CONVERSION OF CITRULLINE TO ORNITHINE BY PLEUROPNEUMONIALIKE ORGANISMS[1]

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A study of the breakdown of amino acids in an attempt to demonstrate their role as energy sources of pleuropneumonialike organisms has shown that glutamine can be deamidated in the presence of adenosine diphosphate (ADP) with the generation of adenosine triphosphate (ATP). The equilibrium of this reaction has been found to favor the generation of high energy phosphate (Smith, 1957). Previous demonstration of the quantitative conversion of arginine to citrulline (Smith, 1955*a*) prompted the assumption that citrulline might undergo conversion to ornithine also with the generation of ATP similar to that observed in some species of bacteria (Oginsky, 1955; Slade, 1955; Korzenovsky, 1955). Subsequently, an examination of the degradative activity of pleuropneumonialike organisms of human origin toward citrulline revealed that such a reaction occurs. The results of the study are presented in this paper.

MATERIAL AND METHODS

Strains 39 and 07 were grown and harvested as previously described (Smith, 1955*a*). Cell free extracts were prepared by subjecting suspensions of washed whole cells, containing one mg cellular nitrogen per ml, in saline or M/15 pH 7.0 tris (hydroxymethylaminomethane) buffer to sonic vibrations in a 9-Kc Raytheon magneto-constriction oscillator for a period of 20 min. Cellular debris was removed by centrifugation at 13,000 rpm for 5 min in a Servall angle centrifuge. Cell extracts were dialysed against pH 7.0 tris buffer at 4 C for 72 hr.

Enzymatic reactions were carried out at 37 C in Thunberg tubes or in the conventional Warburg apparatus employing the appropriate acetate or tris buffers for the time intervals of 30 min to 3 hr. Reactions were stopped by the addition of 10 per cent trichloracetic acid except for those reactions in which ATP was measured. In these instances aliquots of the reaction mixture were removed prior to the addition of acid and heated for 1 min in a boiling water bath.

Citrulline and ornithine were measured by quantitative paper chromatography as previously described (Smith, 1955*a*). Values of both amino acids were corrected for the amount of either amino acid contaminating a given commercial preparation. Ammonia was measured by the nesslerization reaction or by the Conway diffusion technique (Conway and Byrne, 1933). Inorganic phosphate was measured by the method of Fiske and SubbaRow (1925). ATP was measured by the enzymatic method of Kornberg (1950). Carbon dioxide evolution and oxygen uptake were measured in the conventional Warburg apparatus using air as the gas phase. Detection of hydroxamic acid formation was accomplished by measurement of the brown color which appears on the addition of ferric chloride (Lipmann and Tuttle, 1945). Carbamyl phosphate was determined by the method given by Jones *et al.* (1955).

All compounds except carbamyl phosphate were obtained from commercial sources. The lithium salt of carbamyl phosphate (51 per cent purity) was kindly supplied by Dr. S. Friedman, Harvard School of Dental Medicine. The impurity consisted of inorganic phosphate which was readily removed by centrifugation of the carbamyl phosphate solution in the cold.

EXPERIMENTAL RESULTS

Initial attempts to demonstrate the disappearance of citrulline and the formation of ammonia by whole cells were unsuccessful. These failures were assumed to be the result of the impermeability of whole cells to citrulline, since upon disruption of washed resting cells by sonic oscillation, liberation of ammonia occurred. Ornithine was identified as an end product of this reaction. This conversion of citrulline to orni-

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TABLE 1

Cofactor requirement for the conversion of citrulline to ornithine

Condition	ATP Formed
	μmoles
No ADP	0.2
No phosphate	0.6
No cell extract	0
Complete, 3.3 μmoles ADP	3.0
Complete, 6.6 μmoles ADP	7.0

Per tube: DL-citrulline, 50 μmoles; KH_2PO_4, 50 μmoles; ADP, sodium salt, 3.3 μmoles; L-cysteine, 10 μmoles; NaF, 10^{-3}M; M/15 pH 5.5 acetate buffer; dialyzed cell extract equivalent to 1 mg whole cell nitrogen. Final volume, 3.0 ml. Reaction time, 3 hr.

