and T. C. Gibb [*Mössbauer Spectroscopy* (Chapman & Hall, London, 1971)] contains an extensive discussion of the literature on the Mössbauer effect, emphasizing the chemical aspects. *Chemical Applications of Mössbauer Spectroscopy*, V. I. Gol'danskii and R. H. Herber, Eds. (Academic Press, New York, 1968) contains good review articles on most of the commonly used isotopes. An older book [G. K. Wertheim, *Mössbauer Effect: Principles and Applications* (Academic Press, New York, 1964)] is still very popular as an introductory text, but it predates the bulk of the literature. A brief review by C. E. Johnson [*Phys. Today* **24** (No. 2), 35 (1971)] summarizes applications of Mössbauer spectroscopy to biological systems. It is impossible to list here the review articles (more than 40 in just the past few years) involving Mössbauer spectroscopy.

3. E. Matthias and D. A. Shirley, Eds., *Hyperfine Structure and Nuclear Radiations* (North-Holland, Amsterdam, 1968); I. J. Gruverman, Ed., *Mössbauer Effect Methodology* (Plenum, New York, 1965–1971), vols. 1–6.
4. Length limits preclude discussion of the "recoil energy loss." Before 1958 it was thought that the recoil of the emitting nucleus would result in a gamma ray of substantially less energy than the transition energy. Mössbauer discovered that, for a nucleus bound in a solid, this recoil energy loss was quantized and could be effectively zero for some fraction of the transitions. This is now commonly called the "recoil-free fraction." The Mössbauer effect has also been used to investigate lattice dynamics and diffusion by way of the dependence of the recoil-free fraction on the binding of the nucleus in the solid.
5. P. G. Huray, L. D. Roberts, J. O. Thomson, *Phys. Rev. B* **4**, 2147 (1971); R. L. Cohen and K. W. West, *J. Phys.* **32** (Suppl. C1), 781 (1971); R. J. Borg, D. Y. F. Lai, C. E. Violet, *Phys. Rev. B* **5**, 1035 (1972).
6. Z. Mathalone, M. Ron, J. Pipman, S. Niedzwiedz, *J. Appl. Phys.* **42**, 687 (1971); W. A. Ferrando, R. Segnan, A. I. Schindler, *Phys. Rev. B* **5**, 4657 (1972).
7. R. L. Cohen and K. W. West, *J. Electrochem. Soc.* **119**, 433 (1972); in preparation.
8. M. C. Hobson, Jr., and H. M. Gager, *J. Colloid Interface Sci.* **34**, 357 (1970); V. I. Gol'danskii, I. P. Suzdalev, A. S. Plachinda, V. P. Korneev, *Dokl. Akad. Nauk SSSR* **185**, 203 (1969); K. Hennig, W. Hildebrandt, J. Scheve, H. Blume, *Z. Anorg. Allg. Chem.* **377**, 256 (1970); W. N. Delgass, R. L. Garten, M. Boudart, *J. Phys. Chem.* **73**, 2970 (1969).
9. D. A. Channing and M. J. Graham, *J. Electrochem. Soc.* **117**, 389 (1970).
10. P. K. Gallagher and F. Schrey, *Therm. Anal. Proc. Int. Conf. 2nd* (1969), p. 929.
11. This is also called the "nuclear volume effect" and is an electric monopole interaction. The energy of the perturbation can be considered to arise from the product of an electronic term (electronic density at the nucleus) and a nuclear term (proportional to the change in nuclear charge radius between the ground state and the excited state); since the nuclear term is the same for all surroundings, a measurement of the product establishes the value of the electronic density. This interaction is described in detail in the books listed in (*2*).
12. A. H. Muir, Jr., R. M. Housley, R. W. Grant, M. Abdel-Gawad, M. Blander, in *Mössbauer Effect Methodology*, I. J. Gruverman, Ed. (Plenum, New York, 1971), vol. 6, p. 163. A number of reports on the Mössbauer spectroscopy of lunar samples have been presented in the *Science* "Moon Issue" (30 January 1970) and at the various lunar science conferences.
13. R. W. Dundon and S. S. Hafner, *Science* **174**, 581 (1971).
14. See C. E. Johnson (*2*).
15. See P. H. Swerdlow, J. F. Bertles, Y. W. Chow, R. H. Howes, G. C. Papaefthymiou, C. S. Yen, C. S. Wu, *Bull. Amer. Phys. Soc.* **16** (Ser. II), 641 (1971); in preparation.
16. V. I. Gol'danskii, O. Yu. Okhlobystin, V. Ya. Rochev, V. V. Khrapov, *J. Organomet. Chem.* **4**, 160 (1965).
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21. A. Simopoulos, A. Kostikas, D. Petrides, H. H. Wickman, *Chem. Phys. Lett.* **7**, 615 (1970).
22. M. Blume, in *Hyperfine Structure and Nuclear Radiations*, E. Matthias and D. A. Shirley, Eds. (North-Holland, Amsterdam, 1968), p. 911; H. H. Wickman, in *ibid.*, p. 928; M. J. Clauser and M. Blume, *Phys. Rev. B* **3**, 583 (1971); A. M. Afanas'ev and Yu. Kagan, *Sov. Phys. JETP* **18**, 1139 (1964).
23. H. H. Wickman and C. F. Wagner, *J. Chem. Phys.* **51**, 435 (1969).
24. N. D. Heiman, J. C. Walker, L. Pfeiffer, *Phys. Rev.* **184**, 281 (1969).
25. L. Pfeiffer, N. D. Heiman, J. C. Walker, *Phys. Rev. B* **6**, 74 (1972); L. Pfeiffer, *J. Appl. Phys.* **42**, 1725 (1971).
26. A. J. F. Boyle and G. J. Perlow, *Phys. Rev.* **180**, 625 (1969), and the references therein.
27. Research on the Mössbauer effect in the actinides has been reviewed by G. M. Kalvius, in "Proceedings of the Fourth International Conference on Plutonium and Other Actinides," Santa Fe, New Mexico, 5–6 October 1970 [published as *Nuclear Metallurgy*, W. M. Milner, Ed. (Metallurgical Society, New York, 1970), part 1, vol. 17].

