NH_4^+ metabolism and the intracellular pH in isolated perfused rat liver

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The effects of NH_4^+ on the intracellular pH (pH_i) and on the ATP content in isolated perfused rat liver were studied by ^{31}P n.m.r. spectroscopy. In the initial phase of perfusion an average pH_i of 7.29 ± 0.04 was estimated. The presence of low (0.5 mmol/l) and high (10 mmol/l) doses of NH_4Cl induced significant intracellular acidification by -0.06 ± 0.03 and -0.11 ± 0.03 pH unit respectively. This effect was in contrast with the transient intracellular alkalinization observed in preliminary studies on isolated hepatocytes, which was caused by a passive entry of NH_3 by non-ionic diffusion and subsequent conversion into NH_4^+ . During application of 0.5 mmol/l NH_4Cl the liver released 0.54 ± 0.06 μmol of urea/min per g into the perfusate. When the intracellular availability of HCO_3^- was decreased by acetazolamide (0.5 mmol/l) or by removal of HCO_3^- from the perfusion medium, the decrease in pH_i by NH_4Cl application was significantly lower than under control

conditions. Furthermore, synthesis of urea was significantly inhibited by the decrease in intracellular HCO_3^- . Under these conditions, 10 mmol/l NH_4Cl caused the transient alkalinization that was expected because of the passive uptake of uncharged NH_3 . Therefore, it is concluded that the intracellular acidification induced by NH_4Cl is caused by the continuous utilization of intracellular HCO_3^- via the synthesis of urea. This metabolic effect on pH_i dominates the effects of passive NH_3 entry. The rate of urea formation depends on continuous efflux of H^+ , which is strictly limiting the degree of intracellular acidification within a small range. If the extrusion of H^+ by the Na^+/H^+ exchanger was inhibited by amiloride (0.5 mmol/l) during the NH_4Cl application, the decrease in pH_i was amplified and the formation of urea was significantly inhibited. The application of NH_4Cl at 0.5 or 10 mmol/l decreased the ATP content by 11% or 22% respectively.

INTRODUCTION

NH_4Cl -pulse experiments have recently been performed on isolated cultured hepatocytes in order to identify the mechanisms involved in the regulation of intracellular pH (pH_i) [1–3]. In these studies an application of NH_4Cl (10–25 mmol/l) induced an initial, transient, alkalinization by passive entry of non-ionic NH_3 and its subsequent conversion into NH_4^+ . The removal of NH_4Cl , however, caused a transient acidosis by the passive efflux of NH_3 and the formation of H^+ from NH_4^+ . This acidification was corrected by the action of Na^+/H^+ exchange and $\text{Na}^+/\text{HCO}_3^-$ symport.

In the intact liver the synthesis of urea is coupled to an equimolar utilization of NH_4^+ and HCO_3^- . Therefore, synthesis of urea has also been postulated to be involved in the regulation of pH_i in liver cells [4,5]. Häussinger and Gerok [6] found that in isolated perfused rat liver the release of urea stimulated by NH_4Cl (1 mmol/l) was decreased by 90% if the intramitochondrial carbonic anhydrase was inhibited by acetazolamide. Furthermore, the pH of the perfusate was decreased by up to 0.2 pH unit if urea synthesis was stimulated by NH_4Cl (1 mmol/l), suggesting increased efflux of protons from the cells [7]. However, the effect of NH_4Cl on pH_i was not determined in these studies.

In this ^{31}P -n.m.r. study on the intact isolated perfused rat liver, low (0.5 mmol/l) and high (10 mmol/l) doses of NH_4Cl were applied in order to investigate the effects of urea formation and the passive influx of non-ionic NH_3 on pH_i . Furthermore, it was demonstrated that urea formation depends on a continuous extrusion of H^+ via the Na^+/H^+ exchanger. Finally, the effects of NH_4Cl on the content of ATP were discussed.