TABLE 2

Substitution of AMP for ADP in conversion of citrulline to ornithine

Condition	Change in Inorganic Phosphate
	μ moles
AMP	+1.5
AMP + citrulline	−7.1
ADP	+1.1
ADP + citrulline	−9.5

Per tube: Same as table 1, except 10 μmoles ADP or AMP.

thine was found not to be influenced by aerobic or anaerobic environments.

Dialysed cell extracts were shown to require ADP and inorganic phosphate as cofactors (table 1). When ADP was the limiting cofactor, increase of the amount of this compound resulted in an identical increase in ATP formation. Adenosine-5′-monophosphate (AMP) could be substituted for ADP as a phosphate acceptor although slightly better activity was noted with ADP. Uptake of inorganic phosphate in the presence of these two compounds is shown in table 2. Surprisingly, the addition of Mg^{++} was found to inhibit the reaction. Even after prolonged dialysis, levels of Mg^{++} as low as 10 μmoles completely inhibited the reaction. Attempts to substitute Mg^{++} with other cations or anions resulted in either inhibition of activity or no effect. In no instance was there stimulation of the reaction (table 3). It is possible that Mg^{++} is a requirement and dialysis was not sufficient to remove it. However, the great inhibitory effect of low doses of Mg^{++} either as the sulfate or chloride salt would seem to preclude such a possibility. The inhibitory activity probably could be attributed to stimulation of the reverse reaction, i. e., synthesis of citrulline, described later. The addition of cysteine to the reaction mixture frequently enhanced activity. Thus, it was routinely added to ensure a reduced environment. 2-Mercaptoethanol was slightly inhibitory and therefore could not replace cysteine satisfactorily.

The optimum pH for the conversion of citrulline to ornithine as measured by the amount of ATP formed, was pH 5.5 although almost equal activity was obtained at pH 6.0 (table 4).

TABLE 3

Effects of several anions and cations on conversion of citrulline to ornithine

Anion or Cation	ATP Formed
	μmoles
None	2.6
Mg^{++}, 50 μmoles	0
Mg^{++}, 10 μmoles	0
Mn^{++}	0
Co^{++}	0.5
$MoO_4^{=}$	0.9
Fe^{++}	1.3
Cu^{++}	1.3
Zn^{++}	1.3
Ca^{++}	2.4
$SO_4^{=}$	1.3

Per tube: Same as table 1, except 5.0 μmoles ADP; 10 μmoles anion or cation.

TABLE 4

Effect of pH on conversion of citrulline to ornithine

pH	ATP Formed
	μ moles
4.0	1.1
4.5	2.3
5.0	4.5
5.5	5.3
6.0	5.1
6.5	3.8
7.0	1.3
7.5	0.6
8.0	0.2

Per tube: Same as table 1, except 10 μmoles ADP; appropriate M/15 acetate or tris buffer.

The addition of various inhibitors to the reaction mixture gave results typical of phosphorylations. Those compounds which interfere with phosphorylation, arsenate, 2,4-dinitrophenol and fluoride completely inhibited the reaction, while sodium azide was without effect. All inhibitors were added to give a final concentration of 10^{-2} M, except fluoride which was 10^{-1} M. Iodoacetic acid was also inhibitory at a level of 10^{-2} M, a result which conforms to the finding that cysteine was required to permit the reaction to proceed satisfactorily and indicated that the enzyme system possessed sulfhydryl groups.

Substitution of phosphate with arsenate resulted in a typical arsenolysis reaction. As shown in figure 1, the addition of 10 μmoles of arsenate brought about rapid liberation of ammonia from citrulline in the presence of cell extract. Addition of sodium fluoride at 30 min caused complete cessation of further ammonia liberation. In the absence of arsenate and phosphate, no ammonia was evolved.

