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Pyrimidine Nucleotide Biosynthesis in *Phaseolus aureus*

ENZYMIC ASPECTS OF THE CONTROL OF CARBAMOYL PHOSPHATE SYNTHESIS AND UTILIZATION

By B. L. ONG and J. F. JACKSON
Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S. Austral. 5064, *Australia*

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1. Carbamoyl phosphate synthetase activity of *Phaseolus aureus* extracts was assayed by coupling it to the catalytic subunit of *Escherichia coli* aspartate transcarbamoylase and determining the [^{14}C]carbamoylaspartate so formed. The stability of the activity was improved by the addition of ornithine and dimethyl sulphoxide to the extraction medium. 2. The synthetase activity was found to utilize either glutamine or ammonia as amino donor, the Michaelis constants being 0.17 ± 0.03mM and 6.1 ± 1.0mM respectively. *N*-Acetylglutamate did not significantly alter the rate with either substrate, and azaserine inhibited the reaction with both amino donors to the same extent. 3. Ornithine was shown to stimulate the activity, and to counteract inhibition by UMP. The purine nucleotides IMP and GMP enhanced carbamoyl phosphate formation, whereas AMP had an inhibitory effect. 4. The Michaelis constant for carbamoyl phosphate was determined in concentrated extracts for both aspartate transcarbamoylase and ornithine transcarbamoylase activities, and was 0.13 ± 0.03mM and 1.58 ± 0.16mM respectively. The ratio of the activities of these two enzymes, determined at near-saturating substrate concentrations, was 1:3 (aspartate transcarbamoylase/ornithine transcarbamoylase). 5. It is concluded that in this plant tissue there is one enzyme, carbamoyl phosphate synthetase, supplying carbamoyl phosphate to both the pyrimidine and arginine pathways, that the pyrimidine pathway claims most of the available carbamoyl phosphate (depending on the concentration of the nucleotide effectors) when this intermediate is present at low concentrations; and that when the carbamoyl phosphate concentration is increased, possibly by ornithine stimulation, a larger proportion can be taken up by the arginine pathway.

Enzymes synthesizing carbamoyl phosphate from CO_2, ATP and ammonia or glutamine have been the subject of many investigations (Lacroute, 1968; Tatibana & Ito, 1969; Hartman, 1970; O'Donovan & Neuhard, 1970; Tramell & Campbell, 1970; Jones, 1971). Less attention has been paid to carbamoyl phosphate synthesis in higher plants, notwithstanding its importance as the starting point for both the pyrimidine and arginine pathways. In the Alaska pea the presence of a carbamoyl phosphate synthetase utilizing glutamine (rather than ammonia) has been demonstrated by O'Neal & Naylor (1968, 1969). Although the nature of their assay procedure requires relatively high concentrations of ornithine in incubation mixtures, they were also able to show that increasing the concentration of ornithine tended to overcome inhibition by UMP. This, and other results, indicated that the plant enzyme may have some similarities to the synthetase in bacteria, where the one enzyme is thought to produce carbamoyl phosphate for both the pyrimidine and arginine pathways. In contrast, mammalian and fungal cells appear to have a separate carbamoyl phosphate synthetase for each pathway (Jones, 1971; O'Donovan & Neuhard, 1970). Thus in mammals there is a synthetase utilizing ammonia as the amino donor and activated by *N*-acetylglutamate, serving the arginine pathway, and a glutamine-requiring synthetase, producing carbamoyl phosphate for the pyrimidine pathway. The latter is subject to feedback inhibition by UTP.

In the present paper we describe a sensitive, coupled assay for carbamoyl phosphate synthetase based on the conversion of the product, [^{14}C]-carbamoyl phosphate, into the more stable [^{14}C]-carbamoylaspartate by means of the catalytic subunit of aspartate transcarbamoylase from *Escherichia coli*. Because this assay system does not require ornithine, and because the catalytic subunit is not affected by nucleotides (Gerhart & Holoubek, 1967), we were able to study the effect of a range of nucleotides and of ornithine and other urea-cycle intermediates on the synthetase activity of *Phaseolus aureus* extracts. Together with the results of a study of the amino-donor substrates, our results are consistent with there being only one carbamoyl phosphate synthetase in *P. aureus*, as in bacteria, which produces

carbamoyl phosphate for both the pyrimidine and arginine pathways, and which is subject to control by various end-products and intermediates from each pathway. Thus UMP acts as a feedback inhibitor and ornithine will activate the enzyme and counteract UMP inhibition. We have also determined in *P. aureus* extracts the relative activities of the two enzymes, ornithine transcarbamoylase and aspartate transcarbamoylase, utilizing carbamoyl phosphate and leading to arginine and pyrimidine biosynthesis respectively. To investigate possible control at this branch-point in the pathways by substrate-saturation effects, we have measured the Michaelis constant for carbamoyl phosphate with each of these activities.

Materials and Methods

Chemicals

Dimethyl sulphoxide (Grade 1, m.p. 18.4°C, b.p. 189°C) was usually purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), but sometimes from Ajax Chemicals Ltd. (Sydney, Australia). $Na_2{}^{14}CO_3$ (38.4mCi/mmol) and L-[U-^{14}C]aspartate (229mCi/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). [^{14}C]Carbamoylaspartate (DL-ureido[^{14}C]succinic acid) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Plant material

Phaseolus aureus seeds were purchased from the local seed store. Batches (50g) of seeds were surface-sterilized with ethanol for 30s and then washed with sterilized water and soaked in 0.2% (w/v) bleaching powder (May and Baker, Dagenham, Essex, U.K.) for 2–3h. They were then rinsed twice with sterilized water and allowed to imbibe overnight. Then the beans were grown on blotting paper, which had been previously irradiated with u.v. light. The paper was supported by sterile upturned Petri dishes (20cm diam.), placed in covered cake dishes. The radicles were harvested after germination at 20°C in the dark for 2.5–3 days.

