*Perspectives*

# Beadle's progeny: Innocence rewarded, innocence lost*

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## 1. Introduction

In the history of various sciences, we find periods of static tradition punctuated by discoveries that ignite rapid reorientations and novel lines of investigation. The dinosaurs die off and the shrews take over, beady-eyed and ignorant of the past as they spread into new, habitable niches. So it was in the early 1940s when Beadle and Tatum (1941) announced the isolation of their first metabolic mutants of the fungus *Neurospora*, followed by papers that elaborately bore out the promise of the "one gene, one enzyme" hypothesis. The promise of finding the actual role of genes in the structure of proteins and enzymes, and how mutations might be used to dissect metabolic and other complex sequences was clear. *Neurospora* workers, small in numbers, sensed that a new day had come, a day in which the undiscovered country between the molecule and the cell surface might soon be revealed. When we were young, things were simple, and for some time thereafter, we retained our innocence.

## 2. Creative innocence

Two paths lie before a new graduate student. One is to discover something new; the other is to refine doggedly what is known, usually on someone else's grant money. I was forced to do the former, inasmuch as grant funds were not the way my mentor, Paul Levine, supported his students. Moreover, my fascination with *Neurospora* would contribute nothing whatever to his studies of *Drosophila* population genetics or his later work on recombination in *Chlamydomonas*. I had discovered early on that a student without purpose could acquire one simply by relaxing and waiting for mutations to appear. Such accidents, as Pasteur had said, favour the prepared mind, even if the mind was prepared only with a need to discover something, however trivial.

I entered graduate school in 1954, a year after Watson and Crick's discovery of DNA structure (1953) condensed the gene's abstract properties into a molecular model. It took some years for me to appreciate its significance, because my interests favoured the use of microorganisms to illuminate the genetics of haploid organisms and the workings of simple cells. But work on DNA developed in parallel with microbial and biochemical genetics, the three areas probing the fundamental structures and activities of living cells. Genetics would give us the means to dismantle the cell and to teach us, like Vesalius, to ignore the ancient texts. My graduate work had begun with a mutation affecting heterokaryons in *Neurospora*. My innocent delight in demonstrating its 1:1 segregation in a cross with wild-type confirmed my faith in genetics. After all, Beadle and Tatum had done this with somewhat more interesting mutations in 1941 and published it to wide acclaim.

In 1958 I went to Caltech as a postdoctoral fellow, knowing that I must have a passing acquaintance with biochemistry, of which I was then wholly ignorant. My motives were base: I wanted to do biochemistry by mutational techniques rather than having to follow the demanding, fussy chemical approaches that characterized the field. Perhaps I too could become, as Chargaff later remarked, an unlicensed biochemist, using clumsy, random mutations to explore the china shop of metabolism.

At Caltech, I joined the seminal group that had adumbrated the one gene, one enzyme motto. Apparent counterevidence had been assimilated into the theory. For instance, a mutation affecting the isoleucine and valine pathways was found to affect a single enzyme common to both. A problem remained, however: Max Delbrück insisted that the theory

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*This article is dedicated to the memory of two mentors: Herschel K Mitchell, who gave me opportunity and independence, and Norman H Horowitz, who gave me background and insight into *Neurospora* biochemical genetics.

was not falsifiable by Beadle's and Tatum's methods–it wasn't solid science. Mutation of a gene with two roles, one indispensable to life, would never be recovered. The Beadle group did not much care; they went on with their work. But in 1951, Norman Horowitz and Urs Leupold (1951) isolated many mutants unable to grow at a high temperature. Among these temperature-sensitive mutants, many could grow at high temperature on single amino acids or vitamins; they were, in short, monofunctional. Analysis of the spectrum of normal and temperature-sensitive auxotrophs indicated no fundamental difference; multifunctional genes were, at best, rare. In this indirect way, the one gene, one enzyme theory survived Delbrück's challenge. But already a great many problematic mutants had accumulated, ignored in favour of the easy ones.

Soon the theory began to dissolve. The first chink came as Vernon Ingram (1957) showed that genes specified individual polypeptides of multimeric proteins: from then on it was "one gene, one polypeptide." By 1958 – indeed, even by 1948 – one gene, one enzyme was no longer a hypothesis to be resolutely defended; it was simply the name of a research program. The program had been so productive that no one seemed to care whether the motto was affirmed, modified, or abandoned. After all, what are hypotheses for if not to provoke penetrating experiments?

## 3. The pyrimidine mutant quandary

At Caltech, I came under the wing of Herschel Mitchell, a good-natured, taciturn professor in Beadle's group. "So, you want to learn some biochemistry?" he said. "Here." He handed me culture tubes of wild-type and a small collection of pyrimidine-requiring mutants. "This one, *pyr-3*, is funny. I think it's blocked in forming orotidine." What, I asked myself, was orotidine? Naturally, I disguised my ignorance with a sage nod, took the cultures, and went away. So it's think or thwim, I said to myself, and his kind, silent look confirmed it.

