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Allosteric Inhibition of the Ca^{2+}-Activated Hydrophilic Channel of the Mitochondrial Inner Membrane by Nucleotides

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Summary. The control by nucleotides of the Ca^{2+}-activated channel which regulates the nonspecific permeability of the mitochondrial inner membrane has been investigated quantitatively. The cooperative binding of two molecules of ADP to the internal (matrix) side of the channel causes a mixed-type inhibition of channel activity. ATP, AMP, cAMP and GDP are all ineffective. NADH shows a pattern of inhibition similar to that of ADP, though the apparent K_I is higher by a factor of 200. NADPH relieves the inhibition by NADH. NAD^+ also inhibits, but its affinity is a factor of 10 less than that of NADH. When ADP and NADH are added together, they act synergistically to inhibit the Ca^{2+}-activated channel. It is concluded that the concept of the modification of enzyme activity by the allosteric binding of nucleotides, which is well established for soluble enzyme systems, also has application to the regulation of channels that control membrane permeability.

We have previously given evidence that the permeability of the mitochondrial inner membrane can be modulated over a wide range by the mere binding of Ca^{2+} to a membrane channel (Haworth & Hunter, 1979). The evidence in favor of such a channel mechanism is fourfold. Firstly, the Ca^{2+}-dependent permeability change is transitional in nature, i.e., it is discontinuous in time, characteristic of the opening and closing of a channel. This change in permeability, which in intact mitochondria causes a change in configuration from the aggregated[1] to the orthodox[2], we termed the Ca^{2+}-induced transition. Secondly, the permeability change is reversible. Removal of Ca^{2+} by chelation with EGTA immediately eliminates the possibility of further transitions, until more Ca^{2+} is added. Thirdly, the induced permeability change is nonspecific, with a cut-off determined by molecular weight rather than molecular type. Fourthly, the shape of the curve describing the dependence of permeability on the concentration of free Ca^{2+} inside the mitochondria appears to be independent of the nature of the permeating solute. This is a generalized conclusion drawn from the observation that the permeability of the membrane to salts exhibits a dependence on Ca^{2+} concentration identical to that of the permeability of the membrane to $NADP^+$, even though the permeabilities were measured by totally independent methods, one under a pressure gradient and the other at zero volume flow. This last observation has a further significance. Not only does it suggest that the channel can open only when Ca^{2+} is bound, but also it implies that the Ca^{2+}-dependence of permeability to salts, as measured by the shrinkage assay (*see* Materials and Methods), is a good measure of the extent to which the channel site is saturated with Ca^{2+}. More precisely, the measured shrinkage rate is proportional to the concentration of active Ca^{2+}-channel complexes. This conclusion allows us to use our measurements of permeability to investigate closely the mode of action of Ca^{2+} and inhibitors at the active site, using the concepts and equations of enzyme kinetics.

During our studies on intact mitochondria it became apparent that the slow rate at which they undergo the transition is in part a consequence of inhibitory agents contained in the matrix space (Hunter & Haworth, 1979*a*). When a transition occurs, these agents leave the matrix space, and the mitochondria thereafter undergo Ca^{2+}-dependent transitions in permeability at an uninhibited rate (Haworth & Hunter, 1979). It was inferred from the effect of various agents

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[1] The aggregated configuration is characterized by a shrunken, darkly staining matrix space and a large intracristal space.

[2] The orthodox configuration is characterized by an expanded matrix space which stains much less intensely, and a much decreased intracristal space. (*See* Hunter et al. (1976) for an illustration.)

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on intact mitochondria that internal NADH and ADP were two of these agents active in modulating the action of Ca^{2+} (Hunter & Haworth, 1979*a*), and this inference was confirmed by the observation of an inhibitory action of added NADH and ADP on the Ca^{2+}-dependent permeability of mitochondria which had already undergone a transition (Haworth & Hunter, 1979). Such mitochondria in the presence of Ca^{2+} will allow the ready access of added nucleotides to sites of inhibition in the matrix space (*see* Materials and Methods). To us these observations of nucleotide inhibition raised the exciting possibility that the Ca^{2+}-regulated channel could be modified by the mere binding of these nucleotides in some definite stoichiometry, in a manner analogous to the allosteric modification of soluble enzymes. To investigate this possibility, we have examined the inhibition of Ca^{2+}-induced permeability by nucleotides in a quantitative fashion, using mitochondria which have already undergone a transition.

