method for the estimation of ornithine. There is practically quantitative recovery of added ornithine by the paper-electrophoretic method, so that the low recovery by Chinard's method cannot be ascribed to binding of amino acid by liver solids.

Comparison between the regression coefficients and the percentage recoveries shows that in all instances the two agree within the limits of experimental error. Little or no weight can be placed on the differences which are shown, but it is of interest that the trend is for regression coefficients to be less than recoveries for ornithine and greater than recoveries for citrulline, so that there is a slight suggestion that citrulline penetrates into liver water more rapidly than ornithine.

We may conclude from this work that, at the concentrations of ornithine and citrulline ordinarily used in the study of urea synthesis, the intracellular concentration of both amino acids becomes indistinguishable from the external concentration within 15 min. of addition of the amino acid. In experiments such as those of Gornall & Hunter (1943) involving incubations of 2 hr. there can be no question of differences of cell permeability contributing appreciably to the differences in response to ornithine and citrulline.

It might, however, appear to be possible to invoke a permeability factor in connexion with penetration of the amino acids into intracellular particles, but such an explanation of the lower effectiveness of citrulline has to contend with the demonstration by Cohen & Hayano (1948) that the enzymes converting citrulline into arginine occur in the non-particulate fraction of the cytoplasm. That is, any intracellular permeability barrier would be expected to operate to the disadvantage of ornithine.

SUMMARY

1. Methods are described for concentration and separation by paper electrophoresis of ornithine and citrulline, and for their subsequent determination by the ninhydrin procedure of Harris *et al.* (1954).

2. These methods, and those of Chinard (1952) and Fearon (1939), have been applied to the study of the penetration of ornithine and citrulline into rat-liver slices.

3. Citrulline appears to penetrate a little more readily than ornithine. Both amino acids have almost the same concentration in liver water as in medium water after incubation for 15 min. at 37°.

4. No significant changes in the concentrations of the amino acids in liver water occur when the period of incubation is prolonged to 30 min.

5. The data do not support the view that ornithine is a more effective catalyst of urea synthesis than citrulline because it has easier access to liver cells.

REFERENCES

- Bronk, J. R. & Fisher, R. B. (1955). *Biochem. J.* **60**, xxix.
Chinard, F. P. (1952). *J. biol. Chem.* **199**, 91.
Christensen, H. N., Riggs, T. R., Fischer, H. & Palatine, H. M. (1952). *J. biol. Chem.* **198**, 17.
Cohen, P. P. & Hayano, M. (1948). *J. biol. Chem.* **142**, 405.
Fearon, W. R. (1939). *Biochem. J.* **33**, 902.
Gornall, A. G. & Hunter, A. (1941). *Biochem. J.* **35**, 650.
Gornall, A. G. & Hunter, A. (1943). *J. biol. Chem.* **147**, 593.
Harris, H., Mittwoch, U., Robson, E. R. & Warren, F. L. (1954). *Ann. hum. Genet., Lond.*, **19**, 196.
Krebs, H. A. (1952). *The Enzymes, Chemistry and Mechanism of Action*, vol. 2, part 2, p. 866. Ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
McIlwain, H. & Buddle, H. L. (1953). *Biochem. J.* **53**, 412.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
Stämpfli, R. (1945). *Helv. physiol. Acta*, **3**, 628.

The Role of Ornithine and Citrulline in Urea Synthesis

By J. R. BRONK* AND R. B. FISHER

Department of Biochemistry, University of Oxford

(Received 3 October 1955)

We have shown (Bronk & Fisher, 1956) that the anomalously greater effectiveness of ornithine, compared with that of citrulline, in catalysing synthesis of urea in rat-liver slices is not due to greater ease of access of ornithine to liver cells, as suggested by Krebs (1952). A possible alternative

explanation is that ornithine and citrulline catalyse separate processes producing urea. This paper describes experiments made to test this possibility.

METHODS

Biological material. Liver slices from rats and guinea pigs (average weights were 250 and 600 g. respectively) were used. The animals were fed on laboratory stock diet until

* Present address: Laboratory of Cellular Physiology, National Heart Institute, Bethesda, Maryland, U.S.A.

required. Liver slices were cut at a nominal thickness of 0.225 mm. on a McIlwain & Buddle (1953) tissue slicer, and were rapidly washed with about 250 ml. of warm, oxygenated Krebs's bicarbonate-lactate medium (Krebs & Henseleit, 1932) before use.