I struggled for a year to learn how to isolate biochemical intermediates, synthesize several unstable compounds for use in enzyme assays, and of course assay enzymes. I had discovered nothing of interest. Herschel supported my efforts, always taciturn, always usefully skeptical of impulsive hypotheses. He encouraged my independence even further by taking a sabbatical in Switzerland for the whole of my second and final year. By then I had shown that with one exception, all known enzymes of the pyrimidine pathway could be identified with one or another of the *pyr* mutants. In fact, my first real finding was that one of the *pyr-3* mutations, *pyr-3d*, caused a complete deficiency for the enzyme aspartate transcarbamylase (ATCase), the earliest enzyme dedicated specifically to pyrimidine synthesis.

ATCase had not been tested before, because one substrate of the enzyme, carbamyl phosphate (CAP), had been discovered only in 1953, after the Mitchell laboratory had discontinued work on the *pyr* mutants. (ATCase joins CAP and aspartic acid to make carbamyl aspartate, with the elimination of inorganic phosphate.) Moreover, CAP, a very unstable compound, was not commercially available. Fortunately, it was easily synthesized in quantity, giving me the opportunity to identify the enzymatic deficiency of *pyr-3d*.

Many more *pyr-3* mutations had been collected over the years by the Mitchells (Herschel and his then wife, Mary [Houlahan] Mitchell), by Val Woodward at Kansas State University, and by José Reissig, working in Paris in 1960, soon to become professor of genetics at the University of Buenos Aires. Many of these mutants, unlike *pyr-3d,* had normal or increased activity for ATCase, and the properties of the enzyme activity were normal. This category of *pyr-3* mutation was exemplified by the allele *pyr-3a.* Even more interesting, as Woodward's laboratory found, the two types of *pyr-3* mutation were interspersed on the fine-structure genetic map of the gene.

If two mutations of a single gene differed such that one completely lacked an enzyme activity (ATCase) for which the other was completely normal, what was to be made of the one gene, one enzyme hypothesis? There must be another important role of the gene, mutations of which caused a pyrimidine requirement. Indeed, mutants such as *pyr*-3a, having ATCase, were known to complement in heterokaryons with some, such as *pyr*-3d lacking it, further supporting the fact that *pyr*-3a and *pyr*-3d affected different functions. At the time, my data were was news of some interest, and Beadle smiled as he encouraged me to publish on the "dual function" of the *pyr-3* gene.

To follow this up, I was obliged to discover the actual biochemical deficiency of the *pyr-3a*-type mutations. A possible explanation was that the *pyr-3a*-type mutants lacked the ability to make CAP or aspartic acid, the substrates for ATCase. Very unlikely: aspartic acid is used in multiple metabolic reactions and is a protein amino acid. CAP is required in the synthesis of arginine in the ornithine transcarbamylase (OTCase) reaction, which converts ornithine to citrulline. Mutants unable to make CAP would require both arginine and pyrimidines. In fact, well-known bacterial mutants lacking the ability to make CAP had exactly that phenotype. But the unforgiving fact was that *pyr-3a*-type mutants had no need for arginine.

## 4. The role of a suppressor gene

Historians learn that life is too complicated to report faithfully. Selective memory, even collective memory, erases details. The historian must eliminate much more if she is to present a readable account. The result is a myth awaiting

reincarnation in competing, revisionist accounts, the gossip of future generations. What follows, then, may be more flavor than substance, but faithful at least to the literature.

The account so far presents two kinds of *pyr-3* mutations lying at one locus, one having no ATCase, the other having normal ATCase. More had been learned about both before I encountered them. The Mitchells had studied interactions of each *pyr-3* mutant with a number of mutations affecting arginine, lysine, and proline synthesis. The more the facts accumulated, the more confusing the picture had become, an understandable outcome in the late 1940s and 1950s, when so little was yet known of metabolism. In fact, it was a startling example of the principle that facts do not speak for themselves, especially if there are too many of them. I brought my ignorance of biochemistry to bear on the problem, setting aside information that I could not comprehend and, as it turned out, that no one else could interpret.

The arginine pathway includes the intermediate ornithine, which is converted to citrulline, which in turn is converted to arginine. Much earlier, Srb and Horowitz (1944) had presented the pathway as a clear example of the one gene, one enzyme principle, locating metabolic blocks in this sequence, steps presided over by various genes. Several different mutants (*arg-4, arg-5, arg-6*) seemed to be blocked in steps before ornithine, since they could grow on ornithine or citrulline in place of arginine. Two other mutants, *arg-2* and *arg-3*, carrying unlinked mutations, were blocked between ornithine and citrulline, and grew only on citrulline or arginine. Still other mutants could grow only on arginine, blocked in the last steps of the pathway.

