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UREA SYNTHESIS BY PERFUSED RAT LIVER— STUDIES OF CCl_4-INDUCED CIRRHOSIS

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Abstract—Previous studies in man and experimental animals suggest that urea production is decreased in cirrhosis. In the present study, NH_4Cl solutions of increasing concentrations were infused into isolated, perfused livers of normal rats and the rates of ammonia uptake and urea production were calculated for each pre-hepatic ammonia concentration. A pattern of saturation kinetics was generated and characterized through computer analysis. The V_{max} was 7.4 μmoles/min and K_m was 86.3 μmoles/100 ml. The livers of rats with experimental cirrhosis induced by carbon tetrachloride produced less urea than controls (controls: 0.40 ± 0.03; cirrhotic 0.25 ± 0.02 μmole/min/g of wet liver weight; $P < 0.005$) at infusion rates of NH_4Cl, which resulted in ammonia levels approximately twice the K_m value. Simultaneously performed taurocholate transport studies also revealed significant functional differences between cirrhotic and control livers. In summary, this study delineates a model to evaluate the kinetics of urea synthesis from NH_4Cl in the isolated perfused rat liver. It was found that the livers of rats with CCl_4-induced cirrhosis exhibited impaired ammonia utilization and urea production together with abnormalities of taurocholate transport.

Urea is the principal metabolic end product of nitrogen metabolism. In patients with liver disease an impairment of urea synthesis is indicated by elevated concentrations of blood ammonia and by depressed concentrations of urea in blood. Two investigators [1, 2] have attempted to measure in man the maximal rates of urea synthesis in liver disease after oral protein ingestion or amino acid infusion. The impairment in urea synthesis measured in this manner has been found to be proportional to the severity of liver disease, and the results [2] have been used as criteria to select patients for surgery designed to decompress gastroesophageal varices. In these studies, however, the amount of substrate presented to the liver could not be carefully controlled.

The present studies were designed to develop an experimental model to characterize urea synthesis in cirrhosis. In order to assure strict control of the amount of substrate presented to the liver, the studies were performed in the isolated perfused rat liver; ammonium chloride (NH_4Cl) was used as the metabolic precursor. It was found that isolated perfused cirrhotic livers produced less urea than normal livers at NH_4Cl infusion rates, which resulted in ammonia levels twice the K_m value.

METHODS

Sprague–Dawley rats (50–60 g) were subjected to a 23-week course of injections of carbon tetrachloride to induce cirrhosis according to the methods of Rubin *et al.* [3] and Aterman [4]. Half of the rats were injected subcutaneously with 0.15 ml of 50% carbon tetrachloride in mineral oil and the other half (which served as the control group) were injected similarly with mineral oil alone. In both control and experimental rats the injections were given twice a week. Approximately 90 per cent of the animals developed cirrhosis as assessed by gross and and microscopic inspection of the liver, and occasionally, the presence of ascites. The animals were given food and water *ad lib.* and were studied at least 4 weeks after the cessation of the injections.

Liver perfusion. The animals were anesthetized with sodium pentobarbital i.p. and surgically prepared for liver perfusion using a liver perfusion system previously reported from this laboratory [5]. The perfusion media (100 ml) consisted of washed dog red blood cells (20% hematocrit) and 4 g/100 ml of fraction V bovine serum albumin (Pierce Chemical Co., Rockford, IL) in Kreb's bicarbonate buffer, pH 7.4. The perfusion rate was 6.5 ml/min. The perfusate was oxygenated with humidified oxygen (95% with 5% CO_2), and the system was maintained at 38°. Substrate infusions were made with a Braun pump (B. Braun Apparatebau, Melsungen, West Germany).

In each experiment, a pair of test livers was attached to two separate recycling perfusion systems, 30 min prior to the starting of the experimental infusion. Perfusate samples were drawn at 10-min intervals during this pre-infusion period. At zero time, a 50-μmole NH_4Cl bolus was injected into each reservoir and the appropriate infusion begun. Pre- and post-hepatic samples were drawn and equal volumes of perfusate replaced for three 30-min infusion periods.

Taurocholate transport studies. These studies were performed simultaneously with those of urea production in order to demonstrate functional differences between cirrhotic and normal rat livers independent of urea metabolism. To characterize the kinetics of

taurocholate transport, a systematically varied solution containing a given concentration of tauro-[carbonyl-^{14}C]cholic acid, sp. act. 54 mCi/m-mole (Amersham/Searle, The Radiochemical Centre, Amersham, England), was infused into liver perfusions of both control and cirrhotic rats. Simultaneous samples of bile and pre- and post-hepatic perfusate were collected for measurement of radioactivity using a liquid scintillation spectrometer and for calculation of rates of hepatic uptake and biliary secretion for taurocholate. Uptake was determined utilizing the Fick principle by multiplying the perfusate flow rate times the difference in the pre- and post-hepatic perfusate concentration. Biliary secretion was estimated by multiplying bile flow times the calculated concentration of taurocholate in the sample.