Incubation procedure. The liver slices were incubated in Krebs's bicarbonate-lactate medium containing NH_3 (100 $\mu\text{moles}/10\text{ ml.}$) which was equilibrated with $\text{O}_2 + \text{CO}_2$; (95:5). Vessels of the type shown in Fig. 1 were used for the incubation. Each vessel normally contained 10 ml. of medium and 100 mg. of slices, which were kept suspended by a gentle stream of gas from the capillary at the bottom of the tube. A trace of tributyl citrate prevented frothing, and did not interfere with urea synthesis.

At the end of incubation (in a water bath at 38°) the gas flow was stopped and a ground-glass stopper inserted into the mouth of the tube in place of the air condenser. The tube could then be disconnected from the gas line without loss of medium. The capillary was inserted into a half-bored rubber bung and the glass stopper removed. The tubes, which were designed to fit a standard 25 ml. centrifuge cup, were then centrifuged. The supernatant fluid was poured off, and the tube, which had previously been weighed, was reweighed after thorough draining to give the wet weight of slices. The tube was dried in an oven at 105° overnight and reweighed to give the dry weight of slices.

The technique used here for obtaining liver weights probably gives higher estimates of weight, and therefore lower estimates of metabolic activity, than do more usual methods of recovering slices. We have washed slices which had been incubated for 60 min. in such a way that anything which can pass a 0.2 mm. mesh is carried off in the washings. About 10% of the solids can be separated from these washings by mild centrifuging. This fraction of the

solids is included in the liver weight which we measure, but would not be included in an estimate of liver weight made by picking slices out of the medium.

Estimation of urea. Urea was estimated by the method of Conway (1947) on 0.5 ml. samples of medium diluted with an equal volume of 0.2M phosphate buffer, pH 6.8. A 1:10 dilution of a glycerol extract of melon-seed meal ('Urease active meal from melon seeds', British Drug Houses, Ltd.) made by the method of Schmidt (1928) was used.

Ornithine and citrulline. L-Ornithine hydrochloride and L-citrulline (Roche Products, Ltd.) were used. These amino acids were estimated by the method of paper electrophoresis described by Bronk & Fisher (1956).

Units. Rates of urea synthesis are expressed in this paper as μmoles of urea formed/100 mg. dry wt. of tissue/hr. This unit is denoted by R_{urea} .

Reliability of the estimates of rates of urea synthesis. Since the method adopted for urea estimation depends on three independent measurements (free NH_3 , total NH_3 after hydrolysis by urease, and NH_3 in the urease preparation) it was thought advisable to check the reliability of the analytical procedure by an analysis of variance of some of the observations. Three sets of data have been analysed. These consist of 9, 6 and 6 pairs of duplicate determinations in three different sets of conditions, three pairs of observations having been made on the same animal in each set. The three sets of data yielded mean R_{urea} values of 21.24, 18.95 and 2.53 respectively, with standard errors of means of duplicates of 1.69, 1.78 and 0.36. The standard errors are all about 10% of the corresponding mean R_{urea} . Thus, in general, the standard error of the mean effect of a treatment based on three sets of duplicate determinations should have a standard error of about 6% of the mean rate of urea synthesis. Some of the comparisons between different experiments reported in this paper differ more widely than would be expected from this analysis, but this is due to differences between animals, which can be quite large.

RESULTS

Relative effectiveness of ornithine and citrulline as catalysts of urea formation

We found, as Table 1 shows, that if 20 μmoles of urea is added to the 10 ml. of incubation medium it suppresses the urea formation in the absence of added catalyst almost completely, without appreciably affecting the rates of urea formation in the presence of added ornithine or citrulline.

This indicates that the effect of added catalyst is not properly measured by taking the difference between the rate in the presence of catalyst and the rate observed in the blank. We have made a practice in this work of using the addition of urea as a routine and disregarding any correction for the blank.

