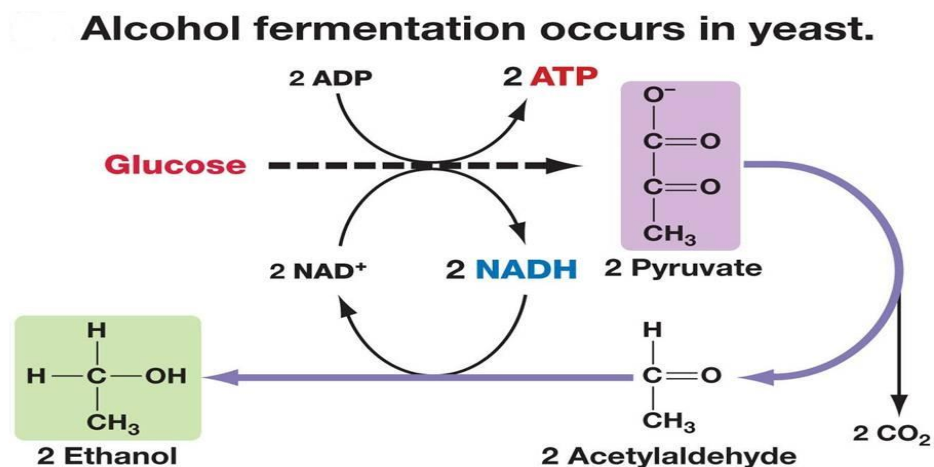
Unit - 2 Bacterial fermentations and Biosynthesis

❖ Alcoholic fermentation

In alcoholic fermentation pyruvate is converted to *ethanol* and *carbon dioxide.* This process is characterise of yeasts, particularly strains of *Saccharomyces cerevisiae.* It is also found in some moulds and in the Mucorales, but is comparatively rare in bacteria. In the bacterium *Pseudomonas,* pyruvate is produced through the Entner-Duodoroff pathway. It is then metabolized to *ethanol* through *acetaldehyde.*

A molecule of *glucose* yields two molecules of pyruvate through the EMP pathway. Pyruvate metabolism takes place in two steps.

i. *Pyruvate* is first decarboxylated, yielding *acetaldehyde* and *carbon dioxide.* The reaction is catalysed through the enzyme *pyruvate decarboxylase,* with *thiamine pyrophosfhate* (TPP) as the coenzyme.
ii. *Acetaldehyde* is then reduced to *ethanol* by NADH+H+ ($NADH_2$) and NAD+ is regenerated. The catalysing enzyme is *alcohol dehydrogenase.*



It will be seen that the hydrogens removed during glucose metabolism are accepted by NAD, which is reduced to $NADH_2$. It is essential, that NAD be regenerated so that it can pick up more hydrogens. If this did not happen glycolysis would stop, resulting in the death of *he organism.

Regeneration of NAD takes place when *acetaldehyde* is reduced to *ethanol.* Alcoholic fermentation is of economic importance in the production of beverages and in raising bread.

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❖ **Lactic acid fermentation**

Lactic acid fermentation is a one-step reaction similar to glycolysis of mammalian cells. *Pyruvic acid* is reduced to *lactic acid,* the reaction being catalysed *by pyruvate reductase.* Lactic acid fermentation is characteristic of the lactic acid bacteria *(Lactobacillaceae)* which cause spoilage of food. Although morphologically heterogeneous, the bacteria are characterized by the fact that they produce *lactic acid* as the end product.

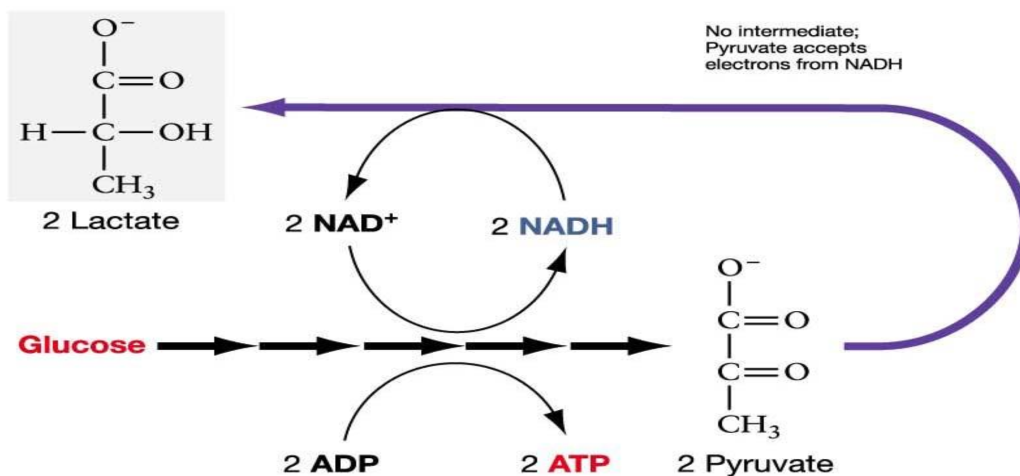
Glucose and other six-carbon sugars are converted into cellular energy and the metabolite lactate. It is an anaerobic fermentation reaction that occurs in some bacteria and animal cells, such as muscle cells.

The *lactobacilli* are divided into two groups, *homofermentative* and *heterofermentative* strains. The demarcation between the two groups is indefinite in some cases.

a) Homolactic fermentation

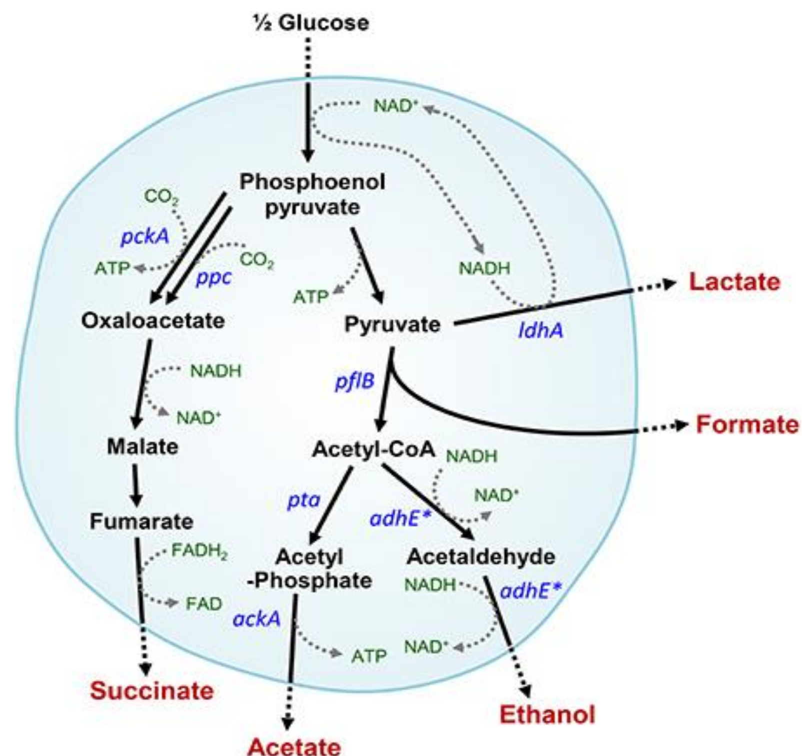
Homolactic fermentation is found in members of the genera *Strepto-coccus, Leuconostoc, Pediococcus* and *Lactobacillus.* In *homolactic fermentation*, one molecule of glucose is ultimately converted to two molecules of lactic acid.