MATERIALS AND METHODS

Liver perfusion

Livers (4.5–5.5 g) were taken from male Wistar rats (90–130 g body wt.), which were fed *ad libitum* on a standard diet. The organs were cannulated at the portal vein, excised, and placed into a 20 mm-diameter n.m.r. tube as described previously [8]. The livers were perfused at a constant rate of 4–4.5 ml/min per g wet wt. in an open non-recirculating mode. The perfusate was a modified Krebs–Henseleit bicarbonate buffer (pH 7.4) containing dextran 70 (1%), glucose (5 mmol/l), L-lactate (1 mmol/l), pyruvate (0.2 mmol/l), L-glutamine (0.5 mmol/l) and L-alanine (0.5 mmol/l). The perfusate was gassed with O_2/CO_2 (19:1). The temperature was 37 °C. In bicarbonate-free perfusion medium HCO_3^- was replaced by Hepes (25 mmol/l, pH 7.4). The bicarbonate-free medium was gassed with 100% O_2 . NH_4Cl was added from neutralized stock solutions by precision micro-pumps.

Enzymic determinations

NH_4^+ and urea were estimated by an enzymic optical test [9] in perfusate samples taken from the effluent in the initial phase of perfusion and 65–66 min after the onset of NH_4Cl (0.5 mmol/l) application.

Perfusate samples taken from the effluent were also measured for the activities of lactate dehydrogenase and glutamate-oxaloacetate transaminase, by standard enzymic methods [9].

^{31}P -n.m.r. spectroscopy

All ^{31}P -n.m.r. spectra were recorded at 161.2 MHz on a Bruker

Abbreviations used: pH_i , intracellular pH; DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid.

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AM 400 WB spectrometer. Typical acquisition parameters were: sweep width 10000 Hz, 4096 data points, pulse width 14 μ s (60°), acquisition time 0.205 s, pulse repetition time 0.455 s. For each spectrum 256 or 1024 scans were accumulated.

Determination of pH_i

The pH_i was determined from the pH-sensitive chemical shift of intracellular P_i relative to the pH-insensitive shift of intracellular glycerophosphocholine (+0.49 p.p.m.; Figure 1). The calibration curve of pH versus the chemical shift of P_i (δ) was established by titrating a 50% (w/v) homogenate of liver tissue. The data points could be fitted optimally by using the following equation:

$$\text{pH}_i = 6.802 - \log [3.298 - \delta / (\delta - 0.720)]$$

This equation was used to calculate pH_i from the chemical shifts (δ) determined in the experiments.

Determination of ATP content

The cellular content of ATP was calculated from the integrals of the β -phosphate signals of ATP (Figure 1) obtained in spectra with 1024 scans. Values are expressed as percentages of the initial value.

Statistical evaluation

The significance of difference between two values (means $\pm$ S.D.) was tested with the appropriate Student's *t* test ($P < 0.05$).

RESULTS

Viability of the perfused liver

Over a period of 115 min perfusion inside the spectrometer, only slight deterioration of the liver was observed, indicated by a slow decrease in ATP content to $91 \pm 6\%$ ($n = 4$) of its initial value. In addition, in perfusate samples taken from the effluent, no activities of lactate dehydrogenase and glutamate-oxaloacetate

transaminase could be detected, indicating that cell damage was negligible during the whole period of perfusion.

Steady-state pH_i

A typical ^{31}P -n.m.r. spectrum is shown in Figure 1. In the initial phase of perfusion an average pH_i of 7.29 ± 0.04 ($n = 31$) was determined. In three experiments, in which the liver was perfused without any further treatment, pH_i remained constant for a period of 2 h.

Effects of NH_4Cl perfusion on pH_i

The infusion of 0.5 mmol/l NH_4Cl induced a decrease in pH_i , which after 20 min reached a minimum value of -0.06 ± 0.03 pH unit. pH_i slowly recovered during the next 46 min of perfusion (Figure 2). The ATP content fell slowly; after 66 min it decreased by 17% (Table 1). Furthermore, 0.5 mmol/l NH_4Cl stimulated the release of urea to a rate of 0.54 ± 0.06 $\mu\text{mol}/\text{min}$ per g wet wt. (Table 2). This was about 13-fold higher than the basal rate (0.04 ± 0.02 $\mu\text{mol}/\text{min}$ per g). When NH_4Cl was removed, pH_i increased almost immediately to its initial value (Figure 2). No significant recovery of ATP content was observed during this time (Table 1).

