EFFECT OF AMMONIA ON DECREASE OF PYRIDINE NUCLEOTIDE LEVELS IN THE ISOLATED RAT LIVER MITOCHONDRIA

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The authors have found that intramitochondrial pyridine nucleotide levels markedly decreased by adding ammonia to the isolated rat liver mitochondria. This does not mean the oxidation of the reduced pyridine nucleotides to their oxidized forms, but means destruction of them in their reduced forms, since no oxidized forms increased and elevation of nicotinamide was observed.

The respiratory inhibition by ammonia which has been reported from our laboratory is thought to be related to the change of the pyridine nucleotide levels. In the preceding paper (Katunuma and Okada, 1963), it has been reported that inhibition of the mitochondrial respiration by ammonia is not due to the glutamate formation by glutamate dehydrogenase, but due to the depression of α-ketoglutarate production through the TCA cycle. We have found that ammonia was most inhibitory for the respiration of citrate or isocitrate. Furthermore, accumulation of isocitrate and inhibition of citrate or pyruvate utilization were observed when citrate or pyruvate in the presence of ammonia was used as a substrate of the TCA cycle(Katunuma et al., 1963).

METHODS

Rat liver mitochondria were prepared by the method of Schneider. Incubation was carried out in a Warburg bath and

oxygen uptake was measured manometrically. The incubation mixture was the same as described before(Katunuma and Okada, 1962), but nicotinamide was usually added unless otherwise stated to inhibit pyridine nucleotidase.

Extraction of pyridine nucleotides from the mitochondria was followed by the method of Bassham et al(1959) except that the neutral extraction was used for the oxidized forms and then the extract was acidified to destroy the reduced forms. Pyridine nucleotides were determined by the alkaline fluorimetric method. Bioassay using Lact. arabinosus was carried out for the estimation of total pyridine compounds, nicotinamide, or pyridine nucleotides on a microscale. Nicotinamide and pyridine nucleotides were separated by ascending paper chromatography. The solvent system used was 80 % n-propanol. The chromatogram was cut into 10 pieces and each of them was analyzed by Lact. arabinosus.

RESULTS AND DISCUSSION

Mitochondria were incubated in the presence and absence of ammonium sulfate and their intramitochondrial pyridine nucleotides were determined. As shown in Fig. 1 the presence of ammonium sulfate resulted in a marked decrease of reduced pyridine nucleotides in 5 minutes while no oxidized forms increased. It will be suggested that disappearance of the reduced forms does not mean the oxidation of pyridine nucleotides by the dehydrogenases. When citrate was added to the mitochondrial system, the decrease of both the reduced and oxidized forms were followed by the respiratory inhibition in the presence of ammonia. It is considered that use of a generating system of reduced pyridine nucleotides by adding citrate also caused the decrease of oxidized forms.

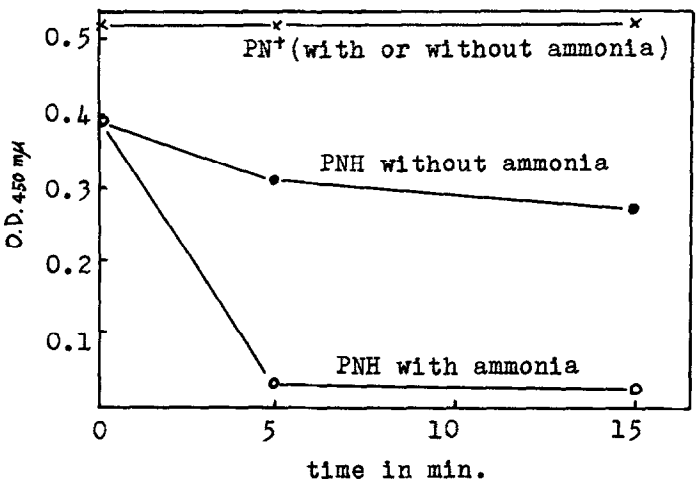


Fig. 1. Behavior of Pyridine Nucleotides during the Incubation Periods of Mitochondria with and without adding Ammonia.

PN^+; Pyridine nucleotides in oxidized form, PNH; Pyridine nucleotides in reduced form.
Mitochondrial suspension in 10 % sucrose was incubated in a test tube at 37°C. Each system contained 50 μmoles of nicotinamide and 10 μmoles of ammonium sulfate if added in the final volume of 2 ml. The reaction was terminated by boiling in alkali or neutral buffer solution.

The disappearance of pyridine nucleotides is probably the result of a reaction of the reduced forms. Amytal which is known to be an inhibitor of oxidation of the reduced pyridine nucleotides, was added to the above mitochondrial system. Reduced forms of pyridine nucleotides were increased at certain concentrations of amytal, but too high levels of amytal also inhibited reduction of pyridine nucleotide by isocitric dehydrogenase. Two to three micromoles amytal per 2.5 ml of reaction mixture was found to be optimal for maintaining the reduced forms of pyridine nucleotide at high level, when 20 to 30 mg protein of mitochondria was used in 2.5 ml of the reaction mixture. At this level of amytal, the reduced pyridine nucleotide disappearing reaction will be able to work most effectively. In Fig. 2, 2.5 μmoles of amytal were used in the mitochondrial system, citrate was added as the substrate. As this amytal level caused about 70 % of

the respiratory inhibition, further ammonium effect on the respiration was not observed, but the reduced pyridine nucleotides were remarkably decreased by adding ammonia. High levels of ammonia(above 2 μmoles/2.5 ml.), however, had no more effect to decrease pyridine nucleotides content, but caused glutamate formation by probably glutamate dehydrogenase under the conditions used, because the Km value of glutamate dehydrogenase for ammonia is rather high.

