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Use of External, Biosynthetic, and Organellar Arginine by *Neurospora*

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The fate of very low amounts of ^{14}C-arginine derived from the medium or from biosynthesis was studied in *Neurospora* cells grown in minimal medium. In both cases, the label enters the cytoplasm, where it is very briefly used with high efficiency for protein synthesis without mixing with the bulk of the large, endogenous pool of ^{12}C-arginine. The soluble ^{14}C-arginine which is not used for protein synthesis is sequestered in a vesicle with the bulk of the endogenous arginine pool. After this time, it is selectively excluded from use in protein synthesis except by exchange with cytoplasmic arginine. The data suggest that in vivo, the non-organellar cytoplasm contains less than 5% of the soluble, cellular arginine. The cellular organization of *Neurospora* described here also prevents the catabolism of arginine. Our results are discussed in relation to previous work on amino acid pools of other eukaryotic systems.

Previous papers from this laboratory (1; R. L. Weiss, J. Biol. Chem., 1973, in press; R. L. Weiss and R. H. Davis, J. Biol. Chem., 1973, in press) show that the enzymes and some intermediates of arginine metabolism are not randomly distributed in *Neurospora* cells. Certain expectations about the compartmentation of arginine metabolism in vivo are tested in this paper.

The enzyme which forms arginine-specific carbamyl phosphate, the enzyme which forms ornithine, and the enzyme which uses ornithine and carbamyl phosphate to make citrulline are mitochondrial (1; R. L. Weiss and R. H. Davis, J. Biol. Chem., 1973, in press). The two enzymes which act in sequence to transform citrulline to arginine are cytoplasmic, and the enzyme which converts ornithine to putrescine is also cytoplasmic. The two catabolic enzymes which act in sequence to degrade arginine, via ornithine, to glutamic-γ-semialdehyde are cytoplasmic (R. L. Weiss and R. H. Davis, J. Biol. Chem., 1973, in press). Both enzymes have substantial activity in cells grown in minimal medium (9).

Over half of the large ornithine and arginine pools in gently disrupted cells are associated with a sedimentable organelle; simple corrections for escape of these compounds after cell breakage lead us to estimate that the organelle contains over 90% of the intracellular arginine and ornithine (about 20 and 30 nmol per mg, dry weight, respectively) (R. L. Weiss, J. Biol. Chem., 1973, in press). The organelle is distinct from mitochondria, but in the absence of identransformed to putrescine; the rest is sequestered in the vesicle. The vesicular pool of ornivesicular pool.

These data have been used to formulate the metabolic scheme in Fig. 1. Some of the expectations of the scheme are as follows:

(i) Whereas most of the ornithine made by *Neurospora* is probably transformed to citrulline before it can leave the mitochondrion, the remainder (about one-sixth) escapes to the cytoplasm. About half of the latter is then transformed to putrescine; the rest is sequestered in the vesicle. The vesicular pool of ornithine equilibrates only slowly with other cellular compartments. Experiments on ornithine will be reported in a subsequent paper.

(ii) Citrulline departs the mitochondrion freely, and is transformed to arginine in the cytoplasm.

(iii) Most of the arginine is fixed into protein via arginyl-tRNA. The remainder (about one-eighth) is withdrawn from the cytoplasm into the vesicle. The steady-state concentration of arginine in the cytoplasm is adequate for protein synthesis, but not for catabolism.

(iv) Catabolism of arginine occurs when high cytoplasmic levels of this compound are present, such as after addition of high levels of arginine to the medium.

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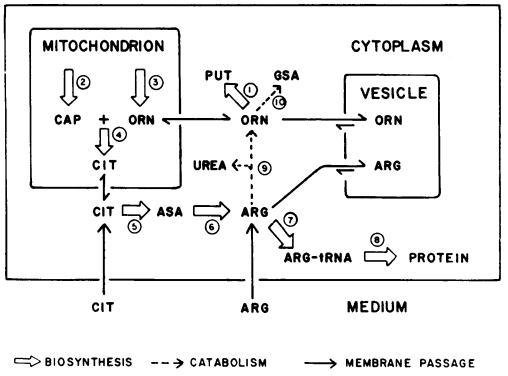

