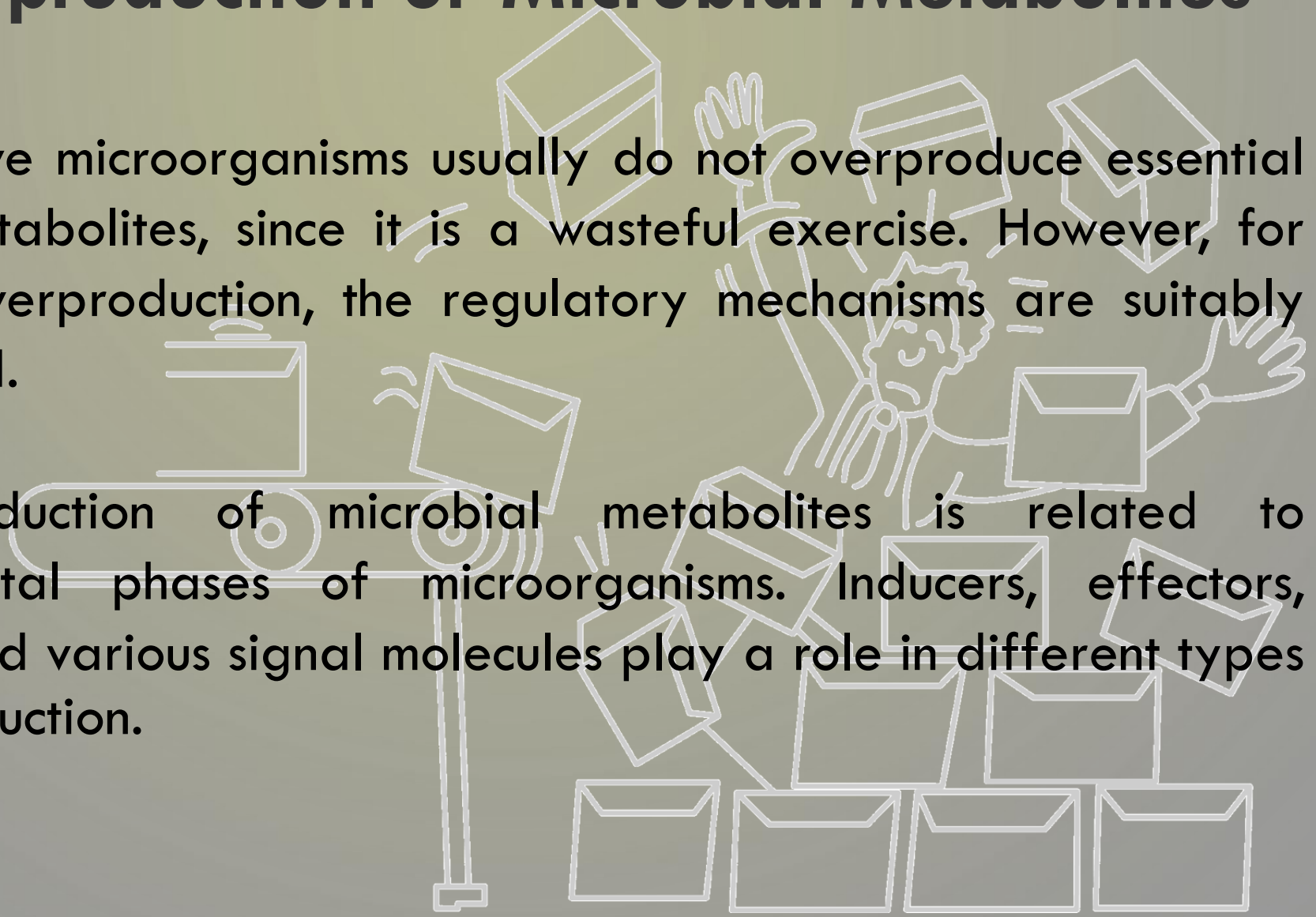
Processes for Overproduction of Microbial Metabolites for Industrial Applications

Overproduction of Microbial Metabolites

The native microorganisms usually do not overproduce essential primary metabolites, since it is a wasteful exercise. However, for industrial overproduction, the regulatory mechanisms are suitably manipulated.

Overproduction of microbial metabolites is related to developmental phases of microorganisms. Inducers, effectors, inhibitors and various signal molecules play a role in different types of overproduction.



1. Mechanisms Enabling Microorganisms to Avoid Overproduction of Primary Metabolic Products Through Enzyme Regulation

Some of the regulatory mechanisms enabling organisms to avoid over-production are

1. Substrate Induction
2. Catabolite Regulation
    2.1 Repression
    2.2 Inhibition
3. Feedback Regulation
    3.1 Repression

- 3.2 Inhibition
- 3.3 Modifications used in branched pathways
  - 3.3.1 Concerted (multivalent) feedback regulation
  - 3.3.2 Cooperative feedback inhibition
  - 3.3.3 Cumulative feedback regulation
  - 3.3.4 Compensatory feedback regulation
  - 3.3.5 Sequential feedback regulation
  - 3.3.6 Isoenzyme feedback regulation

4. Amino acid Regulation of RNA synthesis
5. Energy Charge Regulation
6. Permeability Control

1.1. Substrate Induction

Some enzymes are produced by microorganisms only when the substrate on which they act is available in the medium.

Such enzymes are known as *inducible* enzymes. Analogues of the substrate may act as the inducer.

- When an inducer is present in the medium a number of different inducible enzymes may sometimes be synthesized by the organism.
- This happens when the pathway for the metabolism of the compound is based on sequential induction.
- In this situation the organism is induced to produce an enzyme by the presence of a substrate.

- The intermediate resulting from the action of this enzyme on the substrate induces the production of another enzyme and so on until metabolism is accomplished.
- The other group of enzymes is produced whether or not the substrate on which they act, are present. These enzymes are known as constitutive.
- Enzyme induction enables the organism to respond rapidly, sometimes within seconds, to the presence of a suitable substrate, so that unwanted enzymes are not manufactured.