Infusion of 10 mmol/l NH_4Cl caused pH_i to decrease by -0.11 ± 0.03 pH unit ($n = 3$) within 20 min; it remained constant during the next 46 min of perfusion. Removal of NH_4Cl resulted in a transient further decrease in pH_i (-0.16 ± 0.02 pH unit). Thereafter, pH_i increased rapidly to its initial value and subsequently showed a slight alkalization (Figure 2). During the 66 min of perfusion in the presence of 10 mmol/l NH_4Cl , ATP content decreased continuously. ATP content partly recovered after the removal of NH_4Cl . The decrease in ATP content

Figure 1 A typical ^{31}P -n.m.r. spectrum from an isolated perfused rat liver

The isolated rat liver was perfused with a modified Krebs–Henseleit bicarbonate buffer. The n.m.r. spectrum was measured by using pulse parameters as follows: 161.5 MHz, 60° pulse, 1024 scans in 8.2 min. Abbreviations: SP, sugar phosphates; P-Cho, phosphocholine; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine (+0.49 p.p.m.); P_i , intracellular inorganic phosphate; NAD^+ , dinucleotides, predominantly NAD^+ ; UDP-Glc, nucleoside sugars, predominantly UDP-glucose.

Figure 2 Effects of NH_4Cl application and removal on the pH_i

Livers were perfused with a modified Krebs–Henseleit bicarbonate buffer. NH_4Cl was added to the perfusate from a stock solution by a precision micropump. The ΔpH_i values are calculated for this and subsequent Figures as follows: $\Delta\text{pH}_i(t) = \text{pH}_{i(t)} - \text{pH}_{i(t=0)}$. Data are given as means $\pm$ S.D. from 3 experiments.

Table 1 Effects of NH₄Cl on the content of ATP

Livers were perfused with a modified Krebs–Henseleit bicarbonate buffer. In bicarbonate-free medium HCO₃⁻ was replaced by Hepes. The ATP content was measured 66 min after the onset of NH₄Cl application and a further 49 min after its subsequent removal. Data are given as percentages of the initial value, as means $\pm$ S.D. from 3–4 experiments: *significantly different from the control ($P < 0.05$, Student's *t* test).

		ATP content (%)	
		Application 66 min	Removal 115 min
No treatment	Control	94 $\pm$ 3	91 $\pm$ 6
	0.5 mmol/l NH ₄ Cl	83 $\pm$ 13	86 $\pm$ 15
	10 mmol/l NH ₄ Cl	72 $\pm$ 12*	84 $\pm$ 13
Acetazolamide (0.5 mmol/l)	Control	93 $\pm$ 5	87 $\pm$ 9
	0.5 mmol/l NH ₄ Cl	89 $\pm$ 9	89 $\pm$ 15
	10 mmol/l NH ₄ Cl	73 $\pm$ 4*	75 $\pm$ 3*
No HCO ₃ ⁻	Control	96 $\pm$ 4	92 $\pm$ 3
	0.5 mmol/l NH ₄ Cl	89 $\pm$ 10	87 $\pm$ 5
	10 mmol/l NH ₄ Cl	71 $\pm$ 12*	75 $\pm$ 8*
Amiloride (0.5 mmol/l)	Control	104 $\pm$ 5	105 $\pm$ 6
	0.5 mmol/l NH ₄ Cl	108 $\pm$ 4	110 $\pm$ 5
	10 mmol/l NH ₄ Cl	34 $\pm$ 17*	11 $\pm$ 4*

induced by 10 mmol/l NH₄Cl was almost twice the effect caused by 0.5 mmol/l NH₄Cl (Table 1).

The intracellular acidification caused by NH₄Cl in the intact perfused liver is in contrast with the alkalization observed in isolated hepatocytes [1–3]. A similar phenomenon was recently described in a renal cell line from the Thick Ascending Limb of Henle ('TALH'), which was attributed to an uptake of NH₄⁺ via a NH₄⁺/Na⁺/Cl⁻ cotransport system. In the absence of NH₄⁺ this system mediates K⁺/Na⁺/Cl⁻ cotransport. One of its particular characteristics is its sensitivity to 'loop diuretics' such as bumetanide [10,11]. Therefore, we first investigated the question whether intracellular acidification is blocked by bumetanide. However, even at 0.1 mmol/l bumetanide did not alter the response of the isolated perfused liver to NH₄Cl. The time course and the maximum pH_i change caused by 10 mmol/l in the presence of bumetanide (-0.10 ± 0.04 pH unit) was similar to that under control conditions.

Effects of acetazolamide on pH_i, ATP and urea production

The other possibility, that NH₄Cl infusion stimulates urea synthesis which would consume intracellular bicarbonate and thereby decrease pH_i, was investigated. Therefore, we modified the availability of intracellular bicarbonate in the experiments and studied the effects of such treatments on pH_i, ATP and urea formation.