As noted above, work on other organisms showed that a transcarbamylase reaction (OTCase) converted CAP and ornithine to citrulline. Testing for this reaction, I discovered that both *arg-2* and *arg-3* displayed abundant activity for this reaction. Why, then, could they not use ornithine to satisfy their arginine requirement? Indeed, it was the same problem presented by the *pyr-3a* mutant, whose ATCase activity should have assured its ability to grow without pyrimidines.

Mary Mitchell had discovered a mutation in 1948 with a curious phenotype (Mitchell and Mitchell 1952). It had arisen at a new genetic locus in a *pyr-3a* mutant strain, allowing the double mutant to grow without pyrimidine supplementation as a result. The mutation was initially called *s,* for "suppressor" (of the pyrimidine requirement of *pyr-3a* mutations). When separated by recombination from the *pyr-3a* mutation, the single mutant carrying only the *s* mutation grew just as well as wild-type *Neurospora* on unsupplemented medium. In fact, it displayed no aberrant phenotype at all.

The *pyr-3a, s* double mutant also grew as well as wild-type on unsupplemented medium. However, it revealed a most peculiar trait: it acquired a pyrimidine requirement when grown in the presence of arginine. Even to those living in 1960, this attribute suggested that pyrimidine synthesis in the double mutant depended on a negatively controlled reaction of the arginine pathway. It might seem logical now that I should test the *s* strain for OTCase activity. I did, but I was sufficiently confused at the time that the decision to do so was capricious. The colorimetric enzymic assay racks of the *arg-2* and *arg-3* mutants had room for one more strain. At the end of the reaction, the *s* mutant strain displayed vividly a profound, but incomplete, deficiency (97%) for this enzyme. In the few seconds it took to visualize the qualitative result, the picture developed below began to take shape rapidly. The OTCase deficiency of the *s* strain clearly did not impose an arginine requirement. It was "on the edge of auxotrophy," as we would later say, once we had isolated allelic, completely OTC-less mutants that were entirely dependent upon arginine supplementation. These defined the structural gene, *arg-12*, for OTCase, and the *s* mutation was renamed *arg-12*$^{s}$.

One more fact: the Mitchells' past work had uncovered another unusual phenomenon. The *pyr-3d* mutant, lacking ATCase, when combined with the *arg-2* mutation, had no arginine requirement. The same was true of the *pyr-3d, arg-3* double mutant. These findings were later confirmed during a long, informal collaboration with José Reissig.

To summarize, an OTCase deficiency overcame a pyrimidine requirement of *pyr-3a*, and an ATCase deficiency overcame the arginine requirements of *arg-2* and *arg-3*.

## 5. The model

By the time we knew this, I had taken a job in the Department of Botany at the University of Michigan. The university's excellent Department of Biochemistry in the medical school was nevertheless conservative beyond measure, largely unwilling to grant credence to mutational analyses of metabolism. How was I, still relatively ignorant of biochemistry, to talk believably to a biochemist about these matters? I concluded that I must make a model consistent with the facts and do some real biochemistry. Unfortunately, the model was a towering work of imagination, laughable to geneticists and biochemists alike. It survived only because I was not a member of the Department of Biochemistry or of a genetics department, and was thereby free to pursue metabolic problems as a geneticist in a botany department. Most of my taxonomist colleagues in the latter were uncomprehending, but in awe of both biochemistry and genetics. This brings me to the model, one which would easily have derailed Beadle and Tatum had they believed it could be true in 1941. It came to me in an hour of free association as I tried to explain my results in a letter to José.

