{2+}$, and substrate concentrations in the medium and are higher when oxalate (5 m*M*) is present in the reaction mixture.

Hydrolysis of *p*-NPP is catalyzed by SR, parallel to $Ca^{2+}$ uptake. As shown in Fig. 2, slow hydrolysis is observed before the addition of $CaCl_2$ [ethylene glycol-bis-(oxyethylenenitrilo)-tetraacetic acid (EGTA) is present to chelate contaminating traces of $Ca^{2+}$]. On addition of $CaCl_2$, there is a burst of activity which lessens as the maximum $Ca^{2+}$ accumulation is reached. These results are similar to those obtained with ATP.

The slow activity obtained in the presence of *p*-NPP allows estimation of the initial rates of $Ca^{2+}$ accumulation and *p*-NPP hydrolysis. Initial rates of 120 to 130 $\mu$mole of $Ca^{2+}$ per gram of protein per minute and of 60 to 68 $\mu$mole of *p*-NPP per gram of protein per minute were obtained; the ratios of $Ca^{2+}$ to *p*-NPP were slightly higher than 2, as described for a variety of experimental conditions where $Ca^{2+}$ accumulation was measured in the presence of ATP (*2–4*). As $Ca^{2+}$ accumulation proceeds and the SR vesicles are filled, ratios of $Ca^{2+}$ to *p*-NPP decrease because of the leak of the accumulated $Ca^{2+}$. If the rate of $Ca^{2+}$ transport is defined as $V_a$, the rate of net accumulation as $V_c$, and the rate of passive diffusion (leak) as $V_d$, we may write $V_c = V_a - V_d$ (*7*). Since $V_d$ must be a function of membrane permeability ($P$) and concentration gradient ($C$) ($V_d = P \cdot \Delta C$), it is reasonable that leak of accumulated $Ca^{2+}$ should increase as the vesicles become filled.

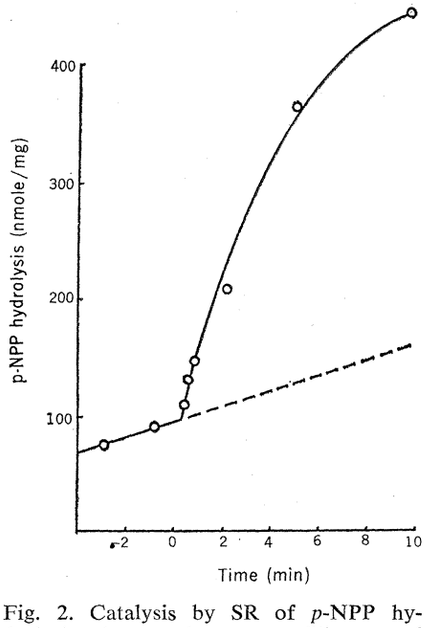


Fig. 2. Catalysis by SR of *p*-NPP hydrolysis (nanomoles per milligram of protein). The reaction mixture was the same as that in Fig. 1A. At zero time $CaCl_2$ was added.

On the assumption that a ratio of 2 is constantly maintained between $Ca^{2+}$ transport ($V_a$) and *p*-NPP hydrolysis ($V_h$), we may write $V_a = 2V_h$. Therefore, the amount of $Ca^{2+}$ leaked at any time can be estimated by subtracting the $Ca^{2+}$ accumulated from twice the *p*-NPP hydrolyzed. These values, plotted in a graph with time curves of accumulation of *p*-NPP hydrolysis (Fig. 3), reveal that leak sharply increases until maximum filling of the vesicles is reached and then gradually decreases. Since $V_d$ is equal to $P \cdot \Delta C$ and at steady state levels $\Delta C$ is maintained constant, the reduction of leak observed in the latter part of the curve must reflect a decrease in $P$, probably because of binding of intravesicular $Ca^{2+}$ ($\sim 10^{-2}M$) to low-affinity binding sites in the membrane, with consequent increase in structural rigidity. Membrane permeability $P$ is a very labile parameter that can be selectively affected by mild-temperature denaturation (40°C) or other procedures (*8, 9*).

