Ca^{2+} is a specific Regulator of Amino Acid Transport and Protein Synthesis in the Water-Mould Achyla.

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SUMMARY

Transport of amino acids, uracil, thymidine, and Ca^{2+} in some cellulose-walled fungi is energy-mediated. Ca^{2+} enhances amino acid uptake and protein synthesis. Uracil and thymidine uptake and synthesis of RNA and DNA proceed independently of Ca^{2+} metabolism. The mucopolysaccharide dye ruthenium red ($Ru(NH_3)_4(OH)Cl_2$) known to selectively inhibit the activity of a Ca^{2+} - stimulated Mg^{2+} - ATPase, specifically inhibits the transport of amino acids and Ca^{2+} in one water-mould, *Achyla*. Saturation kinetics show that amino acids, ruthenium red, and Ca^{2+} may share close or common sites in the transport system for amino acids in these moulds.

Ruthenium red ($Ru(NH_3)_4(OH)Cl_2$) is a mucopolysaccharide stain (1,2) that binds to some mitochondrial component and inhibit Ca^{2+} transport in these organelles (3). Watson, Vincenzi and Davis (4) recently showed that this inorganic dye can selectively inhibit a Ca^{2+} - stimulated Mg^{2+} - ATPase in the membrane of aged red blood cells. We have observed that bivalent cation chelators, ethylenediamine tetraacetic acid (EDTA), ethleneglycolbis-(β-aminoethyl ether) N,N'-tetraacetic acid

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(EGTA) and citrate interrupt the transport of amino acids and protein synthesis at all stages during the development of the coenocytic water-mould, *Achlya*, but that they had no immediate effect on uracil or thymidine transport and the synthesis of RNA and DNA (5). The inhibitory action of these chelators was antagonized by several bivalent cations, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Ba^{2+}.

Ca^{2+} was the most effective antagonist of all the bivalent cations, the others being toxic at relatively low concentrations. We also demonstrated that *Achlya* lacks the requisite transport system for most organic acids of the citric acid cycle. The dicarboxylic amino acids, aspartate and glutamate, were transported with some difficulty (5).

The present study was undertaken to determine whether Ca^{2+} alone or in association with other cations was necessary for amino acid transport and protein synthesis in *Achlya*. We were also interested in finding out whether a coupling between the two processes, amino acid transport and protein synthesis, occurred at the membrane level.

MATERIALS AND METHODS

The strain of *Achlya* used has been described previously by us (6). Procedures for growth of the fungus, harvesting of spores, preparation of synchronized germinated spores, and determination of cell density and viability have been described (5).

Transport Studies. Transport of radioactive metabolites into germinated spores of *Achlya* was carried out in a basal medium composed of 5 mM tris-acetate, 1 mM KCL, 1 mM NaCl, and 5 mM glucose, pH 7 (TKNaG). Uptake was measured by rapidly filtering 1-ml sample of cells (5×10^3 germlings) through 0.45 μm Millipore HAWP filters (25mm) at the specified times, washed 3 times with a total of 15 ml of unlabelled medium at room temperature, sucked dry before counting. Filtration

