The acceptor strength of the present compound is intermediate between that of the related but weaker π-acid, 1,2,3-indanetrione (II) and that of tetracyanoethylene (III) ($E_A = 1.70$ ev).

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9. Solvent was dried before use. The polarographic measurements were made with a Sargent polarograph Model 21, a Sargent IR Compensator Model A, and a dropping-mercury electrode.
10. In Eq. 3, $E_{\frac{1}{2}}$ is taken as positive.
11. Measurements of ESR were taken with a standard Varian V-4500 spectrometer (with a modulation of 100 kc/sec) and a magnet (9 cm). Electrolysis was conducted between platinum and silver-silverperchlorate reference electrodes at 1.0 to 1.12 volts.
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24 May 1967

Lungfish Neoceratodus forsteri: Activities of Ornithine-Urea Cycle and Enzymes

Abstract. *The level of activity of the ornithine-urea cycle is low in the liver of the permanently aquatic Australian lungfish. The rate of incorporation of ^{14}C-bicarbonate into urea by liver slices was only 100th of that previously observed in the estivating African lungfish* Protopterus dolloi. *The activities of enzymes of the ornithine-urea cycle were similarly reduced. The low activity of this cycle in* Neoceratodus *is consistent with its exclusively aquatic nature.*

Members of the two surviving families of lungfishes, Lepidosirenidae and Ceratodontidae, differ from one another in ecological behavior. The African *Protopterus* and the South American *Lepidosiren*, belonging to the former family, must breathe air at rather frequent intervals and they estivate in burrows out of water during dry seasons. The sole surviving member of the latter family, the Australian *Neoceratodus forsteri*, appears to use its lung only as an accessory respiratory organ during periods of high activity, and it cannot survive deprivation of water by burrowing and breathing air as can the African and South American dipnoans (*1*). Ability of the liver to synthesize urea by way of the ornithine-urea cycle was recently demonstrated in the African lungfish *Protopterus* (*2, 3, 4*). This cycle has survival value in *Protopterus* during its periods of estivation, when ammonia cannot be excreted and is incorporated into urea. We tried to determine whether *Neoceratodus*, which never leaves the water and therefore has no need to detoxify ammonia, possesses a functional ornithine-urea cycle.

Two adult *Neoceratodus* were obtained from Australia (*5*). The fish, weighing 2.8 and 5.4 kg and in good condition, were immediately placed in separate fiberglass tanks containing aged tap water at 20° to 30°C. During the next 2 days samples of the water were analyzed for ammonia (*6*) and urea (*7*). The rates of excretion of ammonia and urea in fish *X* were 98 and 5.5 μmole kg^{-1} hour^{-1}, respectively. In fish *Y* the rate of excretion of ammonia was 156 μmole kg^{-1} hour^{-1}; no urea was detectable in the bath water. *Neoceratodus* appears to be much more "ammoniotelic" than *P. ethiopicus*, which eliminates up to 50 percent of its nitrogenous end products as urea when in water (*8*).

Within 2 days of arrival the animals were pithed and the livers were removed immediately and assayed for (i) the presence of a functional ornithine-urea cycle in liver slices, and (ii) the individual enzymes of the cycle. Urea-cycle activity of liver slices was assayed by following the incorporation of ^{14}C-bicarbonate into urea (Table 1; *4*). The activities of carbamoyl phosphate synthetase, ornithine carbamoyltransferase (*Enzyme Nomenclature*, 2. 1.3.3), argininosuccinate synthetase (6. 3.4.5), argininosuccinate lyase (4.3.2.1), and arginase (3.5.3.1) were assayed in liver homogenates according to Janssens and Cohen (Table 1; *3*). Carbamoyl phosphate synthetase, being barely detectable by the colorimetric assay, was assayed by another method (*9*). Enzyme activities were calculated from the ^{14}C incorporated into citrulline, relative to the specific activity (counts per minute per micromole) of the ^{14}C-bicarbonate substrate.

