## III. The History of Glycolysis: An Example of a Linear Metabolic Pathway.

GLYCOLYSIS is Greek for "the splitting of sugar". It is a process whereby glucose, the most abundant monosaccharide in living systems, is converted to simpler products. The identity of these products depends upon the identity of the living system and the conditions under which the pathway proceeds. Thus in the **absence** of oxygen (under anaerobic conditions) the principal product is lactate when the reaction is carried out in muscle (and almost all other systems) but ethanol when it is carried out in yeast. However in the **presence** of oxygen (under aerobic conditions) the final product is carbon dioxide and water in both muscle and yeast, although the immediate product of glycolysis is always a 3 carbon compound, pyruvic acid. This 3C compound is subsequently processed (by the pathway that we will consider in Ch.VIII) to yield carbon dioxide and water.

The elucidation of the steps of the glycolytic pathway was the result of research which proceeded simultaneously in two apparently unrelated areas.

The first area was that of muscle biochemistry which was being investigated by physiologists for its academic interest.[1]

The second area of research was alcoholic fermentation, the process by which yeast converts glucose to ethanol and carbon dioxide. The elucidation of this process was a major preoccupation of the French wine industry in the late 19'th century. In 1860 Pasteur showed that whenever alcoholic fermentation occurred yeast, or another micro-organism, was present in the fermenting fluid. Pasteur demonstrated that fermentation did not occur in sterile solutions or with dead micro-organisms, and these facts were the basis for his assertion that fermentation was a manifestation of a living cell. He said "I am of the opinion that alcoholic fermentation never occurs without ..... the continued life of the cells which are present". This viewpoint was formalized as VITALIST theory----LIVING CELLS HAVE A VITAL FORCE.

In 1897 the Buchner brothers made a key discovery which led to the downfall of this theory and which opened the door, not only to the investigation of the mechanism of fermentation, but also to the whole of modern biochemistry. The Buchners, who were German biologists, were attempting to make an extract of yeast for therapeutic purposes. They mixed yeast cells with sand and ground the mixture in a pestle and mortar to disrupt the tough cell walls. The resultant juice was then filtered through cheesecloth to remove the debris composed of sand, unbroken cells and fragments of cell walls. Having obtained this liquid they were faced with the problem of preserving it. Because it was to be used for nutritional studies with animals they did not want to use conventional antiseptics such as phenol but instead drew upon their culinary knowledge and used the method so familiar to the cook. They added sugar to the extract! It wasn't long before they noticed that the yeast extract was bubbling furiously and it was to their credit that they realized that they had demonstrated alcoholic fermentation in a cell-free extract, and in so doing demolished Pasteur's point of view. However they did not pursue their fundamental observation and thus the broader significance of their discovery was not appreciated for almost 10 years.

The first important exploitation of the Buchner's yeast preparation was made in 1905 by Harden and Young. These investigators incubated glucose with fresh yeast juice and measured the amount of carbon dioxide which was generated..