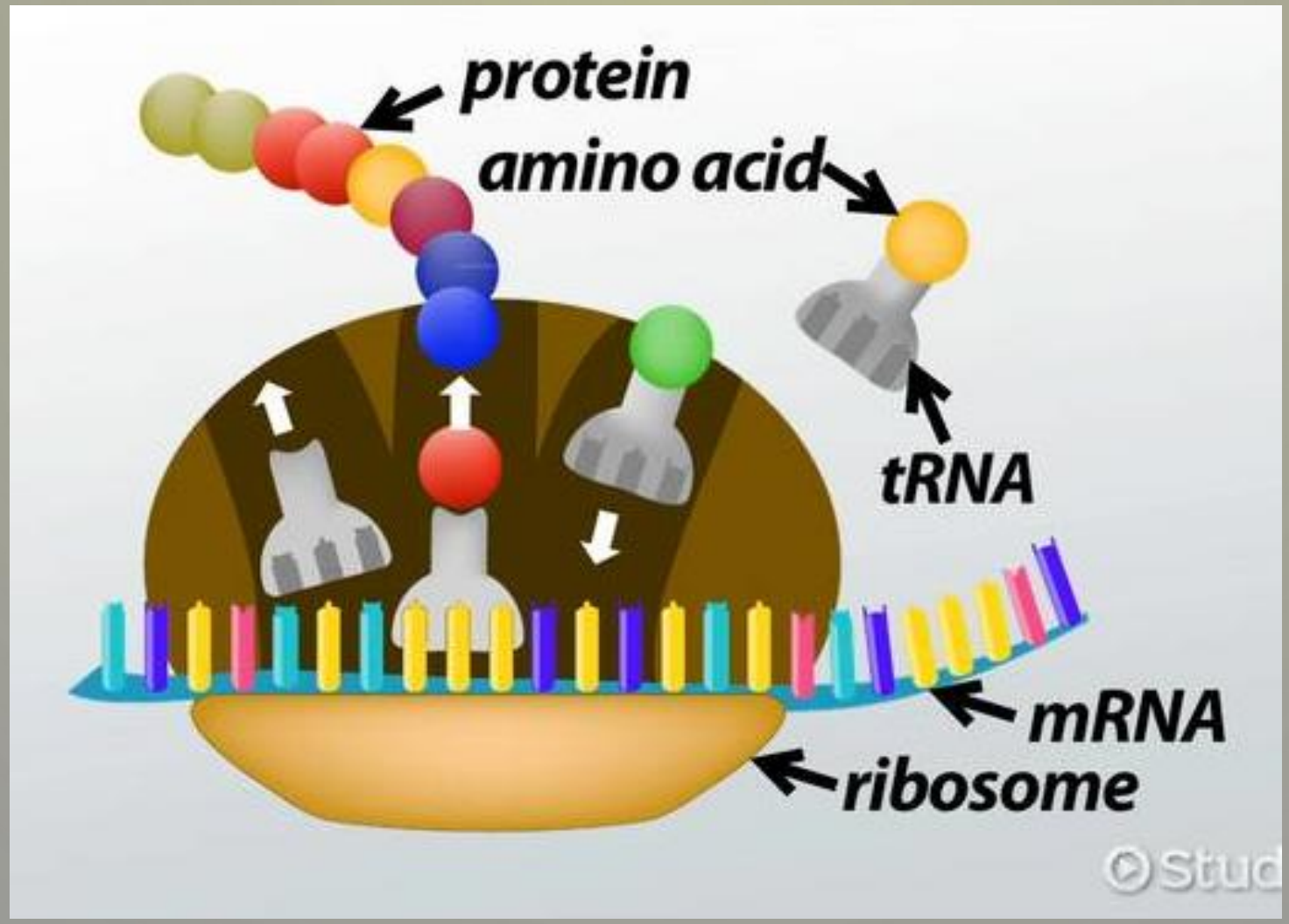
Molecular basis for enzyme induction:

The molecular mechanism for the rapid response of an organism to the presence of an inducer in the medium relates to protein synthesis since enzymes are protein in nature.

Two models exist for explaining on a molecular basis the expression of genes in protein synthesis:

1. The Jacob-Monod Model of the (negative) control of protein synthesis
2. Positive control of protein synthesis

Protein synthesis



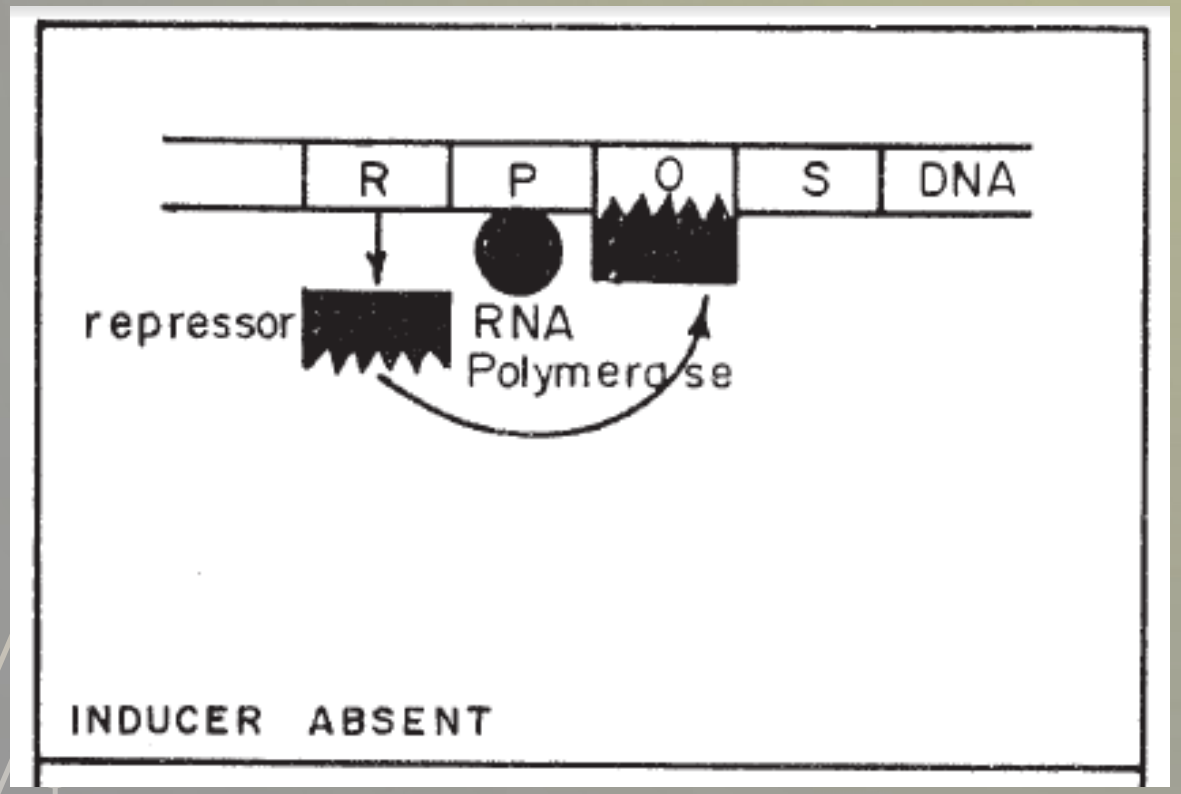
1.1.1 The Jacob-Monod Model of the (negative) control of protein synthesis