Metabolite Distribution in Cells

Two carbamyl phosphate gradients and their sources can be discerned in *Neurospora*.

Rowland H. Davis

Many small molecules are not uniformly distributed within cells in the course of their metabolism. Metabolic channeling, as this phenomenon is called, is hard to demonstrate with certainty or, once demonstrated, is hard to explain in terms of cell structure. The embryologist, the cell biologist, and the mitochondrial physiologist take it for granted that gradients exist in cells and influence cell function (*1*). Many biochemists fear metabolic channeling because it stultifies predictions of metabolic rates based on cellular metabolite concentrations. Sophisticated biochemists and bacterial physiologists contend with the reality of channeling with complex kinetic diagrams and equations (*2*). The disdain among the three groups continues because there is little common background or means of discourse.

In this article I describe the work done in my laboratory on the channeling of carbamyl phosphate (CAP) in *Neurospora*. My colleagues and I have proceeded with the conviction that the kinetics of channeling is secondary to a knowledge of the structures that make it possible. My goal here is to analyze this case of metabolic channeling in terms that biologists in all specialties can understand, enjoy, and possibly apply. The work is unique only in the clarity of the preliminary evidence, in the use of genetic techniques, and in the number of possible explanations for the channeling observed.

The CAP molecule is small in size and is used in the synthesis of the amino acid, arginine, and the pyrimidine nucleotide, uridylic acid. In the arginine pathway, ornithine transcarbamylase (OTC) (E.C. 2.1.3.3) uses CAP to form citrulline from ornithine. In the pyrimidine pathway, aspartate transcarbamylase (ATC) (E.C. 2.1.3.2) uses CAP to form ureidosuccinate from aspartic acid (Fig. 1). In *Neurospora*, two separate carbamyl phosphate synthetases (E.C. 2.7.2.5) catalyze the formation of CAP (*3*). Carbamyl phosphate synthetase A (CPS-A) provides a pool of CAP specifically for the arginine pathway, while carbamyl phosphate

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Table 1. CAP pools and their sources in *Neurospora* strains. The CAP content is calculated as nanomoles of CAP per gram, dry weight of mycelium. [Data from (*16*)]

Strains	Enzyme deficiency	Nutritional requirement	CAP content	Source of CAP pool
Wild type	None	None	6.0	CPS-A and CPS-P
pyr-3a	CPS-P	Uridine	3.8	CPS-A
arg-3	CPS-A	Arginine	0.4	CPS-P
pyr-3a, arg-3	CPS-P and CPS-A	Uridine, arginine	0.0	None
arg-3, pyr-3d	CPS-A and ATC	Uridine	6.6	CPS-P
pyr-3a, arg-12^s	CPS-P and OTC (3%)	None	29.3	CPS-A

synthetase P (CPS-P) provides a CAP pool specifically for the pyrimidine pathway. The path-specificity of the CPS's and the CAP pools was first suggested by the behavior of mutants lacking CPS-A or CPS-P The *arg-2* and *arg-3* mutants, deficient in CPS-A, require arginine, even though their CPS-P is normal. The *pyr-3a* mutants, lacking CPS-P, require uridine despite normal amounts of CPS-A. It appeared that the CAP pool of each pathway was confined in such a way that it could not be used in the other pathway (*3*). The cellular basis of this apparent channeling phenomenon has since been investigated by a variety of techniques.