Lactate dehydrogenase catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+.



b) Heterolactic fermentation (mixed acid fermentation)

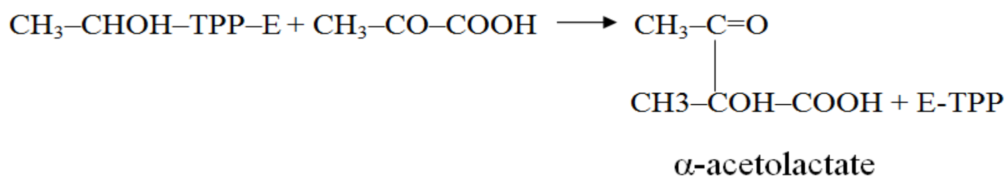
Heterolactic fermentation is done by Lactobacillus and Leuconostoc. Early cleaving of the glucose molecule into ribulous 5 phosphate prevents glycolysis from happening in the first place. CO_2, ethanol, and lactate are all eventual products of heterolactic fermentation. Mixed-acid fermentation starts with glycolysis, then the pyruvate is reduced to Acetyl CoA which is reduced to acetate and ethanol. Other pyruvate is reduced to lactate and succinate in the presence of CO_2. CO_2 and H_2 are released from formate produced from the reduced pyruvate. Mainly the pathway of the *Enterobacteriaceae.* End products are a mixture of **lactic acid**, **acetic acid**, **formic acid**, **succinate** and **ethanol**, with the possibility of gas formation (**CO_2** and **H_2**) if the bacterium possesses the enzyme formate dehydrogenase, which cleaves formate to the gases.



❖ Butandiol fermentation

- Some *Erwinia, Klebsiella* and *Serratia* species produce 2, 3-butanediol in addition to lactate and ethanol from pyruvate, the EMP pathway product.
- Pyruvate is the substrate for one of three enzymes in these bacteria. These are lactate dehydrogenase, pyruvate:formate lyase and α-acetolactate synthase.

- The reactions catalyzed by these enzymes are similar to those of the mixed acid fermentation except for α-acetolactate synthase. This enzyme condenses two molecules of pyruvate to α-acetolactate that is further decarboxylated and reduced to 2, 3-butanediol.
- A similar metabolism is found in *Bacillus polymyxa* during vegetative growth and in lactic acid bacteria fermenting citrate.
- The first enzyme of this metabolism, α-acetolactate synthase, is best characterized in Gram-negative facultative bacteria. This enzyme has thiamine pyrophosphate as a cofactor to catalyze the following reactions:



α-acetolactate

- Under anaerobic conditions, 2, 3-butanediol-producing facultative anaerobes produce acidic products, lowering the external and intracellular pH.
- α-acetolactate synthase, which catalyzes the first reaction to produce 2,3-butanediol, has an optimum at pH 6.0.
- When the intracellular pH drops, this enzyme becomes active to divert carbon flux from acid production to the neutral solvent.
- An enzyme catalyzing the same reaction catalyzes the first reaction of valine synthesis from pyruvate.
- This enzyme is referred to as the pH 8.0 enzyme while the enzyme involved in 2, 3-butanediol synthesis is referred to as the pH 6.0 enzyme.
- *Klebsiella pneumoniae, Klebsiella oxytoca* and *Enterobacter aerogenes* ferment glycerol to various products including 2, 3-butanediol.

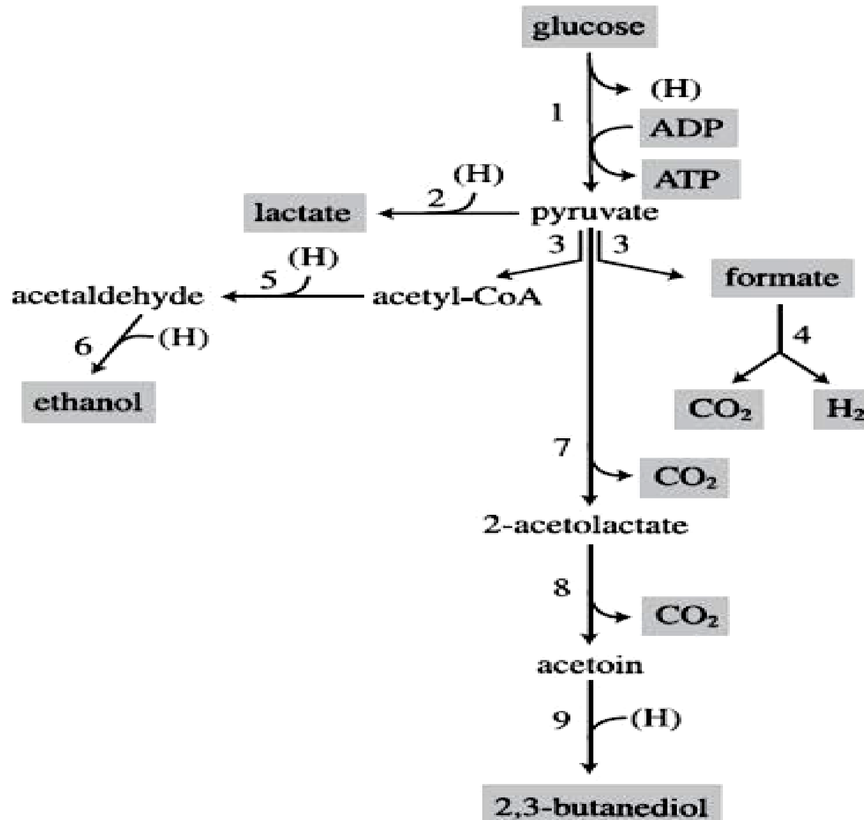


Fig. Butanediol fermentation by some Gram negative facultative anaerobic bacteria. Facultative anaerobes belong to the genera Erwinia, Klebsiella and Serratia and produce 2, 3-butanediol in addition to lactate and ethanol. 1, EMP pathway; 2, lactate dehydrogenase; 3, pyruvate:formate lyase; 4, formate:hydrogen lyase; 5, acetaldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, α-acetolactate synthase; 8, α-acetolactate decarboxylase; 9, 2,3-butanediol dehydrogenase.