Materials and Methods

Preparation of Treated Mitochondria

The preparation of hypotonically swollen beef heart mitochondria which had undergone a Ca^{2+}-induced transition (treated mitochondria) was as previously described (Haworth & Hunter, 1979). Hypotonic treatment was employed in order that the Ca^{2+}-dependent permeability could be measured conveniently, using the light-scattering assay of PEG[3]-induced mitochondrial shrinkage (*see* below).

Equilibration of Treated Mitochondria with Nucleotides

Treated mitochondria have lost their endogenous nucleotides, and are freely permeable to added nucleotides in the presence of Ca^{2+}. Figure 1 shows how quickly ADP can inhibit when added to these mitochondria, even though it must enter the mitochondria in the presence of carboxyatractyloside to reach a site of inhibition which is internal (Hunter & Haworth, 1979*a*). Carboxyatractyloside is a potent and specific inhibitor of the membrane ADP/ATP translocase (Vignais, Vignais & Defaye, 1973), and was included in the incubation medium in order to eliminate the effect of ADP binding at the translocase on the Ca^{2+}-induced permeability. It was shown previously (Hunter & Haworth, 1979*a*) that such binding causes an inhibition of the transition in intact mitochondria. A similar inhibition can be observed in treated mitochondria and details of this will be reported separately. Care was taken to ensure that nucleotides had equilibrated under all conditions used; at the lower levels of Ca^{2+} this sometimes required incubation for ten minutes. Thus the level and type of nucleotide in the matrix space could be precisely controlled. Possible loss of added ADP from residual myokinase activity was ruled out by the observation that the degree of inhibition produced by a given concentration of ADP was constant in time (Fig. 1). Any myokinase activity, combined with the oligomycin-sensitive ATPase activity, would have caused a gradual decrease in the effectiveness of added ADP due to conversion to AMP. Added AMP caused no inhibition. Significant ATPase activity was demonstrated by the observation that added ATP caused a time-dependent inhibition which could be prevented by prior addition of oligomycin, which may therefore be attributed to the ADP produced by the ATPase activity. The actual rapid penetration of treated mitochondria by ^3H-labeled NAD^+ in the presence of Ca^{2+} was also demonstrated directly, using the methodology previously developed to measure the permeation of mitochondria by ^{14}C-labeled sucrose (Hunter, Haworth & Southard, 1976). Oxidation of added pyridine nucleotides was prevented with rotenone; other possible nucleotide transformations were precluded by the absence of required substrates.

Complications due to energy-linked processes were excluded, since the mitochondria burn up their endogenous substrate prior to going through a Ca^{2+}-dependent transition in the preparation procedure (Hunter & Haworth, 1979*a*); in any case, they also lose cofactors at this time.