The availability of intracellular HCO₃⁻ was diminished by inhibition of the carbonic anhydrase with acetazolamide (0.5 mmol/l). After 115 min of perfusion in the presence of acetazolamide, pH_i and ATP content (Table 1) were found to be not significantly different from those estimated in the absence of acetazolamide.

During the infusion with 0.5 mmol/l NH₄Cl, the changes in pH_i were distinctly smaller compared with the intracellular acidosis observed in the absence of acetazolamide. At no time was a significant decrease in pH_i observed. The release of urea into the perfusate was decreased from 0.54 ± 0.06 to 0.16 ± 0.09 μ mol/min per g, a 70% inhibition (Table 2).

In the presence of 0.5 mmol/l acetazolamide, 10 mmol/l

Table 2 Release of urea from the isolated perfused rat liver

Livers were perfused with a modified Krebs–Henseleit bicarbonate buffer. In bicarbonate-free medium HCO₃⁻ was replaced by Hepes. The rate of urea release was estimated in perfusate samples from the effluent taken before and during the application of 0.5 mmol/l NH₄Cl. Data are given as means $\pm$ S.D. from 3 experiments: *significantly different from the untreated control ($P < 0.01$, Student's *t* test).

		Urea release (μ mol/min per g)
No treatment	Basal rate	0.04 $\pm$ 0.02
	+ 0.5 mmol/l NH ₄ Cl	0.54 $\pm$ 0.06
Acetazolamide (0.5 mmol/l)	Basal rate	0.05 $\pm$ 0.01
	+ 0.5 mmol/l NH ₄ Cl	0.16 $\pm$ 0.09*
No HCO ₃ ⁻	Basal rate	0.03 $\pm$ 0.01
	+ 0.5 mmol/l NH ₄ Cl	0.04 $\pm$ 0.02*
Amiloride (0.5 mmol/l)	Basal rate	0.04 $\pm$ 0.01
	+ 0.5 mmol/l NH ₄ Cl	0.16 $\pm$ 0.01*

Figure 3 Effects of NH₄Cl on the pH_i during inhibition of the carbonic anhydrase by acetazolamide

Livers were perfused with a modified Krebs–Henseleit bicarbonate buffer in the absence (○) and the presence (●) of 0.5 mmol/l acetazolamide. NH₄Cl was added to the perfusate from a stock solution by a precision micropump. Data are given as means $\pm$ S.D. from 3 experiments; *significantly different from the value estimated under control conditions ($P < 0.05$).

NH₄Cl elicited initially a transient alkalization ($+0.06 \pm 0.01$ pH unit, $n = 3$) which was greater than the initial effect of NH₄Cl in control experiments ($+0.02 \pm 0.01$ pH unit). After the initial increase, pH_i decreased to significantly lower values (-0.05 ± 0.01 pH unit). From 8 to 41 min of NH₄Cl application, the induced acidosis in the presence of acetazolamide was significantly smaller than the effect of 10 mM NH₄Cl in controls (Figure 3).

The relative changes of ATP content during addition and

Figure 4 Effects of removal and addition of HCO_3^- on pH_i

Livers were perfused with either a modified Krebs–Henseleit bicarbonate buffer or a bicarbonate-free Hepes buffer. In both cases, the pH of the medium was 7.4. Data are given as means $\pm$ S.D. from 3 experiments.

removal of NH_4Cl (0.5 or 10 mmol/l) in the presence of acetazolamide (0.5 mmol/l) were not significantly different from those found in the absence of the inhibitor (Table 1).

Effects of removal of extracellular HCO_3^- on pH_i , ATP and urea production

The removal of HCO_3^- from the perfusion medium resulted in a rapid reversible intracellular acidosis (-0.08 ± 0.03 pH unit, $n = 3$; Figure 4). The ATP content was not significantly altered by this treatment (Table 1).

In the absence of extracellular HCO_3^- , neither 0.5 nor 10 mmol/l NH_4Cl induced a further intracellular acidosis. Instead, the addition of 10 mmol/l NH_4Cl resulted in a marked transient alkalization ($+0.17 \pm 0.01$ pH unit, $n = 4$), followed by a re-acidification to the initial pH value within 10 min. In the presence of 10 mmol/l NH_4Cl , ΔpH_i values estimated in the absence of HCO_3^- were significantly smaller than those determined in the presence of HCO_3^- . The subsequent removal of NH_4Cl (10 mmol/l) induced a pronounced transient acidosis of -0.23 ± 0.06 pH unit. In the absence of HCO_3^- the rate of recovery from the acidosis caused by NH_4Cl removal was distinctly slower than in its presence (Figure 5b).