Metabolic channeling is a seductive idea: it is easy to understand and hard to prove. Sometimes investigators, as a last resort, offer it as an explanation for a problematic metabolic finding without much direct evidence. The danger of doing so lies in the unrecognized alternatives. First, where a compound has more than one metabolic destination, regulatory or kinetic factors may be just as important as physical compartmentation in determining the compound's fate. This problem is obvious where anabolic and catabolic enzymes coexist in the same cell as potential competitors (*4*). While the catabolic enzyme may have no physical access to an anabolic substrate, it may equally well have physical access but need a high concentration of substrate, an activator, or the removal of an inhibitor before it will function. Second, the addition of radioactive tracers often reveals preferential uses of exogenous and endogenous substrate by two enzyme systems (*5*). The tracer gradient that occurs in such experiments indicates differential localization of the enzymes that use the compound in question, but it does not establish with certainty that the endogenous substrate itself is normally disposed in a gradient. Third, where enzymes are shown to be localized in an organelle, it is often difficult to show that the substrates are similarly confined. For example, in the rat liver, one CPS is associated in mitochondria with OTC, the enzyme that uses CAP in the arginine pathway (*6*). The other CPS is associated in an enzyme complex with ATC (*7*) in the soluble fraction of the cytoplasm (*8*). It is widely assumed that mitochondrial CAP is used exclusively in arginine and urea synthesis. Two recent studies, however, reveal that mitochondrial CAP can escape at least under certain circumstances to the ATC reaction (*9*).

In the metabolism of CAP in *Neurospora*, some of the problems encountered in studies of channeling are avoided. First, both sources of CAP are intracellular and are needed simultaneously during growth. Second, the nutritional characteristics of mutants can be used as a test of CAP supply for each pathway in living cells. Third, homogeneous populations of exponentially growing cells may be grown in bulk to determine their enzyme constitution and their CAP content. The conclusion that CAP is channeled in *Neurospora* is based on data from the following types of studies: (i) enzyme and nutritional studies of mutant strains, (ii) a direct study of CAP pools, (iii) the analysis of an enzyme aggregate having both CPS-P and ATC activities, and (iv) a histochemical determination of transcarbamylase localization. Eucaryotic cells, like those of *Neurospora*, have many subcellular structures that participate in the organization of metabolism. All the techniques mentioned above still leave a choice of mechanisms to be explored in detail.

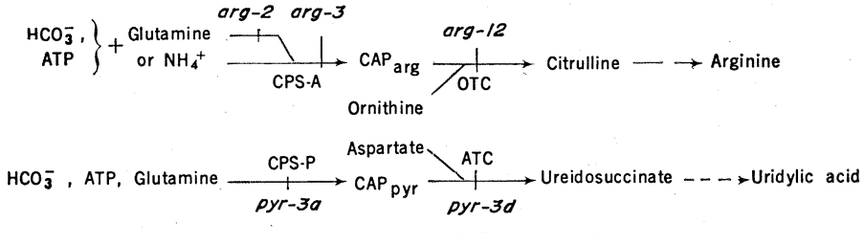

Fig. 1. Scheme of carbamyl phosphate (CAP) metabolism in the arginine and pyrimidine pathways of *Neurospora*. Two CAP pools, CAP_{arg} and CAP_{pyr}, are maintained in vivo. The biological nitrogen donor of the two CAP-synthesizing enzymes (CPS-A and CPS-P) is glutamine. Ammonium ion can serve as an alternate donor for both enzymes when it is present in high concentrations. This is depicted only for the arginine-specific enzyme because the ammonium-accepting ability of this enzyme is more efficient than that of the pyrimidine-specific enzyme, and because this ability persists in the absence of glutamine-accepting ability, as in *arg-2* mutants (*3*).

Gene-Enzyme Studies

Figure 1 is a diagram of CAP metabolism in *Neurospora*, showing the genetic determination of the enzymes involved. Both of the path-specific CPS enzymes catalyze CAP formation from adenosine triphosphate, bicarbonate, and the amide nitrogen of glutamine in the presence of magnesium ion. This reaction was first described by Levenberg (*10*) and has since been found to occur in all types of organisms.