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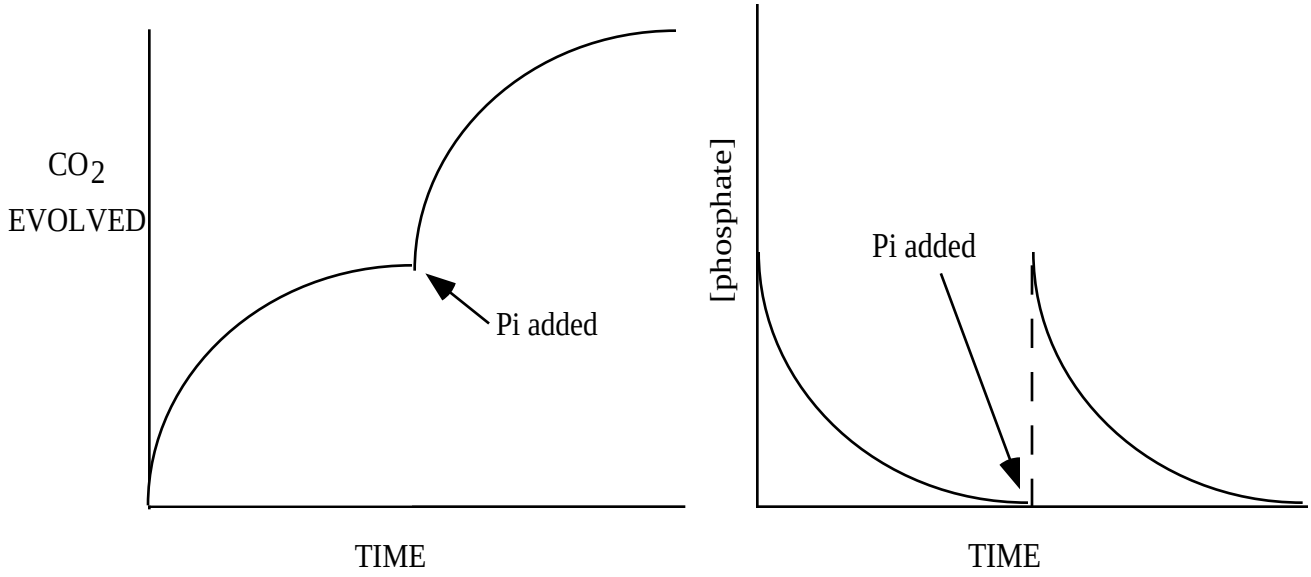
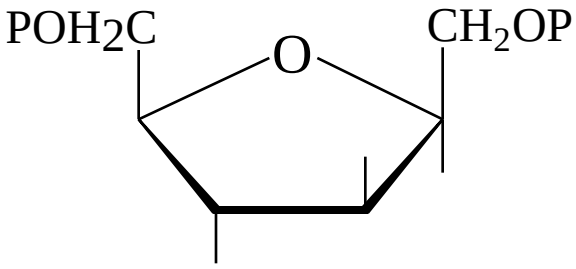
[1] If we exercise vigorously for a prolonged period then the demands of our muscle for chemical energy (ATP) are so large that the ability of oxygen-based energy production to cope is overpowered, principally because we cannot transport oxygen fast enough to our muscles. Under these conditions the muscle tries to SUPPLEMENT its energy supply using the anaerobic catabolism of glucose which, as we will soon see, also generates a small amount of energy. In muscle, however, in the absence of oxygen, the product of glycolysis is lactic acid, and the production of large amounts of this compound leads to an acidification of the tissue (pH 7.2 6.4). Our muscles loose the ability to contract and we are forced to stop exercising. As time proceeds the continuing oxygen supply begins to restore the status-quo, oxygen-based metabolism begins to dominate and the lactate which has accumulated in the absence of oxygen is metabolized allowing the muscle to recover its original tone. The study of the pathway whereby glucose is converted to lactate in muscle was an important early focus of biochemists.



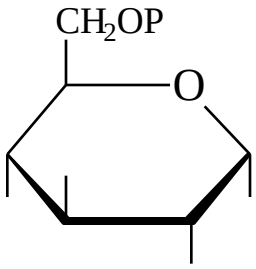

They discovered that the $CO_2$ was initially evolved briskly but that the rate of production decayed and eventually slowed to zero. At this point the addition of phosphate (Pi is the common abbreviation for inorganic phosphate) lead to a repetition of the events. This lead them to monitor the behavior of inorganic phosphate during these events. The disappearance of **inorganic** phosphate from the incubation mixture suggested to Harden & Young that **organic** phosphate esters were being produced[2] and they were able to isolate from the reaction mixture a compound which had the structure:



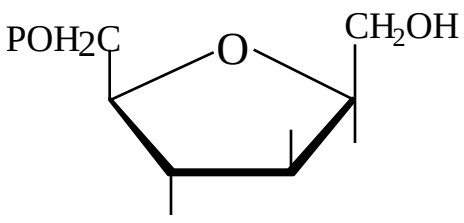

This sugar phosphate is called fructofuranose 1,6-diphosphate or, more commonly, fructose diphosphate (FDP; more recently fructose bis-phosphate, FBP). Harden & Young then demonstrated that FBP could be added to fresh yeast juice with the concomitant production of ethanol and carbon dioxide. This observation established FBP as a probable intermediate:

Glucose + Pi FBP ethanol + $CO_2$

At about the same time Robison discovered a second sugar phosphate in fermenting yeast juice; he showed that this was a hexose **mono**phosphate which, on detailed study, was found to be an equilibrium mixture of two compounds, glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P), in the proportions of 3:1




glucose-6-phosphate (G6P)




Fructose-6-phosphate (F6P)

[2] The popular analytical method only detected inorganic, and not organic, phosphate.