The results of a series of determinations of rates of urea synthesis in these conditions, in the presence of different concentrations of ornithine and citrulline, are shown in Fig. 2. The number by each point refers to the number of observations at each concentration of catalyst.

Fig. 1. Apparatus used for incubation and quantitative recovery of liver slices.

Table 1. *Effect of the addition of urea on the rate of urea synthesis by rat-liver slices*

About 100 mg. quantities wet wt. of liver in 10 ml. of medium were incubated at 38° for 30 min. in the presence of 100 μ moles of ammonia. R_{urea} = μ moles of urea/100 mg. dry wt. of tissue/hr.

Amount of urea added to 10 ml.	R_{urea} in the presence of		
	No catalyst	Ornithine (5.9 μ moles)	Citrulline (22.8 μ moles)
None	8.7 $\pm$ 0.54 (21)	16.9 $\pm$ 0.72 (11)	26.7 $\pm$ 1.13 (9)
20 μ moles	2.3 $\pm$ 0.27 (10)	16.4 $\pm$ 0.59 (18)	25.5 $\pm$ 0.91 (20)

Fig. 2. Relation between catalyst concentration and rate of urea synthesis. Incubation for 30 min. of 100 mg. of liver in 10 ml. with 20 μ moles of urea and 100 μ moles of ammonia. Figures by points are numbers of observations. Vertical lines are 95 % fiducial limits.

These data may be considered in two ways. In the first place, in the one instance in which nearly identical concentrations of the two catalysts can be compared (11.4 μ moles of citrulline and 11.8 μ moles of ornithine/10 ml.), the rate with ornithine as catalyst is highly significantly in excess of the rate with citrulline as catalyst, being roughly twice as great. In the second place, an R_{urea} in the region of 17 is obtained with 5.9 μ moles of ornithine/10 ml., but requires 11.4 μ moles of citrulline/10 ml., and the mean rates found with 22.8 and 34.2 μ moles of citrulline/10 ml. bracket the value found with 11.4 μ moles of ornithine/10 ml. Thus in the conditions of these experiments ornithine is roughly twice as effective as citrulline in catalysing urea synthesis.

Time course of urea synthesis

Nothing is known of the speed at which a steady state is attained in the synthesis of urea by liver slices. Experiments were therefore made with equimolar concentrations (11.4 μ moles/10 ml.) of ornithine and citrulline, and measuring the urea

Table 2. *Time course of urea formation*

Catalyst concentration was 11.4 μ moles/10 ml. in all experiments. Urea (20 μ moles) and ammonia (100 μ moles) were added initially to each tube.

Catalyst	Expt.	Urea formed (μ moles/100 mg. dry wt. of tissue)		
		0-15 min.	0-30 min.	0-45 min.
Ornithine	1	3.28	10.46	20.85
		3.29	9.54	15.97
	2	2.24	9.22	14.34
		1.98	8.95	13.96
Citrulline	3	3.54	5.17	10.27
		1.58	5.02	9.38
	4	1.94	7.90	11.48
		1.06	7.38	10.07

Fig. 3. Rates of urea synthesis in three successive 15 min. periods. The concentration of catalyst was 11.4 μ moles/10 ml. 100 mg. of liver, 20 μ moles of urea, and 100 μ moles of ammonia were used in all experiments. The figures are means of two duplicate experiments.

synthesized in 15, 30 and 45 min. periods. The data are given in Table 2, and the rates for the successive 15 min. periods derived from these data are shown in Fig. 3. An analysis of variance of the data in Table 2 yields estimates of the standard errors of the mean R_{urea} in each 15 min. interval. These are ± 3.43 for the ornithine series and ± 2.34 for the citrulline series. There is, therefore, no significant difference between the rates in the second and third 15 min. periods for either catalyst, and we may take the

mean rates over the 15–45 min. interval as estimates of steady-state rates. The analysis of variance yields a value of ± 2.93 for the standard error of the difference between these steady-state rates for the two catalysts, and the observed difference is 10.82. The estimate of error is based on 18 degrees of freedom and the difference is therefore highly significant.

Since, as Fig. 3 shows, the mean rate in the first 15 min. is about half the steady-state rate for citrulline but only about one-third of the steady-state rate for ornithine, these observations indicate that the process catalysed by citrulline attains a steady state more rapidly than does the process catalysed by ornithine. However, the steady-state rate attained is only about half as great with citrulline as with an equimolar concentration of ornithine.

