Chapter 9

The Elucidation of a Biochemical Pathway

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Introduction

The objectives of this exercise are to elucidate a biochemical pathway and to order a series of mutant alleles in the pathway. The pathway used is the synthesis of prodigiosin in *Serratia marcescens*. This exercise is used in a large class (325 students) at Stanford University. This is the first college-level biology lab the students have, although they have had some chemistry lab and several quarters of biology lecture. This experiment is ideal for someone who is not sure of his or her sterile technique and wants some practice. The experimental procedure is very simple and if a plate becomes contaminated, nothing except that plate is lost.

The experiment takes less than an hour to set up but the students must check the plates several times during the week. This could take one to two more hours. They could even take the plates home with them as they are incubated at room temperature. The preparation time for the instructor is that time required to pour 16 peptone-glycerol agar plates per group of students. We work in groups of four students but smaller groups would work well. The instructor needs to start work on the experiments several weeks in advance in order to provide one set of cultures per two groups of students (see Instructor's Materials).

Student Materials

One of the most widely used methods of elucidating the sequence of reactions (pathway) leading to the synthesis or degradation of a given compound by a given organism utilizes mutants of that organism which do not have the ability to synthesize or utilize that compound. The method is simple and elegant. If the compound in question is an essential one, such as the amino acids or nucleotides are, the mutation is lethal—that is, the organism soon ceases to reproduce—unless the compound in question is supplied to it in its growth medium. Mutants of this kind are called *auxotrophs* (for the compound in question) and their normal or wild-type counterparts are called *prototrophs*. More generally, an auxotroph is any mutant which is unable to grow (or grows poorly) on a medium on which the wild-type can grow.

Auxotrophs can be produced from prototrophs as a result of treatment with mutagenic agents such as UV and X-ray irradiation or the chemicals nitrous acid and hydroxylamine, etc. After treatment, many organisms die and many are unchanged, but a few organisms will be found that require a particular compound for growth that they could formerly synthesize for themselves. That the alteration in nutritional requirements is genetic in nature is illustrated by the observation that progeny of the new auxotrophs have the same nutritional needs as their parent cells.

Various selective techniques have been developed to enable one to isolate a very few auxotrophic mutants from the vast excess of prototrophs with which they will be found after mutagenic treatment. Most of these techniques depend on the fact that, in a particular medium, the prototrophs will be able to grow, while the auxotrophs, because they are unable to synthesize a compound necessary for growth, a compound not supplied in the medium, will not grow. (They do not die, at least not for some time.) Various agents, such as penicillin, are lethal only to growing cells and have no effect on non-growing ones. Thus, by incubating a mixture of auxotrophs and prototrophs—in a medium in which the prototrophs can grow but the auxotrophs cannot—in the presence of an appropriate concentration of penicillin for several hours, one can selectively kill the prototrophs. If one then washes the incubation mixture free of penicillin and incubates portions of it in a medium which now *does* contain the compound which is necessary for growth, the auxotrophs will now grow and can thus be selected for.

When one actually performs a selection experiment of this kind and isolates a number of auxotrophs of independent origin (ones which did not arise from an identical mutational event) for a particular compound, let us say the amino acid tryptophan, one almost invariably finds that the mutants isolated can be separated into groups. That is, though all the mutants have in common the inability to synthesize tryptophan, one may find that upon genetic analysis the mutational sites will map in several different but linked regions of the chromosome (in the case of *E. coli)* or even in unlinked regions (in the case of *Neurospora).* Furthermore, if one supplements the medium in which the tryptophan auxotrophs are incubated with various compounds which one might suspect of being involved in the biosynthesis of tryptophan, one can demonstrate biochemical differences among the mutants. Some auxotrophs will be found which are capable of growing on indole, for instance, as well as tryptophan, while others will not grow on indole. Still others will be found which will grow on anthranilic acid, indole, or tryptophan.

The fact that indole and anthranilic acid can, in some tryptophan auxotrophs, satisfy the requirement for tryptophan (see following formulas), suggests that these compounds may play a rather direct role in the biosynthesis of tryptophan, that they may in fact be intermediates in the biosynthesis of this amino acid from simple nitrogen and carbon sources, such as NH_4^+ and glucose. Such intermediates are termed precursors of the final compound in the biosynthetic pathway. (One must be careful not to conclude that because a compound can be utilized to overcome an auxotrophic deficiency, it is therefore a normal intermediate in the biosynthesis of the final compound. The cell could simply be converting it to a normal intermediate. This process in fact appears to be what is happening in the case of indole utilization in tryptophan biosynthesis.)