Like FBP both of these sugars could be fermented to ethanol and consideration of the structures suggested the sequence:

G + Pi  G6P  F6P + Pi  FBP  ethanol + $CO_2$

**Dialyzed Yeast Juice: The discovery of ATP and hexokinase.**

The next important development was also due to Harden and Young. They discovered that although fresh yeast juice was perfectly competent at fermentation, **dialyzed** juice was completely incapable of fermentation and *couldn't even make a sugar phosphate.*

This inactive, dialyzed juice could be rejuvenated in two ways:

a) Addition of dialysate (the liquid outside the sac). This signified that the factor(s) that caused reactivation were of low-molecular weight.
b) Addition of undialyzed yeast extract that had been inactivated by boiling.

These observations demonstrated that the activating agents were small and non-protein in character.

The observation that dialyzed juice plus glucose neither ferments nor makes sugar phosphates *even when Pi is present* suggests that a **cofactor** is needed for the phosphorylation step(s).

Conclusion: Fermentation requires low-molecular weight non-protein cofactors. Today we know that a number of such factors are necessary, including NAD, ADP/ATP, TPP, Mg, Pi, K.

> The identity of the crucial cofactor (ATP) was discovered in 1929 by Fiske and SubbaRow. It is needed for the early phosphorylation steps. The relevant enzyme had in fact been discovered earlier by Meyerhof, from studies with fresh rabbit muscle. The ability of fresh extracts of muscle to convert glucose to lactate decreases rapidly upon aging. However if the aged, inactive, *muscle* extract is supplemented with dialyzed *yeast* juice the muscle activity is restored. Using this fact Meyerhof was able to isolate from yeast a muscle activating factor. This proved to be an enzyme which he called HEXOKINASE (muscle hexokinase is inherently unstable and loses activity on standing). Hexokinase catalyzes the first step of glycolysis.

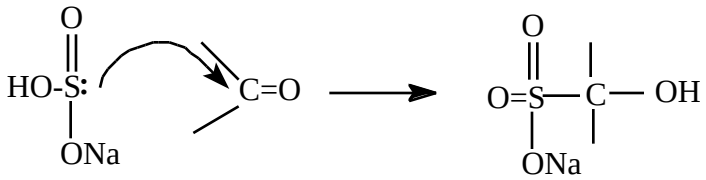
So:

ADP + Pi  ATP  by an unspecified mechanism

hexokinase  ATP
Glucose + ATP  G6P  F6P  FBP  ethanol}

**Contributions from Inhibitors.**

The first application of metabolic inhibitors to this system (and Biochemistry as a whole) was by NEUBERG in 1918. In the years following the Buchner's discovery, many compounds had been incubated with yeast juice, as part of surveys of the ability of yeast juice to metabolize simple organic compounds. One result of these surveys was the discovery that yeast juice could reduce acetaldehyde to ethanol. Neuberg wanted to test whether this reaction was the last reaction of the fermentation process. So he took yeast juice and added glucose plus SODIUM BISULFITE, a carbonyl trap:

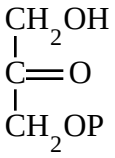



Neuberg found that the presence of bisulfite changed the products from ethanol plus $CO_2$ to equal amounts of glycerol, carbon dioxide and the bisulfite adduct of acetaldehyde. NO alcohol was formed! Thus it seemed that the reduction of acetaldehyde to ethanol was indeed the last reaction.

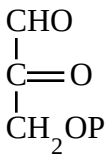
The appearance of carbon dioxide at this stage suggested that the precursor of acetaldehyde was pyruvate, for it was already known that yeast contained an enzyme we now call pyruvate decarboxylase that oxidatively decarboxylates pyruvate to acetaldehyde and carbon dioxide. {The origin of the glycerol will become clear later}. It thus appeared that:

Glucose ===  FBP  2 (3C fragments)  2 $CH_3COCOOH$  2 $CH_3CHO$ + 2 $CO_2$  2 $CH_3CH_2OH$.