Another set of time-course observations was made in which a lower concentration of ornithine (5.9 μ moles/10 ml.) and a higher concentration of

citrulline (22.8 μ moles/10 ml.) were compared. In this set it is again true that the rate in the first 15 min. is a distinctly larger fraction of the rate in the second 15 min. for citrulline than for ornithine. Thus over the range of catalyst concentrations that is of interest, it seems likely that the steady-state rate is always more rapidly attained with citrulline, the less effective catalyst. The results of this second set of observations are given in Fig. 4, together with a series of observations in which liver slices were incubated with ammonia for 15 min. before the addition of 5.9 μ moles of ornithine/10 ml. Although this pre-incubation with ammonia markedly increased the rate of urea synthesis it did not affect the lag in attainment of the steady-state rate: the ratio of mean rate in the second 15 min. to that in the first 15 min. was 1.61:1 when the slices were pre-incubated with ammonia and 1.67:1 in the absence of pre-incubation.

*Effect of pre-incubation with ammonia
on urea synthesis*

Three sets of experiments were performed. In all sets the rate of urea formation over 30 min. periods was measured in controls and in the presence of ornithine (5.9 μ moles) or citrulline (22.8 μ moles), one set of observations being made in which catalyst and ammonia were added simultaneously and the reaction stopped at 30 min., whilst in the other set catalyst was added 15 min. after addition of ammonia, the reaction being stopped after a further 30 min. The control in this set was incubated with ammonia for 45 min., instead of 30 min. as in the first set. In the first series (series A), no urea was added to the medium. In the second series (series B), 20 μ moles of urea was added with the catalyst. In the third series (series C), urea was added 15 min. before the catalysts. The results of these experiments are given in Table 3. It will be seen that the effect of

Fig. 4. Rates of urea synthesis in two successive 15 min. periods; 5.9 μ moles of ornithine and 22.4 μ moles of citrulline were used. 'Pre-incubation' means that ammonia was added 15 min. before the catalyst. Other conditions as in Fig. 3.

Table 3. *Effect of pre-incubation with ammonia on the rate of urea synthesis in rat-liver slices*

In all experiments, liver slices were incubated for 15 min. before adding catalyst, and for 30 min. after adding it. P, addition of ammonia 15 min. before catalyst; N, addition of ammonia at same time as catalyst. In series A, no urea was added; in series B 20 μ moles of urea was added at the same time as catalyst; in series C 20 μ moles of urea was added at the beginning of incubation.

Series		R_{urea} in the presence of		
		No catalyst	Ornithine (5.9 μ moles)	Citrulline (22.8 μ moles)
A	P	15.2 $\pm$ 0.67 (7)	31.7 $\pm$ 2.06 (7)	31.7 $\pm$ 4.82 (2)
	N	8.7 $\pm$ 0.54 (21)	16.9 $\pm$ 0.72 (11)	26.7 $\pm$ 1.13 (9)
	P - N	6.5	14.8	5.0
	Difference from control	—	8.3	-1.5
B	P	7.0 $\pm$ 1.61 (3)	20.5 $\pm$ 1.61 (3)	30.3 $\pm$ 3.40 (3)
	N	2.0 $\pm$ 0.51 (3)	16.9 $\pm$ 0.96 (3)	28.8 $\pm$ 3.83 (3)
	P - N	5.0	3.6	1.5
	Difference from control	—	-1.4	-3.5
C	P	3.3 $\pm$ 2.23 (2)	15.5 $\pm$ 0.36 (2)	22.7 $\pm$ 2.90 (2)
	N	2.6 $\pm$ 1.12 (2)	11.6 $\pm$ 0.51 (2)	18.6 $\pm$ 0.96 (2)
	P - N	0.7	3.9	4.1
	Difference from control	—	3.2	3.4

Table 4. *Ornithine disappearance, urea formation and changes in citrulline content of medium and liver*

Liver slices (about 100 mg.) were incubated for 30 min. in 10 ml. of medium containing 100 μ moles of ammonia, with and without ornithine.