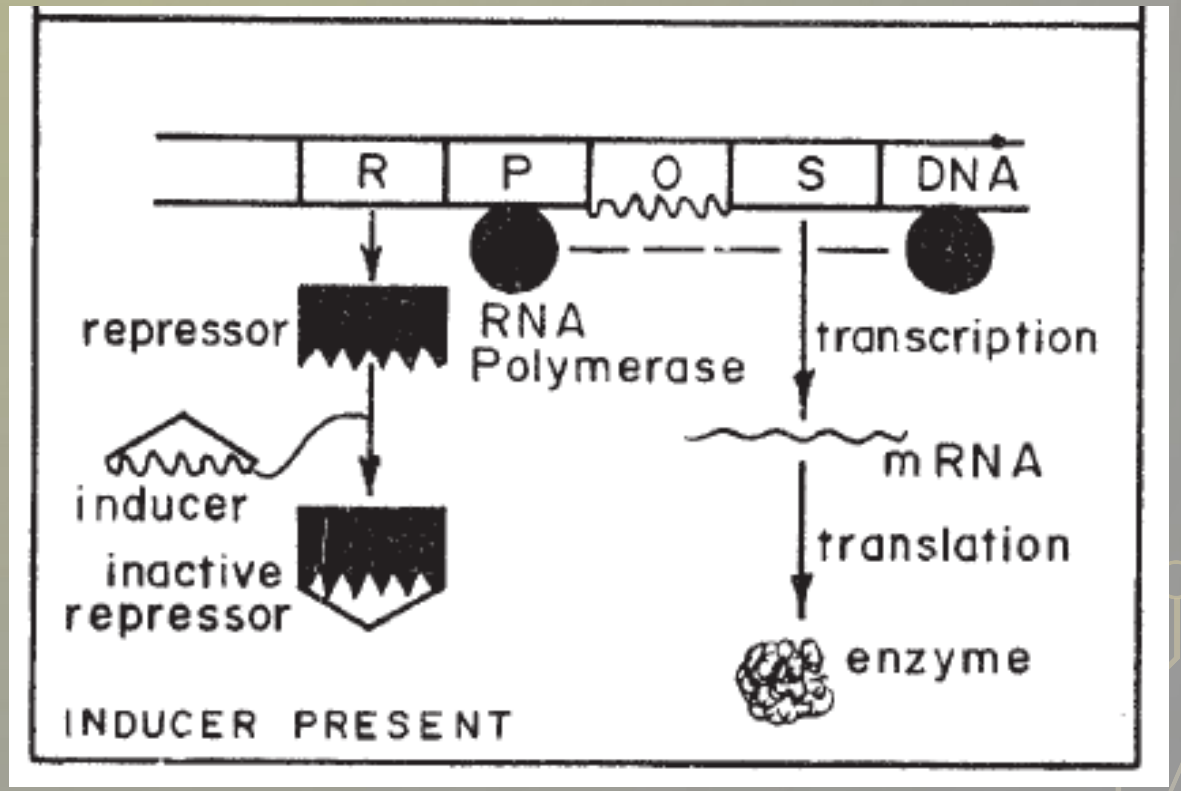
The synthesis of polypeptides and hence enzymes protein is regulated by a group of genes known as the operon and which occupies a section of the chromosomal DNA.

Each operon controls the synthesis of a particular protein. An operon includes a regulator gene (R) which codes for a repressor protein. The repressor can bind to the operator gene (O) which controls the activity of the neighboring structural genes (S).

The production of the enzymes which catalyze the transcription of the message on the DNA into mRNA (namely, RNA polymerase) is controlled by the promoter gene (P).

Diagram Illustrating Negative Control of Protein Synthesis According to the Jacob and Monod Model





If the repressor protein is combined with the operator gene (O) then the movement of RNA polymerase is blocked and RNA complementary to the DNA in the structural genes (S) cannot be made.

Consequently no polypeptide and no enzyme will be made. In the absence of the attachment of the repressor to the operator gene, RNA polymerase from the promoter can move to, and transcribe the structural genes, S.

Inducible enzymes are made when an inducer is added. Inducers inactivate or remove the repressor protein thus leaving the way clear for protein synthesis. Constitutive enzymes occur where the regulator gene (R) does not function, produces an inactive repressor, or produces a repressor to which the operator cannot bind. Often more than one structural gene may be controlled by a given operator.

Mutations can occur in the regulator (R) and operator (O) genes thus altering the nature of the repressor or making it impossible for an existing repressor to bind onto the operator. Such a mutation is called constitutive and it eliminates the need for an inducer.

The structural genes of inducible enzymes are usually repressed because of the attachment of the repressor to the operator. During induction the repressor is no longer a hindrance, hence induction is also known as de-repression. In the model of Jacob and Monod gene expression can only occur when the operator gene is free. (i.e., in the absence of the attachment of the repressor protein the operator gene O. For this reason the control is said to be negative.

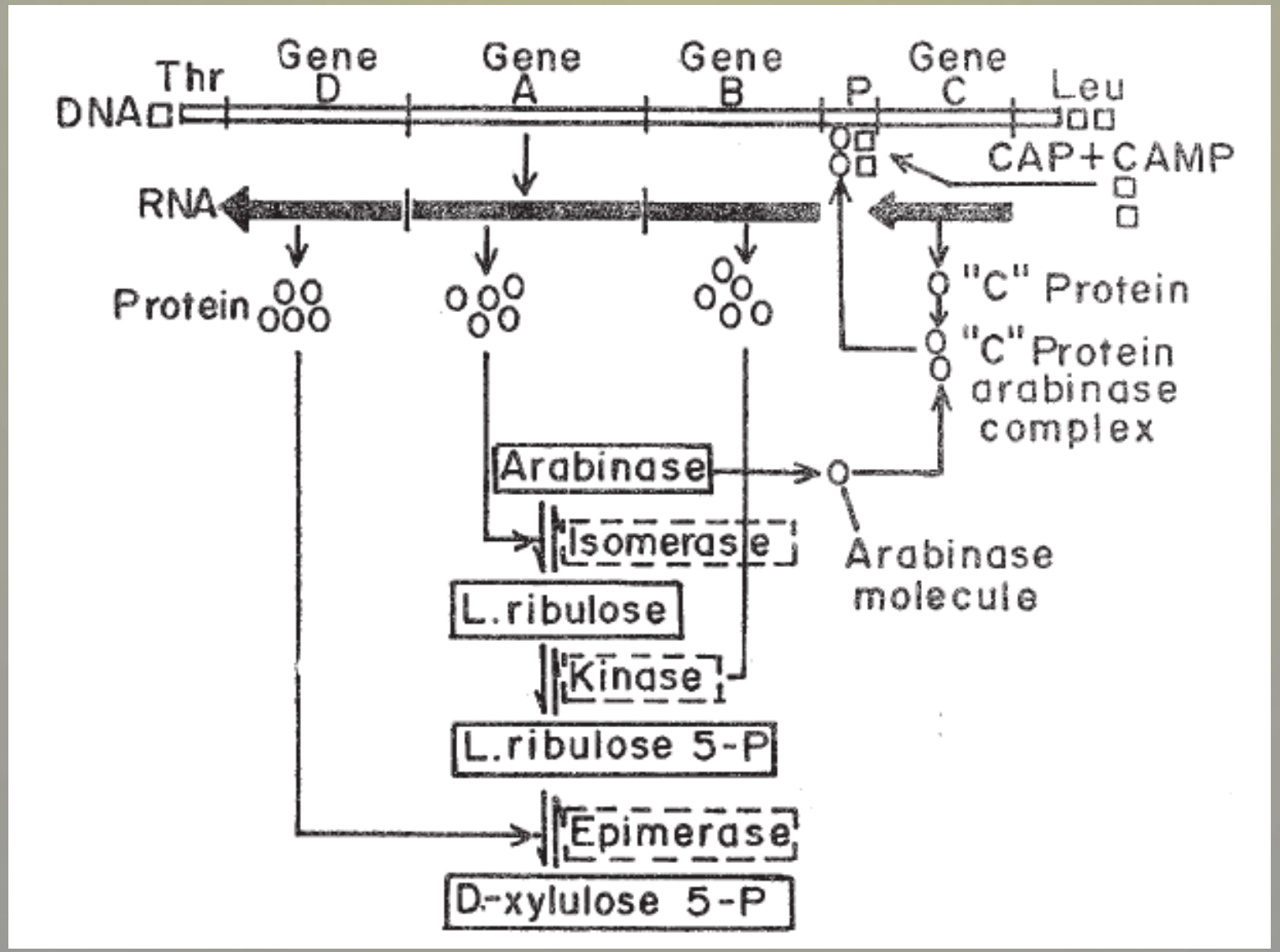
1.1.2 Positive control of protein synthesis

Positive control of protein synthesis has been less well studied but has been established in at least one system, namely the *ara* operon, which is responsible for L-arabinose utilization in *E. coli*.

In this system the product of one gene (ara C) is a protein which combines with the inducer arabinose to form an activator molecule which in turn initiates action at the operon.

