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METABOLISM OF *N*-ACETYL-L-CYSTEINE

SOME STRUCTURAL REQUIREMENTS FOR THE DEACETYLATION AND CONSEQUENCES FOR THE ORAL BIOAVAILABILITY

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Abstract—Rat liver, lung and intestine homogenates deacetylated *N*-acetyl-L-cysteine. Nearly stoichiometric amounts of L-cysteine were recovered. In rat liver, the enzyme activity was associated with the cytosolic fraction. Liver cytosolic fractions from rat, man and mouse hydrolysed *N*-acetyl-L-cysteine at similar rates, while dog liver cytosol was much less active. *N*-Acetyl-D-cysteine or the disulphide of *N*-acetyl-L-cysteine were not deacetylated or in other ways consumed *in vitro*. Isolated, perfused rat liver did not retain or metabolize *N*-acetyl-L-cysteine to any measurable extent during single-pass experiments. *N*-Acetyl-L-cysteine or *N*-acetyl-D-cysteine were injected into a ligated segment of rat intestine *in situ*. After 1 hr 2% of the L-isomer and 35% of the D-isomer remained in the intestinal lumen. Systemic plasma levels were $<3\ \mu M$ of the L-form and $\simeq 40\ \mu M$ of the D-form. We conclude that deacetylation in the intestinal mucosa and possibly in the intestinal lumen is the major factor determining the low oral bioavailability of *N*-acetyl-L-cysteine. The deacetylation is discussed on the basis of the subcellular localization and the structural requirement of the reaction.

N-Acetyl-L-cysteine (L-NAC)‡ is used against liver toxicity caused by paracetamol overdose, and against chronic bronchitis [1, 2]. The mechanism of action with regard to liver toxicity is believed to be that L-NAC provides the liver with glutathione precursors [3, 4]. Less is known about the mechanisms of the effects observed in the treatment of chronic bronchitis. Thus, it is not known whether L-NAC itself acts as an antioxidant, reductant and radical scavenger, or whether any of its metabolites, notably L-cysteine, glutathione and sulphate, is the active molecular species.

The pharmacokinetics [5, 6] and metabolism [7–10] of L-NAC have been investigated in several studies, and the oral bioavailability is known to be low, around 10%. Little specific attention, however, has been devoted to the deacetylation reaction, which we assume is the most important primary metabolic step responsible for the low bioavailability. In the present study we have studied the kinetics of deacetylation of L-NAC in rat and dog tissues and in liver preparations also from man and mouse. In some experiments we included D-NAC and the disulphide of L-NAC for comparison. The plasma levels of the thiols in the rat after intraintestinal injection of L-NAC and D-NAC were also investigated.

MATERIALS AND METHODS

Biological material. Sprague–Dawley rats (200–250 g), NMRI mice (20–25 g), and three beagle dogs (15 kg) (one male, two females) were used. Liver samples from three humans (two females, one male) were kindly provided by Dr Christer von Bahr, Huddinge, Sweden.

The mucosa from intestine samples (jejunum) was scraped off with an objective glass. The organs (liver from man, rat, dog and mouse; lung and intestinal mucosa from rat and dog) were homogenized in 4 vol. of 0.1 M KH_2PO_4, pH 7.5, containing 0.25 M sucrose, with a Potter-Elvehjem homogenizer, equipped with a Teflon pistle. The lung and intestinal samples were used only in the form of whole tissue homogenates. Aliquots of the liver homogenates were used for preparation of various subcellular fractions as follows. The liver homogenates were centrifuged at 4° at 10,000 *g* for 15 min. The supernatants were further centrifuged at 4° for 90 min at 100,000 *g*. The supernatant obtained after this ultracentrifugation is called the cytosolic fraction (prepared from livers from man, rat, dog and mouse). The pellet was washed three times with phosphate buffer containing 0.15 M KCl (1 ml/g liver) by homogenizing the pellet at 4°. After each wash the suspension was centrifuged for 60 min at 4° at 100,000 *g* and the supernatant discarded. After the last wash the pellet was suspended and homogenized in phosphate buffer with sucrose (1 ml/g liver), and this suspension is called the microsomal fraction (prepared only from rat liver). Protein concentrations in the various fractions were measured using the method described by Lowry *et al.* [11].

Compounds. D-NAC and the disulphide of L-NAC were synthesized at AB Draco, Sweden. Other chemicals were obtained from commercial sources.

Incubations. All incubations were performed at 37°. The buffer used was 0.1 M K_2HPO_4, pH 7.5. The incubation mixture, without substrate, was equilibrated for 5 min, and the reaction started by addition of the substrate, which was dissolved in water. The substrate concentration was 100 μM (except in the experiments shown in Fig. 2), and the

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‡ Abbreviations: L-NAC, *N*-acetyl-L-cysteine; D-NAC, *N*-acetyl-D-cysteine.

