Control of the flux in the arginine pathway of *Neurospora crassa*

Modulations of enzyme activity and concentration

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The influence of particular enzyme activities on the flux of metabolites in a pathway can be estimated by 'modulating' enzymes (i.e. changing turnover or concentration) and measuring the response in various parts of the system. By controlling the nuclear ratio of two genetically different nuclear types in heterokaryons, the enzyme concentrations at four different steps in the arginine pathway were decreased over a range. This range was extended by the use of bradytrophs, mutant strains specifying enzymes with greatly diminished enzyme activities. Strains altered simultaneously at more than one step were also constructed by genetic recombination. By measuring the outputs of the pathway and the steady-state concentrations of intermediate pools, the fluxes in different parts of the pathway were calculated. This allowed the construction of flux/enzyme relationships, the slope of which is a measure of the sensitivity of a flux to the change in enzyme activity at that step. All fluxes were found to be considerably buffered for quite substantial decreases in the activities of all enzymes. Mass action plays an important part in this phenomenon, as do inhibition and repression. Because of the existence of expansion fluxes in growing systems, we find quantitatively different fluxes in different parts of the single pathway. For the same reason some enzyme modulations give decreased fluxes in one part and increased fluxes in another. The understanding of control in the pathway thus involves consideration of many mechanisms operating simultaneously and the estimation of changes in the whole system. The concept of a 'rate-limiting step' is found to be inadequate and is replaced by a quantitative measure, the Sensitivity Coefficient, which takes account of all the interactions. It is shown that control of the flux is shared among all the enzymes of the pathway. The results are discussed in terms of the theory of flux control.

In a previous publication (Flint *et al.*, 1980) the roles of reversibility of reactions and of saturation, inhibition and induction of enzymes in affecting the flux in part of the arginine pathway of *Neurospora crassa* were established. In the work reported in the

present paper we investigate how variation in the concentration or activity of enzymes affects the flux through the pathway. Since *Neurospora*, grown on minimal medium, shows maximal repression for all but one of the pathway enzymes (Barthelmless *et al.*, 1974; Cybis & Davis, 1975), quantitative modulation of enzyme activity cannot be achieved by the addition of metabolites, such as arginine, to the medium. Two experimental approaches have been adopted to overcome this difficulty.

The first depends on the possibility of constructing heterokaryons where one of the nuclear types carries a non-functional allele for a specific enzyme activity while the other nuclear type carries the normal form of the gene. By controlling the ratio of the two nuclear types in a number of heterokaryons, an effective control over the concentration of the enzyme is achieved.

Abbreviations used (in subscripts): ASAase, arginino-succinase; OTCase, ornithine carbamoyltransferase; SYNase, argininosuccinate synthetase.

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The second approach uses 'bradytrophic' strains, i.e. mutants with greatly diminished enzyme activities at those loci for which the heterokaryon method achieved decrease in enzyme concentrations. By this method it is possible to obtain much greater decreases in enzyme activities, since extreme nuclear ratios are, for technical reasons, difficult to achieve in heterokaryons.

As a consequence of the experimental approaches two negative-feedback mechanisms, inherent in the metabolic system, are brought into operation. Firstly, arginine is the end product of the pathway as well as the signal for de-repression of all the pathway enzymes (Barthelmess *et al.*, 1974). As a consequence of the decrease in flux, there is the possibility that the arginine concentration becomes lowered. This, in turn, may cause the de-repression mechanism to operate if the arginine concentration moves into the 'control range' (Fig. 1). The net result is a new steady state with a decreased flux to arginine and, except for the mutationally 'throttled' step, elevated enzyme concentrations. Thus both decreases and elevations in enzyme activities are achieved, albeit not independently of one another. The second feedback is on an early enzyme in the pathway, acetylglutamate kinase, that is subject to inhibition by arginine (Cybis & Davis, 1974).

The observed decrease in flux will depend on the magnitude of the change in enzyme activity as well as on the 'sensitivity' of the flux to changes at this particular step. Different steps along the pathway will, in general, differ in this respect.

By imposing a series of modulations at four steps in the pathway (Scheme 1) a corresponding range of quantitatively different steady-state fluxes is obtained that can be estimated experimentally by suitable methods. The present paper attempts a quantitative analysis of the effects of changes in particular enzyme activities on the flux in various parts of the arginine pathway and discusses the results in terms of the general theory of control (Kacser & Burns, 1973, 1979, 1981).

Materials and methods

Strains

These are described in Barthelmess *et al.* (1974) and Flint *et al.* (1980). In addition the following mutants were used: *arg-5⁻* (27947) (F.G.S.T. no. 408) and *ad-4⁻* (F48) (F.G.S.T. no 443). All strains had been back-crossed seven to ten times to strain 74a or 74A (St. Lawrence).

Heterokaryons

Four types of heterokaryons were constructed: *ad-4/arg-5*; *ad-4/arg-12*; *ad-4/arg-1*; *ad-4/arg-10*.

Heterokaryons of defined nuclear ratio and hence defined enzyme concentration can be obtained by the 'pellet' method of Pittenger *et al.* (1955). This was not found to be very reproducible, and hence the following method was adopted. A heterokaryon was produced by superimposition of homokaryotic conidia on the surface of minimal slants. The resulting mycelium was allowed to conidiate, and conidia

Fig. 1. De-repression of enzymes

The ordinate shows the specific activity of the last three enzymes of the arginine pathway for various steady-state concentrations of arginine, the repression signal, divided by the value of the enzymes at wild-type arginine concentration. □, Ornithine carbamoyltransferase; △, argininosuccinate synthetase; ○, argininosuccinase; ●, value for all three enzymes (wild-type, on minimal medium). The abscissa shows the arginine concentration on a logarithmic scale. [From Barthelmess *et al.* (1974).]

were plated at low density on minimal-sorbose plates. Each colony represents a single heterokaryotic conidium. These conidia would contain 2, 3, 4, 5 . . . n number of nuclei, with most having 2 or 3. The nuclear proportions of these conidia would represent 80%, 75%, 66%, 50%, 33%, 25% etc. of one nuclear type, the majority class being 50% and 66% or 33%. The frequency of the different conidial types is determined by the nuclear ratio of the original parent mycelium. Each colony is transferred to a minimal slant, and the nuclear ratio of the resulting mycelium is determined, after conidiation, by appropriate platings. These cultures were found to be quite stable both on slants and in liquid culture. From these results, a number of slants representing a wide range of ratios was then chosen for further work.

The conidia from any slant represent the composition of the mycelium from which they arose. Each conidium, on the other hand, will be the result of 'segregation' into the conidial classes described above as well as homokaryotic conidia of various nuclear numbers. To regenerate the composition of the slant, the mass inoculation of conidia must ensure that complete anastomosis of all conidia has taken place. This was achieved by the following procedure.