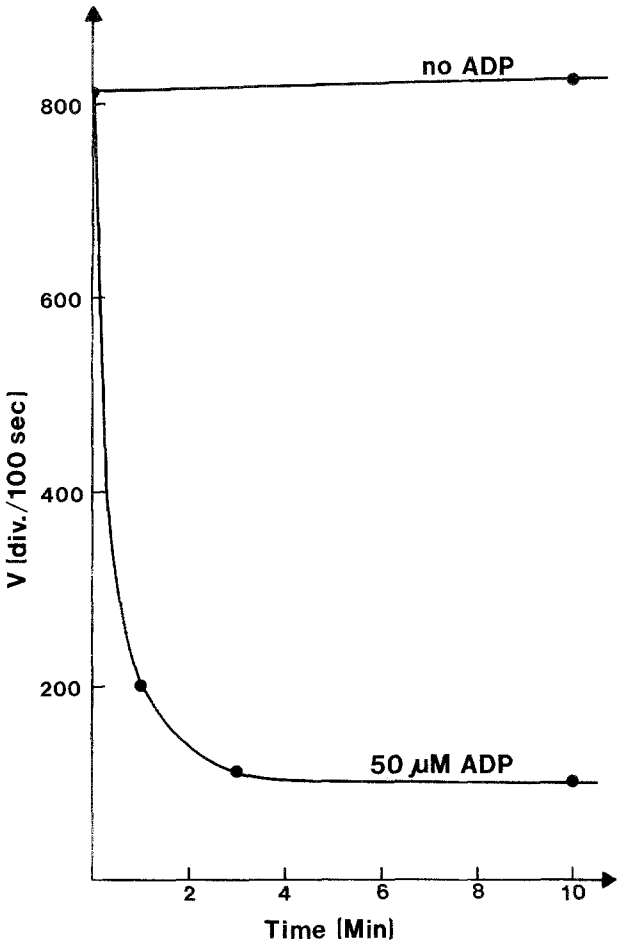

Fig. 1. Rate of influx of ADP into treated mitochondria in the presence of Ca^{2+}, as shown by the rate at which added ADP inhibits at an internal site. To 2.5 ml 5 mM K^+MOPS, 25 mM K^+ cacodylate, 50 mM KCl at pH 6.8 was added 0.15 ml (3 mg) treated mitochondria, 1 μM carboxyatractyloside, and 500 μM $CaCl_2$. The suspension was mixed. After 15 sec, 50 μM ADP was added, at time 0 on the graph. After incubation for the time shown, shrinkage was induced by the addition of 0.3 ml 35% PEG (mol wt 1500). The rate of shrinkage (*V*) is expressed in arbitrary units.

[3] *Abbreviations:* PEG, polyethyleneglycol; MOPS, morpholinopropane sulfonate; TMA, tetramethylammonium.

Permeability Measurements

Light scattering was measured on a Perkin Elmer MPF 3 fluorescence spectrophotometer with excitation and emission wavelengths set at 520 nM. The instrument was fitted with a mechanical stirrer which gave complete mixing of added solutions within approximately two seconds. The cell holder was maintained at 30° in all experiments by a flow-through water jacket. The rate of shrinkage *(V)* was measured from the slope of the 90° light-scattering curve after the addition of 0.3 ml 35% PEG (mol wt 1500) to 2.7 ml mitochondrial suspension (Haworth & Hunter, 1979). In brief, the principle of this assay is as follows. When a solute which cannot permeate the inner membrane is added to a mitochondrial suspension, a rapid efflux of water from the matrix space occurs which is completed within a few seconds. At the new matrix volume, the increased osmotic pressure of internal solutes just balances the increased osmotic pressure of external solutes. Now consider the case of treated mitochondria, where the membrane has a significant (Ca^{2+}-dependent) degree of permeability to solutes of low molecular weight as well as to water. When an impermeable solute such as PEG (mol wt 1500) is added to a suspension of treated mitochondria, the subsequent shrinkage continues beyond that achieved when the membrane is impermeable to internal solutes; these internal solutes are now also leaving, and shrinkage will continue until the osmotic pressure of the PEG is balanced by the osmotic pressure of the matrix proteins and associated Donnan ions. Under the conditions used here, this final matrix volume is almost immeasurably small. It is clear that the more permeable the membrane is to internal solute, the faster the mitochondria will shrink during this extra shrinkage phase. This rate of volume flow is measured by light scattering (Haworth & Hunter, 1979).