Neither CPS requires acetylglutamate, a cofactor needed by the ammonium-dependent CPS-1 of vertebrates (*11*). Both use ammonia less efficiently than they do glutamine as a nitrogen donor (*12*). CPS-A and CPS-P are physically separable by gel filtration and are determined by separate genetic loci (*12*). [CPS-A, in fact, is composed of two polypeptides determined by the unlinked *arg-2* and *arg-3* loci (*3*).] CPS-P is determined by the *pyr-3* locus which, as will be shown, also determines ATC activity. The path-specificity of the two CPS enzymes is clearly indicated by the specific requirements for arginine displayed by *arg-2* and *arg-3* strains, and for uridine by the *pyr-3a* strain.

Further evidence for path-specificity of the two CPS enzymes is that CPS-P is feedback-inhibited by uridine triphosphate, a pyrimidine end product, and

that it is derepressed five- to tenfold upon starvation of mutant mycelia for uridine (*12*). CPS-A is derepressible by arginine starvation, but it is not feedback-inhibited in vitro by arginine.

The CAP produced by the synthetases is used by the two transcarbamylases. The *arg-12* locus determines OTC, and the *pyr-3* locus determines ATC. At both transcarbamylase loci, mutations that eliminate activity entirely and mutations causing only incomplete enzyme deficiencies have been isolated (*13*).

The specific requirements of the two types of CPS mutants strongly suggest that two channeled pools of CAP exist in *Neurospora* (*14*). Further evidence indicates that accumulation of CAP in one pathway leads to CAP overflow into the other pathway. The *pyr-3a* mutants have no CPS-P, but have normal amounts of ATC. Their pyrimidine requirement can be abolished if they carry a second mutation, *arg-12^s*, which reduces OTC activity drastically (*14*). The double mutant is a prototroph (Fig. 2). It appears that arginine-specific CAP accumulates in these cells as a result of the OTC deficiency and can be used in the ATC reaction. This suggests that channeling of CAP occurs, if at all, only when it is present in low concentrations. In a symmetrical fashion, *arg-2* and *arg-3* mutants, lacking CPS-A, are relieved of their arginine requirements by *pyr-3d* mutants (*15*), which lack ATC activity (Fig. 2).

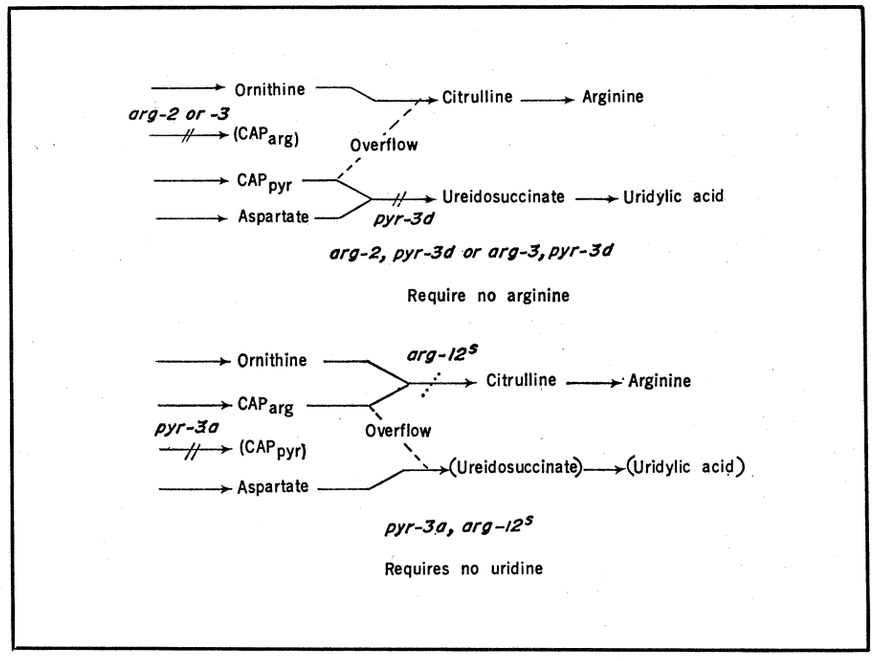

Fig. 2. CAP overflow between pathways in two types of double mutant of *Neurospora*. Mutations *pyr-3d* (top) and *arg-12^s* (bottom) block CAP utilization in one pathway and thereby relieve nutritional requirements imposed by CPS mutants in the other. Parenthesized compounds are not synthesized. Abbreviation: CAP, carbamyl phosphate (arginine- or pyrimidine-specific). [Courtesy of Academic Press (*3*)]

Carbamyl Phosphate Pools

The nutritional behavior of the single and double mutants described above suggests three questions that might be asked about the CAP pools of *Neurospora*. (i) What are the relative contributions of the two CPS enzymes to the steady-state CAP pool? (ii) Is the apparent diversion from one pathway to the other associated with a high intracellular CAP concentration? (iii) Does a mutant lacking one CPS, and starving as a result, nevertheless have a detectable CAP pool made by the other CPS? CAP is a very labile compound, and it is found in exceedingly low concentrations in *Neurospora*. We devised a method of making cold acid extracts of mycelia, and of converting the CAP in the neutralized extracts (with labeled ornithine and OTC) to radioactive citrulline (*16*). The citrulline was measured after it had been purified and chromatographed. Our data for normally growing cultures of various strains are summarized in Table 1 and provide answers to questions (i) and (ii) above (*16*).