Urea synthesis. Normal rat livers were perfused with NH_4Cl at increasing rates (0.8 to 20 μmoles/min). Pre- and post-hepatic samples were obtained to determine ammonia uptake and urea production for each pre-hepatic ammonia concentration.

The rate of NH_4Cl infusion chosen for measurement of the urea production in normal and cirrhotic livers was 8.3 μmoles/min. This rate of infusion achieved perfusate ammonia concentrations approximately twice the K_m value.

Ammonia was determined using the Berthelot color reaction as modified by Chaney and Marbach [6, 7]. Urea and glucose were determined simultaneously by automated methods of chemical analysis using the methods of Hoffman [8] and Marsh *et al.* [9]. Perfusate potassium was analyzed using an IL flame photometer. The data are presented as mean ± S. E. and were evaluated by means of Student's *t*-test [10]. The pH was measured with a Corning pH meter.

RESULTS

Typical light microscopic changes of cirrhosis were found in sections taken from the liver of all the animals given carbon tetrachloride. The weight of cirrhotic animals at the time of perfusion averaged 377 ± 17 g and the controls 415 ± 35 g ($P < 0.05$). Liver weights were 15 ± 1 g in cirrhotic and 12 ± 1 g in control animals ($P < 0.02$).

Taurocholate transport studies. A pattern of saturation kinetics was apparent (Fig. 1) in the taurocholate transport studies. The best-fit hyperbolic curve was determined by computer analysis [11]. The maximal secretory rate determined (V_{max}) was 234 nmoles/g of liver weight/min and the K_m was found to be 260 nmoles/ml. Hepatic uptake of taurocholate significantly exceeded biliary secretion at a taurocholate infusion rate of 1.7 μmoles/min.

Studies were then carried out in six additional control and six cirrhotic rat livers in a manner similar to that described previously in normal livers using a taurocholate infusion rate of 1.7 μmoles/min. In cirrhotic animals (Table 1), both heptatic uptake (60 ± 4 nmoles/min/g) and biliary secretion (74 ± 6 nmoles/min/g) of taurocholate were lower ($P < 0.001$ and < 0.005 respectively) than in controls.

Measurement of urea synthesis. Figure 2 describes the changes in urea production when NH_4Cl was infused at varying rates (0.8 to 20 μmoles/min) into

Table 1. Transport of taurocholate by control and cirrhotic livers

	Control* (N = 6)	P value	Cirrohotic* (N = 6)
Bile flow (μl/min)	202 ± 11	< 0.01	156 ± 10
Pre-hepatic concn (nmoles/ml)	527 ± 33	< 0.01	750 ± 63
Biliary secretion (nmoles/min/g)	112 ± 9	< 0.001	60 ± 4
Hepatic uptake (nmoles/min/g)	141 ± 15	< 0.005	74 ± 6

* Values represent mean ± S.E.

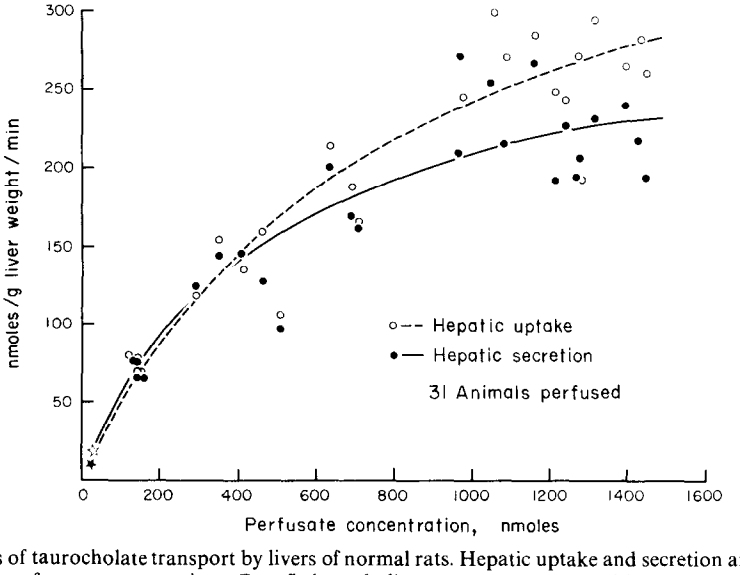

Fig. 1. Kinetics of taurocholate transport by livers of normal rats. Hepatic uptake and secretion are plotted vs pre-hepatic perfusate concentrations. Best-fit hyperbolic curves were generated by computer analysis.