Preparation of extracts

Radicles (5–10g) were separated from the endosperm and immediately placed in a beaker on ice. The radicles were ground in a pre-chilled mortar and pestle for 10min with 0.5ml of buffer/g containing 0.1M-tris–glycine (pH8.4), 0.05M-KCl, 0.05M-$MgCl_2$, 0.2M-ornithine, 0.002M-dithiothreitol, 30% (v/v) dimethyl sulphoxide and 20% (v/v) glycerol, and then were homogenized in an all-glass homogenizer kept at 0°C. The buffer was prepared just before use. The homogenate was squeezed through two layers of muslin and the filtrate centrifuged for 10min at 2000*g*. To remove endogenous substrate, 1.0ml of the supernatant (referred to below as the crude extract) was then passed through a column (0.8cm^2 × 6.3cm) of Sephadex G-25, which had been previously equilibrated with at least four column volumes of the extracting buffer used above. The fraction which emerged at 2.8–4.0ml (Sephadex G-25 fraction) was collected and used for enzyme assays, unless otherwise stated. The flow rate was 4ml/h and all operations were carried out at 2–4°C. To concentrate the Sephadex G-25 fraction for ornithine transcarbamoylase and aspartate transcarbamoylase assays, a 30–80%-satn. $(NH_4)_2SO_4$ fraction was collected, dissolved in a small vol. of 0.02M-potassium phosphate buffer (pH8.0) containing 0.002M-2-mercaptoethanol and 0.001M-EDTA, and dialysed against this same solution.

Enzyme assays

Carbamoyl phosphate synthetase. The assay mixture, in a total volume of 0.15ml, contained 10μmol of ATP, two units of aspartate transcarbamoylase (one unit synthesizes 1μmol of carbamoylaspartate/h at 30°C and pH8.5) from either *E. coli* (catalytic subunit) or *P. aureus*, 0.05ml of plant extract and radioactive substrate, $Na_2{}^{14}CO_3$ or [^{14}C]aspartate. When 0.7μmol of $Na_2{}^{14}CO_3$ (5μCi/μmol) was used as the labelled substrate, 1μmol of aspartate was also added, and the reaction was initiated by the addition of 10μl of $Na_2{}^{14}CO_3$ solution delivered from a syringe. When 0.5μmol of [^{14}C]aspartate (1μCi/μmol) was used, 0.7μmol of Na_2CO_3 was added also, and the reaction was initiated by the addition of plant extract. The test tubes were capped securely and incubated at 28°C for 10–15min. The reaction was terminated by acidification with 10μl of 150% (w/v) trichloroacetic acid and the precipitate was removed by centrifugation. Paper electrophoresis followed by radioassay with a liquid-scintillation spectrometer was used to determine the [^{14}C]carbamoylaspartate formed, as described by Ong & Jackson (1972).

Ornithine transcarbamoylase. The assay mixture contained, in a total volume of 1.1ml, 100μmol of tris–acetate buffer, pH8.5, 10μmol of ornithine, carbamoyl phosphate as indicated, and 0.05ml of conc. plant extract (1mg of protein). After 5min at 30°C, the reaction was stopped with 0.1ml of 5M-acetic acid. The colorimetric method of Prescott & Jones (1969) was used to determine the citrulline formed.

Aspartate transcarbamoylase. Incubation mixtures for the determination of this activity contained 100μmol of tris–acetate buffer, pH8.5, 10μmol of aspartate, carbamoyl phosphate as indicated, and 0.05ml of conc. *P. aureus* extract (1mg of protein),

in a total volume of 1.1ml. After 15min at 30°C, the reaction was terminated by adding 0.1ml of 5M-acetic acid. The colorimetric procedure of Prescott & Jones (1969) was used to determine the amount of carbamoylaspartate formed.

Preparation of aspartate transcarbamoylase

The catalytic subunit of aspartate transcarbamoylase was partially purified from *E. coli* B by the method of Gerhart & Holoubek (1967). The enzyme used in the coupled assay had a specific activity of 40μmol of carbamoylaspartate/h per mg of protein. Where *P. aureus* aspartate transcarbamoylase was used for the coupled assay, the enzyme was partially purified by $(NH_4)_2SO_4$ precipitation, followed by DEAE-cellulose and DEAE-Sephadex A-50 chromatography, and finally by gel filtration through a column of Sephadex G-200 (Ong & Jackson, 1972). It had a specific activity of 8μmol of carbamoylaspartate/h per mg of protein at pH8.5.

Determination of Michaelis constants

Computer programs for both hyperbolic and sigmoidal forms of substrate-concentration curves were developed for use on the CDC 6400 computer by using the FORTRAN IV language. The statistical method of Wilkinson (1961), assuming equal variance, was used for the hyperbolic curves (Atkinson *et al.*, 1961), and Cleland's (1967) treatment for the sigmoidal plots.