FIG. 1. *Diagram of biochemical steps and cellular structures involved in arginine synthesis in Neurospora. The vesicle is a cytoplasmic inclusion different from mitochondria, but whose possible identity with the vacuole or microbodies has not yet been successfully tested. Abbreviations: ARG, arginine; ASA, argininosuccinic acid; CAP, carbamyl phosphate (arginine-specific); CIT, citrulline; GSA, glutamic-γ-semialdehyde; ORN, ornithine; PUT, putrescine (1,4-diaminobutane). Metabolic steps: 1, ornithine decarboxylase (EC 4.1.1.17); 2, carbamyl phosphate synthetase A (EC 2.7.2.5, arginine-specific); 3, N^{α}-acetylornithine-glutamate transacetylase; 4, ornithine transcarbamylase (EC 2.1.3.3); 5, argininosuccinic acid synthetase (EC 6.3.4.5); 6, argininosuccinic acid lyase (EC 4.3.2.1); 7, arginyl-tRNA synthetase; 8, polypeptide formation; 9, arginase (EC 3.5.3.1); 10, ornithine-α-ketoglutarate transaminase (EC 2.6.1.13).*

In this study, we used minimal-grown cultures to show that catabolism of arginine is insignificant; that ^{14}C-arginine entering the cytoplasm at tracer levels largely bypasses the resident arginine pool as it is incorporated into protein; and that the isotope, if not used immediately in protein synthesis, is sequestered and substantially excluded from protein synthesis thereafter.

MATERIALS AND METHODS

Materials. The ureaseless strain, *ure-1 A* (allele *ur9* of Kølmark [11]); obtained from the Fungal Genetics Stock Center, was used throughout this investigation. Vogel minimal medium N (20) was used for growth. L-Arginine, L-citrulline, and cycloheximide were purchased from Sigma Chemical Co. The isotopically labeled compounds L-arginine-*guanidino*-^{14}C (25 mCi/mmol) and L-citrulline-*ureido*-^{14}C (32.5 mCi/mmol) were purchased from Calatomic and purified by column chromatography prior to use.

Growth and sampling. The *ure-1* strain was grown at 25 C from a conidial inoculum (about 5×10^6 conidia per ml) in 500 or 1,000-ml cultures grown exponentially in minimal medium with forced air (8). Cultures were used when they had attained a density of 0.30 mg (dry weight) per ml. Dry weights were determined by harvesting and acetone-drying 10- or 20-ml volumes of culture. In the major experiments, ^{14}C-arginine or ^{14}C-citrulline were added to a final concentration of about 0.2 μM (11×10^3 to 15×10^3 counts per min per ml). The additions had no detectable influence on the metabolism of the cells.

Fast harvests of 3 ml were made with wide-bore, 5-ml pipettes. The following parameters were measured, as indicated. (i) The total radioactivity taken up by whole cells was measured in cells pipetted initially into an ice-cold solution of unlabeled arginine or citrulline such that the final concentration of the unlabeled compound was 10 mM. No further uptake or exchange of isotope took place in these conditions. The cells were later collected by suction on Gelman GA-6 cellulose acetate membrane filters (0.45-μm pore size) and washed with ice-cold 10 mM arginine or citrulline, as appropriate. The filters, with the cells, were placed in scintillation counting vials with 15 ml of dioxane counting fluid and counted. (ii) Radioactivity fixed into protein at the time of harvesting was measured in 3-ml samples of culture pipetted into an ice-cold solution of trichloroacetic acid such that the final concentration of the acid was 5%. The insoluble material was collected on membrane filters and washed with cold, 5% trichloroacetic acid. The filters were placed in scintillation vials and counted. (iii) Where acid-soluble pools were to be extracted, 10- to 20-ml samples of the culture were harvested directly on membrane filters and washed with ice-cold distilled water. No loss of intracellular counts took place in this process. The mycelial pad was scraped from the filter and placed in 4 ml of cold, 5% trichloroacetic acid. After centrifuging the insoluble material, the supernatant fluid was poured off, extracted five times with ethyl ether, and used for column and paper chromatography. (iv) Where the identity of radioactivity in proteins was to be determined, the protein was isolated from acetone-dried pads or from trichloroacetic acid-extracted residues by the procedure of Roberts et al. (17). The protein was hydrolyzed in 6 N HCl in evacuated tubes for 18 h at 110 C. The hydrolysates were evaporated, filtered, and redissolved for chromatography. Measurements of radioactivity during the isolation procedure established that radioactivity was associated with the protein fraction and that it was all liberated as soluble, chromatographically identifiable arginine during hydrolysis.

