

Intracellular Compartmentation and Transport of Metabolites

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The intracellular locations of enzymes and metabolites were determined for ornithine metabolism in *Neurospora*. Pulse label experiments were used to measure the rates of intracellular translocations and the sizes of compartmented pools of metabolites in the mitochondrial, cytosolic and vesicular compartments. The results indicate that rapid equilibration occurs between these pools during growth in minimal medium, although the vast majority of the ornithine is confined to the vesicular compartment. Arginine, the biosynthetic end-product of ornithine metabolism, regulates ornithine utilization through a combination of feedback inhibition, repression, and control of intracellular translocations. The last phenomenon plays a decisive role indicating that the regulation of intercompartmental translocations may be a common mechanism in rapid adaptation responses in eukaryotic cells.

Key words: compartmentation, tracers, *Neurospora*, arginine, vacuoles, ornithine

Eukaryotic cells are not homogeneous volumes of protoplasm. The most cursory look at cells with a microscope reveals many structures, such as mitochondria, vacuoles, lysosomes, plastids, lipid droplets, and the nucleus. Histochemical staining of intact cells and isolation and biochemical characterization of these organelles has demonstrated the non-random distribution of enzymes and other macromolecules and thus the unique functions of organelles. The existence of morphological compartments has made it easy to imagine that small molecules are not distributed uniformly. In addition, enzyme aggregates appear to serve as molecular compartments which confine intermediates of a reaction sequence to the vicinity of enzymes which use them.

Many advantages have been ascribed to the compartmental nature of eukaryotic protoplasm. First, high intermediate concentrations are achieved at appropriate reaction sites. This improves the speed of response to changes in concentrations of substrates and controlling molecules. Second, compartmentation protects intermediate pools from loss by diffu-

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sion and degradation, allows a smaller investment in the absolute size of the pools in the cell, and thus effects a conservation of the solvent capacity of cell water [1]. Third, compartmentation offers the possibility of storing large amounts of small molecules within the cell without disrupting control mechanisms which normally respond to these molecules.

It is only recently that the nonrandom distribution of small molecules has been widely supported by experiment. The difficulty of proving the existence of the phenomenon is a consequence in part of the rapid diffusion of small molecules. Small molecules do not remain localized in standard histochemical preparations. In differential centrifugation experiments, small molecules often diffuse out of subcellular particles with which they are associated *in vivo*. The most compelling, long-standing evidence for compartmentation of small molecules is based upon the behavior of radioactive tracers added to living cells. In such experiments, tracers appear in products faster or more slowly than would be expected if they were randomly distributed throughout the cell. Storage pools of compounds, for instance, were suggested by unexpectedly low turnover rates of a radioactive tracer once it was inside the cell [2]. Interpretation of the early tracer work was difficult. Kinetic descriptions of the data were complex, and models had to be built without certainty of the location of enzyme activities or of the label itself. The inability to connect reactions and locations, and the arbitrariness of models has delayed acceptance of the concept of small-molecule compartmentation by biochemists outside the areas of plant cell biology and mitochondrial physiology. Even now, the relationship between cellular anatomy and the functioning of major metabolic systems is only slowly becoming understood.

Our thesis here is that intracellular compartments are important controlling elements in eukaryotic cell metabolism. This idea, however well documented in general terms, is best served by an example. We have provided such an example in recent years by an investigation of the arginine pathway of *Neurospora* [3–10]. Among the phenomena we summarize below are the regulatory roles of the mitochondrial and vesicular (vacuolar) compartments or their membranes; storage without catabolism; and differing cellular distributions of arginine pathway metabolites in anabolic and catabolic conditions.

ARGININE METABOLISM

Arginine biosynthesis (Fig. 1) involves the synthesis of two precursors, ornithine and carbamyl phosphate. These two compounds are made by separate enzyme systems in the mitochondria [3, 11]. In the case of ornithine, at least three of the four required enzymes are known to be mitochondrial [3, 12]. Carbamyl phosphate is made by the mitochondrial carbamyl phosphate synthetase A, a glutamine-dependent enzyme wholly devoted to the arginine pathway. (The enzyme is distinct from carbamyl phosphate synthetase P, a pyrimidine biosynthetic enzyme located in the nucleolus [11, 13].) Carbamyl phosphate synthetase A, despite its rate-controlling role in the arginine pathway, is not inhibited by arginine (R. H. Davis, unpublished data). This situation may have evolved because the logical feedback effector, cytosolic arginine, is separated from the enzyme by the mitochondrial membrane. Instead, carbamyl phosphate synthetase A is greatly repressed in cells grown in arginine-supplemented medium [12]. Negative control of this step by arginine is therefore slow. Preexisting enzyme is diluted by growth after the addition of arginine to cultures growing in minimal medium (R. H. Davis, unpublished data). As we will show, the mitochondrial membrane may play a significant role in controlling carbamyl phosphate utilization until carbamyl phosphate synthetase A is diluted to its fully repressed level [10].

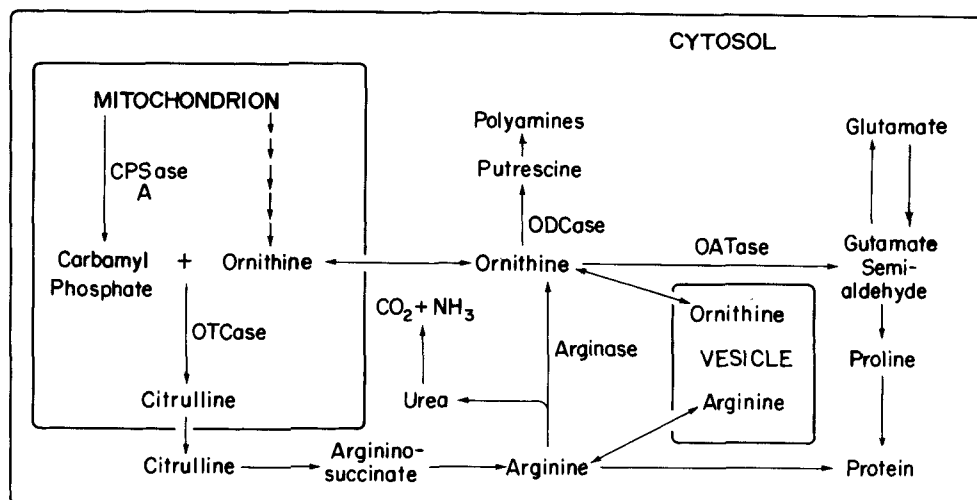


Fig. 1. Diagram of arginine metabolism in *Neurospora*, including locations of major enzymic steps. Proline synthesis has not been definitively localized, but is shown in the cytosol (non-organellar cytoplasm). Interconversion of glutamate and glutamate semialdehyde requires two enzyme paths, a catabolic enzyme (glutamate semialdehyde to glutamate) and a poorly known biosynthetic path (glutamate to glutamic semialdehyde). Certain key enzymes are named. Abbreviations: CPSase A, carbamylphosphate synthetase A; OATase, ornithine aminotransferase; ODCase, ornithine decarboxylase; OTCase, ornithine transcarbamylase.