Results

Coupled assay for carbamoyl phosphate synthetase

An assay method for determining carbamoyl phosphate synthetase activity has been developed based on the continuous conversion of [^{14}C]carbamoyl phosphate into [^{14}C]carbamoylaspartate by the addition of excess of aspartate and aspartate transcarbamoylase. The stable product was then separated by high-voltage paper electrophoresis (Ong & Jackson, 1971). Co-chromatography was used to establish that this product was likely to be [^{14}C]-carbamoylaspartate. The four solvent systems used were (i) ethanol–methanol–formic acid–water (6:6:3:5, by vol.) as described in a New England Nuclear Corp. information sheet for DL-ureido[^{14}C]-succinic acid (NEC-159), lot no. 330-039 (1968), (ii) butan-1-ol–formic acid–water (12:3:5, by vol.) (Smith, 1960), (iii) butan-1-ol–pyridine–water (14:14:17, by vol.) (Smith, 1960), and (iv) propan-1-ol–water (10:3, v/v) (Lieberman & Kornberg, 1954).

The rate of formation of carbamoylaspartate by *P. aureus* extracts was shown to be linear with time and was proportional to the amount of extract added (up to 0.4mg of protein/assay). Although the extracts possessed a relatively high ATPase activity, in no case was there less than 65% of original ATP remaining after completion of the routine assay described here. The requirements for carbamoyl phosphate synthetase activity in crude extracts and in the Sephadex G-25 fraction are shown in Table 1. Although crude extracts do not show a requirement for glutamine, it was necessary for the Sephadex G-25 fraction, indicating that glutamine may be present in crude extracts. Table 1 also shows that some activity is observed in the absence of added aspartate transcarbamoylase, suggesting that in these crude extracts there is sufficient endogenous coupling enzyme to give substantial product formation. A large excess of partially purified aspartate transcarbamoylase was added as a routine to ensure linearity. When studying effectors, use of the catalytic subunit of the *E. coli* enzyme was essential, since both the native *E. coli* transcarbamoylase (Porter *et al.*, 1969) and endogenous *P. aureus* transcarbamoylase (Ong & Jackson, 1972) are known to be inhibited by some of these effectors.

Table 1. *Requirements for carbamoyl phosphate synthetase activity*

The complete system contained 70mM-tris–HCl buffer (pH8.7), 6.5mM-glutamine, 6.5mM-ATP, 23mM-$MgCl_2$, 10mM-aspartate, 4.5mM-$Na_2{}^{14}CO_3$ (5μCi/μmol), 3 units of the catalytic subunit of *E. coli* aspartate transcarbamoylase and 0.05ml of extract from 3-day-old *P. aureus* radicles. Total volume was 0.15ml.

System	Carbamoylaspartate formed (nmol/h per mg of protein)	
	Crude extract	Sephadex G-25 fraction
Complete	6.2±0.3	6.1±0.3
Minus ATP	0.6±0.1	0.5±0.1
Minus glutamine	5.3±0.3	0.4±0.1
Boiled extract	0 ±0.1	0 ±0.1
Minus added aspartate transcarbamoylase	4.3±0.3	4.5±0.3

Table 2. *Stability of carbamoyl phosphate synthetase activity*

The complete extraction medium contained 0.1 M-tris–glycine buffer (pH 8.4), 0.002 M-dithiothreitol, 0.05 M-KCl, 0.05 M-$MgCl_2$, 0.02 M-ornithine, 30% dimethyl sulphoxide and 20% glycerol. Activity was determined as described in the Materials and Methods section.

Extraction medium	Half-life (h)	
	Crude extract	Sephadex G-25 extract
Complete	13.0–14.5	14.0
Minus dimethyl sulphoxide	3.5	4.0
Minus ornithine	4.5	3.5
Minus dimethyl sulphoxide and minus ornithine	4.0	—
0.1 M-Tris–glycine (pH 8.4) and 0.1 M-mercaptoethanol only	3.5	—

Stability of carbamoyl phosphate synthetase activity

Carbamoyl phosphate synthetase in *P. aureus* extracts was most stable when prepared in 0.1 M-tris–glycine buffer, pH 8.4, containing 0.002 M-dithiothreitol, 0.5 M-KCl, 0.05 M-$MgCl_2$, 0.02 M-ornithine, 30% (v/v) dimethyl sulphoxide and 20% (v/v) glycerol, although even in this medium an average loss in activity of 4%/h over the first 12 h after extraction was usual. This compares with a 0.1% loss/h found for the synthetase from pea (O'Neal & Naylor, 1969). Omission of dimethyl sulphoxide or ornithine, or both, resulted in greatly decreased stability (Table 2).

pH profile of synthetase activity

The pH optimum in 0.1 M-tris–HCl buffer at 30°C, with 5 mM-glutamine as substrate, was similar to that found for the pea carbamoyl phosphate synthetase (O'Neal & Naylor, 1969), which is around pH 8.1–8.2 in 0.05 M-tris–glycine–KOH buffer. At pH 8.0 an activity of 12.5 nmol of carbamoyl phosphate/h per mg of protein was obtained, and at pH 7.5 and 9.1 activities observed were 6.2 and 3.9 nmol/h per mg of protein respectively.

Determination of the Michaelis constant for glutamine and NH_4Cl

The ability of glutamine and NH_4Cl to serve as amino donors was tested in the presence and absence of *N*-acetylglutamate (Fig. 1). At pH 8.7, both substrates will serve as donors, but the apparent K_m for glutamine is approximately 45 times lower than that for NH_4Cl in the presence and absence of *N*-acetylglutamate. The K_m for glutamine would thus appear to be more physiological, making it more likely that glutamine serves as the substrate within the plant cell. The presence of *N*-acetylglutamate with NH_4Cl as the substrate did not enhance the activity of the enzyme significantly. Moreover, no additive effect of activities could be observed when both substrates (glutamine, 3.7 mM, and NH_4Cl, 7.4 mM) were present together with 1.5 mM-*N*-acetylglutamate, when using a crude extract preparation, as with each amino donor alone.