In the scheme 'C' protein combines with arabinose to produce an arabinose – 'C' protein complex which binds to the Promoter P and initiates the synthesis of the various enzymes isomerase, kinase. epimerase) which convert L-arabinose to D-xylulose-5-phosphate, a form in which it can be utilized in the Pentose Phosphate pathway. Positive control of protein synthesis also operates during catabolite repression.

Diagram Illustrating Positive Control of Protein Synthesis



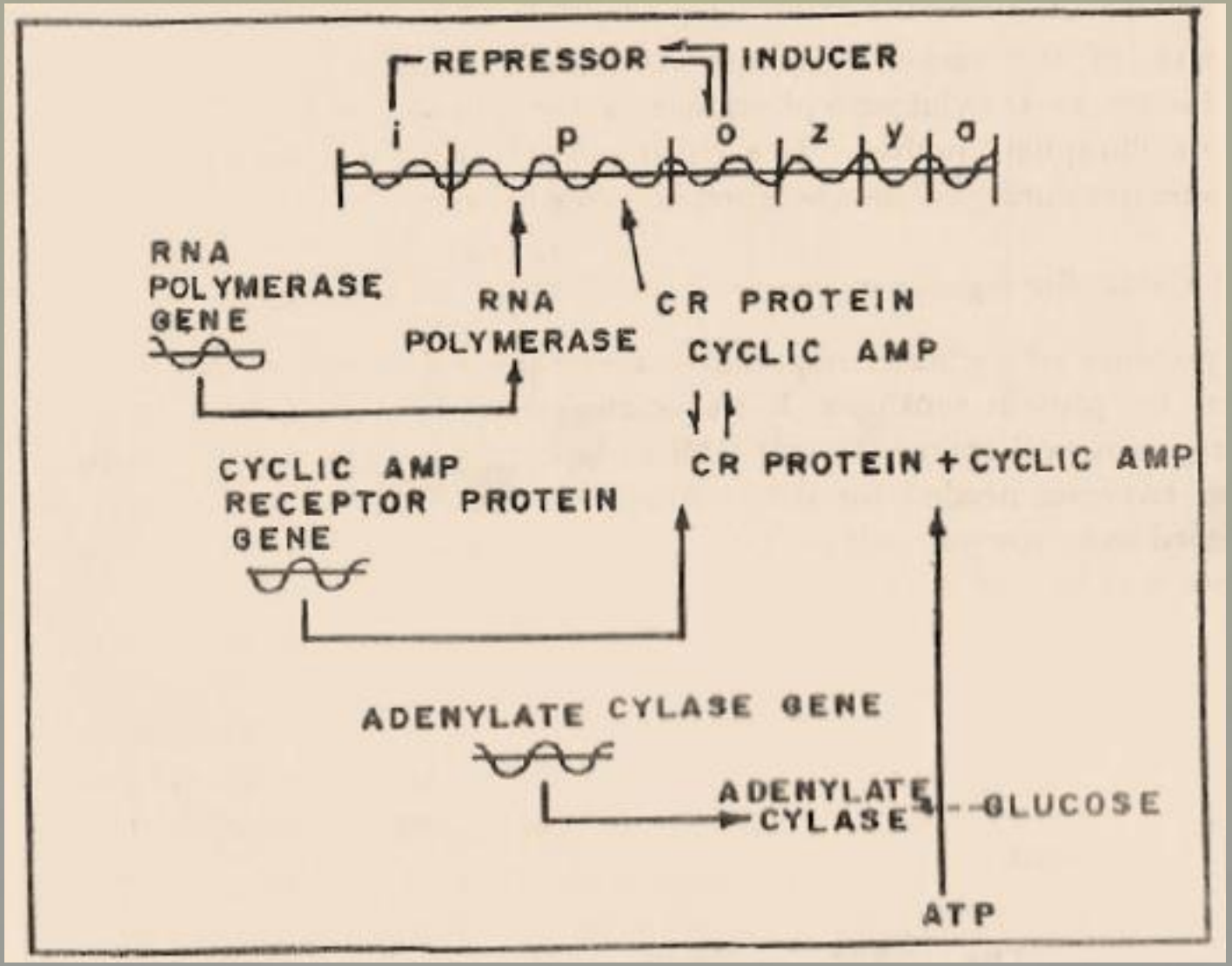
1.2 Catabolite Regulation

The presence of carbon compounds other than inducers may also have important effects on protein synthesis. If two carbon sources are available to an organism, the organism will utilize the one which supports growth more rapidly, during which period enzymes needed for the utilization of the less available carbon source are repressed and therefore will not be synthesized.

- As this was first observed when glucose and lactose were supplied to *E. coli*, it is often called the 'glucose effect', since glucose is the more available of the two sugars and lactose utilization is suppressed as long as glucose is available.
- It soon became known that the effect was not directly a glucose effect but was due to some catabolite.

- The term *catabolite repression* was adopted as more appropriate.
- The active catabolite involved in catabolite repression has been found to be a nucleotide cyclic 3’5’-adenosine monophosphate (cAMP).
- In this model an increased concentration of c-AMP is a signal for energy starvation.
- When such a signal is given, c-Amp binds to an intracellular protein, c-AMP-receptor protein (CRP) for which it has high affinity.
- The binding of this complex to the promoter site of an operon stimulates the initiation of operon transcription by RNA polymerase.
- The presence of glucose or a derivative of glucose inhibits adenylate cyclase the enzyme which converts ATP to c-AMP.
- Transcription by susceptible operons is inhibited as a result.
- In short, therefore, catabolite repression is reversed by c-AMP.

Action of Cyclic Amp on the Lac Operon



1.3 Feedback Regulation

- Feedback or end-product regulations control exerted by the end-product of a metabolic pathway.
- Feedback regulations are important in the control over anabolic or biosynthetic enzymes whereas enzymes involved in catabolism are usually controlled by induction and catabolite regulation.
- Two main types of feedback regulation exist:
    1. feedback inhibition
    2. feedback repression
- Both of them help adjust the rate of the production of pathway end products to the rate at which macro-molecules are synthesized

1.3.1 Feedback inhibition

In feedback inhibition the final product of metabolic pathway inhibits the action of earlier enzymes (usually the first) of that sequence. The inhibitor and the substrate need not resemble each other, hence the inhibition is often called *allosteric* in contrast with the *isosteic* inhibition where the inhibitor and substrate have the same molecular conformation. Feedback inhibition can be explained on an enzymic level by the structure of the enzyme molecule. Such enzymes have two type of protein sub-units. The binding site on the sub-unit binds to the substrate while the site on the other sub-unit binds to the feedback inhibitor. When the inhibitor binds to the enzyme the shape of the enzymes is changed and for this reason, it is no longer able to bind on the substrate. The situation is known as the allosteric effect.

1.3.2 Feedback Repression

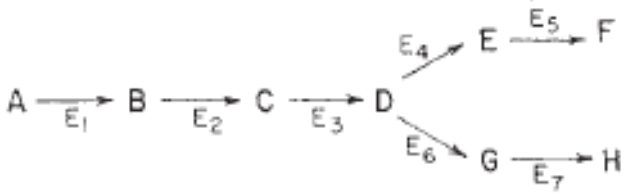
Feedback repression deals with a reduction in the rate of synthesis of the enzymes. In enzymes that are affected by feedback repression the regulator gene (R) is said to produce a protein aporepressor which is the end-product of the biosynthetic pathway. The activated repressor protein then interacts with the operator gene (O) and prevents transcription of the structural genes (S) on to mRNA. A derivative of the end-product may also bring about feedback repression. It is particularly active in stopping the over production of vitamins, which are required only in small amounts. Feedback repression acts more slowly both in its introduction and in its removal than feedback inhibition . About two generations are required for the specific activity of the repressed enzymes to rise to its maximum level when the repressing metabolite is removed; about the same number of generations are also required for the enzyme to be repressed when a competitive metabolite is introduced.