Wild-type mycelia contain about 6 nanomoles of CAP per gram, dry weight, if they are grown in minimal medium. A double mutant, lacking both CPS enzymes (*arg-3, pyr-3a*; see Fig. 1) has, as expected, no detectable CAP even when it is starved for one or the other of its nutritional requirements. By comparing the CAP pools of the single mutants *arg-3* (having only CPS-P) and *pyr-3a* (having only CPS-A), the contributions of the CPS enzymes can be separately determined. The amount of CAP associated with the pyrimidine pathway (seen in *arg-3*) is 0.4 nmole/g dry weight, while CAP of the arginine pathway (seen in *pyr-3a*) is 3.8 nmole/g. The pools are measured in strains grown only in the presence of the nutritional supplement needed as a result of the deficient pathway. Therefore, the CAP pool in the single mutants—that of the intact pathway—is not influenced by the presence of an end product in the medium. The sum of the CAP pools of the single mutants is consistent, within error, with what is found in a wild type grown on minimal medium.

Table 1 also indicates that CAP concentration is elevated under conditions where CAP appears to overflow from one path to the other (Fig. 2). Two comparisons can be made to demonstrate this phenomenon. First, *arg-3* lacks CPS-A, it has an arginine requirement, and contains, at steady-state, 0.4 nmole of CAP per gram, dry weight, made by CPS-P. In contrast, *arg-3, pyr-3d* has no arginine requirement (Fig. 2) and contains 6.6 nmole of CAP per gram, also made by CPS-P. Thus the abolition of the arginine requirement is correlated with elevated "pyrimidine-specific" CAP. Second, *pyr-3a* lacks CPS-P, it has a uridine requirement, and contains 3.8 nmole of CAP per gram, made by CPS-A. In contrast, the *pyr-3a, arg-12^s* strain has no pyrimidine requirement (Fig. 2), and this is correlated with a CAP content of 25 to 35 nmole/g. Once again, a high CAP concentration leads to its being used in the other pathway.

The determinations of CAP pools are made in acid extracts and, while the enzymic source of the CAP in each strain is known, the physical discreteness of the pools is unproven. Data on this problem were obtained (*16*) from an experiment designed to answer question (iii) posed earlier: Do mutants starving for CAP in one pathway still contain CAP of the other pathway?

The critical point of this experiment was to establish whether the larger pool of CAP, associated with the arginine path (3.8 nmole/g) still existed in a starving *pyr-3a* mutant. (A normally functioning pyrimidine sequence has only 0.4 nmole of CAP per gram.) The *pyr-3a* strain was grown on limited amounts of uridine. It grew well until the uridine was exhausted, at which time growth ceased. The CAP pool (originating in the arginine pathway) actually increased more than twofold, to 8 to 10 nmole/g, as growth stopped. This concentration of CAP is 20-fold higher than the normal CAP levels of the pyrimidine pathway. Thus pyrimidine synthesis stops not because CAP is absent during starvation, but because it is made by the arginine pathway. We tentatively conclude that the amount of CAP found is physically confined to the arginine pathway in the starving *pyr-3a* mutant. Only at higher concentrations, as in *pyr-3a, arg-12*^{s} (Table 1), does CAP escape at a rate sufficient to support pyrimidine synthesis.

Several features of these data suggest we should not be wholly complacent about the conclusions offered. The most pertinent question is why a strain carrying *pyr-3a* should grow at the same rate as the wild type when its arginine-specific CAP pool is 30 nmole/g (as in *pyr-3a, arg-12*^{s}) and not at all when it is 8 to 10 nmole/g (as in the starving single mutant). Is the overflow of CAP really such a discontinuous function of concentration? Or is it growth that responds discontinuously to CAP concentration? Or, finally, is the apparent "channeling" really due to an obscure kinetic parameter of the ATC reaction in the *pyr-3a* mutant?

The Pyrimidine Enzyme Aggregate

The data reviewed so far are only indicative of CAP's being physically channeled. The conclusion that such channeling does, in fact, occur acquires more force if the possible mechanisms for the phenomenon are examined.

