

The Penicillin Method of Mutant Selection

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Pasteur's famous aphorism, 'in the fields of observation chance favors only the prepared mind', was emblazoned on the ceiling of the entry to the dormitory at my medical school, Harvard. The most obvious meaning of 'prepared' is having a reservoir of relevant information that permits the observer to recognize the significance of an unexpected finding. But the history that I am about to relate illustrates another aspect of preparation: a persistent preoccupation or desire that rolls around in the recesses of the mind and leads the possessor to create an opportunity from a chance event.

My preoccupation was with the new field of biochemical genetics, and it arose in the following way. In medical school I undertook research in protein chemistry with Edwin J. Cohn. Shortly afterwards, when I entered the US Public Health Service in World War II, this background led to my being assigned to the laboratory of Elvin Kabat, to seek an immunochemical explanation for false positive serological tests for syphilis. We did not succeed in finding any difference between the antibodies in syphilitics and the molecules in some non-syphilitics that similarly reacted with the non-specific antigen of the Wassermann reaction. But after the war, this immunological experience led to the opportunity to set up an independent laboratory for a newly formed Tuberculosis Control Division in the USPHS.

Since I had never done any research with bacteria I sought some background by spending a year in the laboratory of Rene Dubos. Within a few months I had become tuberculin-positive and had developed a spontaneous pneumothorax, which was treated with 18 months of rest, in 1945 and 1946. (I have described elsewhere the incredibly macho and casual attitude of investigators at that time toward working with virulent organisms⁽¹⁾.)

During this period, which was a sort of sabbatical just before setting up an independent laboratory, I was captivated by a review written by George Beadle on 'biochemical' mutants of *Neurospora*, which were blocked in various biosynthetic reactions. These mutants had proved to be immensely valuable in two ways. First, they established the one-gene-one-enzyme relationship (though the exceptions generated far too much scepticism, until the relationship was later recognized more precisely as one-gene-one-polypeptide). Second, these mutants were powerful tools for the dissection of biosynthetic pathways, since each accumulated

copious quantities of the substrate of the blocked enzyme – a substance present only in undetectable quantities in cells with a normally functioning pathway.

I developed the conviction that work on such universal problems of biology would be much more satisfying than work on distant twigs on the evolutionary tree, such as improving growth media for the tubercle bacillus, or trying to distinguish true from false positive serologic tests for syphilis. Edward Tatum had isolated a few mutants in *E. coli* similar to those of *Neurospora*; bacteria would clearly be more convenient than *Neurospora* as tools in biochemical investigations, and bacterial genetics was just opening up, with the discovery of gene transfer by transformation, transduction or conjugation. But how to enter this field of biochemical genetics, as it was then called? I knew no genetics. Moreover, it hardly seemed practicable to initiate genetic studies with the tubercle bacillus, which required three weeks to grow out and did not yield nice colonies, each derived from a single cell.

Nevertheless, the conviction created the opportunity, when I set up a Tuberculosis Research Laboratory, directly funded by the USPHS, in space provided by Cornell University Medical College. My personal experience with the tubercle bacillus probably discouraged me, unconsciously, from inviting tuberculin-negative people, who lacked immunity, to work with the virulent organism. In any case, I decided to work on the mechanism of action of the first anti-tuberculous agent, streptomycin, using *E. coli*.

The choice of topic was not a timely one. We now know that streptomycin acts primarily on the ribosome, and secondarily, via the resulting misread proteins, on membrane permeability. At that time, biochemistry had not yet provided a foundation in either of these areas. It was not until 40 years later that I could fit together various pieces to produce a coherent, multi-step explanation for the bactericidal action of streptomycin⁽²⁾.