Further experimental evidence for the case that the activity in the *P. aureus* extracts is a glutamine-utilizing enzyme is the fact that azaserine (diazoacetyl-L-serine), an analogue of glutamine, inhibits both the glutamine- and ammonia-dependent activities (Table 3). It has been observed that glutamine analogues will inhibit the activities of glutamine-dependent carbamoyl phosphate synthetase in various tissues (Hager & Jones, 1967) with either glutamine or ammonia as substrate.

Effect of variation in Mg^{2+} and ATP concentration on the synthetase activity

Although normal Michaelis–Menten kinetics were observed when attempting to obtain a K_m value for ATP, the resulting value had little meaning because of the high ATPase activity in the extracts. At low concentrations of ATP (<0.5 mM), most of the ATP was hydrolysed to AMP and ADP by the end of the incubation period. The effect of varying Mg^{2+} concentration at two fixed ATP concentrations is shown in Fig. 2. At the lower ATP concentration (1.8 mM), the curve is hyperbolic, but at the higher concentration (6.7 mM-ATP), it becomes sigmoidal. Thus, at 5 mM-$MgCl_2$, the activity is 2.75 nmol/h per mg of protein at 1.8 mM added ATP, but only 0.5 nmol/h per mg of protein at 6.7 mM added ATP, suggesting inhibition at the higher ATP/Mg^{2+} ratios. It seems likely that the Mg^{2+}–ATP complex is the true substrate and that free ATP acts as an inhibitor of the *P. aureus* carbamoyl phosphate synthetase, as with the pea enzyme (O'Neal & Naylor, 1969).

Regulatory effects of nucleotides on the synthetase and aspartate transcarbamoylase activities

As shown in Table 4, the carbamoyl phosphate synthetase activity of the Sephadex G-25 fraction is

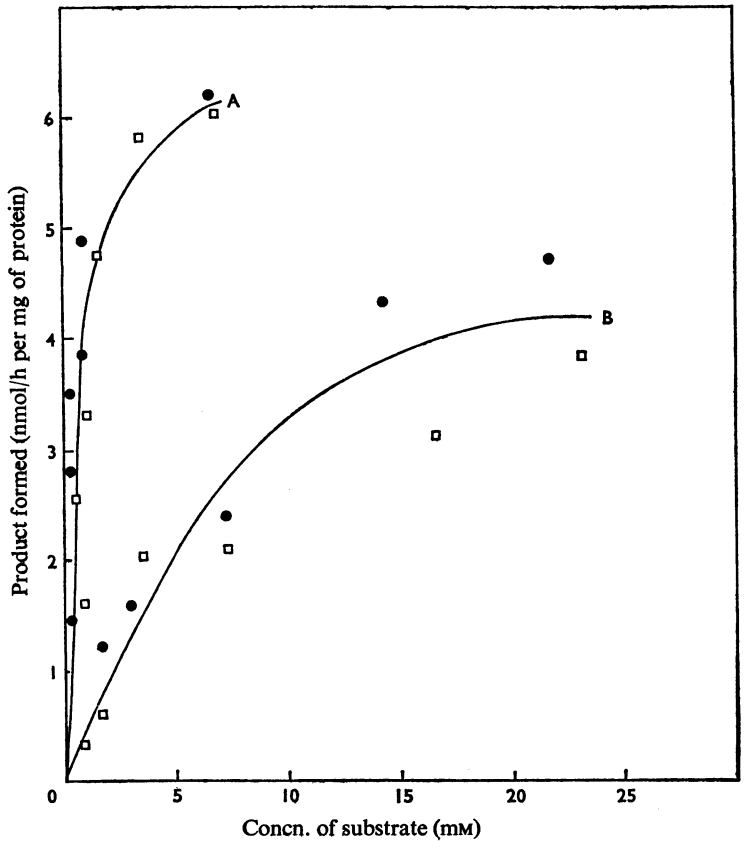


Fig. 1. *Substrate-saturation curves for glutamine and ammonia in the synthetase reaction*

The assay mixture in a total volume of 0.17 ml consisted of 60 mM-tris–HCl buffer (pH 8.5), 10 mM-$MgCl_2$, 5.9 mM-ATP, 4.1 mM-$Na_2{}^{14}CO_3$ (5 μCi/μmol), 6.0 mM-aspartic acid and 2 units of *P. aureus* aspartate transcarbamoylase. The concentration of glutamine or NH_4Cl was varied in the presence or absence of 5.0 mM-*N*-acetylglutamate. The reaction was initiated by the addition of 0.05 ml of Sephadex G-25 extract containing 0.4 mg of protein, and completed as described in the Materials and Methods section. By the method of Wilkinson (1961), the following apparent K_m and $V_{max.}$ values with their standard errors were calculated from the results.

Substrate	Apparent K_m (mM)	Apparent $V_{max.}$ (nmol/h per mg of protein)
Glutamine	0.167 ± 0.026	6.09 ± 1.05
Glutamine + 5.0 mM-*N*-acetylglutamate	0.146 ± 0.018	5.92 ± 0.35
NH_4Cl	6.09 ± 1.04	6.25 ± 0.36
NH_4Cl + 5.0 mM-*N*-acetylglutamate	6.57 ± 1.95	5.02 ± 1.10

The rates obtained with glutamine are shown in curve A, and with NH_4Cl in curve B. ●, No added *N*-acetylglutamate; □, with added *N*-acetylglutamate.

activated by the purine nucleotides IMP, thio-IMP, GMP and to a smaller extent by GTP. AMP, in contrast, was found to be inhibitory.