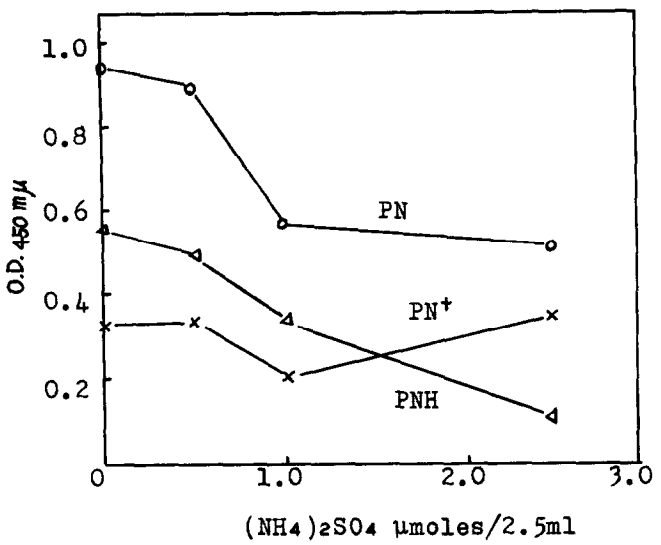


Fig. 2. Decrease of Pyridine Nucleotides corresponding to Ammonia added in the Presence of Amytal.

PN; Pyridine nucleotides(total of oxidized and reduced forms) The system contained 5 μmoles citrate, 2.5 μmoles amytal and 50 μmoles nicotinamide in the final volume of 2.5 ml. Incubation was carried out for 15 minutes in a Warburg bath at 37°C.

The product of this disappearing reaction of pyridine nucleotides was analyzed without adding nicotinamide as shown in Table I. Although a decrease in pyridine nucleotide corresponding to the respiratory inhibition was observed, there was no difference of pyridine ring contents between the systems with and without adding ammonia. Results obtained by paper chromato-

graphy showed that the reaction product which increases by adding ammonia, is probably nicotinamide or its analogue.

System	Citrate -	Citrate $AmSO_4$(1)	Citrate $AmSO_4$(10)
O_2 uptake, µl	95.2	18.7	13.6
PN mµmoles*	50	10	8
PNH mµmoles*	41	1	0
total PN mµmoles*	91	11	8
Pyridine Ring mµmoles**	61	68	78
PN(Rf 0-0.3) **	150	53	53
NA(Rf 0.8-1.0)**	320	462	445

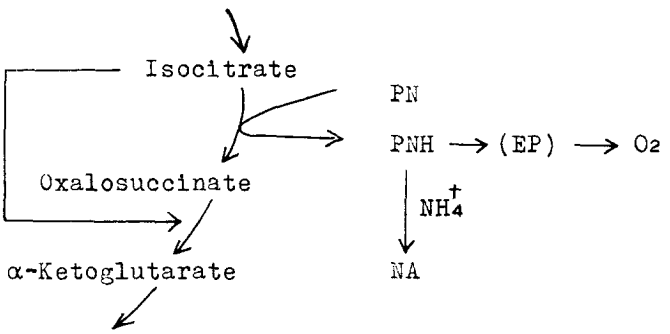
Table I. The Reduced Pyridine Nucleotides Disappearing Reaction and Its Product.

NA; nicotinamide. * determined by fluorimetric method, ** determined by bioassay(measured O.D.$_{660}$ mµ).
The system contained 5 µmoles of citrate in the final volume of 2.5 ml. and incubation was carried out for 15 minutes at 37°C. The neutral extract was used for bioassay.

The first step of the ammonium inhibition of the TCA cycle is considered to be the splitting of reduced pyridine nucleotides. This results in a decrease of pyridine nucleotides contents and the respiration is necessarily inhibited. Pyridine nucleotides linked dehydrogenases in the TCA cycle will be all inhibited and their substrates will be accumulated, because of the shortage of their coenzymes. But it is considered that the isocitrate dehydrogenase is the most sensitive to ammonium inhibition from the following reasons. According to the report by Ochoa et al. (1948), isocitrate inhibits the decarboxylation of oxalosuccinate and we have observed an accumulation of isocitrate which is considered to be enough to inhibit the decarboxylation, in the

presence of ammonia. The other is that the reduced level of NADP which is the coenzyme of isocitrate dehydrogenase, is much higher than that of NAD in mitochondria. The inhibitory mechanism will be understood as shown in Scheme I.

On the nucleotide disappearing reaction some details are under inverstigation using solubilized enzyme systems and will be discussed in another paper.



Scheme I. Inhibitory Mechanism of TCA Cycle by Ammonia.

ACKNOWLEDGMENT

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