The direct demonstration of this possibility was provided by EMBDEN using the compound IODOACETATE ($ICH_2COOH$); this compound is now known to be an inhibitor of enzymes that contain sulfhydryl groups. When iodoacetate was added to juice actively fermenting either glucose or FBP the principal products were found to be:

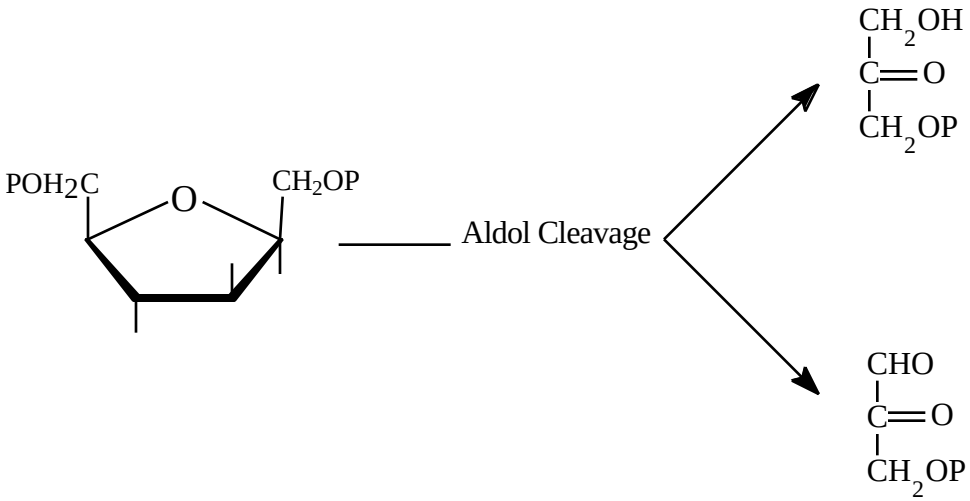


Dihydroxy-acetone phosphate (DHAP)



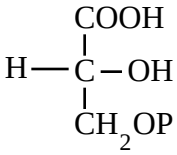
D-glyceraldehyde 3-phosphate (G3P or 3PGA)

Embden therefore suggested the sequence:

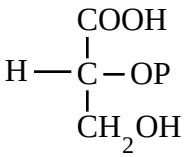



Aldolase, the enzyme which catalyses this reaction, was subsequently isolated by Meyerhof.

The third example of the use of an inhibitor was also provided by EMBDEN. He added FLUORIDE to actively fermenting yeast juice and found that 3 new three-carbon compounds accumulated. These were phosphoglycerol together with a mixture of the 2- and 3- isomers of phosphoglyceric acid:



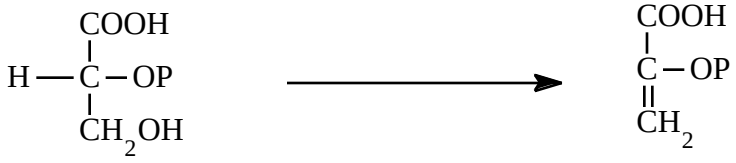
3-phosphoglyceric acid



2-phosphoglyceric acid

These presumably arose through a coupled oxidation-reduction reaction in which DHAP was reduced to glycerol-3-P with the concomitant oxidation of G3P to 3-phosphoglyceric acid; 3-phosphoglycerate is in equilibrium with 2-phosphoglycerate. {It was subsequently established that this coupled reaction requires two dehydrogenases with NADH serving as the mediator of reducing equivalents; the NAD is reduced by triose-P dehydrogenase and reoxidized by α-glycerophosphate dehydrogenase.}

Whole yeast juice converts 2-phosphoglycerate to ethanol. However with dialyzed juice a new intermediate accumulates (due to removal of ADP):



2-phosphoglycerate --- presumably dehydrates  phospho-enolpyruvate (PEP)

If ADP is now added to the dialyzed juice containing PEP, the PEP disappears and ATP and pyruvate appears (pyruvate, not ethanol, for pyruvate decarboxylase requires TPP and this was removed by the dialysis).

PEP + ADP  ATP + pyruvate  { ETOH}

With this outline we have touched on the salient observations which led to the elucidation of the steps that comprise the pathway of fermentation. There were three basic experiments:

1). The identification of possible intermediates and the demonstration that they could be converted to products.

2). Removal of Cofactors by dialysis.

3). Use of Inhibitors.