Ornithine, which arises in the mitochondria, has four fates. First, ornithine transcarbamylase, a mitochondrial enzyme [3, 11], uses ornithine and carbamyl phosphate to make citrulline, the next intermediate of the arginine pathway. Citrulline diffuses out of the mitochondria and is transformed to arginine by a sequence of two cytosolic enzymes [3]. The other three fates of ornithine require that it leave the mitochondria. In the cytosol, ornithine is used at a low rate by ornithine decarboxylase to form putrescine, an indispensable polyamine precursor [3, 14]. Ornithine can also be catabolized by the cytosolic enzyme of arginine catabolism, ornithine aminotransferase [15]. This enzyme has substantial activity in cells which have not been exposed to arginine or ornithine. However, its affinity for ornithine is low, and as we shall see, it fails to "bleed" ornithine from the cytosol at significant rates in cultures grown in the absence of ornithine or arginine [7, 15]. Finally, ornithine is stored in membrane-bound vesicles, to which we will return [4]. Thus under anabolic conditions, where the cell must synthesize arginine *de novo*, arginine precursors made in the mitochondria are destined largely for arginine synthesis, with a small amount of ornithine being diverted in the cytosol to putrescine synthesis, catabolism, and storage [7].

Catabolism of arginine takes place in the cytosol when arginine is provided to cells [8]. The first catabolic enzyme, arginase, yields urea and ornithine (Fig. 1). Under these conditions, ornithine is formed in the cytosol in high concentrations [10]. Ornithine thus becomes readily available for catabolism by ornithine aminotransferase, and in addition continues to support putrescine synthesis. The latter is of some significance, because ornithine biosynthesis in the mitochondria is feedback-inhibited by high arginine concentrations [14, 16]. Thus under catabolic conditions, much of the ornithine is destined for the proline pathway (see Fig. 1) and for glutamate synthesis, with a small amount again being diverted to putrescine

synthesis. It might be asked whether, under these conditions, any cytosolic ornithine enters the mitochondria and if so, what happens to it. Experiments to be described will answer this question.

An intriguing property of *Neurospora* is that cells growing in minimal medium have the unexpectedly large amounts of 25–30 nmoles per milligram dry weight of both arginine and ornithine. Neither compound, however, is catabolized to a significant extent [15, 17]. The lack of catabolism, despite the presence of catabolic enzymes, led us to seek a mechanism for the segregation of enzymes and substrates. We found that most of the ornithine and arginine are confined to membrane-bound vesicles [4]. With gentle methods of disrupting protoplasts and differential centrifugation, the vesicles can be separated easily from mitochondria. However, leakage and breakage of organelles in this procedure preclude accurate determination of the amino acid pool sizes in the vesicles, the mitochondria and the cytosol.

INTRACELLULAR POOL SIZES AND FLUXES: TRACER METHODOLOGY

We wished to know in our system how much ornithine and other amino acids there were in the vesicles, the mitochondria and the cytosol. In addition, we wished to know what the amino acid fluxes were across the membranes of the organelles. Finally, we wished to know whether compartmentation and intracellular fluxes changed in response to altered environmental circumstances; that is, whether compartmentation had any regulatory or adaptive function.

Isotopic tracers have been widely used to answer questions of this sort. We will first describe our system and what results might be predicted in tracer experiments, based on several compartmental models of increasing complexity. We will then discuss the actual data, drawing from them the quantitative estimates of pool size and flux of ornithine across intracellular membranes.

Neurospora can be made to grow exponentially if a large number of spores (conidia) are introduced into liquid culture medium. In the exponential phase, we have confirmed the expectation that the pool sizes per milligram dry weight remain constant. We also know that proteins and other macromolecules accumulate in proportion to dry weight. Increments of mass can be expressed in terms of the growth equation, $\Delta A = A_0(e^{kt} - 1)$, where A_0 is the mass at the beginning of time interval t , and k is the growth constant derived from the mass doubling time (ca 175 minutes in *Neurospora* at 25°C). Thus we can draw up a balance sheet of the cells' "arginine economy." At steady state, the amount of ornithine stored, and that used to make arginine, polyamines, and catabolic products, must equal the rate of ornithine synthesis. By using the growth equation, we can calculate how many nanomoles of each product are made per milligram dry weight every minute. This is an important parameter to know in tracer experiments, because by relating the number of radioactive molecules being used by any reaction to the total number of molecules going through that reaction at the same time, we may judge whether the reaction in question uses radioactive or unlabeled molecules preferentially.

In the experiments to be described later we add a very small amount of highly radioactive ornithine. The amount is so small that it has no metabolic impact; it is for all intents and purposes chemically negligible. However, it is a "pulse" absorbed by the cells by a high-affinity uptake system in less than five minutes. This is a very short time in relation to the mass doubling time of 175 minutes. In these first few minutes of the experiment, the radioactivity finds its way into all of the reactions which use ornithine, as well as into the vesicles [7, 9]. The time course by which the isotope enters each reaction is used to draw our conclusions about intercompartmental exchange and pool size.

For purposes of illustration, let us consider some idealized situations as illustrated in Fig. 2. Let us assume (Fig. 2A) that there is a negligible pool of ornithine; each molecule is used in further reactions as it is produced. Assume that ^{14}C -ornithine is introduced into the medium and that the radioactive molecules are used immediately upon entry into the cells. The time course of entry and of ^{14}C -product formation will be the same, and no radioactive ornithine pool would be detectable. In this experiment, we can also determine the specific radioactivity of new product molecules, that is, the counts per minute of new product divided by the amount of new product made in any time interval. In the situation pictured in Fig. 2A, the ratio would be the counts entering the product divided by the amount (labeled and unlabeled) entering the product. It will be at a maximum initially, and will decline as the rate of uptake of labeled molecules falls to zero. It is important to remember that throughout this period, the system is in a steady-state chemically; it is only the label which displays variation in use.

Now let us assume (Fig. 2B) that there is a single, soluble ^{12}C -ornithine pool within the cell. In this case, we see the appearance of radioactivity in an extractable pool, which reaches a maximum at or before the end of the uptake period. The rate of appearance of radioactivity in products shows a different time course. It starts with a lag and increases until the soluble pool from which products are being made reaches maximal radioactivity. Then, as radioactive molecules are replaced by endogenous, unlabeled molecules, ^{14}C -ornithine enters product at a declining rate until the radioactivity is washed out of the pool. If we look now at the specific radioactivity curves for the soluble ornithine pool and new molecules of product made from it (Fig. 2B, bottom), we see that they are the same. As the precursor pool changes its radioactivity due to uptake or utilization, the new product specific radioactivity reflects this exactly. From this observation, we may conclude that intracellular molecules of the precursor are used at random to make the product.