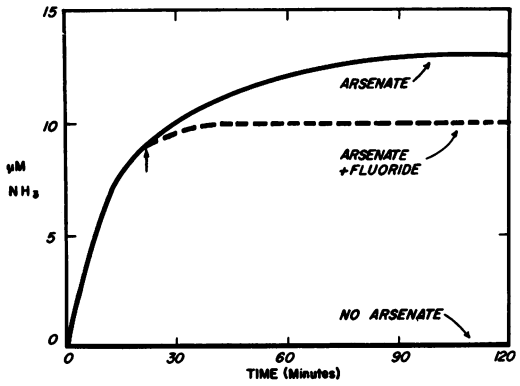

Figure 1. Arsenolysis of citrulline. DL-citrulline, 30 μmoles; ADP, 10 μmoles; L-cysteine, 10 μmoles; sodium arsenate, 10 μmoles; sodium fluoride, M, added at 30 min; M/15 pH 5.5 acetate buffer; cell extract equivalent to 1.0 mg whole cell N. Final volume, 3.0 ml.

TABLE 5

Reaction equilibrium of conversion of citrulline to ornithine

Reaction component...	CO_2	O_2	ATP	NH_3	PO_4	Citrulline	Ornithine
Change in μmoles	+3.1	0	+3.3	+2.9	−3.0	−3.6	+3.6

20 μmoles DL citrulline, 10 μmoles each of ADP and KH_2PO_4 at start; 10^{-3}M NaF. Final volume, 3.0 ml. Reaction time, 3 hr.

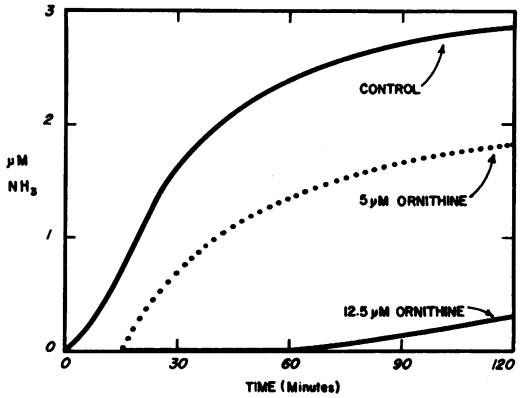

Figure 2. Inhibitory effect of ornithine on citrulline degradation. DL-citrulline, 50 μmoles; KH_2PO_4, 50 μmoles; ADP, 10 μmoles; L-cysteine, 10 μmoles; M/15 pH 5.5 acetate buffer; L-ornithine, as indicated; cell extract equivalent to 1.0 mg whole cell N. Final volume, 3.0 ml.

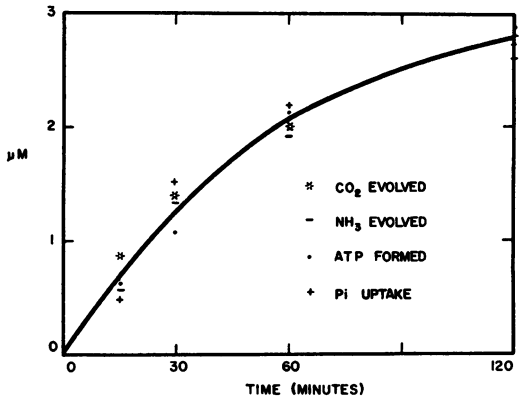

Figure 3. Rates of change of reaction components during citrulline degradation. DL-citrulline, 20 μmoles; ADP, KH_2PO_4 and L-cysteine, 10 μmoles each; sodium fluoride, 10^{-3} M; M/15 pH 5.5 acetate buffer; cell extract equivalent to 1.0 mg whole cell N. Final volume, 3.0 ml.