Fractionation and chromatography. Arginine and citrulline were purified on Dowex-50 X8 columns (Na^+-form, 200–400 mesh, 0.7 by 15 cm) eluted with 0.116 M Na_3 citrate adjusted to pH 5.3 with concentrated HCl. Citrulline, argininosuccinic acid, and urea emerged at the front; after 40 ml of citrate buffer was passed through the columns, arginine was displaced with 10 ml of 0.2 N NaOH. Fractions containing citrulline and arginine were desalted on columns of the same type in the H^+-form, and eluted with successive additions of 10 ml of 0.1 N HCl, 20 ml of 1.5 N HCl, and 10 ml of 6 N HCl. Amino acids emerged in the last fraction; salts and urea were removed in the 1.5 N HCl fraction. Amino acids were determined after evaporation of HCl.

To estimate urea, volumes of whole culture were

brought to 5% trichloroacetic acid, and centrifuged. The supernatant fluids were ether extracted, applied to Dowex-50 columns in the H^+-form and eluted as in the desalting procedure. Urea emerged in the second 10 ml of 1.5 N HCl and was estimated after evaporation of HCl.

Radioactivity was estimated with a Beckman CPM-100 liquid scintillation counter with quench standardized or corrected. Arginine was estimated colorimetrically by the method of Van Pilsum et al. (19); citrulline and urea were estimated by the method of Koritz and Cohen (12), with chloride added to intensify and standardize color values (6).

The identity of samples was checked on a number of occasions by paper chromatography in phenol-water (100:20 vol/vol) in an HCN atmosphere, or in propanol-acetic acid-water (70:5:20). In the case of fractions isolated as arginine or citrulline, at least 90% of the radioactive compound in question was indistinguishable from authentic arginine or citrulline. Citrulline fractions also contain all of the small amount of argininosuccinic acid found in cells. Radioactivity was detected by an Actigraph III paper strip scanner (Nuclear-Chicago Corp.), and amino groups were detected with ninhydrin.

Calculations. The raw data for the ^{14}C-arginine experiments were the total isotope taken up by cells and the trichloroacetic acid-insoluble isotope in samples of the same size, taken simultaneously. The acid-soluble radioactivity was calculated by subtracting the best-fit curve for acid-insoluble radioactivity from the best-fit curve for total radioactivity. Specific radioactivity (counts per min per μmole) of the soluble pool compounds was determined directly, or was calculated with a knowledge of the radioactivity and the average value for moles of soluble arginine in these cells. The two methods agreed within 15% or less. Where the radioactivity of citrulline was measured, no attempt was made to free it of argininosuccinic acid.

The specific radioactivity of arginine entering new protein in a given interval was determined as follows: The increase of radioactivity (counts per min per 3 ml of culture) was determined from raw data on trichloroacetic acid precipitates. The constant doubling time (150 min) and the constant amount of proteinaceous arginine per unit, dry weight, (0.187 μmol per mg, dry weight) allowed calculation of the increase of μmoles of arginine fixed into protein in t minutes according to the formula $\Delta X = X_o (e^{kt} - 1)$. where X_o is the value of protein arginine in a 3-ml volume of culture at the beginning of the interval. For a 150-min doubling time, the value of k was 0.0046/min. The specific radioactivity was determined as the increase in counts per minute divided by ΔX.

RESULTS

Routes of ^{14}C-guanidino-arginine utilization. The several fates of ^{14}C-arginine can be inferred from the results in Table 1. In this experiment, ^{14}C-arginine (about 33,000 counts/min per 3 ml) was added to exponentially growing cultures of the *ure-1* strain. Samples were taken at 15 and 30 min after the tracer was added. The arginine was added to a final concentration of 2×10^{-7} M (carrier free) with or without pretreatment of mycelia for 5 min with 10 μg of cycloheximide per ml. Total uptake, acid-insoluble radioactivity, acid-soluble radioactivity in arginine, and radioactivity in urea were measured.