Ornithine added (μ moles)	Ornithine disappearing (μ moles)	Citrulline found (μ moles)		Urea formed (μ moles)
		Medium	Liver	
None	Nil	0.72 ± 0.051 (6)	0.22 ± 0.028 (6)	5.47 ± 0.49 (6)
5.9	1.52 ± 0.073 (6)	0.60 ± 0.062 (6)	0.16 ± 0.023 (6)	10.40 ± 1.03 (6)
Difference	1.52	-0.16	-0.06	4.93

Table 5. *Citrulline disappearance, urea formation and changes in ornithine content of medium and liver*

Liver slices (about 100 mg.) were incubated for 30 min. in 10 ml. of medium containing 100 μ moles of ammonia, with and without citrulline.

Citrulline added (μ moles)	Citrulline disappearing (μ moles)	Ornithine found (μ moles)		Urea formed (μ moles)
		Medium	Liver	
None	Nil	0.10 ± 0.017 (6)	0.065 ± 0.003 (6)	5.47 ± 0.49
22.8	2.35 ± 0.615 (4)	0.55 ± 0.011 (4)	0.24 ± 0.014 (4)	13.15 ± 0.12
Difference	2.35	0.45	0.17	7.68

pre-incubation cannot be described simply. In the absence of urea, pre-incubation naturally increases the urea formation in the control since the liver slices are in contact with ammonia for a longer period. In these circumstances the effect of pre-incubation on the ornithine reaction catalysed by ornithine is much greater than can be accounted for by its effect on the control, but there is no greater effect on the reaction catalysed by citrulline than can be accounted for by effect on the control. Series *B* and *C* show that the longer the time for which added urea is present in the system, the less the effect of pre-incubation on the controls. Urea also abolishes the differences in response between the reaction catalysed by ornithine and the reaction catalysed by citrulline. The effects of urea in these experiments are of as much interest as those of pre-incubation with ammonia, since the urea concentration used was very low, being about 40 % of the physiological concentration in rat-tissue water.

Fate of ornithine and citrulline in liver slices

With methods available (Bronk & Fisher, 1956) for the estimation of ornithine and citrulline that are more specific than those of Chinard (1952) and Fearon (1939) we have used them to study the fate of these amino acids in liver slices.

Table 4 shows the changes in apparent citrulline content of slices and medium in the presence and absence of added ornithine, together with the amount of ornithine disappearing and the amount of urea formed. Table 5 gives the corresponding changes in apparent ornithine content of slices and medium in the presence and absence of added citrulline, together with the amount of citrulline disappearing and the amount of urea formed.

It has already been shown (Bronk & Fisher, 1956) that the amounts of preformed apparent ornithine and citrulline found in liver slices are not affected by prolonging the period of incubation from 15 to 30 min. On the other hand, the data of Tables 4 and 5 show that appreciable amounts of added amino acid are destroyed in 30 min., these amounts being 10–20 times as great as the total preformed apparent ornithine or citrulline in the slices. Thus the preformed apparent ornithine and citrulline behave quite differently metabolically from authentic amino acid added to the medium. This strengthens our previous conclusion that the apparent ornithine and citrulline found preformed in liver slices is contaminant material.

The data in Tables 4 and 5 give conclusive evidence that both ornithine and citrulline act catalytically in increasing the rate of urea synthesis. In both groups of observations more than three times as much urea was produced as amino acid lost.

The percentage of added ornithine disappearing (Table 4) is 26 % (1.52 out of 5.9 μ moles added), and the absolute amount disappearing is very little less than the amount of citrulline which disappears (Table 5) at a much higher initial concentration (2.35 out of 22.8 μ moles, or 10 %). These changes are large relative to the amount of contaminant material present and can be safely attributed to real changes in ornithine and citrulline content. Thus in both instances the mean concentration of catalyst does not change greatly from the initial value during an incubation period of 30 min., and citrulline probably disappears more slowly in comparable conditions than does ornithine.

In the presence of added ornithine (Table 4) there is no indication of an increase in the apparent citrulline content of the liver or medium. On the other hand, there is an increase in apparent ornithine content when citrulline is added (Table 5). However, this increase is small and cannot be attributed with certainty to a true increase in ornithine content.