Depending on the experimental conditions, either too low $Ca^{2+}$ concentrations in the outside medium or too high $Ca^{2+}$ concentrations inside the vesicles can be shown to be a limiting factor for further $Ca^{2+}$ transport. On the other hand, in experiments where comparably low $Ca^{2+}$ concentrations are maintained in the outside medium at steady state levels of maximum filling, the asymptotic accumulation depends on the amount of free energy made available by hydrolysis of either substrate. If the difference between the standard $\Delta G^0$ values for *p*-NPP and ATP is overcome by changing their concentrations in the reaction mixture, similar steady state levels of intravesicular $Ca^{2+}$ concentrations are obtained in the presence of *p*-NPP and ATP.

In experimental conditions (Fig. 1), the steady state $Ca^{2+}$ concentrations inside the vesicles can be estimated if it is assumed that 1 g of SR protein corresponds to 10 ml of inner volume (*3*), and that most of the accumulated $Ca^{2+}$ is in the form of free ion (*2, 3*). Furthermore, the calcium concentration in the outside medium can be measured and the $Ca^{2+}$ concentration may be calculated, based on a Ca·EGTA affinity constant of $10^{6.5}$ (*p*H 6.8). Consequently, the energy required to translocate 2 moles of $Ca^{2+}$ against the steady state concentration gradient ($\Delta G = RT \ln C_{in}/C_{out}$) can be estimated to be 10.10 kcal at the maximum levels obtained in the presence of *p*-NPP, and 11.80 kcal at the maximum levels obtained in the presence of ATP. These values match remarkably well with the $\Delta G$ values calculated (*10*) for *p*-NPP (−10.5 kcal/mole) and ATP (−12.4 kcal/mole) in the same experimental conditions. These estimates do not take into account the possible thermodynamic interference of a membrane electrical potential. In fact, on the as-

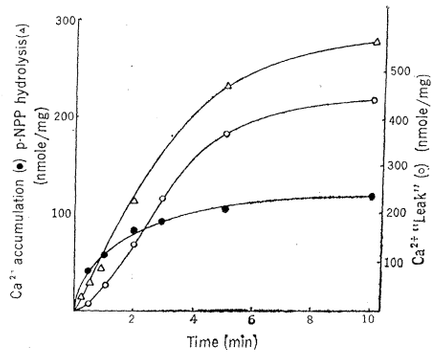


Fig. 3. Accumulation of $Ca^{2+}$, *p*-NPP hydrolysis, and leak of accumulated $Ca^{2+}$ (nanomoles per milligram of protein), derived from the experiments illustrated in Figs. 1A and 2. The values of hydrolysis express the $Ca^{2+}$-dependent activity (total to $Ca^{2+}$ independent). Leak of $Ca^{2+}$ was estimated by subtracting accumulated $Ca^{2+}$ (net) from twice the *p*-NPP hydrolyzed (on the assumption that $V_a = 2V_h$).

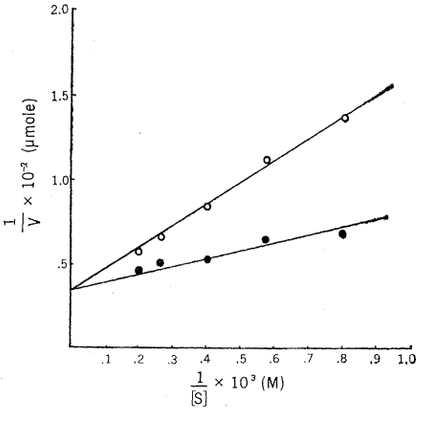


Fig. 4. Competitive inhibition of 0.1 m*M* ATP (○) on hydrolysis of *p*-NPP. Reaction mixtures: 20 m*M* tris-maleate (*p*H 6.8), 80 m*M* KCl, 10 m*M* $MgCl_2$, 0.21 mg of SR protein per milliliter and various concentrations of *p*-NPP. Before the reaction was started, SR was treated with 5 percent (by volume) diethyl ether (*9, 11*) to avoid $Ca^{2+}$ accumulation and to obtain constant rates of hydrolysis.

sumption of a potential of 70 mv across the membrane, 6.44 additional kilocalories would be required to transport 2 moles of $Ca^{2+}$, therefore exceeding the $\Delta G$ values of *p*-NPP and ATP. This suggests that either no electrical work is accomplished during $Ca^{2+}$ transport (for example, equivalent negative charge produced on the inner side of the membrane, simultaneous to cation release), or no membrane electrical potential is formed in fragmented sarcoplasmic reticulum.