Uptake of carrier-free ^{14}C-arginine was complete within 15 min, and cycloheximide did not significantly affect this process. At both times, in the absence of cycloheximide, all label was found in acid-soluble and protein arginine. Between 15 and 30 min, acid-soluble arginine decreased as the label in protein increased. Virtually no ^{14}C-urea was detected. (The basal level of urea appears during germination, and does not increase appreciably during prolonged growth [7].) Cycloheximide blocks entry of soluble ^{14}C-arginine into the acid-insoluble fraction of the cells (Table 1). This identifies the bulk of the acid-insoluble radioactivity of untreated cultures as arginine in proteins made on cytoplasmic ribosomes. As expected, cyclohex-

TABLE 1. *Isotope distribution after addition of ^{14}C-arginine to Neurospora culture*[a]

Arginine (M)	Cycloheximide (μg/ml)	Time (min)	Whole cells (counts per min per 3 ml)	Protein (counts per min per 3 ml)	Soluble arginine		Urea	
					μmoles	counts/min	μmoles	counts/min
					3 ml	3 ml	3 ml	3 ml
0	0	0			23		11	
2×10^{-7}	0	15	30,500	10,400	25	18,600	10	35
	0	30	30,600	15,800	27	14,300	10	46
	10	15	28,000	179	33	29,100	13	82
	10	30	28,600	229	38	28,000	14	153

[a] A 33,000 counts/min amount of ^{14}C-guanido-arginine was added per 3 ml; the cultures, grown in minimal medium, contained 1.0 mg (dry weight) of mycelium per 3 ml.

imide causes an accumulation of arginine, in terms of both counts and μmoles, in the soluble arginine pool. This is correlated with a slight increase in ^{14}C-urea formation, though the data do not allow precise interpretation.

From these data, we infer that minimal cultures catabolize virtually no arginine and that carrier-free ^{14}C-arginine may be used to explore the relationship of cellular arginine to protein synthesis.

Incorporation of ^{14}C-arginine (carrier-free) into pool and protein. After administration of carrier-free ^{14}C-arginine to cells, uptake is completed within 3 min (Fig. 2 and 3, inset). As the cell acquires isotope, the isotope enters protein rapidly. After 3 min, however, and for the remaining 2 h, protein synthesis removes counts only slowly from the pool. The two phases of isotope incorporation into protein may be considered in turn with the specific radioactivity data shown in Fig. 3.

(i) The raw data for the first 15 min of the experiment are shown on expanded axes in the inset of Fig. 3, and specific radioactivities of soluble and new-protein arginine are shown in the body of the figure. The specific radioactivity of arginine being incorporated into protein in short intervals over the first 2 min is much higher than that of the total acid-soluble arginine. In the first interval, 0 to 10 s, new protein arginine averages about 15×10^6 counts per min per μmol, whereas the pool averages about 0.25×10^6 counts per min per μmol; that is, 60-fold less. We infer that only about 1.7% of the arginine of the cell is exposed to protein synthesis, and that this is preferentially labeled in the first 10 s. The figure is an overestimate of the steady state, inasmuch as significant flux occurs through this pool in 10 s.

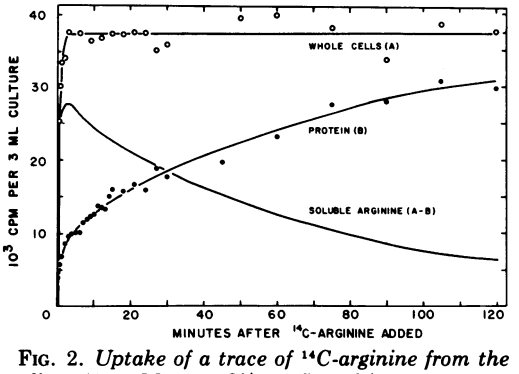

FIG. 2. *Uptake of a trace of ^{14}C-arginine from the medium (0.2 μM, 25 mCi/mmol) and its appearance as soluble arginine or proteinaceous arginine thereafter. The line describing soluble arginine was derived by subtracting the line for protein from the line for total cellular ^{14}C. Data for the first 15 min are shown in detail in the inset of Fig. 3.*

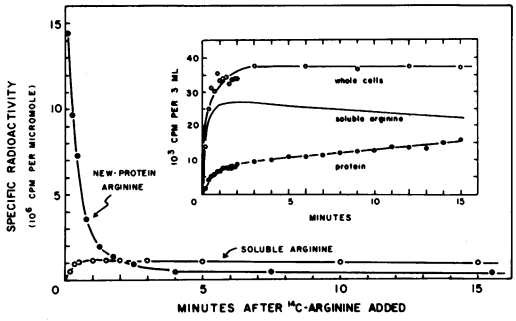

FIG. 3. *(Main axes) Specific radioactivities of soluble arginine and the arginine being used for protein synthesis after ^{14}C-arginine addition (see Fig. 2). New-protein arginine data are plotted at the midpoints of individual, successive intervals considered. Points representing soluble arginine describe the specific radioactivities at the sampling times indicated on the abscissa. (Inset). Raw data for the ^{14}C-arginine in whole cells, soluble arginine, and protein arginine from 0 to 15 min.*