Results

Inhibition of Ca-Induced Permeability by ADP

The existence of an internal site of ADP inhibition was inferred previously from the correspondence between the depletion of endogenous ADP of intact beef heart mitochondria by phosphoenolpyruvate and the stimulation of the Ca^{2+}-dependent transition (Hunter & Haworth, 1979*a*). With the shrinkage assay the action of ADP could be demonstrated directly (Fig. 2). It should be noted that this and other experiments reported here used a buffer at pH 6.8. This is because at this pH the apparent K_m for Ca^{2+} is sufficiently high to eliminate the need for Ca^{2+} buffers. The apparent K_m for Ca^{2+} is very sensitive to pH: at pH 7.2 the apparent K_m for Ca^{2+} (in the absence of ADP or NADH) is <10 μM and is still dropping with increasing pH (Haworth & Hunter, 1979). Endogenous levels of mitochondrial Ca^{2+} *in vivo* may therefore be expected to be active in the mechanisms described here. It may be seen in Fig. 2 that ADP reduces the maximum Ca^{2+}-induced shrinkage rate and also increases the apparent K_m for Ca^{2+}. 100 μM ATP, AMP, cAMP and GDP were all found to be inactive at this site. This pattern of inhibition by ADP is analogous to the action of a mixed-type inhibitor. The solid lines in Fig. 2, which

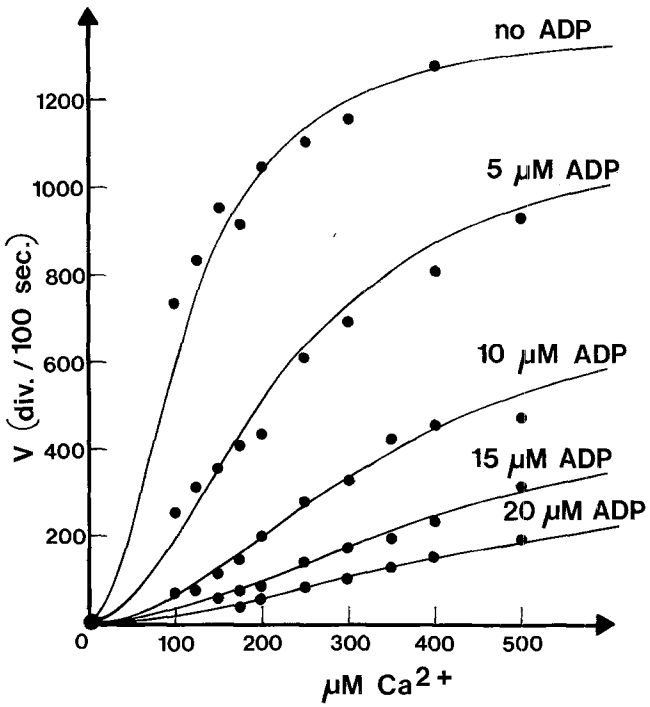

Fig. 2. Inhibition by ADP of Ca^{2+}-induced permeability. To 2.5 ml buffer containing treated mitochondria (*see* Fig. 1) was added 2.5 μg A23187, 1 μM carboxyatractyloside, ADP and $CaCl_2$ to give the levels of free ADP and Ca^{2+} shown. (This required only a small correction, as ADP does not bind Ca^{2+} significantly under these conditions.) After 5 min incubation, 0.3 ml PEG was added. A23187, a Ca^{2+} ionophore, was added to facilitate equilibration of Ca^{2+} across the membrane. Its inclusion was not essential, but at high levels of ADP and low levels of Ca^{2+} it served to shorten the time needed for equilibration of both Ca^{2+} and ADP.

are an excellent fit of the experimental data points, are generated from the velocity equation which describes the pattern of mixed-type inhibition shown in Fig. 3. The precision of the fit to such a simple formulation is a striking result. Since the effects of concentrations of Ca^{2+} <100 μM were not measured in this experiment, it might be thought that the conformity of the "zero ADP" curve to the formulation of Fig. 3 is open to doubt in this region. We have, however, reported previously that a Hill Plot of the Ca^{2+} dependence of shrinkage rate was linear with a slope of almost 2 (Haworth & Hunter, 1979), and this curve was measured at eleven different Ca^{2+} concentrations between zero and 200 μM. From Fig. 2 we may therefore conclude that either two molecules of ADP or two atoms of Ca^{2+} may bind with a high degree of cooperativity; the binding of either substantially inhibits the binding of the other (by the coefficient α), and the channel may open only when Ca^{2+} and not ADP is bound (the complex EL_2 in Fig. 3). Attempts to fit the curves with formulations based on numbers of ADP molecules binding other