About 10^7 conidia were suspended in 50 ml of Vogel's minimal medium (Vogel, 1956) supplemented with 0.05% arginine and 0.01% adenine and incubated at 28°C with gentle shaking for 6 h. The germinated conidia were spun on a bench centrifuge, washed in minimal medium and finally resuspended in 10 ml of minimal medium, without shaking, for 16 h. The anastomosis was performed on the bottom of 1-litre flat-bottomed flasks that had three 'dimples' blown into their sides. After 16 h 400 ml of minimal medium was added and further growth took place on a rotary shaker (Flint *et al.*, 1980). The dimples had the effect of breaking up the initial mat and keeping the growing mycelium from clumping. Five to seven flasks were used for each heterokaryon, and complete flasks were harvested for each time point. Enzyme assays and pool determinations were conducted on freeze-dried samples of the harvests.

Assays of enzyme activities

Enzyme activities were determined as described in Flint *et al.* (1980) and Barthelmess *et al.* (1974). Acetylornithine aminotransferase activity was determined as described by Vogel & Jones (1970).

Determination of amino acid pools

Amino acid pools were determined as described by Flint *et al.* (1980).

Determination of protein arginine

A 10 mg portion of freeze-dried mycelium was

extracted with cold 5% (w/v) trichloroacetic acid, and the precipitate was washed three times in 5% trichloroacetic acid and finally washed five times in ether. The precipitate was hydrolysed in 6 M-HCl in a de-gassed closed Pyrex tube at 105°C for 20 h. The contents were dried over NaOH in a vacuum desiccator, redissolved in distilled water, acidified and loaded on to an amino acid analyser (Flint *et al.*, 1980).

Results and discussion

Estimation of fluxes

Four series of heterokaryons were produced, by using, as one nuclear type, one of four different arginine auxotrophic strains in each series: the mutants *arg-12⁻* (lacking ornithine carbamoyltransferase activity) *arg-1⁻* (lacking argininosuccinate synthetase activity), *arg-10⁻* (lacking argininosuccinase activity) and *arg-5⁻* (lacking acetylornithine aminotransferase activity). The second nuclear type in each case was wild-type at all four loci but had *ad-4⁻* (lacking adenylosuccinate lyase) as a forcing marker. The relevant portion of the arginine pathway is shown in Scheme 1. Heterokaryons belonging to a particular series carried the same arginine auxotrophic mutation, but differed in their ratios of *arg⁺* and *arg⁻* nuclei and hence in the concentration of the enzyme specified by the *arg⁺* locus. The methods used to generate and grow heterokaryons of the desired nuclear ratio are given in the Materials and methods section. In addition, four bradytrophic strains are discussed in this section: *arg-12s* (decreased ornithine carbamoyltransferase activity, approx. 4% of wild-type), *arg-1r26* and *arg-1r3* (decreased argininosuccinate synthetase activity, approx. 36% and 23% respectively) and *arg-10r1* (decreased argininosuccinase activity, approx. 5%).

Bradytrophs are strains that, as a result of a mutation affecting some enzyme activity, show various degrees of slow growth from 'leakiness' to almost full growth. They can be produced by 'forward' mutation or, as in the present case, by reversion from auxotrophy. All our strains grow with only slightly diminished rates in spite of severe decreases in enzyme activity (as do our extreme heterokaryons). One difficulty in combining results from heterokaryons and bradytrophs concerns the establishment of a common scale for 'enzyme activity'. Whereas heterokaryons, containing 'null' alleles for a particular enzyme, will have decreased concentrations of this enzyme (and hence decreased $V_{\max}$ only), bradytrophs may be mutationally altered in turnover (affecting $V_{\max}$) as well as in any of the Michaelis constants occurring in the rate expression. The net effect on the enzyme activity is therefore more complex than the simple $V_{\max}$ values

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). The loci specifying the structural genes for these enzymes are given by their genetic designations.

appropriate to heterokaryon comparisons. There is, of course, a notional change in enzyme concentration that is equivalent to any mutational change no matter how many kinetic parameters are affected. For a monomolecular transformation without saturation this is given by the ratio $V_{\max}/K_m$, but more complex algebraic expressions apply to other cases. Since the major effect of the mutations in our bradytrophic strains is on the turnover, and since we have shown that the wild-type enzymes operate at low saturation (Flint *et al.*, 1980), we shall use the above expression as a good approximation for 'equivalent enzyme activity'.

Table 1 shows the sizes of intermediate pools, the protein arginine and the activities of 'modulated' enzymes observed in these heterokaryons and bradytrophic strains during growth on minimal medium. Also shown are the exponential growth constants. [It should be noted that these refer to exponentially growing liquid cultures. When these strains are grown on solid medium in so-called race tubes, no difference in growth rates are discernible (Kacser & Burns, 1968). Such tubes measure essentially the rate of hyphal extension and not the total flux to biomass.] The bradytrophic strains have been tabulated along with the appropriate series of heterokaryons according to the enzyme activity affected.

The data in Table 1 have been used to calculate

fluxes. There are two types of fluxes in such a system: (1) the metabolic outputs (protein and urea) and (2) the internal 'expansion fluxes'. They are shown in Table 2. The arginine flux to protein will depend on the mean arginine content of the proteins and the rate of synthesis of these proteins. This flux to protein is therefore obtained by the product of the protein arginine per g dry wt. of mycelium and the exponential growth constant (k). [All concentrations are expressed as $\mu\text{mol/g}$ dry wt. of mycelium (instead of $\mu\text{mol/l}$) since the effective volume relevant to any metabolite is unknown.] The urea flux under the conditions of the present experiments is negligible, for reasons discussed below. The sum of these two metabolic output fluxes is, of course, carried by every step in the pathway.

In addition, 'expansion fluxes' are generated in all growing systems (Flint *et al.*, 1980). Since the experiments were conducted under conditions of exponential growth, the time-invariant steady-state value of each intermediate pool implies a flux to each metabolite that balances the continuous volume expansion. This flux to expansion is therefore obtained by the product of the steady-state pool concentration and the exponential growth constant ($F^{\text{xp}} = [S] \times k$). The growth constant, k , obtained from weight/time data, is, of course, also the constant for the growth of the volume.

Thus the flux to arginine (which is the flux carried

Table 1. *Changes in growth constant and pools*

The V_{max} values are expressed as a percentage of the value found for wild-type homokaryon. The exponential growth constant is obtained from weight/time data. $k = \ln 2/\text{doubling time}$. For argininosuccinate most of the values recorded are a common mean. Argininosuccinate is difficult to determine accurately in low concentrations because of its conversion into its two anhydrides on ion-exchange columns. Full details are given in the Materials and methods section. Abbreviations: Orn, ornithine; Cit, citrulline; Asa, argininosuccinate.

