INTRODUCTION

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1.1: Importance of Nucleic acids

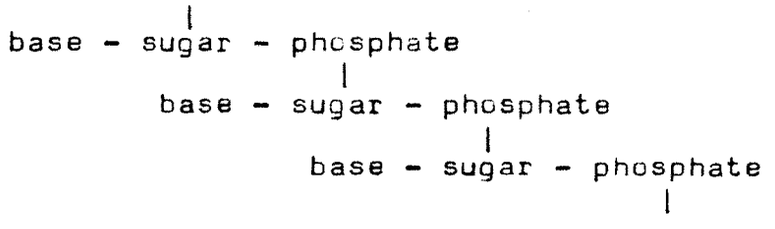
Nucleic acids, DNA and RNA are quite essential to the maintainance of life processes. Occurance of DNA is universal except in some viruses. Function of DNA is to restore the complete genetic information required to specify the structure of all the proteins. DNA acts as a genetic material, which is responsible for transfer of hereditory characters from one generation to the next generation (1).

In all types of organisms, whether microbes, plants or animals, the primary role of RNAs is in the protein biosynthesis. Different types of RNAs, m-RNA, r-RNA and t-RNA perform specific functions during biosynthesis of proteins (2).

Some viruses have RNA as the genetic material e.g. tobaco mosaic virus, poliovirus etc. (6).

1.2: Nucleic acids

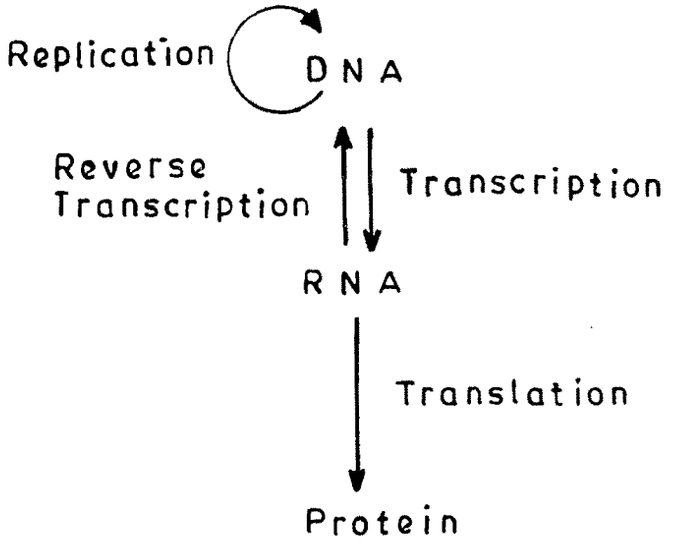
In 1869 Meisher isolated nucleic acids, which he called nuclein (2). The nucleic acids are linear polymers in which the nucleotide residues are linked by phosphodiester bonds. The general structure of this is,



Nucleic acids are present in all living organisms, whether plants, animals or viruses. They are associated with proteins to form nucleoproteins. There are two types of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

DNA on hydrolysis yields the purine bases, adenine and guanine; the pyrimidine bases, cytosine and thymine; a2-deoxyribose sugar and phosphoric acid. Similarly RNA on hydrolysis yields bases, adenine,guanine, cytosine and uracil; a pentose sugar, D-ribose and phosphoric acid (3).

In 1953, James Watson and Francis Crick postulated the double helicle structure of DNA. The Watson and Crick hypothesis also shows the central dogma of molecular genetics, which defines three major steps in the processing of genetic information.



In the first step, replication, the copying of parent DNA occurs to form daughter DNA molecules having nucleotide sequences identical to those of the parent DNA. The second step is transcription, the process in which parts of the genetic message in DNA are rewritten in the form of RNA. The third step is translation, in which the genetic message coded by RNA is translated by the ribosomes into the 20 - letter alphabet of protein structure.

1.3: Structure of Nucleic acids

1.3.1 : Structure of DNA

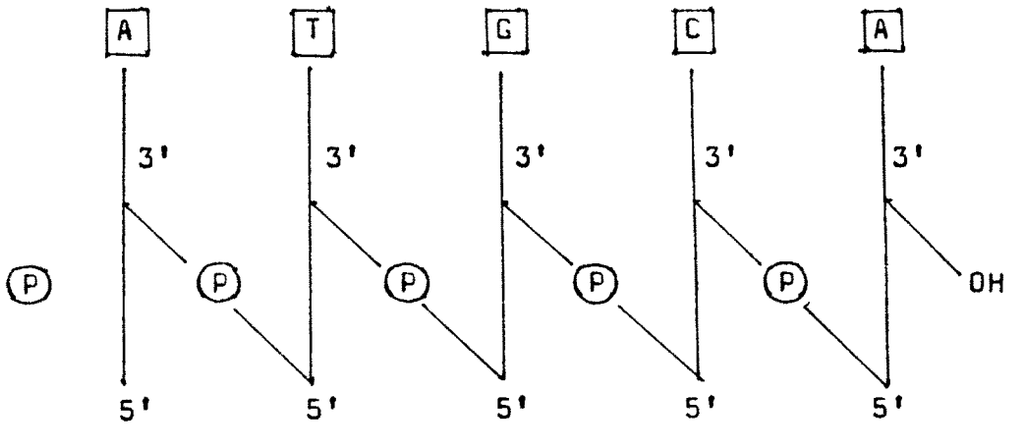
DNAs are extremely long chained double stranded molecules. DNA consists of many thousands of deoxyribonucleotides of four different kinds, joined in a sequence that is characteristic of each organism (4).

