Biochem. J. (1980) **190**, 1–15
Printed in Great Britain

Control of the Flux in the Arginine Pathway of *Neurospora crassa*

THE FLUX FROM CITRULLINE TO ARGININE

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(*Received 24 August 1979*)

The arginine pathway is a complex one, having many branch points and effector interactions. In order to assess the quantitative role of the various mechanisms that influence the flux in the pathway, the system was divided experimentally into two moieties by the introduction of a genetic block abolishing ornithine carbamoyltransferase activity. This normally produces citrulline from ornithine within the mitochondria. The endogenous citrulline supply was replaced by citrulline in the growth medium, and control of the influx rate was achieved by using glycine or histidine as uptake inhibitors. By modulating the influx rate over a large range of values, the importance of such factors as reversibility, saturation, inhibition and induction in affecting the flux and the sizes of intermediate pools between citrulline and arginine was assessed. The role of expansion fluxes as important controls in the exponentially growing system was established.

The investigation and understanding of biochemical events *in vivo* are constrained by a fundamental dilemma: experimental manipulation disturbs the system investigated, sometimes drastically, and it is often not clear how far the conclusions from the disturbed system can be applied or extrapolated to the undisturbed condition. The extreme case is the complete dissection of the system into its parts, which are then subjected to a detailed and sophisticated analysis. In so doing many interactions of the parts cease to exist and the problem of 'synthesizing' the whole from its parts becomes intractable both in logic and algebra. The methodological questions that arise and their possible solutions have been discussed before (Kacser & Burns, 1973, 1979), and some of the concepts and approaches are now applied in the present paper.

The arginine pathway in *Neurospora* has been the subject of considerable investigations (Davis, 1962; Barthelmess *et al.*, 1974; Cybis & Davis, 1975; Bowman & Davis, 1977; Davis *et al.*, 1978). A simplified diagram of the pathway is shown in Scheme 1. It symbolizes only the known metabolic transformations (which are believed to be complete), but leaves out much that is known about the cellular distribution of enzymes and substrates and any effector interactions such as inhibitions, inductions and repressions. We do, however, take these into account when interpreting our experimental results.

The flux through the arginine pathway may be said to 'begin' at glutamate and 'end' at arginine. It is, however, clear that the glutamate is derived from a product of the tricarboxylic acid cycle, thus extending into glycolysis, and that arginine goes into protein, which is directly related to growth and hence to the rate of synthesis of enzymes. In addition, there are connections to the proline and pyrimidine pathways. All these interactions, in principle, affect the flux in the pathway and, in turn, are affected by it. Within the pathway there are metabolic transformations with various degrees of reversibility and saturation. In addition, there are effector interactions, principally inhibition of acetylglutamate kinase (enzyme 3 in Scheme 1) by arginine (Cybis & Davis, 1974), inductions of arginase (enzyme 10) and ornithine aminotransferase (enzyme 13) by arginine (Weiss & Anterasian, 1977; Weiss & Davis, 1977) and de-repression of most of the biosynthetic enzymes (enzymes 2–9) by arginine or arginyl-tRNA (Cybis & Davis, 1975; Barthelmess *et al.*, 1974). Finally, arginine and ornithine are compartmentalized by a reversible distribution between vesicle and cytosol. Only the

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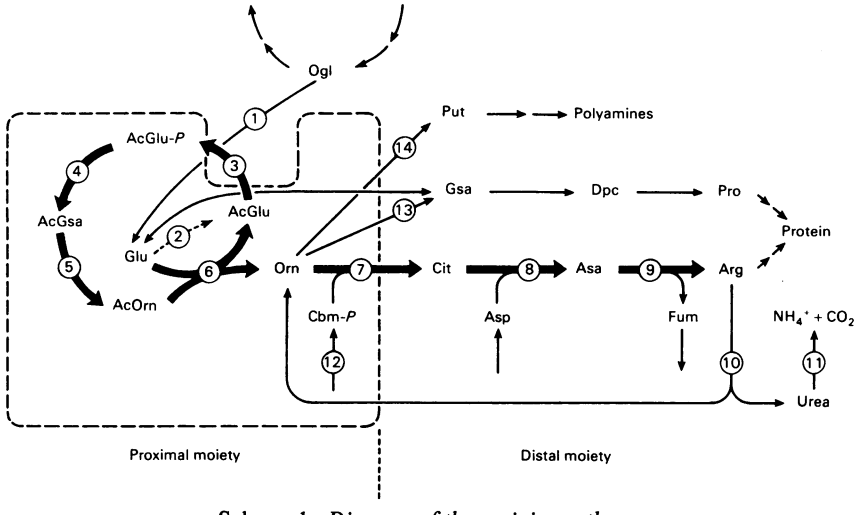
cytosolic arginine pool takes part in the interactions discussed in the present paper (Subramanian *et al.*, 1973; Karlin *et al.*, 1976; Weiss, 1976). It is therefore seen that the concentration of arginine plays a critical role in influencing the rate of its own synthesis and degradation.

Most of the information about the system derives from observations and experiments with mutants (both null and partial for particular enzymes) and from experiments involving the use of large changes in the concentrations of nutrients and intermediates. By its nature this approach yields mainly qualitative information about the 'structure' of the system and the mechanisms operating, e.g. what types of interaction, such as de-repression or compartmentalization, exist. More quantitative conclusions derive from tracer experiments (e.g. Bowman & Davis, 1977), which allow some observations to be made on the relatively undisturbed system. To assess the importance in quantitative terms of the various types of interactions, however, requires the method of modulation, i.e. the determination of the response to small movements around the steady state (Kacser & Burns, 1973, 1979). In practice a series of finite changes may be made in some system parameter and the response curves defined by interpolation. The responses are often decidedly non-linear (see, e.g., Barthelmess *et al.*, 1974). By adopting such a quantitative approach, the experiments, of which the present paper is the first report, aim to determine the relative importance of the various interactions and 'controls', and to obtain insight into the net outcome of the simultaneous activity of many molecular processes.

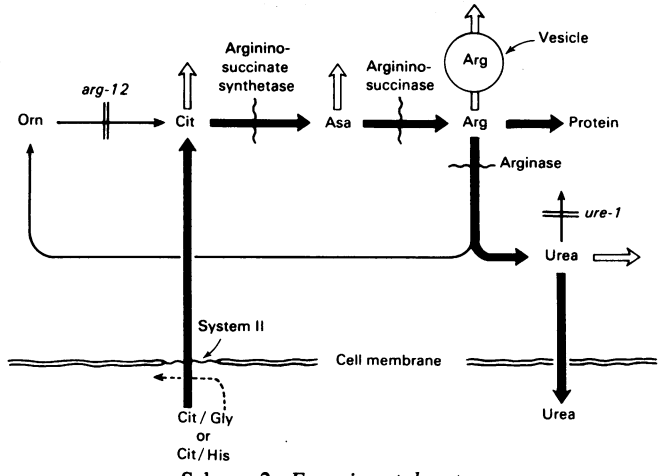
Experimental System

The approach adopted depends on 'cutting' the system into moieties by the introduction of a genetic block in the middle of the pathway, making the strain auxotrophic. The normal endogenous supply of arginine precursors can be replaced by an exogenous supply whose rate is amenable to modulation in a manner not practicable from within the system. In addition, this allows the two resulting moieties ('distal' and 'proximal' with respect to the enzyme deficiency) to be studied in partial independence of one another. There is an obvious and convenient place for such a severance. All enzymes proximal to, and including, ornithine carbamoyltransferase (enzyme 7) are located within the mitochondria (with the exception of enzyme 3),



Scheme 1. *Diagram of the arginine pathway*

Abbreviations used: Ogl, 2-oxoglutarate; Glu, glutamate; AcGlu, acetylglutamate; AcGlu-*P*, acetylglutamate phosphate; AcGsa, acetylglutamate semialdehyde; AcOrn, acetylornithine; Orn, ornithine; Cbm-*P*, carbamoyl phosphate; Cit, citrulline; Asp, aspartate; Asa, argininosuccinate; Arg, arginine; Fum, fumarate; Gsa, glutamic semialdehyde; Dpc, Δ^1-pyrrolinecarboxylate; Pro, proline; Put, putrescine. The common names of the enzymes numbered (1)–(14) are as follows: (1) glutamate dehydrogenase (EC 1.4.1.2); (2) glutamate acetyltransferase (EC 2.3.1.35); (3) acetylglutamate kinase (EC 2.7.2.8); (4) *N*-acetyl-γ-glutamyl phosphate reductase (EC 1.2.1.38); (5) acetylornithine aminotransferase (EC 2.6.1.11); (6) acetylornithine deacetylase (EC 3.5.1.16); (7) ornithine carbamoyltransferase (EC 2.1.3.3); (8) argininosuccinate synthetase (EC 6.3.4.5); (9) argininosuccinase (argininosuccinate lyase, EC 4.3.2.1); (10) arginase (EC 3.5.3.1); (11) urease (EC 3.5.1.5); (12) carbamoyl phosphate synthetase (ammonia) (EC 6.3.4.16, formerly EC 2.7.2.5); (13) ornithine–oxo acid aminotransferase (EC 2.6.1.13); (14) ornithine decarboxylase (EC 4.1.1.17).



