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LIVER CARBONIC ANHYDRASE AND UREA SYNTHESIS

THE EFFECT OF DIURETICS*

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Abstract—1. In isolated perfused rat liver, urea synthesis is rapid and reversibly inhibited not only by the well-known carbonic anhydrase inhibitors acetazolamide, methazolamide and ethoxzolamide, but also by diuretics, like xipamide, mefruside, chlortalidone, and chlorothiazide. Furosemide was without effect.

2. Similar to findings with isolated perfused rat liver, acetazolamide inhibits urea synthesis from ammonium ions in normal and cirrhotic human liver slices.

3. Inhibition of urea synthesis by xipamide and acetazolamide is accompanied by a 70% decrease of the cellular citrulline content and the tissue levels of 2-oxoglutarate and citrate, suggesting a block of urea synthesis at a step prior to citrulline formation.

4. At a constant extracellular pH (7.4), inhibition of urea synthesis by xipamide, mefruside and acetazolamide was overcome by increasing the extracellular concentrations of HCO_3^- and CO_2 to above twice the normal values. This shows that inhibition of urea synthesis by these diuretics is not due to an unspecific inhibition of one of the urea cycle enzymes but is due to an inhibition of mitochondrial carbonic anhydrase and therefore due to an impaired HCO_3^- provision for mitochondrial carbamoylphosphate synthesis.

5. It is concluded that the activity of mitochondrial carbonic anhydrase is required for urea synthesis also in human liver and that several diuretics impair urea synthesis by inhibition of mitochondrial carbonic anhydrase. The pathophysiological significance of these data is discussed with respect to the development of diuretics-induced hyperammonemia and alkalosis in liver disease.

Diuretic therapy is a frequent and long-known precipitant factor of hepatic encephalopathy in patients with liver cirrhosis [1]. This is largely thought to be due to hyperammonemia resulting from an increased renal ammonia production following diuretics-induced hypokalemia and alkalosis [2, 3]. In view of recent work, however, there may be alternative explanations. These studies have shown the existence of a hepatic mitochondrial carbonic anhydrase [4], a new isoenzyme [5]. This enzyme, in contrast to the cytosolic liver carbonic anhydrase III [6] is sensitive to sulfonamide inhibition. Acetazolamide largely inhibits citrulline synthesis in guinea-pig liver mitochondria [7] and urea synthesis in isolated perfused rat liver [8], pointing to a role of mitochondrial carbonic anhydrase for bicarbonate provision for biosynthetic pathways like urea synthesis. This is important, because the mitochondrial membrane is not freely permeable for HCO_3^- in contrast to CO_2 [9, 10]. Thus, CO_2 must be converted into HCO_3^- inside the mitochondria in order to become available as substrate for carbamoylphosphate synthetase with its comparatively high K_m (HCO_3^-) = 5.3 mM [11]. Evidence supporting the concept of a rate control of hepatic urea synthesis by mitochondrial HCO_3^- provision was given in experiments with isolated perfused rat liver [8], demonstrating that mitochondrial carbonic anhydrase can adjust urea cycle flux according the requirements of bicarbonate and acid base homeostasis [8] in line with the proposed role of the liver in systemic pH regulation [8, 12–15]. In addition, these studies showed a linear dependence of urea synthesis from the extracellular CO_2 concentration, when carbonic anhydrase was inhibited by acetazolamide [8]. Further, the acetazolamide inhibition of urea synthesis was fully overcome in the presence of unphysiologically high extracellular CO_2 concentrations (at a constant extracellular pH 7.4), indicating that under these conditions the uncatalyzed intramitochondrial HCO_3^- formation became sufficient for carbamoylphosphate synthesis [8] and ruling out the possibility of an unspecific inhibition of urea cycle enzymes by acetazolamide.

Many diuretics are structurally related to the classical carbonic anhydrase inhibitor acetazolamide (see Scheme 1). Although the mechanism of diuretic action of thiazides, xipamide and mefruside is not due to an inhibition of renal carbonic anhydrase [16, 17], the possibility of a still inherent inhibitory activity on hepatic mitochondrial carbonic anhydrase with the consequence of an inhibition of ureogenesis and hyperammonemia must be considered.