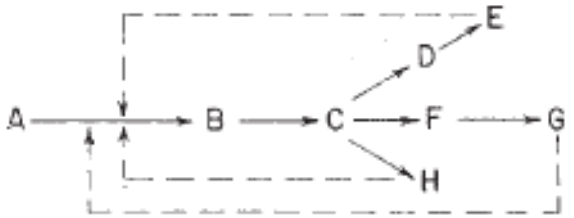
1.3.3 Regulation in branched pathway

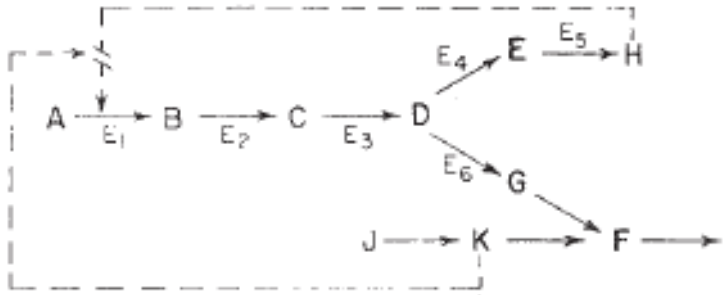
In a branched pathway leading to two or more end-products, difficulties would arise for the organism if one of them inhibited the synthesis of the other. For this reason, several patterns of feedback inhibition have been evolved for branched pathways such as

(i) Concerted or multivalent feedback regulation:
(ii) Cooperative feedback regulation:
(iii) Cumulative feedback regulation:
(iv) Compensatory antagonism of feedback regulation:
(v) Sequential feedback regulation:
(vi) Multiple enzymes (isoenzymes) with specific regulatory effectors:

Feedback Regulation (Inhibition and Repression) of Enzymes in Branched Pathways

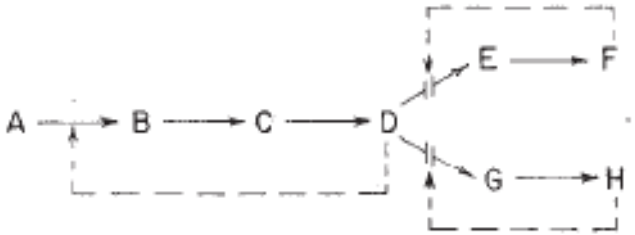


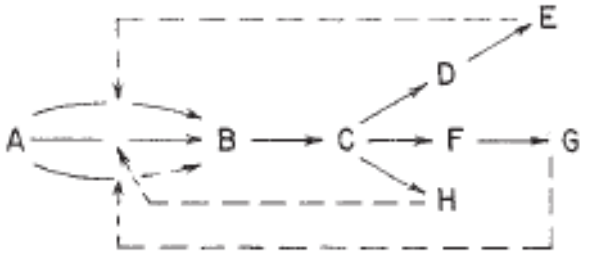




A, B, C, D. . . . K = Substrates
E_1, E_2, E_3, E_4, E_5 = Enzymes
———— = Biosynthetic routes
- - - - - - - - - - - - = Feed inhibition/repression
= Interruption of biosynthetic route.

Top left = Generalized scheme illustrating substrates, enzymes, biosynthetic routes
Top Right = Cumulative feedback regulation
Center = Compensatory antagonism of feedback inhibition
Bottom left = Sequential feedback inhibition
Bottom Right = Multiple enzymes (isoenzymes)





(i) ***Concerted or multivalent feedback regulation***: Individual end-products F and H have little or no negative effect, on the first enzyme, E1, but together they are potent inhibitors. It occurs in *Salmonella* in the branched sequence leading to valine, leucine, isoleucine and pantothenic acid.

(ii) ***Cooperative feedback regulation***: In this case the end-products F and H are individually weakly inhibiting to the primary enzyme, E1, but together they act synergistically, exerting an inhibition exceeding the sum of their individual activities.

(iii) ***Cumulative feedback regulation***: In this system an end-product for example (H), inhibits the primary enzyme E1 to a degree which is not dependent on other inhibitors. A second inhibitor further increases the total inhibition but not synergistically. Complete inhibition occurs only when all the products (E, G, H) are present.

(iv) ***Compensatory antagonism of feedback regulation***: This system operates where one of the end-products, F, is an intermediate in another pathway J, K, F . In order to prevent the other end-product, H, of the original pathway from inhibiting the primary Enzyme E1, and thus ultimately causing the accumulation of H, the intermediate in the second pathway J, K is able to prevent its own accumulation by decreasing the inhibitory effect of H on the primary enzyme E1.

(v) ***Sequential feedback regulation***: Here the end-products inhibit the enzymes at the beginning of the bifurcation of the pathways. This inhibition causes the accumulation of the intermediate just before the bifurcation. It is the accumulation of this intermediate which inhibits the primary enzyme of the pathway.

(vi) ***Multiple enzymes (isoenzymes) with specific regulatory effectors***: Multiple primary enzymes are produced each of which catabolyzes the same reaction from A to B but is controlled by a different end-product. Thus if one end-product inhibits one primary enzyme, the other end products can still be formed by the mediation of one of the remaining primary enzymes.

1.4 Amino Acid Regulation of RNA Synthesis

Both protein synthesis and RNA synthesis stop when an amino acid requiring mutant exhausts the amino acid supplied to it in the medium. In this way the cell avoids the overproduction of unwanted RNA. Such economical strains are 'stringent'. Certain mutant strains are however 'relaxed' and continue to produce RNA in the absence of the required amino acid. The stoppage of RNA synthesis in stringent strains is due to the production of the nucleotide guanosine tetraphosphate (PpGpp) and guanosine pentaphosphate (ppGpp) when the supplied amino acid becomes limiting. The amount of ppGpp in the cell is inversely proportional to the amount of RNA and the rate of growth. Relaxed cells lack the enzymes necessary to produce ppGpp from guanosine diphosphate and ppGpp from guanosine triphosphate.

1.5 Energy Charge Regulation

The cell can also regulate production by the amount of energy it makes available for any particular reaction. The cell's high energy compounds ATP, ADP, and AMP are produced during catabolism. The amount of high energy in a cell is given by the adenylate charge or energy charge. This measures the extent to which ATP-ADP-AMP systems of the cell contains high energy phosphate bonds, and is given by the formula.

$$\text{Energy charge} = \frac{(\text{ATP}) + 1/2\,(\text{ADP})}{(\text{ATP}) + (\text{ADP}) + \text{AMP}}$$

Using this formula, the charge for a cell falls between 0 and 1.0 by a system resembling feedback regulation, energy is denied reactions which are energy yielding and shunted to those requiring it. Thus, at the branch point in carbohydrate metabolism phosphoenolpyruvate is either dephosphorylated to give pyruvate or carboxylated to give oxalocetate. A high adenylate charge inhibits dephosphorylation and so leads to decreased synthesis of ATP. A high energy charge on the other hand does not affect carboylation to oxoloacetate. It may indeed increase it because of the greater availability of energy.