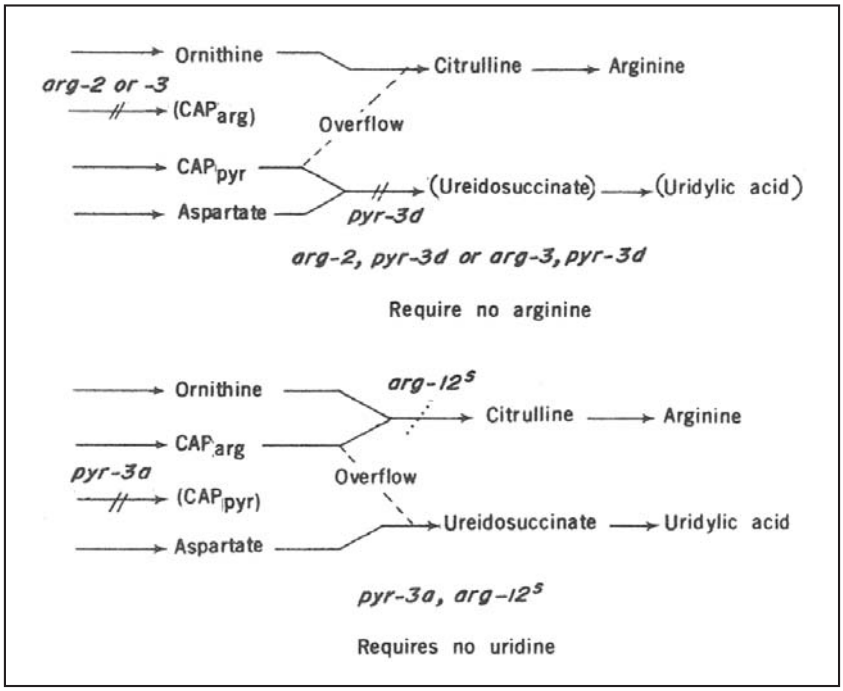


**Figure 1.** CAP overflow between pathways in two types of double mutant of *Neurospora.* Mutations *pyr-3d* (top) and *arg-12*$^{s}$ (bottom) block CAP utilization in one pathway and thereby relieve nutritional requirements imposed by CPS mutants in the other. Parenthesized compounds are not synthesized. CAP: carbamyl phosphate (arginine- or pyrimidine-specific) (From Davis 1972; a correction to an editorial error in the figure has been incorporated here.)

- Two carbamyl phosphate synthetases (CPS), one specifically devoted to pyrimidine synthesis (CPS-P), the other devoted to arginine synthesis (CPS-A), coexist in *Neurospora*. The *pyr-3a* mutation eliminates CPS-P. The *arg-2* and *arg-3* mutations eliminate CPS-A (figure 1).
- The product (CAP) of each CPS is normally destined exclusively to its own pathway.
- The OTCase deficiency of *arg-12*$^{s}$ leads to an accumulation of CAP produced by CPS-A and diverts much of it to the pyrimidine pathway. This permits the *pyr-3a, arg-12*$^{s}$ double mutant to grow without pyrimidine supplementation.
- Similarly, CAP produced by CPS-P accumulates in the *pyr-3d* mutant, because of the mutant's deficient ATCase, the normal consumer of CAP. The CAP is thus diverted to OTCase, overcoming the CAP deficiency of *arg-2* and *arg-3* mutants. Thus *arg-2, pyr-3d* and *arg-3, pyr-3d* double mutants do not have an arginine requirement. (They do retain their need for pyrimidine supplementation.)
- The allelism of *pyr-3d* (lacking ATCase) and *pyr-3a* mutations (lacking CPS-P in the model) suggests that ATCase and CPS-P are enzymatic activities of the same protein. This might allow the intermediate, CAP, to remain enzyme-bound under normal circumstances, and thus not free to diffuse to OTCase of the arginine pathway.

## 6. Implications of the model

The model (Davis 1962; Davis and Woodward 1962), developed in 1961, could not be described to anyone but friends. Traditional biochemists would choke on most of its elements, given the lack of any direct (meaning biochemical) evidence. The model had these unusual implications:

- *One gene, two enzymes.* The *pyr-3* gene appeared to encode a polypeptide with two quite different enzyme activities. While heteromultimeric proteins were known, few examples of multifunctional polypeptides had been demonstrated, largely because the enzymatic domains of multifunctional polypeptides are easily separated by proteolysis during extraction, a point appreciated fully only in the 1970s (Gaertner and Cole 1976; Stark 1977).
- *Three genes, one enzyme activity.* Granting that *arg-2* and *arg-3* could specify polypeptides of a single enzyme, as in the case of the α- and β-chains of hemoglobin, there was nevertheless a third gene, *pyr-3*, that might be involved with CAP synthesis.

Biochemists might say that one CPS enzyme protein could have multiple locations, or several enzymes might yield chemically different carbamylating substrates for the use of OTCase and ATCase. A great deal of speculation on this very point had preceded the discovery of CAP.

- *Compartmental confinement of CAP*. The bag-of-enzymes view of the cell still prevailed in many biochemical quarters, even in view of recent work on mitochondria. Many biochemists' recent views arose from a background in physical chemistry, where simplified visions of the cell omitted internal membranes (on the blackboard, cells were roughly drawn circles, leaving space for names of metabolic intermediates, reverse arrows, $k_1$ and $k_{-1}$). No barriers to diffusion of small molecules were included. This was especially true in the minds of biochemists working with bacteria, flecks of protoplasm without obvious internal structure. Even geneticists could only speculate about the significance of intracellular structures of eukaryotic cells, as yet beyond the reach of mutational rationales.

## 7. The outcome

For one committed to a simple view of life, I found myself in an exposed position, able to defend my evidence but not my model. Gradually, direct evidence began to emerge from experiments as I forced myself to learn more biochemistry. In the process, I was retrospectively embarrassed by what I had so naively proposed, but there was no turning back. Soon I was rewarded with support from biochemists themselves.