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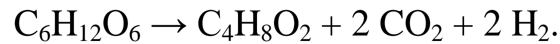
❖ Butyric acid fermentation

- Butyrate is produced as end-product of a fermentation process solely performed by obligate anaerobic bacteria.
- Examples of butyrate-producing species of bacteria: *Clostridium butyricum, Clostridium kluyveri, Clostridium pasteurianum, Fusobacterium nucleatum, Butyrivibrio fibrisolvens, Eubacterium limosum.*
- The pathway starts with the glycolytic cleavage of glucose to two molecules of pyruvate, as happens in most organisms. Pyruvate is then oxidized into acetyl coenzyme A using a unique mechanism that involves

an enzyme system called pyruvate-ferredoxin oxidoreductase. Two molecules of carbon dioxide (CO_2) and two molecules of elemental hydrogen (H_2) are formed as waste products from the cell.

- Then, ATP is produced, as can be seen, in the last step of the fermentation. Three molecules of ATP are produced for each glucose molecule, a relatively high yield. The balanced equation for this fermentation is

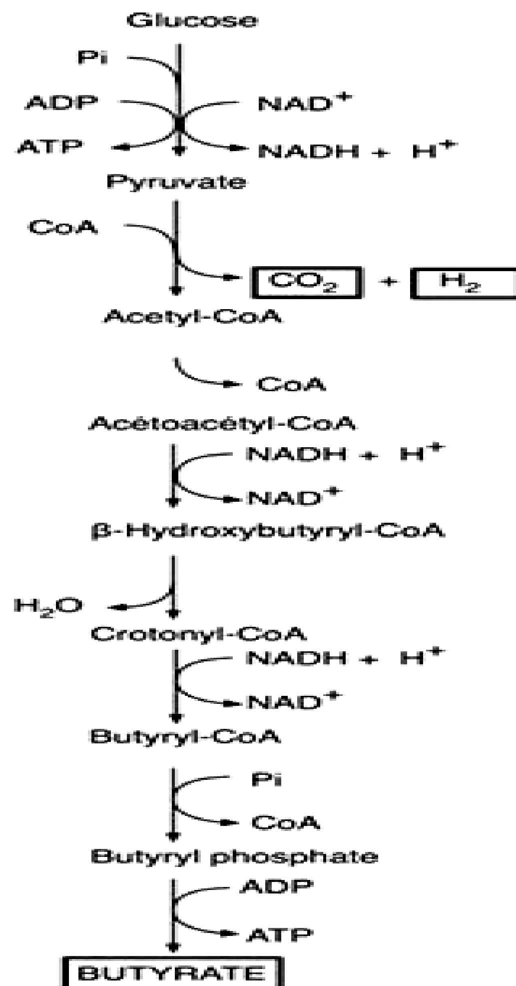


$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2\ CO_2 + 2\ H_2.$$

- Several species form acetone and *n*-butanol in an alternative pathway, which starts as butyrate fermentation. Some of these species are: *Clostridium acetobutylicum*, *Clostridium beijerinckii, Clostridium tetanomorphum, Clostridium aurantibutyricum*
- These bacteria begin with butyrate fermentation, as described above, but, when the pH drops below 5, they switch into butanol and acetone production to prevent further lowering of the pH. Two molecules of butanol are formed for each molecule of acetone.
- The change in the pathway occurs after acetoacetyl CoA formation. This intermediate then takes two possible pathways:

acetoacetyl CoA → acetoacetate → acetone

acetoacetyl CoA → butyryl CoA → butyraldehyde → butanol

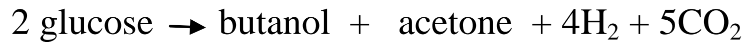


❖ Acetone Butanol fermentation

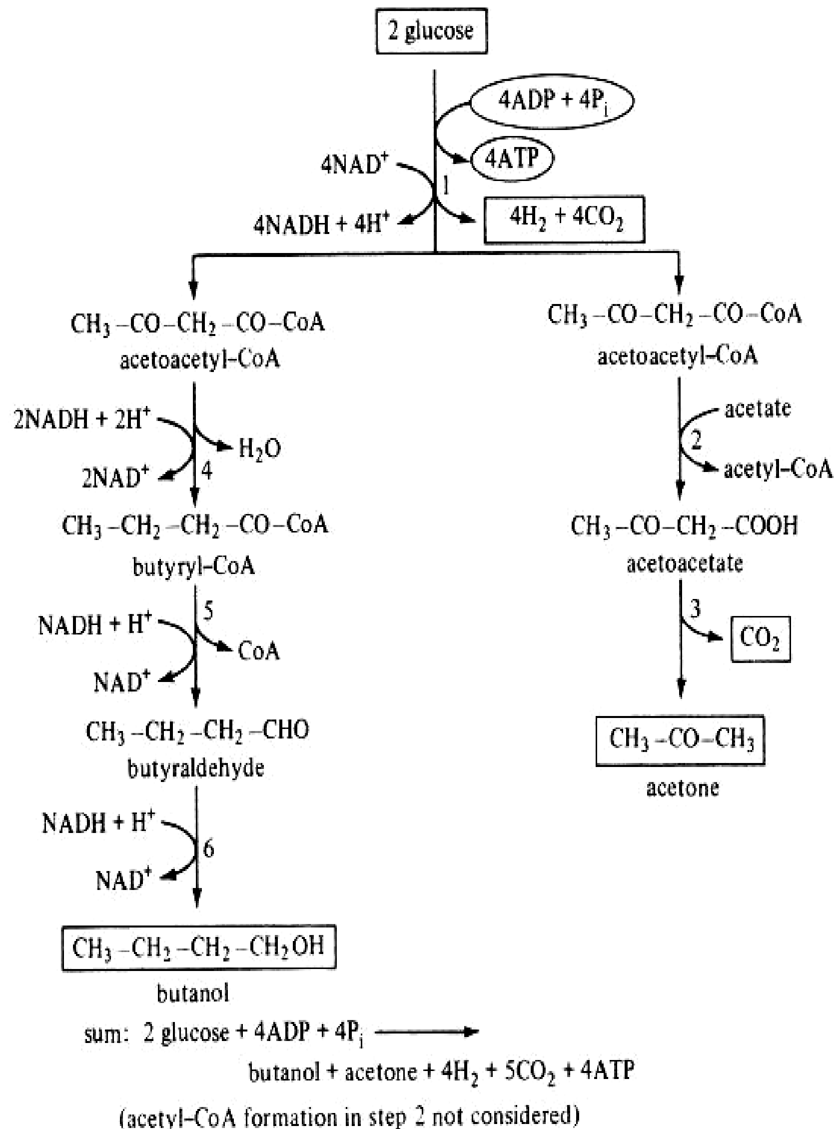
- A number of butyrate producing clostridia form small amounts of *n*-butanol.
- With a few species, however, a real shift from butyrate production to solvent production (*n*-butanol and acetone or isopropanol) can be observed under certain conditions.
- These species include *C. acetobutylicum*, *C. beijerinckii*, *C. tetanomorphum*, and *C. aurantibutyricum*.
- The most prominent species among these is *C. acetobutylicum*, which has been used on an industrial scale for the synthesis of *n*-butanol and acetone from molasses.
- During butanol fermentation the glycolytic reducing equivalents are reoxidized by reduction of butyryl-CoA to butanol via butyraldehyde. Therefore, 2 mol hexose have to be oxidized to gain the electrons required. The spare acetoacetyl-CoA is converted to acetoacetate and the

CoA is transferred to acetate, giving rise to acetyl-CoA and opening the opportunity for additional ATP synthesis in the acetate kinase reaction.