1.6 Permeability Control

While metabolic control prevents the overproduction of essential macromolecules, permeability control enables the microorganisms to retain these molecules within the cell and to selectively permit the entry of some molecules from the environment. This control is exerted at the cell membrane. A solute molecule passes across a lipid-protein membrane only if there is driving force acting on it, and some means exists for the molecule to pass through the membrane. Several means are available for the transportation of solutes through membranes, and these can be divided into two:

1.passive diffusion,
2.active transport via carrier or transport mechanism.

1.6.1 Passive transport

The driving force in this type of transportation is the concentration gradient in the case of non-electrolytes or in the case of ions the difference in electrical charge across the membrane between the internal of the cell and the outside. Yeasts take up sugar by this method. However, few compounds outside water pass across the border by passive transportation.

1.6.2 Transportation via specific carriers

Most solutes pass through the membrane via some specific carrier mechanism in which macro-molecules situated in the cell membrane act as ferryboats, picking up solute molecules and helping them across the membrane. Three of such mechanisms are known:

(i) ***Facilitated diffusion***: This is the simplest of the three, and the driving force is the difference in concentration of the solute across the border. The carrier in the membrane merely helps increase the rate of passage through the membrane, and not the final concentration in the cell.

(ii) ***Active transport***: This occurs when material is accumulated in the cell against a concentration gradient. Energy is expended in the transportation through the aid of enzymes known as permeases but the solute is not altered. The permeases act on specific compounds and are controlled in many cases by induction or repression so that waste is avoided.

(iii) ***Group translocation***: In this system the solute is modified chemically during the transport process, after which it accumulates in the cell. The carrier molecules act like enzymes catalysing group-transfer reactions using the solute as substrate. Group translocation can be envisaged as consisting of two separate activities: the entrance process and the exit process. The exit process increases in rate with the accumulation of cell solute and is carrier-mediated, but it is not certain whether the same carriers mediate entrance and efflux.

Carrier-mediated transportation is important because it is selective, and also because it is the rate-limiting step in the metabolism of available carbon and energy sources. As an increased rate of accumulation of metabolisable carbon source can increase the extent of catabolite repression of enzyme synthesis, the rate of metabolisable carbon transport may have widespread effects on the metabolism of the entire organism.

2.Derangement or Bypassing of Regulatory Mechanisms for the Overproduction of Primary Metabolites

The methods used for the derangement of the metabolic control of primary metabolites including:

(1) Metabolic control
    (a) feedback regulation
    (b) restriction of enzyme activity
(2) Permeability control.

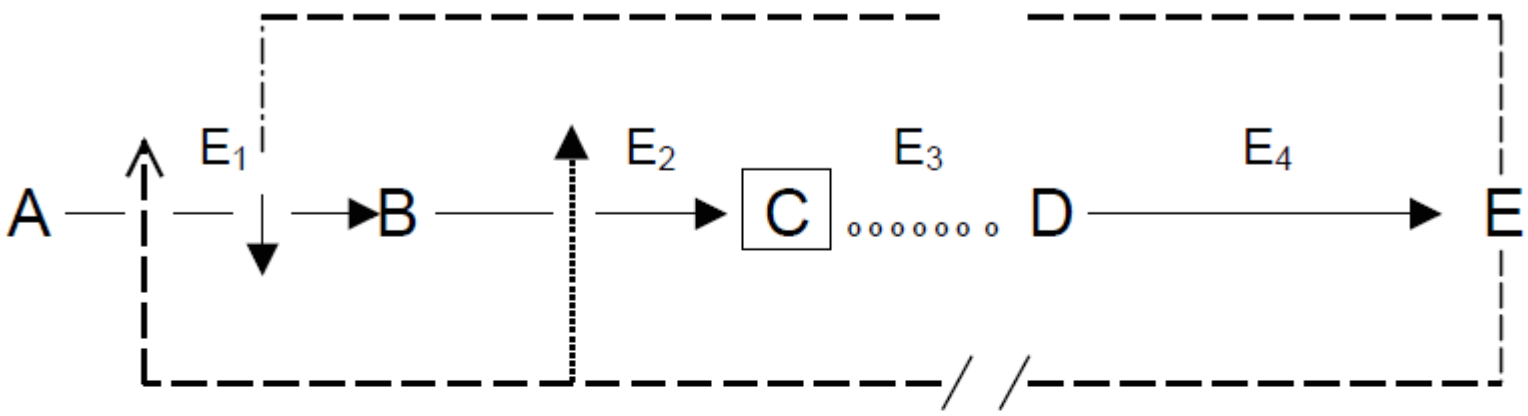
2.1 Metabolic Control

2.1.1 Feedback control

Feedback control is the major means by which the overproduction of amino acids and nucleotides is avoided in microorganisms. The basic ingredients of this manipulation are knowledge of the pathway of synthesis of the metabolic product and the manipulation of the organism to produce the appropriate mutants.

(i) **Overproduction of an intermediate in an unbranched pathway:**

The accumulation of an intermediate in an unbranched pathway is the easiest of the various manipulations to be considered. Consider the production of end-product E following the series



A, B, C, D	=	Intermediates
E_1, E_2, E_3, E_4	=	Enzymes
----------	=	Biosynthetic routes
....	=	Feedback inhibition/repression
ooooo	=	Interrupted biosynthetic route
--/ / --	=	Feedback interruption
[C]	=	Overproduced intermediate

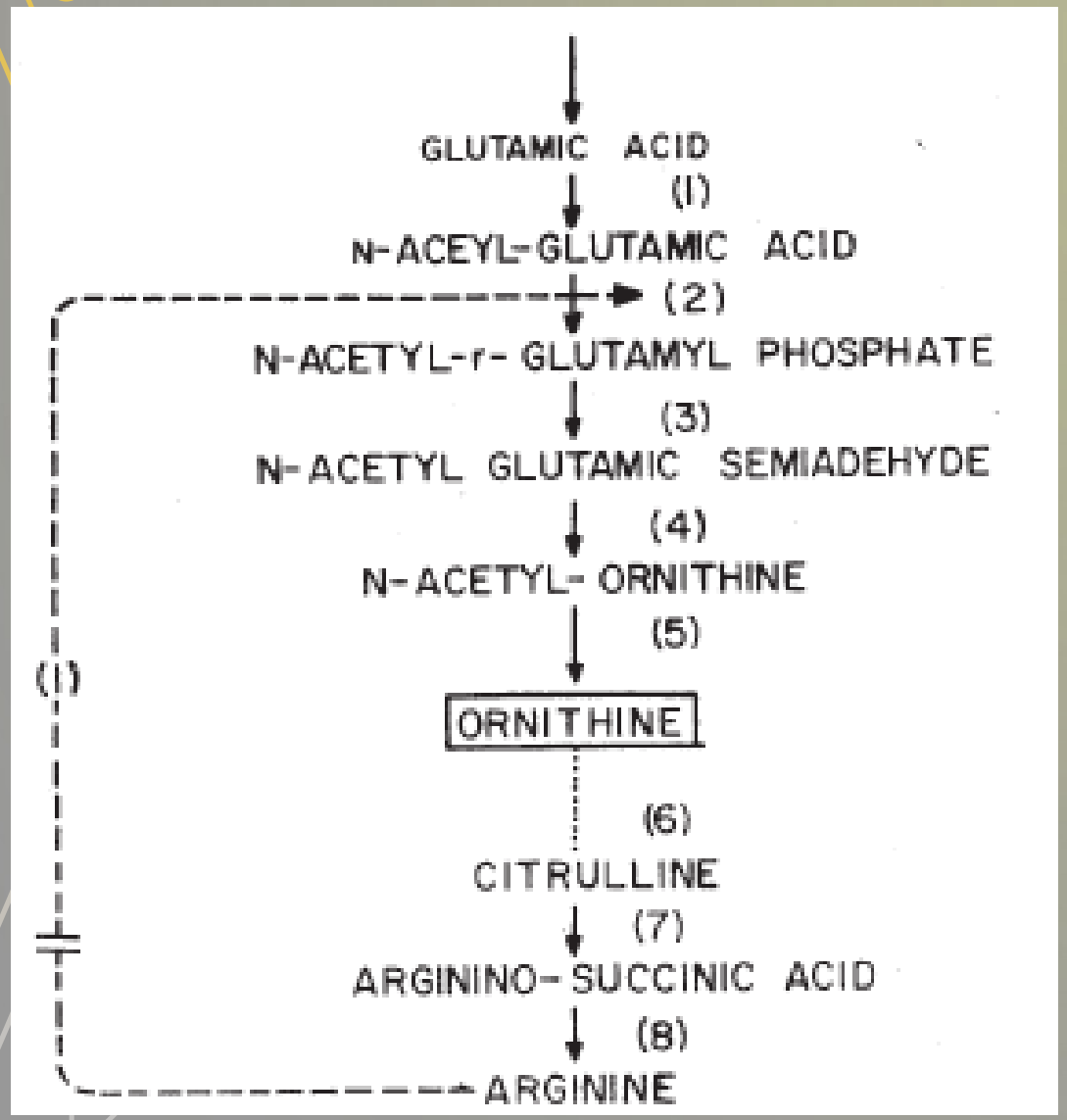
End-product E inhibits Enzyme 1 and represses Enzymes 2, 3, and 4. An auxotrophic mutant is produced which lacks Enzyme 3. Such a mutant therefore requires E for growth. If limiting (low levels) of E are now supplied to the medium, the amount in the cell will not be enough to cause inhibition of Enzyme 1 or repression of Enzyme 2 and C will therefore be over produced, and excreted from the cells. This principle is applied in the production of ornithine by a citrulline-less mutant of *Corynebacterium glutamicum* to which low level of arginine are supplied