This study shows that mitochondrial carbonic anhydrase is required for urea synthesis also in human liver and that the above-mentioned diuretics inhibit mitochondrial carbonic anhydrase resulting in an impaired urea synthesis.

* Dedicated to Prof. Dr. P. Schölmerich on the occasion of his 70th birthday.

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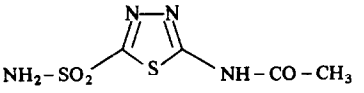
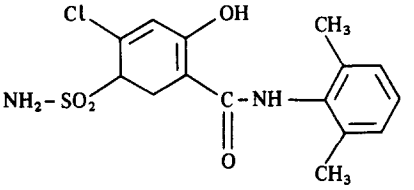
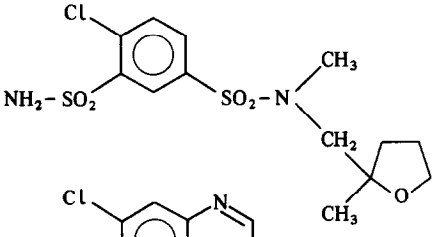
Acetazolamide



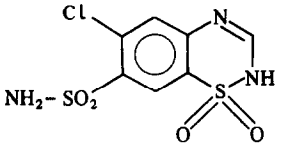
Xipamide



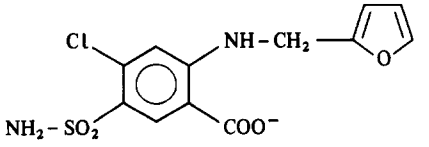
Mefruside



Chlorothiazide



Furosemide



Scheme 1. Chemical structure of acetazolamide, xipamide, mefruside, chlorothiazide and furosemide.

MATERIALS AND METHODS

Hemoglobin-free liver perfusion. Livers of male Wistar rats of 120–220 g body weight, fed *ad libitum* on stock diet (Altromin) were perfused as described previously [18] in an open (non-recirculating) system from portal to caval vein. If not indicated otherwise, the medium contained bicarbonate (25 mM) and was equilibrated with O_2/CO_2 (95/5, v/v). In other experiments, the medium bicarbonate and CO_2 concentrations were so varied that the HCO_3^-/CO_2 ratio was kept constant (pH 7.4). This was achieved by mixing two different media being delivered by two separate systems, each consisting of a perfusion fluid reservoir, an oxygenator and a precise roller pump [8]. This apparatus allowed the adjustment of desired HCO_3^- and CO_2 concentrations in entering perfusate by precise mixing of two fluids with different HCO_3^- concentrations, each being equilibrated separately with different O_2/CO_2 gas mixtures. The perfusion fluid was the Krebs–Henseleit saline plus L-lactate (0.3 mM), pyruvate (0.3 mM) and ornithine (2 mM), except that the HCO_3^- concentration was varied over 3–50 mM with isoosmolarity being achieved by corresponding additions or reductions of sodium chloride. The temperature was 37°. Perfusion flow was approx. 4 ml/(g liver)/min and was kept constant throughout the individual perfusion experiment.

Incubation of human liver slices. Slices prepared from human liver according [19] were incubated at 37° in a Krebs–Henseleit bicarbonate buffered (25 mM) saline (5–10 mg liver/ml), containing L-lactate (4.2 mM), pyruvate (0.6 mM), ornithine (2 mM) and NH_4Cl (10 mM). The incubation mixture was continuously gassed with O_2/CO_2 (95/5, v/v). Under these conditions, urea synthesis linearly increased with the incubation time up to 180 min and the amount of incubated tissue, expressed as mg wet weight or as mg protein. Normal human liver was obtained from kidney transplant donors and was used for the metabolic studies after a 20–30 min hemoglobin-free preperfusion according a surgical protocol; cirrhotic human liver was obtained by needle biopsy during laparoscopy, where a histological liver examination was required from a medical point of view and the amount of biopsy material exceeded that necessary for histological examination. The diagnosis of liver cirrhosis was established histologically. Preparation and incubation of the slices were performed immediately after biopsy.