The well-known CPS of mammals, associated with the urea cycle, uses ammonia as a nitrogen donor for CAP synthesis. This is how mammals, as ureoteles, detoxify ammonia. The reaction seemed to be universal, since the CPS of bacteria, as known at the time, used the same substrates. The CPS of other organisms showed that non-ureotelic CPS enzymes could, like those of mammals, use ammonia as a nitrogen donor. But Bruce Levenberg, a friend in the Michigan Biochemistry Department, discovered a CPS in the fungus *Agaricus bisporus* that, *in vitro*, could use glutamine as an alternate nitrogen donor (Levenberg 1962). We quickly found this to be true of the CPS-A of *Neurospora.* Ultimately, we found that the *arg-2* gene encoded a glutaminase polypeptide, and the *arg-3* gene encoded a larger polypeptide carrying the remaining binding sites and core catalytic activity for CAP synthesis, the two combined in an unstable, heterodimeric protein. Both the *arg-3* product and the heterodimer could use ammonia as a substrate *in vitro*.

Mary Ellen Jones, the discoverer of CAP itself, found with some difficulty that mammals possessed a glutamine-dependent CPS-P enzyme, distinct from the known CPS required in the urea cycle (Hager and Jones 1967). After difficulties of our own, we and José's laboratory also detected a second glutamine-dependent CPS (Davis 1967; Reissig *et al* 1967), which we later identified as the long-sought CPS-P of *Neurospora*. It was biochemically and physically distinct from CPS-A, and was indeed lacking in *pyr-3a* mutants (Williams and Davis 1970). The model was becoming more believable: two CPS enzymes, one the product of two genes.

In 1970, Larry Williams, a postdoctoral fellow in the laboratory, demonstrated that ATCase and CPS-P activities co-purified, suggesting they were properties of a single protein (Williams *et al* 1970). The evidence included defining three types of mutants at the *pyr-3* locus: those lacking ATCase, those lacking CPS-P, and those lacking both, many of each due to revertible mutations. Indeed, those lacking only one enzyme activity tended to lay at one or the other end of the fine-structure genetic map, a point clearly shown by Alan Radford, then at Stanford University, using inventive genetic techniques (Radford 1970). That the enzyme was constituted of a multifunctional polypeptide lay wholly in genetic, rather than biochemical evidence. In the meantime, biochemists had increasingly defined multifunctional polypeptides on biochemical criteria, an early example being the two aspartokinase–homoserine dehydrogenases of *E. coli* K12 (Truffa-Bachi *et al* 1968).

Our culminating finding was that the two trans-carbamylases, ATCase and OTCase, were located in different cellular compartments (Bernhardt and Davis 1972)*. Electron microscope histochemical techniques revealed that ATCase (and, perforce, CPS-P) lay in the nucleolus (where ribosomal RNA is made). OTCase, on the other hand, lay in mitochondria. Richard Weiss, a later postdoctoral fellow in the laboratory (figure 3), confirmed this with cell fractionation studies, and showed, in addition, that CPS-A and an earlier enzyme of the arginine pathway were also in mitochondria (Weiss and Davis 1973). The same studies showed that the two enzymes that converted citrulline to arginine lay in the cytosol. The compartmental hypothesis – that two sites of CAP production and use were both membrane-bound – could

*Recent evidence (Benoist *et al* 2000) in yeast demonstrates that in contrast with earlier studies, the multifunctional CPS-P-ATCase protein accumulates in the cytoplasm, not in the nucleus. The findings, based on immunological detection of the protein and fluorescent signals from a green fluorescent protein (GFP) derivative thereof. The nuclear signal (lead phosphate precipitate) based on the ATCase reaction itself, found in previous studies, is probably artifactual. This finding, if confirmed in *Neurospora*, actually simplifies, rather than undercuts, the physiological model of CAP compartments we proposed, since the mitochondrial membrane is sufficient to segregate CPS-P and CPS-A and their products in wild-type cells (see main text).

not have received better support. Biochemical experiments confirmed that there were indeed two discrete pools of CAP in the cell (Williams *et al* 1971; reviewed in Davis 1972). Later still, we showed that the arginine-specific CAP pool, as well as CPS-A, was confined to mitochondria (Davis and Ristow 1987). Others showed, in yeast, that the pyrimidine-specific CAP pool appeared indeed to be enzyme-bound, whatever the role of the nuclear membrane (Lue and Kaplan 1970).

What would Beadle have thought about all this? He was an open-minded man, affable and energetic. He expected the most of his group, which he inspired to explore the unknown terrain of genetics and metabolism. He was untroubled by Delbrück's skepticism, preferring to carry the flag for the idea that genes had fundamental roles in the cell, that enzymes could be targeted by mutation, that biochemical sequences and, by extension, any biological sequence could be dissected by genetic techniques. Unlike those pursuing a specific hypothesis, such as the form of energy leading to mitochondrial ATP synthesis, or the structure of the gene, Beadle had refined a rationale of investigation, welcoming new findings. He famously said, "Hypotheses have to make sense, but facts don't." His flag carried a slogan: "one gene, one enzyme." But he smiled as it became tattered in the ecstasy of investigation that he had unleashed.