We may now introduce a storage pool of ornithine as a further complication. Here, we assume that only a few percent of a large cellular pool of soluble ornithine (that is, cytosolic plus storage pools) is accessible to the enzymes making product (Fig. 2C). The rest of the ornithine is in the vesicles, which show restricted exchange with the dynamic cytosolic pool. If radioactive ornithine is introduced into the medium, uptake delivers it to the cytosol. Because the radioactive molecules mix freely only with the small pool in the cytosol, the label will quickly enter products of ornithine-utilizing reactions. The labeled molecules will also enter the vesicular pool, where they become greatly diluted by unlabeled molecules. In Fig. 2C, the curves are drawn to indicate that about one-third of the label initially enters the product, and two-thirds enters the vesicles. After label is fully absorbed from the medium, all of the ^{14}C -ornithine which has not been used for product formation is in the vesicles. Later in the experiment, the product acquires label only slowly as exchange occurs with the vesicular pool, which has a low specific radioactivity. If we look at the specific radioactivity curves (Fig. 2C, bottom) we notice that the specific radioactivity of the cellular ornithine pool rises until uptake is complete, and falls slowly thereafter. New product molecules, on the other hand, are much more radioactive initially because they are made from a cytosolic pool of high specific radioactivity. We can conclude that products are made initially by nonrandom use of cellular ornithine. As uptake ends, the specific radioactivity of new product falls. In fact, this curve ultimately falls below that of the pool, because the vesicle, containing the bulk of the ornithine, has sequestered most of the remaining radioactive ornithine. At this point, products are made preferentially from unlabeled ornithine made endogenously in the cell.

The data of Fig. 2C can be used to determine the size of the cytosolic ornithine pool and the flux of ornithine across the vesicular membrane. The pool size can be approximated

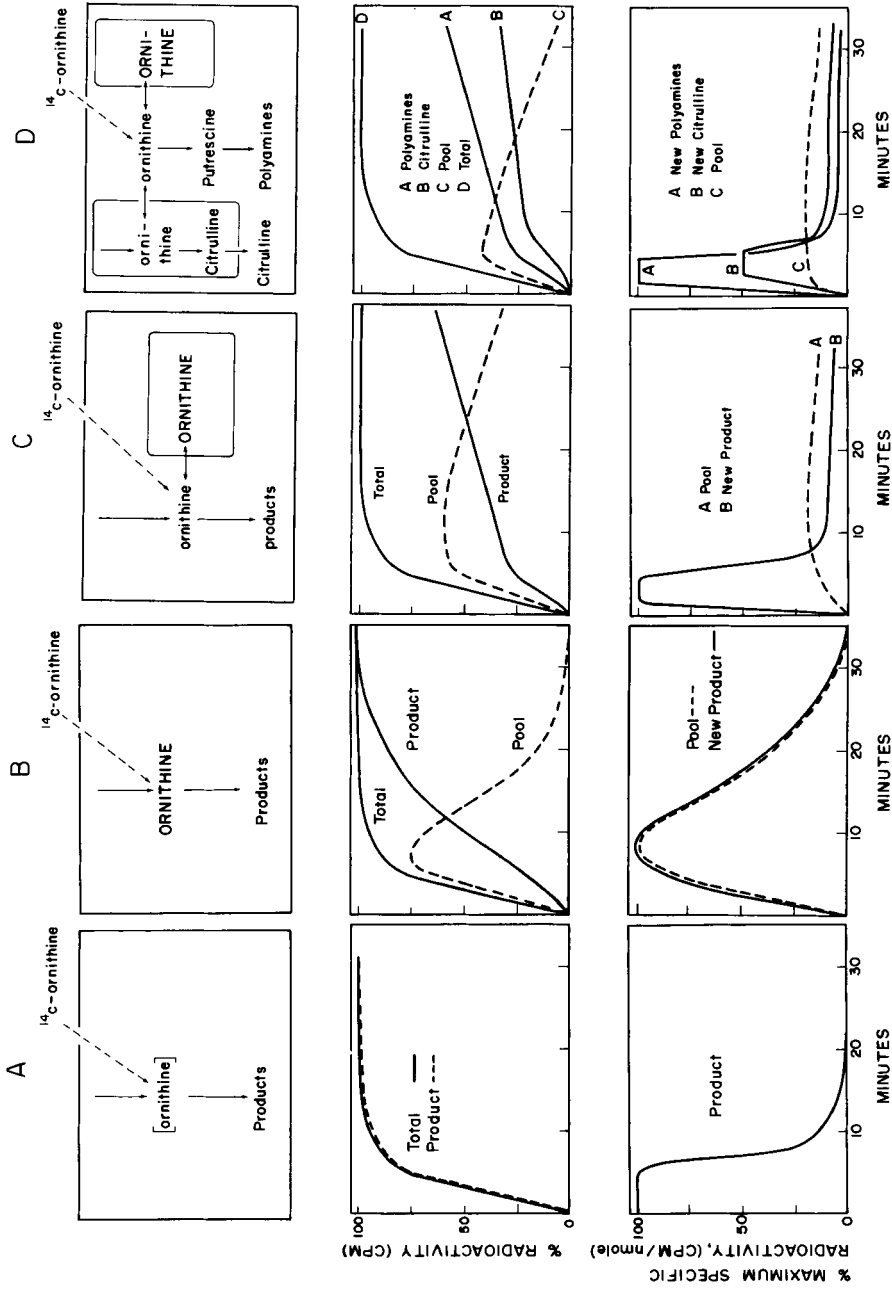


Fig. 2. Four models of the ornithine pool (top), the expected patterns of radioactivity (counts per min) in pool and products (middle) and of specific radioactivity (counts per min per nmole) in pool and products (bottom). (A) The cell has no ornithine pool; product is made as fast as endogenous or exogenous, ^{14}C -ornithine appears. (B) The cell has a large, randomly distributed ornithine pool, with which ^{14}C -ornithine mixes as it enters. (C) The cell has a very small ornithine pool in the cytosol, with which ^{14}C -ornithine mixes freely, and a large, sequestered pool which mixes (equilibrates) slowly with the cytosolic pool. (D) The cell has three ornithine pools. One small one is in the mitochondria, where ornithine is made and used for citrulline synthesis; a second small one is in the cytosol. The second pool is the one which mixes immediately with ^{14}C -ornithine as it enters. The third pool is a large, sequestered pool which behaves as in the previous model, (C). See text for explanation of graphs.