Scheme 2. *Experimental system*

The distal moiety begins at the block in ornithine carbamoyltransferase, the *arg-12* mutation. The metabolic fluxes are indicated by solid arrows, the expansion fluxes by open arrows. The synthesis of citrulline from ornithine is replaced by the transport of citrulline from the medium controlled by inhibitors of System II, namely glycine (Gly) or histidine (His). For abbreviations see Scheme 1.

whereas the remaining enzymes are cytosolic (Cybis & Davis, 1975). Thus the added problems of transport or diffusion into and out of the mitochondria are avoided when the distal moiety is investigated. The division is indicated by the broken line in Scheme 1. The mutant *arg-12*, which lacks ornithine carbamoyltransferase activity, fails to supply citrulline to the pathway, but this supply can be replaced by providing citrulline in the growth medium. The system distal to ornithine carbamoyltransferase is therefore metabolically isolated from the proximal moiety, and hence the fate of citrulline and the effects of varying its rate of uptake can be studied. Citrulline is taken up by the mycelium through a transport system (system II; Thwaites & Pendyala, 1969) and reaches an internal concentration 60-fold higher than that found in the wild-type grown on minimal medium. Uptake cannot be controlled by varying the concentration in the medium at any but extremely low values, when the quantity is so small that depletion of citrulline severely limits the length of time during which steady exponential growth can be sustained. Its uptake, however, can be controlled by the presence of competitive inhibitors of the process such as glycine or histidine (Thwaites & Pendyala, 1969). In this way mycelial concentrations of citrulline both above and below those of the wild-type can be achieved. Thus the rate of supply of citrulline by the proximal moiety, subject to the influence of a large number of interactions arising within the system, is replaced by uptake rates under the experimenter's control. Modulation of the influx into the experimental system is thus achieved by the manipulation of a single parameter, namely the inhibitor/citrulline concentration ratio.

To enable measurement of the rate of urea formation, which constitutes an important exit flux of the pathway, a second lesion, in urease, was introduced into the *arg-12* strain. The introduction of the *ure-1* mutation has no effect on growth, and little effect on metabolism (Flint, 1977), since the minimal medium contains high concentrations of ammonium. Scheme 2 shows the distal moiety with the transformations that are the subject of the present paper.

Materials and Methods

Strains

The *arg-12,ure-1* strain was generated by Dr. R. Tateson, from the ureaseless mutant *ure-47* (Kølmark, 1969) and the *arg-12* mutant U.M. 107 (Davis & Thwaites, 1963), which had both previously been back-crossed repeatedly into St. Lawrence ST 74a background (Tateson, 1971).

Growth conditions and harvesting

Mycelium was grown in special 1-litre dimpled flasks, under constant agitation on a New Brunswick gyrorotary shaker, at 200 rev./min, in a 29°C constant-temperature room. Each flask contained 400 ml of Vogel's minimal medium (Vogel, 1956), containing 8.75 g of glucose (separately autoclaved) and the appropriate amino acid supplementation. Glycine or histidine, used as competitive inhibitors of citrulline uptake, have severe effects on germination and growth rate if added at the time of inocula-

tion. If inhibitor addition is progressively delayed these effects become progressively less severe. At 8h after inoculation into citrulline medium the effect of glycine addition is only slight, and this procedure was used for the 'steady-state' experiments, sufficient time being allowed for the system to adjust to the new uptake rate. The time for histidine addition required a delay of about 14h.

Since accurate sampling from a growing mycelium is not possible, complete flasks were harvested for each time point. Mycelia were harvested by filtration through three layers of filter paper in a Buchner funnel, and the mycelial pads were rapidly frozen in an acetone/solid CO_2 mixture. Each pad was then freeze-dried and used to determine dry weight, enzyme activities, amino acid pools and mycelial acid-soluble and acid-insoluble radioactivity, as described below. Samples of medium, when required, were withdrawn from the filtrate of each harvest and rapidly frozen.

Amino acid pools

A 25mg portion of freeze-dried powdered mycelium was homogenized in 1.2ml of 5% (w/v) sulphosalicylic acid, containing 0.4μmol of α-amino-β-guanidinopropionic acid as an internal standard and kept on ice for 30min before centrifugation at 1000*g* for 15min. Suitable volumes of supernatant were used to determine amino acid pool sizes on an amino acid analyser (Burns *et al.*, 1965), with the usual ninhydrin/$SnCl_2$ colour development. Alternatively the detection system was changed to the BUN method of Marsh *et al.* (1965), which detects urea as well as citrulline. This was achieved by appropriate mixing of the streams of (1) column effluent, (2) acid reagent (20% H_2SO_4, 6% H_3PO_4 and 0.003% $FeCl_3,6H_2O$) and (3) colour reagent (0.25% diacetylmonoxime and 0.0125% thiosemicarbazide) and development at 100°C for 20min. An abbreviated procedure was also used in preference to the complete analyser programme for the routine rapid separation of urea and citrulline before detection by the BUN method. Samples were added to a 15cm (6mm diam.) column containing Dowex 50W X8 (200–400 mesh; H^+ form) resin pre-equilibrated at pH2.8 with sodium citrate buffer (0.2g-ion of Na^+/litre) and eluted by passing through the pH2.8 buffer for 20min, followed by a pH4.9 sodium citrate buffer (0.8g-ion of Na^+/litre) for 30min, at a flow rate of 0.32ml/min.

Urea accumulation and citrulline depletion in the medium

Urea and citrulline present in samples of medium were separated by ion-exchange chromatography, and their concentrations were determined either colorimetrically, by using the BUN procedure, or by scintillation counting of radioactivity for those cultures grown on [*ureido*-^{14}C]citrulline. Urea was not completely resolved by chromatography from small quantities of other BUN-positive or labelled substances thought to be derived from citrulline *in vivo*. Therefore each sample of medium was split, and one portion treated with 2mg of urease/ml (30min incubation at 37°C in 0.1M-sodium/potassium phosphate buffer, pH7.0) to remove all urea. The value obtained for the urea region of the chromatogram in the urease-treated portion was then subtracted from the value obtained in the untreated portion to give the true urea concentration. For a particular steady-state condition, the regression of total urea or citrulline in the medium on mycelial dry weight at the time of sampling was used to calculate the rate of increase in urea in the medium, or the rate of decrease in citrulline in the medium, per g increase in weight. These values were converted into absolute fluxes by multiplying by the exponential growth constant obtained from the regression of dry weight on time.

Total mycelial acid-soluble and acid-insoluble radioactivity

All radioactivity determinations were performed by liquid-scintillation counting, with NE260 scintillant (Nuclear Enterprises, Sighthill, Edinburgh, Scotland, U.K.), on a Nuclear–Chicago Isocap 300 counter. [*ureido*-^{14}C]Citrulline (sp. radioactivity 60mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Mycelial extracts were prepared as described for amino acid pools. Mycelial acid-soluble radioactivity was determined on these supernatants. Mycelial acid-insoluble radioactivity was determined on the whole precipitate, which was washed and resuspended three times in 5% (w/v) sulphosalicylic acid, and then incubated for 2–3h in NCS™ protein solubilizer (Amersham/Searle, High Wycombe, Bucks., U.K.). The digest was neutralized with acetic acid and its radioactivity counted.