Would he still smile today? Even the operational definition of a gene has disappeared as we learn of introns, nested genes, alternative starts and alternative splicing, overlapping genes, fused transcripts, a variety of cis-regulatory elements, chromatin modifications and non-coding, regulatory RNAs. He would smile, I am sure, knowing that a productive hypothesis is simple at the outset, and must ultimately succumb under the weight of its progeny. And the greater that weight, the prouder the grandfather can be.

## 8. A brief look into our bag of enzymes

Finding two discrete pools of CAP emphasized a question that had plagued biochemists and cytologist for decades. First, are many small molecules compartmentalized in eukaryotic cells? It would seem obvious that intermediates of the Krebs cycle must be confined to the mitochondrion. Similarly, botanists had shown that the plant vacuole contains a different array of small molecules from the cytoplasm. Such observations and inferences based on them made it clear that the eukaryotic cell was not a bag of enzymes. Many biologists and biochemists stressed this at the beginning of their seminars to assure their audiences of the speaker's sophistication.

The statement was too easy to understand to evoke any objection. Of course enzymes might be localized; they might even be organized and aggregated in the cytoplasm. As long as the focus was on enzymes and proteins, all those membranes in the early electron micrographs made the idea of their intracellular localization reasonable. But small molecules were, well, *small*. In a small cell, diffusion would easily get small molecules around the membranes to the active sites of enzymes, most of which were cytoplasmic. So the idea of localizing small molecules (*pace* the mitochondrion) seemed a more difficult proposition than localizing enzymes. And there you have it again: the cytoplasm was a bag of enzymes!

Scientists understandably ignore complications beyond the reach of experimental design. We call this "simplification." As noted above, the willful disregard of metabolic compartmentation arose from the study of metabolism in *E. coli*, an organism without "much" internal structure. This work gave confidence that the rest of the living world was irrelevant in learning the "basic" principles of metabolism. Even mitochondria could be ignored once their bacterial origins were accepted – a special case. And so mathematical formulations of metabolic sequences, with their vast array of summation signs and partial differential equations involving $V_{max}$s, $K_m$s, [S1]…[Sn]s, $A_0^{kt}$s and [I]s accumulated, with enormous benefit to understanding the properties and kinetics of simplified systems. Much of this applied well to glycolytic sequences and to bacterial biosynthetic pathways with feedback inhibition and repression. But some investigators strayed into the eukaryotic cell, blind to the barriers to diffusion that might render the entire kinetic approach unworkable from the start.

Cell fractionation studies designed to prove compartmental distribution of small molecules had either failed to show it or, if suggestively positive, had drawn the criticism that small molecules could redistribute themselves during cell breakage. Therefore, demonstration of metabolic compartmentation in living cells was essential. The advent of radioactive amino acids after World War II had generated substantial provocative, though contradictory, evidence of different intracellular "pools" of metabolites, of different specific radioactivities in eukaryotic microorganisms (Subramanian *et al* 1973). The evidence was not at all satisfactory to discerning biochemists. Moreover, the "compartments" involved were kinetic (and very mathematical) abstractions, designated as "metabolic pools," "endogenous pools," or "biosynthetic pools," few or none identified with a visible structure. And so the question of compartmental attributes of metabolism lingered, fading with the growing knowledge of metabolic and regulatory information from the primitive *E. coli*, already more of a reagent than an organism.

Our laboratory was faced with some new, stubborn facts. We had shown that arginase, which breaks down arginine to urea and ornithine, was located in the cytosol. Mutants lacking arginase showed that the enzyme was dispensable to normal growth in minimal medium. In wild-type cells, arginine, the substrate, was present at the apparent concentration of

