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REVERSAL OF THE INHIBITORY ACTION OF AMMONIA ON THE RESPIRATION OF RAT-LIVER MITOCHONDRIA

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SUMMARY

1. Glutamate reverses the inhibitory effect of ammonia on respiration of rat-liver mitochondria by preventing further amination of α-ketoglutarate.

2. Succinate also abolishes this inhibition but without interfering with the reductive amination of α-ketoglutarate.

3. A method of calculating the ratio: reduced/oxidized mitochondrial pyridine nucleotides, based on the equilibrium constant of the glutamate dehydrogenase reaction, is discussed.

INTRODUCTION

In a previous paper[1], the inhibitory action of ammonia upon the respiration of rat-liver mitochondria in the presence of α-ketoglutarate was discussed and as explanation a competition for DPNH between glutamic dehydrogenase and electron transport chain was postulated. It also was stated that ammonia markedly depresses oxygen uptake when citrate is used as substrate. In the present paper a further investigation of this effect is reported and furthermore, on the basis of our previous results, attempts were made to reverse the inhibitory effect of ammonia on respiration of rat-liver mitochondria.

EXPERIMENTAL

The preparation of mitochondria and manometric procedure were as previously described[1].

α-Amino acids were determined according to the method of YEMM AND COCKING[2], ammonia by the CONWAY diffusion technique followed by nesslerization[3], citric acid by a modification of the method of PERLMAN *et al.* as described by BEUTLER AND YEH[4]. Protein was determined by the biuret method[5]. Spectrophotometric assays of pyridine nucleotide reactions were carried out at 340 mμ in a Beckman DK-2 spectrophotometer at 30°.

Abbreviations: PN$^+$ = DPN$^+$ + TPN$^+$; PNH = DPNH + TPNH.

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RESULTS

Ammonia and citrate oxidation

Ammonia markedly inhibits respiration when citrate is used as substrate (Table I). The oxygen uptake of controls, without ammonia, is much higher than expected from the amount of citrate used up: since malonate was added to block oxidation of succinate, it follows that under such conditions two oxidative steps are taking place: isocitrate → ketoglutarate → succinate. In the presence of ammonia reductive amination occurs and is proportional to the depression in oxygen uptake. This depression, in the presence of malonate, is about twice as high as glutamic acid formation (or ammonia incorporation), which is in close agreement with what would be expected theoretically. When ammonia is present, each molecule of α-ketoglutarate which is diverted from succinate to glutamate, sacrifices one reduced pyridine nucleotide, and at the same time a second reduced pyridine nucleotide is used for reductive amination. In the absence of malonate, oxidation proceeds beyond the succinate step and accordingly the depression in oxygen uptake caused by ammonia is found to be much higher than the amino acid formation.

TABLE I

EFFECT OF AMMONIA ON CITRATE OXIDATION

Incubation mixture: phosphate buffer (pH 7.4), 100 μmoles; NaF, 30 μmoles; $MgCl_2$, 20 μmoles; ATP, 5 μmoles; cytochrome *c*, 0.5 mg; citrate, 20 μmoles; malonate, 20 μmoles; NH_4Cl, 30 μmoles; glucose, 90 μmoles and hexokinase in a total volume of 2.8 ml. Incubation 80 min at 30°. Mitochondrial suspension corresponding to 16.3 mg protein per cup.

	Ammonia	*Oxygen (μatoms)*		*Citrate (μmoles)*		*NH_4^+ (μmoles)*		*α-NH_2 (μmoles)*	
		Uptake	*Δ*		*Δ*		*Δ*		*Δ*
	Without	19.2		7.6	11.9			3.6	1.3
	With	5.0	14.2	9.2	10.3	24.1	6.1	10.4	6.6
"0" Time	Without			19.5				2.3	
	With			19.5		30.2		2.5	

Reversal by glutamate of inhibition of respiration caused by ammonia

The depression in oxygen uptake caused by ammonia is partially reversed by the addition of 50 μmoles and completely reversed by the addition of 150 μmoles of glutamate (Table II) when α-ketoglutarate is used as substrate; under these conditions, glutamate is not oxidized and reductive amination does not take place (there is neither ammonia production nor incorporation). The utilization of glutamate via transaminase is prevented by the addition of malonate.

When citrate is used as substrate (10^{-2} M) even less glutamate is required to reverse the depression in oxygen uptake produced by ammonia. This would be expected from thermodynamic considerations since under these conditions α-ketoglutarate is not present initially.

