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Energetics of Proline Transport in Corn Mitochondria[1]

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ABSTRACT

The mechanism of proline entry into the matrix region of isolated corn mitochondria (*Zea mays* L. Mo17 × B73) was investigated by measuring osmotically induced changes of mitochondrial size (changes in A_{520}) in combination with oxygen uptake measurements. Using NADH oxidation to generate the electrochemical gradient, we have determined that proline transport is stereospecific and that it can be inhibited by the proline analog L-thiazolidine-4-carboxylic acid.

The energetics of proline transport was investigated by measuring the effects of FCCP (*p*-trifluoromethoxycarbonyl cyanide phenylhydrazone) and valinomycin on mitochondrial swelling and substrate oxidation. Proline transport and resulting oxidation were found to be partially dependent upon the energy of the electrochemical gradient. At low proline concentrations, entry was found to be primarily independent of the gradient (based on insensitivity to FCCP), whereas at higher proline concentrations a gradient-dependent mechanism became involved. Results with valinomycin indicated that proline transport and oxidation are dependent upon the pH potential across the membrane rather than the electrical (membrane) potential.

Proline has recently been shown to be oxidized by a dehydrogenase bound to the matrix side of the inner mitochondrial membrane. Proline dehydrogenase feeds electrons and protons directly into the electron transport chain near its beginning, and is thus an integral part of the chain similar to malate dehydrogenase, succinate dehydrogenase, and the NADH dehydrogenases (5, 6).

During water stress in some plants, proline accumulates because of increased proline synthesis and an inhibition of proline oxidation (13). Since proline must pass through the mitochondrial inner membrane into the matrix for oxidation to occur, it is possible that proline oxidation *in vivo* may be regulated at either the transport, dehydrogenase, or electron transport chain level. Thus, it is of interest to determine the mechanism of proline entry into the matrix in unstressed tissue, in order to evaluate if transport becomes limiting during stress.

Of the techniques available to investigate mitochondrial transport, we have chosen to use light scattering (changes in A_{520}) in combination with oxygen uptake measurements. Mitchondria undergo osmotically induced changes in size as a result of net ion movement across the inner membrane. As the mitochondria increase in size, they scatter less light. This response is followed spectrophotometrically by measuring either changes in the amount of light scattered or transmitted. An appropriate wavelength (typically 520 nm) is chosen to minimize interfering absorbance of substrates, cofactors, or other media additions.

Proline transport has been investigated in deenergized mung bean mitochondria (1) and was found to be partially stereospecific and reversibly inhibited by sulfhydryl reagents. These are properties consistent with a protein-mediated mechanism of transport. Proline transport has also been investigated in mitochondria isolated from spinach leaves (15).

In this paper, we have followed oxidation and transport at substrate concentrations normally used to determine ADP:O ratios. By following these processes in energized mitochondria, we have been able to show that proline enters the mitochondrial matrix in a stereospecific and partially energy-dependent manner. In addition, we provide evidence that proline transport is dependent upon the pH potential across the membrane and not upon the electrical potential.

MATERIALS AND METHODS

Corn seedlings (*Zea mays* L. Mo17 × B73) were grown in the dark at 30 ± 2°C in moist vermiculite. Mitochondria were isolated from 3- to 4-d-old seedlings as before (6), except that often Tes was used rather than KH_2PO_4 and EDTA rather than EGTA[2] in the grinding medium. In addition, a Moulinex blender was sometimes used rather than a mortar and pestle to disrupt the tissue. Protein was estimated by the method of Lowry *et al.* (11) using BSA (fraction V) as the standard. FCCP (2.0 mM) and valinomycin (0.9 mM) were solubilized in 100% ethanol. Residual valinomycin was removed from the oxygen electrode, cuvette, and stirrer by rinsing with 100% ethanol.