Stoichiometric analysis of the reaction revealed that for each mole of citrulline degraded, one mole of inorganic phosphate disappeared and one mole each of ornithine, ammonia, carbon dioxide and ATP were formed. The best activity obtained under optimal conditions was the conversion of about 5 μmoles of citrulline. The significance of this reaction as a means for supplying high energy phosphate did not appear to be great. Consequently, the equilibrium of the reac-

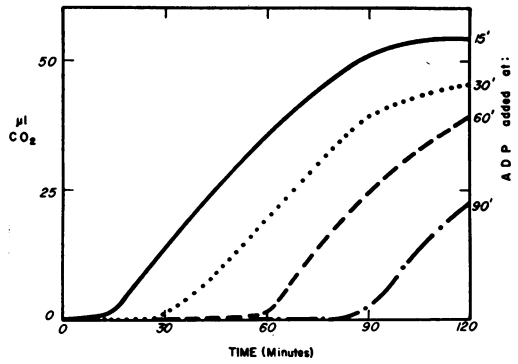

Figure 4. Effect of delayed addition of ADP on CO_2 evolution from citrulline. Contents of reaction vessels same as in figure 3.

tion was determined. Cell extracts were incubated in the presence of a low level of fluoride, with 10 μmoles each of KH_2PO_4 and ADP and 20 μmoles of DL-citrulline for 3 hr. An equilibrated state was apparent at the end of this time interval as demonstrated by the cessation of evolution of carbon dioxide. Analysis of the reaction components was performed on several samples from six determinations. Changes in the concentration of these components is shown in table 5. The equilibrium values for the conversion of citrulline to ornithine varied from 4.6×10^{-1} to 8.4×10^{-1}.

A further attempt was made to establish the significance of the conversion of citrulline to ornithine as a source of high energy phosphate by coupling the reaction with a system requiring ATP, i. e., glucose plus hexokinase. Since Mg^{++} was inhibitory to the citrulline conversion, none was added to the reaction mixture. Rather, it was hoped that by use of undialysed extracts enough Mg^{++} would be present to allow the phosphorylation of glucose to proceed. Indeed, in the presence of 0.5 μmoles of ADP, 2.2 μmoles of phosphate disappeared, while in the presence of 1.0 μmoles of ADP, 4.4 μmoles of phosphate was taken up. Although this activity did not appear great it was significant that phosphate turnover occurred in the presence of low levels of ADP.

An explanation of the poor activity with citrulline was obtained upon further study. Oginsky (1955) reported that ornithine inhibited the phosphorolysis of citrulline by *Pseudomonas aeruginosa*. Incorporation of varying levels of ornithine in the reaction mixture resulted in progressively reduced activity. Five μmoles of ornithine significantly inhibited the reaction and produced a lag, while 12.5 μmoles was almost completely inhibitory as measured by ammonia liberation (figure 2). From these results the degradation of more than 5 to 7 μmoles of citrulline could not be expected unless some means for removal of ornithine was present. Under the conditions optimal for citrulline conversion little or no ornithine was lost.

Accumulation of a phosphorylated intermediate would be indicative of an equilibrium favoring citrulline degradation. An analysis, made to determine the rates of appearance of carbon dioxide, ammonia, and ATP and the disappearance of inorganic phosphate, showed that the change in all four components occurred at essentially the same rate (figure 3). In the absence of any high energy phosphate acceptor, such as ADP, no change in the concentration of the initial components was detectable, nor was any ornithine formed. Upon addition of ADP at varying time intervals after the addition of citrulline to the reaction mixture, carbon dioxide was liberated at identical rates regardless of the period of preincubation (figure 4). If a phosphorylated intermediate had accumulated it would be expected that evolution of carbon dioxide would occur much more rapidly with increased preincubation time. These results tend to disfavor the accumulation of a phosphorylated intermediate.