Of the pyrimidine nucleotides tested, UMP was the most effective inhibitor of the synthetase, whereas dUMP, CMP and CTP did show some degree of inhibition, and dTTP showed none at all (Table 4). The inhibition by UMP was shown to be partially overcome by the purine nucleotides IMP and thio-IMP (Fig. 3). Although UDP and UTP were also

Table 3. *Effect of azaserine on the synthetase activity*

Synthetase activity was determined as described in Table 2, except that *P. aureus* aspartate transcarbamoylase was used as coupling enzyme. Each assay contained 0.4mg of protein of *P. aureus* extract.

Substrate	Concn. of azaserine (mM)	Activity (nmol/h per mg of protein)	Activity remaining (%)
Glutamine (0)	0	0.96	13.4
Glutamine (5.5mM)	0	7.14	100
Glutamine (5.5mM)	5.8	4.05	57
NH_4Cl (0)	0	1.10	16.3
NH_4Cl (30mM)	0	6.80	100
NH_4Cl (30mM)	5.5	4.80	70

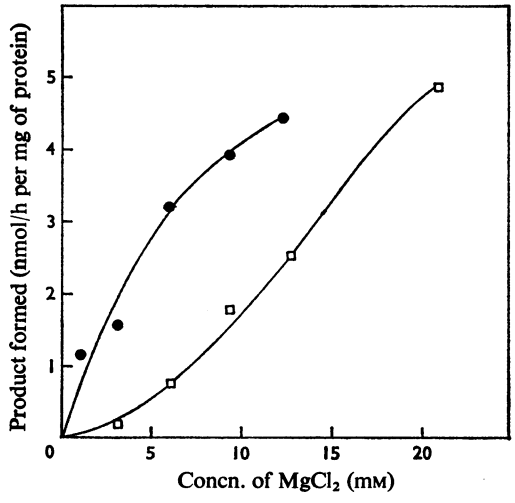


Fig. 2. *Effect of $MgCl_2$ concentration on the synthetase reaction*

The assay mixture, in a total volume of 0.15ml, contained 70mM-tris–HCl buffer (pH8.5), 6.5mM-glutamine, 3 units of *P. aureus* aspartate transcarbamoylase and 6.5mM-aspartate. The reaction was initiated by the Sephadex G-25 extract containing 0.3mg of protein, and completed as described in Fig. 1. ●, 2.0mM-ATP; □, 6.0mM-ATP.

Table 4. *Regulatory effects of nucleotides on the synthetase activity*

Activity was determined as described in Table 1. With no regulatory compound added, synthetase activity was 3.2nmol of carbamoylaspartate formed/h per mg of protein.

Regulator	Concentration (mM)	Relative activity (% of control)
None	—	100
IMP	1.5	125
Thio-IMP	1.3	134
GMP	1.3	121
AMP	1.4	78
Uridine	1.5	91
Dihydro-orotate	1.3	87
CMP	1.3	75
CTP	1.4	91
dTTP	1.3	103
dUMP	1.8	59
UMP	0.2	65
UMP	0.7	47
UMP	3.0	19
UMP (+2.7mM-ornithine)	0.2	86
UMP (+2.7mM-ornithine)	0.7	64
UMP (+2.7mM-ornithine)	3.0	44

found to inhibit the synthetase activity (50% inhibition given by 1.5 and 1 mM respectively), it is uncertain just how effective these nucleotides are, since they were degraded to UMP to some extent in the presence of the Sephadex G-25 fraction. However, the above results suggest that they cannot be as inhibitory as UMP.

The multiplicity of effectors demonstrated for *P. aureus* carbamoyl phosphate synthetase is not a property shared with the next enzyme in the pyrimidine pathway, aspartate transcarbamoylase. The latter appears specifically to utilize uridine nucleotides as allosteric inhibitors (Ong & Jackson, 1972). It does so in a way that has important implications for the pathway. As shown in Fig. 4, the pattern of inhibition produced by increasing concentrations of UMP at a constant carbamoyl phosphate concentration is not markedly changed by a change in aspartate concentration. Low UMP concentrations, however,

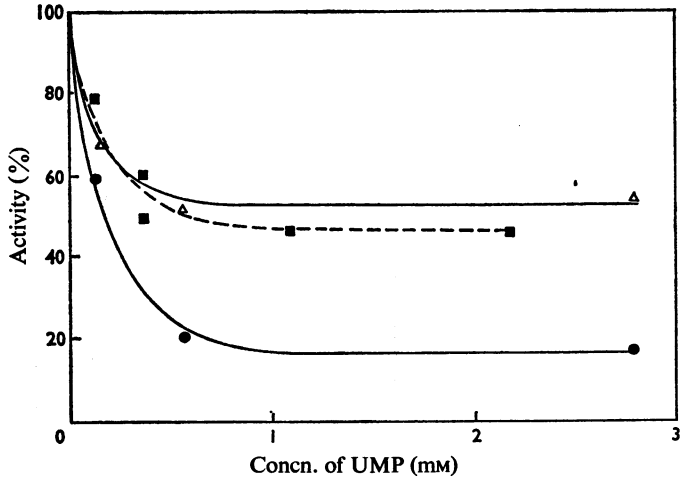


Fig. 3. *Reversal of UMP inhibition of carbamoyl phosphate synthetase activity by IMP and thio-IMP*

The reaction was carried out as described in Table 4. ●, UMP alone; ■, UMP with 4.0mM-IMP; △, UMP with 4.0mM-thio-IMP.