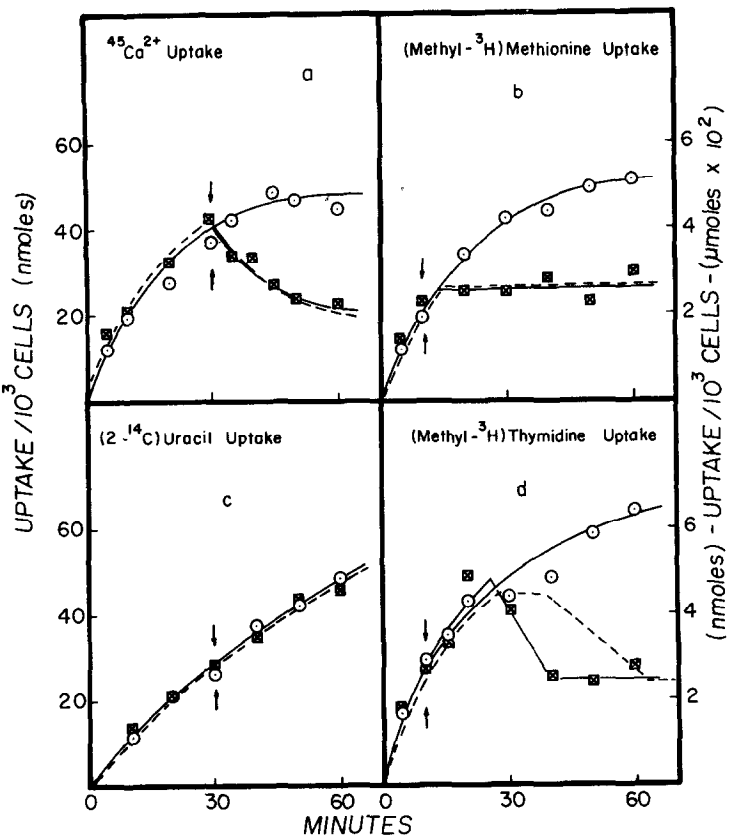

Fig. 1. Influence of ruthenium red and citrate on the transport of (a) Ca^{2+} (b) methionine (c) uracil and (d) thymidine into *Achlya* germlings. At the times indicated by arrows, either 25 μM ruthenium red or 10 mM citrate was added to the cultures. 1-ml samples were collected, washed as prescribed in the Methods at the specified times. Broken lines represent uptake patterns for cultures treated with citrate; (⊙) control; and (⊠) ruthenium red-treated cultures.

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was carried out in a 30-chamber Millipore filtration manifold attached to vacuum pumps. Sampling and washing took about 20 sec. The washed filters were transferred to scintillation vials, dissolved in Bray's fluid (7) and radioactivity determined with a Packard TriCarb liquid scintillation spectrometer.

Substrate saturation kinetics were done at a constant incubation

time of 10 min at 24° in 2-ml volumes at a cell density of 2.5 x 10^3 germlings per ml.

Radioactive labelling of macromolecular materials was determined in cells treated with ice-cold CCl_3COOH, 5% final concentration, containing 1000 fold excess of the unlabelled carrier.

Radioisotopes were obtained from Amersham/Searle. The radioactive isotopes used were (2-^{14}C) uracil, 60 mCi/mM; (L-3-ring 4-^{3}H) phenylalanine, 12.5 Ci/mM; (methyl-^{3}H) methionine, 7.5 Ci/mM; purified ^{14}C- protein hydrolysate containing 15 of the 20 common amino acids, 54 mCi/mAtom; and $^{45}CaCl_2$, 0.5 mCi/15 µg Ca^{2+}. Other biochemicals were obtained from Sigma Chemical Co., Calbiochem., and Nutritional Biochemicals. Only the L-optical isomers of amino acids were used.

RESULTS AND DISCUSSION

Inhibition by Citrate and Ruthenium Red. It was shown by us that citrate is not transported by *Achlya* at any stage of its deveopment (5). Instead, citrate binds to the cellular membrane and inhibits the transport of amino acids and bivalent cations such as Ca^{2+}, Mg^{2+} and Mn^{2+}. Cellular protein synthesis was also hindered by citrate. The transport of uracil and thymidine proceeded unperturbed by citrate interaction with the cells and RNA and DNA syntheses were not immediately prohibited.

The results presented in Fig. 1 compare the inhibitory action of citrate with that of ruthenium red. Uptake of Ca^{2+} and methionine were terminated immediately upon addition of citrate or ruthenium red to the cultures (Fig. 1 a and b). Both inhibitors induced an efflux of $^{45}Ca^{2+}$ but the pool of amino acids remained undisturbed. There was no efflux of methionine. Experiments done with uniformly labelled mixture of 15 amino acids gave the same result; consequently, the