Most of this research occurred in the period 1890-1930.

**Additional Material (Not Required).**

**Some Methods used in Metabolic Research.**

The objectives of any metabolic investigation are:

1. To establish the sequence of reactions.
2. To isolate and characterize the responsible enzymes.
3. To reconstruct the pathway using purified components.
4. To understand the control mechanisms.

For the metabolic processes which we will be considering:

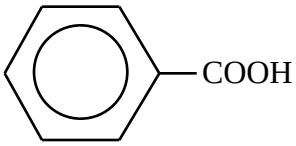
Glycolysis and Tricarboxylic Acid Cycle are at stage 4
Electron Transfer System is at stage 3
Oxidative phosphorylation is at stage 2, though stage 1 is still not unequivocally established.

**Methods**

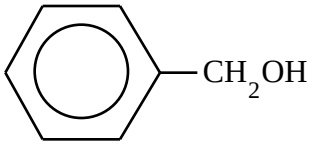
The simplest experimental approach uses intact organisms which for mammals means whole animals. The most basic experiment consists of feeding the animal a defined food and analyzing the chemical composition of the excreta.

Usually such analysis is not very revealing about the function of the BOX and, in general, it is necessary to interfere with the normal function of the BOX - to throw a spanner in the works - if one is to obtain any useful information. The classic example of such a study is provided by the work of Knoop (1904) who was trying to understand the pathways of fatty acid metabolism.

Knoop synthesized a series of phenylated fatty acids of the general structure $\Phi CH_2\text{-}(CH_2)_n\text{-}COOH$ (n = 1 to 10) and fed these to rabbits and dogs. The function of the phenyl group was to provide a label for the fatty acid and it was his assumption that the bond between the phenyl group and the adjacent methylene was not cleaved during metabolism. He subsequently collected the urine from the animal and analyzed it for phenylated fatty acids. He discovered that if the dog was fed fatty acids with an even number of methylene groups e.g. $\Phi CH_2\text{-}(CH_2)_9\text{-}COOH$ then benzoic acid accumulated in the urine.




Benzoic acid

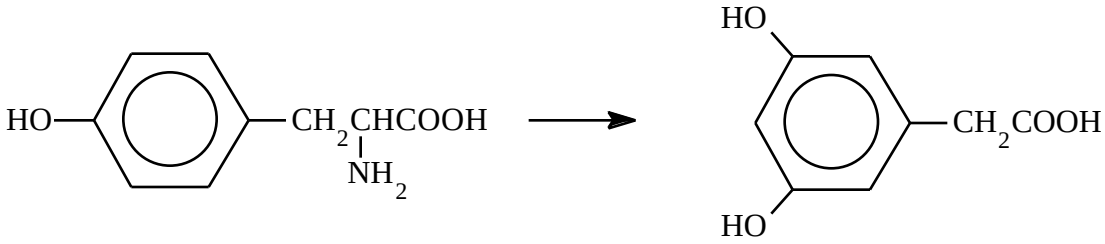



Phenylacetic acid

However when the dogs were fed fatty acids with an odd number of methylene groups then phenylacetic acid was found in the urine. From this simple observation Knoop deduced that fatty acids were metabolized by some process which lopped off two-carbon fragments from the carboxyl terminus: when n is odd the sidechain (methylenes plus carboxyl) has an odd number of C atoms and removing carbons two at a time will leave a single C at the end which become the carboxyl of benzoic acid. Conversely, when n is even the side chain has an even number of carbon atoms; however, because the bond between the phenyl label and the first sidechain carbon is not broken the final two-carbon fragment is not eliminated and phenylacetate is the excreted product.

The metabolic pathway discovered by Knoop is called the **beta-oxidation** pathway for fatty acids and the two carbon fragment that he deduced was split off we now call acetyl-CoA, a metabolite that plays an extremely important role in metabolism in general.