The percentage incorporation of ^{14}C-bicarbonate into urea was very low (Table 1), being 0.01 to 0.02 percent of the radioactivity present in the medium —about 100 count/min (above background) as ^{14}C-urea. The average production of ^{14}C-urea by liver slices from both fish was 480 count/min g^{-1} hour^{-1}. The boiling of liver slices for 5 minutes reduced the incorporation of ^{14}C-bicarbonate into urea to one-tenth of the value observed in untreated slices (fish *X*, Table 1). Addition of unlabeled citrulline to the incubation medium, which was expected to reduce the ^{14}C incorporated into urea by competing with ^{14}C-citrulline formed from ^{14}C-bicarbonate, had little effect on the rate of synthesis of ^{14}C-urea. On the other hand, unlabeled arginine reduced the rate of synthesis of ^{14}C-urea to about one-third of that for untreated slices.

The difference in effectiveness of unlabeled citrulline and arginine as inhibitors of synthesis of ^{14}C-urea may be related to relative rates of uptake or to differences in pool size of the two

Table 1. Incorporation of ^{14}C-labeled bicarbonate into urea by liver slices of *Neoceratodus*. Approximately 200-mg slices were incubated in 3.0 ml of Krebs-Ringer solution (diluted 2 parts to 3 with water) containing $10^{-2}M$ ammonium chloride, $10^{-2}M$ sodium lactate, $10^{-3}M$ L-ornithine, and ^{14}C-labeled bicarbonate (6.8×10^5 count/min). The mixtures were shaken for 1 hour in 100-percent oxygen at 30°C.

Fish	Modification of assay	Incorporation (%)*	^{14}C-urea (count min^{-1} g^{-1} hr^{-1})	Urea synthesis† (μmole g^{-1} hr^{-1})
Neoceratodus X	None	0.01‡	382	3.8×10^{-3}
Neoceratodus X	Slices boiled	.001	34	
Neoceratodus X	L-Citrulline, $10^{-2}M$	.01	352	
Neoceratodus X	L-Arginine, $10^{-2}M$	.004	120	
Neoceratodus Y	None	.02	576	5.8×10^{-3}
Protopterus (4)§				6.9×10^{-1}

* [Count min^{-1} in urea/count min^{-1} in incubation medium] $\times$ 100. † Calculated by dividing (count min^{-1} g^{-1} hr^{-1}) by specific activity of ^{14}C-bicarbonate (10^5 count min^{-1} μmole^{-1}) in medium. ‡ Average of duplicate assays. § Six fish, average.

Table 2. Activities of enzymes of the ornithine-urea cycle in *Neoceratodus* liver. Carbamoyl phosphate synthetase determined by radioactive assay (see text). BLD, below limit of detection.

Fish	Carbamoyl phosphate synthetase	Ornithine carbamoyl-transferase	Arginino-succinate synthetase	Arginino-succinate lyase	Arginase
Micromoles of product per gram of tissue per hour					
Neoceratodus X	0.45	156	BLD	12.6	1530
Neoceratodus Y	.30	154	4.3	8.5	1048
Protopterus (*3*) (two fish, av.)	31.2	1675	6.6	56.8	34,800
Micromoles of product per milligram of protein per hour					
Neoceratodus X	0.006	2.88	BLD	0.231	28.1
Neoceratodus Y	.004	1.94	0.067	.134	16.4
Protopterus (*3*) (two fish, av.)	.63	37.7	.16	1.33	795

amino acids in the liver slices. In any event the inhibitory effect of unlabeled arginine suggests that ^{14}C-bicarbonate is entering urea by way of the ornithine-urea cycle. The rate of synthesis of urea by liver slices of *Neoceratodus* is 3.8 to 5.8 $\times$ 10^{-3} μmole g^{-1} $hour^{-1}$ (Table 1)—approximately 100th of that observed in *P. dolloi* (Table 1) and consistent with the low rate of excretion (production) of urea in *Neoceratodus*. Since the ratios of liver weight to body weight are similar in *Neoceratodus* and *Protopterus* (0.01), the rate of synthesis of urea per kilogram of body weight in *Neoceratodus* is 100th of that in *Protopterus*.

The presence of all enzymes of the ornithine-urea cycle was demonstrated in extracts of livers from *Neoceratodus* (Table 2; Fig. 1). The levels of these enzymes are very low compared to those previously found in *P. ethiopicus* (Table 2). The activity of carbamoyl phosphate synthetase, which appears to be rate-limiting for synthesis of urea in *Neoceratodus* liver, is about 100th of that of *Protopterus*. The difference in activity of carbamoyl phosphate synthetase between *Neoceratodus* and *Protopterus* corresponds to the difference in rate of incorporation of ^{14}C-bicarbonate into urea by liver slices (Table 1).