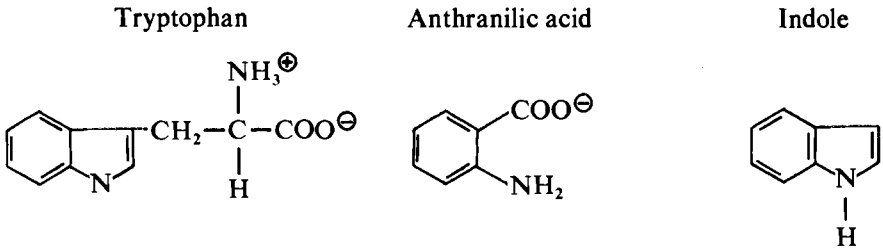
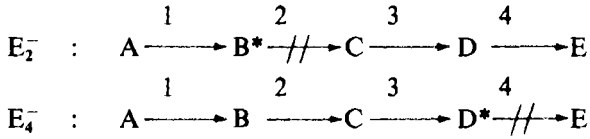


Figure 9.1. The amino acid tryptophan and two related compounds.

Consider now the following generalized scheme for a biosynthetic pathway: $A \xrightarrow{1} B \xrightarrow{2} C \xrightarrow{3} D \xrightarrow{4} E$ where A is the initial compound in the pathway, E is the final compound or end-product, and B, C, and D are intermediates. Enzyme 1 catalyzes the conversion of A to B, 2 catalyzes the conversion of B to C, and so forth. In this pathway, E, the end-product, normally accumulates in the cell where, if it is an amino acid, it is used for protein synthesis. In line with the one-gene—one-enzyme hypothesis, one would expect that many mutants which required E for growth (E^- auxotrophs) would have a *single* reaction in the pathway which was blocked due either to a missing or to a non-functional enzyme. Moreover, one would expect that a mutation which caused any one of the enzymes 1 through 4 to be non-functional would result in what would be a phenotypically E^- auxotroph. However, it is important to note that at least four biochemically distinct classes of E^- auxotrophs can exist, one blocked in each of the reactions 1 through 4. Furthermore, since E normally accumulates, one might expect that the intermediate which immediately precedes any reaction in the pathway which is blocked would also accumulate and, since it in general will not be used up as E would be, one might expect extremely large excesses of such intermediates to accumulate.

This line of reasoning, then, suggests a way in which (1) a minimum number of steps in a biosynthetic pathway can be established, (2) a group of mutants can be at least partially separated into biochemically distinct types, (3) the groups of mutants can be used to establish the order in which the steps in the biosynthetic pathway occur, (4) with the addition of biochemical techniques, the chemical nature of the intermediates can be established. That all of these things should be possible simply because of the nature of the pathway is suggested by the following line of reasoning: Consider two E^- auxotrophs, E_2^- and E_4^-. Suppose that E_2^- is blocked in reaction 2 and E_4^- in reaction 4. In light of the previous discussion, one would expect E_2^- to accumulate B and E_4^- to accumulate D. Their pathways would look as follows: in which $-//-$ indicates a blocked enzyme and D* indicates that the asterisked compound is accumulated in large excess and very probably excreted into the medium.



Comparison of these pathways reveals the following very useful fact: E_2^- is functional in step 4, exactly the one in which E_4^- is blocked. (The converse is also true with respect to step 2.) Since E_2^- can clearly convert D to E and since E_4^- accumulates and excretes D, one would predict that a cell-free filtrate of E_4^-, grown on sufficient but limiting E, would be able to support the growth of E_2^- *without* any addition of E, since it contains D which E_2^- can convert to E. In fact, such filtrates do in many cases support the growth of another biochemically distinct auxotroph. Note, however, that not every auxotroph will support the growth of every other biochemically distinct auxotroph. A cell-free filtrate of E_2^- will *not* support the growth of E_4^- because, although E_4^- is quite capable of performing the reaction in which E_2^- is deficient, it is itself blocked in a subsequent reaction in the pathway. This property of the system allows one to order the biochemically distinct auxotrophs in the same order as the enzymic steps in which they are deficient, that is, in the same order as the biosynthetic pathway. Close examination of the system will convince you of the generality of the following statement: in a linear pathway in which only single enzymic blocks exist, if a mutant X is able to feed (has a cell-free filtrate which supports the growth of) a mutant Y, then X is blocked in a reaction in the pathway which is subsequent to the reaction in which Y is blocked. The converse of this statement (that if X is unable to feed Y it is blocked in a reaction prior to that of Y) is not necessarily true. Why not?

Thus, without any knowledge of the detailed biochemical nature of the steps involved, one can, by the procedure described above, order a series of mutants for the production or degradation of a given compound in the same order as the reactions in which the mutants are blocked occur in the normal pathway.