Summation experiments with ornithine and citrulline

If the two catalysts participate in the same cycle, it is to be expected that, in a system not saturated with one catalyst, the addition of the other will increase the rate of urea synthesis. The same effect would be expected, but more markedly, amounting probably to an exact summation, if the two catalysts

Table 6. *Comparison of rates of urea synthesis in rat-liver slices in the presence of ornithine alone, of citrulline alone, and of ornithine and citrulline*

All incubations were for 30 min. in the presence of 20 μ moles of urea and 100 μ moles of ammonia. In Expts. 1 and 2, 5.9 μ moles of ornithine and 22.8 μ moles of citrulline were used. In Expts. 3 and 4, 11.8 μ moles of ornithine and 11.4 μ moles of citrulline were used.

Expt.	Ornithine	R_{urea} in the presence of	
		Citrulline	Citrulline and ornithine
1	17.4	21.3	21.2
	18.0	20.0	18.4
2	15.7	30.9	25.7
	15.9	28.3	24.7
Mean*	16.8 $\pm$ 1.48	25.1 $\pm$ 1.48	22.5 $\pm$ 1.48
3	24.8	19.3	26.0
	23.6	17.4	20.1
4	32.5	18.1	24.1
	31.4	22.8	25.7
Mean*	28.1 $\pm$ 1.31	19.4 $\pm$ 1.31	24.0 $\pm$ 1.31

* In both series of experiments there is a significant difference between the pairs of animals used. The standard errors of means given here are based on the residual variance, after eliminating effects of treatments and of difference between animals. In each series there are 8 degrees of freedom available for calculation of residual variance.

Table 7. *Comparison of rates of urea synthesis in guinea-pig liver in the presence of ornithine alone (5.9 μ moles), of citrulline alone (22.8 μ moles) and of citrulline and ornithine*

Incubations were for 30 min. in the presence of 20 μ moles of urea and 100 μ moles of ammonia.

Ornithine	R_{urea} in the presence of	
	Citrulline	Ornithine and citrulline
15.5	15.7	15.0
15.1	18.1	14.4

participated in completely independent cycles. However, as Table 6 shows, the rate of urea synthesis with the two catalysts present is lower in rat liver than the higher of the rates found with one of them alone. The first pair of experiments shown in this table was designed so that the higher rate was obtained with citrulline, the second pair so that the higher rate was obtained with ornithine. In both instances, the mean rate obtained with the two catalysts is intermediate.

Table 7 shows that in guinea-pig liver the rate with the two catalysts together is lower than with either alone. Although we have not made a full study of the relation between catalyst concentration and rate of urea synthesis for guinea-pig liver, the data in Table 6 suggest that ornithine is again the more effective catalyst here. With a citrulline concentration four times that of ornithine, the two rates of urea synthesis are the same.

DISCUSSION

The work described in this paper confirms the finding of Gornall & Hunter (1943) that ornithine is more effective than citrulline as a catalyst of urea synthesis in rat liver, and suggests that the same is true of guinea-pig liver. The conditions and technique of the present experiments differ from those of Gornall & Hunter at many points. Urea was added to our incubation media, our experiments lasted for 30 min. instead of 120 min., our procedure for estimating liver-slice weight gave a maximum estimate, and we used four times as much liver per unit volume of medium as did Gornall & Hunter. It is not surprising therefore that our estimates of activity of liver differ from theirs. Despite the many differences, the same relation between the activities of the catalysts appears.

The observations on time-course are of the greatest importance, since they effectively exclude hypotheses which seek to account for the greater effectiveness of ornithine on a basis of differential access to a single cycle. Whether the hypothesis takes the form of differential access to the interior of cells (which we have already excluded, Bronk & Fisher, 1956), differential access to intracellular inclusions, or differential rates of conversion into derivatives which are the effective catalysts, the expectations are: (1) that the difference in observed rates of urea synthesis will be caused by a greater lag in the attainment of the steady state in the reaction catalysed by citrulline; and (2) that when equimolar concentrations of the catalysts are used the same steady-state rate will be attained in both instances.