Assays. The concentrations of ammonia and urea in the perfusion or the incubation fluid were determined after deproteinization in enzymatic optical tests, based on the procedures laid out in [20].

Samples of perfused rat liver (0.5–1 g) were taken by the freeze-stop technique and neutralized perchloric extracts were prepared as described in [21]. The concentrations of glutamate, 2-oxoglutarate and citrate were determined in an enzymatic optical test

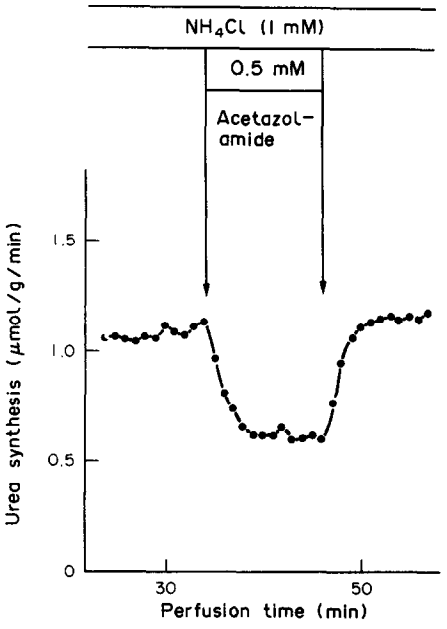


Fig. 1. Effect of acetazolamide (0.5 mM) on urea synthesis from NH_4Cl (1 mM) in isolated perfused rat liver.

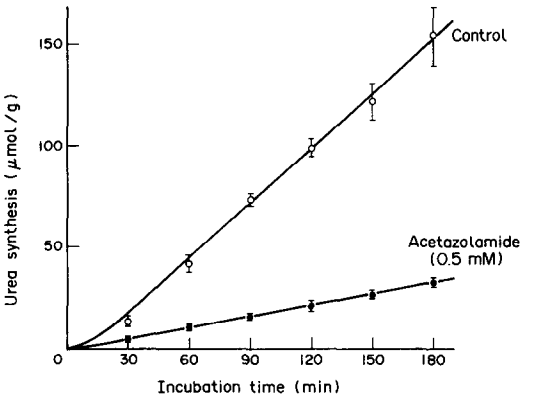


Fig. 2. Effect of acetazolamide (0.5 mM) on urea synthesis from NH_4Cl (10 mM) in human liver slices. For incubation conditions see Materials and Methods. Data are given as means ± SEM from 3 different incubations for each condition.

as described in [20]. Citrulline was measured with an automatic amino acid analyzer (LKB 4151 alpha plus).

Chemicals. All enzymes, NADH, NAD^+, pyruvate and 2-oxoglutarate were from Boehringer (Mannheim). Acetazolamide was from Sigma (Munich). L-Lactic acid was from Roth (Karlsruhe). Xipamide (provided as solvent), mefruside, chlortalidone and amiloride, chlorothiazide, hydrochlorothiazide were generous gifts from Beiersdorf (Hamburg), Bayer (Leverkusen), Ciba Geigy (Grenzach Whylen) and Sharp & Dohme (Munich), respectively. Ethoxzolamide was a generous gift from Upjohn Company (Kalamazoo). Methazolamide was from Cyanamid (Wolfratshausen). All other chemicals were from Merck (Darmstadt).

RESULTS

Effect of acetazolamide on urea synthesis in rat and human liver

In agreement with previous data [8], in isolated perfused rat liver, urea synthesis is rapid and reversibly inhibited by acetazolamide (Fig. 1). This is also observed with the other inhibitors of carbonic anhydrase methazolamide or ethoxzolamide (Table 1). With extracellular HCO_3^- and CO_2 concentrations of 25 mM and 1.2 mM, respectively, double reciprocal plots yielded a half-maximal inhibition of urea synthesis by acetazolamide, ethoxzolamide and methazolamide at influent concentrations of about 30 μM, 10 μM and above 110 μM, respectively.