Similar, although less striking, effects were also observed with 0.5 mmol/l NH_4Cl (Figure 5a). It is noteworthy that the release of urea from the liver could not be stimulated by 0.5 mmol/l NH_4Cl in HCO_3^- -free medium (Table 2).

In liver perfused with HCO_3^- -free medium the changes in ATP content induced by NH_4Cl (0.5 or 10 mmol/l) were not significantly different from those found in livers perfused with HCO_3^- -containing medium (Table 1).

Effects of amiloride

In previous studies reported by other authors, a strong Na^+/H^+ -exchange activity has been described in liver cells [2,3]. Therefore, amiloride (0.5 mmol/l) was used to investigate the activity of the Na^+/H^+ exchanger [12]. After 115 min of perfusion in the presence of amiloride pH_i was not altered. The ATP content,

Figure 5 Effects of (a) 0.5 mmol/l NH_4Cl and (b) 10 mmol/l NH_4Cl on pH_i in the absence (●) and the presence (○) of HCO_3^-

Livers were perfused with either a modified Krebs–Henseleit bicarbonate buffer (○) or a bicarbonate-free Hepes buffer (●). In both cases the pH of the medium was 7.4. NH_4Cl was added to the perfusate from a stock solution by a precision micropump. Data are given as means $\pm$ S.D. from 3–4 experiments: * significantly different from the value estimated in the presence of HCO_3^- ($P < 0.05$).

Figure 6 Effects of NH₄Cl on the pH_i during inhibition of the Na⁺/H⁺ exchanger by amiloride

Livers were perfused with a modified Krebs–Henseleit bicarbonate buffer in the absence (○) or the presence (●) of 0.5 mmol/l amiloride. NH₄Cl was added to the perfusate from a stock solution by a precision micropump. Data are given as means ± S.D. from 3–4 experiments: * significantly different from the value estimated under control conditions ($P < 0.05$).

however, was increased and was significantly higher compared with control conditions (Table 1).

In the initial phase of NH₄Cl application (0.5 mmol/l, 25 min) the pH_i decrease observed in the presence of amiloride (0.5 mmol/l) was not significantly different from that found in the absence of the inhibitor (-0.04 ± 0.01 pH unit, $n = 3$, versus -0.05 ± 0.02 pH unit, $n = 4$; Figure 6). After 66 min of NH₄Cl application in the absence of amiloride, pH_i recovered slowly to -0.03 ± 0.01 pH unit. In the presence of amiloride, however, pH_i continued to decrease to -0.06 ± 0.01 pH unit. After removal of NH₄Cl, the recovery of pH_i was almost completely blocked by amiloride (Figure 6).

In the presence of 0.5 mmol/l NH₄Cl and amiloride, ATP content increased, reaching $108 \pm 4\%$ after 66 min. After removal of NH₄Cl, ATP content remained constant (Table 1).

The formation of urea in the presence of NH₄Cl (0.5 mmol/l) was significantly inhibited by amiloride (Table 2).

In the presence of 0.5 mmol/l amiloride, the application and moreover the subsequent removal of 10 mmol/l NH₄Cl caused a severe injury to the liver. The addition of 10 mmol/l NH₄Cl induced a rapid fall in pH_i of -0.14 ± 0.01 pH unit. ATP continuously decreased until it reached $34 \pm 17\%$ of its initial value. The removal of NH₄Cl amplified the decrease in pH_i

(-0.46 ± 0.10 pH unit), and ATP content diminished, reaching only $11 \pm 4\%$ of the initial levels. When the liver was removed from the n.m.r. tube an increase in its weight by about 40% was observed, indicating a dramatic swelling.

Effects of DIDS

DIDS (4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid, 0.5 mmol/l) was used as a probe for trans-membrane uptake of HCO₃⁻. The addition of DIDS elicited a decrease in pH_i by -0.09 ± 0.02 pH unit ($n = 3$) within 10 min. The pH_i was almost constant thereafter. However, the viability of the liver was decreased by DIDS. The ATP content decreased to 50% within 30 min. If the effects of NH₄Cl on pH_i and urea formation were altered by DIDS, it could not be ascertained as to whether the lower viability or a decreased uptake rate of HCO₃⁻ was responsible for the observed results. Therefore this series of experiments was discontinued.