Oxygen Uptake and Mitochondrial Swelling. These processes were measured simultaneously using a Clark type oxygen electrode mounted in the light path of either a Cary 210 spectrophotometer as reported previously (8), or in a Zeiss PMQ II. For measurements with the Zeiss spectrophotometer, the oxygen electrode was placed in the top of a stirred 1.6-ml cuvette which was mounted 5 cm from the detector window. A slit width of 0.33 mm was used and changes in relative absorbance were measured with a period of 1 s and a full scale of 0.1 *A*. Oxygen utilization within this cuvette was measured at 26 ± 1°C in 1.1 to 1.2 ml of SRM consisting of 250 mM sucrose and 30 mM MTT (10 mM each Mes, Tes, Tricine). Other additions are indicated in the figure legends.

RESULTS AND DISCUSSION

Effects of FCCP on Mitochondrial Swelling and Substrate Oxidation. When substrates are being oxidized during substrate

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[2] Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethyl ether)*N,N'*-tetraacetic acid; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; SRM, standard reaction medium; MTT, Mes, Tes, and Tricine; T4C, L-Thiazolidine-4-carboxylic acid.

state, their rate of oxidation is often limited by the magnitude of the electrochemical gradient present across the inner mitochondrial membrane (7). When this limiting gradient is diminished by phosphorylation or addition of uncoupler, the oxidation rate increases. For example, when a saturating amount of the uncoupler FCCP is added to mitochondria oxidizing exogenous NADH (in Fig. 1B the NADH plus FCCP trace), the oxidation rate increases dramatically. This increased rate is maintained until the medium is depleted of oxygen. Mitochondrial swelling occurs to a limited extent during exogenous NADH oxidation (Fig. 1A), with this swelling being reduced by FCCP addition. If FCCP is added to the mitochondria before NADH (the FCCP, plus NADH traces), the uncoupled rate of oxidation is rapidly established and little swelling occurs. The mitochondrial swelling that occurs with NADH is assumed to result from uptake of ions from the reaction medium and from some uptake of NAD (4).

When FCCP is added to mitochondria oxidizing succinate, a different response is obtained (Fig. 1D). Initially, a rapid rate of oxidation is observed; however, this is followed by a gradual transition to a rate lower than that during substrate state. The changes in size that the mitochondria undergo during this transition are considerable (Fig. 1C). It is apparent that the rapid uncoupled rate of oxidation is maintained only until the matrix volume is depleted to a certain extent. If FCCP is added before succinate, mitochondrial swelling is severely reduced and the rate of oxidation is very low. Mitochondrial swelling and oxidation with proline as substrate (Fig. 1 E, and F) yields results similar to those with succinate, except that with proline more swelling occurs. In addition, the initial rapid rate of oxidation upon addition of FCCP that is observed with succinate is often not observed with proline. Instead (as shown), a gradual reduction in the oxidation rate occurs.

Because proline and succinate are oxidized on the matrix side of the inner mitochondrial membrane, their entry into the matrix is essential for oxidation to occur. Since addition of FCCP results in a slower oxidation rate, that correlates with a decrease in mitochondrial size, it is reasonable to propose that this slower oxidation rate is due to depletion of substrate from the matrix. Thus, transport of these substrates is partially dependent upon the electrochemical gradient. In contrast, exogenous NADH oxidation does not become limited upon relief of the electrochemical gradient since this oxidation is not dependent upon transport processes. Results consistent with the above interpretation have been reported by Day and Hanson for NADH, succinate, and malate oxidation (2). In addition, FCCP's effects on the oxidation of malate and succinate have been published by Lima and De Melo (10) and by Ikuma and Bonner (9).