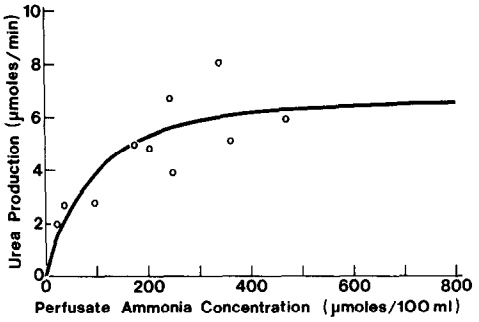

Fig. 2. Kinetics of urea production from NH_4Cl by perfused livers of normal rats. Urea production (in μmoles/min) is plotted vs pre-hepatic perfusate ammonia concentrations. K_m and V_{max}, and hyperbolic plot were determined by computer analysis.

the recycling perfusate of normal rat livers. It is apparent that, when the hepatic production of urea was plotted against inflow perfusate ammonia concentration, a pattern of saturation kinetics was obtained. The V_{max} was 7.4 μmoles/min and the K_m was 86.3 μmoles/100 ml.

The average rate of urea production achieved during the 90 min of perfusion at an NH_4Cl infusion rate of 8.3 μmoles/min was higher in control perfusions as compared to those performed using livers of cirrhotic rats (control: 0.40 ± 0.03; cirrhotic: 0.25 ± 0.02 μmoles/min/g; $P < 0.005$). When the results were not corrected for wet liver weight, control livers also had a higher ($P < 0.025$) rate of urea synthesis (control: 4.5 ± 0.2; cirrhotic: 3.7 ± 0.23 μmoles/min). Although the mean body weight of the cirrhotic rats was lower than that of controls, when individual animals were matched for body weight (three controls and four cirrhotics), urea production, again, was significantly lower ($P < 0.02$) in the cirrhotic group (control: body weight 360 ± 16, urea production 0.42 ± 0.02; cirrhosis: body weight 361 ± 10 g, urea production 0.25 ± 0.04 μmoles/min/g).

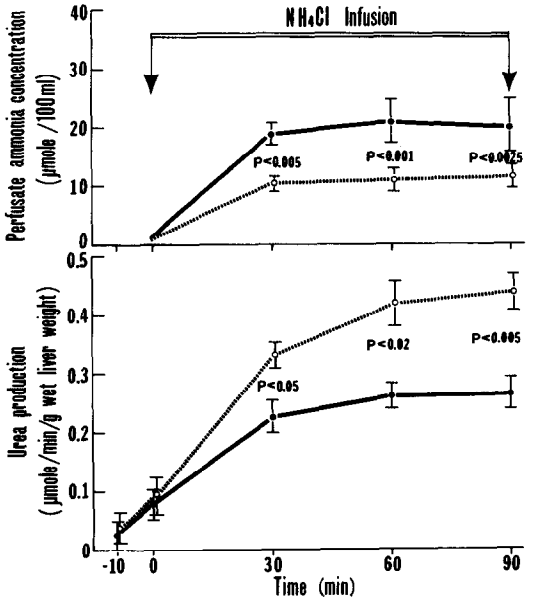

Fig. 3. Sequential changes in urea production and perfusate ammonia concentration during NH_4Cl infusion. Each point represents the mean ± S.E. of six experiments. The solid lines connect the points (closed circles) of the cirrhotic livers, the dashed lines those of controls (open circles).

Figure 3 illustrates the sequential changes in mean urea production and perfusate ammonia concentration during the 90-min perfusion. It can be appreciated that, at each sampling period, urea production was higher in normal than in cirrhotic livers, while the opposite was true for ammonia concentrations.

There was no apparent deterioration of the liver perfusions, as evidenced by measurements of glucose output [12], pH changes [13] and A-V potassium differences [14]. The viability of the livers used in our experiment was also demonstrated by the stability of urea synthesis with time (Fig. 3).

DISCUSSION

The present study describes an animal model designed to assess urea synthesis. This model utilizes the technique of isolated liver perfusion, which permits strict control of substrate presented to the liver. Using NH_4Cl as substrate, a pattern of saturation kinetics was observed. The model was applied to the study of urea synthesis in experimental cirrhosis. The results confirm previous studies suggesting that the rate of urea synthesis is decreased in cirrhosis.