- Acetoacetate is decarboxylated to acetone, the second product of this fermentation. The overall reaction is:



$$2\ \text{glucose} \rightarrow \text{butanol} + \text{acetone} + 4H_2 + 5CO_2$$



Formation of acetone and butanol by C. acetobutylicum. 1, Reactions as in butyrate fermentation pathway (Phosphotransferase system and Embden-Meyerhof-Parnas pathway, pyruvate-ferredoxin oxidoreductase, hydrogenase, acetyl-CoA-acetyltransferase (thiolase)); 2, acetoacetyl-CoA: acetate coenzyme A transferase; 3, acetoacetate decarboxylase; 4, L(+)-,ß-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase; 5, butyraldehyde dehydrogenase; 6, butanol dehydrogenase.

❖ Nucleotide Biosynthesis

I. Biosynthesis of purine nucleotides: -The precursors of the purine ring were first established in the laboratory of Buchanan by administering **labeled** isotopes of carbon and nitrogen compounds to pigeons. These studies revealed the following general picture of the origin of the purine nucleus. Carbon atoms 2 and 8 are derived from *format* or the 1-carbon unit arising from various compounds, e.g., *serine* and *glycine*. Carbon atom 6 is derived from CO**2**. Carbon atoms 4 and 5 come from *carboxyl* and *methylene carbons* of *glycine*, respectively. Nitrogen 7 is also derived from *glycine*. It is apparent that glycine is incorporated as a whole. Nitrogen atom 1 is derived from *amino nitrogen* of *aspartic acid* and nitrogens at position 3 and 9 come from the *amide nitrogen*

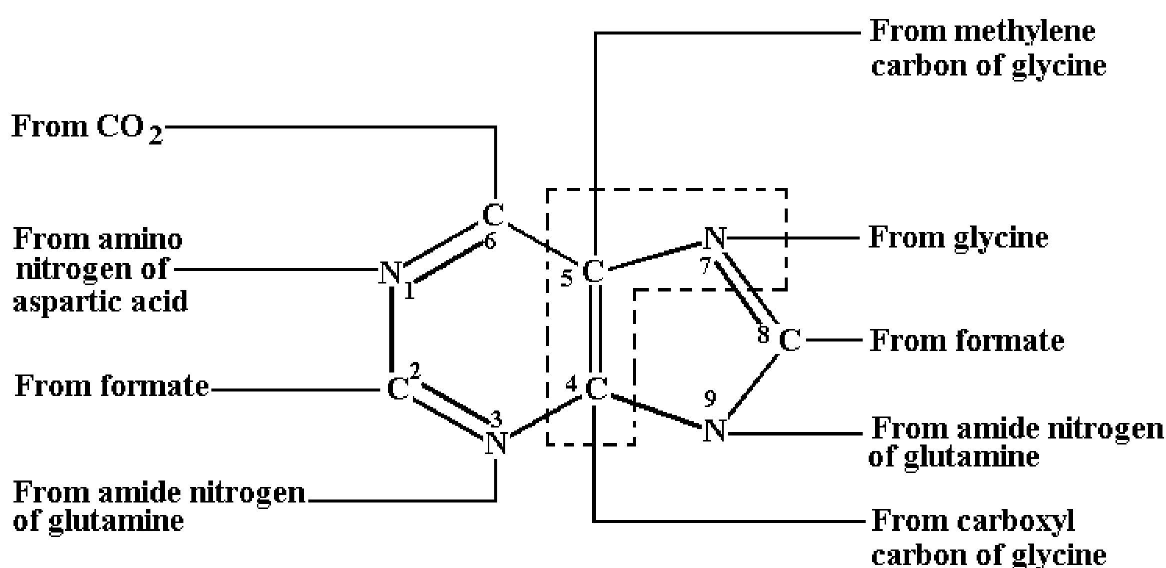


Fig. - Origin of carbon and nitrogen atoms of the purine ring from different precursors.

of *glutamine* (fig.).

• De novo synthesis of inosinic acid.

Inosinic acid is the first product formed with a complete purine ring structure in the biosynthesis of purine nucleotides. The general route for purine biosynthesis has been studied in many species and is essentially the same in all organisms. The detailed biosynthetic pathway, for which the enzymes have now been isolated and studied, is indicated in above Fig. The pathway consists of a series of successive reactions by which the purine ring system is formed on *carbon-1* of *ribose 5-phosphate*. This directly leads to the formation of

purine ribonucleotides. Free purines or nucleosides never appear as intermediates in this pathway.

(a) **5-Phosphoribosyl-1-pyrophosphate(PRPP):**

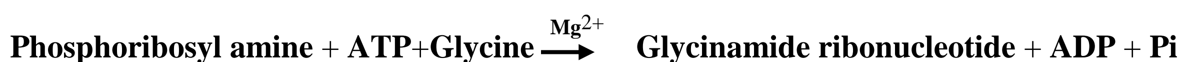
ATP-dependent pyrophosphorylation of ribose-5-phosphate gives *5-phosphoribosyl-1-pyrophosphate*. This compound is a key substance in the biosynthesis of both purine and pyrimidine nucleotides. The reaction is interesting in that it is catalysed by a *kinase that transfers pyrophosphate* rather than phosphate from ATP.

(b) **5-Phosphoribosyl-1-amine:**

The second step of this pathway involves the glutamine-dependent amination of PRPP, yielding a labile amino sugar, 5-*phosphoribosyl-1-amine* in presence of *glutamine phosphoribosyl pyrophosphate amido-transferase.* The reaction incorporates a nitrogen atom into position 9 of the purine ring. The amination step result in the inversion of the configuration at carbon 1 of ribose, since *purine ribosides* are of the β-configuration while *5-phosphoribiosyl-pyrophosphate has the* ∝-configuration. Hence, an N-glycosidic bond of proper stereochemistry is introduced early in the reaction sequence. This reaction is the "*committed step"* in purine biosynthesis, and is subject to feed back inhibition. The enzyme purified from *A. aerogenes* was inhibited by low concentrations of purine ribonucleotides.