One possibility is that pyrimidine-specific CAP may normally be enzyme-bound in its metabolism. A number of years ago, it was apparent that both CPS-P and ATC were determined by the *pyr-3* locus (*3, 14, 17*). It was conjectured that the enzymes might be physically associated and that CAP molecules might never leave the aggregate between the time they were made and the time they were consumed.

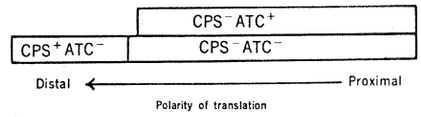

Fig. 3. Distribution of phenotypically different classes of *pyr-3* mutations within the locus. [After Radford (*19*)]

Mutants of the *pyr-3* locus, as noted previously, may lack CPS-P, ATC, or both activities. The distribution of phenotypically different groups of mutations on a recombination map of the locus (*18, 19*) is shown in Fig. 3. While there is a clear differentiation of the locus into CPS-specific (right) and ATC-specific (left) parts, the majority of mutations abolishes both activities. At least one of the doubly deficient mutants is the result of an amino acid substitution. This is good evidence for a physical association between the enzymes. Furthermore, mutations that eliminate one activity may alter the characteristics of the other (*20*). A number of the doubly deficient types are frame-shift or nonsense mutants. By means of reversion tests with various mutagens, as well as complementation tests, Radford established that the two enzyme activities arise by translation from a common messenger RNA, starting with the CPS-specific region (Fig. 3) (*21*).

The hypothesis that the two enzymes were physically aggregated was then tested directly (*22*). Purification of CPS-P led to the concomitant purification of ATC, the more stable enzyme of the two. Both activities were associated with a species having a molecular weight of 630,000. On sucrose gradients, uridine triphosphate (the feedback effector of CPS-P) reduced the apparent molecular weight of both to 385,000. With a purified preparation, the major protein peak also shifted when uridine triphosphate was in the gradient. By determining the amount of protein that responded in this way to uridine triphosphate, the purified preparations were estimated to be 50 percent pure. The reduction in sedimentation value induced by uridine triphosphate may signify an asymmetric dissociation of the large complex into separate CPS and ATC moieties whose sedimentation profiles are superimposable. In any case it is probable that the complex is multimeric, possibly heteromultimeric.

In the course of these investigations, Lue and Kaplan (*23*) independently established that the CPS-P and ATC of yeast were very similar to the *Neurospora* enzymes in genetic determination and in macromolecular structure. Using a purified preparation of the complex, they found that the CAP made by CPS-P was used by ATC as though it were enzyme-bound. In one experiment, "endogenous" CAP, made from radioactive bicarbonate, was used preferentially over a low concentration of added, unlabeled CAP. In another experiment, ATC had a clear kinetic advantage over added OTC for CAP produced by the pyrimidine enzyme complex.

Channeling of CAP, if it does occur, does not manifest itself at the nutritional level in yeast. Nevertheless, the results of Lue and Kaplan may help to explain the *Neurospora* system. Thus it is possible that pyrimidine-specific CAP is confined to a "molecular compartment" (*23*) provided by the CPS-ATC complex. This would explain why CAP is not used in the arginine pathway unless ATC activity is missing (as in a strain carrying *pyr-3d*; see Fig. 2). Consistent with this view is the finding that the amount of pyrimidine-specific CAP in *Neurospora* (0.4 nmole/g) is two to five times less than the calculated molar concentration of the CPS-ATC complex (1.2 to 2.0 nmole/g) (*16*).

Another, less obvious possibility suggested by the Lue and Kaplan experiments is that the CPS-ATC complex might exclude "exogenous" CAP, that is, CAP not originating in the CPS-P reaction. Only the experiments in which radioactive tracers were used to reveal preferential uses of exogenous and endogenous substrates (*5*) suggest this, but such a phenomenon may also be operating in the experiments of Gaertner *et al.* on the multienzyme complexes of the aromatic and tryptophan pathways of *Neurospora* (*24*). These workers found that intermediates of an aggregated series of enzymes failed to support reaction rates as well as the initial compound of the sequence. If low concentrations of exogenous CAP were excluded by the CPS-ATC complex, the complex could formally explain channeling of both CAP pools of *Neurospora*.

There is no evidence for or against a molecular aggregate containing both CPS-A and OTC activities. The genes determining the enzymes assort inde-

pendently, and the enzymes themselves are independently mutable and easily separable. Moreover, the CAP pool of the arginine pathway is several times greater than the pool of OTC molecules.