The in-out approach that I have just described is more revealing when applied to some abnormal organism, such as individuals that suffer from and INBORN ERROR OF METABOLISM (= genetic defect). For instance, sufferers of ALCAPTONURIA are unable to properly metabolize tyrosine; consequently they excrete an intermediate in the catabolism of tyrosine called homogentisic acid which, because it is a diphenol, reacts rapidly with air to form a black pigment similar to melanin. As a consequence the urine of individuals afflicted with this condition rapidly turns black upon exposure to air. The sequence is:

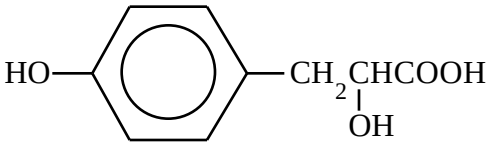



Tyrosine

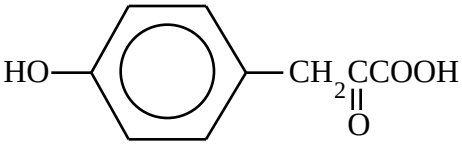
Homogentisic Acid

The homogentisic acid is normally metabolized by oxidation and ring opening. However alcaptonuriacs lack the oxidase enzyme that catalyses this reaction and as a consequence the homogentisic acid is excreted in the urine.

Are there intermediates between tyrosine and homogentisic acid? True intermediates should also be metabolized to yield homogentisic acid whereas compounds related to tyrosine which are not intermediates will not produce homogentisic acid. For example, feeding ρ-hydroxyphenyllactate to an alcaptonuriac does not give rise to urine that turns black whereas feeding ρ-hydroxyphenylpyruvate does!




ρ-hydroxy-phenyllactate




ρ-hydroxy-phenylpyruvate

We thus deduce the sequence {Lehninger Ch 21-11}

Tyrosine ρ-hydroxyphenylpyruvate homogentisic acid ring opening
Transaminase hydroxylase oxidase

Another well known example of an inborn error is DIABETES, a condition induced by insulin deficiency. A diabetic will excrete glucose when placed on a carbohydrate diet; however, on a fat diet a diabetic excretes the so-called ketone-bodies (acetoacetate, acetone and β-hydroxybutyrate). (A diabetic lacks glucokinase (liver) and impaired glucose transport (via insulin deficiency)).

The relation of amino acid catabolism to the metabolism of carbohydrates and fats can thus be deduced simply by examining the composition of the urine after feeding a diabetic one-or-another amino acid. For example

Ala,Glu,Ser..... Glucose
Phe,Tyr..... Ketone Bodies

**Perfused Organs.** (1 stage less complicated than a whole animal)

Certain organs (notably heart, liver, kidney) can be surgically removed from an animal and "kept alive" for several hours by providing the organ with suitable nourishment such as fresh blood or synthetic substitutes (such as the physiological salines), using the vascular system to transport the fluids to the tissues. By making additions to the blood and then analyzing the fluid as it exits the organ it is possible to deduce important metabolic functions for that organ. In this way it was established that the liver is a major site of both lipid and carbohydrate metabolism.

**Tissue Slices and Homogenates.**

Either: Cut at thin slice of the organ (0.3mm thick, diameter. = a quarter)
Or: Perform a carefully controlled homogenization of the tissue in suitable buffers.

The advantage of the former is that damage to the cells is confined to the surface layer where the cut was made. Thus if the metabolic process requires the participation of well-integrated reactions the metabolic activity has the best chance of surviving in a slice. The slice is made as thin as possible to facilitate the diffusion of nutrients from the incubation buffer to the cells of the tissue

Homogenates are advantageous when there are permeability barriers to the metabolite being studied for, in the tissue-homogenate, cell membranes have been broken down. However because the cells have been disrupted pathways that require the coordinated action of sub cellular components will not proceed as efficiently. Clearly the two experimental materials are complementary.

These materials are used in Metabolite --> Product analysis experiments and are especially convenient because of the potential for interfering with the system under study. In particular one may be able to:

1. Remove an enzyme or enzyme system (separation of glycolysis from the TCA cycle)
2. Selectively inhibit or destroy a specific enzyme .
3. Trap intermediates with group specific reagents.

We came across examples of such manipulations when we discussed the background to glycolysis.

Finally, we can work with sub cellular fractions such as mitochondria or nuclei or with soluble enzymes. Tissue fractionation was described earlier in this course and there will be an exercise in cell fractionation in the lab course.

## BACTERIAL MUTANTS

(Ref.: The Bacteria, Vol. VIII. An article by Umbarger and Davis in "Pathways of Amino Acid Metabolism").