(c) **Glycinamide ribonucleotide:**

The next reaction is catalysed by *phosphoribosyl Glycinamide synthetase.* This is an ATP-dependent reaction wherein the entire structure of glycine is conjugated through the formation of an amide linkage with 5-phosphoribosyl-1-amine.the enzyme isolated from A.*aerogenes* is not inhibited by purine ribonucleotides.



(d) N-Formylglycinamide ribonucleotide:

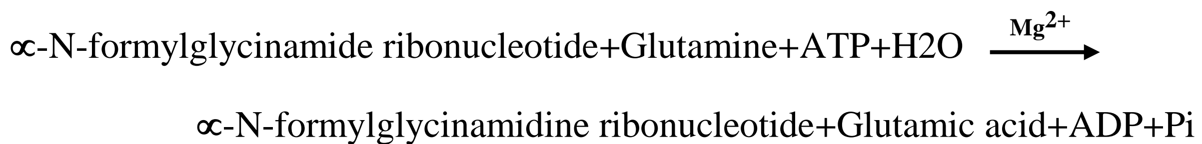
Glycinamide ribonucleotide is formulated to yield **α-N-formylglycinamide ribonucleotide**. This reaction is catalysed by *Glycinamide*

ribonucleotide transformylase, the enzyme transfers a formyl residue from N5, and N10-methenyltetrahydrofolate.

Glycinamide ribonucleotide + N^5, N^{10}-methenyltetrayrofolate+H_2O

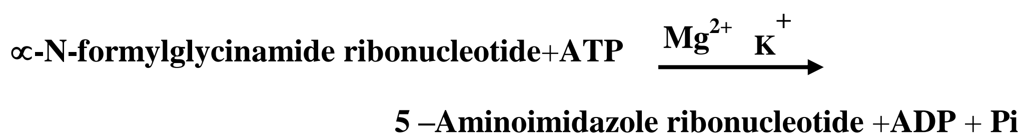
(e) **∝-N-formylglycinamide ribonucleotide:**

The next step involves transfer of the amide group from glutamine to ∝–*N-formylglycinamide ribonucleotide* to from ∝–*N-formylglyciamidine ribonucleotide.* This is the second glutamine-dependent amination, which inserts the nitrogen atom at position 3 of the purine ring.



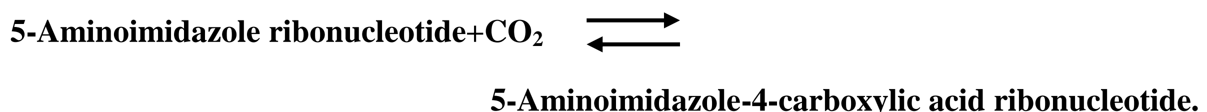
(f) **5-Aminoimidazole ribonucleotide:**

∝-N-formylglycinamidine ribonucleotide has all the structural features of the imidazole ring of purines. This ring is closed by an ATP-dependent dehydration catalysed by the enzyme *5'-phosphoribosyl formylglycinamidine cycloligase* to yield *5-aminoimidazole ribonucleotide.*



(g) **5-Aminoimidazole-4-carboxylic acid ribonucleotide:**

A molecule of CO**2** is now incorporated through a reaction catalysed by *aminoimidazole ribonucleotide carboxylase* to yield *5-aminoimidazole-4-carboxylic acid ribonucleotide.*



(h) **5-Aminoimidazole-4-N- succinocarboxamide Ribonucleotide:**

The conversion of 5-aminoimidazole-4-carboxylic acid ribonucleotide to the corresponding amide occurs in a two-step process. First, an *amide linkage* between the substrate ad aspartic acid is introduced in an ATP-dependent

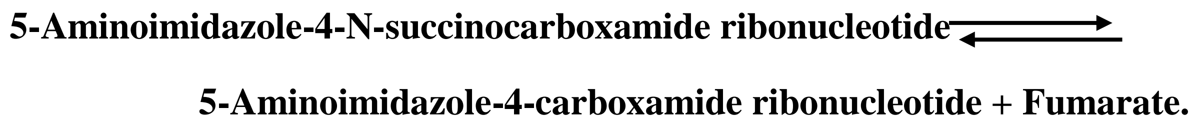
reaction catalysed by the enzyme *5'-phosphoribosyl-4-carboxy-5-aminoimidazole-L-aspartate ligase.* This produces the intermediate *5-aminoimidazole-4-N-succinocarboxamide ribonucleotide.*

5-Aminoimidazole-4-N-suceinocarboxamide ribonucleotide + Aspartic acid + ATP

$\xrightleftharpoons{Mg^{2+}}$ 5-Aminoimidazole-4-N- succinocarboxamide ribonucleotide + ADP + Pi

(i) **5-Aminoimidazole-4-carboxamide ribonucleotide:**

5-Aminoimidazole-4-N-succinocarboxamide ribonucleotide subsequently undergoes an *elimination reaction* wherein the carbon skeleton of the asparatate molecule is eliminated as *fumarate*. This inserts the nitrogen atom at position 1 of the purine ring.

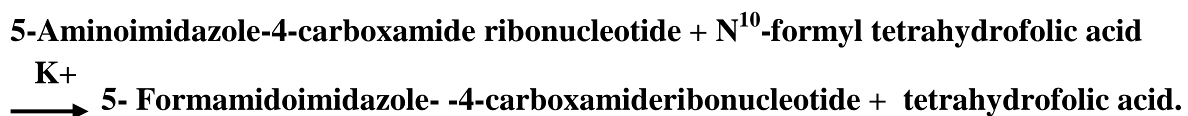


5-Aminoimidazole-4-N-succinocarboxamide ribonucleotide $\rightleftharpoons$

5-Aminoimidazole-4-carboxamide ribonucleotide + Fumarate.

(j) **5-Formamidoimidazole-4-carboxamide ribonucleotide:**

The last carbon atom required to yield all elements of the purine ring system is now introduced by the formylation of 5-aminoimidazole-4-carboxamide ribonucleotide. The reaction is catalysed by *transformylase* with N^{10}-formyltetrahdrofolate as formyl donor.



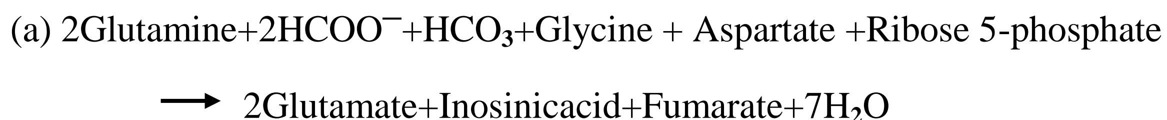
5-Aminoimidazole-4-carboxamide ribonucleotide + N^{10}-formyl tetrahydrofolic acid

$\xrightarrow{K+}$ 5- Formamidoimidazole- -4-carboxamideribonucleotide + tetrahydrofolic acid.