The sort of investigation described above generally involves a moderate amount of technical manipulation, particularly in the case of auxotrophs where cell-free filtrates are often required. Several systems exist, however, in which the technical aspects are extremely simple and the procedures are very fast. One such system, involving the production of the bright red pigment prodigiosin by the bacterium *Serratia marcescens*, will be used in this experiment.

Prodigiosin is not a compound essential for survival of the organism. Mutants defective at various steps in the biosynthetic pathway are recognized, not by their inability to grow on minimal medium (as auxotrophs are recognized), but rather by their lack of the organism's normal red color. Similarly, feeding in this system is not the ability of an extract of one mutant strain to

support the growth of another. In this system, feeding is indicated by the ability of one mutant strain to synthesize prodigiosin (and thus look red) when grown in the presence of another. (The second mutant strain is said to feed the first.) However, the theoretical considerations developed earlier apply to this system as they do to biosynthetic pathways producing compounds necessary for cell growth. Feeding tests can therefore be used to order steps in the pathway.

Instructor's Materials

In order to elucidate the relationship of each mutant to the other a series of feeding tests are done on peptone-glycerol agar plates. The feeding test consists of streaking each of two strains on the surface of the medium in a petri dish so that they form a V not quite closed at the bottom. It should be a V with a narrow opening and wide arms, so as to insure maximum precursor production by each strain (see Figure 9.2). A positive feeding test consists of the production of red (not orange) pigment. Basically a positive test makes the mutant look like the wild type. After preparation the plates should be incubated at room temperature and examined every few days, i.e., after 2, 4, and 7 days. Some feeding patterns are slow to develop. The plates may be incubated, if desired, at temperatures up to 33°C. Above that temperature the color does not develop.

The plates should be incubated right-side up. This is contrary to normal microbiological practice and should be emphasized. The reason for this means of incubation is that some of the mutants (XII–114, 9–3–3, XII–20) produce a volatile product which is heavier than air. If the plates are incubated upside down the precursor collects in the lid of the petri plate and is not as effective in feeding auxotrophic strains.

The mutants which produce a volatile product cause the mutant streaked opposite it to turn red all along its length. The mutants which produce a soluble product (WF, 3–14, OF) cause the opposite mutant to change color at the bottom of the V. An observant student will note these differences. A less observant student will still be able to deduce the pathway but won't realize some products are volatile, some soluble.

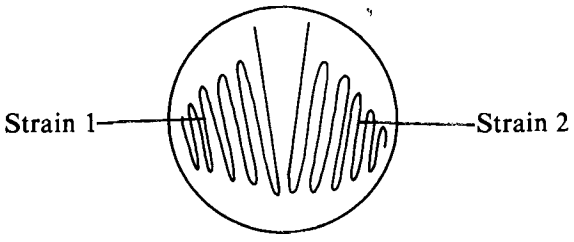


Figure 9.2. Streaking pattern.

Streaking the plates is done with a loop held parallel to the surface of the plate. Be sure to flame the loop before picking up a bit of the stock culture and again after streaking it. A set of new stock plates should be made. Mutants develop more color as they age. The students will need these stock plates for comparison. Only then can they tell if 9–3–3, for example, is darker when streaked against WF than when it's on its own.

The strains we have and use in this experiment and their phenotype are as follows:

Strain	*Color*
OF	dark orange
WF	light orange
C–11	purplish
XII–20	light purple
9–3–3	dark purple
Hy (wild-type)	dark red

Pour plates at least one week before the experiment, because color development is best when plates at least this old are used. The biochemical pathway for prodigiosin production and the feeding results are shown in Figure 9.3.

The table is read in the following manner. If the numbers of two mutants appear in the box they both feed each other e.g., 9–3–3 and WF, but if just one number appears then the number appearing is *fed,* e.g., C–11 feeds 9–3–3.