The data of Figs. 3 and 4 make it quite clear that the process catalysed by citrulline does not lag behind the one catalysed by ornithine in the approach to a steady state, and that the steady-state

rate with citrulline is of the order of half that attained with an equimolar concentration of ornithine.

The only other hypothesis which would permit one to retain the notion of a single cycle involving ornithine and citrulline is that the catalysts enter the cycle in the form of derivatives, and that free citrulline can be destroyed rapidly in liver cells. Tables 4 and 5 provide evidence on this point. They indicate that neither amino acid disappears very rapidly and that citrulline probably disappears more slowly than ornithine. Thus we may say that it is difficult, if not impossible, to account for the facts on a single-cycle hypothesis.

Pre-incubation with ammonia before addition of the catalyst produces an increase in the rate of the reaction catalysed by ornithine, but has no effect on the reaction catalysed by citrulline. It is difficult to see how this treatment could affect one catalysed process without affecting the other if ornithine and citrulline were directly concerned in the same cycle.

Against this background, the observation that ornithine and citrulline can mutually inhibit each other's catalysis of urea synthesis is positive evidence for two cycles. This phenomenon is inconsistent with any explanation of either catalytic process which involves, as an essential step, the conversion of either ornithine or citrulline into the other. Every revolution of a chemical catalytic cycle involves equally all of its chemical components, so that an increase in the rate of catalysis involves an increase in the concentration of all the components. In conditions such as those of the present experiments, in which it is possible to increase the rate of the reaction catalysed by ornithine by increasing the concentration of ornithine, there must be a corresponding increase in the concentration of all the components of the ornithine cycle. If citrulline, as such, were a component of the ornithine cycle, we should have this position: that increasing the concentration of citrulline by adding ornithine increased the rate of urea synthesis although, as experiment shows, increasing the concentration of citrulline by adding citrulline to ornithine decreases the rate of urea synthesis. There seems, therefore, to be no escape from the conclusion that citrulline itself is not a component of the ornithine cycle. Similar arguments indicate that ornithine is not a member of the citrulline cycle.

On the other hand, the same observations that lead to this conclusion point to a close relation between the two cycles. If the two were totally distinct the mutual inhibition observed in our experiments should not occur. It is also very difficult to believe that the mass of evidence for the partial reactions of the Krebs & Henseleit (1932)

scheme which has been gathered in recent years (see for instance, Cohen, 1954; Krebs, 1952; Ratner, 1954) is without relevance to the nature of the cycles operating in the intact cell. Although we can adduce no specific evidence at the present juncture, we believe that all the observations could be reconciled if we were to postulate two cycles, each of the general nature of the Krebs & Henseleit (1932) cycle, but differing in that in both instances the catalysts ornithine and citrulline enter the cycle after a specific transformation. It is known that [α -N]-substituted arginine derivatives can be hydrolysed by arginase (Felix, Müller & Dirr, 1928; Hunter, 1938), so that if ornithine were to undergo transformation to one such derivative and if citrulline were to undergo some different transformation, then a basis could be found both for the anomalous ratio of effectiveness of the catalysts and for their mutual interference.

This type of explanation is shown diagrammatically in more detail in Fig. 5. In this figure the postulated two series of derivatives of ornithine, citrulline and arginine are shown as OX, CX, AX and OY, CY, AY respectively, and it is suggested that, whilst both ornithine and citrulline can enter each set, ornithine enters the one set more readily and citrulline the other. If ornithine enters the set having the higher natural rate of catalytic cycling, this will account for its greater effectiveness. The assumption that both ornithine and citrulline can compete for the two transforming enzymes, though only one of them can be transformed at a high rate by each enzyme, will mean that the net rate of transformation of the two, and therefore the total rate of entry of them into the cycles, will be reduced when both ornithine and citrulline are added to the system.

Fig. 5. Scheme of two catalytic cycles for formation of urea, devised to account for the greater effectiveness of ornithine and for the competition between ornithine and citrulline. Continuous lines indicate relatively rapid reaction; broken lines indicate relatively slow reaction. OX, CX, AX are one set of derivatives of ornithine, citrulline and arginine capable of interconversion into one another, and OY, CY, AY is another, similar set.