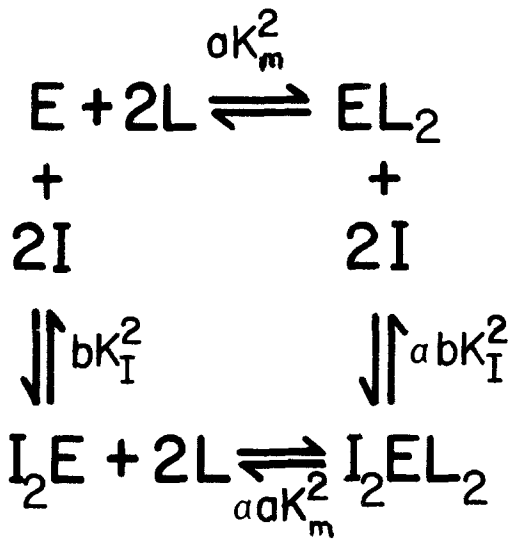

Fig. 3. Equilibria which describe the mixed-type inhibition shown in Fig. 2. The velocity equation describing these equilibria is:

$$\frac{1}{v}=\frac{1}{V_{max}}\left[1+\frac{[I]^2}{b\alpha K_I^2}\right]+\frac{aK_m^2}{V_{max}[L]^2}\left[1+\frac{[I]^2}{bK_I^2}\right]$$

where

$$aK_m^2=\frac{[E][L]^2}{[EL_2]} \quad \text{and} \quad bK_I^2=\frac{[E][I]^2}{[EI_2]}.$$

The solid curves in Fig. 2 are derived from this equation using the values $\sqrt{(aK_m^2)}=113\,\mu M$, $\sqrt{(bK_I^2)}=2.57\,\mu M$, $\alpha=19.59$. The above formulation is only valid if a and $b \ll 1$; that is, if there is a high degree of cooperativity of both Ca^{2+} and inhibitor binding, so that the concentration of singly-occupied species is negligible. The validity of this condition for Ca^{2+} binding has already been shown (Haworth & Hunter, 1979)