Modulated enzyme	Strain	V_{max} of modulated enzyme (%)	Exponential growth constant (h^{-1})	Intermediate pools ($\mu\text{mol/g dry wt.}$)				Protein arginine ($\mu\text{mol/g dry wt.}$)
				Glu	Orn	Cit	Asa	
None	Wild-type	100	0.29	121.6	10.8	3.7	1.3	130.1
	arg-12 ⁻ ad-4 ⁺ /arg-12 ⁺ ad-4 ⁻ heterokaryons	73	0.24	121.7	17.2	2.0	1.3	154.0
Ornithine carbamoyltransferase		56	0.24	110.2	18.2	2.8	1.3	150.5
		48	0.26	105.0	20.5	3.4	1.3	148.6
		47	0.23	111.0	17.7	2.0	1.3	141.4
		42	0.26	131.3	30.3	3.0	1.3	142.4
		21	0.26	99.0	20.7	2.6	1.3	143.7
	arg-12s bradytroph	4	0.22	75.8	34.4	0.6	0.8	130.1
Argininosuccinate synthetase	arg-1 ⁻ ad-4 ⁺ /arg-1 ⁺ ad-4 ⁻ heterokaryons	89	0.25	90.6	17.6	11.6	1.3	132.3
		47	0.27	90.5	7.8	24.8	1.3	129.0
		46	0.25	84.2	6.5	58.4	1.3	131.9
	arg-1r26 bradytroph	43	0.20	82.5	7.2	65.2	1.3	130.8
Argininosuccinase	arg-1r3 bradytroph	36	0.22	106.3	9.5	76.7	1.3	130.0
	arg-10 ⁻ ad-4 ⁺ /arg-1 ⁺ ad-4 ⁻ heterokaryons	23	0.19	81.5	16.1	241.8	1.0	130.1
		55	0.30	134.7	14.8	5.3	5.7	128.4
		54	0.29	127.1	14.5	6.0	6.6	126.0
Acetylornithine aminotransferase	arg-5 ⁻ ad-4 ⁺ /arg-5 ⁺ ad-4 ⁻ heterokaryons	26	0.28	88.2	8.1	34.0	58.7	126.7
	arg-10r1 bradytroph	10	0.20	59.8	10.6	78.7	132.3	105.2
		5	0.19	54.4	6.5	54.5	186.6	130.1
		68	0.27	128.0	30.3	8.8	1.3	137.7
		52	0.30	119.6	17.9	6.2	1.3	138.9
		45	0.26	127.2	15.6	6.6	1.3	130.5
		42	0.33	124.3	11.6	7.2	1.3	128.6
		10	0.24	106.9	2.6	5.3	1.3	121.4

Table 2. *Changes in metabolic and expansion fluxes*
The $V_{\max}/K_m$ values are expressed as percentages of the value found for wild-type homokaryon. The fluxes are calculated from the values in Table 1. Full details are given in the text.

Modulated enzyme	Strain	$V_{\max}/K_m$ of modulated enzyme (%)	Fluxes ($\mu\text{mol/h per g}$)									
			F_{Protein}	$F_{\text{Arg}}^{\text{NP}}$	F_{ASAase}	$F_{\text{Asa}}^{\text{NP}}$	F_{SYNase}	$F_{\text{Cit}}^{\text{NP}}$	F_{OTCase}	$F_{\text{Orn}}^{\text{NP}}$		
None	Wild-type	100	37.8	7.0	44.8	0.3	45.1	1.1	46.2	3.1		
Ornithine carbamoyltransferase	arg-12 ⁻ ad-4 ⁺ /arg-12 ⁺ ad-4 ⁻ heterokaryons	73	37.0	2.3	39.3	0.3	39.6	0.5	40.1	4.1		
		56	36.1	2.5	38.6	0.3	38.9	0.7	39.6	4.4		
		48	38.6	2.2	40.8	0.3	41.1	0.9	42.0	4.9		
		47	32.5	2.1	34.6	0.3	34.9	0.5	35.4	4.1		
		42	37.0	2.1	39.1	0.3	39.4	0.8	40.2	7.9		
		21	37.4	1.6	39.0	0.3	39.3	0.7	40.0	5.4		
Argininosuccinate synthetase	arg-12s bradytroph	4	28.0	0.3	28.3	0.2	28.5	0.1	28.6	7.6		
	arg-1 ⁻ ad-4 ⁺ /arg-1 ⁺ ad-4 ⁻ heterokaryons	89	33.1	6.2	39.3	0.3	39.6	2.9	42.3	4.4		
		47	34.8	6.1	40.9	0.3	41.2	6.7	47.9	2.1		
		46	32.5	4.5	37.0	0.3	37.3	9.9	47.2	1.6		
		43	26.2	2.7	28.7	0.3	29.1	13.0	42.1	1.4		
	arg-1r26 bradytroph	52	28.6	2.6	31.2	0.3	31.5	16.9	48.4	2.1		
Argininosuccinase	arg-1r3 bradytroph	24	25.7	0.5	26.2	0.2	26.4	45.9	72.3	3.0		
	arg-10 ⁻ ad-4 ⁺ /arg-10 ⁺ ad-4 ⁻ heterokaryons	55	38.5	6.9	45.4	1.7	47.1	1.6	48.7	4.4		
		54	36.5	6.9	43.5	1.9	46.4	1.7	48.1	4.2		
		26	35.5	3.3	38.7	16.4	55.1	9.5	64.6	2.3		
		10	21.0	1.2	22.3	26.5	48.8	15.7	64.5	2.1		
	arg-10r1 bradytroph	5	24.7	0.5	25.2	35.3	60.5	10.4	70.9	1.2		
Acetylornithine aminotransferase	arg-5 ⁻ ad-4 ⁺ /arg-5 ⁺ ad-4 ⁻ heterokaryons	68	37.2	7.0	44.2	0.3	44.5	2.4	46.9	8.2		
		52	41.7	5.9	47.6	0.3	47.9	1.9	49.8	4.7		
		45	33.9	4.2	38.1	0.3	38.4	1.7	40.1	4.1		
		42	42.4	4.4	46.8	0.3	47.1	2.4	49.5	3.8		
		10	29.1	0.8	29.9	0.3	30.2	1.3	31.5	0.6		

by argininosuccinase) is equal to the flux to protein plus the arginine expansion flux (neglecting the urea flux).

Similarly, the flux carried by argininosuccinate synthetase, the prior step, is equal to F_{ASAase} plus the argininosuccinate expansion flux (Scheme 2). In this way, working 'backwards', all the fluxes in the system are calculable, provided the concentrations of all intermediates are known so that each expansion flux can be added.