We suggest in Fig. 5 that arginase is common to the two cycles. Although the remaining steps of the two cycles are shown separately in this figure, there seems to be no objection to the assumption that the enzymes catalysing the remaining steps are common to the two cycles, provided that in physiological conditions these enzymes, as is the case with arginase, are likely to be far from the point of saturation. Thus provided that the enzymes of the partial systems that have been investigated in the last ten years are capable of transforming simple derivatives of ornithine and citrulline corresponding to our OX, OY, etc., this scheme provides a way of reconciling the whole body of work up to date on the mechanisms of urea synthesis.

SUMMARY

1. It has been confirmed that ornithine is a more effective catalyst of urea synthesis in rat liver than citrulline, and some evidence has been obtained that the same is true of guinea-pig liver.

2. Low concentrations of urea (2 mM) suppress urea formation in the absence of added catalyst and differentially affect the urea synthesis catalysed by ornithine and that catalysed by citrulline.

3. The greater effectiveness of ornithine is not due to a smaller susceptibility to destruction by liver.

4. Despite the greater effectiveness of ornithine, the reaction catalysed by ornithine reaches a steady state more slowly than the reaction catalysed by citrulline, and therefore measurements of mean rate

of synthesis from the time of addition of catalyst underestimate the relative effectiveness of ornithine. The steady-state rate attained with ornithine is significantly higher than that attained with an equimolar amount of citrulline.

5. Incubation of liver slices with ammonia before adding catalyst has different effects on the reaction catalysed by ornithine and reaction catalysed by citrulline.

6. Ornithine and citrulline interfere with each other's catalytic reactions.

7. A new scheme of urea synthesis is proposed, based on these observations.

REFERENCES

- Bronk, J. R. & Fisher, R. B. (1956). *Biochem. J.* **64**, 106.
 Chinard, F. P. (1952). *J. biol. Chem.* **199**, 91.
 Cohen, P. P. (1954). *Chemical Pathways in Metabolism*, vol. 2, p. 36. Ed. by Greenberg, D. M. New York: Academic Press Inc.
 Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed., p. 133. London: Crosby Lockwood and Son.
 Fearon, W. R. (1939). *Biochem. J.* **33**, 902.
 Felix, K., Müller, H. & Dirr, K. (1928). *Hoppe-Seyl. Z.* **178**, 192.
 Gornall, A. G. & Hunter, A. (1943). *J. biol. Chem.* **147**, 593.
 Hunter, A. (1938). *Biochem. J.* **32**, 826.
 Krebs, H. A. (1952). *The Enzymes, Chemistry and Mechanism of Action*, vol. 2, part 2, p. 866. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press, Inc.
 Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
 McIlwain, H. & Buddle, H. L. (1953). *Biochem. J.* **53**, 412.
 Ratner, S. (1954). *Advanc. Enzymol.* **15**, 319.
 Schmidt, E. G. (1928). *J. biol. Chem.* **69**, 435.

The Relation of Ammonia Concentration to Urea Synthesis

By J. R. BRONK* AND R. B. FISHER
Department of Biochemistry, University of Oxford

(Received 3 October 1955)

In the course of attempts to establish that the processes catalysed by ornithine and by citrulline which lead to urea synthesis in rat-liver slices were distinct, some experiments were made on the effect of variation of ammonia concentration on the rate of urea synthesis. These experiments were made in a restricted range of conditions, but it is not possible at present to extend the observations. The data show that the relation between ammonia concentration and rate of urea synthesis is more complex than has hitherto been thought.

We have also made observations on the relation between ammonia utilization by liver and the rate of

urea synthesis in a variety of conditions. These experiments disclose a number of new features of ammonia metabolism in liver.

METHODS

The methods used have been described in detail in the two previous papers (Bronk & Fisher, 1956*a, b*). Urea was estimated as NH_3 released by melon-seed urease, and the determination of free NH_3 was an essential part of this method. Thus, provided that the amount of NH_3 originally added to the incubation medium containing liver slices was known, NH_3 utilization could be computed from the data obtained during the urea determinations.

Glutamic acid and aspartic acid were separated from each other and from other amino acids by the paper-electro-

* Present address: Laboratory of Cellular Physiology, National Heart Institute, Bethesda, Maryland, U.S.A.