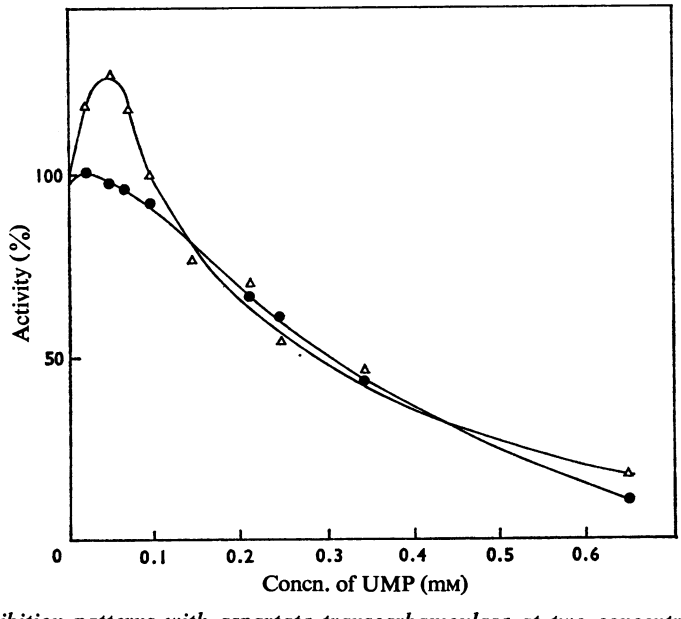


Fig. 4. *UMP inhibition patterns with aspartate transcarbamoylase at two concentrations of aspartate*

Activity was determined by the colorimetric technique. Carbamoyl phosphate concentration was kept at 2mM. The partially purified enzyme was used (0.09mg of protein/assay); in the absence of UMP, activity was determined as 3.5μmol of carbamoylaspartate/h per mg of protein. △, 0.7mM-Aspartate; ●, 3.3mM-aspartate.

stimulated activity over and above that given in the absence of UMP, when activity was measured at low concentrations of aspartate. This is similar to the stimulation given by low concentrations of the inhibitor, succinate (Ong & Jackson, 1972). In contrast, changing the concentration of carbamoyl phosphate has a large effect on the inhibition by UMP (Fig. 5). At low carbamoyl phosphate concentration,

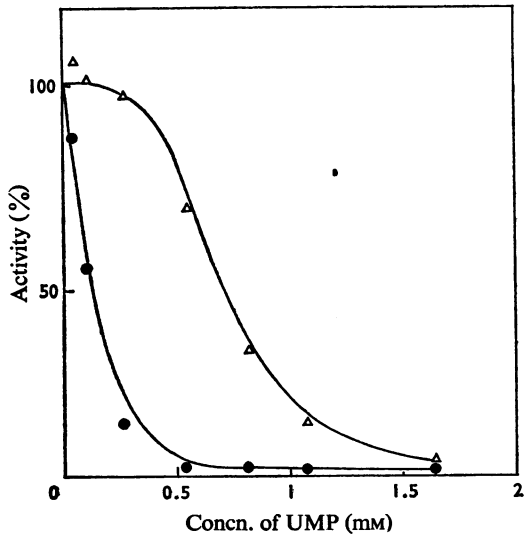


Fig. 5. *UMP inhibition patterns with aspartate transcarbamoylase at two concentrations of carbamoyl phosphate*

The colorimetric assay was used in this experiment and aspartate concentration was kept at 3.3mM. ●, 0.55mM-Carbamoyl phosphate; △, 2.78mM-carbamoyl phosphate. At the former substrate concentration, the concentration of UMP giving 50% inhibition was found to be 0.11mM (and the Hill coefficient, $n = 2.3$), and at the latter, 0.67mM ($n = 3.8$). Enzyme used was capable of synthesizing 3.5μmol of carbamoylaspartate/h per mg of protein; 0.09mg of protein was added to each assay mixture.

inhibition by UMP is much more severe. It appears, then, that in this sense, UMP inhibition is competitive with the first substrate, carbamoyl phosphate, and not with the second substrate, aspartate.

Regulatory effects of the urea-cycle intermediates

The Sephadex G-25 synthetase activity is enhanced by addition of ornithine, reaching maximal activation at concentrations of ornithine exceeding 10mM (Fig. 6). In addition, ornithine was shown partially to overcome inhibition by UMP (Table 4). We also found that the UMP inhibition of *P. aureus* aspartate transcarbamoylase activity of crude extracts or of the partially purified fraction is not reversed by 4mM-ornithine, or by any of the purine nucleotides listed in Table 4. Nor do these compounds activate or depress this activity in the absence of UMP. Other intermediates of the urea cycle, such as fumarate, urea, arginine and citrulline, were without effect on the

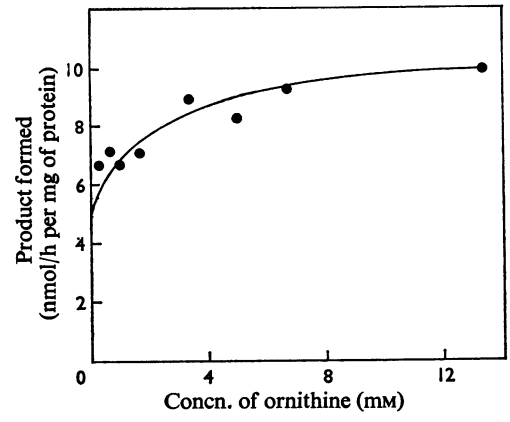


Fig. 6. *Activation of the synthetase activity by ornithine*

The Sephadex G-25 column, used to prepare the Sephadex G-25 fraction for this series of experiments, was previously equilibrated with the usual extraction medium without added ornithine. The reaction mixtures contained ornithine at the indicated concentrations and the reaction was initiated by addition of $Na_2{}^{14}CO_3$.

synthetase activity or on aspartate transcarbamoylase of *P. aureus*.