(k) **Inosinic acid:**

Closure of the ring with dehydration by the enzyme *inosinicase* yields inosinic acid (hypoxanthine ribonucleotide).

The synthesis of inosinic acid from elementary precursors can be regarded as the result of the sum of the following artificial, composite equations:

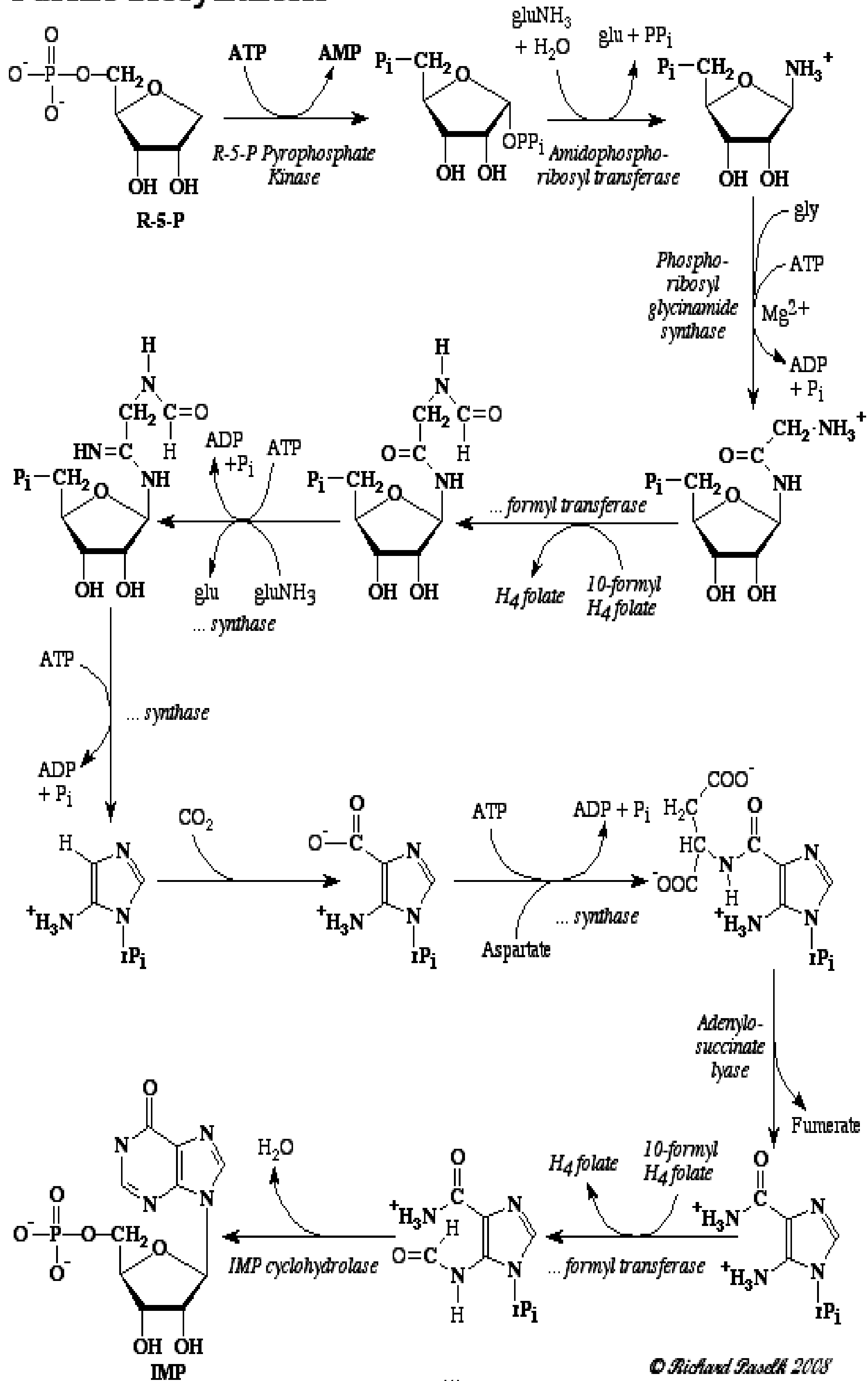


(a) 2Glutamine+2$HCOO^-$+HCO_3+Glycine + Aspartate +Ribose 5-phosphate

$\longrightarrow$ 2Glutamate+Inosinicacid+Fumarate+7H_2O

(b) 7ATP +7H_2O $\longrightarrow$ 6ADP + 6Pi + AMP + PPi + $7H^+$

Purine Biosynthesis



B.

Conversion of inosinic acid to adenine and guanine ribonucleotides.

Formation of adenylic acid from inosinic acid proceeds via a two-step reaction. In the first step the formation of adenylosuccinate *(IMP: L-aspartate ligase),* with participation of aspartic acid and GTP. In the second step adenylosuccinate is cleaved to yield adenylic acid and fumaric acid. This reaction is catalysed by adenylosuccinate (*adenylosuccinate AMP-lyase*). This is probably the same enzyme, which cleaves fumarate from 5-aminoimidazole-4-N-succinocarboxamide ribonucleotide.

Conversion of inosinic acid to guanylic acid also proceeds in a two-step process. In the first step inosinic acid is oxidized to xanthylic acid by the enzyme *inosinic acid dehydrogenase*

(*IMP: NAD oxidoreductase*) with NAD as the electron acceptor. In the second step xanthylic acid is converted to guanylic aced with ATP and ammonia or glutamine. The reaction is catalysed by *guanylic acid synthetase* (xanthosine-5'-phosphate: ammonia ligase). The biochemical transformations converting inosinic acid to adenylic and guanylic acids are indicated in Fig.