by use of the lag time in the curve (Fig. 2C, middle) describing the entry of radioactivity into the product [2, 7, 9]. The lag time is a period in which the cytosolic pool is increasing in specific radioactivity. During the transient linear phase which follows (theoretically it is exponential, but it is effectively linear in the short period considered), the product is being made from a pool of constant specific radioactivity (see plateau of the product specific radioactivity in Fig. 2C, bottom). Extrapolation of the transient linear portion of the product curve in Fig. 2C, middle panel, to the time axis gives the time for one effective replacement of the pool. Because we know the sum of all enzyme reaction rates drawing upon this pool, the pool size can be crudely calculated by multiplying the time in minutes by the sum of the reaction rates. In our example, the lag time is drawn as being very short, and thus the cytosolic pool is very small: it turns over rapidly. To be accurate, one must know also the rate of ^{14}C -ornithine entry into the vesicle, as well as into products of ornithine metabolism. One should also know the rate at which ^{12}C -ornithine emerges from the vesicle, because it will erroneously enlarge the estimate of cytosolic pool size. This is a more complex calculation to which we shall return.

In the latter part of the experiment the sole source of the label is the vesicle. Because the cytosolic pool is very small, we can assume that the specific radioactivity of extractable ornithine late in the experiment (shown by the pool curve in Fig. 2C, bottom) is a good measure of the specific radioactivity of vesicular ornithine. At this time, the products made in the cytosol have two sources of ornithine: endogenous (^{12}C) and vesicular (^{14}C), for which the specific radioactivity can be estimated. The products in Fig. 2C late in the experiment have one-third the specific radioactivity of vesicular ornithine. Therefore the efflux from the vesicles is one-third the known rate of ornithine formation and can be calculated. The influx of ornithine into the vesicles must be slightly more than this rate to account for the observed growth of the pool as it keeps up with mass increases of the system. Thus the entry of ornithine into the vesicles is equal to the efflux plus the increase of the pool during growth. In this way, we have calculated transport rates across an intracellular membrane.

Our last, most complex model introduces a factor known to be true of ornithine metabolism: it is made and used for citrulline synthesis in the mitochondria (Fig. 2D). The mitochondrial ornithine pool exchanges with the cytosolic pool. The latter exchanges with the vesicular pool as in the previous model. We also have two products to deal with separately: citrulline and polyamines. For purposes of illustration only, we will assume equal rates of synthesis of these two compounds. If the radioactive ornithine is administered to the cells, we see rapid uptake of label, with ^{14}C -ornithine entering the cellular pool and polyamines similarly to the previous model. However, we also see citrulline being made from radioactive ornithine. First, the lag time in the citrulline curve (Fig. 2D, middle) is greater than in the polyamine curve because the labeled molecules must equilibrate not only with the cytosolic ornithine, but subsequently with mitochondrial ornithine. Second, despite our assumption of equal rates of synthesis, the two products acquire label at different rates. This indicates that the mitochondrial membrane limits the flow of label from the cytosol. If we look at the specific radioactivities of the new molecules of polyamines and citrulline (Fig. 2D, bottom), we see that there is a period during which both are constant. The ratio is, in our example, 2:1, with the polyamines having the greater specific radioactivity. We infer from this that the specific radioactivity of mitochondrial ornithine is one-half that of cytosolic ornithine because of dilution by ^{12}C -ornithine made in the mitochondria during this period. Because the rate of ornithine synthesis is known, the influx from the cytosol can be calculated; in this case, it is equal to the rate of ornithine synthesis. The efflux from the mitochondria is equal to the influx rate plus the sum of rates of consumption and storage outside the mitochondria. Again, we have calculated intercompartmental exchange rates within the cell.

A complex mathematical treatment is required to derive the sizes of the mitochondrial and cytosolic pools. The solution requires knowledge of the intercompartmental exchange rates, the rates of synthesis of ornithine and its products, and the simplifying assumptions that entry of label is linear in the first minutes and that no label, once in the vesicles, emerges from them in the first few minutes. Crude estimates of pool sizes, however, can be obtained in two more straightforward ways. The first is to use the short lags, or "washout" times of polyamines and of citrulline, and to calculate the amount of ornithine known to be used by the cell in these times. The second is to compare the specific radioactivities of the cellular pool with those of citrulline and polyamines in the earliest time points. If, for example, the new polyamines have a specific radioactivity of ten, while that of the cellular pool at the same time is one, we infer that label in the polyamine precursor pool (cytosol) is being diluted by unlabeled molecules to a much lesser extent than if polyamines drew randomly on all cellular ornithine molecules. The 10:1 disparity indicates that the pool of ornithine from which polyamines are made can be no larger than 1/10th the total ornithine in the cell.

ORNITHINE COMPARTMENTATION IN NEUROSPORA GROWN IN MINIMAL MEDIUM

We have performed the experiment described above with *Neurospora* cultures growing in minimal medium [7, 9]. Samples were withdrawn at short intervals after adding the small pulse of ^{14}C -ornithine and added to trichloroacetic acid. Protein was hydrolyzed and radioactivity in amino acids derived from ornithine was determined. Similarly, the soluble materials were fractionated and the radioactivity in ornithine and all compounds made from it were measured in each sample. With knowledge of pool sizes and the rates of small molecule and macromolecular synthesis, specific radioactivities of pools and new molecules of product were calculated. It should be noted that in addition to citrulline and polyamines, another enzymic fate of ornithine is measured. This is the catabolism of ornithine via ornithine aminotransferase to glutamic- γ -semialdehyde and its derivatives, mainly proline (Fig. 1) [7]. The data assembled in Fig. 3A show the sums of radioactivity entering the ornithine pool, the mitochondrial ornithine transcarbamylase reaction, and the cytosolic ornithine decarboxylase and aminotransferase reactions. Figure 3B shows the specific radioactivity of the cellular ornithine pool and new products.