Arginase assay

Arginase activity was assayed in extracts of freeze-dried mycelial powder by the method of Davis & Mora (1968), except that (i) the incubation was conducted at 30°C (as opposed to 37°C) and for up to 20min (as opposed to 5min) and (ii) the rate of formation of the reaction product, urea (as opposed to L-ornithine), was determined colorimetrically, by using the BUN method. The rate of increase in total BUN-positive material in stopped timed reaction mixes was used after it had been shown that the total increase in BUN-positive material was attributable only to enzymically derived urea. The specific activities were calculated from the concentration of protein in the mycelial extracts determined by the

Folin phenol method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co.) as standard.

Results and Discussion

Steady state

Figs. 1–5 illustrate the type of experiment used to estimate the steady-state input and exit fluxes of the distal moiety of the system. Fig. 1 shows the growth of the *arg-12,ure-1* strain on four different glycine/citrulline concentration ratios. The growth rates are all exponential, although there are small differences in the values of the growth constants. These differences are not due to arginine limitation, since the arginine pool is in all cases larger than the minimal grown wild-type value, and glycine has been found to inhibit the growth of the wild-type even in the presence of exogenous arginine (Flint, 1977). We have no explanation for this effect of glycine, but the growth-rate differences are taken into account in the subsequent calculations.

Fig. 2 shows how citrulline influx from the medium (based on medium-depletion data), as a function of dry weight, is affected by the glycine/citrulline concentration ratio provided. The

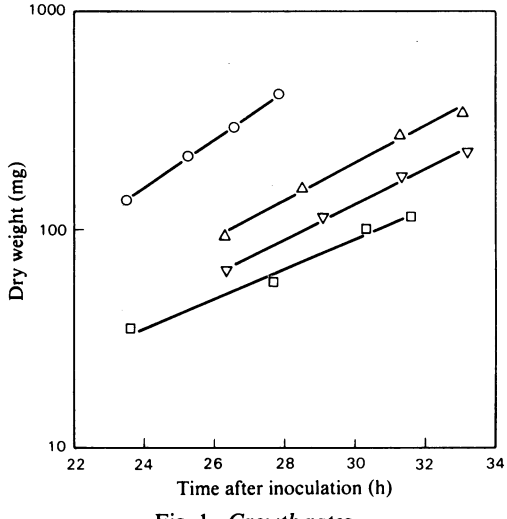


Fig. 1. *Growth rates*

The effect of glycine on the growth of the *arg-12,ure-1* strain on a growth medium supplemented with citrulline is shown. O, No glycine, 0.5 mM-citrulline, $k = 0.26$; △, 1.5 mM-glycine, 0.5 mM-citrulline, $k = 0.20$; ▽, 2.5 mM-glycine, 0.5 mM-citrulline, $k = 0.19$; □, 3.5 mM-glycine, 0.5 mM-citrulline, $k = 0.15$. Growth rate constants, in h^{-1}, were obtained by regression of weight (logarithmic scale) on time. The amino acid concentrations are the initial values in the medium. Full details are given in the text.

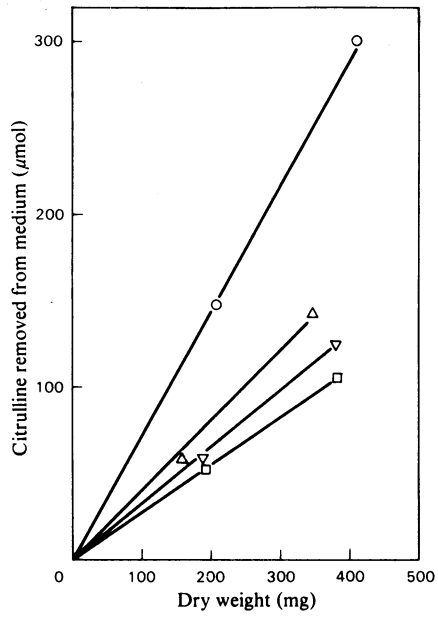


Fig. 2. *Citrulline influx*

The rate of citrulline uptake as a function of mycelial dry weight was determined from medium-depletion data with [*ureido*-^{14}C]citrulline. The [glycine]/[citrulline] ratios and symbols are as given in Fig. 1. Full details are given in the text.

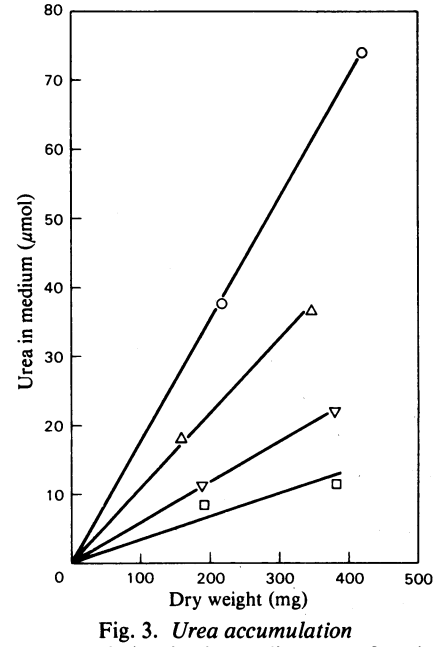


Fig. 3. *Urea accumulation*

Urea accumulation in the medium as a function of dry weight was determined from the radioactivity of [*ureido*-^{14}C]citrulline-derived urea as described in the Materials and Methods section. Symbols are as given in Fig. 1. Full details are given in the text.

depletion rate is constant for a given ratio despite the fact that the external citrulline concentration falls, in some cases, to almost one-quarter of the initial external value. This argues for the transport system being saturated by citrulline and/or glycine over the range of experimental conditions. Transport system II has K_m values in the micromolar range (Pall, 1969), whereas citrulline and glycine concentrations in the medium are in the millimolar range.

The two final exit fluxes of the pathway are the catabolism of arginine by arginase and the incorporation of arginine into protein. In *ure-1* strains accumulation of urea in the medium accounts for most of the flux through the enzyme arginase, the remainder being accounted for by the mycelial urea pool (discussed below). This accumulation is shown in Fig. 3, as a function of dry weight. To measure the other exit of the pathway, that to protein synthesis, [^{14}C]citrulline was added to the medium at an early stage of growth to label the arginine entering protein (Fig. 4). [*ureido*-^{14}C]Citrulline was used so that the arginine formed via argininosuccinate became labelled only in the guanido carbon atom, and therefore did not give rise to any labelled ornithine after catabolism by arginase. Although labelled urea is formed, it is not metabolized further because of the urease deficiency. Thus arginine is the only protein-forming amino acid to become labelled, and the increase in total mycelial acid-insoluble radioactivity provides a measure of the incorporation of arginine into protein. Incorporation into protein, per unit dry-weight increase, was found to be unaffected by the glycine/citrulline concentration ratio used, being 127.2 μmol/Δg (Fig, 4). (Calculations of concentrations require a knowledge of the internal volumes accessible to the molecular species in question. Since this involves a variety of assumptions, none of which is readily verifiable, we use, throughout the text, dry weight as the unit. Thus 'concentrations' are μmol/g, 'accumulations' are μmol/Δg and 'fluxes' are μmol/h per g. Most of the arguments are concerned with relative changes where the particular units are not relevant.) This is in good agreement with values obtained previously from protein hydrolysis (130 μmol/Δg; Tateson, 1971). The flux to protein arginine for each treatment is obtained by multiplying this value by the growth constant. This calculation is based on the assumption that all acid-insoluble arginine is 'protein' and that all other arginine will be detected as acid-soluble arginine by column chromatography. This will give the net flux to protein irrespective of any protein turnover. If, however, there are protein-breakdown products in the form of small peptides that do not appear in either the above fractions, the calculated flux would be an underestimate. No data on protein turnover are available for *Neurospora*, but for growing yeast cells Halvorson (1958) reports a value of 0.03%/h. This would correspond to a 'back' flux of arginine from protein of less than 0.3% of the net flux into protein. The neglect of any possible small peptide compartment is therefore not likely to affect the estimated value significantly. Mycelial acid-soluble radioactivity, for the reasons