Ornithine transcarbamoylase and aspartate transcarbamoylase activities

Carbamoyl phosphate saturation curves for both ornithine transcarbamoylase and aspartate transcarbamoylase were determined on the same concentrated *P. aureus* extract. They show (Fig. 7) that the Michaelis constant for the ornithine-utilizing enzyme is more than 10 times that for the aspartate enzyme (1.58±0.16mM compared with 0.13±0.03mM); the maximal rate attainable with the former enzyme is also higher (358.1±14.7, compared with 121.3± 10.4nmol of product formed/h per mg of protein). Assuming no relative loss of activity during the concentration procedure, we have calculated that the proportions of activities of the three enzymes at the branch-point of the pyrimidine and arginine pathways in our *P. aureus* extracts is: synthetase/aspartate transcarbamoylase/ornithine transcarbamoylase, 1.0:18.5:55.5.

Discussion

Several assay methods are available for determining carbamoyl phosphate (Jones, 1962; Anderson & Meister, 1965). However, owing to the unstable

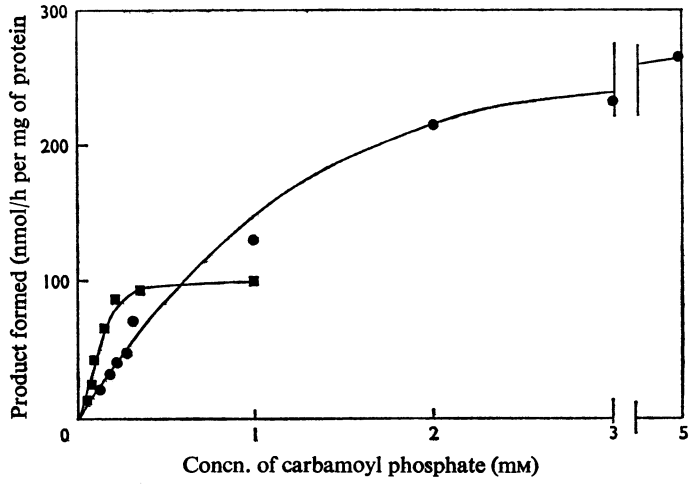


Fig. 7. *Carbamoyl phosphate saturation curves for ornithine transcarbamoylase and aspartate transcarbamoylase*

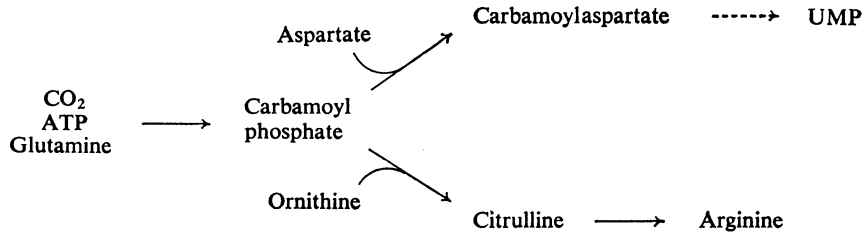
The colorimetric method of Prescott & Jones (1969) was used to determine these activities at various carbamoyl phosphate concentrations in a concentrated *P. aureus* extract. For the ornithine transcarbamoylase reaction, the Michaelis constant for carbamoyl phosphate was calculated to be 1.58 ± 0.16 mM and the apparent $V_{max.}$ was 358.1 ± 14.7 nmol/h per mg of protein. The corresponding values for aspartate transcarbamoylase were 0.13 ± 0.03 mM and 121.3 ± 10.4 nmol/h per mg of protein. ●, Ornithine transcarbamoylase; ■, aspartate transcarbamoylase.

nature of the compound under physiological conditions [according to Allen & Jones (1964), at 37°C in 0.06M-tris buffer (pH 8.4) the half-life of carbamoyl phosphate is approx. 38min], it is desirable that carbamoyl phosphate be converted into a more stable compound, which can be easily determined either spectrophotometrically or by the radiochemical assay of label incorporated. It is difficult to determine the synthetase in crude extracts of plant material spectrophotometrically by coupling it with either ornithine transcarbamoylase or aspartate transcarbamoylase, because the colorimetric method used in both cases relies on the reaction of a ureido moiety. Plant materials are known to contain high concentrations of ureido compounds (Eagles *et al.*, 1971), which would interfere with this reaction. However, if $Na_2{}^{14}CO_3$ is used as substrate, and the reaction is coupled with aspartate transcarbamoylase, as described in the present paper, to yield [^{14}C]carbamoylaspartate, the product can be conveniently separated by paper electrophoresis and counted for radioactivity in a liquid-scintillation spectrometer (Ong & Jackson, 1971).