When I started, however, it was known that streptomycin irreversibly inhibits protein synthesis, and the kinetics suggested that this was what killed the cells. To pursue this action further it seemed possible that streptomycin might specifically interact with the incorporation of certain amino acids. I therefore isolated several amino acid auxotrophs (as I subsequently named this class of mutants) from a cell suspension mutagenized by ultraviolet irradiation. For the isolation, I developed (as did Joshua Lederberg independently) a screening method in which limited enrichment of minimal medium would allow the occasional auxotroph on a plate to yield a small colony, while the predominant wild type, not requiring any enrichment, would yield large colonies. But even though depriving an auxotroph of its required amino acid slowed its killing by streptomycin, different amino acid deprivals did not yield sufficiently different kinetics to provide a promising lead.

Meanwhile, I attended a seminar at which one of the early workers on bacterial mutants, Robert Guthrie, mentioned that it would be of great value if one could find a way not merely to *screen* for auxotrophic mutants, on the basis of the appearance of their colonies, but to *select* rare mutants from a large population, as one can do with drug or phage resistance.

Having just written a chapter on chemotherapy for a textbook of microbiology edited by Dubos, I was very much aware that the lethal and lytic action of penicillin requires growth of the bacterial cells. It instantly occurred to me, during the seminar, that in minimal medium, in which the wild type could grow but mutants could not, penicillin should permit selective survival of the latter, and they could then be recovered by removing or diluting the penicillin and providing the required growth factors.

Back in the laboratory I immediately set up a reconstruction experiment, exposing to penicillin in minimal medium a mixture of wild-type cells and a few cells of an auxotroph. Overnight incubation yielded excellent selection of the auxotrophs. But when I applied the same treatment to an irradiated suspension of wild-type cells no mutants were recovered. And despite manipulation of many variables, the results remained consistently negative.

This was very disappointing. In casting about for an explanation, I realized that the reconstruction experiments had been done with a mixture in which all the cells were alive, while in the procedure seeking new mutants the cells killed by the UV irradiation were the predominant population. The metabolites released by their lysis might feed the mutants, which would then grow and be killed, along with the wild type.

To circumvent this interference, I added to the procedure a step of intermediate cultivation, in which the irradiated cells were incubated overnight in rich medium, allowing the survivors (both wild type and mutant) to go through enough generations to greatly dilute out the dead cells in the inoculum. One would then free the cells of the enrichment medium and expose them to penicillin in minimal medium.

Just at that time I was attending a meeting at Cold Spring Harbor for a few days, so I gave instructions for this experiment to a technician. She phoned to let me know that the experiment was phenomenally successful: the plates of the survivors after exposure to penicillin were crowded with mutants. I recall vividly that I instantly developed a migraine headache, which I rarely experience – I knew I had a really significant discovery, and my excitement overloaded some circulatory or other cerebral function.

Only after some weeks did I realize that I had done the right experiment for the wrong reason. The real reason involved a novel relationship between genotype and phenotype, of a kind that had not been seen in classical genetics. In the early experiments I had exposed the cells to penicillin immediately after mutagenic radiation, so a cell with a mutation that inactivated the gene for a particular biosynthetic enzyme would still be the wild type in its enzymatic composition and hence in its growth requirements. During the intermediate cultivation, however, the wild-type enzyme molecules would be diluted out in the multiplying cells, because they were no longer being replenished; hence the mutation would become phenotypically expressed.

I later suggested the terms *phenome*, and *phenomic lag*, to refer to the parts of the cell that lag in expression of the consequences of a mutation – i.e. all of the cell except its genome. But the phenomic lag turned out to be an incomplete explanation: it is part of a broader *phenotypic lag*, which has

other components. For while bacteria are haploid, and have only one kind of chromosome, rapidly growing cells have several copies. Hence after irradiation has induced a particular mutation there is a *segregation lag* before the affected chromosome is separated from companion chromosomes, allowing the enzymatic deficiency then to be phenotypically expressed after the phenomic lag. (Still further complications involve the kinetics of repair of mutations.)