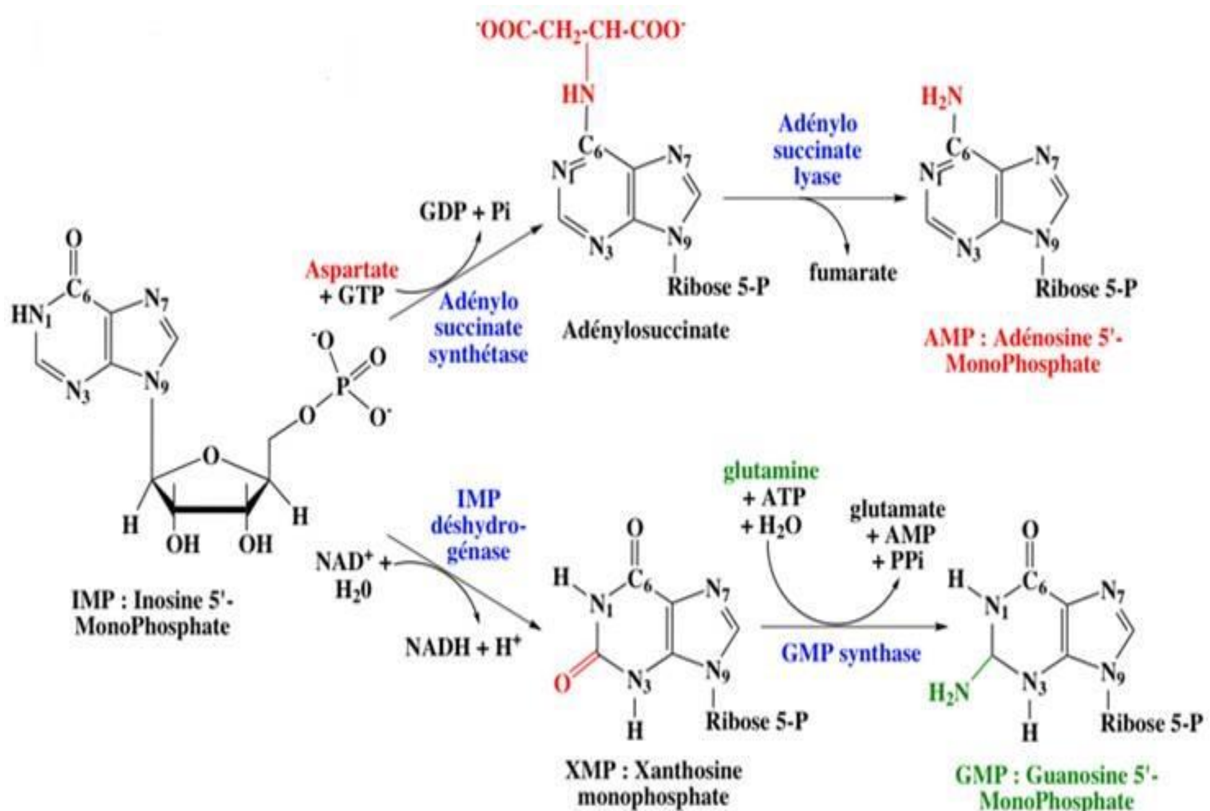


Fig. **Biosynthesis of adenylic and guanylic acids from inosinic acid.**

II. **BIOSYNTHESIS OF PYRIMIDINE NUCLEOTIDES**

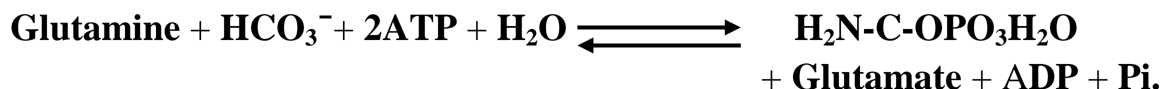
A major distinction between the metabolic routes leading to the synthesis of purine and pyrimidine nucleotides is the timing in the formation of the N-glycosidic bond. This bond is formed in the very early steps in purine synthesis, and the ring system is built upon this foundation. In contrast, the complete pyrimidine nucleus is first synthesized and then attached to ribose-5-phosphate. *Orotic acid,* which contains the pyrimidine nucleus, is the key intermediate introduced in the N-glycosidic linkage. The main enzymatic pathways leading to the formation of orotic acid and its conversion to uridylic acid have been elucidated.

A. **De novo synthesis of orotate.**

In the formation of orotic acid, ammonia is incorporated into the nitrogen atom at position 1 of orotate, carbon dioxide into position 2 and L-asparate into the remainder of the orotate molecule.

(a) Carbamoyl phosphate:

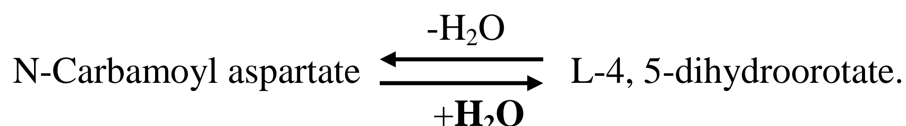
The initial step in pyrimidine biosynthesis is the formation of Carbamoyl phosphate. Enzymes utilizing glutamine as the amino group donor synthesize carbamoyl phosphate. *Carbamoyl phosphate synthetase* isolated from *E. coli* catalyses a reaction in which two molecules of ATP are consumed per molecule of Carbamoyl phosphate synthesized. The enzyme utilizes ammonia as well as glutamine as the amino group donor. However, the affinity for glutamine is much greater than for ammonia.



Glutamine + $\mathbf{HCO_3^-}$ + **2ATP** + $\mathbf{H_2O}$ $\rightleftharpoons$ $\mathbf{H_2N\text{-}C\text{-}OPO_3H_2O}$ + **Glutamate** + **ADP** + **Pi.**

(b) L-4, 5-Dihydroortate:

N-Carbamoyl aspartate is converted to L-4, 5-dihydroorotic acid. This brings about ring closure catalysed by *dihydro-orotase.*



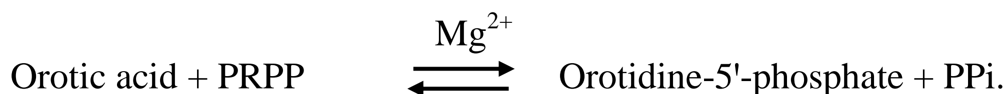
N-Carbamoyl aspartate $\underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}}$ L-4, 5-dihydroorotate.

(c) Orotic acid:

L-4, 5-dihyroorotate is oxidised to orotic acid by the enzyme *dihydro-orotate dehydrogenase.* The enzyme is flavoproteins and transfers the electrons to NAD^+.