Let us look first at the polyamines (ornithine decarboxylase) and citrulline (ornithine transcarbamylase) data in order to derive the fluxes of ornithine across the mitochondrial membrane. Citrulline is known to be made at about ten times the rate of polyamines [7]. Both the polyamine and citrulline curves show lags before the transient linear portions (Fig. 3C), the lag for the cytosolic polyamine curve (6 sec) being shorter than that for the citrulline curve (20 sec). The difference in lag times is consistent with the knowledge that the two reactions take place in different compartments. The two reactions show linear phases between 1.5 and 3.0 minutes after addition of ^{14}C -ornithine to the culture. Citrulline, during this period, accumulates label at 5.3 times the rate radioactivity enters polyamines. Because the ratio of the actual rates of formation of the two compounds is 10:1, we infer that there is a barrier to free exchange between the sites of citrulline and polyamine synthesis. This is probably the mitochondrial membrane. Looking now at specific radioactivities of the new citrulline and new polyamines (Fig. 3B), we see that new citrulline is 53% as

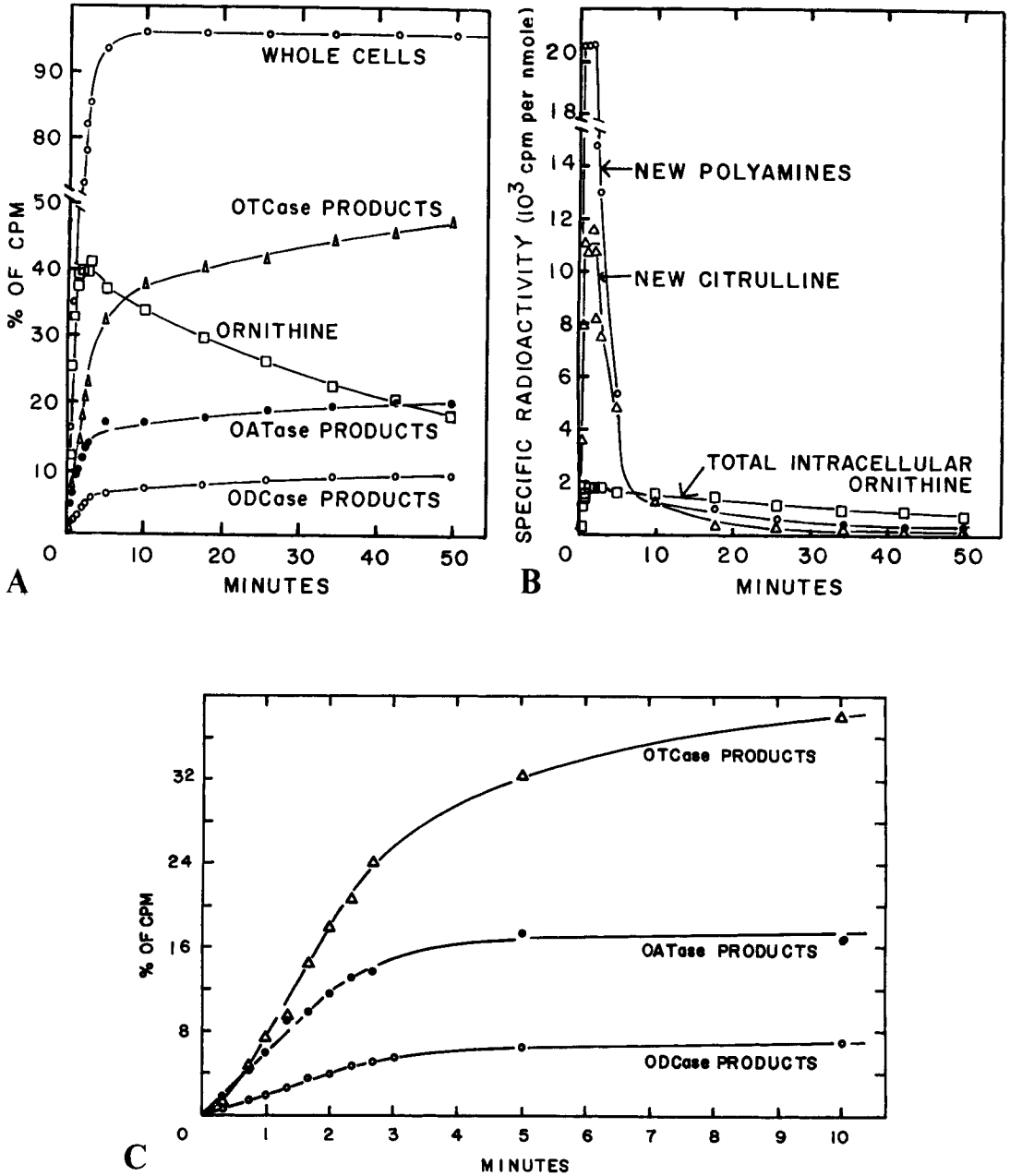


Fig. 3. Data from an experiment in which ¹⁴C-ornithine was added at a concentration of 0.1 μM to a logarithmic culture of Neurospora at time zero. (A) Curves showing uptake of ¹⁴C by cells ("whole cells") and the distribution of ¹⁴C in the acid extractable pool ("ornithine") and in the products of the three enzymic reactions using ornithine. (B) Specific radioactivities of the ornithine pool, newly formed polyamines and newly-formed citrulline as they vary with time in the experiment shown in (A).

Fig. 3. (C) Data of Figure 3A for the first 10 minutes on an expanded time scale. Abbreviations as in Figure 1.

radioactive as polyamines in this period. This tells us that unlabeled ornithine is diluting cytosolic, radioactive ornithine (the source of polyamines) as it enters the mitochondria, where ornithine is used for citrulline synthesis. The known rate of ornithine synthesis in the mitochondria ($1.17 \text{ nmole mg dry weight}^{-1} \text{ min}^{-1}$), therefore, is 47% of the total ornithine appearing each instant in the mitochondria. The influx of ornithine from the cytosol is therefore $1.17 \times (0.53/0.47) = 1.32 \text{ nmole min}^{-1} \text{ mg}^{-1}$. Knowing that $0.31 \text{ nmole min}^{-1} \text{ mg}^{-1}$ is consumed or stored outside the mitochondria, the efflux from the mitochondria is $1.63 \text{ nmole min}^{-1} \text{ mg}^{-1}$ [9].

The ratio of counts entering citrulline and polyamines in the early, transient linear phase of the experiment, as noted above, is 5.3. During this time, the medium is the source of label entering the cytosol. If our assumptions about steady-state and compartmental relations are correct, we should expect to find the same ratio late in the experiment (after 10 min), when the vesicles are the source of label entering the cytosol. Utilization of labeled molecules entering the cytosol should be independent of the source of such molecules. When we calculate the ratio for the later period, we find again that the citrulline to polyamine ratio is 5.3. This satisfying correspondence supports the assumed relation of vesicles, medium, cytosol, and mitochondria pictured in Fig. 4 [9].

Using the rate of loss of counts from the ornithine pool later in the experiment, we infer that the vesicles exchange ornithine with the cytosol. The efflux is $0.66 \text{ nmole min}^{-1} \text{ mg}^{-1}$, if we assume virtually all extractable ornithine (labeled and unlabeled) is vesicular at this time. Because there is a net storage of $0.09 \text{ nmole min}^{-1} \text{ mg}^{-1}$, influx must be $0.75 \text{ nmole min}^{-1} \text{ mg}^{-1}$. The flux figures correspond well with independent calculations made on the basis of figures drawn from the early part of the experiment [9].