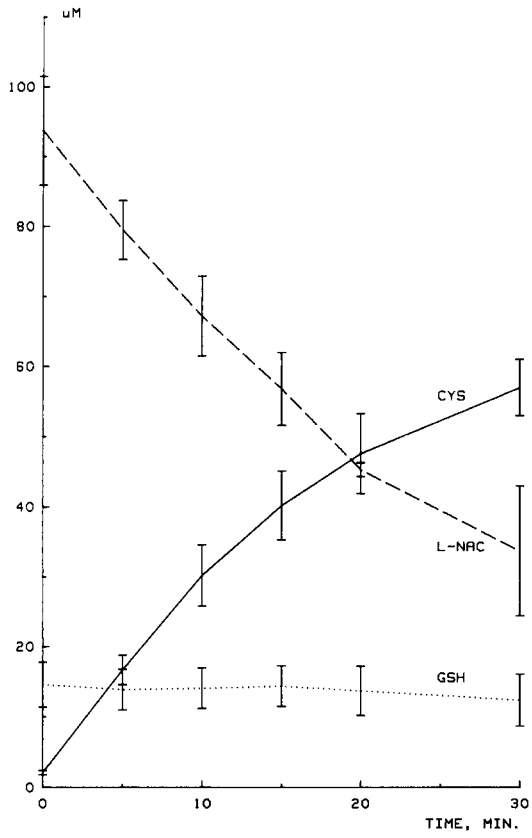

Fig. 1. Hydrolysis of L-NAC in homogenates of rat liver. The protein concentration was 1 mg/ml. Three experiments were performed, and means ± SD are indicated.

protein concentration was 1 mg/ml. The total volume of the incubations was 5 ml. The incubations were terminated by transferring 125 μl of the incubation mixture to 25 μl 50% trichloroacetic acid. After centrifugation the supernatants were neutralized with $NaHCO_3$ and then prepared for analysis as described below.

Rat liver perfusion. Rats were anaesthetized with pentobarbital 60 mg/kg i.p., and the livers perfused with an oxygenated (95% O_2/5% CO_2) Krebs-Ringer buffer (pH 7.35) containing 0.1% glucose according to standard procedures. The perfusion flow was 4 ml/min/g liver. After 15 min of equilibration perfusion, the livers were perfused with 500 ml medium containing 14, 75 of 150 μM L-NAC, followed by control medium to make a total perfusion time of 60 min. Every 5 min, samples (≈1 ml) were taken from the perfusion medium on the venous side. Bile was collected over 5-min-intervals.

Intraintestinal injection. The rats were fasted overnight and anaesthetized with 60 mg/kg pentobarbital injected intraperitoneally. The abdomen was opened by a midline incision. The small intestine was covered *in situ* with a saline-soaked gauze and kept at 37°. A 20–30 cm segment of the small intestine, starting 2–3 cm from pylorus, was ligated at both ends *in situ*. Left or right arteria carotis was cannulated with a Pe-50 polyethylene tubing (Clay Adams, U.S.A.). L- or D-NAC was dissolved in physiological saline at a concentration of 6.13 mM (1 mg/ml), and pH adjusted to 7.5 by NaOH. Two millilitres of the solutions were injected into the ligated small intestine segment.

Blood samples were collected from arteria carotis into heparinized Eppendorf tubes, before administration of the drugs, and after 15, 30, 45, and 60 min. Plasma was prepared by centrifugation at 10,000 *g* for 1.5 min. In some experiments the segment of the small intestine was dissected out after 60 min, the luminal content collected, and the lumen rinsed with 25 ml of phosphate buffer. Plasma samples and intestinal content were analysed as described below.

Analysis. Total thiols (i.e., free and disulphide-bound thiols) were analysed after reduction with dithiothreitol and derivatization with monobromobimane as described by Cotgreave and Moldéus [12]. The HPLC-systems consisted of two Waters M-45 pumps, a Waters Intelligent Sample Processor 712, a Waters Automated Gradient Controller model 680, a Shimadzu Fluorescence HPLC Monitor RF 530 (emission at 480 nm and excitation at 394 nm), and a Waters 740 Data Module. The column was a Supelco C_{18} column, 3 μm (150 × 4.6 mm i.d.). Peak heights were determined. The flow rate was 1 ml/min. Solvent A: 9% acetonitrile, 0.25% glacial acetic acid, 0.25% perchloric acid (70%), pH 3.70. Solvent B: 75% acetonitrile. The elution started with 100% A, and after 4 min this was changed to 88% A/12% B. Cysteine, glutathione and NAC had retention times of 4, 6 and 10 min, respectively. The column was washed with 100% B for 7 min and equilibrated with 100% A for 7 min between each run.