Flint *et al.* (1980) found the flux to urea (through catabolism of arginine by arginase) to be substantial only under conditions for which arginine concentrations are high. The *ure-1* mutation (which results in urease deficiency) was initially introduced to estimate this flux by measuring the rate of urea accumulation. In all heterokaryons so examined in the present work, the total flux of urea formation was found to be very small: at least 20-fold lower than the flux of arginine to protein (Tateson, 1972; Flint *et al.*, 1980). This can be attributed to the fact that these strains have arginine pool concentrations lower than those found in wild-type *Neurospora* grown in minimal medium. At wild-type concentrations around 98–99% of the total arginine pool is present in vesicles where it is unavailable for catabolism (Subramanian *et al.*, 1973). The concentration of available cytoplasmic arginine is consequently very low. This, in turn, results in the arginase being minimally induced (Flint *et al.*, 1980), so that no appreciable catabolism occurs. Further, low rates of arginine catabolism cannot be measured accurately by the urea-accumulation method because purine catabolism also contributes to urea formation (Davis, 1970). The neglect of this minor flux to arginine catabolism therefore does not affect the conclusions in the present study. It is now possible to consider the relationships that exist between these fluxes and the activities of the modulated enzymes (Table 2).

Flux/enzyme relationships

Distal fluxes and Sensitivity Coefficients. We first consider only fluxes distal to the point of modu-

Scheme 2. *Metabolic fluxes and expansion fluxes*

The metabolic fluxes (solid arrows) are catalysed by the respective enzymes. The expansion fluxes (open arrows) are generated by the exponential increase in the volume of the system. Abbreviation: Asa, argininosuccinate.

lation. Fig. 2 shows the flux to arginine carried by final enzyme of the pathway (F_{ASAase}) as a function of the various activities of argininosuccinase, ornithine carbamoyltransferase, argininosuccinate synthetase and acetylornithine aminotransferase. Enzyme activities are expressed as percentages of the value found for the wild-type homokaryon grown on minimal medium. It should be noted that for the points derived from bradytrophic strains (open symbols) account is taken of changes in the Michaelis constants of the mutant (Chilcott, 1966) to the wild-type enzyme. This is given by $100 \times (V_{\text{max.}}/K_m)_{\text{brad}}/(V_{\text{max.}}/K_m)_{\text{wild}}$. Modulation of enzyme activity by mutation and by variation of the gene dosage can therefore be treated as equivalent procedures.

It is clear that the flux/enzyme relationships are decidedly non-linear for all four modulations, and that quite substantial decreases in the wild-type enzyme activity cause only a marginal decrease in the flux. It has been shown by Kacser & Burns (1973, 1979, 1981) that the response of a given flux to small changes in a particular enzyme activity is best expressed by the Sensitivity Coefficient (Z). This is obtained by dividing the fractional change in flux by the corresponding fractional change in the enzyme causing the change. It provides a measure of 'control' exerted by the enzyme over the flux for the metabolic steady state in question:

$$Z \approx \frac{\partial F}{F} \bigg/ \frac{\partial E}{E}$$

In the limit:

$$Z = \frac{dF/dE}{F/E} = \frac{dF}{dE} \times \frac{E}{F}$$

It can be seen that the coefficient is the slope of the flux/enzyme relationship, dF/dE , multiplied by the scaling factor, E/F (Fig. 3). The four enzymes examined all exhibit Sensitivity Coefficients with respect to the flux to arginine that are without exception very small when they act in the wild-type strain grown on minimal medium. None of them, individually, therefore appears to exert any significant control over the flux, i.e. $Z \ll 1$. From the known recessivity of all the loci determining enzymes in the pathway, the same conclusion applies to the other steps not directly investigated (Kacser & Burns, 1981). The significance of this is discussed later below.

If the activity of any one enzyme is decreased sufficiently, its Sensitivity Coefficient will approach the value of 1, i.e. a given fractional change in enzyme will cause an almost equal fractional change in flux. It is clear from the relationships shown in Fig. 2 that each of the modulated enzymes shows an increased coefficient when present at low activities.

Fig. 2. Relationship between enzyme activity and flux to arginine

The flux to arginine, i.e. the flux carried by the enzyme argininosuccinase (F_{ASAse}), was calculated as described in the text (Table 2). The enzyme activities were measured as given in the Materials and methods section: (a) argininosuccinase; (b) ornithine carbamoyltransferase; (c) argininosuccinate synthetase; (d) acetylornithine aminotransferase. For the heterokaryons (●) they are the direct specific activities. For the bradytrophs (○) they are divided by the ratios of the Michaelis constants (see the text). All values are given as percentages of the wild-type value (⊙). The curves are fitted by eye.

Fig. 3. Sensitivity Coefficient as a function of the enzyme/flux relationship

The Sensitivity Coefficient is defined as:

$$Z = \frac{dF/dE}{F/E} = \frac{dF}{dE} \times \frac{E}{F}$$

where F , the flux, has the units $\text{mol} \cdot \text{h}^{-1} \cdot \text{v}^{-1}$ and E stands for enzyme activity and is a function proportional to enzyme concentration with units $\text{mol} \cdot \text{v}^{-1}$.

The results further indicate that a decrease in the argininosuccinate synthetase activity to around 30% of the wild-type value results in a lower F_{ASAse} value than do comparable decreases in the other enzyme activities. It would appear that at low activities the argininosuccinate synthetase carries greater 'control' (i.e. has higher Sensitivity Coefficients) than is the case for the other enzymes.

The Sensitivity Coefficients, derivable from the tangent by interpolation from these curves, are positive (decrease in enzyme decreases flux) and rise from a very low value of <0.1 at high enzyme activities to ≈ 1.0 at very low enzyme activities. As the coefficient approaches unity the enzyme may unquestionably be said to be 'controlling' the flux or be 'rate-limiting'. It is, however, clear that this is an extreme condition, and that, in general, any value between zero and unity may obtain. With a coefficient of, say, 0.5 it would not be appropriate to describe such an enzyme either as 'rate-limiting' or as 'not limiting'. It is for this reason that such classificatory terms are not useful in what is a problem in quantitative control theory (Kacser & Burns, 1973, 1979). The magnitude of Z completely describes a particular enzyme's role in a particular system. The mechanisms and the factors that contribute to this response characteristic are discussed later below.