It appears then, that in appropriate experimental conditions the maximum filling capacity of the vesicles corresponds to a certain gradient ($Ca^{2+}{}_{in}$/$Ca^{2+}{}_{out}$), where the energy required for transport of 2 moles of $Ca^{2+}$ matches $\Delta G$ of the substrate used in the hydrolytic reaction. Therefore, the active transport is energetically limited and near the asymptote the free energy efficiency approaches 100 percent. Furthermore, independent of the substrate structure, a ratio of a $Ca^{2+}$ to substrate of 2 is maintained, and an identical amount of work is required for $Ca^{2+}$ transport at a given gradient. In contrast, the structure of the substrate (quite different for *p*-NPP as opposed to ATP) has a profound influence on the reaction rates and the affinity of the system for $Ca^{2+}$. This can be easily demonstrated from a study of the linear rates of adenosine triphosphatase and *p*-nitrophenyl phosphatase catalyzed by Triton-solubilized SR (*11*), whose half-maximum activation is obtained in the presence of $6 \times 10^{-7}$ and $7 \times 10^{-6} M$ $Ca^{2+}$, respectively.

As opposed to *p*-NPP, *p*-nitrophenyl acetate does not sustain appreciable activity, in spite of a high negative $\Delta G$ value. This suggests that the presence of a phosphate ester is necessary to substrate utilization for $Ca^{2+}$ transport.

In view of the apparently identical mechanism of $Ca^{2+}$ transport in the presence of *p*-NPP and ATP, it would be interesting to know whether hydrolysis of the two substrates is catalyzed by the same enzymatic site or whether preliminary phosphorylation of intermediates is required for utilization of different substrates. We know that both activities require $Mg^{2+}$ and $Ca^{2+}$ and that ATP is a competitive inhibitor of *p*-nitrophenyl phosphatase (Fig. 4). The fact that adenosine diphosphate (ADP) is also an inhibitor of *p*-nitrophenyl phosphatase indicates that intermediate phosphorylation of trace amounts of endogenous ADP is an improbable step in the mechanism of *p*-NPP hydrolysis catalyzed by SR. However, a definite answer to the identity of the enzyme or enzymes will only be obtained after solubilization and purification of the involved protein. At any rate, *p*-NPP is a convenient chromogenic substrate that, because of coupling of its relatively slow hydrolysis to $Ca^{2+}$ transport, allows interesting kinetic and thermodynamic observations.

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## Chromosome Lesions Produced with an Argon Laser Microbeam without Dye Sensitization

Abstract. *Improvements in the argon laser microbeam have made it possible to cause damage to chromosomes of tissue culture cells without prior treatment of the cells with a photosensitizing agent. These results have been confirmed independently in two laboratories.*

In previous studies of argon laser microirradiation it has been necessary to treat the cells with a photosensitizing agent (*1*). When an appropriate vital dye, such as acridine orange, is introduced into a tissue culture chamber for 5 minutes, the dye molecules intercalate into the DNA helix, making the chromosome (and specifically the DNA) light-sensitive. Subsequent irradiation of specific chromosomes with intense laser light has permitted physical deletion of selected portions of chromosomes, with concomitant functional loss. However, the necessity of using a vital dye increases the chance of secondary effects attributable to the dye itself, with the result that long-term experiments are difficult, if not impossible.

Improvements on the prototype argon laser microbeam system (*2*), as well as the construction of a new system in which a high-power argon laser is used (*3*), have permitted microirradiation with much more laser energy than before. This capability permits the production of discrete chromosomal lesions without the addition of a vital dye. These results have been confirmed independently in two separate laboratories.

The tissue culture techniques are similar to those described earlier (*1*, *4*). Primary explants of salamander (*Taricha*) lung are established in Rose multipurpose culture chambers (*5*). The culture medium is minimum essential Eagle's (*6*) medium with 10 percent fetal calf serum, penicillin, and streptomycin added. Mitotic chromosomes are clearly visible 1 to 3 weeks after establishment of the culture. Chromosomes are irradiated in prophase, metaphase, or anaphase.

The two microbeam systems consist of argon lasers combined with Zeiss photomicroscopes and closed circuit television receivers. In the system used at the Pasadena Foundation for Medical Research (*2*) a 1.5-watt (peak power per pulse, 25-$\mu$sec half pulse width) argon laser in multimode, multiline operation is used; in the system used at the University of Michigan (*3*) a 12-watt (peak power per pulse, 30-$\mu$sec half pulse width) argon laser in single mode, multiline operation is used. Energy measurements in the Michigan system are made with a calibrated vacuum photodiode (spectral response of 5), fitted with a narrow beam adapter, and