As shown in Fig. 2 urea synthesis from NH_4Cl in slices from normal human liver is inhibited by about 80% in the presence of acetazolamide, the inhibitor of carbonic anhydrase. Similarly, when human cirrhotic livers were studied, acetazolamide (0.5 mM) decreased the rate of urea synthesis by about 50% from 5.0 ± 0.5 (N = 5) to 2.6 ± 0.6 μmol/hr/g wet weight (N = 5). These data suggest that urea syn-

Table 1. Effect of several diuretics on urea synthesis from NH_4Cl by isolated perfused rat liver

Diuretic	% Inhibition of urea synthesis	N
Acetazolamide (0.5 mM)	54 ± 2	6
Xipamide (0.3 mM)	36 ± 4	5
Mefruside (0.3 mM)	34 ± 2	5
Chlortalidone (0.3 mM)	34 ± 3	3
Chlorothiazide (0.3 mM)	15 ± 2	3
Hydrochlorothiazide (0.3 mM)	9 ± 2	3
Furosemide (0.3 mM)	0 ± 0.5	3
Amiloride (0.3 mM)	−2	2
Methazolamide (0.5 mM)	19	1
Ethoxzolamide (0.1 mM)	65	1

Livers were perfused with NH_4Cl (1 mM) and ornithine (2 mM). In the absence of added diuretics, urea synthesis was 1.42 ± 0.05 μmol/g/min (19 different perfusion experiments). In the individual perfusion experiment, the rate of urea synthesis in the absence of diuretics was set to 100%. Data are given as means ± SEM (N = number of different perfusion experiments).

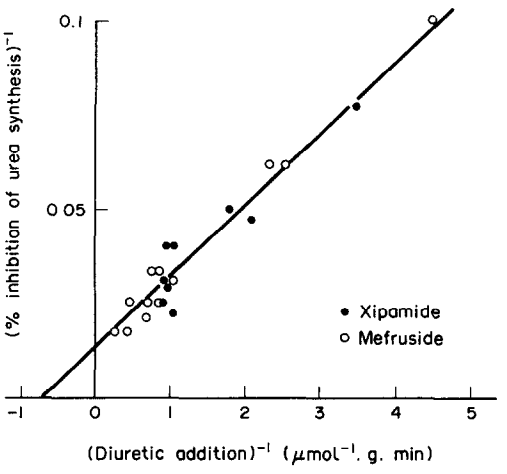


Fig. 3. Double-reciprocal plot analysis of the inhibition of urea synthesis from NH_4Cl (1 mM) in isolated perfused rat liver by xipamide (●) and mefruside (○). Data were obtained during metabolic steady states and represent 4–8 separate measurements. A xipamide or mefruside addition of 1 μmol/g/min corresponds to a concentration in influent of about 0.25 mM. Perfusate concentrations of HCO_3^- and CO_2 were 25 mM and 1.2 mM, respectively.

thesis also in human liver requires the activity of mitochondrial carbonic anhydrase, supplying the bicarbonate for mitochondrial carbamoylphosphate synthesis, as it was shown for rat and guinea-pig liver [7, 8]. The decreased rate of urea synthesis in cirrhotic compared to normal human livers is explained by the decreased urea cycle enzyme activities in the diseased liver [22].

Effect of diuretics on urea synthesis in perfused rat liver

As shown in Table 1, urea synthesis from NH_4Cl (1 mM) in isolated perfused rat liver is not only inhibited by the classical carbonic anhydrase inhibitors, but also by diuretics without clinically overt carbonic anhydrase inhibiting potency, like xipamide, mefruside and chlortalidon. No inhibition was observed with furosemide and amiloride, and the chlorothiazides had only a slight inhibitory effect on urea synthesis. The inhibition of urea synthesis by xipamide and mefruside (Fig. 3) showed a similar concentration dependence; double-reciprocal plot analysis showed a half-maximal inhibitory effect on urea synthesis at mefruside and xipamide additions of about 1.5 μmol/g/min, corresponding to influent concentrations of 0.35–0.4 mM. In the presence of physiological portal HCO_3^- and CO_2 concentrations of 25 mM and 1.2 mM, respectively, a maximal inhibition of urea synthesis of 70–80% is calculated from the data of Fig. 3.