So far only the flux through the 'final' step in the pathway has been considered. Because the pools 'distal' to the modulated enzymes (apart from arginine itself) have very low molar concentrations, their expansion fluxes constitute only a very small fraction of the flux through each step. As a result, the relationships, for example, between ornithine carbamoyltransferase activity and the flux F_{OTCase} or F_{SYNase} , and between argininosuccinate synthetase activity and the flux F_{SYNase} , are almost identical (see

Table 2) with those shown in Figs. 2(a) and 2(b) respectively and have not been shown separately. The Sensitivity Coefficients with respect to any enzyme for its 'own' flux and for all other fluxes distal to that step are therefore small and positive and increase as the activity is decreased.

Proximal fluxes and Sensitivity Coefficients. On the other hand, the fluxes through steps proximal to, i.e. 'before', the point of modulation show quite different relationships with the activity of the modulated enzyme. They increase sharply at low enzyme activities, as shown in Figs. 4 and 5 for the argininosuccinase and argininosuccinate synthetase modulations respectively.

Fig. 4. Relationship of enzyme activity to proximal fluxes: argininosuccinase modulation

(a) The fluxes carried by argininosuccinate synthetase ($\square$) and ornithine carbamoyltransferase ($\circ$) proximal to the modulated enzyme activity (argininosuccinase) are shown. In the lower half the respective expansion fluxes of citrulline (Δ) and argininosuccinate (∇) are shown. Data are from Table 2. Open symbols, heterokaryons; black symbols, bradytrophy. (b) The steady-state arginine concentration is plotted against argininosuccinase activity. Also shown is the de-repression function (from Fig. 1) relevant to these arginine values.

Fig. 5. Relationship of enzyme activity to proximal fluxes: argininosuccinate synthetase modulation

(a) The flux carried by ornithine carbamoyltransferase ($\square$) and the citrulline expansion flux (Δ) are plotted against the argininosuccinate synthetase activity. Symbols are as indicated in Fig. 4 legend. (b) Arginine concentration is plotted against argininosuccinate synthetase activity and the portion of the de-repression function is shown.

We therefore find the apparently paradoxical result that modulating one enzyme activity decreases the fluxes distal to it whereas it increases the fluxes proximal to it. Inspection of Table 2 shows that this increase in the proximal fluxes is entirely due to the steep rise in expansion fluxes when the modulated enzyme activity reaches low values.

The two negative-feedback mechanisms are responsible for this phenomenon. The first, de-repression of all the biosynthetic enzymes, has already been referred to (Fig. 1). This begins to come into effective operation when the arginine concentration falls to about half its wild-type value (Barthelmess *et al.*, 1974; Burns & Kacser, 1977). The mechanism for this response is likely to be by way of a 'general' or 'cross-pathway' rather than by a 'pathway-specific' effect (Flint & Kemp, 1981; Barthelmess, 1982).

The position of the de-repression curve (taken from Fig. 1) is shown in the (b) portions of Figs. 4 and 5, together with the corresponding arginine concentrations in the modulated strains. Clearly the elevation of enzyme concentrations of that part of the pathway proximal to the throttled step will increase the metabolic fluxes and will cause higher steady-state concentrations of the proximal pools.

The second feedback loop is the de-inhibition of the early pathway enzyme acetylglutamate kinase (3 in Scheme 1) by the falling arginine concentration (Cybis & Davis, 1974). Although the de-repression mechanism plays a very minor role at values above about 20 μmol of arginine/g, the de-inhibition control curve has a significant slope at higher arginine concentrations. The reverse is true at low arginine concentrations (Fig. 6; D. J. Porteous & H. Kacser, unpublished work).

These two mechanisms reinforce each other in causing a substantially increased flux into the proximal pools that greatly exceeds the decrease in metabolic flux caused by the distally diminished enzyme activity. The increased fluxes are reflected in the high concentrations of pools proximal to the modulated step, implying appropriate increased expansion fluxes. Since changes in the concentrations of some of the pools proximal to ornithine are, however, not readily determinable experimentally, no values for these fluxes are calculated. It was, however, observed that at low concentrations of the modulated enzymes the concentration of the glutamate pool is progressively decreased (Table 1), suggesting that the synthesis of glutamate, from 2-oxoglutarate, is 'limited'. Since the tricarboxylic acid-cycle enzymes and glutamate dehydrogenase are not de-repressed in response to the arginine signal, an increasing demand on the glutamate $\rightarrow$ arginine flux at a constant rate of 2-oxoglutarate synthesis would lead to a progressive decrease in the glutamate concentration.

Fig. 6. *Relative contribution of inhibition and repression* The flux to ornithine was measured in a strain (*arg-12⁻,arg-7⁻,aga⁻*) while the arginine concentration was falling. For details see the text. Enzyme activity data allow the assignment of the de-repression and de-inhibition contribution to the total change in flux (D. J. Porteous & H. Kacser, unpublished work).

Theory of control

It is now possible to interpret, at least in part, the changes in fluxes and coefficients as revealed by the above experiments. Reference to the complete theory of control in multi-enzyme systems should be made (Heinrich & Rapoport, 1974, 1975; Kacser & Burns, 1973, 1979, 1981). Only the general foundations and principal conclusions are sketched out in the present paper below.

Since changes at any step in a system will have consequences that spread, in principle, to all other parts by the linking of substrates and products, we have to consider the system as a whole. Each flux (such as, e.g., the F_{ASAase} flux) has 'attached' to it Sensitivity Coefficients with respect to every enzyme in the system that measures the outcome of these interactions:

$$Z_i^j = \frac{dF_i/dE_i}{F_j/E_i}$$

By modulation experiments, such as described in the present paper for four enzymes, the magnitude of such coefficients can be estimated for infinitesimal movements around their existing values. They turned

out generally to be small, which is precisely what is predicted from the theory. One of its principal conclusions is the establishment of the Summation Property. This states that, if the Sensitivity Coefficients for any flux, say F_j , are determined by modulating, in turn, every enzyme, E_i ($i = 1$ to n), in the system, their sum will be found to be equal to unity:

$$\sum_{i=1}^n Z_i^j = 1$$

Every other flux in the same system will have another set of coefficients, different in magnitudes but equal in number, n , and constrained by an identical summation relation by summing over all the enzymes. Thus, the more enzymes involved in a flux, the lower the coefficient of the 'average' enzyme, and, since n is very large, this average coefficient will be very small. For any one flux, most enzymes will be in a different part of the 'metabolic map' and will have coefficients very much smaller than average, approaching close to zero for this flux. Although it is, in principle, not possible to divide a system in this way, there will, on the other hand, be at least nine enzymes, those intimately involved in arginine synthesis, that are likely to have higher-than-average coefficients. The observation that these enzymes individually display coefficients that are small with respect to F_{ASase} is therefore entirely consistent with the expectations of the Summation Property. None of the enzymes 'controls' this flux, but all of them share a small part of it.