Carbamoyl phosphate synthetase from *Rana* (*10*) and *Protopterus* (*3*) is dependent on *N*-acetyl-L-glutamate for activity, but *Neoceratodus* liver showed activity even in the absence of added *N*-acetyl-L-glutamate (Fig. 1). Liver extracts did not activate purified carbamoyl phosphate synthetase from frogs. Enzyme activity in *Neoceratodus* liver in the absence of added *N*-acetyl-L-glutamate, therefore, cannot be explained by a high concentration of activator in the liver of this species unless the K_m for *N*-acetyl-L-glutamate in *Neoceratodus* is very much below that in the frog. Resolution of this problem must await purification of carbamoyl phosphate synthetase from *Neoceratodus* liver, which was rendered impossible by the extremely low activity of this enzyme and the small amount of tissue available.

It is significant from an ecological point of view that *Neoceratodus* has the ability, albeit very limited, to incorporate ammonia into urea by way of the ornithine-urea cycle present in its liver. On the other hand, the enzymes of this cycle also function in biosynthesis of pyrimidine and amino acid (*11*), and their presence in this dipnoan may be more related to the latter functions. It is interesting that carbamoyl phosphate synthetase of mouse spleen, which is thought to function mainly in biosynthesis of pyrimidine in this organ, is not activated by *N*-acetyl-L-glutamate (*12*).

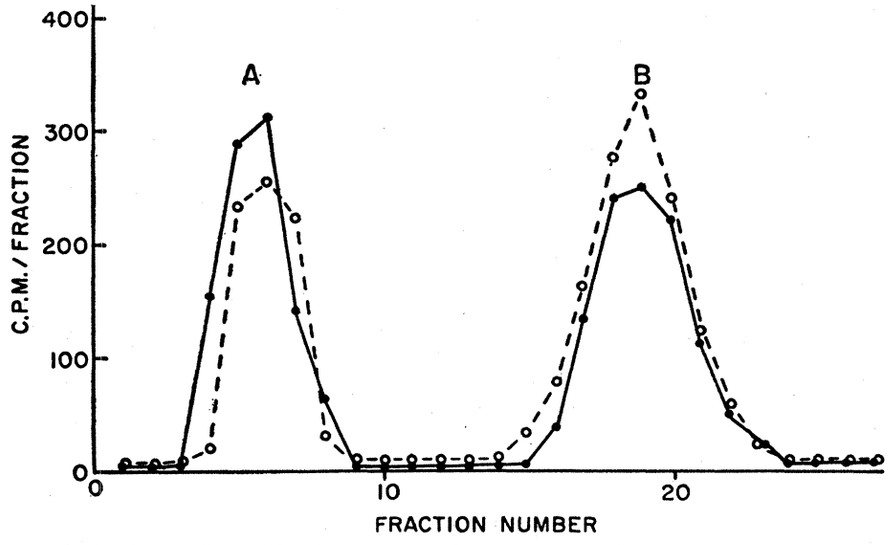

Fig. 1. Synthesis of citrulline by extracts of *Neoceratodus* liver. The complete system contained 500 μmole of ammonium bicarbonate, 42 μc of ^{14}C as 1.55 μmole of sodium bicarbonate, 50 μmole of adenosine triphosphate, 50 μmole of L-ornithine, 50 μmole of *N*-acetyl-L-glutamate, 100 μmole of $MgSO_4$, 300 μmole of glycylglycine buffer (*p*H 7.5), and 600 units of purified beef ornithine carbamoyltransferase in a final volume of 10 ml. The incubation (30 minutes at 38°C) was terminated by addition of 10 ml of 10-percent trichloroacetic acid, and CO_2 was bubbled through the medium for 1 hour. After centrifugation, 20 μmole of L-citrulline was added to the supernatant, which was chromatographed on a 1- by 10-cm column of Dowex 50 (H^+). Gradient elution with HCl was employed (*9*). Fractions (5-ml) were collected and assayed for citrulline (*13*) and radioactivity (liquid scintillation). Solid circles, complete system; open circles, complete system minus *N*-acetyl-L-glutamate. The identity of peak *A* was not investigated; peak *B* corresponds to the elution pattern of citrulline.

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14. Supported by NSF grants GB5613 and G-21237 and PHS grant HE-04457.

28 April 1967