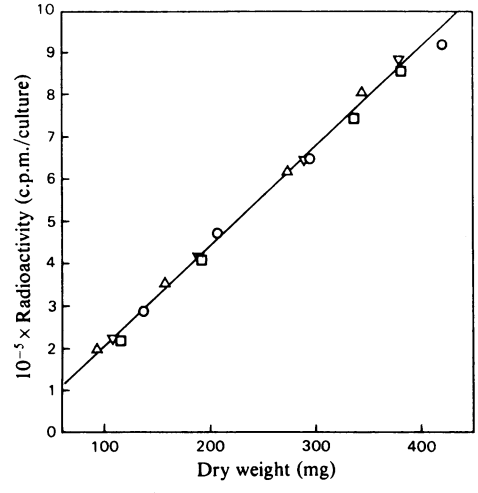


Fig. 4. *Protein arginine*
The rate of the arginine incorporation from the radioactivity of acid-insoluble precipitates of mycelium grown in [*ureido*-^{14}C]citrulline (see the text) is shown. The regression gives a value of 127.2 μmol of arginine/Δg dry wt. of mycelium. Symbols are as given in Fig. 1. Full details are given in the text.

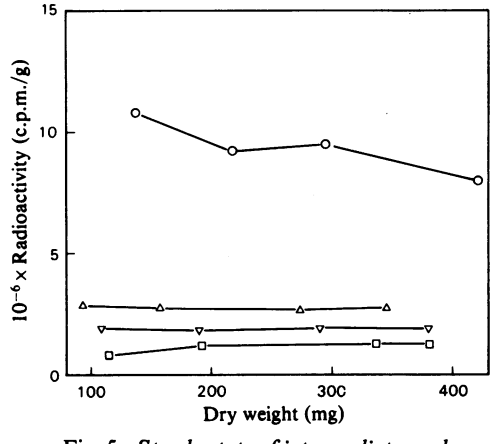


Fig. 5. *Steady state of intermediate pools*
Acid-soluble radioactivity from mycelium grown on [*ureido*-^{14}C]citrulline is shown. Only citrulline, argininosuccinate, arginine and urea are labelled. Symbols are as given in Fig. 1. Full details are given in the text.

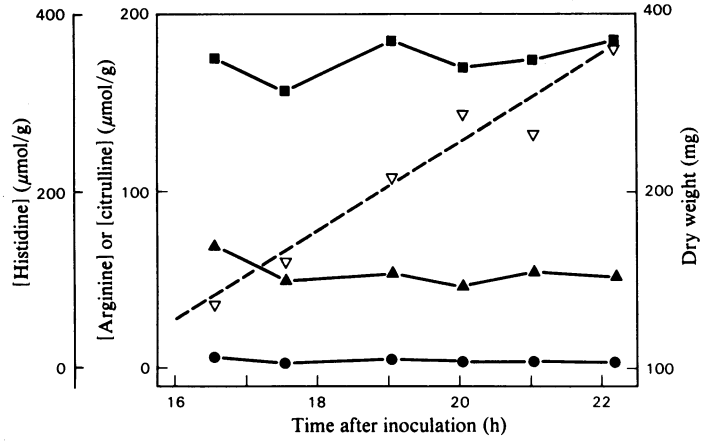


Fig. 6. *Steady state of individual pools*

Growth of mycelium on medium supplemented with 1.2 mM-histidine and 0.4 mM-citrulline is shown. Amino acid determination was by column chromatography and ninhydrin. ▽, Dry weight (logarithmic scale); ■, histidine; ▲, arginine; ●, citrulline. Full details are given in the text.

Table 1. *Influxes and effluxes*

Tabulated results of the experimentally determined fluxes into and out of the system shown in Scheme 2 at different compositions of the medium. Column (*a*), ratios of millimolar concentrations of citrulline and glycine in the medium; column (*b*), exponential growth constants from regression of log(dry weight) on time (cf. Fig. 3); column (*c*), citrulline depletion of medium from [*ureido*-^{14}C]citrulline (cf. Fig. 4); column (*d*), values (*b*) × values (*c*); column (*e*), urea accumulation in medium (determinations as described in the Materials and Methods section) (cf. Fig. 5); column (*f*), intramycelial urea determinations as for (*e*); column (*g*), values (*b*) × values [(*e*) + (*f*)]; column (*h*), values (*b*) × 127.2 (cf. Fig. 6); column (*i*), values (*g*) + values (*h*); column (*j*), values (*d*) − values (*i*).

(*a*) Composition of medium [Citrulline]/[glycine] (mM/mM)	(*b*) Growth constant (h^{-1})	(*c*) Citrulline depletion (μmol/Δg)	(*d*) Citrulline influx (μmol/h per g)	(*e*) Urea accumulation (μmol/Δg)	(*f*) Mycelial urea (μmol/g)	(*g*) Flux to urea (μmol/h per g)	(*h*) Flux to protein (μmol/h per g)	(*i*) Sum of effluxes (μmol/h per g)	(*j*) Apparent flux deficit (μmol/h per g)
1/0	0.245	675	165.4	222	10.2	56.9	31.2	88.1	77.3
1/1	0.22	663	145.9	287	9.6	65.1	28.0	93.1	52.8
1/2	0.20	652	130.4	219	10.1	45.8	25.4	71.2	59.2
0.5/1.5	0.20	413	82.6	106	6.0	22.4	25.4	47.8	34.8
0.5/2.5	0.19	329	62.5	47	4.7	9.8	24.2	34.0	28.5
0.5/3.5	0.15	276	41.4	31	2.0	5.0	19.1	24.1	17.3

given above, is restricted to the citrulline, argininosuccinate, arginine and urea pools, arginine and citrulline accounting for more than 85% of the label (on the basis of column chromatography; see the Materials and Methods section). The constant value of acid-soluble radioactivity (Fig. 5) therefore provides confirmation that the system is at metabolic steady state for each of the growth conditions.

That individual pools reach steady state is shown in Fig. 6 in an experiment employing growth on histidine/citrulline instead of glycine/citrulline medium, followed over a 6h period. Growth is exponential while the histidine, citrulline and arginine pools (as well as others not shown) are all substantially time-invariant. Although the inhibitor concentrations required to achieve a given influx are different for histidine and glycine, the two procedures give equivalent results (see also Fig. 8*c*).

Balance of fluxes

It is possible to construct a balance sheet of influxes and effluxes of the system. This is shown in Table 1. The depletion rate in the medium (column *c*) when multiplied by the growth constant (column *b*) gives the flux of citrulline into the mycelium. Similarly the accumulation rate of urea in the

medium gives one of the effluxes. To this, however, must be added the urea that is produced, but held, in the mycelium. Although the intramycelial urea has a time-invariant steady-state concentration (for any set of conditions), the mycelium is expanding exponentially and therefore the rate of urea production must have equalled that expansion to keep the mycelial concentration at a steady value. This 'flux to expansion' is of course simply the steady-state concentration (in μmol/g) multiplied by the exponential rate constant, k, (in h^{-1}) of growth. Columns (*e*) and (*f*) must therefore be added before multiplying by k to give the total flux to urea (column *g*). Finally, column (*h*) gives the flux of arginine into protein as described above. The sum of columns (*g*) and (*h*) then gives the net efflux of the system (column *i*). Comparison of the influx and efflux reveals an apparent deficit.