Aspartate transcarbamoylase, rather than ornithine transcarbamoylase, is the logical choice of coupling enzyme for several reasons. The product is more mobile on paper electrophoresis; and the Michaelis constant for carbamoyl phosphate is lower with aspartate transcarbamoylase, whether the *P. aureus* (Fig. 7) or *E. coli* (Porter *et al.*, 1969) enzyme is used, compared with that for ornithine transcarbamoylase from *P. aureus* (Fig. 7), *E. coli* (Rogers & Novelli, 1962), *Staphylococcus aureus* (Zaharia & Soru, 1971) or *Streptococcus faecalis* (Ravel *et al.*, 1959). It also means that ornithine is not an essential component of the assay mixture; thus if the catalytic subunit of *E. coli* aspartate transcarbamoylase is used in the coupled assay, a simultaneous study can be made of effectors such as ornithine and the various nucleotides. [^{14}C]Aspartate may also be used as a source of label for the production of [^{14}C]carbamoylaspartate in this coupled assay. There may be an advantage in using this label over $Na_2{}^{14}CO_3$ since there is the possibility that the latter may alter in specific radioactivity by exchange with CO_2 from the atmosphere. In the present experiments we found similar results with either source of labelled material.

The properties of the *P. aureus* carbamoyl phosphate synthetase described here are similar in many respects to those observed with the purified *E. coli* enzyme, in that the activity shows a distinct preference for glutamine (over ammonia) as the amino group donor, and that it is subject to feedback inhibition by UMP. This has also been observed for the partially purified pea enzyme (O'Neal & Naylor, 1969). We have extended these observations and shown stimulation of the *P. aureus* activity by IMP and ornithine, including partial reversal of UMP inhibition, further properties shared with the bacterial enzyme (Anderson & Meister, 1965; Anderson & Marvin, 1968; Pierard, 1966).

In animal tissues, the available evidence suggests



Scheme 1. *Utilization of carbamoyl phosphate for pyrimidine and arginine biosynthesis in P. aureus*

the presence of two enzymes producing carbamoyl phosphate, one for the arginine pathway, the other for pyrimidine biosynthesis (Jones, 1971). The former activity depends on ammonia as the amino-group donor and requires *N*-acetylglutamate as cofactor, whereas the latter utilizes glutamine. That there is only one enzyme supplying carbamoyl phosphate to both pathways in *P. aureus* is suggested not only by the above effector properties of ornithine and/or UMP, but more particularly by studies on the Michaelis constants for glutamine and ammonia (the latter has a Michaelis constant approx. 45 times that of the former), together with the observation that *N*-acetylglutamate has no significant effect on the rate with either amino donor. An added point in the favour of a single enzyme is that the glutamine analogue azaserine was found to inhibit both the glutamine and ammonia reactions equally well, suggesting that ammonia binds at the same site as glutamine. We have not been able to extend our observations by purification of the synthetase, mainly owing to the extreme lability of the *P. aureus* activity. The pea enzyme described by O'Neal & Naylor (1969) is approx. 40 times more stable than the activity described here. However, work on crude extracts does have the advantage that all enzymes are present, and investigations with *P. aureus* can make use of information about the *P. aureus* aspartate transcarbamoylase (Ong & Jackson, 1972) when examining the pyrimidine pathway as a whole.

All the above phenomena can be explained on the basis of the one enzyme in *P. aureus* producing carbamoyl phosphate for two pathways, one leading to pyrimidine nucleotide synthesis, and the other to arginine (Scheme 1). Thus UMP is acting as a feedback inhibitor from the pyrimidine pathway, and IMP (and, as observed here, GMP), being an intermediate in the complementary purine pathway, stimulates carbamoyl phosphate synthesis. The inhibition observed here by AMP, also observed with the pea enzyme by O'Neal & Naylor (1969) and with the synthetase from land snail (Tramell & Campbell, 1970), is rather more difficult to explain from a regulatory viewpoint. In terms of mechanism, it is possible that AMP is combining at the same site as Mg–ATP and (unchelated) ATP. As in the bacterial system, the activiation of the synthetase activity by ornithine and the reversal of UMP inhibition by ornithine are presumably necessary for stimulation of glutamine-dependent carbamoyl phosphate production for arginine synthesis.

Aspartate transcarbamoylase in *E. coli* is subject to feedback inhibition by CTP, whereas the equivalent enzyme in *P. aureus* is inhibited by UMP. Thus the first two enzymes of the pyrimidine pathway in *P. aureus*, unlike in *E. coli*, are subject to feedback inhibition by the same end product. Further, the inhibition of the second enzyme, aspartate transcarbamoylase, by UMP is competitive with the product of the first, carbamoyl phosphate. This 'in-series' type of inhibition must represent an extremely effective and fine control over pyrimidine nucleotide synthesis. The low K_m for carbamoyl phosphate displayed by aspartate transcarbamoylase, together with the conclusion from kinetic studies (Ong & Jackson, 1972) that the binding of carbamoyl phosphate to this enzyme is essentially an irreversible process, should lead to a channelling of carbamoyl phosphate into the pyrimidine pathway, particularly as none of the intermediates in arginine biosynthesis appear to have any inhibitory action on aspartate transcarbamoylase. Thus it would appear that in *P. aureus*, the pyrimidine pathway takes precedence over the arginine pathway in the utilization of carbamoyl phosphate, especially at low concentrations of carbamoyl phosphate. At higher carbamoyl phosphate concentrations, attained perhaps in response to higher ornithine concentrations, there could be considerably more spill-over into the arginine pathway, as the relative activity of ornithine transcarbamoylase in our crude extracts of *P. aureus*, measured at near-saturating substrate concentrations, is greater than that for aspartate transcarbamoylase.

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