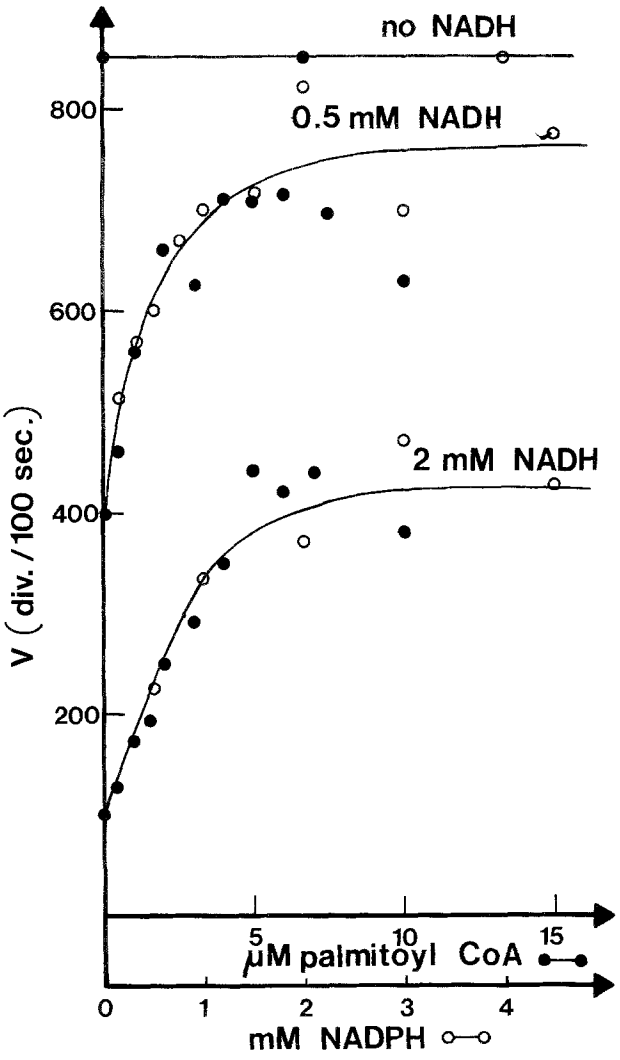

Fig. 4. Relief of NADH-induced inhibition of Ca^{2+}-induced permeability by NADH and palmitoyl CoA. To 2.5 ml 5 mM K^+MOPS, 25 mM TMA cacodylate, 50 mM KCl at pH 6.8 was added 0.15 ml (3 mg protein) treated mitochondria, 5 μM rotenone, 500 μM $CaCl_2$, and nucleotides as shown. After incubation for 1 min, shrinkage was induced by the addition of PEG as usual.

than 2 produced gross discrepancies; any single curve of ADP inhibition could be fitted, but all other curves would then be wildly different from those predicted. It might be added that the competitive inhibition of the Ca^{2+} effect by Sr^{2+} (Haworth & Hunter, 1979) also may be formulated in kinetic terms, and be shown to act by the cooperative binding of two atoms of Sr^{2+} (data not shown).

Inhibition of Ca^{2+}-Induced Permeability by NADH

The existence of an internal inhibitor which was in redox equilibrium with endogenous NADH was inferred previously from the effects of modulators of the NADH/NAD^+ couple on the rate of the Ca^{2+}-induced transition (Hunter, 1977; Hunter & Haworth, 1979*a*). A similar effect of the NADH/NAD^+ couple on the rate of mitochondrial Ca^{2+} efflux was observed by Lehninger, Vercesi and Bababunmi (1978), an observation we attribute to the role of this couple in controlling the transition rate (Hunter & Haworth, 1979*b*). Using the shrinkage assay, the inhibitor can be identified as NADH itself. The pattern of mixed-type inhibition was similar to that caused by ADP at the internal site (Fig. 2), except that NADH was found to be much less potent: 1 mM NADH in the presence of rotenone was approximately equivalent to 5 μM ADP. The same dependence on the square of inhibitor concentration was found. NAD^+ was also found to inhibit, but with an affinity lower than that of NADH by an approximate factor of 10. Of considerable interest was the observation that the inhibition exerted by NADH was relieved by NADPH but not by similar levels

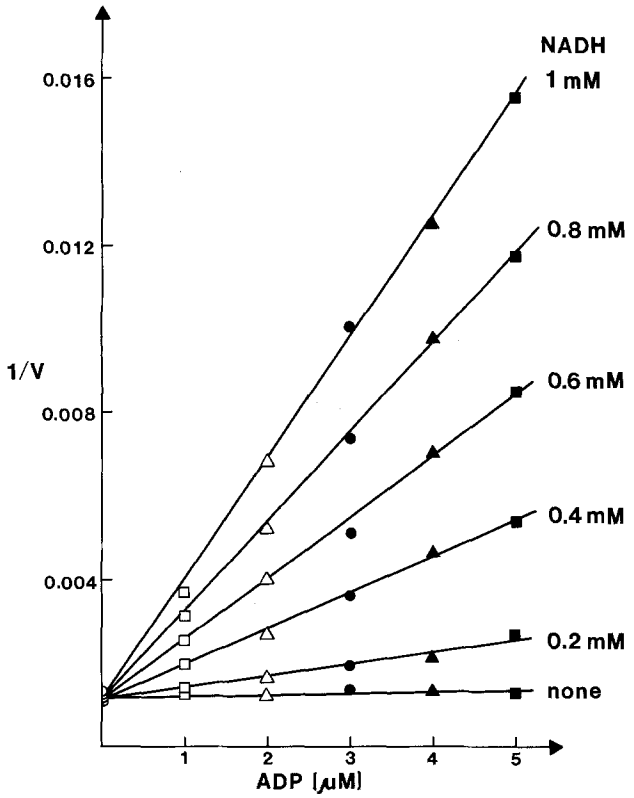

Fig. 5. Synergistic action of ADP and NADH. To 2.5ml buffer containing mitochondria (*see* Fig. 1) was added 5 μM rotenone, 1 μM carboxyatractyloside, ADP and NADH as shown. After 15 sec, 500 μM $CaCl_2$ was added. After incubation for 10 min, shrinkage was induced by addition of PEG as usual.