Expansion fluxes

Just as it was necessary to add the urea expansion flux to the accumulation of urea in the medium, so all the intermediate pools held in the growing system use up real fluxes, which the metabolic transformations must supply. Since we find time-invariant pool concentrations in conjunction with an exponential growth rate, fluxes to expansion for each pool must exist, which exactly balance the volume expansion due to growth. The deficit is therefore due to these internal expansion fluxes of citrulline, argininosuccinate and arginine, i.e. their steady-state concentrations multiplied by the growth rate constant. Table 2 gives the balance of all the fluxes and a comparison with the independently determined influx. There is good agreement over the whole range. It is worth noting that, at the highest influx rate, the second largest single flux in the system is in fact the citrulline expansion flux (47.0 μmol/h per g).

An interpretation of flux relationships in steady state growing systems such as bacteria and fungi must therefore take account of all expansion fluxes, unlike the steady-state analysis of non-growing systems such as mammalian liver preparations or erythrocytes. It should be noted that these expansion-flux calculations are independent of any possible compartmentalization, such as is the case with arginine. Irrespective of whether a pool is held in one phase or is distributed between several, with or without active transport between them, the finding that there is a steady state of the total pool implies a flux to expansion of that pool proportional to its concentration.

Another consequence of the expansion fluxes is that, again in contrast with the non-growing system, successive enzymes carry different fluxes. In particular, the largest flux is carried by the 'first' enzyme and each subsequent flux decreases by the proximal expansion flux. Table 3 demonstrates this. It should be noted that the sum of the effluxes is only between 53 and 64% of the influx over the range, which includes, at the lowest influx rate, conditions not dissimilar to the normal, wild type, flux. In the wild-type this flux, measured here by citrulline depletion, will, of course, be supplied by the proximal system, which has to satisfy all the expansion fluxes of its own intermediate pools. Of these pools the largest is probably glutamate (approx. 80 μmol/g). The 'first' enzyme of the pathway, glutamate dehydrogenase (enzyme 1), will therefore carry a very much higher flux than even the requirements to supply proline and polyamines in addition to arginine would suggest. Glutamate is also one of the principal transamina-

Table 2. *Expansion fluxes and balance sheet*

The expansion fluxes are calculated from the steady-state pool concentrations and the growth constants. These are added to the sum of the effluxes and compared to the citrulline influx. Columns (*a*), (*i*) and (*c*), as in Table 1; columns (*k*), (*l*) and (*m*), values (*b*) from Table 1 multiplied by pool concentrations from Fig. 10; column (*n*), sum of values (*i*), (*k*), (*l*) and (*m*).

(*a*) Composition of medium	(*i*) Sum of effluxes	(*k*) Arginine flux to expansion	(*l*) Argininosuccinate flux to expansion	(*m*) Citrulline flux to expansion	(*n*) Sum of all fluxes	(*c*) Citrulline influx
1/0	88.1	40.6	2.8	47.0	178.5	165.4
1/1	93.1	34.1	2.4	17.6	147.1	144.9
1/2	71.2	33.3	2.2	7.6	114.3	130.4
0.5/1.5	47.8	22.2	0.38	1.6	72.0	82.6
0.5/2.5	34.0	15.4	0.34	1.1	50.8	62.5
0.5/3.5	24.1	7.6	0.29	0.5	32.5	41.4
arg-12$^+$,*ure-1* on MIN	33.3	5.5	0.3	0.6	39.7	
arg-12$^+$,*ure-1* on glycine	26.6	3.84	0.26	0.52	33.6	

Table 3. *Fluxes through successive enzymes*

Because of the expansion fluxes successive steps carry decreasing fluxes in an exponentially growing steady-state system. Columns (*d*) and (*i*), as in Table 1; column (*p*), values (*i*) plus values (*k*) from Table 2; column (*o*), values (*i*) plus values (*k*) plus values (*l*) from Table 2.

(*d*) Flux* through system II	(*o*) Flux through argininosuccinate synthetase	(*p*) Flux through argininosuccinase	(*i*) Flux through arginase plus protein flux
165.4	131.5	128.7	88.1
145.9	129.5	127.1	93.1
130.4	106.7	104.5	71.2
82.6	70.4	70.0	47.8
62.5	49.7	49.4	34.0
41.4	32.0	31.7	24.1

* The values show only the citrulline influx. There is, in addition, a substantial glycine or histidine influx through the same transport system.

tion donors, and this implies an additional flux at least 20-fold that to arginine.

Pool relationships

By controlling the rate of influx through the inhibition by glycine or histidine, it is now possible to consider the relationship between the intermediate pools. They will settle to their steady-state values when the fluxes into and out of each pool balance, which will depend on the enzyme parameters of each flanking step.

The first pool to consider is the citrulline pool. We have already shown (Fig. 2) that citrulline influx is quite insensitive over a wide range of citrulline concentrations in the medium and depends largely on the inhibitor/citrulline concentration ratio. There are two fluxes that, at steady state, must balance this influx, namely the flux to argininosuccinate via argininosuccinate synthetase and the expansion flux of citrulline. The steady-state concentration of citrulline will settle to its value depending on these three fluxes and their relative changes. The response of the steady-state citrulline concentration to changing influxes is shown in Fig. 7. There is a small rate of rise up to about 110 flux units, after which the pool begins to rise sharply to more than 20-fold over the next 50 units. This means that the flux to argininosuccinate responds progressively less to increases in the influx, suggesting saturation of the argininosuccinate synthetase. Also shown in Fig. 7 are the citrulline expansion fluxes, and it is clear that from about 110 influx units the rate of rise of citrulline concentration is parallel to that of the influx relation. When the metabolic removal of citrulline by argininosuccinate synthetase is being progressively saturated, further, irreversible, injection of citrulline will result in an increasing rise of the pool concentration. Since the flux to expansion is

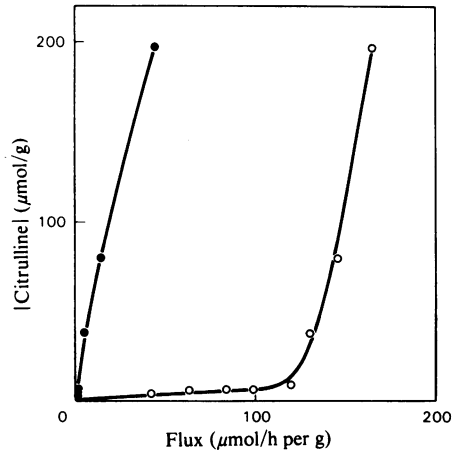


Fig. 7. *Intramycelial citrulline*

The change of the citrulline pool at different rates of influx (O) is shown. The citrulline pool is also plotted against the flux to expansion of citrulline (●), which is calculated by $F^{l}_{xp} = [\text{citrulline}] \cdot k_l$. The rate of change of the citrulline pool as a function of influx rate at high influx values can be almost entirely accounted for by the change in expansion flux. Full details are given in the text.

proportional to the citrulline concentration ($F_{xp} = [\text{citrulline}] \cdot k$), the concentration will increase until this internal flux reaches the same value as the 'excess' of the influx that is not being metabolized by the enzyme. No further rise in citrulline concentration will then take place at this point of balance, and the steady state will have been automatically reached. It is thus seen that, at high influx values, the 'control' of the citrulline concentration is by its own expansion flux. No feed-

back on the rate of influx is involved. The citrulline concentration 'controls' itself.

At lower concentrations of citrulline both expansion flux and metabolic conversion jointly determine the steady-state value. The increment in influx between the two highest inputs is 31.4 units (Table 2, column *n*), which is distributed between an increase in expansion flux of 29.4 units (93.6%) (Table 2, column *m*) and an increase in flux through argininosuccinate synthetase of 2 units (6.4%) (Table 3). The corresponding values for the increments between the two lowest inputs are: 18.3 units influx increment, 0.7 expansion (3.3%), 17.7 argininosuccinate synthetase (96.7%). The share of the control of the citrulline concentration is therefore seen to change substantially over the range of influxes.

Although the metabolic conversion of citrulline into argininosuccinate (the flux through argininosuccinate synthetase) is a major factor in determining the steady-state concentration of citrulline at the lower values of the flux, this flux in turn will be 'controlled' in part by the Mass Action effect of the pool itself. Both flux and concentration are interdependent variables, and neither can be said to be the 'cause' of the other. They are both 'effects'. The same is, of course, true for the subsequent steps.

