A PROCESS REQUIRING MITOCHONDRIAL NADPH: UREA FORMATION FROM AMMONIA

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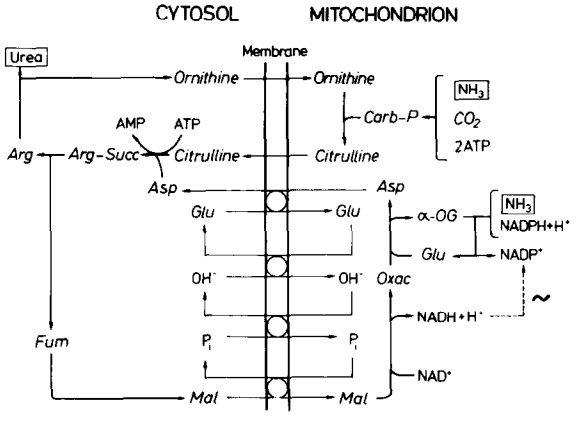
1. Introduction

It has been generally thought that the main NADPH requiring process in liver is that of fatty acid synthesis [cf.1]. In the present communication we want to point out that synthesis of urea from CO_2 and NH_4^+ is a process which, in the normal liver, utilizes NADPH at a comparable rate. Thus, reductive amination of 2-oxoglutarate to glutamate may be considered to be one of the major NADPH requiring reactions of the liver cell, and it is of particular interest that this step is localized in the mitochondrial matrix whereas the other major NADPH requiring reactions are localized extramitochondrially.

The necessity of a redox transition of nicotinamide nucleotides during NH_4^+ elimination into urea may, at first sight, not be straightforward. As illustrated in scheme 1, one half of the ammonia must ultimately appear as aspartate in the cytosol, and it is this part which is implicated with glutamate dehydrogenase. In previous studies with perfused liver by Chamalaun and Tager [3] and by Sies et al [4] a large oxidation in the NADPH/$NADP^+$ system during urea formation from NH_4Cl was demonstrated. Furthermore, the nicotinamide nucleotide pool responding to ammonia was shown to be qualitatively different from that reacting with NAD-linked substrates in situ. Thus, the fluorescence enhancement of nicotinamide nucleotides being oxidized during ammonia metabolism was less than half of that observed for those being reduced during ethanol metabolism [4], and secondly a peak shift towards the red with respect to 340 nm was demonstrated for the NH_4Cl-linked transition, compare to a blue shift for the ethanol-linked transition [5].



Scheme 1. Metabolic pathway from ammonia to urea. Modified from ref. [2].

We are now presenting further support for the possibility of a sustained utilization of NADPH during ureogenesis from NH_4Cl by, a) competition with another NADPH utilizing process and, b) inhibition of energy-linked transhydrogenase.

2. Materials and methods

Hemoglobin-free perfusion of livers from male Wistar rats of 150–180 g body weight was performed as previously [4,6], and rates of ammonia uptake and urea release were determined in effluent perfusate [6]. Hepatocytes were isolated from rat livers perfused with a medium containing collagenase, a method deriving from the original suggestion of Berry and Friend [7] as described in detail elsewhere [8]. Urea, ammonia, $NADP^+$ and NAD^+ were assayed by standard methods laid out in [9].

t-Butyl hydroperoxide was a gift from Peroxid-Chemie München, Höllriegelskreuth. Rhein was generously provided from Sandoz, Basel. Chemicals were from Merck, Darmstadt, and biochemicals, including clostridial collagenase, were from Boehringer, Mannheim.

3. Results and discussion

3.1. *Rates of NADPH requiring biosynthetic processes in rat liver*

Maximal rates of the major NADPH requiring processes occurring under conditions of the intact cell have been collected from the literature (table 1). From these data, rates of NADPH utilization have been calculated. These must be considered tentative because assumptions of the underlying stoichiometry have to be made. However, it is apparent that ureogenesis from ammonia may rank among the major NADPH requiring processes.

Although studies with isolated mitochondria have early led to the proposal that glutamate dehydrogenase operates preferentially with NADPH [14–16], those investigators working with more intact systems generally have included only NADH in their considerations [cf.17] or have made no functional distinction between NADH and NADPH with respect to glutamate dehydrogenase [18]. A main reason for the uncertainty in this problem appears to reside in the difficulty to assess the role of the energy-linked transhydrogenase and glutamate dehydrogenase under physiological conditions. Recently, considerable advances in this respect have been made by Hoek and Ernster [1] and by Hoek et al. [19] with isolated rat liver mitochondria.