Similar to findings with acetazolamide, the inhibition of urea synthesis by xipamide is accompanied by a considerable decrease in the citrulline tissue levels, indicating a block of the urea cycle prior to the step of ornithine transcarbamoylase (Table 2). Simultaneously, there is an increase in glutamate tissue contents, but decreased levels of the citric acid cycle intermediates 2-oxoglutarate and citrate.

The extent of inhibition of urea synthesis by xipamide is strongly dependent on the extracellular HCO_3^- and CO_2 concentrations. HCO_3^- provision for mitochondrial carbamoylphosphate synthesis occurs not only by mitochondrial carbonic anhydrase, but also by the uncatalyzed hydratation of CO_2, which penetrates the mitochondrial membrane in contrast to HCO_3^-. Thus, the inhibition of urea synthesis by acetazolamide as a consequence of an inhibition of mitochondrial carbonic anhydrase is fully overcome in presence of unphysiologically high extracellular CO_2 (and also HCO_3^- concentrations, in order to maintain a constant extracellular pH) and under these conditions the uncatalyzed intramitochondrial HCO_3^- formation becomes sufficient for the requirements of urea synthesis [8]. As shown in Fig. 4A–C, the inhibition of urea synthesis by acetazolamide, mefruside and xipamide can be overcome by increasing the extracellular HCO_3^- and CO_2 concentrations above 50 mM and 2.4 mM, respectively. Linear regression analysis of the data given in Fig. 4A–C yielded similar threshold HCO_3^- additions, required to overcome fully the inhibitory effect of the diuretics: these values were 237, 269 and 233 μmol/g/min for acetazolamide, mefruside and xipamide, respectively, corresponding to

Table 2. Effect of xipamide (0.3 mM) and acetazolamide (0.5 mM) on the tissue levels of citrulline, 2-oxoglutarate, glutamate and citrate

Condition	Citrulline	2-Oxoglutarate	Glutamate	Citrate
	(μmol/g wet weight)			
Control	0.84 ± 0.25	0.251 ± 0.023	1.72 ± 0.17	0.107 ± 0.013
Xipamide	0.27 ± 0.01*	0.035 ± 0.007	2.46 ± 0.26*	0.044 ± 0.009
Acetazolamide	0.18 ± 0.03	0.053 ± 0.009	2.72 ± 0.15	0.062 ± 0.003

Livers were perfused with Krebs–Henseleit buffer containing NH_4Cl (1 mM), ornithine (2 mM), lactate (0.3 mM) and pyruvate (0.3 mM). Freeze-stops were taken at the 30 min of perfusion (control). In the case of xipamide (0.3 mM) or acetazolamide (0.5 mM), these compounds were added from min 15 to 30. Data are given as means ± SEM and are from 4 to 6 different perfusion experiments. Comparison of control data with the data obtained in presence of xipamide or acetazolamide, respectively, by Student-*t*-test analysis yielded high statistical significance (if not indicated otherwise $P < 0.01$).

* $P < 0.05$.