The response observed upon addition of FCCP is dependent upon the concentration of substrate used (Fig. 2A). With increasing proline concentration, an increase in mitochondrial size is observed. At low concentrations, mitochondrial size is not affected by addition of FCCP, which indicates that most proline entry into the matrix at this concentration is not dependent upon the energy of the electrochemical gradient. However, with higher

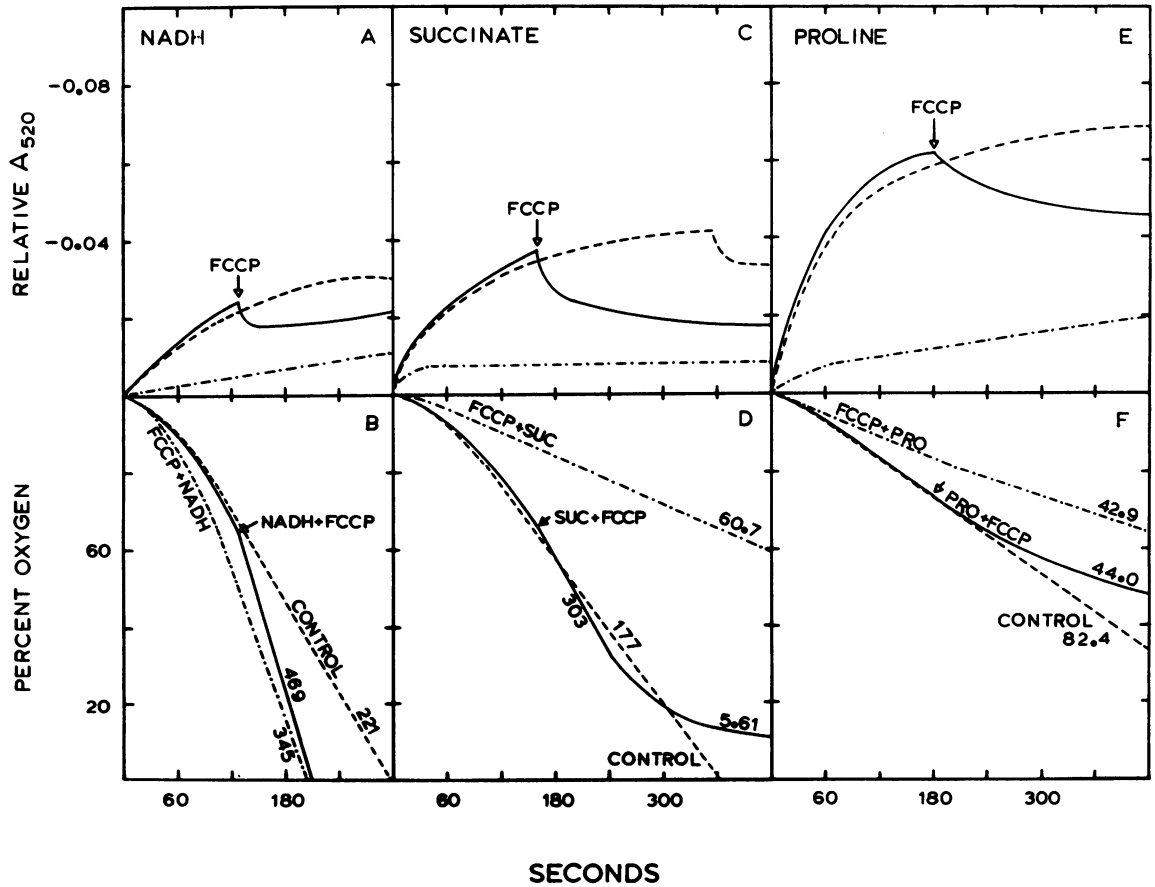

FIG. 1. The effect of FCCP on mitochondrial swelling and substrate oxidation. Changes in A_{520} and substrate dependent oxygen uptake were measured simultaneously in 1.1 ml of SRM containing approximately 650 μg of mitochondrial protein. The arrows indicate additions of 2 μM FCCP. The concentrations of other additions were 1 mM NADH, 10 mM succinate, and 10 mM L-proline. For the FCCP plus substrate assays, FCCP was added just before addition of substrate (0 s). The numbers on the traces represent natoms O/min·mg protein. These figures are representative of results obtained with three separate mitochondrial isolations. Controls (- - -); FCCP added before substrate (-·-·-); FCCP added after substrate (——).