RESULTS

Hydrolysis of L-NAC in subcellular fractions from different tissues and species

Hydrolysis of L-NAC was tested in homogenates of rat liver, intestine and lung. The results from incubations with liver homogenates are shown in Fig. 1. The rate of hydrolysis of L-NAC was 2.4 nmol/mg protein/min during the first 20 min under these conditions. Almost stoichiometric amounts of cysteine were found. The glutathione levels were not changed during the incubations. In homogenates of lung and intestinal mucosa the rates of hydrolysis were 1.1 and 3.7 nmol/mg protein/min, respectively. Also in these two tissue homogenates stoichiometric amounts of cysteine were formed, but in the intestinal homogenates the glutathione initially present was consumed during the incubations.

The activity to hydrolyse L-NAC was measured in whole homogenate, 9000 *g*-pellet, 9000 *g*-supernatant, cytosol and microsomes from rat liver. Around 70% of the activity was present in the cytosol, and the rest of the activity in the 9000 *g*-pellet and in the microsomes. The activity associated with these particulate fractions could be removed almost completely (>98%) by washing the pellets, followed by ultracentrifugation. The washing procedure was repeated three times, and the third "wash supernatant" contained virtually no activity. Thus, the entire hydrolytic activity towards L-NAC in rat liver was present in the soluble fractions.

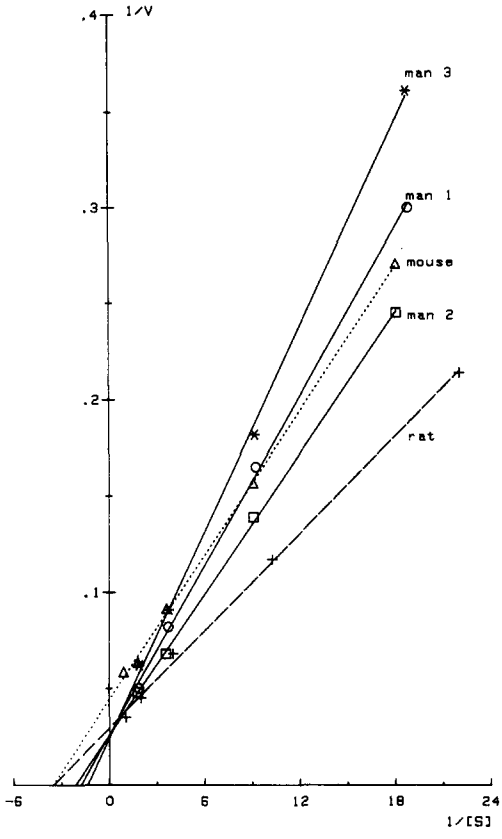

Fig. 2. Hydrolysis of L-NAC in liver cytosolic fractions from man, rat and mouse. The dependence on substrate concentration. The unit for substrate concentration is mM, and the unit for rate of hydrolysis μM/min. The protein concentration was 1 mg/ml.

Table 1. Kinetic parameters for the hydrolysis of L-NAC in liver cytosol*

	V_{max} (nmol/min)	K_m (mM)
Man 1	38	0.56
2	39	0.47
3	41	0.73
Rat	34	0.29
Mouse	23	0.28
Dog	<3	—

* The protein concentrations were 1 mg/ml. The numbers are calculated from the data given in Fig. 2.

Cytosolic liver fractions were prepared from rat, mouse, dog and man. V_{max} and K_m for the hydrolyses of L-NAC were determined from the equations of the Lineweaver–Burk plots shown in Fig. 2. These results are summarized in Table 1. Surprisingly, the dog liver cytosol hydrolysed L-NAC at a barely detectable rate (<10% of the other species). To clarify further the capacity of dog tissues to hydrolyse L-NAC, we prepared homogenates of liver, lung and intestinal mucosa from two female beagles. The activity to hydrolyse L-NAC was very low in liver and lung homogenates (~0.2 nmol/mg protein/min), while in intestinal mucosa the activity was 0.9 nmol/mg protein/min (compare with rat tissue homogenates above).

Substrate specificity of the hydrolysis

D-NAC was incubated with rat tissue homogenates, but was not hydrolysed. If rat liver homogenate was preincubated for 6 min with 100 μM D-NAC before addition of 100 μM L-NAC, the hydrolysis of L-NAC was not inhibited. The disulphide of L-NAC was not hydrolysed by any of the rat tissue homogenates.

Metabolism of L-NAC in isolated rat liver

Isolated rat livers were perfused with L-NAC in single-pass experiments as described above. There was no sign of any uptake or metabolism of L-NAC. Neither could we detect any L-NAC in bile. Cysteine or glutathione levels in perfusate or bile did not change as a reult of L-NAC perfusion.