Cellular Localization of Enzymes

Mizutani (*25*) and Spors and Merker (*26, 27*) have devised a histochemical method by which transcarbamylases of the rat hepatocyte may be observed with the electron microscope. When the carbamyl group is transferred from CAP to aspartate or ornithine, inorganic phosphate is released. In the presence of lead ion, the phosphate precipitates in situ as an electron-dense lead salt. (This principle is used in many histochemical tests for phosphatases.) Mizutani (*25*) and Merker and Spors (*27*) found OTC in the mitochondria, as expected from previous centrifugation studies on this tissue. Spors and Merker (*26*) found ATC mainly in the endoplasmic reticulum, a localization consistent with centrifugation work (*8*).

We applied the Merker-Spors technique, with little modification, to *Neurospora* (*28*). Incubation of glutaraldehyde-fixed *Neurospora* with CAP and ornithine in the presence of lead ion yielded a reaction product only inside the mitochondria (Fig. 4a). Similar incubations with aspartate in place of ornithine yielded reaction product confined almost entirely in the nucleus (Fig. 4b). It appeared that OTC was in the mitochondria and ATC was in the nucleus. Controls, in which cells of the wild type and mutants lacking one or both transcarbamylases were incubated with CAP and both aspartate and ornithine, confirmed our observations (*28*). The conclusions were substantiated by our finding that OTC and CPS-A appeared in the mitochondrial fraction after gently disrupting the cells and subjecting them to differential centrifugation (*16, 29*). Localization of ATC (and, by inference, CPS-P) to nuclei by differential centrifugation is difficult for technical reasons, so the histochemical evidence cannot be taken as conclusive. It might be noted here that the last two arginine biosynthetic enzymes are in the soluble fraction of the cytoplasm (*29*), and one of the pyrimidine enzymes (dihydro-orotate dehydrogenase) is in the mitochondria (*30*). Thus the two organelles, nuclei and mitochondria, do not each enclose an entire pathway.

Speculations and Conclusions

The evidence obtained by electron microscopy suggests that the apparent confinement of CAP might be attributed to the mitochondrial or nuclear membranes or both. As noted previously, the localization of enzymes does not demonstrate that their substrates are localized in the same places. In the case of CAP, furthermore, the evidence for discreteness of the two pools is not wholly satisfactory. In view of this, two additional points should be made. First, there is evidence against channeling of CAP produced by CPS-A in rat hepatocytes and in yeast. In rat liver slices (*9*), substantial amounts of CAP produced in mitochondria can enter the ATC reaction. In yeast, strains lacking CPS-P have no pyrimidine requirement (*31*), indicating that in this organism, ATC normally has access to CAP produced by CPS-A. Lue and Kaplan (*32*) have noted that if ATC has physical access to CAP produced by CPS-A, the feedback inhibitor of the pyrimidine pathway should logically inhibit not only CPS-P but ATC as well. Indeed, both CPS-A and ATC of yeast are inhibited by uridine triphosphate (*32*). Conversely, if ATC has no physical access to CAP of the arginine pathway, feedback sensitivity of ATC to uridine triphosphate need not be expected. We have never found ATC of *Neurospora* sensitive to pyrimidine nucleotides or nucleosides (*12*). The observations as a whole suggest that yeast achieves by regulation what *Neurospora* accomplishes by compartmentation; the latter phenomenon is therefore not universal in CAP metabolism.

The second point concerns ornithine channeling. Endogenous ornithine is used almost exclusively by the OTC reaction; little or none is consumed by ornithine transaminase, a catabolic enzyme always present at significant con-