A confirmatory calculation may be made. The isotope added to the medium has a specific radioactivity of 50×10^6 counts per min per μmol (25 mCi/mmol). The isotope is diluted in the first 10 s only about fourfold as it moves from medium to protein. In the first 10 s, 13.5×10^4 counts/min, or 0.27 nmol, arginine entered 1.14 mg of cells from the medium. There, it mixed with three parts (0.81 nmol) of unlabeled, intracellular arginine in the course of its exposure to arginyl-transfer ribonucleic acid (tRNA) synthetase. The unlabeled pool accessible to arginyl-tRNA synthetase is thus about 0.81/1.14, or 0.71 nmol of arginine per mg (dry weight) of cells. This is only 3.5% of the total cellular arginine (20 nmol per mg, dry weight), and again, it would be an overestimate because it disregards flux. This is similar to the figure of 1.7% calculated on a different basis above. Although these calculations may be inaccurate to a small degree, they demonstrate a highly selective use of arginine entering the cell for protein synthesis.

(ii) From the first sampling time onward, the specific radioactivity of arginine used for protein synthesis falls rapidly. After 2 min, the soluble ^{14}C-arginine which remains is selectively excluded from protein synthesis. This is shown by the fact that the specific radioactivity of new-protein arginine is lower, in defined intervals, than the total intracellular arginine present at the same times (Fig. 3). This observation has been confirmed in experiments in which the specific radioactivity of purified,

soluble arginine was determined directly rather than by calculation.

In more quantitative terms, we may ask whether the entire arginine pool (20 nmol per mg, dry weight) is a direct intermediate between its synthesis and its use. If so, it should turn over about eight times per doubling, to generate the formation of an additional 20-nmol pool and 180 nmol of protein arginine. The soluble label remaining after a generation should be $(\frac{1}{2})^8$, or 0.004 of that at the beginning. At 2 min, there is about 26,000 counts/min of soluble intracellular arginine per 3 ml of culture. We would expect only 100 counts/min to remain 150 min (one generation) later, where in fact there are about 3 to 7,000 counts/min. The result demonstrates a sequestration of isotope in this phase of the experiment.

The pattern of isotope utilization seen in Fig. 2 and 3 may be rationalized by reference to the diagram in Fig. 1, and the interpretation may be used to design a confirmatory experiment. In the first phase of the experiment (0–1.5 min), ^{14}C-arginine from the medium is taken up and finds itself mainly in the cytoplasm. There, it is diluted little as it is used for protein synthesis, since over 95% of the resident, unlabeled pool is in the vesicle. The label not incorporated immediately into protein moves into the vesicle, after which it is selectively bypassed, in protein synthesis, by biosynthetic arginine formed in the cytoplasm from citrulline. Thereafter, exchange of cytoplasmic and vesicular arginine permits the latter to be used only slowly for protein synthesis. Arginine is not transformed to other compounds in this process. The central feature of this explanation is the equivalence of external and biosynthetic arginine in their immediate relation to protein synthesis. This hypothesis is tested in the next experiment.

Utilization of ^{14}C-citrulline (carrier-free). ^{14}C-guanidino-arginine may be introduced into the cell by endogenous conversion of added ^{14}C-ureido-citrulline. The conversion of citrulline to arginine is accomplished by a sequence of two enzymes, both cytoplasmic (Fig. 1). If the interpretation of the previous experiment is correct, this ^{14}C-arginine would first be used selectively for, and later selectively excluded from, protein synthesis.

Figure 4 shows uptake of 36,000 counts/min of carrier-free ^{14}C-citrulline (about 0.5 nmol) per 3 ml of culture and the radioactivity subsequently associated with fractions corresponding to protein arginine, soluble arginine, and citrulline plus argininosuccinate. Uptake of citrulline is completed at about 8 min. In the cells, the isotope appears most rapidly in citrulline plus argininosuccinate, then in soluble arginine, then in protein. The citrulline pool behaves in a regular manner, falling exponentially from its peak value to less than 10% of the peak value within 20 min. (The residual 6–7% of the isotope disappears more slowly thereafter; this may represent derivatives which need not be considered here.)

