PROPAVANE AN INHIBITOR OF OXIDATIVE PHOSPHORYLATION CONNECTED WITH MITOCHONDRIAL GLUTAMATE METABOLISM

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Studies of oxidative reactions of mitochondria and particularly studies of oxidative phosphorylation have taken much advantage of the availability of specific inhibitors. Antibiotics such as oligomycin and aurovertin (Lardy et al., 1958 and Lardy, 1961) as well as the guanidines (e.g. Pressman, 1963, Chance and Hollunger, 1963 b) have served as useful reagents in the studies of the complex reactions of energy transfer.

This report describes a new inhibitor, propavane (2-propionyl-10-(2'dimethylaminopropyl)phenthiazinmaleate) which in very low concentrations (10 uM) is an effective inhibitor of glutamate oxidation in pigeon heart mitochondria. With higher propavane concentrations (200 uM) respiration due to other tricarboxylic acid cycle compounds is inhibited as well. Like amytal (Chance, Hollunger and Hagihara, 1962 and Chance and Hollunger, 1963a) propavane is considered to interfere with both the electron transfer mechanism and with the energy transfer mechanism. In contrast to amytal however propavane exerts its major effect in the phosphorylating system although the inhibition is not counteracted by uncouplers such as 2,4-dinitrophenol. Fluorescence measurements suggest that propavane reacts at the pyridine nucleotide level.

Materials and Methods.

Pigeon heart mitochondria (PHM_w) were prepared according to the method of Hagihara (Chance and Hagihara, 1963). Oxygen uptake was determined either with a Clark type electrode as described by Chappell (1961) or by means of a vibrating platinum electrode (e.g. Chance and Hagihara, 1963) placed in the cuvette of an Eppendorf photometer with fluorescence attachment (Netheler and Hinz, Hamburg). In the latter

case it was possible by fluorescence measurements to follow the reduction of pyridine nucleotides and oxygen uptake simultaneously.

The assay medium contained mannitol (225 mM), sucrose (75 mM), tris (20 mM), EDTA (o.5 mM) usually supplemented with 10 mM orthophosphate, pH: 7.4. Protein concentration as determined by the biuret method was usually 4oo-6oo ug/ml in the experiments.

Results.

The influence of varying concentrations of propavane on the rate of glutamate respiration is shown in Fig. 1. Pigeon heart mitochondria were diluted in the assay medium and propavane and glutamate (7 mM) added. After incubation for about two minutes ADP or ADP and 2,4-dinitrophenol were added to the reaction chamber. The data summarized in Fig 1 show that the rate of O_2 uptake in the absence of ADP or 2,4-dinitrophenol (half open circles) is little affected by increasing concentration of propavane while the rate of O_2 uptake in the presence of ADP (open circles) or ADP + 2,4-dinitrophenol (closed circles) is markedly inhibited. The action of 2,4-dinitrophenol is observed to be inhibitory for dinitrophenol concentrations between 0 and 115 uM. This was examined by adding the uncoupler continuously to the reaction mixture. The propavane concentrations causing a 50% inhibition of the rate of oxygen uptake are 14 uM in the presence of ADP and 6 uM when 2,4-dinitrophenol is present in the concentration (14 uM) which causes maximal uncoupling in the absence of propavane.

The rates of oxygen consumption with a number of other substrates and substrate combinations have been examined and found to be insensitive to presence of propavane in the concentrations employed here, e.g. glutamate in combination with malate or succinate. Concentrations of propavane as high as 2oo-5oo uM, however, did inhibit the respiration of such substrates to about half maximal.

The sensitivity to propavane apparently is not directly related to the turnover in the respiratory chain. An aged mitochondrial preparation with a high respiratory activity which could not be further increased by ADP has a low propavane sensitivity whether or not ADP is present. In

normal preparations uncoupling of oxidative phosphorylation without pronounced stimulation of respiration (e.g. with arsenate) preserves the high propavane sensitivity.