The liver weight in the cirrhotic animals was significantly greater that in the controls, due in part to increased fibrous and/or fatty tissue. Thus, it is possible that expressing the data of cirrhotic animals per gram of wet liver weight could have underestimated urea production. However, when urea production was not corrected per liver weight, the differences in urea production were still significant.

In general, our data cannot be compared with previously reported studies of urea synthesis in the isolated perfused rat liver [15–19] since experimental protocols differ widely and, except for one study [19], the rates of urea production were not characterized kinetically. Saheki and Katunuma [19] studied urea synthesis by isolated perfused rat liver using NH_4Cl and glutamine as nitrogen sources. In their studies, however, NH_4Cl was not given by constant infusion and their liver perfusion technique using recycled perfusate did not permit a constant rate of perfusate delivery to the liver. They found that the rate of urea formation increased with administration of NH_4Cl concentration up to 5 mM. Their observed K_m value of 70 μmoles/100 ml was in close agreement with the K_m value of 86.3 μmoles/100 ml found in the present study. At higher NH_4Cl infusion rates, removal of ammonia from the medium exceeded the formation of urea nitrogen, a finding that was also observed in the present study.

Hems *et al.* [17] studied the time course of urea formation and ammonia removal in the perfused rat liver. Addition of 10 mM NH_4Cl led to a rapid removal of ammonia from the medium at a rate of 1.4 μmoles/min/g and urea appeared in the medium at a steady rate of 0.8 μmole/min/g. Chamalaun and Tager [16]

showed that the basic rate of urea synthesis by perfused rat liver was stimulated by nitrogen donors. Alanine stimulated the system by a factor of about 1.5, glutamine by a factor of approximately 2 and NH_4Cl by a factor of about 4.5. The rates of urea synthesis found by these authors are similar to those reported in the present study and far exceeded those which can possibly be reached under physiologic or liverless conditions, where rates are limited by the quantity of substrate available [20].

Barak and Beckenhauer [15] found that, in general, fatty livers and livers from azo-dye-fed animals were not as efficient as normal livers in producing urea from ammonium salts, amino acids or a combination of both. Henley *et al.* [18], using a different model of experimental cirrhosis (induced by feeding rats a low content of protein, choline and vitamin B_{12} and a high content of fat), studied nitrogen metabolism in the perfused cirrhotic liver. In their experiment, cirrhotic livers produced greater quantities of ammonia from endogenous sources and from added alanine than controls.

Two studies in man have attempted to measure maximum rates of urea synthesis immediately after protein ingestion. Rudman *et al.* [2] found a maximal rate of urea synthesis of 65 mg urea N/hr/kg of body weight in normal subjects given either an oral protein formula or an intravenous infusion of amino acids. In the patients with cirrhosis, the maximal rate of urea production was 27 mg urea N/hr/kg or body weight. Among patients fed different protein intakes, the occurrence of hyperammonemia and encephalopathy at each level of protein intake was related inversely to the maximal rate of urea synthesis. On the other hand, Raforth and Onstad [1] could not demonstrate a maximal rate of urea synthesis after a single protein meal in subjects, even at rates higher than the maximum rate reported by Rudman *et al.* [2]. Patients with cirrhosis demonstrated impairment of urea synthesis proportional to the degree of impairment of liver function.

The present *in vitro* studies using the perfused rat liver are in agreement with the studies of Rudman *et al.* [2] establishing that urea synthesis is decreased in liver disease. The amount of substrate delivered to the liver in the present studies was controlled carefully, unlike the above-mentioned studies in humans utilizing exogenous amino acids or proteins. The study also confirms and amplifies studies in isolated perfused cirrhotic livers [15–18] which demonstrated decreased urea synthesis after a single bolus injection of a precursor.

The data presented in this paper provide no information as to the pathogenesis of the observed abnormalities or to the particular step(s) of urea biosynthesis impared in cirrhosis. It is possible that the nutritional state of the animals influenced our results. For instance, it is possible that a more abundant glycogen supply in normal as compared to cirrhotic livers could have resulted in a more readily available source of metabolic energy for urea production, In addition, it remains undefined whether K_m and/or V_{max} is changed in livers of cirrhotic rats.

In summary, the present studies delineate an *in vitro* animal model that permits a kinetic evaluation of urea production in hepatic disease states and the study of the effect of drugs, hormones, and protein intake on protein metabolism as well. Several aspects of hepatic function were evaluated in perfused livers of rats with CCl_4-induced cirrhosis. Using NH_4Cl infusion rates which resulted in perfusate ammonia levels twice the K_m value, it was found that the livers of rats with experimental cirrhosis produced less urea than controls.

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