of $NADP^+$, in a manner which appears to be partially competitive in nature (Fig. 4). A similar relief could be achieved by micromolar amounts of palmitoyl CoA (Fig. 4); higher levels caused aggregation of the treated mitochondria. CoA was ineffective at relieving inhibition. The low levels needed to give significant relief of NADH-induced inhibition suggest that long-chain acyl-CoAs could play an important role in regulating the Ca^{2+}-induced transition *in vivo*. Palmitoyl CoA caused a similar relief of inhibition by ADP, and at similar levels; NADPH, by contrast, was almost ineffective at relieving inhibition by ADP.

Synergistic Inhibition of Ca-Induced Permeability by ADP and NADH

When ADP and NADH together are incubated with treated mitochondria plus Ca^{2+} it is found that the nucleotides are much more potent in combination than separately. This effect is shown in Fig. 5, where the data is expressed as a Dixon plot. It is seen that levels of ADP and NADH which separately cause insignificant inhibition[4] can together cause very potent inhibition of Ca^{2+}-induced permeability. The point of intersection of the lines at positive values of $1/v$ indicates that ADP and NADH bind cooperatively (Segel, 1975). It is possible that the two cooperative binding sites for ADP and the two NADH binding sites are identical. The synergistic action of ADP and NADH could then be readily explained by one site favoring ADP and the other site favoring NADH.

Discussion

The characteristics of the Ca^{2+}-dependent channel, some of which have now been defined with a fair degree of accuracy, set it apart from other regulated membrane channels that are presently characterized. It does, however, combine the features of many. It lacks the specificity of the ion channels of the nerve membrane, but shares the attribute of specificity of activation, with its remarkable discrimination between Ca^{2+} and Sr^{2+} (Hunter et al., 1976). It requires the binding of two atoms of Ca^{2+} for activation (Haworth & Hunter, 1979); the acetylcholine-activated channel likewise is activated by the binding of two molecules of ligand (Dionne, Steinbach & Stevens, 1978). A nonspecific but (apparently) unregulated channel has been isolated from the outer membrane of bacteria (Nakae, 1976), and a similar but voltage-dependent channel has been found in the outer membrane of mitochondria (Colombini, 1979). But the channel which is most similar to the inner membrane channel, in being both nonspecific and chemically gated, is perhaps the channel which spans the intercellular gap junction. This channel has a molecular weight cut-off (Simpson, Rose & Loewenstein, 1977; Flagg-Newston, Simpson & Loewenstein, 1979) in a range similar to that demonstrated for the inner membrane Ca^{2+}-activated channel (Haworth & Hunter, 1979), and it can be gated by Ca^{2+} (Rose, Simpson & Loewenstein, 1977). It is tempting to speculate that the opening of the inner membrane channel could entail fusion with the outer membrane, producing a transient gap junction. The feature which at present makes the inner membrane channel unique is the overriding of the activating action of Ca^{2+} by the stoichiometric binding of nucleotides. This property undoubtedly ensures

[4] In Fig. 5 1 mMNADH alone has little effect at all, whereas in Fig. 4 the effect of 1 mM NADH is considerable. The difference is related to the age of the preparation of treated mitochondria. The effect of Ca^{2+} and ADP on treated mitochondria were generally reproducible on preparations stored for up to four or five days in the refrigerator. The inhibition produced by NADH, however, declined with time of storage. It may well be that this decline is related to the gradual loss of residual ADP in the preparation.

that the action of Ca^{2+} is normally substantially inhibited, allowing the mitochondria to maintain large gradients across the inner membrane. The circumstances under which the transition would be required to occur *in vivo* are presently unknown and are under active investigation.

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