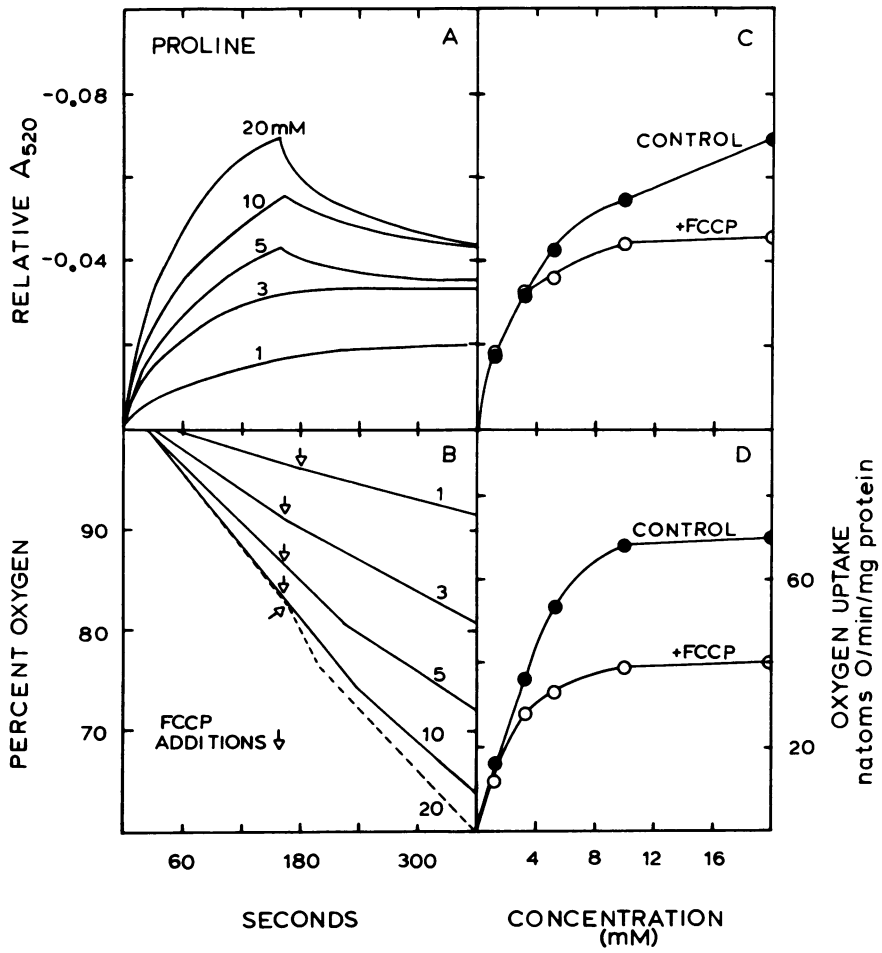

FIG. 2. The effect of proline concentration on the response obtained with FCCP. Assays were conducted as in Figure 1, except that mitochondrial protein was near 610 μg/assay. The numbers on the traces (A and B) represent mM proline concentrations. In Figures C and D, the results (of A and B) are replotted to better show the response. Mitochondrial size and oxidation rates just before FCCP addition (at 160 s in A and B) were taken as the control rates. Mitochondrial size and oxidation rates in the presence of FCCP were taken at 360 s. Similar results were obtained in two other experiments, although variation existed which seemed to depend primarily on the proline concentration required to saturate oxygen uptake. For example, the response observed here with 20 mM proline (the increased rate of oxidation upon addition of FCCP) has been observed in some preparations at a proline concentration as low as 5 mM.

concentrations a large portion of proline entry is dependent upon the gradient. These results contrast those obtained by Yu *et al.* using a silicone oil centrifugation technique and spinach leaf mitochondria (15). The oxygen uptake data (Fig. 2B) support the swelling results since sensitivity to FCCP increases with an increase in proline concentration. As the proline concentration was increased from 3 to 10 mM, an increased lag period after addition of FCCP was observed before inhibition occurred. This corresponds to the time required for oxidation to decrease the matrix concentration of proline to that maintained in the absence of an electrochemical gradient.