(d) Orotidine-5'-phosphate*:*

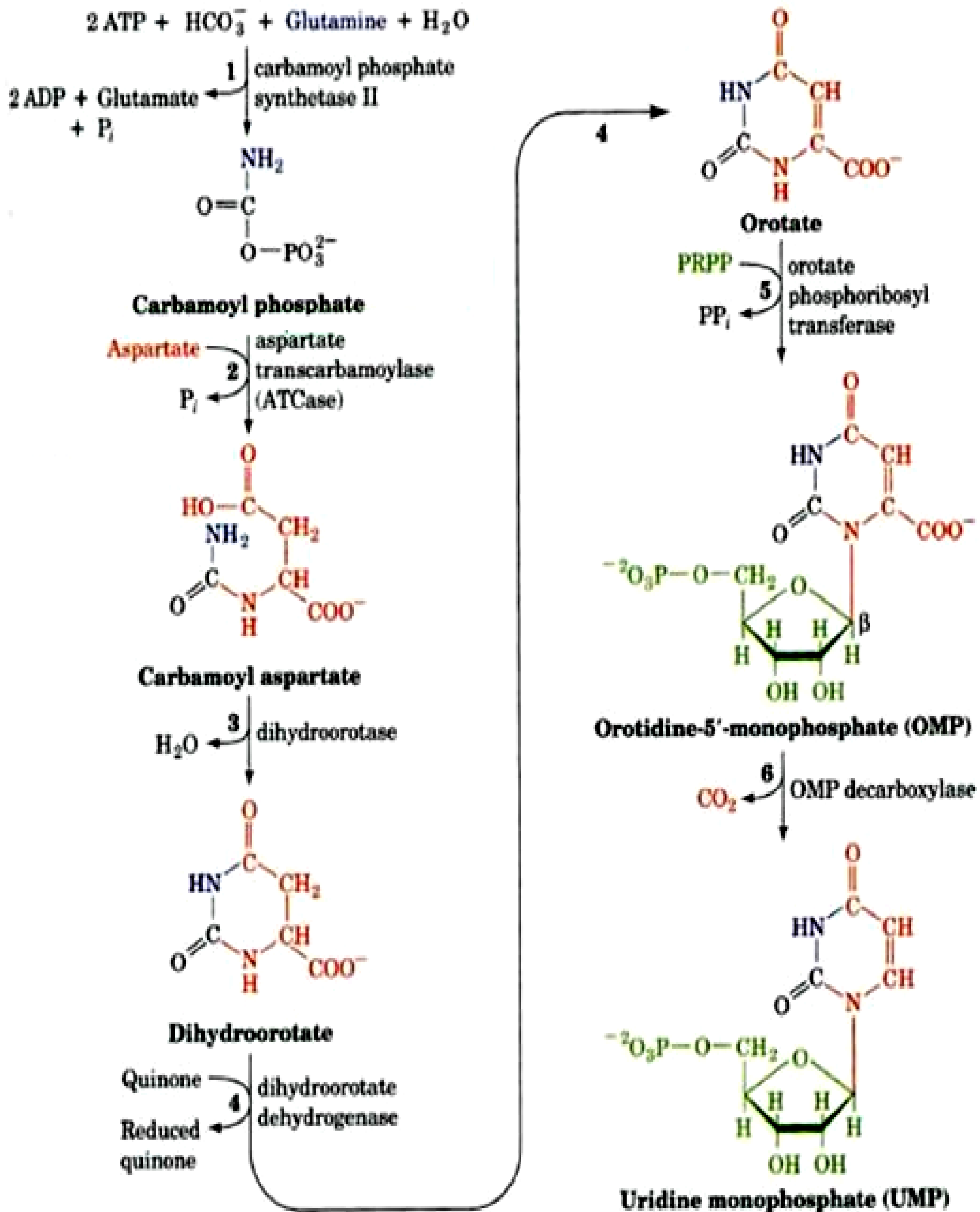
Orotic acid combines with a phosphoribosyl group from PRPP to form the first nucleotide orotidine-5'-phosphate. The reaction is catalysed by *orotate phosphoribosyl transferase.* The enzyme is specific for orotic and does not react with precursors of orotic acid or related pyrimidines.



(e) Uridylic acid:

Orotidine-5'-phosphate is decarboxylated to yield uridylic acid. This irreversible reaction is catalysed by *oretidine-5'-phosphate decarboxylase.*

The metabolic pathway leading to the synthesis of uridylic acid is given in Fig.



III. Biosynthesis of deoxyribonucleotides

The purine and pyrimidine deoxyribonucleotides of DNA are derived by direct conversion from corresponding ribonucleotides. The formation of deoxyribonucleotides. Thus formation of deoxyribose from ribose occurs at the nucleotide level. Two distinct classes of *ribonucleotide reductases* have been

described. The enzyme isolated from E.coli, and the other by the enzyme represents one class from *L.leichmannii.* Both enzymes catalyse the substitution of the –OH group at position2' of ribose by a hydrogen, with retention of configuration at the carbon atom.

Reichard and his co-workers have investigated synthesis of deoxyribonucleotides in E. coli. The basic aspects of this system can be summarised as follows:

1. The substrates for reduction are *ribonucleoside diphosphates.*
2. The reduction is catalysed by *ribonucleoside reductase.*
3. The specific hydrogen donor for the reduction is *thioredoxin* in the sulphydryl form. Thioredoxin is a small protein (108 amino acids MW=1,200 in *E. coli),* containing an oxidation-reduction active disulphide with the sequence (-Cys-Gly-Pro-Cys). This sequence represents the active centre of thioredoxin isolated from all sources.
4. The oxidised form of thioredoxin is reduced in the cell by a specific FAD-protein, *thioredoxin reductase,* with NADPH as the hydrogen donor. *Thioredoxin reductase* from *E. coli* contains two moles of FAD, and has a molecular weight of 66,000, with the general structure $(FAD)_2$. Each polypeptide chain contains one oxidation-reduction active disulphide (-Cys-Ala-Thr-Cys). The reaction mechanism involves a stepwise reduction of FAD and oxidation-reduction disulphides. Since oxidation-reduction active disulphides also appear in *ribonucleotide reductase* the overall sequence of electron transfer from NADPH to ribonucleotides involves a shuttle of S_2 / $(SH)_2$ interchanges as described in Fig.
5. All four nucleoside diphosphates, ADP, GDP, CDP and UDP, are corresponding deoxyderivatives by the same *reductase system.*

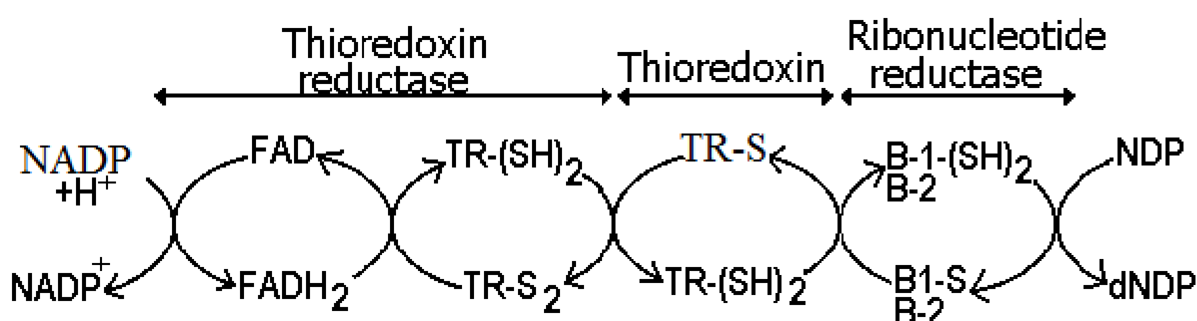


Fig. Involvement of oxidation-reduction active disulphides in ribonucleotide reduction in *E.coli.*