The reason for this general unresponsiveness lies in the inherent interactions of the pathway enzymes that must arise from the linking of the steps by their common substrates. The product of one enzyme step is the substrate for the next. How much of the small movement of substrate concentrations consequent on the modulation of an enzyme activity is transmitted along the pathway will determine the net effect on the measured flux. This response in transmission is almost invariably less than proportional. Suppose that a small decrease in enzyme activity, V_{max} , at one step is effected. This results in a slight increase in the preceding substrate concentration (S) and a slight decrease in the succeeding product concentration (P). There will then be an increase in the thermodynamic pressure across the step, given by an expression of the form:

$$(S - P/K_E)$$

Thus the rate, given by the product

$$v \approx V_{\text{max}} (S - P/K_E)$$

has two terms moving in opposite directions tending to cancel each other. Not included in the above formulation is the effect of saturation of enzymes by

substrate or product. A more complete expression for the rate is:

$$v = \frac{V_{\text{max}}/K_m^s (S - P/K_E)}{1 + S/K_m^s + P/K_m^p}$$

Depending on the parameters of the saturation term (denominator), this will also tend to diminish the net transmitted effect. The values that S and P settle to will be affected by the response of the preceding and succeeding enzymes.

There is thus built into a linked enzyme system an inherent buffering by mass action alone quite independent of any possible negative feedbacks that may also operate. These, in the present case, will depend quantitatively on the nature of each control function for repression and inhibition respectively (Fig. 6). The effect on inhibition of lowering the arginine concentration becomes increasingly important over most of the range. At very low values de-repression of all the enzymes will add significantly to the tendency to cancel the effect of the modulated enzyme decrease. The buffering inherent in the pathway is thus reinforced by the buffering due to the feedbacks. The net result of all these mechanisms is a prolonged 'plateau phase', as demonstrated in Fig. 2. The magnitude of the Sensitivity Coefficient, generated by these various effector responses, measures this net effect.

In fact, these 'local' responses to effector changes constitute the fundamental aspects of the system's functional units, the individual enzyme rates. They are expressed, in a manner similar to the Sensitivity Coefficients, by the 'Elasticity Coefficients' (Kacser & Burns, 1973, 1979):

$$\varepsilon_i^j = \frac{dv_j/dS_i}{v_j/S_i}$$

i.e. the fractional response of the 'isolated' rate, v , over the fractional change in the concentration of one of the substrates, products or other effectors, S , participating in it. There are as many Elasticity Coefficients for a given rate as there are participating effector molecules. Their magnitudes, usually between 0 and 1 (or -1 for products), will depend on the local milieu, on such factors as saturation, disequilibrium ratio, inhibitor concentration and other conditions. It is these 'local' Elasticity Coefficients that generate the 'global' Sensitivity Coefficients, and we discuss some of these effects in a later section below.

Turning then to changes in the proximal fluxes affected by the modulations, it will be seen that, for enzyme activity values around the wild-type value, the Sensitivity Coefficients are also small and positive when de-repression and de-inhibition play only a minor role.

Inspection of Figs. 4 and 5 reveals, however, that,

at low values of enzyme activity, the coefficients become negative (decrease in enzyme increases flux). We have already seen that the combined steep control functions of de-repression and de-inhibition (Fig. 6) over small movements of the arginine concentration are responsible for this reversal of the flux/enzyme relationship. It should perhaps be reiterated that, although the feedbacks are a necessary condition for this reversal, it is the fact that they are operative in an expanding system that creates this unusual phenomenon. The same mechanisms in a non-expanding system, such as liver or erythrocytes, would generate only positive coefficients, since no expansion fluxes exist, and would simply produce further buffering, thus diminishing the effect of enzyme variation on the flux. This joint control by a repression/inhibition system is not unique to the arginine pathway of *Neurospora*. Donachie (1964) has shown that both mechanisms operate in pyrimidine synthesis acting on aspartate carbamoyltransferase.

Fig. 5 shows, perhaps most clearly, the different 'phases' of the response. In 'running down' the enzyme activity from 100% to about 50%, the concentration of the arginine pool decreases slightly, and the flux through the proximal step, F_{OTCase} , appears to follow (small positive coefficient). There is evidence that de-inhibition has begun to act, as the small rise in the concentration of the citrulline pool (and hence expansion flux, F_{Cit}^p ; Fig. 5a) over this range indicates. This rise, however, is less than the decrease in metabolic flux. Further decrease in arginine concentration brings the steep de-repression function into prominence, as well as further de-inhibition. The flux to citrulline increases sharply, so that the net result is the reversal of the slope of the F_{OTCase} relationship. The Sensitivity Coefficient is, of course, negative, and may approach the value of -1 . Unlike the situation for enzymes and fluxes distal to the modulation, there is no upper bound for these negative coefficients, whose magnitude is conditional on the steepness of the control functions. All fluxes proximal to ornithine carbamoyltransferase, probably up to glutamate, also have negative coefficients under these conditions, since they all carry the steeply rising citrulline expansion flux as well as their 'own' expansion fluxes. The existence of a bank of negative coefficients under conditions of a very low enzyme activity of one of the steps must be related to the Summation Property, which states that the coefficients of one flux summed over all the enzymes in the system must equal $+1$. For, say F_{OTCase} there will be negative coefficients at low values of the argininosuccinate synthetase and argininosuccinase activities. Whatever the magnitude of the sum of these negative coefficients may be, it follows that the sum of all the positive coefficients for this flux must exceed the negatives by $+1$.

This leaves it open, but not necessary, that one or more of the positive coefficients can be >1 . We have no evidence that this is so in the present case.

Forcing marker

The discussion of the results has been entirely in terms of the changed activity of the modulated enzyme and the consequential effects on pools, fluxes and enzyme activities dependent on these. Although this is adequate for the bradytrophic strains, the use of heterokaryons raises an additional question. The allele *ad-4⁻* was used as a 'forcing marker' in order to maintain the mycelium heterokaryotic on minimal medium. This inevitably leads to a modulation of the *ad-4⁺* product (adenylosuccinate lyase), since variation in the *ad-4⁻/ad-4⁺* nuclear ratio is the inverse of the *arg⁻/arg⁺* variation. The pathway of adenine synthesis is metabolically 'remote' from that of arginine synthesis, so that in conformity with the Summation Property even large changes are not likely to affect it ($Z \ll 1$). It is nevertheless possible that general changes (e.g. growth-rate changes) might accompany the variation in this enzyme. In fact, heterokaryons possessing percentages of *ad-4⁺* nuclei as low as 10% showed growth rates indistinguishable from that of the wild-type homokaryon (Tateson, 1972). This suggests that the enzyme adenylosuccinate lyase carries a low Sensitivity Coefficient with respect to the flux to adenine as well as to arginine. Since most of the heterokaryons examined carried more than 40% *ad-4⁺* nuclei, the effect of variation in the *ad-4⁺* product on the flux/enzyme relationships in the arginine pathway can be regarded as negligible. The fact that the bradytrophic strains form a coherent group with the heterokaryons emphasizes this point.