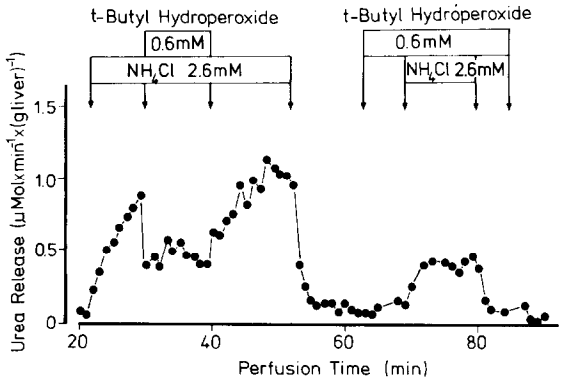


Fig.1. Rate of urea release from perfused rat liver into effluent perfusate. As indicated on the top, NH_4Cl and *t*-butyl hydroperoxide were infused during the time intervals given by the arrows.

In the following sections, we will present results obtained with the intact cells.

3.2. *Competition of t-butyl hydroperoxide with urea formation*

In hemoglobin-free perfused rat liver, maximal rates of urea production from added NH_4Cl are 1.1 μmol × min^{-1} × g^{-1} of liver (table 1, [6]). As shown in fig.1, the infusion of *t*-butyl hydroperoxide at a concentration of 0.6 mM during the interval from 30 to 40 min results in a marked decrease of urea release which then is only 0.4–0.5 μmol × min^{-1} × g^{-1} of liver. During this interval, ammonia uptake by the liver is decreased accordingly (not shown). Urea release re-acquires the higher initial rates in the time from 40 to 52 min. In the later part of this experiment (62–84 min), it is shown that unlike in controls the addition of NH_4Cl

Table 1
Estimated rates of NADPH utilization in 'normal' (not-pretreated) rat livers

Process	Rate μmol × min^{-1} × g^{-1} of liver	Ref.	Calculated rate of NADPH utilization μmol × min^{-1} × g^{-1} of liver
Lipogenesis (from glucose, C_2-units into fatty acids)	0.4–0.5	10	0.7–0.9
Ureogenesis (from ammonia)	1.1	6	up to 1.1
Monooxygenation (hexobarbital)	0.2	11	0.2
Hydroperoxide reduction	up to 3	12, 13	up to 3

does not lead to high rates of urea release when *t*-butyl hydroperoxide is already present in the perfusate.

This experiment demonstrates a reversible inhibition of urea formation from ammonia by *t*-butyl hydroperoxide. The residual rate of urea formation is 35–40% of that in absence of the hydroperoxide.

t-Butyl hydroperoxide-linked redox transitions were shown to consist of a substantial oxidation in the NADPH system, due to coupling of glutathione peroxidase and glutathione disulfide reductase, whereas no indication for a participation of the NAD^+-system was obtained [12,20]. Therefore, our interpretation of the experiment shown in fig.1 is that the inhibition of urea release is in agreement with a limitation of NADPH supply for ammonia fixation at glutamate dehydrogenase.

3.3. *Effect of rhein on urea formation*

It has been previously shown by Moyle and Mitchell [21] and by Hoek et al. [19] in isolated mitochondria that rhein at a concentration of 0.1 mM inhibits the energy-linked NAD[P] transhydrogenase. It was, therefore, of interest to compare the inhibition of energy-linked NADPH formation by rhein with the stimulation of NADPH utilization by GSSG reductase regarding their effects on urea formation from ammonia. For this purpose, experiments were carried out with isolated hepatocytes. As shown in fig.2, a linear rate of accumulation of urea is observed, amounting to 1.4 μmol $\times$ min^{-1} $\times$ g^{-1} of liver cells.