The amount of ornithine in the cells used in the experiment is large: $29 \text{ nmole/mg dry weight}$. We may ask how the ornithine is distributed within the cell. If the pool were distributed uniformly, such that label mixed freely with all of it during entry, the specific radioactivities of new polyamines and citrulline would have resembled the cellular pool throughout. No early steady state could have been achieved before the label had been wholly absorbed from the medium. In fact, however, transient steady-state labeling in new polyamines and citrulline was achieved quickly as we have seen, with lag times of 6 and 20 seconds. Moreover, specific radioactivities of the two compounds are each over 30-fold the specific radioactivity of the cellular ornithine pool at the same time. Calculations on the basis of specific radioactivities suggest that label entering polyamines has been diluted by only 1.5% of cellular ornithine. Label entering citrulline has been diluted by another 1.5% of cellular ornithine. Other methods yield similar results. Pool sizes calculated from the lag times are 1% and 2% of the cellular ornithine for the cytosolic and mitochondrial pools respectively. If the compartmental diagram of Fig. 4 is used and if exchanges between compartments are taken into account, a complex mathematical derivation of the pool sizes can be obtained as discussed above [F. C. Williams and R. H. Davis, unpublished calculations]. It yields pool sizes of 1% for both cytosolic and mitochondrial pools. Thus there is agreement among the methods to the effect that approximately 1% of the cellular ornithine is in each of the two metabolically active compartments, and 98% is in the vesicles. Making assumptions about the distribution of cell water ($2.5 \text{ ml/mg dry weight}$, [18]), we may obtain a rough idea of ornithine concentrations. If 13% of cell water is in mitochondria [19], the 0.29 nmole of mitochondrial ornithine has a concentration of 0.9 mM . If 80% of cell water is cytosolic, ornithine in the cytosol is about 0.125 mM . The concentration of ornithine in the vesicles cannot be calculated, because their collective volume is unknown.

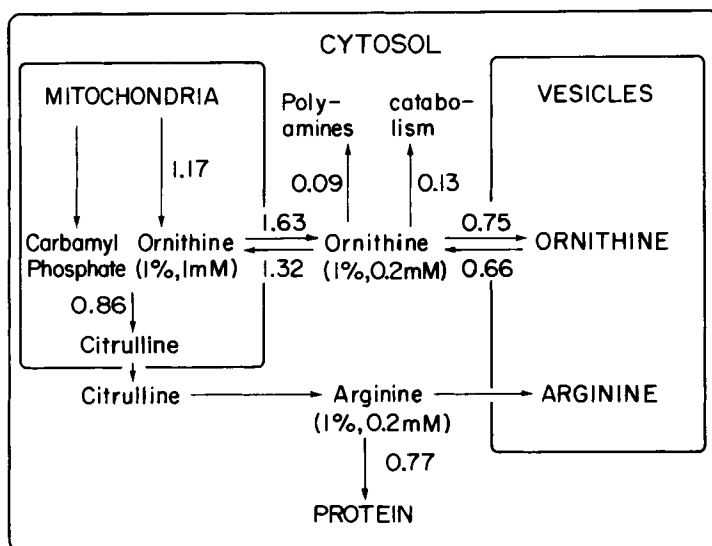


Fig. 4. Fluxes of enzyme reactions and intercompartmental exchange, in units of $\text{cpm min}^{-1} \text{mg}^{-1}$, determined from steady state values in a growing culture (enzyme reactions) and from data of Fig. 3 (intercompartmental exchange). Pool size estimates for ornithine and arginine are given as percent of cellular ornithine or concentration. See text for details.

In the experiment described in Fig. 3, labeled molecules enter not only polyamines and citrulline, but also the ornithine aminotransferase reaction. This is a catabolic reaction, yet it is taking place in unsupplemented medium under "anabolic" conditions. The entry of labeled molecules into this reaction occurs almost immediately upon addition of labeled ornithine to the culture. Moreover, label enters at a high rate initially, some three times the rate into polyamines. Yet in the later part of the experiment, when the vesicles are the sole source of label, the rate is only 1.5 times that into polyamines [9]. The difference in rates suggests that ornithine aminotransferase has better access to label entering through the cell membrane than to label emerging from the vesicles. This pattern has been demonstrated repeatedly, usually with a more extreme difference between early and late times [7]. A hypothesis, yet to be tested, is that some portion of the catabolic enzyme is associated with the cell membrane [7]. However, we have used the later time period of the experiment (10–30 min) to calculate the absolute rate of ornithine catabolism in the culture (steady-state). By dividing the amount of label entering the catabolic reaction by the calculated specific radioactivity of cytosolic ornithine (cpm/nmole), we obtain 0.13 nanomoles catabolized each minute by each mg (dry weight) of cell mass [9].

COMPARTMENTATION OF ORNITHINE DERIVATIVES

As ^{14}C from ornithine finds its way into its derivatives, one can analyze these derivatives as if they were pulse-labeled. By calculations similar to those given for ornithine, we find that proline and citrulline appear not to be significantly compartmented [7]. This is shown by the similarity of pool and product specific radioactivity for these compounds throughout the experiment. When we look at arginine, however, we find it, like ornithine, is highly

compartmented, such that about 1% is cytosolic, the remainder vesicular [5, 7]. This confirms the compartmental behavior for arginine found in the earliest of our experiments, in which ^{14}C -arginine or ^{14}C -citrulline was added directly to cells [5].

Unlike ornithine, endogenously synthesized arginine is not catabolized at all by cultures grown in unsupplemented medium [17]. Studies with native arginase have indicated that the enzyme in crude extracts has a sigmoid concentration-velocity curve, and thus a threshold arginine concentration below which the reaction will not proceed (R. H. Davis, unpublished observations). In unsupplemented cultures, the arginine concentration in the cytosol, where arginase is localized, is probably below this reaction threshold. The calculated cytosolic arginine concentration (0.05–0.12 mM) and the arginase reaction threshold observed *in vitro* (ca 0.05 mM) are in rough agreement [4, 5]. We see, then, that at steady-state, the cytosolic arginine concentration is poised by the dynamics of biosynthesis, vesicular storage, and protein synthesis, to allow optimal protein synthetic rates and storage without catabolism.

RESPONSE OF CELLS TO LARGE INCREASES IN CONCENTRATION OF EXOGENOUS ARGININE

We may now ask how the *Neurospora* cells respond to the addition of a large amount of arginine to the culture medium. One of the striking features of *Neurospora* is the speed of change in the metabolic steady-state: catabolism of arginine and ornithine begins immediately [8]. The earliest events depend in part on changes in flux across intracellular membranes (Fig. 5).