These figures also show that the oxidation rate becomes limited before transport, since mitochondrial size increases considerably between 10 and 20 mM proline while the oxidation rate (before FCCP addition) remains the same. At 20 mM proline, the oxidation rate increases upon addition of FCCP similar to the response obtained with 10 mM succinate (Fig. 1D). This shows that, between 10 and 20 mM proline, the electrochemical gradient began to limit the electron transport chain, and thus limited substrate oxidation. The rapid uncoupled rate of oxidation can only occur when the electrochemical gradient had been limiting oxidation and substrate was loaded into the matrix at a concentration greater than that necessary to maintain the oxidation rate.

In Figure 2, C and D, we have replotted the data of Figure 2, A and B, to indicate the relative electrochemical gradient dependence of the process of proline transport and subsequent oxidation. The areas underneath the +FCCP curves roughly represent transport and subsequent oxidation that does not require an electrochemical gradient. The rates represented by the area between the two curves depend on such a gradient. By using a saturating amount of FCCP, we have reduced the electrochemical gradient as much as possible. However, since FCCP is simply competing for protons with other processes (7), it is clear that we are probably underestimating the degree of energy dependence of transport and oxidation. By presenting the data in this manner (Fig. 2, C and D), we have been able to evaluate and compare the energetics of the process of transport and subsequent oxidation between preparations. If this technique is carefully quantitated, it should also be possible to compare the characteristics of transport and oxidation in different systems.

Since we have shown that a portion of proline entry into the matrix is energy dependent, it is quite reasonable to assume that this transport is protein mediated. However, since diffusion of substrate through the membrane could be enhanced by the presence of an electrochemical gradient, we have also evaluated the stereospecificity of proline transport. If D-proline is added to mitochondria, no swelling is observed. However, D-proline is not oxidized, and therefore no energy is available to drive transport. By adding NADH initially to generate the electrochemical gradient, we were able to compare the ability of mitochondria to transport D- and L-proline (Fig. 3). This technique has been used previously to study transport and oxidation of malate and pyruvate (3) and of malate and succinate (2). When L-proline is added, considerable swelling occurs. In contrast, little swelling occurs with D-proline. These results indicate that proline (at concentrations up to 10 mM) enters the matrix in a stereospecific manner. Stereospecificity and partial energy dependence are characteristics that are not consistent with diffusion of proline through the lipid bilayer. Investigation of proline (200 mM) entry into the matrix of deenergized mung bean mitochondria had indicated less stereospecificity (1).