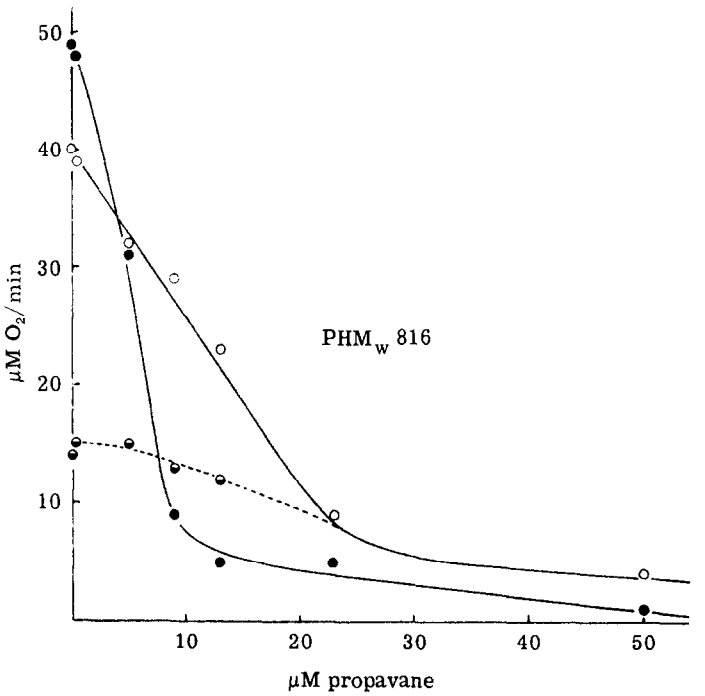


Fig. 1. Glutamate respiratory rates plotted against propavane concentrations. Open circles: ADP present, filled circles: ADP + 14 uM 2,4-dinitrophenol present, half filled circles: neither ADP nor uncoupler present. 465 ug mitochondrial protein pr. ml.

The soluble dehydrogenase from liver (Boehringer, Mannheim) shows little sensitivity to propavane. An enzyme concentration of 4 x 10^{-10}M would cause half the normal turnover of glutamate in mitochondria (ADP present) and half inhibition was obtained with 450 uM propavane (U.F.Rasmussen in preparation).

Figure 2 shows the effects of varying concentrations of propavane upon pyridine nucleotide reduction associated with glutamate oxidation. In a phosphate deficient medium glutamate addition results in a reduction of the pyridine nucleotides (experiment 2o7-11), but without a simultaneous oxygen consumption (not shown in Fig. 2). The extent of re-

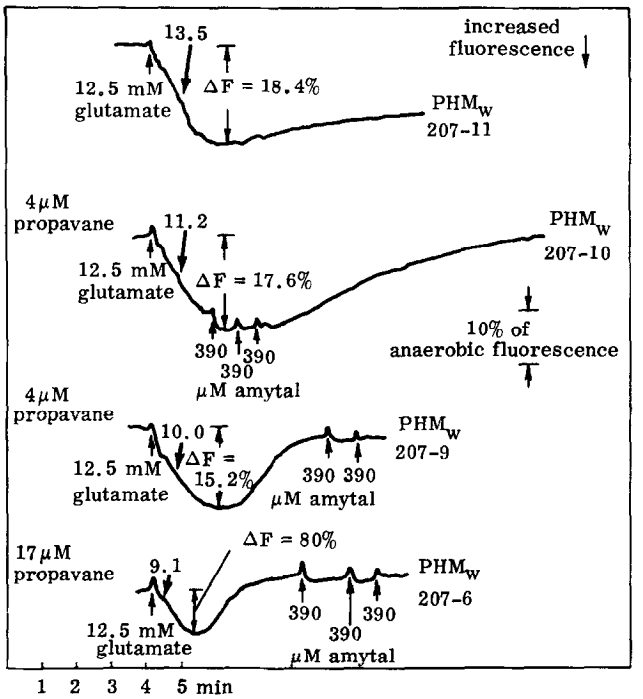


Fig. 2. Pyridine nucleotide reduction by glutamate in a phosphate-deficient medium. Propavane, when present, was added 2 minutes prior to glutamate. Additions as marked. Changes in fluorescence (Δ F) are given as percentages of anaerobic fluorescence. Rates are given as %/min. The initial fluorescence signal (experiment 2o7-11) corresponds to complete oxidation (i.e. same signal is obtained with glutamate, ADP and phosphate present) = 64% of total (anaerobic) fluorescence emission.
610 ug mitochondrial protein pr. ml.

duction by glutamate as shown in this experiment is maximal, i.e. the same as when (succinate + ATP) were present.

The pattern of pyridine nucleotide reduction is markedly influenced by pretreatment with propavane (experiments 2o7-1o-9-6). Experiment 2o7-9 of Fig 2 shows that pretreatment of the mitochondria with 4 uM propavane diminishes both the rate of pyridine nucleotide reduction and the extent of reduction. The most obvious effect of propavane is, however, that the reduced steady state level is transient: the pattern of pyridine nucleotide reduction is followed by an oxidation returning to the original steady state level. This is even more pronounced in the bottom trace (experiment 2o7-6) where a propavane concentration of 17 uM was employed.

In this instance the amount of pyridine nucleotide reduced by glutamate is less than half the original. The inhibition of pyridine nucleotide reduction observed under these conditions apparently occurs at nearly the same concentrations of propavane as those exerting about 50% inhibition in the oxygen consumption experiments. These findings have been taken as evidence that inorganic phosphate is not involved in the inhibited reaction, and that the inhibition occurs primarily at the level of pyridine nucleotides.