The effect of rhein on urea formation is illustrated

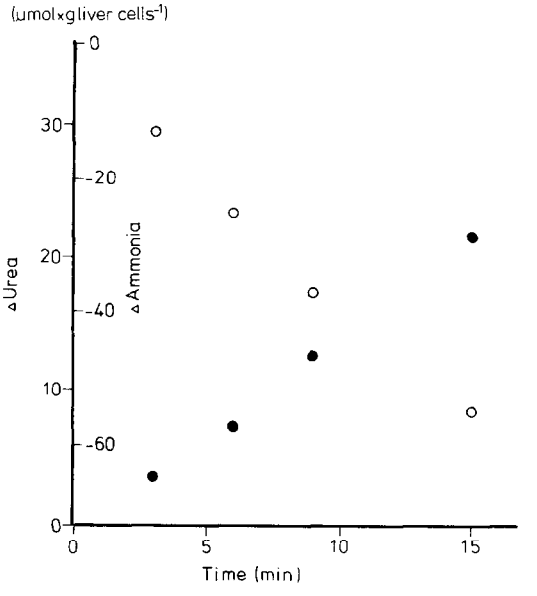


Fig.2. Urea accumulation (•) and ammonia consumption (○) by isolated rat hepatocytes. Hepatocytes (7.2 mg protein per ml) were incubated similar to the conditions given in table 2. NH_4Cl was 3.8 mM at zero time. Data are given per gram of liver cells wet weight (140 mg protein per gram cells wet weight).

in table 2. Subsequent to addition of 5.4 mM NH_4Cl, only approx. 40% of urea accumulated when rhein was present compared to the control. However, under conditions where urea formation did not require the step at glutamate dehydrogenase, rhein did not inhibit.

Table 2
Effect of rhein on urea formation from NH_4Cl and from glutamine in isolated rat hepatocytes

Addition	Urea formed at 15 min (nmol × mg protein^{-1})		Rate with rhein compared to control %
	Control	Rhein [0.1 mM]	
NH_4Cl [5.4 mM]	127	49	39
L-Glutamine [6 mM]	24	23	96

Isolated hepatocytes (6.8 mg protein per ml) were incubated in a medium consisting of 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM Na_2SO_4, 25 mM $NaHCO_3$, 2 mM ornithine, 0.3 mM lactate, 0.3 mM pyruvate, equilibrated with O_2 containing 5 vol % CO_2, pH 7.35, at 37°C. Samples taken before and after addition of NH_4Cl or glutamine were deproteinized with perchloric acid and neutralized with K_2CO_3 buffered with triethanolamine. Rhein was added from a 10 mM stock solution in dimethylsulfoxide.

Table 3
$NADP^+$ and NAD^+ levels in isolated heptocytes 5 min after addition of NH_4Cl [5.4 mM]

	$NADP^+$	NAD^+
	nmol × mg protein^{-1}	
Without rhein	0.38	4.3
With rhein (0.1 mM)	0.95	4.1

Incubation conditions as in table 2. Levels before NH_4Cl addition were 0.08 and 4.3 nmol × mg protein^{-1} for $NADP^+$ and NAD^+, respectively.

This is shown in table 2 for urea formation from endogenous amino acids and added glutamine. Glutamate and ammonia are formed from this precursor, obviating the necessity to convert free ammonia into the ultimate form of aspartate (scheme 1) but, instead, requiring only a transamination step.

Thus, this experiment, too, is in agreement with the dependence of urea formation on NADPH supply for glutamate dehydrogenase when ammonia is the precursor.

Further information is provided in table 3 where $NADP^+$ and NAD^+ levels at 5 min after NH_4Cl addition are shown. Whereas $NADP^+$ rises to values approaching total NADPH + $NADP^+$ in presence of rhein, there is no change in NAD^+.

4. Conclusions

Although we are aware that the evidence provided here is of an indirect nature, taken together with the data referred to above [3–5,14–16,19], it appears reasonable to ascribe to glutamate dehydrogenase the major function in its 'biosynthetic' capacity, i.e. formation of glutamate utilizing NADPH, rather than in the reverse direction, the provision of 2-oxoglutarate and ammonia, as has been thought by some investigators. In fact, Mendes-Mourao et al. [22], in a recent study on the effect of L-leucine on urea synthesis, suggested the possibility that the function of NH_4^+ release from aminoacids might be taken over by adenosine deaminase, based on part of the purine nucleotide cycle as described by Lowenstein [23].

The functional association of glutamate dehydrogenase with a mitochondrial pool of NADPH will shed some further light on the problem of the redox potential of mitochondrial free NADPH. So far, the postulated NADPH redox potentials in the mitochondrial matrix space range from −450 mV [21] and −390 mV [24] up to about −300 mV [18], corresponding to a discrepancy by a factor of 10^5 in the ratio of free NADPH/$NADP^+$. For the energy-linked transhydrogenase to operate effectively, the more negative potentials are required. However, this problem awaits further direct experimental information.

It may be mentioned, finally, that free ammonia concentration in portal blood (0.26 mM [25]) is close to half-maximal stimulation of urea synthesis [6], and caval blood ammonia is 0.03 mM [25]. Therefore, we propose that the process utilizing mitochondrial NADPH discussed in this report is continuously operating in the intact animal.

Acknowledgements

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