Changes in Elasticity Coefficients

It has already been demonstrated that substantial decreases in enzyme activity will result in changes of the Sensitivity Coefficient of the flux through the enzyme, increasing from a very low value to values that may approach unity. Since the Summation Property applies at all constellations of enzymes, it is clear that, when one of the coefficients has been altered, the coefficients of all other steps must automatically have altered as a consequence. In general, the increase in Sensitivity Coefficient of one step will imply the decrease in Sensitivity Coefficient of all other steps for a given flux. Such changes in response of the system when drastic changes are imposed at one step are investigated further in what follows. This will make it possible to gain insight into the mechanisms that produce changes in the Elasticity Coefficients of individual steps that, in turn, generate the changes in Sensitivity Coefficients. Since the ratios of successive Elasticity Coefficients

are inversely related to the ratios of the respective Sensitivity Coefficients (Kacser & Burns, 1979), we have clear expectations of independently observed responses.

We have compared two strains, one carrying the wild-type enzyme at the *arg-10* locus (100% argininosuccinase activity) and the other carrying the mutant, *arg-10r1* (5% enzyme activity). From the slopes and estimated E/F of Fig. 2(a) it is seen that the Sensitivity Coefficients for F_{ASAase} in the two strains are very different: for *arg-10*⁺ (⊙), $Z \approx 0.01$; for *arg-10r1* (○), $Z \approx 0.8$. To understand what factors are responsible for this change in Sensitivity Coefficient we must obtain information on the difference in Elasticity Coefficients of the step when one enzyme is replaced by the other. Experimentally the method of modulating components other than argininosuccinase will give this information. We can investigate how variation, genetic or environmental, proximal to the argininosuccinase step affects the flux and the system in the two strains. The methodology is indicated in Scheme 3 and the results are given in Table 3. As far as the response of the F_{ASAase} is concerned, it is irrelevant how changes proximal to argininosuccinase are effected, since such response is only determined by the Elasticity Coefficients of the step to the participating ligands. Within each strain, variation in argininosuccinate synthetase flux results from the presence of one or several of the mutants *arg-12s* (decreased ornithine carbamoyltransferase), *arg-1r3* or *arg-1r26* (decreased argininosuccinate synthetase), or from growth on citrulline or minimal medium (Barthelmess *et al.*, 1974). The results given for the *arg-10*⁺

group also include data obtained previously by modulating citrulline uptake in an *arg-12*⁻, *arg-1*⁺, *arg-10*⁺ strain (Flint *et al.*, 1980). The production of the desired double-mutant combinations by crossing has been described previously by Barthelmess *et al.* (1974). Despite the diversity in genetic constitution and growth medium, by which the considerable differences in the concentrations of pools (in particular of citrulline and argininosuccinate) are achieved, the relationships obtained for each group are monotonic.

Fig. 7 shows the relationship between the flux through argininosuccinate synthetase and its two component fluxes, i.e. that to argininosuccinate pool expansion and that through argininosuccinase, in the two groups of strains. The slope of the relationship between argininosuccinate expansion flux and the argininosuccinate synthetase flux (Fig. 7a) for the group carrying the *arg-10r1* mutation approaches unity when the argininosuccinate synthetase flux exceeds approx. 40 $\mu\text{mol/h per g}$, at which point the F_{ASAase} flux (Fig. 7b) tends to become constant. Inspection of Table 1 and of Table 3 (from Tables 2 and 3 of Barthelmess *et al.*, 1974) shows that for all these strains or conditions the argininosuccinate concentration in the *arg-10r1* group is between 30- and 140-fold greater than for the corresponding *arg-10*⁺ group. With these values of the substrate concentration the enzyme is nearing saturation. There are no clear differences in the Michaelis constants of the two argininosuccinase enzymes (Donachie, 1962). Only at the highest F_{SYNase} values in the *arg-10*⁺ group do the argininosuccinate concentrations approach the lowest ones in the

Scheme 3. Methodology for estimating Elasticity Coefficients

Different fluxes are generated in the two strains *arg-10*⁺ and *arg-10r1* by genetical or nutritional alterations proximal to the argininosuccinase step. The introduction of decreased enzyme activities in one or both of the proximal steps or the growth of the strains on minimal or citrulline-supplemented medium allows a comparison of the responses of the two enzymes to be made over a large span of inputs. Abbreviations used: Cit, citrulline; Asa, argininosuccinate.

Table 3. Comparison of the two groups of strains *arg-10⁺* and *arg-10r1*

As indicated in Scheme 3, the substitution of different alleles proximal to the argininosuccinase step and/or the addition of citrulline to the medium produces a series of input fluxes that result in differential responses in the two strains. For ease of reference, the different groups are designated by the mutants occurring in them. For *arg-12* three mutants are shown: $- = arg-12^-$, $+ = arg-10^+$, $m = arg-12S$. For *arg-1*: $+ = arg1^+$, $m' = arg-1r26$, $m'' = arg-1r3$. For *arg-10*: $+ = arg-10^+$, $m = arg-10r1$. The first line (*) represents the series of fluxes generated by varying the citrulline/glycine ratio given in Tables 1 and 2 of Flint *et al.* (1980). It will be noted that the values of $F_{Protein}$ differ significantly from those shown in Table 2. The principal reason is the difference in the growth constants, k , caused by slight differences in experimental conditions.