Both amytal and propavane inhibit glutamate respiration. Amytal however causes a reduction of pyridine nucleotide (c.f. Chance, Hollunger and Hagihara, 1962). The action of the two compounds in combination is illustrated in Figure 2 (experiment 207-10). This experiment shows that with 4 uM propavane the subsequent addition of 390 uM amytal causes almost the original total reduction of the pyridine nucleotides (17.6 versus 18.4% in the control experiment). The presence of 1.2 mM amytal cannot prevent the reoxidation of pyridine nucleotide described above, although the rate of reoxidation is decreased by the presence of amytal (cf. experiment 207-10 and 9, Fig. 2). When amytal is added in the reoxidized state it has no effect (two lower traces). In experiments (not shown in Fig. 2) preincubated with 2.7 mM amytal 17 uM propavane still causes a complete reoxidation of the pyridine nucleotides.

Discussion.

The data presented indicate that propavane inhibits glutamate respiration, presumably by preventing glutamate dehydrogenase interaction with pyridine nucleotide associated with the phosphorylating mechanism. The steady state concentration of the fluorescent compound, presumably the bound reduced pyridine nucleotide, was observed to be very low in the presence of propavane (Fig. 2).

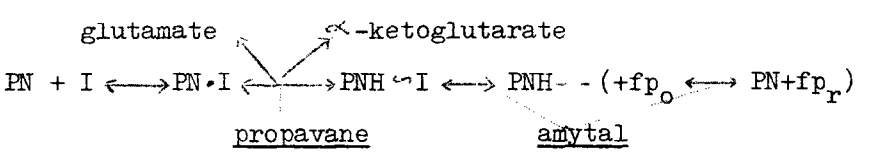
The experiments with the crystalline glutamate dehydrogenase moreover indicated that propavane might inhibit this enzyme although there was a considerable quantitative difference in the sensitivity to propavane of the mitochondrial and the crystalline glutamate dehydrogenase.

Further quantitative differences were observed by comparing the effect of propavane upon glutamate respiration in the absence

and presence of ADP, 2,4-dinitrophenol, arsenate or other compounds known to interfere with the energy transfer mechanism. The sensitivity to propavane was highly increased in the presence of any of these compounds apparently independent of the turnover in the electron transfer mechanism. These results could be explained by a change in sensitivity of glutamate dehydrogenase in the presence of any of the compounds mentioned or perhaps more likely by an interference of propavane with the energy transfer chain in addition to the electron transfer chain. Most likely the point of interaction is the bound pyridine nucleotide compounds. An effect of propavane at this point would account for the observation that dinitrophenol does not uncouple propavane inhibited respiration.

The action of propavane upon an energy transfer compound is substantiated by experiments (not published) which showed pronounced inhibition by propavane of the amount of pyridine nucleotide reduction due to "reversed electron transfer" upon addition of ATP. The experiments were carried out with terminally inhibited mitochondria (c.f. Chance, 1961) in the absence of substrates. The inhibition was half maximal for 8 uM propavane and with 30 uM propavane no pyridine nucleotide reduction occured upon addition of 1.2 mM ATP to the mitochondria. The inhibition of the ATP-effect by the low propavane concentrations in these experiments where participation of any dehydrogenase was excluded strongly indicates that the fundamental effect of propavane is exerted in the phosphorylating system.

In accordance with the mechanism for oxidative phosphorylation proposed by Chance and Williams (1956) propavane most likely impedes the formation of the bound reduced pyridine nucleotide, while amytal very likely inhibits the flavoprotein catalyzed reoxidation of free reduced pyridine nucleotide or the decomposition of the bound reduced pyridine nucleotide compound (Chance, Hollunger and Hagihara, 1962).



This scheme for the action of propavane is able to explain the oxidized pyridine nucleotide level in the presence of propavane and the inability of amytal to change this appreciably. The substrate (dehydrogenase) specificity is accounted for if propavane somehow interferes with the complex between the dehydrogenase, pyridine nucleotide and "I". The overall inhibition will then be determined by the affinity of the dehydrogenase for the bound pyridine nucleotide. The propavane insensitive respiration might then represent the ability of the dehydrogenase to react with free oxidized pyridine nucleotide.

Summary.

A description is given of an inhibitor, propavane, which:

1) is specific for glutamate oxidation in low concentrations,

2) gives half maximal state 3 (i.e. ADP supplemented) respiration for about 12 uM, i.e. for 25 mu moles/mg protein,

3) inhibits state 4 (i.e. ADP limited) respiration only very slightly,

4) is not influenced by the presence of inorganic phosphate,

5) cannot be reversed by uncouplers (2,4-dinitrophenol),

6) maintains a completely oxidized steady state of the pyridine nucleotides in the presence of glutamate even when preincubated with amytal (2.7 mM).

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