(ii) **Overproduction of an intermediate of a branched pathway; Inosine –5- monophosphate (IMP) fermentation:**

This is a little more complicated than the previous case. Nucleotides are important as flavoring agents and the overproduction of some can be carried out.

In the pathway end products adenosine 5- monophosphate (AMP) and guanosine –5-monophsophate (GMP) both cumulatively feedback inhibit and repress the primary enzyme [1].

Scheme for the Overproduction of Ornithine by a Citrulineless Mutant of *Corynebacterium glutamicum*



1, 2, 3 = Enzymes and enzymic steps
→ = Feedback
I = Inhibition
R = Repression
..... = Dotted lines denote absence of enzymic activity
– – –/ /– = Bypass of control mechanism
[ORNITHINE) = Overproduced metabolite

(iii) **Overproduction of end-products of a branched pathway:**

The overproduction of end production of end-products is more complicated than obtaining intermediates. Among end-products themselves the production of end-products of branched pathways is easier than in unbranched pathways.

(iv) **Overproduction of end-product of an unbranched pathway:**

Two methods are used for the overproduction of the end-product of an unbranched pathway. The first is the use of a toxic analogue of the desired compound and the second is to backmutate an auxotrophic mutant.

Use of toxic or feedback resistant analogues:

In this method the organism are exposed to a mutagen then plated in a medium containing the analogue of the desired compound, which toxic to the organism. Most of the mutagenized cells will be killed by the analogue. Those which survive will be resistant to the analogue and some of them will be resistant to feedback repression and inhibition by the material whose overproduction is desired.

This is because the mutagenized organism would have been 'fooled' into surviving on a substrate similar to mutagenesis. As a result it may exhibit feedback inhibition in a medium but may be resistant to feed back inhibition, due to slight changes in the configuration of the enzymes produced by the mutant. The net effect is to modify the enzyme produced by the mutant so that it is less sensitive to feedback inhibition. Alternatively the enzyme forming system may be so altered that it is insensitive to feedback repression.

Use of reverse Mutation:

A reverse mutation can be caused in the structural genes of an auxotrophic mutant in a process known as reversion. Enzymes which differ in structure from the original enzyme, but which are nevertheless still active, often result. It has been reported that the reversion of auxotrophic mutants lacking the primary enzyme in a metabolic pathway often results in revertants which excrete the end-product of the pathway. The enzyme in the revertant is active but differs from the original enzyme in being insensitive to feedback inhibition.

2.1.2 Restriction of enzyme activity

In the tricarboxylic acid cycle the accumulation of citric acid can be encouraged in *Aspergillus niger* by limiting the supply to the organism of phosphate and the metals which form components of co-enzymes. These metals are iron, manganese, and zinc. In citric acid production the quantity of these is limited, while that of copper which inhibits the enzymes of the TCA cycle is increased.

2.2 Permeability

Ease of permeability is important in industrial microorganisms not only because it facilitates the isolation of the product but because of the removal of the product from the site of feedback regulation. If the product did not diffuse out of the cell, but remained cell-bound, then the cell would have to be disrupted to enable the isolation of the product, thereby increasing costs.

The importance of permeability is most easily demonstrated in glutamic acid producing bacteria. In these bacteria, the permeability barrier must be altered in order that a high level of amino acid is accumulated in the broth.

This increased permeability can be induced by several methods including:

***(i) Biotin deficiency*:**

Biotin is a coenyme in carboxylation and transcarboxylation reactions, including the fixation of CO_2 to acetate to form malonate. The enzyme which catalyses this is rich in biotin. The formation of malonyl COA by this enzyme (acetyl-COA carboxylase) is the limiting factor in the synthesis of long chain fatty acids. Biotin deficiency would therefore cause aberrations in the fatty acid produced and hence in the lipid fraction of the cell membrane, resulting inleaks in the membrane. Biotin deficiency has been shown also to cause aberrant forms in *Bacillus polymax*, *B. megaterium*, and in yeasts.

(ii) ***Use of fatty acid derivatives*:**

Fatty acid derivatives which are surface-acting agents e.g. tween 60 and tween 40 have actions similar to biotin and must be added to the medium before or during the log phase of growth. These additives seem to cause changes in the quantity and quality of the lipid components of the cell membrane. For example they cause a relative increase in saturated fatty acids as compared to unsaturated fatty acids.

(iii) ***Penicillin*:**

Penicillin inhibits cell-wall formation in susceptible bacteria by interfering with the crosslinking of acetylmuranmic-polypeptide units in the mucopeptide. The cell wall is thus deranged causing glutamate excretion, probably due to damage to the membrance, which is the site of synthesis of the wall.

3.Regulation of Overproduction in Secondary Metabolites

The physiological basis of secondary metabolite production is much less studied and understood than primary metabolism. Nevertheless there is increasing evidence that controls similar to those discussed above for primary metabolism also occur in secondary metabolites. Some examples will be given below:

3.1 Induction

The stimulatory effect of some compounds in secondary metabolite fermentation resembles enzyme induction. A good example is the role of tryptophan in ergot alkaloid fermentation by *Claviceps* sp. Although the amino acid is a precursor, its role appears to be more important as an inducer of some of the enzymes needed for the biosynthesis of the alkaloid. This is because analogues of tryptophan while not being incorporated into the alkaloid, also induce the enzymes used for the biosynthesis of the alkaloid. Furthermore, tryptophan must be added during the growth phase otherwise alkloid formation is severely reduced. This would also indicate that some of the biosynthetic enzymes, or some chemical reactions leading to alkaloid transformation take place in the trophophase, thereby establishing a link between idiophase and the trophophase. A similar induction appears to be exerted by methionine in the synthesis of cephalosporin C by *Cephalosporium ocremonium*.