Group	Sub-group	Genotype			Growth medium	Fluxes ($\mu\text{mol/h per g}$)						$F_{\text{'into' citrulline}}$
		<i>arg-12</i>	<i>arg-1</i>	<i>arg-10</i>		$F_{Protein}$	F_{Arg}^{xp}	F_{ASAase}	F_{Asa}^{xp}	F_{SYNase}	F_{Cit}^{xp}	
A (<i>arg-10⁺</i>)	1	—	+	+	Citrulline/glycine	*	*	*	*	*	*	*
		+	+	+	Minimal	32.5	5.2	37.7	0.33	38.0	0.73	38.7
		m	+	+	Minimal	28.0	0.34	28.3	0.18	28.5	0.13	28.6
	2	+	m'	+	Citrulline	28.6	4.8	33.4	0.22	33.6	77.0	110.6
		+	m'	+	Minimal	28.6	2.6	31.2	0.29	31.5	16.9	48.4
		m	m'	+	Minimal	25.4	0.16	25.6	0.12	25.7	3.7	29.4
	3	+	m''	+	Citrulline	25.7	0.28	26.0	0.23	26.2	81.5	107.7
		+	m''	+	Minimal	25.7	0.49	26.2	0.19	26.4	45.9	72.3
		m	m''	+	Minimal	26.3	0.04	26.5	0.16	26.7	1.1	27.8
B (<i>arg-10r1</i>)	4	+	+	m	Citrulline	24.7	0.59	25.3	54.7	80.0	50.4	130.4
		+	+	m	Minimal	24.7	0.49	25.2	35.3	60.5	10.4	70.9
		m	+	m	Minimal	27.6	0.28	27.9	10.5	38.4	0.30	38.7
	5	+	m'	m	Citrulline	22.1	0.44	22.5	17.1	39.6	59.3	98.9
		+	m'	m	Minimal	22.1	0.31	22.4	13.8	36.2	33.5	69.7
		m	m'	m	Minimal	23.7	0.40	24.1	8.6	32.8	0.73	33.5
	6	+	m''	m	Citrulline	17.2	0.33	17.5	4.4	21.9	53.1	75.0
		+	m''	m	Minimal	17.2	0.24	17.4	4.1	21.5	34.1	55.6
		m	m''	m	Minimal	24.7	0.56	25.3	7.9	33.2	4.3	37.5

arg-10r1 group, and there is some indication of a decrease in the F_{ASAase} response to the increase in the F_{SYNase} flux. Any increase in the F_{SYNase} beyond these high values would result largely in increasing the flux to expansion of the argininosuccinate pool. At that point, argininosuccinate expansion constitutes less than 2% of the F_{SYNase} flux in strains carrying the *arg-10⁺* allele. In contrast, in the *arg-10r1* group almost 70% of the flux entering argininosuccinate via the argininosuccinate synthetase is taken up by expansion of the pool.

It is thus seen that the substitution of wild-type (*arg-10⁺*) by the *arg-10r1* enzyme, with greatly diminished activity, produces drastic changes in the pool pattern. These are brought about by a number of simultaneously acting mechanisms, e.g. saturation, de-inhibition, de-repression and mass action, which are mutually interactive and are therefore responses of the whole system. Thereby the milieu around the enzyme is changed (principally from an increase in the concentration of argininosuccinate leading to increase in saturation), so that its further responses to changes in substrates is changed, i.e. the 'local' Elasticity Coefficient to

argininosuccinate is greatly decreased. The relative unresponsiveness of the F_{ASAase} in the *arg-10r1* strain to substantial variation in input (Fig. 7b) compared with the response of the *arg-10⁺* strain is a reflection of this changed Elasticity Coefficient. At the same time, it should be noted that the changed Elasticity Coefficient is a factor in affecting the milieu that leads to its own change. Since Elasticity Coefficients are inversely related to Sensitivity Coefficients, the greatly increased Sensitivity Coefficient of this strain (Fig. 2a) finds its explanation. The diminished effectiveness on F_{ASAase} of changes in the proximal steps also implies that their Sensitivity Coefficients have been correspondingly decreased (Summation Property). It is thus seen that a much greater share of the 'control' has been shifted on to the argininosuccinase step by replacing the *arg-10⁺* enzyme by the *arg-10r1* enzyme.

General discussion

Biochemical systems inevitably have interactions both within and between pathways. It is therefore not sufficient to investigate the nature of the individual components or even to establish what

Fig. 7. Comparison of flux response in systems with different Elasticity Coefficients of argininosuccinase. Data are from Table 3 and Flint *et al.* (1980). (a) Response of the argininosuccinate expansions flux to variation in the F_{SYNase} . (b) Response of F_{ASase} to variation in F_{SYNase} . $\bullet$, *arg-10⁺* group; $\square$, *arg-10^{r1}* group. The broken lines are equivalent to proportional responses.

characteristics of the system that cannot be obtained by analytical procedures on 'isolated' steps.

At the same time, the notion that one can assign single 'causes' to the control in such systems must be abandoned and replaced by a conceptual and theoretical framework that is based on the systemic nature of the phenomena. The responses to modulation are defined by two types of differential coefficients. The Elasticity Coefficient, ϵ , which measures the 'local' response of individual enzymes to modulations of its substrates, products and other effectors, constitutes the functional aspect of the component enzymes. These coefficients are determined, not only by the nature and concentration of the enzyme itself, but also by the milieu, which is determined by the system as a whole. We have seen how changes of some enzyme parameters in the system can seriously affect the steady-state concentrations of substrates, products and the inhibitors of many enzymes. Thus one enzyme can display quite different Elasticity Coefficients and hence quite different transmission responses to the flow of metabolites consequent upon changes elsewhere in the system. The second coefficient is the 'global' Sensitivity Coefficient, Z , which measures the response of some flux to changes in one of the enzymes. Since the flux is a systemic variable, such responses will involve, in principle, all enzyme steps and hence the Elasticity Coefficients of every enzyme. We have seen that most Sensitivity Coefficients are small, reflecting the inherent buffering of a linked system. Feedback loops, when they operate significantly, added further (negative) Elasticity Coefficients with respect to changes in the signal. We have seen that, under some circumstances, these can become quantitatively most important.

We have not attempted to assign precise values to the Sensitivity Coefficients and Elasticity Coefficients and the changes they have undergone. Neither the accuracy of the data nor the relatively restricted range of variation would justify this. Short of further massive collection of data, a more realistic approach is the simulation of the system based on the available evidence. This would not require the formulation in terms of the fundamental rate constants of all enzyme steps (see, e.g., Kuchel *et al.*, 1977), but would be adequately represented by the overall rate laws involving maximal velocities and apparent Michaelis constants only. A program can be devised that, apart from generating pools and fluxes, prints out all observed coefficients for any state of the system (Burns, 1969; Kacser & Burns, 1973, 1979). Such a simulation would also reveal the coherence of the system as we have described it. We believe that our method has uncovered all the important interactions. By giving semi-quantitative estimates of coefficients it has given us insight into the mechan-

interaction mechanisms, such as feedbacks, exist. The simultaneous operation of many mechanisms and the spread of their effects throughout the system demand a special methodology to give a quantitative estimate of the role that each plays when embedded in the whole. The method of modulation, of enzymes or of substrates, accompanied by appropriate measurements on the 'intact' system, gives the required information on the response

isms that operate *in vivo*. In particular, it has shown how changes in Elasticity Coefficients (caused by genetic or environmental means) generate changes in Sensitivity Coefficients, constrained by the Summation Property, which are compensatory in manner. Thus the control over the flux is shared among all the enzymes. As we have shown in the present paper, an understanding of the distribution of control can only be obtained by modulation methodology and sensitivity analysis.

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