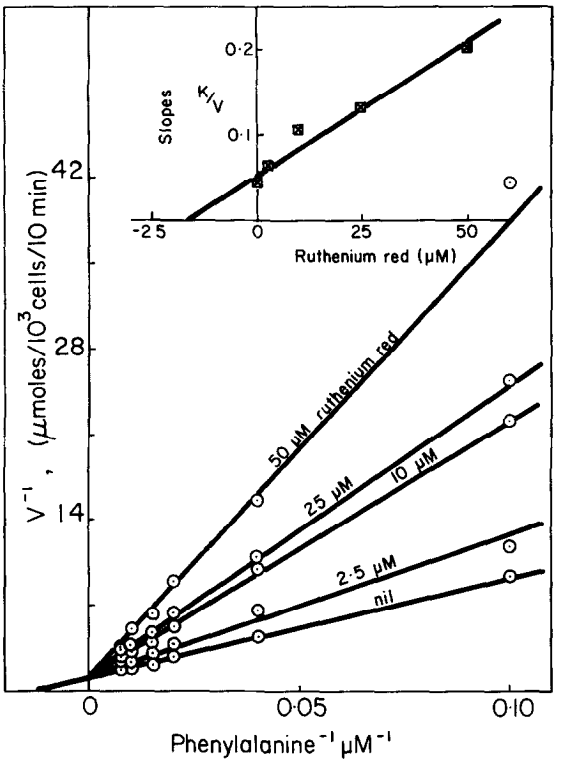

Fig. 2. Lineweaver-Burk representation of rate of uptake of L-3 (ring 4-^{3}H) phenylalanine by *Achlya* as a function of substrate concentration in the presence of ruthenium red at the concentrations specified in the illustration. Inset. Replot of slopes against inhibitor concentration to estimate K_i value for ruthenium red.

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data are not given here. Citrate and ruthenium red did not perturb the uptake mechanisms for uracil and thymidine initially (Fig. 1 c and d), but as protein synthesis was terminated immediately, decline in the uptake of thymidine and uracil which occurred about 30 min after addition of the inhibitors is probably a secondary effect. Superficially, the two inhibitors appear to affect Ca^{2+} and amino acid metabolism in the same way.

Competitive Inhibition of Amino Acid Transport. We reasoned that if Ca^{2+}, citrate, amino acids and ruthenium red were all interacting at common

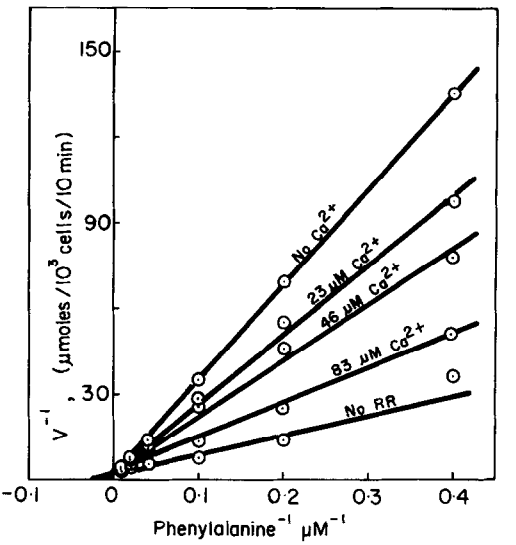

Fig. 3. Lineweaver-Burk representation of the rate of uptake of L-3 (ring 4-^{3}H) phenylalanine by *Achlya* as a function of substrate concentration in the presence of inhibitor, ruthenium red (RR) and activator, Ca^{2+}. Unless specified all reaction systems contained 50 μM ruthenium red and Ca^{2+} at the concentrations indicated in the illustration.

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or closely related sites, then saturation kinetics of amino acid transport with citrate and ruthenium red as inhibitors should show competitive inhibition patterns. Furthermore, inhibition should be reversed by Ca^{2+}. The results shown (in Lineweaver-Burk form (8)) in Fig. 2 indicate that phenylalanine transport was inhibited competitively by ruthenium red. Inset replot of slopes against ruthenium red concentrations gives an estimated K_i value of 1.65 x 10^{-5}M for ruthenium red. The K_m for phenylalanine determined from the control plot is 7.14 x 10^{-5}M. Citrate also inhibited amino acid transport competitively. The K_i value for citrate was estimated as 1.7 x 10^{-3}M. For brevity these kinetic plots are not presented here.

The inhibition caused by ruthenium red was partially reversed by Ca^{2+} as illustrated in Fig. 3 and 4. As the concentration of Ca^{2+} was increased from 0 μM to 100 μM, ruthenium red inhibition was almost