The elucidation of the interdependence of these variables requires special experimental procedures (Kacser & Burns, 1979), which are not reported here. In the present paper we give semi-quantitative arguments that reveal the major operating factors.

The relationship between the three pools, citrulline, argininosuccinate and arginine, are given in Fig.

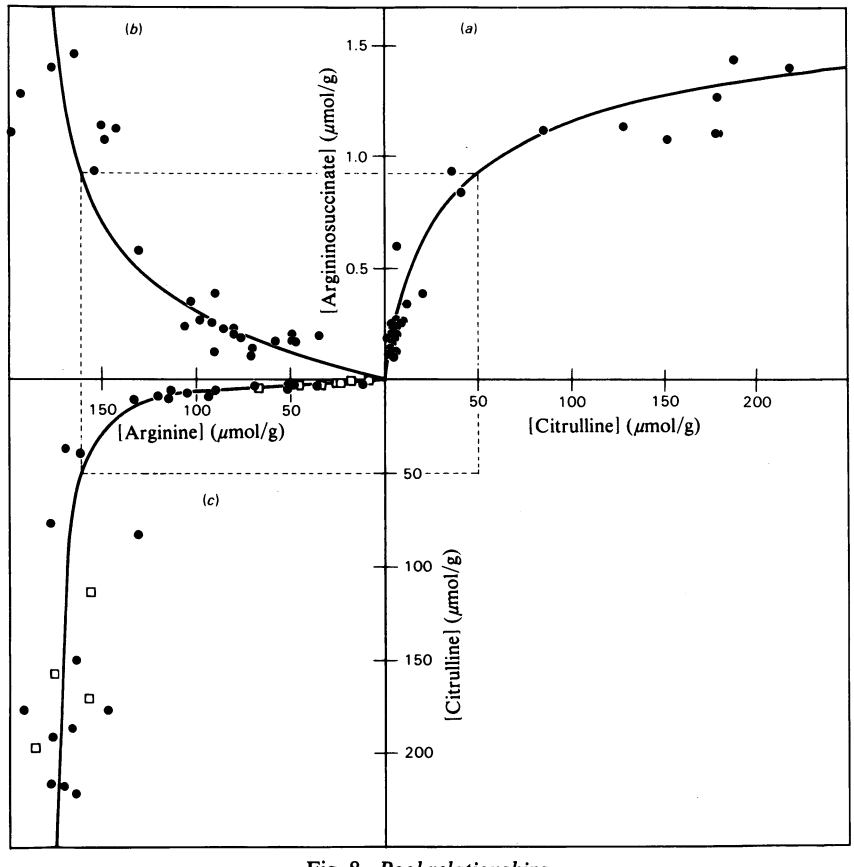


Fig. 8. *Pool relationships*

The substrate–product relationship for the two steps are shown as a reflection diagram. (*a*) Citrulline–argininosuccinate (argininosuccinate synthetase); (*b*) argininosuccinate–arginine (argininosuccinase), (*c*) shows the overall [citrulline]/[arginine] ratios, for which a larger number of determinations was available. ●, Glycine inhibition; □, histidine inhibition. The scale for argininosuccinate concentration was chosen 12-fold that for citrulline and arginine concentrations for graphical expediency and visual clarity. The curves shown are a visual 'best fit'. Any point on a curve is related to two points on the other curves by a rectangle indicated by the broken lines. Full details are given in the text.

8. They are shown as three 'reflection diagrams'. Fig. 8(*a*) is the relationship between the substrate, citrulline, of argininosuccinate synthetase and its product, argininosuccinate. [It should be noted that the concentration scale for the latter is more than 10-fold that of citrulline (and arginine) for visual clarity.] Fig. 8(*b*) shows the corresponding relationship for argininosuccinase, the enzyme catalysing the next step, between argininosuccinate and arginine. These two relations must generate that between citrulline and arginine (Fig. 8*c*). Apart from experimental error of the individual determinations, the three curves are constrained by the necessity that any two define the third. The constraints on the curves are indicated for a single set of values by the rectangle indicated by broken lines.

Since no functions for these relations are algebraically derivable (Kacser & Burns, 1973, 1979), the curves are visual 'best fits' for the data. There is, of course, a narrow family of three curves, all mutually consistent, that will be equally acceptable, but the choice of any other set can only marginally differ from the one given and will not affect the conclusion that may be drawn. Values taken from the curves are therefore a better estimate than any set of experimental points shown.

The rate across the argininosuccinate synthetase involves six molecular species:

$$\text{Citrulline} + \text{aspartate} + \text{ATP} = \text{argininosuccinate} + \text{ADP} + \text{P}_i$$

It is possible to write down an expression for the instantaneous rate for the step in terms of the participating molecules and certain kinetic constants. The concentration of aspartate, however, does not change greatly in comparison with those of citrulline and argininosuccinate over the range of fluxes, and we have no evidence that the [ATP]/[ADP] ratio changes. Since aspartate, ATP and ADP each take part in many other reactions, they are considerably buffered towards changes in any one of their participating fluxes. As a good approximation we can therefore take the following rate law as representing the steady states for any set of values for the concentrations of citrulline and argininosuccinate:

$$v_{CA} = \frac{\dfrac{V_C}{K_m^C}\cdot\left([\text{citrulline}] - \dfrac{[\text{argininosuccinate}]}{K_1}\right)\cdot C_1}{C_2 + \dfrac{[\text{citrulline}]}{K_m^C} + \dfrac{[\text{argininosuccinate}]}{K_m^A}}$$

where v_{CA} is the rate from citrulline to argininosuccinate, $V_C = V_{max.}$ in the 'forward' direction from citrulline, K_m^C and K_m^A are the operational Michaelis constants for this particular milieu with respect to citrulline and argininosuccinate respectively and K_1, C_1 and C_2 are constants involving sums and products of the other, invariant, molecular species and constants.

Reversibility does not contribute significantly to the citrulline–argininosuccinate relation, since we have shown that the *arg-12* mutant, when grown on arginine (and therefore with no endogenous or exogenous supply of citrulline), accumulates substantial concentrations of argininosuccinate (approx. 10-fold wild-type values) owing to the presence of argininosuccinase, but contains less than 3% of wild-type concentration of citrulline in spite of the presence of active argininosuccinate synthetase. Although the 'backflow' from arginine to argininosuccinate is therefore substantial, the flow from argininosuccinate to citrulline is negligible. The use of ^{14}C-labelled arginine in the same type of experiment showed substantial labelling in argininosuccinate but no detectable label in the citrulline region of the chromatographic fraction. In the virtual absence of a back reaction, the numerator term in the equation will therefore contain effectively only the changing citrulline pool concentrations, the argininosuccinate term being negligible. The denominator, on the other hand, being the 'saturation term', will lower the rate by virtue of the 'substrate saturation' ([citrulline]/K_m^C) and 'product inhibition' ([argininosuccinate]/K_m^A) and will therefore depend on the changing molecular concentrations and their respective Michaelis constants. These latter, when known, are determined *in vitro*, and the well-known difficulties apply of extrapolating these values to the conditions *in vivo* where the milieu is likely to be different. An added difficulty in our case is the problem of translating the pool concentrations from mol/g into mol/l, already referred to, when considering the absolute Michaelis values. The ratios of Michaelis constants determined under the same conditions are, however, more likely to reflect the comparative effects of saturation when the ratios of the respective molecular concentrations (in any units) are known.