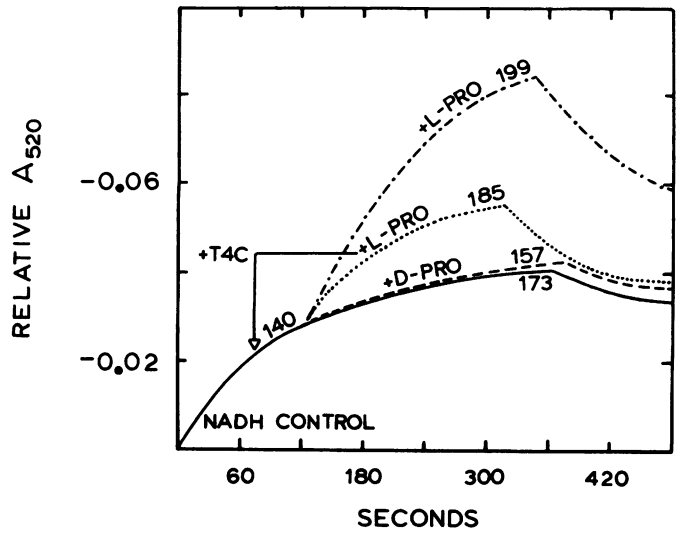

FIG. 3. Stereospecificity of proline entry into the mitochondrial matrix. Assays were conducted as in Figure 1, except that mitochondrial protein was about 610 μg/assay. The numbers on the traces represent natoms O/min·mg protein. Concentration of additions were 1 mM NADH, 1 mM T4C, 10 mM L-proline, and 10 mM D-proline. The reduction in size towards the right of these traces occurs when the medium is depleted of oxygen (as in the other figures). The oxygen uptake rates do not correlate with points of oxygen depletion because of variation in the initial NADH oxidation rate. The value of 140 is the average of the initial rates for the 4 assays, with the average deviation of the mean being 7.3 natoms O/min·mg protein. Similar results were obtained in two other experiments.

The use of NADH oxidation to drive transport processes eliminates many of the problems in interpretation that arise if oxidation of a substrate is driving its own transport. We have used this technique to determine if thioproline (T4C) inhibits proline transport (Fig. 3). These results show that thioproline does inhibit proline-dependent mitochondrial swelling. Our previous research had indicated that thioproline inhibited proline dehydrogenase since proline-dependent oxygen uptake was inhibited before a sufficient reduction in size of the mitochondria had occurred (8). Thus, it seems that thioproline inhibits both transport and oxidation.

Effect of Valinomycin on Mitochondrial Swelling and Substrate Oxidation. Since a portion of proline transport is driven by the electrochemical gradient, we wanted to determine if that portion is dependent upon the electrical potential, the pH potential, or both. Addition of FCCP reduces the pH potential and in doing so also reduces the electrical (membrane) potential. However, when the K^+ ionophore valinomycin is added to mitochondria, it diminishes only the electrical potential. Decreasing the electrical potential may effect an increase in the pH potential so as to maintain a constant electrochemical gradient (12).

When a saturating amount of valinomycin is added to mitochondria oxidizing exogenous NADH, the mitochondria swell instantaneously due to the uptake of K^+ (Fig. 4A). This increase in size should closely reflect the electrical potential that was present. In addition, the oxidation rate is increased (Fig. 4B), with this increase being maintained until the medium is depleted of oxygen. When valinomycin is added before NADH, increased swelling and oxidation are observed. Since NADH transport is not involved in these reponses, the results indicate that the electrical potential was limiting the electron transport chain during substrate state. These results are consistent with those of