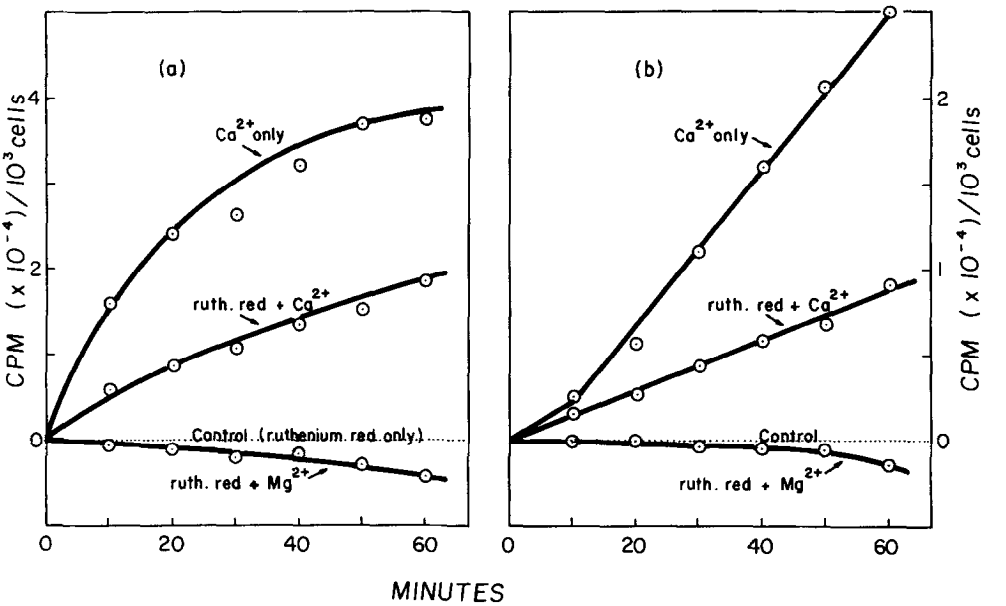

Fig. 4. Reversal of ruthenium red inhibition of amino acids uptake and protein synthesis by Ca^{2+}. Ruthenium red was used at 10 μM and Ca^{2+} at 25 μM. Where specified, Mg^{2+}, the use of Mn^{2+} gave similar results. Uptake of amino acids measured in (a) and protein synthesis in (b). Incubation and assays conducted in TKNaG medium.

completely antagonized. The cellular interactions of ruthenium red and amino acid in the presence of Ca^{2+} remained competitive. A separate kinetic analysis showed that 135 μM Ca^{2+} was required to antagonize the inhibition of amino acid transport caused by 50 μM ruthenium red. The effective neutralizing ratio of 3 parts Ca^{2+} to 1 part ruthenium red may only be the fortuitous (ruthenium is a hexavalent cation!).

Inhibition of Protein Synthesis. Amino acid transport and protein synthesis were inhibited simultaneously by citrate and ruthenium red. When either Ca^{2+}, of Mg^{2+}, or Mn^{2+} (all at 100 μM) were added to inhibited cells, only Ca^{2+} released the cells from inhibition if ruthenium red was the inhibitor. All three bivalent cations were effective antagonists of citrate-inhibited cells. Both Mn^{2+} and Mg^{2+} were even slightly inhibitory

on the residual amino acid uptake and protein synthesis activities (Fig. 4 a and b) that occurs at the low concentration of ruthenium red used. Specificity for Ca^{2+} in both metabolic processes was clearly indicated by these latter results. Thus either high concentrations of amino acids or low concentrations of amino acids in the presence of Ca^{2+} is required for efficient amino acid transport in *Achlya*. An allosteric role of Ca^{2+} is implied and this aspect is under investigation.

CONCLUSIONS

Since citrate does not penetrate the cell membrane of *Achlya* and can arrest protein synthesis by excluding Ca^{2+} and other bivalent cations from these cells it was difficult in our early studies to determine, unequivocally, whether Ca^{2+} plays the role of a regulator of amino acid transport and of protein synthesis. Our previous study (5) did not exclude Mg^{2+} and Mn^{2+} as possible regulators as well. The specificity of ruthenium red for Ca^{2+} -mediated metabolic systems has permitted us to resolve this problem. Ruthenium red and citrate interfere with the transport of at least 15 amino acids (the only ones we have tested so far). In *Achlya* and related water-moulds, it is likely that the transporters for all amino acids are stimulated by Ca^{2+}. It is known that amino acid uptake can proceed in the absence of an exogenous supply of Ca^{2+} but the transport system becomes easily saturated and stops functioning unless Ca^{2+} is present in the medium (5, and Cameron and LéJohn, in preparation). Throughout development of these moulds, Ca^{2+} uptake and protein synthesis proceed in step. We therefore suggest that Ca^{2+} couples amino acids transport and protein synthesis at the membrane level in *Achlya*.

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