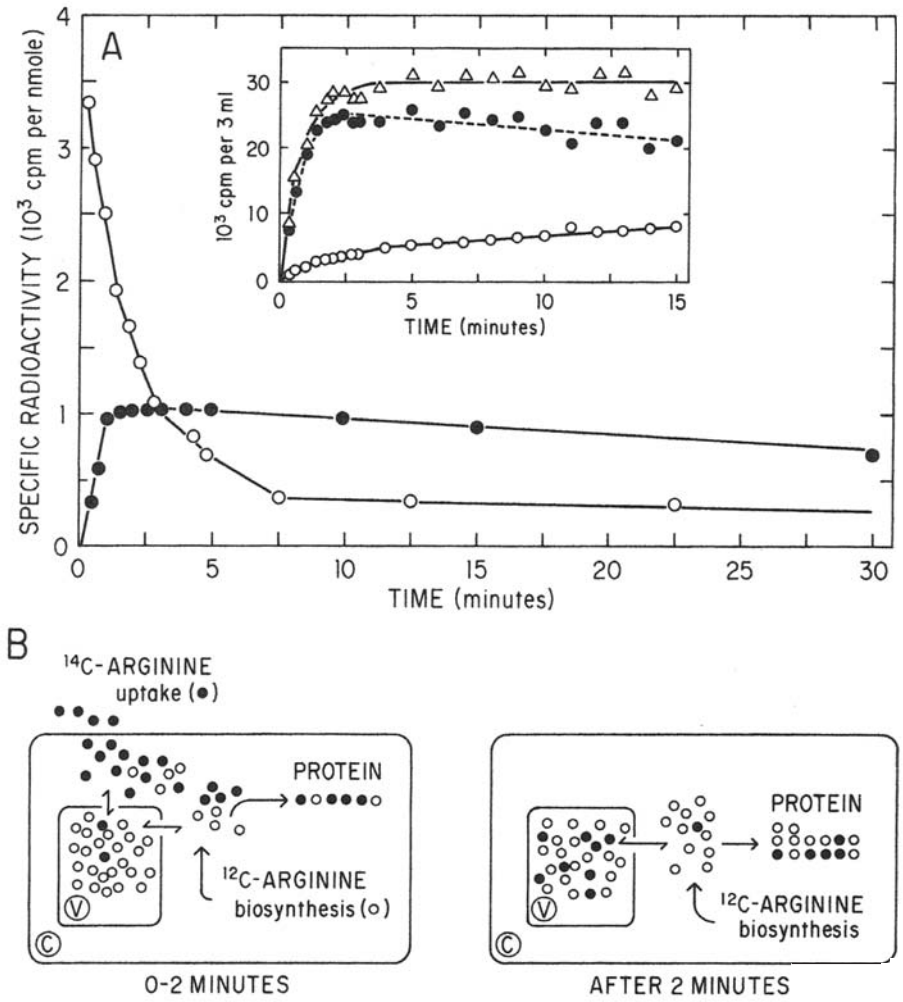


**Figure 2.** Fate of a small pulse of [$^{14}$C]arginine added at time zero to *N. crassa* growing in minimal medium. (**A**) (Main axes) Specific radioactivity of the arginine being used as a protein precursor (open circles) and of the total, trichloroacetic acid-soluble arginine (closed circles) of the same cells. Inset; Raw data from the same experiment, showing radioactivity entering the cell (triangles), protein arginine (open circles), and extractable, soluble arginine (closed circles). (**B**) interpretation of the specific radioactivity data of (**A**). Early in the experiment, [$^{14}$C]arginine (closed circles) is used selectively for protein synthesis over [$^{12}$C]arginine (open circles) confined in the vacuole; the radioactive arginine mixes only with the very small, endogenous, cytosolic pool. At later times, the remaining radioactive arginine is confined to the vacuole and is somewhat excluded as a protein precursor. (From Davis 1986)

20 mM in cell water during exponential growth. That was ridiculous enough; what engaged our interest was that not a trace of it was catabolized during normal growth. (This was shown with a urease-less mutant that allowed us to monitor urea accumulation in the cells and culture medium.)

Dick Weiss had used gentle cell disruption methods to localize OTCase to mitochondria, using enzymes that digested the tough cell wall in the presence of a buffer of high osmotic strength. He felt that they might be gentle enough to isolate other organelles. In deft experiments, he found that centrifugation of a cell-free homogenate of *Neurospora* yielded a pellet that contained not only over 70% of OTCase, but 50% of the cell's soluble arginine as well (Weiss 1973). Controls and corrections for breakage of organelles finally showed that over 95% of the arginine in the cell must be associated with a cell particulate. The same was true of the equally large ornithine pool.

We were tempted to think that the two amino acid pools lay in the mitochondria. But differential centrifugation in sucrose gradients easily separated these amino acids from mitochondrial markers, such as succinate dehydrogenase and OTCase. Isolation (and eventual purification to homogeneity) of the organelles and assay for marker enzymes showed that they were the *Neurospora* vacuole, invisible or unrecognizable in exponentially growing cells owing to their tiny size. Simultaneous isolation and characterization