Fig. 9 shows the argininosuccinate synthetase flux as a function of substrate and product. Since argininosuccinate is directly derived from citrulline, the pools will always be positively correlated and the saturation terms for both in the denominator will be increasing. The fact that the argininosuccinate concentration is an order of magnitude lower than that of citrulline is, of course, not evidence that its effect on the inhibition of the flux is negligible. No values for K_m^A are available. By estimating the Mass Action ratio, [citrulline]/[argininosuccinate], for the range of fluxes from the data of Fig. 8(*a*) we find that it changes from about 1, at lowest fluxes, to about 15 at the highest. This change points to a progressive inhibition of the rate with increasing substrate concentrations. The limitations of the flux

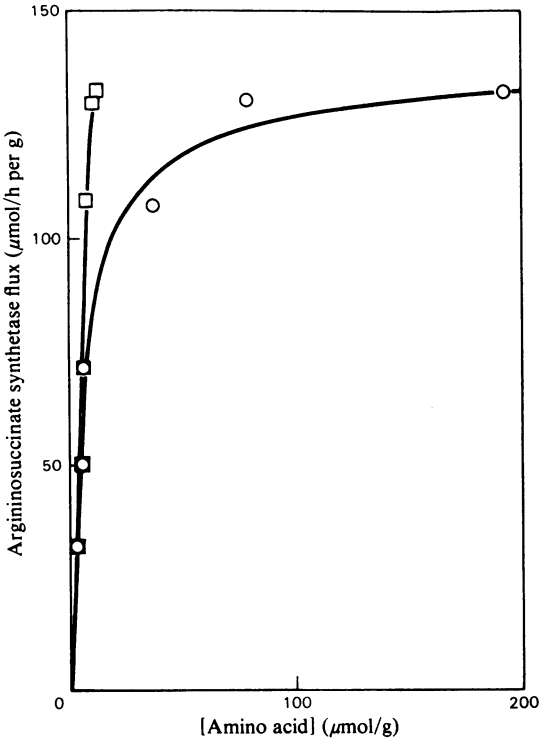


Fig. 9. *Flux–pool relationship: argininosuccinate synthetase*
The flux across argininosuccinate synthetase as a function of citrulline concentration (O) and of argininosuccinate concentration (□) is shown. Full details are given in the text.

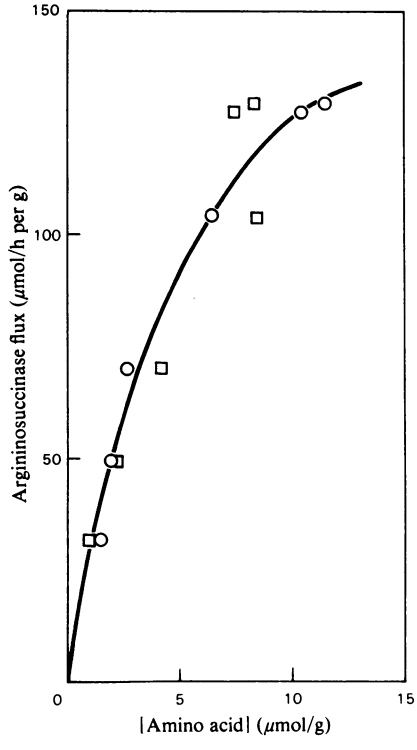


Fig. 10. *Flux–pool relationship: argininosuccinase*
The flux across argininosuccinase as a function of argininosuccinate concentration (O) and of cytosolic fraction of the arginine concentration (□) is shown. Full details are given in the text.

at higher flux values are therefore due to the saturation of argininosuccinate synthetase by citrulline and/or argininosuccinate, as shown by the evidence of Figs. 7 and 9.

Although the rate of argininosuccinate production (argininosuccinate synthetase flux) is thus set by the factors discussed above, its steady-state values will in turn be affected by its rate of removal by the next step. By an argument similar to that for argininosuccinate synthetase, the rate across argininosuccinase can be represented by:

$$v_{AR} = \frac{\frac{V_A}{K_m^A}\cdot\left([\text{argininosuccinate}] - \frac{[\text{arginine}]}{K_2}\right)\cdot C_3}{C_4 + \frac{[\text{argininosuccinate}]}{K_m^A} + \frac{[\text{arginine}]}{K_m^R}}$$

where the symbols have the same meanings as before except that they refer to the enzyme parameters of argininosuccinase. In this case there is reasonable reversibility with an equilibrium constant, $K_E = 1.1 \times 10^{-2}$M (Ratner, 1970). The Michaelis constants *in vitro* are also known (Cohen & Bishop, 1966; Chilcott, 1965) and are: for argininosuccinate, $K_m^A = 2.1 \times 10^{-4}$M; for arginine, $K_m^R = 8.1 \times 10^{-4}$M.

The pool values plotted in Fig. 8(*b*) are the total extractable pools. Whereas argininosuccinate is almost certainly entirely cytosolic, arginine on the other hand is distributed between a vesicle (~99%) and the cytosol (~1%) at wild-type flux values (Subramanian *et al.*, 1973; Davis *et al.*, 1978). By similar methods, using pulse-labelling, we estimate that at 160μmol of arginine/g (derived from citrulline) the cytosolic arginine fraction is 5%. We have used a linear interpolation between these two values to estimate the cytosolic fractions for intermediate values of total extractable arginine. When these estimates, together with the extrapolated curve of Fig. 8(*b*), are used to calculate the cytosolic [argininosuccinate]/[arginine] ratios, the values obtained are about 1.0 for the lowest flux and 1.4 for the highest. Since the Michaelis constant for the forward reaction is 4-fold lower than that for the back reaction, though the cytosolic argininosuccinate and arginine concentrations are roughly equal

over the range of fluxes studied (Fig. 10), it is clear that argininosuccinate will play a greater role than arginine if any saturation of the step occurs.

The fact that the Mass Action ratio ([argininosuccinate]/[arginine]) does not change greatly indicates that, unlike the preceding argininosuccinate synthetase, no substantial progressive inhibition of the argininosuccinase rate takes place, but suggests that reversibility plays an important part. An estimate of this reversibility can be obtained by determining the [argininosuccinate]/[arginine] ratios in mutants that are blocked before argininosuccinase and grown on arginine. These are *arg-12* (ornithine carbamoyltransferaseless) and *arg-1* (argininosuccinate synthetaseless). Both of these, when grown on 3 mM-arginine, have concentrations of 151 μmol of arginine/g and 2.2 μmol of argininosuccinate/g. Under these conditions the cytosolic fraction of arginine is about 10% (Weiss, 1976; Davis *et al.*, 1978), giving an [argininosuccinate]/[arginine$_{cyt.}$] ratio of 0.15. Under these circumstances the net flux across argininosuccinase is arginine$_{cyt.}$ → argininosuccinate → expansion. Although the argininosuccinate expansion flux is small (approx. 0.5 flux unit) and thereby puts the ratio slightly out of equilibrium in favour of arginine, a ratio of argininosuccinate ≒ arginine$_{cyt.}$ of about 0.2 is a reasonable estimate of the equilibrium. This means that the Mass Action ratio of about 1 in our steady-state system is about 5-fold out of equilibrium, but that reversibility contributes significantly to this value.

The effect of sequestration of arginine by the vesicle cannot be discussed in detail in the absence of reliable data on the distribution of arginine between vesicle and cytosol over the full range of fluxes. The values for cytosolic arginine shown in Fig. 10 are based on the assumption of a linear increase in the cytosolic fraction from 1 to 5% over the range of fluxes with increasing total extractable arginine.

Since the total concentration of argininosuccinate is small, the flux through argininosuccinase is only between 1 and 2% smaller than that through argininosuccinate synthetase. For the same reason the flux to expansion of argininosuccinate contributes very little to the control of its pool. Since equilibration plays a major role, it is the rate of removal of arginine that will strongly influence the changes in the argininosuccinate pool. For this we must turn to the final steps in the metabolic chain. Fig. 11 shows the three exit fluxes, to protein, to expansion and to urea. Since the arginine proportion in the protein does not change, the flux to protein reflects only the minor changes in the growth rate constant. The flux to expansion is of course linearly related to the total arginine concentration. It is the flux to urea, however, that requires discussion.