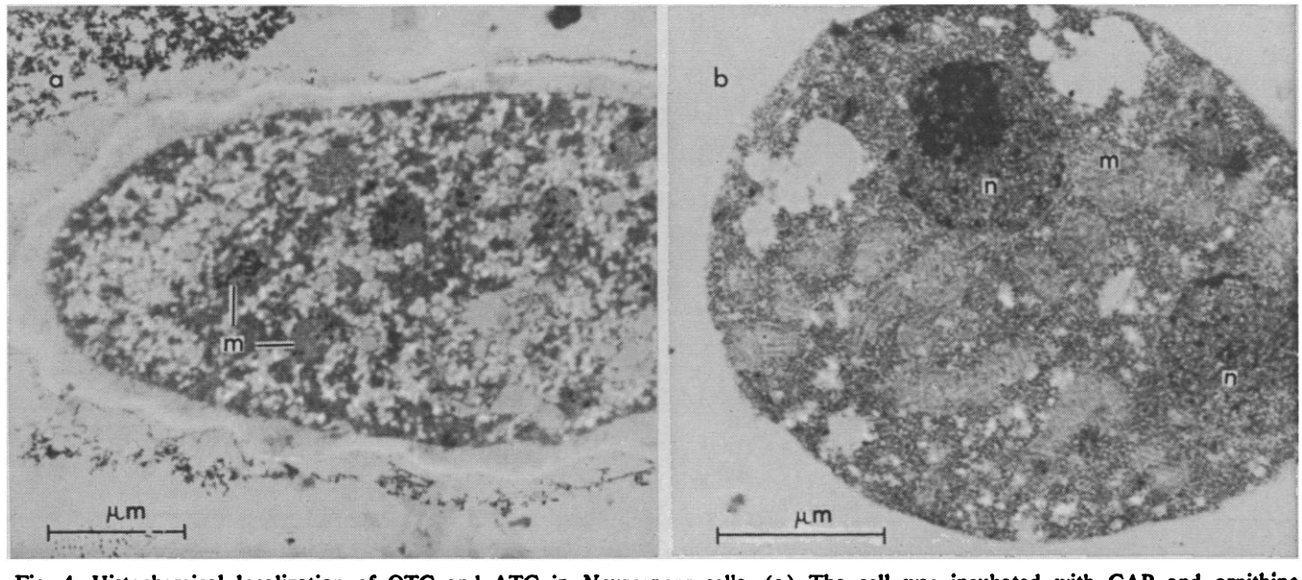

Fig. 4. Histochemical localization of OTC and ATC in *Neurospora* cells. (a) The cell was incubated with CAP and ornithine, and the lead phosphate reaction product that is indicative of OTC activity appeared in the mitochondria (*m*). No such product appeared in the nuclei (not shown) or in the cytoplasm. (b) The cell was incubated with CAP and aspartate, and the ATC reaction product appeared in the nuclei (*n*). None appeared in the mitochondria [for further details see (*28*)].

centrations. In this laboratory, Weiss has found by cell fractionation procedures that the enzyme of ornithine synthesis and OTC are localized in mitochondria. None of the ornithine transaminase is mitochondrial; it is a soluble enzyme. Most significant is the finding that a very large part (80 to 90 percent) of the endogenous ornithine pool of these cells is particulate, and most of it can be centrifuged down with the mitochondria and the vacuoles. The bulk of the large ornithine pool may be in vacuoles, rather than in mitochondria. Nevertheless, the channeling of ornithine to the anabolic reaction (OTC) could be accomplished in part by producing and using most of it in mitochondria, and in part by sequestering the remainder, in vacuoles, from the catabolic enzyme.

To explain the specificity of CAP of the pyrimidine pathway, there are three possible mechanisms, any or all of which might prevent it from entering the arginine pathway: (i) the CPS-ATC complex, explored in detail above; (ii) the mitochondrial membrane, which may be impermeable to entry of CAP; and (iii) the nuclear membrane, which despite the many nucleo-cytoplasmic transactions across it, remains intact during mitosis in *Neurospora*. With the data available it seems reasonable to attribute the confinement of pyrimidine-specific CAP in part to the CPS-ATC complex. The nuclear localization of the CPS-ATC complex may simply reflect an advantage gained by the organism in having the feedback-sensitive enzyme (CPS) placed where the end products (pyrimidines) are used most rapidly.

In conclusion, a systematic view of channeling can be offered: (i) Metabolites may be confined to enzyme aggregates as "enzyme-bound" intermediates. This mechanism may explain the ubiquity of aggregates, even among bacteria: they provide a means by which only minute amounts of intermediates per cell are needed, with aggregates acting as "conductors" (*33*). (ii) Organelles may confine metabolites. The effect is to maintain high local metabolite concentrations without the entire cell being flooded with them and, as in the case of ornithine, without their being exposed to catabolic possibilities. Another effect is to allow regulation of independent metabolic sequences having, as represented by CAP, an identical intermediate. (iii) To the two channeling mechanisms we should add intracellular gradients. Metabolite gradients are expected whenever enzymes are localized in cells. Whether they are sharp or shallow depends upon relative rates of production and consumption and upon the efficiency of channeling mechanisms. In the case of CAP, "overflow" between the pyrimidine and arginine pathways is nutritionally significant at elevated CAP concentrations (Fig. 2). A gradient of CAP is implied by this behavior, and shallower reciprocal gradients of the two pools probably prevail under normal circumstances. If the behavior of CAP is at all typical, one must conclude that channeling within cells is often quite inefficient. This probably reflects the fact that many compounds must diffuse freely within the cell. The difficulties of ensuring strict intracellular compartmentation, then, may have been one of the earliest selective pressures in the development of differentiated, multicellular organisms.

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