DISCUSSION

In this ³¹P-n.m.r. study a steady-state pH_i of 7.29 ± 0.04 was found in the isolated perfused rat liver. This value is in good agreement with pH_i values which have previously been determined by ³¹P n.m.r. in livers of intact animals or in isolated perfused liver [13–17]. The pH_i found in our study is also in good agreement with pH_i values determined with fluorescent dyes in isolated hepatocytes [1,3,18,19].

NH₄Cl was applied at concentrations of 0.5 and 10 mmol/l. The concentration of 0.5 mmol/l was chosen for our experiments, because it might occur in the portal vein *in vivo*. Furthermore, we assumed that such a low concentration of NH₄Cl could not induce detectable effects on pH_i only by a passive flux of NH₃. In studies reported on isolated hepatocytes [1], 10–25 mM NH₄Cl, however, induced a large increase in intracellular pH by the passive influx of non-ionic NH₃ and the subsequent formation of NH₄⁺ (NH₃ + H⁺ → NH₄⁺). The subsequent removal of NH₄Cl resulted in a transient acidification generated by a rapid efflux of NH₃ and the degradation of NH₄⁺ (NH₄⁺ → NH₃ + H⁺). The recovery from the acid load by removing NH₄Cl was performed by Na⁺/H⁺ exchangers and Na⁺/HCO₃⁻ symporters [2,20–22].

Surprisingly, in intact liver the application of 0.5 and 10 mmol/l NH₄Cl induced significant intracellular acidifications. The decrease in pH_i induced by 10 mmol/l NH₄Cl was only twice that with 0.5 mmol/l. Therefore, this acidification was most probably caused by a saturable process. The removal of 0.5 mmol/l NH₄Cl induced a recovery of pH_i to the initial level. In comparison with the experiments on hepatocytes, the removal of 10 mmol/l NH₄Cl induced a further decrease in pH_i by NH₃ efflux and a subsequent recovery.

Two mechanisms for the intracellular acidification induced by NH₄Cl were taken into account, both dominating the effect of passive NH₃ influx: the decrease in pH_i could be the result of a rapid uptake of NH₄⁺ ions via a bumetanide-sensitive Na⁺/K⁺(NH₄⁺)/2Cl⁻ symport which has been established in some renal cells [5,10,11]. Furthermore, the acidification could be elicited by utilization of intracellular HCO₃⁻ via an increased synthesis of urea [4,20].

In agreement with the studies by Petzinger et al. [23], uptake of NH₄⁺ ions via a bumetanide-sensitive transport system could not be established for the isolated liver.

It has been reported that the stimulation of urea synthesis by NH₄Cl (1 mmol/l) is inhibited if the carbonic anhydrase is blocked by acetazolamide, or if the perfusion medium did not contain CO₂ and HCO₃⁻ [6]. If, by analogy with these experi-

ments, the availability of intracellular HCO_3^- was decreased by one of the procedures used, the decreases in both pH_i and the release of urea into the perfusion medium were inhibited. The release of urea was only determined during an application of 0.5 mmol/l NH_4Cl , because urea was not detectable in the presence of 10 mmol/l NH_4Cl by the enzymic optical method adopted. It was concluded that NH_4Cl induced the formation of urea, and that the utilization of HCO_3^- by urea synthesis caused the intracellular acidification.

When urea formation was inhibited, the application of 10 mmol/l NH_4Cl induced an intracellular alkalinization which was expected to be caused by the passive influx of NH_3 . In the absence of CO_2 and HCO_3^- this alkalinization was much more pronounced than in the acetazolamide experiments, because the intracellular buffer capacity was decreased.

The stimulation of urea formation by NH_4Cl (1 mmol/l) was coupled to a decrease in pH of the perfusion medium [7]. This decrease in the extracellular pH could be generated by extrusion of H^+ and/or uptake of HCO_3^- .

In our study amiloride was used to probe for the Na^+/H^+ -exchanger activity. The acidosis induced by application of 0.5 mmol/l NH_4Cl was only slightly amplified by amiloride (0.5 mmol/l). Therefore the extrusion of H^+ by the Na^+/H^+ exchanger in the absence of amiloride seemed to be slow at first sight. However, the stimulation of urea synthesis, which was the origin of the intracellular acidification, was significantly inhibited when the activity of the Na^+/H^+ exchanger was blocked.