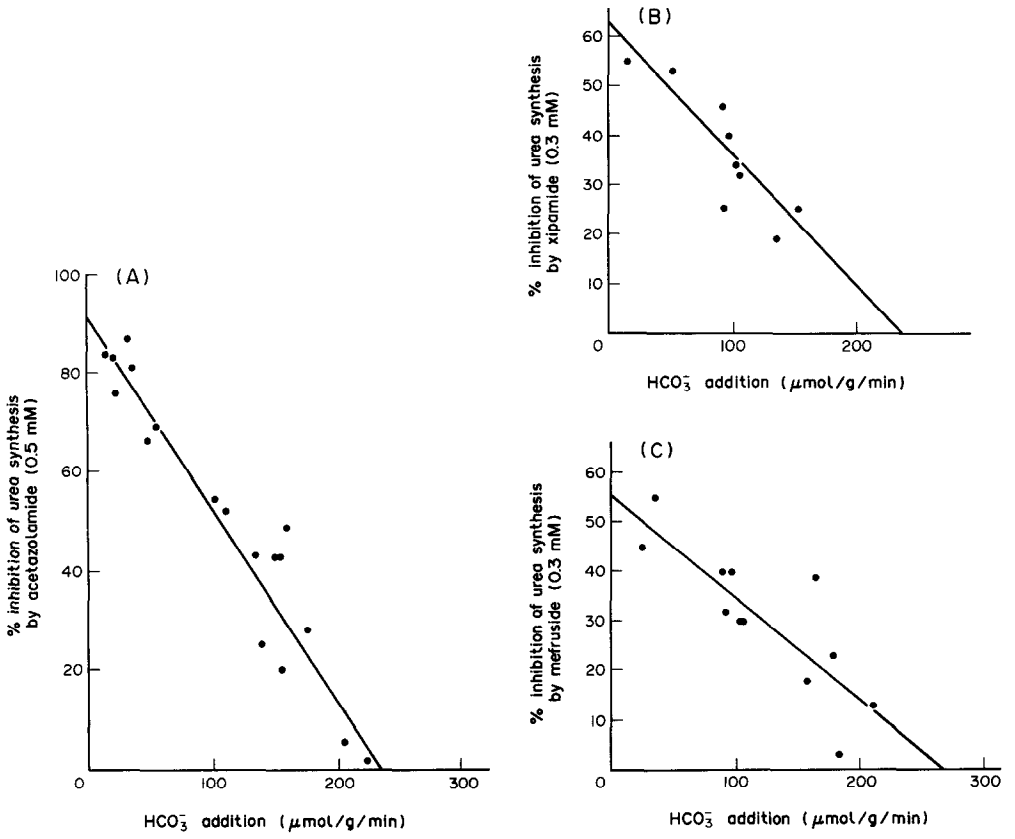


Fig. 4. Effect of extracellular CO_2 and HCO_3^- on the inhibition of urea synthesis by acetazolamide (A), xipamide (B) or mefruside (C). HCO_3^- and CO_2 concentrations in the perfusate were varied in parallel that the HCO_3^-/CO_2 ratio was kept constant (pH 7.4). An HCO_3^- addition of 100 μmol/g/min corresponds to an HCO_3^- concentration of about 25 mM in influent perfusate. The concentrations of acetazolamide, xipamide and mefruside were 0.5 mM, 0.3 mM and 0.3 mM, respectively. Regression analysis of these data yielded correlation coefficients of −0.95 (A), −0.86 (B), and −0.83 (C).

influent HCO_3^- and CO_2 concentrations of 55–65 mM and 2.6–3.1 mM. These data suggest that the inhibition of urea synthesis by xipamide and mefruside is due to an inhibition of mitochondrial carbonic anhydrase and is not explained by an unspecific inhibition, e.g. of one of the urea cycle enzymes. Whereas for acetazolamide a maximal inhibition of urea synthesis of about 90% can be extrapolated from Fig. 4A, only a 50–60% inhibition is observed for xipamide and mefruside. This is explained by the submaximal concentration of the latter diuretics, whereas acetazolamide was used at a maximal inhibitory concentration.

DISCUSSION

The data in this paper indicate that several diuretics like xipamide and mefruside impair hepatic urea synthesis due to an inhibition of mitochondrial carbonic anhydrase, the enzyme providing HCO_3^- for carbamoylphosphate synthesis. This is of interest because the diuretic effect of these drugs is not explained by an inhibition of renal carbonic anhydrase [16, 17]. Although the concentrations of these drugs employed in this study are above the therapeutic plasma concentrations, an inhibition of urea synthesis by about 5–10% is observed (not shown) at xipamide concentrations of about 30 μM, i.e. concentrations found in cirrhotic patients [23]. Such a small inhibition of urea cycle flux, however, may be sufficient to induce hyperammonemia and hepatic encephalopathy in cirrhotic patients, whose urea cycle enzyme activity is already considerably impaired [22]. In agreement with data obtained in animal studies [7, 8] mitochondrial carbonic anhydrase is also required for urea synthesis in human liver (Fig. 2).

Thus, this study suggests that hyperammonemia, induced by several diuretics may be explained by an impaired hepatic urea synthesis, in addition to the proposed increased ammonia production by the kidney [2, 3]. Such an inhibition of urea synthesis will not only lead to hyperammonemia, but also to a decreased HCO_3^- removal by the liver, in line with the proposed role of the liver in HCO_3^- homeostasis and systemic pH regulation [8, 12–15]. To what extent an inhibition of mitochondrial carbonic anhydrase by these diuretics may also affect gluconeogenesis due to a diminished HCO_3^- provision for pyruvate carboxylase is a matter of speculation.