Fate of L-NAC and D-NAC after intraintestinal injection to rats in situ

L-NAC (2 mg) was injected into a ligated segment of the intestine of anaesthetized rats. After 1 hr 2.2 ± 0.6% (N = 3) of the L-NAC remained in the intestine. Plasma levels of NAC (blood was sampled from arteria carotis) were always <3 μM. No significant changes in cysteine or glutathione levels in plasma were observed. D-NAC was injected into rat intestine in the same way. After 1 hr the plasma levels of D-NAC were 41 ± 18 μM (N = 8), and the intestines contained 35 ± 2% (N = 3) of the amount injected.

DISCUSSION

This study demonstrates that L-NAC is effectively hydrolysed to yield L-cysteine by rat, mouse and human tissue. This metabolic step is also probably a prerequisite for most of the protective effects of L-NAC at paracetamol intoxication, since these effects are thought to be dependent on de novo glutathione biosynthesis [3, 4]. Dog tissues deacetylated L-NAC at a much slower rate, and thus L-NAC should not work as efficiently as a glutathione precursor and paracetamol antidote in this species.

The deacetylation seems to be a process with rather specific structural requirements on the substrate, since D-NAC and the disulphide of L-NAC were virtually resistent to hydrolysis. The observation that D-NAC was not hydrolysed is not too surprising, since other N-acetylated amino acids have been shown to be stereoselectively hydrolysed [13]. Several proteolytic enzymes are strictly dependent on the stereochemistry of the amino acids. The most well-studied enzyme is probably α-chymotrypsin [14–16]. The stereoisomers of *N*-acetyl-tryptophanamide bind to this enzyme with similar affinities, but the hydrolytic step is 10^7-fold faster for the L-form. In that case the D-form inhibits the hydrolysis of the L-form. When we preincubated rat liver cytosol with D-NAC, we did not, however, observe any inhibition of the hydrolysis of L-NAC.

Our results do not settle which enzyme is hydrolysing L-NAC, but some discussion on this matter

may be relevant. Firstly, coenzyme A-dependent *N*-acetyl transferases are known to acetylate several xenobiotics [17]. These enzymes are cytosolic, but to our knowledge the reverse reaction, i.e. a deacetylation, has not been shown to be catalysed by these enzymes. It is striking, however, that the dog liver cytosol, compared to this fraction from several other species, has low activity to acetylate several aromatic amines [18]. Secondly, unspecific carboxylesterases/amidases [19, 20], including deacetylases [21], of several sorts are located primarily to microsomes (though soluble enzymes of this class have been described [22]), and are therefore less likely to be the type of enzyme catalysing hydrolysis of L-NAC. In addition, the capacity of the dog to deacetylate arylacetamides is comparable to that of other species investigated [23]. Thirdly, and most interestingly, a large number of proteins in animal and plant cells are N-acetylated on their N-terminal amino acid [24]. An enzyme, acyl-peptide hydrolase from rat liver, liberating the N-acetylated amino acid, has been described [25, 26], and α-*N*-acylamino acid hydrolases have been purified from rat kidney [27] and bovine liver [28]. We therefore suggest that L-NAC is hydrolysed by such α-*N*-acyl-L-amino acid hydrolases, enzymes which have their physiological role in regeneration of free L-amino acids during protein turnover.

Our results show that the intestinal mucosa very effectively hydrolyses L-NAC. This organ is probably the main barrier resulting in the low systemic bioavailability of oral L-NAC, since the isolated perfused rat liver did not retard or metabolize L-NAC to any measurable extent, and since human plasma does not hydrolyse L-NAC (unpublished observations). It cannot, however, be excluded that proteolytic enzymes, excreted into the intestine, also may hydrolyse L-NAC.

In humans, organs other than the intestine most likely contribute to the biotransformation of L-NAC, but the intestine probably plays a major role. Thus, intravenous infusion of 600 mg L-NAC into human volunteers resulted in a recovery of 30% of unchanged drug (or its disulphide) in the urine. After oral intake around 3% was found in the urine [5]. We believe that deacetylation is the major initial metabolic step, but *in vivo* other reactions like S-methylation and S-oxidation may take place as well. Recently, it was reported that the sulphur of ^{35}S-D-penicillamine was largely excreted as sulphate in urine when given to human volunteers [29].

In summary, we have shown that deacetylation of L-NAC is an effective process in man, rat and mouse, probably explaining the low oral bioavailability. If free L-NAC enters a cell, it will be almost instantaneously hydrolysed and the cysteine processed further in various ways. If L-NAC enters a cell in a disulphide form, cleavage of the sulphur bridge must precede deacetylation. We also demonstrate that a stereoisomeric modification of L-NAC will change the metabolism and thus the pharmacokinetic properties drastically.

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