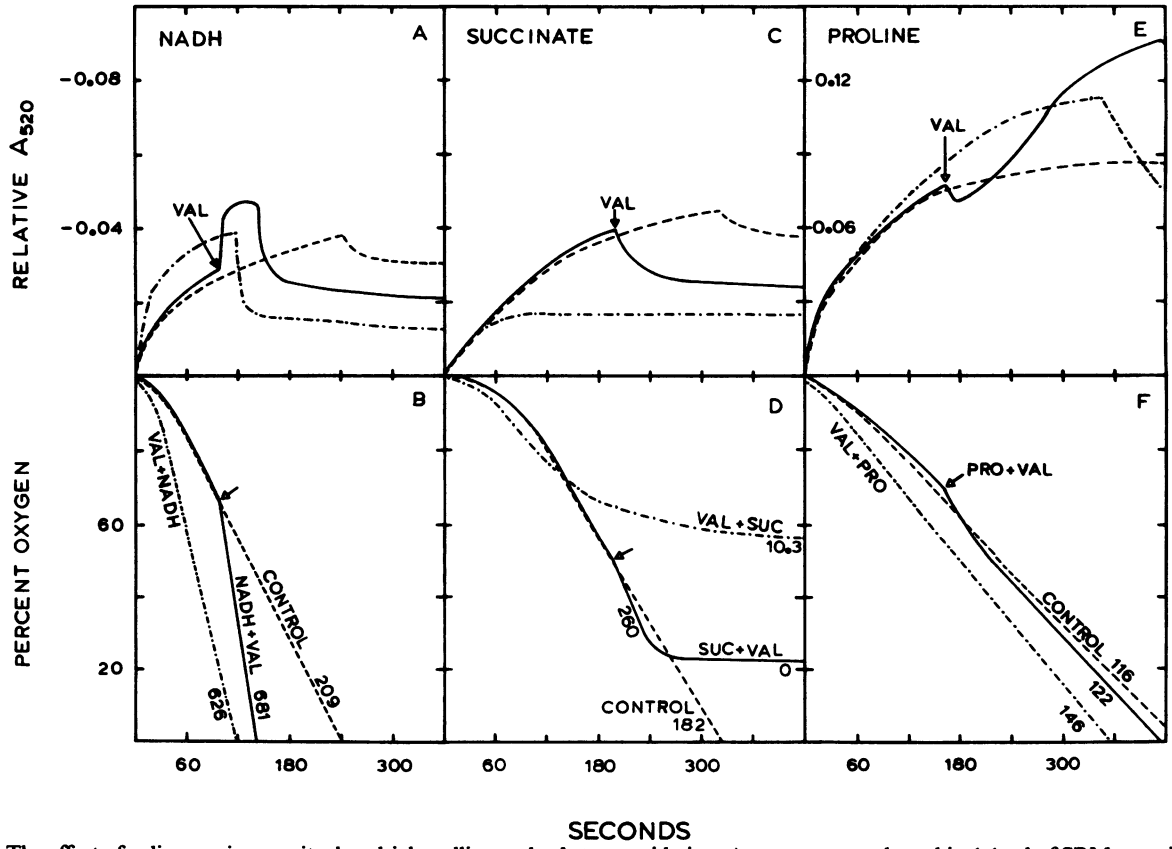

FIG. 4. The effect of valinomycin on mitochondrial swelling and substrate oxidation. Assays were conducted in 1.1 ml of SRM containing 1 mM KCl. Mitochondrial protein was near 560 μg/assay. Arrows indicate additions of 1.1 μM valinomycin. Other conditions are the same as those in Figure 1. The results are representative of three separate experiments. Controls (– – –); valinomycin added before substrate (–·–·–); valinomycin added after substrate (——).

Day and Hanson (2).

When valinomycin is added to mitochondria oxidizing succinate, it effects an increase in the oxygen uptake rate (Fig. 4D) and a gradual reduction in the size of the mitochondria (Fig. 4C). When the mitochondrial size has decreased to a certain extent, oxidation decreases. Thus, for the continued oxidation of succinate, the presence of an electrical potential is essential. When valinomycin is added before succinate, swelling is reduced and the oxidation rate eventually decreases. However, oxidation is not affected much initially. Thus, it seems possible that succinate transport may not be dependent upon the electrical potential, but rather that succinate oxidation may become limited by the inability to remove or exchange oxidation products (perhaps oxaloacetate) with succinate (14).

With proline as substrate, addition of valinomycin yields a different response (Fig. 4, E and F). The mitochondria show increased swelling and the oxidation rate increases. A somewhat increased oxidation rate is maintained until oxygen depletion. When valinomycin is added before proline, the mitochondria also show increased swelling and increased oxygen uptake. Thus, proline transport and continued proline oxidation are clearly not dependent upon the electrical potential across the membrane.

We have purposely avoided discussing the possible role of Pi exchange in proline transport. Several experiments have shown that a 1 mM Pi addition reduces both proline-dependent mitochondrial swelling and oxygen uptake. However, we feel that it would be desirable to do a more extensive treatment of Pi effects than could be included here (similar to that of Day and Hanson for other substrates [2]).

By using NADH oxidation to drive the transport of substrates in combination with transport inhibitors, it should be possible to determine if proline is transported via one of the known carriers. Just as exciting, the determination of FCCP effects on substrate oxidation (as in Fig. 2) will allow for investigation of physiological responses that result from the integration of substrate transport and oxidation.

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