The immediate consequence of adding arginine to a culture growing in minimal medium (Fig. 5A) is a great elevation in the cytosolic pool of arginine. By measurement of specific radioactivity of pool and new protein or new urea, the cytosolic pool is estimated at 15 mM, a 75-fold increase over cultures grown in minimal medium [6]. Catabolism begins as soon as it can be measured, and achieves a constant rate within 5 minutes [8]. This is long before the total cellular arginine pool has reached its peak, and indicates that the cytosolic arginine pool either surpasses the saturating substrate concentration for the enzyme almost immediately or remains constant while the total pool increases. Cycloheximide does not affect this pattern of catabolism. From the earliest moments, it appears that the uptake of arginine for protein synthesis cannot maintain the low cytosolic pool of arginine characteristic of cultures grown in minimal medium. However, uptake into the vesicles does occur, such that most of the soluble arginine of the cell at any instant is found there (see below).

Because the arginase reaction yields ornithine, the ornithine concentration of the cytosol rises. A greatly increased rate of ornithine catabolism via ornithine aminotransferase is immediately seen (Fig. 5A) [10]. But even more important, in the first few minutes after arginine addition, is the discharge of ornithine from the vesicles. One manifestation of this is a drop in the total cellular ornithine content, and if vesicular ornithine is prelabeled, the label begins to be catabolized immediately upon arginine addition (Fig. 5A) [10]. The mechanism of mobilizing the vesicular ornithine is not known. It is clear from specific radioactivity determinations that over 90% of the ornithine behaves as though it were cytosolic after arginine addition. By analogy with the behavior of isolated yeast vacuoles, it is probable that arginine both inhibits ornithine entry into the vesicle and accelerates the efflux of ornithine as well, possibly by a simple exchange reaction on a common carrier [20].

Labeling studies have shown that ornithine from the vesicles is used by cytosolic enzymes in exactly the same fashion as ornithine arising from the arginase reaction [10]. We therefore picture ornithine use mainly from the vesicle in the first 10–20 min, with in-

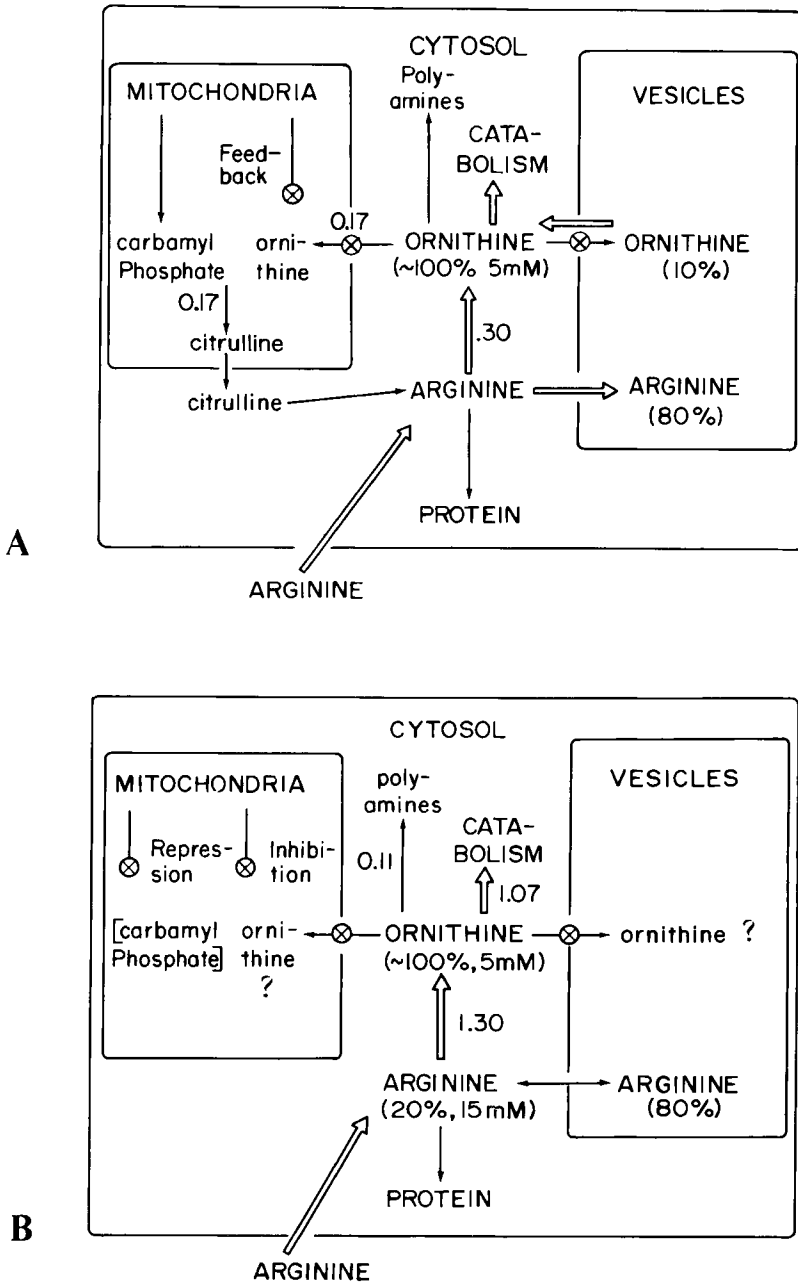


Fig. 5. Diagram of fluxes in cells after addition of a large amount of arginine to the medium. (A) State of the pathway 5–60 min after arginine addition to cells. Immediate effects are feedback inhibition of ornithine biosynthesis and blockade of ornithine entry into mitochondria and vesicles (circled X's). In addition, the arginine catabolic path is activated, and ornithine is discharged into the cytoplasm as arginine enters the cytosol and vesicles (open arrows). (B) Steady state of pathway in cells grown in arginine. In addition to effects seen in Fig. 5A, repression of carbamyl phosphate synthetase A is complete, and catabolic enzymes are fully induced. Certain flux determinations (in $\text{nmoles min}^{-1} \text{mg}^{-1}$) and pool sizes are shown.

creasing and continued use of ornithine from the arginase reaction thereafter. Within a few minutes after arginine addition, therefore, the ornithine concentration of the cytosol has risen to 5 mM, or about 25-fold that of a culture grown in minimal medium. It is clear that the membrane or contents of the vesicle play a vital role in achieving this effect, and thus of diverting ornithine into catabolism.

We may now consider what is happening to mitochondrial processes during this time. First, arginine immediately inhibits ornithine biosynthesis. This has been shown both by "balance-sheet" analysis of total ornithine [10] and its derivatives [15], and by showing that ^{14}C -glutamate no longer enters ornithine or arginine *in vivo* after addition of arginine to the medium (R. L. Weiss, unpublished observations). As a consequence of feedback inhibition, all ornithine produced in the cell after addition of arginine arises in the cytosol (via arginase), instead of in the mitochondria.