Such a phenomenon could partly explain the observed (Table 2) decrease of the tissue levels of citric acid cycle intermediates, like citrate and 2-oxoglutarate, by acetazolamide or xipamide.

Interestingly, furosemide does not inhibit urea synthesis despite its sulfonamide- and chloro-substituted aromatic ring (Scheme 1), a structural feature also shared by mefruside, chlorothiazide and chlorotalidone, i.e. diuretics which inhibit urea synthesis. The reason for this is unclear, it may, however, be related to a different permeability of these drugs across the plasma and mitochondrial membranes.

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REFERENCES

1. H. O. Conn, in *The Liver* (L. Schiff and E. Schiff) (Eds), pp. 847–977. J. Lippincott, Philadelphia (1982).
2. J. M. Baertl, S. M. Sancetta and G. J. Gabuzda, *J. Clin. Invest.* **42**, 696 (1963).
3. G. R. Onstad and L. Zieve, *Gastroenterology* **77**, 803 (1979).
4. S. J. Dodgson, R. E. Forster II, B. T. Storey and L. Mela, *Proc. natl. Acad. Sci. U.S.A.* **77**, 5562 (1980).
5. D. Hewett-Emmett, R. G. Cook and S. J. Dodgson, *Isozyme Bull.* in press.
6. T. H. Maren, A. C. Ellison, S. K. Fellner and W. B. Graham, *Molec. Pharmac.* **2**, 144 (1966).
7. S. J. Dodgson, R. E. Forster II, D. A. Schwed and B. T. Storey, *J. Biol. Chem.* **258**, 7696 (1983).
8. D. Häussinger and W. Gerok, *Eur. J. Biochem.* **152**, 381 (1985).
9. J. A. Elder and A. L. Lehninger, *Biochemistry* **12**, 976 (1983).
10. J. B. Chappel and A. R. Crofts, in *Regulation of Metabolic Processes in Mitochondria* (Eds. J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater), pp. 292–314. Elsevier, Amsterdam (1966).
11. G. Guthörlein and J. Knappe, *Eur. J. Biochem.* **8**, 207 (1969).
12. D. E. Atkinson and M. N. Camien, *Curr. Top. Cell. Reg.* **21**, 261 (1982).
13. D. E. Atkinson and E. Bourke, *Trends Biochem. Sci.* **7**, 197 (1984).
14. D. Häussinger, W. Gerok and H. Sies, *Trends Biochem. Sci.* **7**, 300 (1984).
15. D. Häussinger, W. Gerok and H. Sies, *Biochem. J.* **226**, 261 (1986).
16. J. E. F. Reynolds (Ed.) *Martindale. The Extra Pharmacopoeia.* Pharmaceutical Press, London.
17. R. Greger, in *Diuretika* (Eds. H. Knauf and E. Mutschler) pp. 3–17. Urban & Schwarzenberg, München (1986).
18. H. Sies, *Methods Enzymol.* **52**, 48 (1978).
19. H. A. Krebs, W. Bartley, D. E. Griffiths and L. A. Stoke, in *Tissue Preparations* in vitro (Ed. H. Rauen). p. 522, Springer Verlag. Heidelberg (1964).
20. H. U. Bergmeyer, (Ed.) *Methoden der enzymatischen Analyse*, 3rd. Edn., Verlag Chemie Weinheim (1974).
21. D. Häussinger, L. Weiss and H. Sies, *Eur. J. Biochem.* **52**, 421 (1975).
22. K. P. Maier, H. Talke and W. Gerok, in *Aminosäuren, Ammoniak und hepatische Enzephalopathie* (Eds. F. Wewalka & B. Dragosics), pp. 33–38. Fischer Verlag Stuttgart (1978).
23. E. Mutschler, H. Knauf and H. Spahn, in *Diuretika* (Eds. H. Knauf and E. Mutschler), pp. 57–72. Urban & Schwarzenberg München (1986).