DNA contains two pyrimidine bases, cytosine (c) and thymine (T), and two purine bases, adenine (A) and guanine (G) and a sugar; 2 deoxyribose and a phosphoric acid. In addition to above bases, DNA also contains some minor bases. These are methylated forms of the principal bases.

The nucleotides of DNA and RNA are covalently linked to each other through phosphate group "bridges". The 5'-hydroxyl group of the pentose of one nucleotide unit is joined to the 3'-hydroxyl group of the pentose of the next nucleotide by a phosphodiester linkage.

Thus the covalent backbones of nucleic acids consist of alternating phosphate and pentose groups and the bases are joined to the backbone at regular intervals. The backbone is highly polar, since the phosphate groups are acidic and have negative charges at the cellular pH. The purine and pyrimidine bases are insoluble in water, are hydrophobic. DNA and RNA strands have a specific polarity or direction, because all the internucleotide phosphodiester linkages have the same orientation along the chain. Because of this polarity, each linear nucleic acid strand has a 5' end and a 3' end.

The nucleotide sequences of nucleic acids can be represented as (3)



5'→3'

The bases are symbolized by A,T,G and C each deoxyribose by a vertical line and the phosphate groups by Ⓟ. The structure of a single strand of DNA is written in the 5'→3' direction.

The four bases occur in different ratios in the DNAs of different organisms and that the bases have a relationship to each other. DNA isolated from different tissues of the same species have the same base composition. The base composition of DNA varies from one species to another. The base composition of DNA in a given species does not change with age of the organism, its nutritional state or changes in its environment. The number of adenine residues in all DNAs, is equal to the number of thymine residues (that is A=T), and the number of guanine residues is equal to the number of cytosine residues (G≡C).

Watson and Crick proposed the three dimensional structure of DNA. It consists of two helical DNA chains coiled around the same areis to form a right-handed double helix. In the helix the two chains or strands are antiparallel, i.e. their 5',3' internucleotide phosphodiester bridges run in opposite direction. Their hydrophillic backbones, which consists of alternating deoxyribose and negatively charged phosphate groups, are on the outside of the double helix, facing the surrounding water. The hydrophobic purine and pyrimidine bases of both strands are stacked inside the double helix, so that the planer base molecules are very close together and perpendiculer to the long axis of the double helix. The bases of one strand are paired in the same planes with the bases of the other strands. The bases of each pair are linked to each other by hydrogen bonds. In between G and C, there are three hydrogen bonds, (G≡C) but in between A and T, there are only two bonds (A=T).

The double helix is about 2 nm thick. There are about 10 nucleotide residues in each complete turn of the double helix. The two antiparallel polynucleotide chains of double helical DNA are complementary to each other. All the phosphate groups in the polar backbones of the double helix are ionized and negatively charged at pH 7; thus DNA is strongly acidic.

1.3.2 : Structure of RNA

Ribonucleic acids consist of long strings of ribonucleotides. They have shorter chains than that of DNAs. There are three major classes of RNAs, messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) (5).

Messenger RNA functions as the template used by ribosomes for the translation of the genetic information into the amino acic sequence of proteins. The nucleotide sequence of mRNA is complementary to the genetic message contained in a specific segment of the template strand of DNA. A single eukaryotic cell may contain over 10^4 different mRNA molecules, each coding for one or more different polypeptide chains.

Transfer RNAs consist of a single strand of ribonucleotides but in highly folded conformation. They have 70 to 95 ribonucleotides, corresponding to molecular weights between 23,000 and 30,000. Each of

the 20 amino acids found in proteins has one or more corresponding tRNAs to bind it and carry it to the ribosomes and serve as an "adapter" for translating the genetic code words of the mRNA into the amino acid sequence of proteins. Each tRNA contains a specific trinucleotide sequence, called its anticodon, which is complementary to a codon, the trinucleotide sequence of mRNA that codes for one specific amino acid.

Ribosomal RNAs (rRNAs) are the major components of ribosomes and constitute up to 65 percent of their weight. Ribosomal RNAs play an important role in the structure and biosynthetic function of ribosomes.