A significant economic problem remains. Because carbamyl phosphate synthetase A is not feedback inhibited by arginine, and because its repression is not complete for over eight hours [9, 21], the possibility of a wasteful reentry of ornithine into the anabolic pathway exists. This possibility is heightened by the previous demonstration that ornithine easily enters mitochondria in cultures grown in minimal medium, and that the ornithine concentration of the cytosol is very high under catabolic conditions. When the flux of cytosolic ornithine into citrulline is measured at any time in the first hour after arginine is added, however, it is found to be only $0.17 \text{ nmoles min}^{-1} \text{ mg}^{-1}$ [10]. This is only 20% of the rate of citrulline synthesis seen in minimal medium. There is no evidence whatever that arginine inhibits the ornithine transcarbamylase or carbamyl phosphate synthetase reactions, and in fact carbamyl phosphate levels remain high for several hours after arginine administration [22]. We therefore presume that the high arginine concentration of the cytosol is inhibiting the entry of ornithine into mitochondria. While this inhibition is not complete, the influx rate would be only 13% of that seen in cultures grown in minimal medium ($1.32 \text{ nmoles min}^{-1} \text{ mg}^{-1}$), despite the extremely high ornithine concentration on the cytosolic face of the mitochondrial membrane. This mechanism, which has yet to be confirmed *in vitro*, would be a novel way of controlling the ornithine cycle. It would be an alternative to feedback inhibition of carbamyl phosphate synthesis seen in some bacteria, and to the inhibition of ornithine transcarbamylase by arginase seen in some yeasts [23, 24]. In summary, feedback inhibition of ornithine synthesis and the confinement of catabolic ornithine to the cytosol leads to fast and efficient ornithine catabolism, and to a minimization of wasteful synthesis of new ornithine and new citrulline.

After prolonged growth of cells on arginine, a new steady-state is achieved (Fig. 5B). By that time (about three generations), full repression of carbamyl phosphate synthetase A has taken place [10; R. H. Davis, unpublished observations], thereby *fully* blocking the ornithine cycle, and a 2- to 4-fold induction of arginase and ornithine aminotransferase is complete. The induction of catabolic enzymes, as measured by enzyme activity, begins after 40 minutes, but the rate of enzyme synthetic capacity production (presumably mRNA) increases almost immediately [25]. At catabolic steady-state, arginine catabolism takes place at a rate 2- to 3-fold the rate arginine enters protein, cytosolic concentrations of arginine and ornithine are high, and the vesicles contain a large amount of arginine — 4-fold the amount seen in vesicles of cultures grown in minimal medium [6]. Although this steady-state is achieved only slowly, its important metabolic consequences commence almost instantly: onset of catabolism, and a blocking of the ornithine cycle. Intracellular compartments and their membranes are important aspects of these immediate changes.

RESPONSE TO THE REMOVAL OF ARGININE

Given a catabolic steady-state, what happens when arginine is exhausted or removed from the medium? Here again, *Neurospora* cells adapt almost immediately, and the vesicle again plays an important part. A diagram of this state is shown in Fig. 6.

Upon the exhaustion of arginine from the medium, catabolism ceases within five minutes, despite the high arginine content of the cells [8]. By the logic of the previous discussion, this indicates that the cytosolic arginine concentration has fallen to levels at which arginase is not active. Presumably the arginine present in the cytosol when uptake ceases is quickly absorbed by the vesicles, used for protein synthesis, or catabolized. In turn, ornithine catabolism becomes reduced, and ornithine biosynthesis is reinitiated by relief of feedback inhibition. (No enzyme of ornithine biosynthesis is repressible [12]). The cessation of arginine catabolism has deprived putrescine of a precursor; it is fitting that ornithine synthesis resume quickly.

Arginine synthesis cannot resume quickly, however, because carbamyl phosphate synthetase A must be derepressed. Thus there is a possibility that exhaustion of arginine from the medium would lead to a transient starvation for arginine. This possibility does not materialize, because the efflux of arginine from the vesicles is rapid enough to sustain maximal rates of protein synthesis [8]. That arginine efflux from the vesicles can sustain protein synthesis was proved by growing an auxotroph on arginine until arginine was exhausted from the medium. At that point, it continued to synthesize protein at a maximal rate for over half a doubling at the expense of vesicular arginine until all arginine of the vesicles was gone [8]. We conclude that the efflux from the vesicle can maintain a cytosolic pool sufficient for protein synthesis, but not for catabolism. When seen in the context of the culture grown in minimal medium, this phenomenon is general: the vesicular membrane can regulate – or buffer – the cytosol against radical amino acid deficiencies as long as the vesicle contains amino acid.

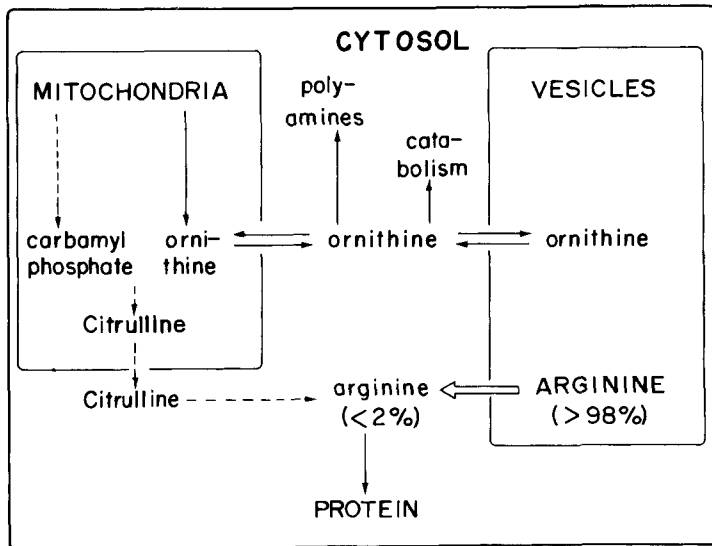


Fig. 6. Diagram of fluxes in arginine-grown cells when arginine is removed from the medium. Dashed arrows signify the slow onset of synthesis of carbamyl phosphate and its derivatives.

CONCLUSION

The complexities of the system described here in *Neurospora* have yielded to tracer methodology and cell fractionation techniques. The intracellular traffic of certain metabolites across internal membranes has been described. It is clear that the eukaryotic cell is unusually well equipped to respond immediately to changes in the external environment by virtue of subcellular structures. In this sense, eukaryotic cells may resemble bacterial cells which depend mainly upon fast growth, repression, enzyme induction, and feedback inhibition for their adaptive responses. It is likely that compartmental features of eukaryotic cells will be increasingly recognized in the future as the basis of much of their regulatory capacity.

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