Attempts to determine the existence of an intermediate phosphorylated compound by the incorporation of hydroxylamine in the reaction mixture to yield a hydroxamic acid resulted in negative findings. However, hydroxylamine proved to be inhibitory to the reaction at a level of 10 μmoles. Since Jones *et al.* (1955) iso-

TABLE 6

Degradation of carbamyl phosphate

Time	CO_2 Evolved
min	*μmoles*
5	5.4
15	6.7
30	7.7
60	8.9

Per cup: carbamyl phosphate, 10 μmoles; ADP, 10 μmoles; M/15 pH 5.5 acetate buffer; 0.6 mg cellular nitrogen as cell extract. Final volume, 3.0 ml.

TABLE 7

Rate of citrulline synthesis from ornithine and carbamyl phosphate

Time	Citrulline Formed
min	*μmoles*
5	1.5
10	6.4
15	13.2
30	12.0
60	14.3

Reaction mixture: L-ornithine, 25 μmoles; carbamyl phosphate, 15 μmoles; M/15 pH 7.0 tris buffer; 0.6 mg cellular nitrogen as cell extract. Final volume, 5.0 ml.

lated carbamyl phosphate as the phosphorylated intermediate in the degradation and synthesis of citrulline, the activity of pleuropneumonialike organisms toward carbamyl phosphate was determined. As shown in table 6, carbamyl phosphate is very rapidly degraded to carbon dioxide, ammonia and ATP in the presence of cell extract and ADP.

In spite of this rapid degradation of carbamyl phosphate, the limiting factors in the conversion of citrulline to ornithine appeared to be the accumulation of ornithine and the necessity for the presence of ADP to allow the reaction to proceed at all. Thus it was important to examine the reverse reaction, i. e., the synthesis of citrulline. Attempts to synthesize citrulline from ornithine, ATP, ammonium chloride and sodium bicarbonate were unsuccessful either in the presence or absence of added Mg^{++}. No change in any of the reaction components was noted. However, when cell extracts were incubated with ornithine and carbamyl phosphate, synthesis of citrulline was rapid and complete (table 7). This reaction occurred over a wide range of pH (5.5–8.0) with better activity at acid pH. Determinations of the equilibrium of the reaction of pH 5.5 resulted in a value of 4.8×10^2, indicating that the synthesis of citrulline was the favored reaction, since in the presence of low levels of ornithine, formation of ATP is inhibited.

DISCUSSION

The phosphorolysis of citrulline is another reaction by which pleuropneumonialike organisms might derive energy. As with certain species of bacteria (Oginsky, 1955) the importance of this reaction is dependent upon the ability of the organisms to utilize ornithine. Preliminary experiments on the utilization of ornithine by pleuropneumonialike organisms have shown that it does disappear from the reaction mixture at alkaline pH.

Nutritional studies with these organisms have demonstrated a requirement for relatively large amounts of arginine in synthetic media (Smith, 1955*b*). Neither citrulline nor ornithine can replace this arginine requirement. As has been pointed out, whole cells are impermeable to citrulline. Ornithine on the other hand, is utilized by whole cells. Arginine is readily converted to citrulline by intact cells (Smith, 1955*a*). This evidence together with the uptake of inorganic phosphate in the presence of small amounts of ADP, when coupled with glucose and hexokinase, indicates that citrulline might be used as a means to produce high energy phosphate.

However, the significance of this reaction to the energetics of these organisms is doubtful because of the decelerating action of accumulated ornithine, the rapidity with which citrulline is synthesized from ornithine and carbamyl phosphate and the inhibitory activity of low levels of Mg^{++}.

SUMMARY

Pleuropneumonialike organisms of human origin have been shown to carry out a phosphorolysis of citrulline giving rise to ornithine and adenosine triphosphate. Carbamyl phosphate is active in this system. Mg^{++} was found to inhibit the reaction strongly. Equilibrium of the reaction was determined and found to be governed by the accumulation of ornithine. Reversal of the reaction, i. e., the synthesis of citrulline, was demonstrable only when carbamyl phosphate was present. Optimum activity for both the forward and reverse reactions was obtained at pH 5.5. The role of the phosphorolysis of citrulline in supplying energy for pleuropneumonialike organisms is discussed.

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