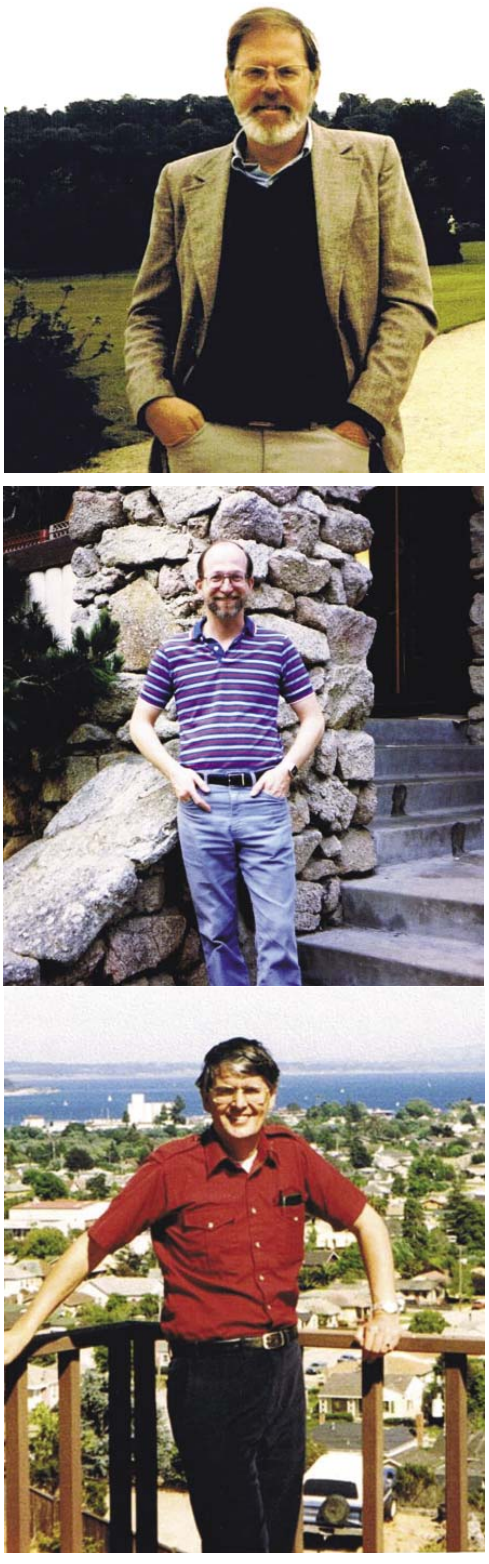

**Figure 3.** Some of the players (photos in the mid-1980s): The author (top); Richard Weiss (middle); Barry Bowman (bottom).

by Wiemken on the prominent yeast vacuole confirmed our work (Wiemken and Nurse 1973).

Was all this an artifact of cell fractionation? Dick had taken care to show that radioactive arginine, added to cells upon cell breakage, did not distribute to the particulates upon centrifugation; it remained wholly soluble. Nevertheless, we had to demonstrate compartmentation of arginine in living cells. Dick and another postdoctoral fellow, K N Subramanian, found that after adding a chemically insignificant (tracer levels) amount of highly labelled [$^{14}$C]arginine to a growing culture, it was rapidly taken up by the cells. By fractionating culture samples placed in trichloroacetic acid every 10 seconds, they showed that most of the radioactivity went from the medium, into the cells, and into protein synthesis, largely bypassing (that is, without dilution by) the huge endogenous arginine pool (Subramanian *et al* 1973). All of this happened in the first minute. The success of the experiments lay in the short time intervals of sampling. Later samples showed that the [$^{14}$C]arginine not used for protein synthesis was sequestered with the vacuolar pool. After that time, arginine arising from biosynthesis, the preferred source of the amino acid for protein synthesis, had flushed the cytoplasm of radioactivity (figure 2). This story was fleshed out by graduate students Joan Karlin and Barry Bowman (figure 3), who determined the biosynthetic, catabolic, and transmembrane fluxes (mitochondrial and vacuolar) of ornithine and arginine in the living cell (Karlin *et al* 1976; reviewed in Davis 1986; 2000).

It was not hard to see that the vacuolar membrane protected arginine from cytosolic arginase, and that cytosolic arginine remained very low in concentration (now estimated at micromolar concentrations) – high enough for arginyl-tRNA synthetase, but not for arginase, which has quite a low affinity for its substrate. Ornithine displays precisely the same behavior: sequestered in the vacuole from its catabolic enzyme, ornithine aminotransferase, it cannot be catabolized. Interestingly, an actual site of feedback inhibition is the mitochondrial membrane: ornithine, the cytosolic product of arginase, cannot pass through the mitochondrial membrane to re-enter the biosynthetic pathway when the concentration of cytosolic arginine is high. Instead, it is appropriately confined to a catabolic fate (ornithine aminotransferase) in the cytoplasm under these conditions.

The experiments of 1971–75 initiated a comprehensive study of arginine, proline and polyamine metabolism that continues with molecular techniques to this day. But the early findings focused us and others on metabolic systems regulated as much by compartmental distribution of enzymes and substrates as by regulatory phenomena such as feedback inhibition and repression.

How did the biochemical community react to these (and similar findings) in the meantime?

"We always knew the cell wasn't a bag of enzymes."

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