3.2 Catabolite Regulation

Catabolite regulation as seen earlier can be by repression or by inhibition. It is as yet not possible to tell which of these is operating in secondary metabolism. Furthermore, it should be noted that catabolite regulations not limited to carbon catabolites and that the recently discovered nitrogen catabolite regulation noted in primary metabolism also occurs in secondary metabolism

3.2.1 Carbon catabolite regulation

- The regulation of secondary metabolism by carbon has been known for a long time. The glucose effect' in which production is suppressed until the exhaustion of the sugar is well known in a large number of secondary products such as penicillin and cephalosporin.
- Although the phenomenon where an easily utilizable source is exhausted before a less available is used has been described as glucose effect, other carbon sources may be preferred in two-sugar systems when glucose is absent.
- Carbon sources which have been found suitable for secondary metabolite production include sucrose (tetracycline and erythromycin), soyabena oil (kasugamycin), glycerol (butirosin) and starch and dextrin (fortimicin).

Secondary metabolites whose production is suppressed by glucose

Secondary Metabolite	*Organism*	*Non-interfering Carbon Sources*
Actinomycin	*Streptomyces antibioticus*	Galoactose
Indolmycin	*Streptomyces griseus*	Fructose
Kanamycin	*Streptomyces kanamyceticus*	Galactose
Mitomycin	*Streptomyces verticillatus*	Low glucose
Neomycin	*Streptomyces fradiae*	Maltose
Puromycin	*Streptomyces alboniger*	Glycerol
Siomycin	*Streptomyces sioyaensis*	Maltose
Streptomycin	*Steptomyces griseus*	Mannan
Bacitracin	*Bacillus licheniformis*	Citrate
Prodigiosin	*Seratia marcescens*	Galactose
Violacein	*Chromobacterium violaceum*	Maltose
Cephalosporin C	*Cephalosporium acremonium*	Sucrose
Ergot alkaloids	*Claviceps purperea*	-
Enniatin	*Fusarium sambucinum*	Lactose
Gibberellic acid	*Fusarium monoliforme*	-
Penicillin	*Penicillium chrysogenum*	Lactose

- It is fairly easy to decide whether the catabolite is *repressing or inhibiting the synthesis.*
- In *catabolite* repression the synthesis of the enzymes necessary for the synthesis of the metabolite is repressed.
- It is tested by the addition of the test substrate just prior to the initiation of secondary metabolite synthesis where upon synthesis is severely repressed.
- To test for catabolite inhibition by glucose or other carbon source it is added to a culture already producing the secondary metabolite and any inhibition in the synthesis noted

3.2.2 Nitrogen catabolite regulation

- Nitrogen catabolite regulation has also been observed in primary metabolism.
- It involves the suppression of the synthesis of enzymes which act on nitrogen-containing substances (proteases, ureases, etc.) until the easily utilizable nitrogen sources e.g., ammonia are exhausted.
- Secondary metabolites which are affected by nitrogen catabolite regulation include trihyroxytoluene production by *Aspergillus fumigatus,* bikaverin by *Gibberella fujikuroi* and cephamycins by *Streptomyces* spp.
- In all these cases nitrogen must be exhausted before production of the secondary metabolite is initiated.

Self-inhibition by secondary meabolites:

- Several secondary products or even their analogues have been shown to inhibit their own production by a feedback mechanism.
- Examples are audorox, an antibiotic active against Gram-positive bacteria, and used in poultry feeds, chloramphenicol, penicillin, cycloheximids, and 6-methylsallicylic acid (produced by *Penicillium urticae*).
- Chloramphenicol repression of its own production also shows chorismic acid inhibition by tryptophan

3.4 ATP or Energy Charge Regulation of Secondary Metabolites

- Secondary metabolism has a much narrower tolerance for concentrations of inorganic phosphate than primary metabolism.
- A range of inorganic phosphate of 0.3-30 mM permits excellent growth of procaryotic and eucaryotic organisms. On the other hand the highest level that favors secondary metabolism is 1.0 mM while the lower quantity that maximally suppresses secondary process is 10 mM
- High phosphate levels inhibit antibiotic formation hence the antibiotic industry empirically selects media of low phosphate content, or reduce the phosphate content by adding phosphatecomplexing agents to the medium.

Several explanations have been given for this phenomenon.

One of them is that phosphate stimulates high respiration rate, DNA and RNA synthesis and glucose utilization, thus shifting the growth phase from the idiophase to the trophophase. This shift can occur no matter the stage of growth of the organisms. Exhaustion of the phosphate therefore helps trigger off idiophase.

Another hypothesis is that a high phosphate level shifts carbohydrate catabolism ways fromHMP to the EMP pathway favoring glycolysis. If this is the case then NADPH would become limiting of ridiolite synthesis.

3.3 Feedback Regulation

- That feedback regulation exists in secondary metabolism is shown in many examples in which the product inhibits its further synthesis.

- An example is penicillin inhibition by lysine. Penicillin biosynthesis by *Penicillium chrysogenum* is affected by feedback inhibition by L-lysine because penicillin and lysine are end-products of a brack pathway.

- Feedback by lysine inhibits the primary enzyme in the chain, homocitrate synthetase, and inhibits the production of -aminoadipate. The addition of aminoadipate eliminats the inhibitory effect of lysine.

4.Empirical Methods Employed to Disorganize Regulatory Mechanisms in Secondary Metabolite Production

- Metabolic pathways for secondary metabolites are becoming better known and more rational approaches to disrupting the pathways for overproduction are being employed.

- More work seems to exist with regard to primary metabolites. Methods which are used to induce the overproduction of secondary metabolites are in the main empirical.

- Such methods include mutations and stimulation by the manipulation of media components and conditions.

(i) Mutations:

Naturally occurring variants of organisms which have shown evidence of good productivity are subjected to mutations and the treated cells are selected randomly and tested for metabolite overproduction. The nature of the mutated gene is often not known.

(ii) **Stimulatory effect of precursors:**

In many fermentations for secondary metabolites, production is stimulated and yields increased by the addition of precursors. Thus penicillin production was stimulated by the addition of phenylacetic acid present in corn steep liquor in the early days of penicillin fermentation. For the experimental synthesis of aflatoxin by *Aspergillus parasiticus*, methionine is required. In mitomycin formation by *Streptomyces verticillatus*, L-citurulline is a precursor.

(iii) ***Inorganic compounds***:

Two inorganic compounds which have profound effects of fermentation for secondary metabolites are phosphate and maganese. The effect of inorganic phosphate has been discussed earlier. In summary, while high levels of phosphate encourage growth, they are detrimental to the production of secondary metabolites. Manganese on the other hand specifically encourages idiophase production particularly among bacilli, including the production of bacillin, bacitracin, mycobacillin, subtilin, D-glutamine, protective antigens and endospores. Surprisingly, the amount needed are from 20 to several times the amount needed for growth

(iv) ***Temperature*:**

While the temperature range that permits good growth (in the trophophase) spans about 25°C among microorganisms, the temperature range within which secondary metabolites are produced is much lower, being in the order of only 5-10°C. Temperatures used in the production of secondary metabolites are therefore a compromise of these situations. Sometimes two temperatures – a higher for the trophophase and a lower for the idiophase are used.