Measurements of the specific radioactivity of citrulline plus argininiosuccinate (calculated as counts per minute per μmole of citrulline), soluble arginine, and arginine in new proteins are shown in Fig. 5. As expected, citrulline has the highest specific radioactivity at first, after which it declines as a result of replacement by endogenous citrulline synthesis. The specific radioactivity of soluble arginine and of new-

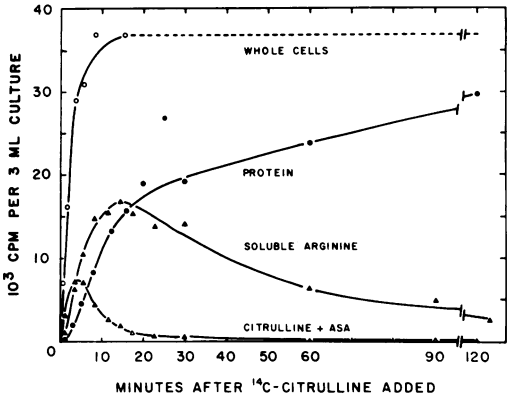

FIG. 4. *Appearance of ^{14}C in whole cells, in citrulline plus argininosuccinic acid, in soluble arginine, and in protein arginine after administration of ^{14}C-ureido-citrulline (0.2 μM, 32.5 mCi/mmol) to the culture.*

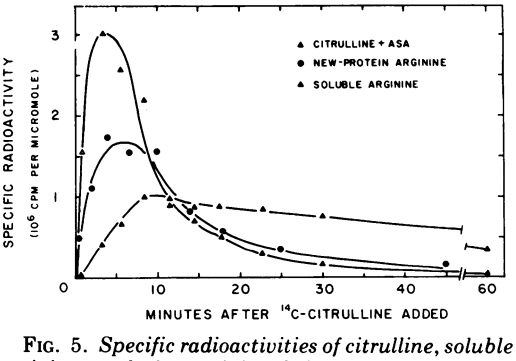

FIG. 5. *Specific radioactivities of citrulline, soluble arginine, and the arginine being used for protein synthesis after ^{14}C-citrulline addition (see Fig. 4). New-protein arginine determinations are plotted at the mid-points of the individual successive intervals considered. Points representing soluble compounds are the specific radioactivities found at the sampling times indicated on the abscissa.*

protein arginine confirms the expectations outlined above. The labeled arginine, made from citrulline, is selectively used for protein synthesis, largely bypassing the unlabeled arginine pool. After about 13 min, however, protein is made from an arginine precursor pool which, like citrulline in the same period, is poorly labeled. The soluble arginine persists at a higher specific activity indefinitely, sequestered from use in protein synthesis.

Only one calculation is germane here. In the interval 0 to 30 s, the new-protein arginine has a specific radioactivity of 5×10^5 counts per min per μmol, whereas the pool averages 0.24×10^5 counts per min per μmol. This suggests that the fraction of cellular arginine exposed in a 30-s interval to arginyl-tRNA synthetase is 0.24/5, or 5% of the total pool. This is consistent with the 1.7 to 3.5% fraction calculated for a 10-s interval in the previous experiment.

DISCUSSION

Whether a ^{14}C-arginine pulse is derived from the external medium or from biosynthesis, its immediate fate in *Neurospora* cells is very similar. In the initial few seconds, it is used efficiently for protein synthesis, mixing with less than 5% of the free, intracellular ^{12}C-arginine. The inference that external and biosynthetic arginine both enter the soluble portion of the cytoplasm (Fig. 1) is supported by three considerations: (i) the cytoplasmic membrane is in contact with the cytoplasm; (ii) the last enzyme of the arginine synthesis is a soluble enzyme (R. L. Weiss and R. H. Davis, J. Biol. Chem. 1973, in press); and (iii) protein synthesis, which immediately uses external and biosynthetic sources of arginine with the same high efficiency, is a cytoplasmic function.