Normal (Wildtype) bacteria are usually nutritionally nonexacting and can be grown on a so-called minimal medium; this usually contains mineral salts, a source of nitrogen ( usually ammonium ion) and glucose as a source of both carbon and energy. Such bacteria are called prototrophs: Escherichia Coli is a typical example.

When a suspension of such bacteria are exposed to ultra-violet light (or to one of a number of mutagenic chemicals (e.g. nitrosoguanidine)) for a time such that about 99.99% of the bacteria are killed, then it is found that a fraction of the 0.01% of the survivors have developed specific nutritional needs. They have become auxotrophs. These auxotrophs, of which there may be several quite different kinds, are unable to synthesize crucial metabolites and, as a consequence, will now only grow if these metabolites are included in their growth medium.

Isolation of an Auxotroph

1 . The irradiated suspension is incubated in a minimal medium to which penicillin has been added. Any surviving wild-type cells grow but, because of the penicillin present in the medium, the new cell-walls that they synthesize are defective and the cells lyse. (Penicillin inhibits the last stages of cell wall-biosynthesis.) The auxotrophs do not grow and hence survive.

2. The cell suspension is washed to remove the penicillin and then the nutritional needs of the auxotrophs are established by brute force screening.

3. It is often the case that the suspension contains several different mutants with the same nutritional requirement. It is a straightforward genetic technique to identify and separate the different varieties of the same auxotroph and indeed the initial objective is to obtain a series of mutants with different genetic constitution (genotype) but with the same nutritional requirement (phenotype).

This family of mutants is then exploited as in the following examples:

Imagine that we have isolated three mutants A, B, and C each of which requires that Compound Z be present in the growth medium, i.e. they are auxotrophs for Z. We infer that Z is a late or final product of a biosynthetic pathway which has been incapacitated:

| | | | |
|---|---|---|---|
| Early Intermediates---------> W ----------> X ----------> Y ----------> Z | | | |
| Mutant: | A | B | C |
| Gene Missing | i | ii | iii |
| Relevant Enzymes | I | II | III |

A, B and C will all show a requirement for Z but for different reasons:

A has gene i mutated, lacks enzyme I and thus can't make X

B..... ii.....II..... Y

C ..... iii.....III..... Z

The basic ways in which these mutants are exploited are as follows:

**1**. It is not strictly necessary that Z be the nutrient provided. Any intermediate in the pathway which is made after the genetic block should serve just as well! So, knowing the structure of Z we guess plausible candidates for X and Y and see if any of the mutants can grow on the putative intermediary metabolite. For example, A and B will both grow on Y but C will not. Thus the order of the mutants would be (A,B)C. A will grow on X and Y but will not etc.

There is an important caveat in this kind of experiment. It is obviously necessary that the added compounds (or subsequently produced products) be able to cross the bacterial cell wall. THERE MUST BE NO PERMEABILITY BARRIERS. This is a common problem in Biochemistry which is only satisfactorily avoided when it is possible to make cell-free extracts which are still competent to carry out the pathway under investigation.

**2a**. Accumulation experiments. The three mutants are incubated in the presence of very low (limiting) concentrations of Z which permits some, but not unlimited, growth. The growing bacteria will try to biosynthesize Z to supplement that provided by the medium. However they cannot carry the pathway to completion. Consequently mutant A will accumulate W, B will accumulate X (etc.) and hopefully these materials will be excreted into the medium from which they can be isolated and identified. The metabolic sequence is then rationalized from the structural relationships between the characterized compounds.

**2b**. Cross-Feeding Experiments.

Mutant B can synthesize X but cannot metabolize it.
Mutant A cannot synthesize X but can metabolize it.

Consequently, mutant A should be able to grow in a medium which previously contained mutant B; "a late mutant should be able to support an early mutant because....."

Thus with combinations of experiments of this kind we should be able to order the mutants A, B, C and to identify W, X, and Y in our scheme.

**Radio-Isotopes: A modern extension of Knoop's approach**

The use of radio-isotopes to tag chemical compounds is now a fundamental technique. By studying the fate of the radio-active label subsequent to presenting the labeled compound to the organism the metabolic path followed by that compound can be partially or wholly deduced. The most famous example of the use of labels is to be found on the experiments which were conducted to deduce the path of carbon in photosynthesis--the lollipop experiment (V&V; p649).