Succinate and inhibition of respiration caused by ammonia

When α-ketoglutarate or citrate is used as substrate, succinate suppresses the inhibitory effect of ammonia on respiration without interfering with the reductive

amination of α-ketoglutarate (Table III). Of all the different substrates of the Krebs cycle tested with α-ketoglutarate and ammonia (pyruvate, citrate, succinate, malate) only succinate was effective.

TABLE II

REVERSAL BY GLUTAMATE OF THE INHIBITION OF RESPIRATION CAUSED BY AMMONIA

Incubation mixture as indicated in the legend to Table I except: α-ketoglutarate 30 μmoles, malonate 30 μmoles, glutamate as indicated. Incubation 30–40 min at 30°.

Expt. No.		Ammonia (μmoles)	Glutamate (μmoles)	Oxygen (μatoms)		NH_4^+ (μmoles)	
				Uptake	Δ		Δ
1		—		20.3			
		30		16.7	3.6	25.3	4.8
		30	50	18.6	1.7	27.2	2.9
	"0" Time	30	50			30.1	
2		—		19.6			
		30		13.5	6.1	25.5	6.9
		30	150	19.3		32.8	
	"0" Time	30	150			32.4	

TABLE III

SUPPRESSION BY SUCCINATE OF INHIBITORY EFFECT OF AMMONIA ON MITOCHONDRIAL RESPIRATION

Incubation mixture as indicated in the legend of Table I. Except: α-ketoglutarate, 40 μmoles or citrate, 20 μmoles; succinate (no malonate), 40 μmoles. Incubation 20–30 min at 30°.

Substrate		Ammonia	Oxygen uptake (μatoms)	α-NH_2 (μmoles)		NH_4^+ (μmoles)	
					Δ		Δ
α-Ketoglutarate		Without	22.5	1.7	0.8		
		With	23.8	10.0	4.3	23.6	4.7
	"0" Time	Without		0.9			
		With		4.9		28.3	
Citrate		Without	18.4	2.1	0.7		
		With	17.5	4.7	2.6	30.1	2.6
	"0" Time	Without		1.4			
		With		1.4		32.7	

Spectrophotometric assays involving TPN with citrate as substrate

To gain another insight into the reactions taking place, spectrophotometric assays were carried out using essentially the same incubation mixture and the same mitochondrial concentration as in the manometric experiments.

After the addition of citrate an increase in absorbancy at 340 mμ occurs due to the reduction of externally added TPN (Fig. 1). When the steady-state level is reached, ammonia is added and a decrease in absorbancy is recorded due to the oxidation of TPNH by glutamic dehydrogenase. Substrate for reductive amination, the α-ketoglutarate, comes from the oxidation of isocitrate. The new steady-state level of TPNH corresponds to about 30% of that obtained in the absence of ammonia.

The effect of succinate on the steady-state level of reduced TPN is recorded in

Fig. 2. In the presence of succinate the steady-state level of TPNH is higher. After the addition of ammonia no significant change in absorbancy occurs, while in the absence of succinate the usual decrease in absorbancy (caused by ammonia) can be observed.

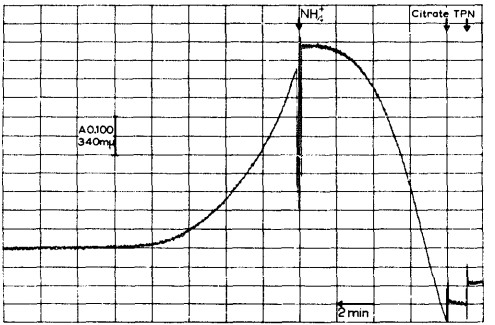

Fig. 1. Incubation mixture: citrate, 20 μmoles; TPN, 0.25 mg; NH_4Cl, 30 μmoles; $MgCl_2$, 10 μmoles; mitochondrial suspension corresponding to 12 mg protein; 0.1 M phosphate buffer (pH 7.4) to 3.0 ml. Blank: mitochondrial suspension, $MgCl_2$ and phosphate buffer.