The importance of an uptake of extracellular HCO_3^- by the $\text{Na}^+/\text{HCO}_3^-$ symporter for the regulation of pH_i in the intact liver was indicated by a decrease in pH_i when HCO_3^- was removed from the perfusion medium. Furthermore, the recovery of pH_i after removal of NH_4Cl from the medium was delayed in the absence of extracellular HCO_3^- . Unfortunately, we did not investigate the involvement of this symporter in the regulation of pH_i during the stimulation of urea formation by NH_4Cl . In HCO_3^- -free medium, urea synthesis and the resulting acidosis were blocked. An attempt to inhibit the transporter by the use of DIDS did not yield reliable results, because DIDS decreased the viability of the livers, probably by entering the cells.

The application of NH_4Cl induced not only a decrease in pH_i but also a small decrease in ATP content in the liver. Only the effect at 10 mmol/l of NH_4Cl was significant. Therefore, it was concluded that NH_4Cl stimulated ATP-consuming processes such as the synthesis of urea or secondary active membrane transport mechanisms involved in the regulation of pH_i , and/or it decreased the rate of ATP formation. The Na^+/H^+ exchange and the $\text{Na}^+/\text{HCO}_3^-$ symport are secondary active because they are driven by the trans-membrane Na^+ gradient built up by the Na^+/K^+ -ATPase.

The decrease in ATP content induced by NH_4Cl was not significantly affected if both the formation of urea and the intracellular acidification were inhibited by acetazolamide or by a HCO_3^- -free perfusion medium. Thus the ATP demand for both the synthesis of urea and for pH_i regulation seemed to be of minor importance for the effect of NH_4Cl on ATP levels in the liver.

In the presence of amiloride (0.5 mmol/l) the ATP content of liver slowly increased, reaching 105 % of its initial value after 2 h. In untreated control liver, however, ATP content decreased slowly to 91 %. The use of amiloride therefore demonstrated the impact of the continuous amiloride-sensitive Na^+ influx and its subsequent removal by the Na^+/K^+ -ATPase in the energy metabolism of the liver. The pH_i was kept constant in the presence of amiloride within the experimental time. The activity of the Na^+/H^+ exchanger seemed to be replaced by other pH_i -

regulating processes. On the other hand, amiloride might inhibit other predominant Na^+ -transporting processes at pH_i 7.3.

In the presence of amiloride the ATP content was not even decreased by the application of 0.5 mmol/l NH_4Cl . For this reason one might conclude that in the absence of amiloride the stimulation of the secondary active Na^+/H^+ -exchanger by a metabolic acidosis might be of some importance for the decrease in ATP levels elicited by 0.5 mmol/l NH_4Cl . However, this assumption is inconclusive because of the conclusions drawn from the results of the acetazolamide experiments and the HCO_3^- removal.

The application, and moreover the subsequent removal, of 10 mmol/l NH_4Cl in the presence of amiloride cause severe injury to the liver. In the presence of NH_4Cl and after its removal, ATP content decreased continuously to 11 % of its initial value. The injury to the liver is probably due to the extreme intracellular acidosis, which also resulted in an increase in its cell volume and wet weight.

The effects of NH_4Cl application on the ATP content of the liver could not finally be explained by the present results. However, these effects should be the subject of future research.

In this study on intact rat liver, ^{31}P n.m.r. was used as a non-invasive method to investigate the interrelations of the synthesis of urea and the regulation of pH_i during the application of NH_4Cl at physiologically low (0.5 mmol/l) and experimentally high concentrations (10 mmol/l). The application of NH_4Cl to the isolated perfused rat liver stimulated, as expected, the synthesis of urea and its release into the perfusate. As a consequence, utilization of intracellular HCO_3^- was increased. The removal of intracellular HCO_3^- led to an initial decrease in the pH_i and was followed by stabilization at a lower steady-state value. The bicarbonate utilized in urea formation must be supplied both by the activity of the carbonic anhydrase and by a continuous uptake of extracellular HCO_3^- . The extracellular acidosis observed by other authors [7] can thus be explained by the extrusion of H^+ via the Na^+/H^+ exchanger and uptake of HCO_3^- from the perfusion medium. The limitation of the intracellular acidosis to a small extent by the action of the Na^+/H^+ exchanger was essentially to keep up the rate of urea formation simulated by NH_4Cl application.

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