Within a few weeks I had more mutants than the Neurospora group had accumulated in years. But I was in no hurry to publish. I wanted to be sure that the penicillin method was applicable to all metabolites, including trace growth factors (vitamins), whose auxotrophs are infrequently recovered because they are more likely to be cross-fed by live cells. So I simply told friends about the discovery. However, I had an unexpected visit from an even younger scientist, Joshua Lederberg, who introduced himself and explained that he had just been describing to Luria a method for isolating mutants that he and his student, Norton Zinder, had developed. Luria informed him that I had done exactly the same. Lederberg had already sent a letter to the *Journal of Biological Chemistry*, and he offered to ask them to hold it up if I would immediately send in a parallel letter.

I accepted this generous offer with pleasure. But within a few days we both were shocked to receive letters from the editors, expressing appreciation for our bacteriological experiments but concluding that they were not of sufficient biochemical interest for their journal. In fact, I now believe the journal editors were right – it was a bacteriological method, with implications for bacterial genetics as well as for studies of biosynthesis.

However, since I viewed the papers primarily in terms of biochemical applications, and since *J. Biol. Chem.* was the most prestigious journal of that day, I was disappointed. I persuaded a reluctant Lederberg to accept the invitation of an editor of the *Journal of the American Chemical Society* to publish the papers in its rapid Communications section⁽³⁾. More interesting: we had the two reprints, though only one page each, bound in a single cover. We have further described this history elsewhere⁽⁴⁾. It may be a useful model, especially since research is not a zero-sum game, and both independent discoverers can get full credit.

Fortunately, my chief in the Tuberculosis Control Division of the USPHS, Dr Carroll Palmer, recognized that I had a gold mine, and he supported me for seven years, during which I exploited the applications of *E. coli* mutants, with almost no work directly related to tuberculosis. I began thinking of problems in the rapidly growing field of bacterial genetics, and after a few months I had accumulated a long list of ideas for projects. I went to Wisconsin to discuss them with Lederberg. At the end of two days it had become clear that he had thought of all these experiments and had either done them or figured out why they were not worth doing. I stuck with the biochemical applications of auxotrophs for the next decade, pursuing especially the pathway to the aromatic amino acids and vitamins, but also scattered steps in many other pathways.

One amusing sidelight illustrates how remarkably even a most accomplished scientist can resist the 'falsification' of a

hypothesis. At the time when bacterial genetics was burgeoning, the eminent physical chemist Sir Cyril Hinshelwood rejected all the evidence for specific mechanisms involving genes in regulating the formation of various enzymes and metabolites. In a series of articles in the *Proceedings of the Royal Society*, and a book, he tried to explain the kinetics of bacterial growth entirely in terms of the mass law. He worked with *Aerobacter aerogenes*, an organism closely related to *E. coli*, and he reported that he had been unable to obtain any mutants with penicillin as we had described. In a letter I informed him that we also had failed to obtain mutants of *Aerobacter*, using the same procedure as with *E. coli*; but when we increased the penicillin concentration threefold we obtained them in abundance. Hinshelwood replied, thanking me for letting him know that at a sufficient concentration penicillin could influence the *formation* [emphasis added] of enzymes in *Aerobacter*, just as in *E. coli*! Evidently the action of penicillin had to be fitted into the framework of physico-chemical kinetics, excluding Darwinian selection from a reservoir of genetic variation.

The principle behind the penicillin method can be applied, of course, to any agent that preferentially kills growing cells. Many years ago Martin Lubin developed a method that is in principle applicable to any kind of cell that can be cultured: exposure to a radioactively labeled nucleoside (or, in fact, any nutrient), followed by a long period to allow suicide of those cells that have been growing and hence have taken up the radioactivity.

Years later I came to feel that I had made a mistake in continuing so long with the prosperous program of isolating one biosynthetic intermediate after another. I relied on excellent associates to identify the intermediates, because trying to think like a bacterium has been more comfortable for me than doing organic chemistry; so I may have missed greater opportunities in microbial genetics⁽⁵⁾. But probably most scientists, looking back at the end of their career, have such misgivings about the roads not taken.

References

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