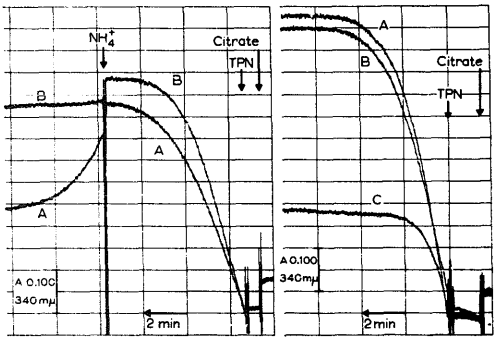

Fig. 2. (A) Incubation mixture as indicated in the legend of Fig. 1. (B) As above plus 20 μmoles of succinate. Mitochondrial suspension corresponding to 10.5 mg protein.
Fig. 3. (A) Incubation mixture as indicated in the legend of Fig. 1 except no NH_4Cl added. (B) As in (A) plus 30 μmoles of NH_4Cl and 20 μmoles of succinate. (C) As in (A) plus 30 μmoles of NH_4Cl. Mitochondrial suspension corresponding to 11.7 mg protein.

When ammonia is present from the beginning (Fig. 3) the steady-state level of TPNH is much lower than that of the control and corresponds to the one recorded in Fig. 1 after the addition of ammonia. Succinate abolishes this effect of ammonia and the curve follows closely that of the control.

DISCUSSION

From the data presented in this and in our previous paper[1] it is concluded that there are two possible mechanisms to reverse the inhibitory effect of ammonia on mitochondrial respiration: (a) the addition of glutamate, which as a product of reductive amination prevents the further amination of α-ketoglutarate, (b) increasing the steady-state level of reduced pyridine nucleotides, either by omitting phosphate acceptor or by addition of succinate.

The equilibrium point of the glutamic dehydrogenase reaction is reached with $5 \cdot 10^{-2}$ M glutamate, beginning with α-ketoglutarate and ammonia in initial concentrations of 10^{-2} M. The assumption that the equilibrium was reached is based upon the findings that there is no depression in oxygen uptake and that ammonia is neither incorporated nor produced. The equilibrium constant for this reaction is[12]:

$$K = \frac{(\alpha\text{-ketoglutarate})\ (NH_4^+)\ (PNH)\ (H^+)}{(\text{glutamate})\ (PN^+)\ (H_2O)} = 1.8 \cdot 10^{-15}$$

The removal of the H^+ and H_2O terms gives a value of about $2 \cdot 10^{-6}$ ($K_{app.}$) at pH 7.4. Notwithstanding the low value of this constant it is possible to reach equilibrium with a relatively low concentration of glutamate. To account for this finding a very low PNH/PN$^+$ ratio must be postulated for these experimental conditions (active state) and the stated concentrations of reactants:

$$\frac{PNH}{PN^+} \simeq \frac{K_{app}(\text{glutamate})}{(\alpha\text{-ketoglutarate})\ (NH_4^+)} \simeq \frac{2 \cdot 10^{-6} \times 5 \cdot 10^{-2}}{10^{-2} \times 10^{-2}} = 10^{-3}$$

The PNH/PN$^+$ represents the ratio of total reduced to total oxidized endogenous pyridine nucleotides. The decrease in α-ketoglutarate concentration during the incubation time due to its oxidation, affects but slightly this ratio and can be neglected for the purpose of this discussion. Various investigators[6-10] have shown that the concentrations of endogeneous TPN and DPN in rat-liver mitochondria are of the same order of magnitude. Therefore under our experimental conditions both ratios TPNH/TPN$^+$ and DPNH/DPN$^+$ must be very low. Respiration of mitochondria in the active state in the presence of α-ketoglutarate, ammonia and glutamate, with this low steady-state level of reduced DPN, proceeds at the same rate as in the controls with α-ketoglutarate alone. In this connection, KLINGENBERG AND SLENCZKA[10] reported a very low DPNH/DPN$^+$ ratio for mitochondria in active state with α-ketoglutarate as substrate.

Succinate completely abolishes the inhibitory effect of ammonia on actively respiring mitochondria. Unlike the action of glutamate, succinate allows reductive amination of α-ketoglutarate to proceed at a high rate with no depression in oxygen uptake.

CHANCE AND HOLLUNGER[15], KULKA *et al.*[13], KREBS *et al.*[14], BIRT AND BARTLEY[11], AVI-DOR *et al.*[16] showed that succinate increases the steady state level of reduced coenzymes. From the spectrophotometric recordings (Figs. 1–3) it can be seen that in the presence of externally added TPN, succinate is able to increase the steady-state level of TPNH even in the presence of ammonia. In these spectrophotometric assays no phosphate trapping system was used but from the manometric experiments it is

evident that succinate also abolishes the effect of ammonia in presence of phosphate acceptor.

When this manuscript was ready for publication the paper by JONES AND GUTFREUND[17] appeared in which the authors showed the same effect of succinate on the steady state level of reduced pyridine nucleotides in presence of ammonia using a fluorometric technique.

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