The efficiency with which external and biosynthetic arginine bypass the resident arginine pool indicates that the bulk of the pool is in a discrete compartment. In fact, Weiss has isolated an arginine-containing organelle from gently disrupted cells and has characterized it to some extent in a separate publication (R. L. Weiss, J. Biol. Chem., 1973, in press). This organelle, which we temporarily call the vesicle, may be considered a third source of arginine, after uptake and bioysnthesis. It appears that biosynthetic or external ^{14}C-arginine which fails to enter protein synthesis within a few seconds enters the vesicular pool. There it persists, exchanging only slowly with cytoplasmic arginine.

The experiments reported here involve a ^{14}C-arginine pulse of very low molar concentration. The amounts added have only a small effect on the size of the cytoplasmic pool and a negligible effect on the size of the vesicular pool. The fact that almost no ^{14}C-urea appears during the passage of tracer through the cytoplasm or its residence in the vesicle suggests two things about arginine catabolism. (i) The concentration of arginine in the cytoplasm, where arginase resides (R. L. Weiss, and R. H. Davis, J. Biol. Chem., 1973, in press), is too low to support catabolism. If 5% of the free arginine and 80% of the cell water (18) are in the nonorganellar cytoplasm, the latter would contain 0.5 mM arginine. The K_m (Michaelis constant) of native arginase is much higher (5 mM) (K. N. Subramanian and R. H. Davis, unpublished data), and there is a suggestion of sigmoid substrate-velocity relations in the range of 0.5 mM arginine (14). (ii) The second conclusion is that the vesicle containing arginine may be adadpted in part to minimize arginine catabolism when biosynthesis is the only source of this compound.

Our basic observations have been anticipated by investigators who have studied the utilization of amino acids in plants (16), animals (15), and eukaryotic microorganisms (4, 10, 13, 21). Halvorson and Cohen (10), working with bakers' yeast, found that shortly after addition of high external levels of ^{14}C-phenylalanine, the label was preferentially used over the endogenous phenylalanine pool. Their simple, formal explanation of this phenomenon is entirely consistent with ours. Zalokar (22) demonstrated the same pattern of initial utilization of external ^{14}C-proline in *Neurospora*. Finally, with a different methodology, Matchett and DeMoss (13) concluded that biosynthesis "channeled" tryptophan to protein synthesis in *Neurospora*, without fully mixing with the endogenous tryptophan pool.

Two major studies of amino acid flow in eukaryotic cells, one in the yeast, *Candida utilis* (3, 4, 5), the other in higher plants (2), bear comment. In both cases, it was shown that amino acids derived from glucose did not equilibrate with substantial "storage" pools of the same amino acids in the same cells. Our results, which show vesicular arginine being withheld from cytoplasmic protein synthesis, support this conclusion. On the other hand, our results appear to conflict with another conclusion derived from the studies cited above. Where we conclude that external and biosynthetic arginine have similar access to protein synthesis, the previous authors were led to the conclusion that biosynthetic amino acids were formed and used for protein synthesis in a compartment which was protected from isotopic competition

with exogenous amino acids. This must remain an open issue for two related reasons. First, label may pass from the medium, through the cytoplasm, to the vesicles very quickly. This may make it difficult to interpret experiments involving longer sampling times. More important, the previous studies cited involved continuous, high levels of external amino acids as competitors, where we have used a pulse of ^{14}C-amino acid at tracer levels. The two experimental regimes, among other things, may impose substantially different types of metabolic flow upon the cells studied. A fuller exploration of a single system in a wider range of conditions is necessary before reliable comparisons can be made.

In a recent publication, Wiemken and Nurse (21) explore the problem of amino acid distribution in *Candida* further. The results, which define a small cytoplasmic and a large vacuolar amino acid pool by the use of tracers, differential extraction, and microscope observations, are wholly in accord with the views presented here and in a related publication (R. L. Weiss, J. Biol. Chem., 1973, in press). Although the results of Wiemken and Nurse do not resolve the apparent contradiction noted above, there is little doubt that the behavior of amino acids in *Candida* and in *Neurospora* reflect very similar cytological mechanisms.

Our further work will concern the capacity of the vesicular pool, competition among amino acids for occupancy of the vesicle, the compartmental regulation of ornithine metabolism and arginine breakdown, and enzyme regulation in the cytoplasm. In addition, the identity of the "vesicle" and its possible homology with vacuoles and lysosomes of other organisms must be sought. Unfortunately, the functional and cytological definitions of such organelles in *Neurospora* are imperfect, and the cells we have used here lack many of the enzymes (and thus the diagnostic criteria) of those that have been described.

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