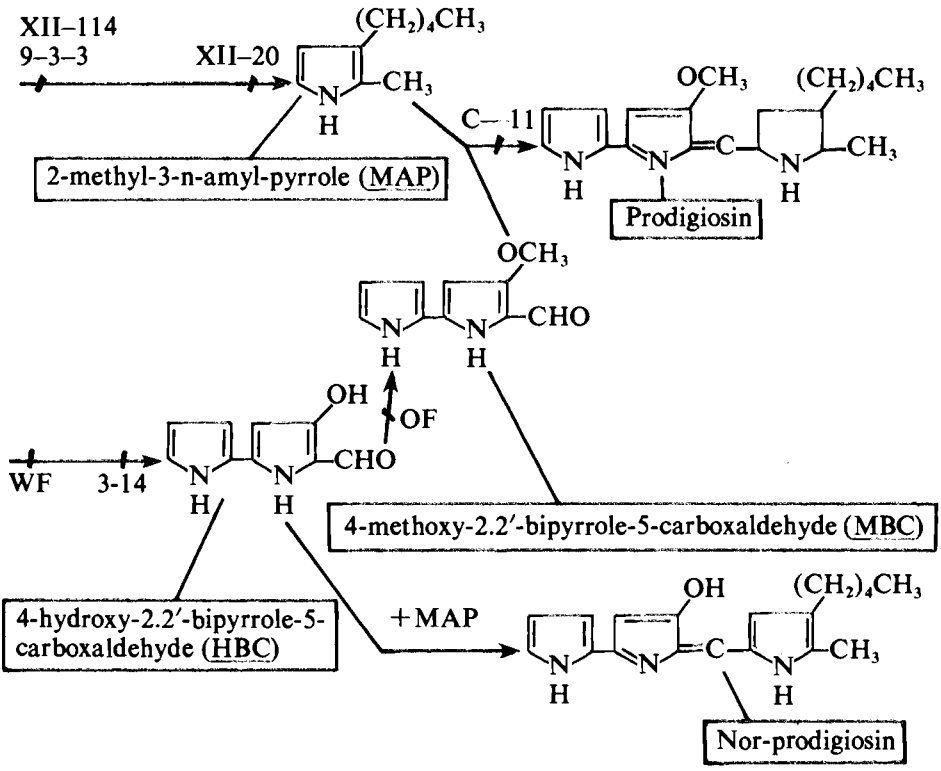
Materials and supplies needed for a class of 20 (if working in groups of four):

Bunsen burners and streakers
Transfer loops
100 peptone-glycerol agar plates
1 set of cultures for every two groups.

The only place I know to obtain the cultures is from us at Stanford University. Other individuals may have them but none of the biological supply houses seem to have more than one or two mutant strains. To obtain cultures please write to:

Ms. Nancy van Zwol, Department of Biological Sciences, Stanford University, Stanford, California 94305. Please include $15.00.

To keep the strains from year to year, slants (fill sterile tube half full of sterile medium and place at angle) should be made of the same media listed in the appendix in 16 × 75-mm screw cap tubes. The tubes should be inoculated and allowed to grow overnight at 25°C. Pipette in sterile mineral oil (heated in oven 160°C for two hours in beaker covered in aluminum foil) to fill tube. Store in refrigerator.



	9–3–3	*WF*	*C–11*	*3–14*	*OF*	*XII–20*	*XII–114*
9–3–3	--						
WF	9–3–3 WF	--					
C–11	9–3–3	WF	--				
3–14	9–3–3 3–14	WF	3–14	--			
OF	9–3–3 OF	WF	OF	3–14	--		
XII–20	9–3–3	XII–20 WF	XII–20	XII–20 (3–14)*	OF XII–20	--	
XII–114	--	XII–114 (WF)	XII–114	XII–114 (3–14)	XII–114 (OF)	XII–114	--

*( ) Indicates a weak reaction.

Figure 9.3. Biochemical pathway and expected feeding results for *Serratia* mutants.

References

Hayes, W. The genetics of bacteria and their viruses. 2nd ed. New York: John Wiley and Sons; 1968: 87–92, A restatement of the theory of biochemical genetics and 108–124, a survey of cases to which these methods have been applied.

Morrison, D.A. Prodigiosin synthesis in mutants of *Serratia marcescens*. J. Bacteriol. 91: 1599–1604; 1966. (We do not give students the Morrison reference as it gives the answer to the problem they are trying to solve.)

Srb, A.M. Genes and metabolic pathways. Editor, Vincent G. Dethier. Topics, the study of life; New York: Harper and Row; 1971: 265–272. An excellent introduction to the material covered in this experiment.

APPENDIX

Preparation of Peptone-Glycerol Agar Plates

Peptone-glycerol	1 liter
Bacto-peptone	5 g
Glycerol	10 ml
Bacto-agar	15 g
H_2O	1 liter

Place all of the above in a 1.5- to 2-liter flask and sterilize at 15 lbs. pressure for 30 minutes. Allow to cool until you can hold the flask comfortably, and pour 25 ml per plate into sterile glass or plastic petri plates. Store the plates upside down for one week to one month before use.

You will need 3 liters of media to make 100 plates, but 1 liter is about all you can pour conveniently by hand. If you have an automatic pipetter you can make a larger quantity in a carbuoy, but then you must increase the time for sterilization to 90 minutes for 10 liters.

This would be a good time to make some slants for storing the strains (see Instructor's Materials) as only 50 ml of media is needed and it's inconvenient to make such a small amount.