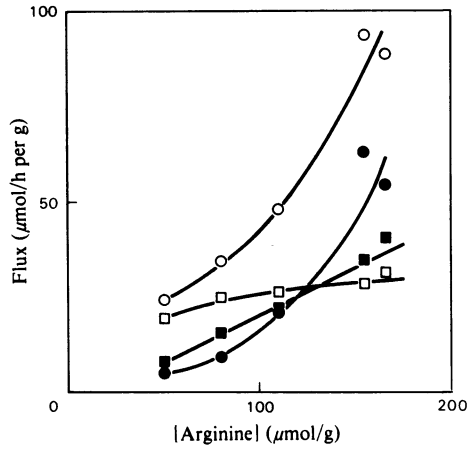


Fig. 11. *Flux–pool relationship: arginase and protein flux*

The protein flux (□) is estimated from the growth constants and the arginine content (127.2 μmol/g) by $F_p = 127.2k$; ●, the flux through arginase from urea determinations; ■, the expansion flux calculated by [arginine]·k. The sum of all the exit fluxes is shown as ○. Full details are given in the text.

The rate increases more than proportionately with increase in the arginine concentration, which is shown as the total extractable value. This is discussed below. The net outcome of the three exit routes of arginine is that the expansion flux and urea flux jointly are the principal factors in controlling the changing arginine concentrations.

Changes in enzyme activity

We must now turn to the possible effects of changes in the enzyme concentrations or activities on the fluxes across each step. It was known (Castaneda *et al.*, 1967; Weiss & Davis, 1977) that arginase was inducible by high concentrations of arginine. Fig. 12 shows arginase activity as a function of total arginine concentration. Specific-activity ratios are plotted, i.e. activities relative to those found in the minimal-medium-grown wild-type.

Although the exact induction kinetics cannot be deduced from these few data, it is evident that they differ considerably from the de-repression kinetics of the biosynthetic enzymes (Barthelmess *et al.*, 1974). For these enzymes de-repression occurs at values of arginine concentration below 10 μmol/g, at which value the enzymes respond sharply to small changes in arginine concentration, as indicated by the broken line. The slope of arginase change over the range of arginine concentration values is never very steep, making an allosteric mechanism with a high Hill coefficient unlikely (Burns & Kacser, 1977).

The combination of Mass Action effects on reaction rate and induction effects on enzyme concentration by the increasing arginine concentrations is expected to produce an accelerating response of the urea flux to arginine concentration. This is in agreement with observation. The precise relations cannot be predicted until the true cytosolic concentration of substrate, the saturation parameters with respect to arginine and urea and the induction signal concentrations are known.

Although both the urea flux (Fig. 11) and the arginase induction (Fig. 12) are shown plotted against total arginine concentration, though for both mechanisms only the cytosolic arginine concentration is relevant, it is the relative changes of the two that are important. No matter what transformation of the concentration of total arginine into one of cytosolic arginine is made, the observed urea flux will be seen to move more than proportional to the arginase values. This must mean that Mass Action and induction act multiplicatively, as would be predicted from the rate expression, and that any changes in saturation or inhibition must be negligible.

Since the enzyme induction response is approximately linear with arginine concentration, the flux response can, at the most, be quadratic with arginine concentration. This situation will obtain when the arginase is unsaturated, such that the mass action effect is also linear with arginine concentration. Citrulline has been reported as being capable of inhibiting arginase *in vitro* (Mora *et al.*, 1972). Inhibition by citrulline *in vivo*, however, must clearly be negligible, since the present study shows that the urea flux increases about quadratically with arginine concentration despite a 60-fold change in citrulline concentration over the range studied (see Fig. 8*c*).

The specific activities of argininosuccinase and argininosuccinate synthetase change little over the range of arginine concentrations examined here. There is a small 'induction' (~20%) from the present lowest to the highest arginine concentrations, a phenomenon already reported by Barthelmess *et al.* (1974) as well as by Karlstrom & Gorini (1969) for *Escherichia coli*. This remains an unexplored phenomenon, but it can play only a minor role in the face of the much larger changes in pools and arginase activity.

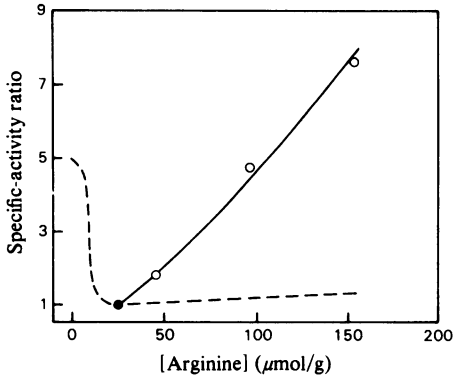


Fig. 12. *Inductions and de-repressions*
Arginase (O) was determined as described in the Materials and Methods section. It is expressed as values relative to those of the wild-type on minimal medium (●). The other values were obtained from various growth conditions involving citrulline, citrulline/histidine or arginine supplements. They are the means of between three and six determinations. The specific-activity ratios are compared with those of the co-ordinately changing last three biosynthetic enzymes (----) from Barthelmess *et al.* (1974). Full details are given in the text.

General Discussion

Evidence has been presented to show that the three steps in our system display quantitatively different relationships for the molecular interactions that control fluxes and intermediate pools. At low influxes the argininosuccinate synthetase is unsaturated and its flux responds to the small rises in its substrate citrulline concentration in a linear fashion. At high influxes the saturation of argininosuccinate synthetase limits the flux whereas the citrulline expansion flux determines the high steady-state value of the pool. For the argininosuccinase-mediated transformation reversibility appeared to be important over the whole range in determining the ratio of substrate to product. Neither expansion nor saturation plays a significant role in influencing the flux, which is virtually the same as for the preceding step. In the arginase step a major factor is the inducibility of the enzyme by its substrate arginine, which results in a more than proportional change of the flux to increases in its substrate.

What role the sequestration of a large part of the arginine into the vesicle plays is not clear, since the mechanism of the reversible interchange between the cytosol and the vesicle is still rather obscure. The isolation of a high proportion of the arginine from participation in metabolic activities does not in itself have any consequences on the control properties of the system at steady state except that it contributes a quantitatively important flux to expansion. This flux not only affects the arginine concentrations but must be carried by all preceding enzyme steps.

Although our 'distal' system is metabolically isolated from events in the 'proximal' moiety, it would still be subject to the influence of possible effectors from that part. If such effectors were changing as a

result of changes in the distal moiety, these additional interactions would have to be taken into account. Ornithine is the principal candidate. Two mechanisms, emanating from arginine, affect its concentration. (i) Arginine catabolism produces equimolar amounts of urea and ornithine and the changes in urea flux are therefore also those for the arginine-derived ornithine. (ii) Arginine acts as an inhibitor for acetylglutamate kinase, an early enzyme in ornithine synthesis.

These two opposing effects, together with the other ornithine-catabolic steps, result, however, in no significant changes in the steady-state concentration of ornithine taking place over the range investigated. Ornithine has been reported to be an inhibitor of arginase (Davis *et al.*, 1978). It is also sequestered into the vesicle, and therefore only its cytosolic concentration, a small fraction of its total concentration ($\sim 10\,\mu$mol/g), is available for this role. If this value *in vivo* is significant in arginase inhibition, it would constitute a constant factor, lowering the rate of urea production. Our evidence on the urea flux suggests that it is of little importance.

Little is known about the mechanism of urea efflux into the medium. There is a small increase in intramycelial urea concentration for large changes in urea efflux. This is consistent with a relatively unsaturated enzyme mechanism or with a diffusion-dependent process.

The device of cutting the system into moieties enabled us to modulate the citrulline flux in a manner not possible with the complete system. Although all steps interact, each influencing each other, we were able to assign semi-quantitative importance to some of the mechanisms by having available response curves over a large range of variation. This range covered, at low flux values, the condition in the wild-type when it is 'linked' to the proximal moiety. Under these conditions the disturbance of the system by our experimental procedures appears to be minimal. A comparison of the fluxes in the wild-type (*arg-12*$^+$) in Table 2 with the lowest influx steady states of our series indicates that we have successfully replaced the endogenous citrulline supply by an equivalent, and measurable, exogenous supply. Reference to the dependence of the flux on the substrates and products (Figs. 9, 10 and 11) shows that, at wild-type flux values, the enzymes in the distal part all operate under essentially unsaturated conditions. This makes the kinetic treatment of the wild-type when grown on minimal medium easier. The experimental separation into the two moieties makes it possible to investigate the 'proximal' part by essentially the same methods as reported in the present paper and allows a 'synthesis' of the whole.

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