1.4 : Enzymes involved in Nucleic acid Metabolism

There are a large number of enzymes which are involved in synthesis and degradation of nucleic acids (3).

1.4.1 : Enzymes involved in Nucleic acid synthesis

DNA polymerase : An enzyme catalysing template dependent synthesis of DNA from its deoxyribonucleoside 5'-triphosphate precursors DNA polymerase requires Mg^{2+} for its action and also contains tightly bound Zn^{2+} in its active site.

Primase - The synthesis of Okazaki fragments requires as a primer a short length of RNA, complementary to the DNA template strand.

The enzyme primase binds to the preprimed strands and catalyzes synthesis of the RNA primers that begins each new strand of DNA.

DNA - Ligase - An enzyme that creates a phosphodiester bond between the 3'-phosphate group at the end of one DNA segment and the 5'-hydroxyl group at the end of the another, while they are base paired to a template strand.

Helicase - This enzyme catalyses energy dependent unwinding of the duplex using two ATPs per nucleotide pair.

DNA gyrase - In the transcription process, an RNA strand having a base sequence complementary to one of the DNA strands is synthesized by an enzyme system.

RNA polymerase - is an enzyme that catalyzes the formation of RNA from ribonucleoside 5'-triphosphates, using a strand of DNA or RNA as a template.

Reverse transcriptase - An RNA directed DNA polymerase is synthesized in retroviruses; that is capable of making DNA complementary to an RNA. The reverse transcriptase requires a primer; they make the DNA newstrand in the 5'-3' direction.

1.4.2 : Enzymes involved in hydrolysis of nucleic acids

A large number of nucleolytic enzymes have been isolated from various sources, including fungi, bacteria, Yeast, plants and

animals. Depending upon the specificity for sugar residues in nucleic acids, these are broadly divided into three main groups, ribonucleases which specifically degrade ribonucleic acids, deoxyribonucleases which cause the cleavage of deoxyribonucleic acids and nucleases which are non specific and hydrolyze both RNA and DNA.

a) Nuclease - An enzyme which is capable of hydrolyzing internucleotide linkages of nucleic acid. There are two types of nucleases i) Endonuclease and ii) Exonuclease

i) Endonuclease - An enzyme capable of hydrolyzing internal phosphodiester bonds of a nucleic acid, e.g. Mung bean nuclease, wheat nuclease, sharkliver nuclease.

ii) Exonuclease - An enzyme that hydrolyzes only a terminal phosphodiester bond of a nucleic acid. e.g. Snake venom phosphodiesterase, spleen phosphodiesterase.

The endonucleases are divided into two main groups, the ribonuclease which attack RNA and the deoxyribonuclease which attack DNA.

b) Ribonuclease

Ribonucleases can be classified according to their 1) mode of action, 2) specificity towards the bases and 3) nature of the product.

A ribonuclease is capable of hydrolyzing certain internucleotide linkages of RNA. The best characterised pancreatic ribonuclease splits the linkage joining the phosphate residue at C-3' in a pyrimidine nucleotide to C-5' in the next nucleotide in sequence.

RNases from animal sources are mostly absolutely specific for pyrimidines, microbial RNases are either strictly guanosine specific or more often purine specific in earlier stages, whereas plant RNases are non-specific enzymes hydrolyzing RNA molecule completely. However plant RNases also may be purine specific in earlier stages.

Ribonuclease H - catalyzes hydrolysis of the RNA strand of a DNA : RNA hybrid duplex,

c) Deoxyribonucleases - There are two main types of deoxyribonuclease (DNase) which have been well characterized; both are endonucleases.

The first is pancreatic deoxyribonuclease (DNase I) is a 5' phosphomonoester formers. The second type (DNase II) which is found in spleen and thymus is a 3'-phosphomonoester former.

d) S_1 Nuclease - It is a single strand - specific endonuclease isolated from Aspergillus oryzae (79) It hydrolyzes primarily single stranded DNA and RNA to yield 5'-nucleoside monophosphates and a very small amount of dinucleotides.

e) Restriction endonuclease(s)- A site specific endodeoxyribonuclease causing cleavage of both strands of DNA at points signaled by two-fold symmetry of base sequence about a center. This enzyme is very important in genetic engineering. (5).

1.5 : Occurance of nucleolytic enzymes in plants

In plants, ribonucleases, nucleases are widely distributed. These are isolated, purified and characterized from various parts of the plants, i.e. roots, e.g. corn roots (36) Vicia faba root cells (26) (63,64), seedlings, e.g. mung bean (11) pea (13), muskmelon (8) oats (12) cucumber (21) and soyabean (17), tubers e.g. potato (14); sweet potato (33), leaves e.g. barley (9), ryegrass (15), spinach (17), tobacco (19) sugarcane (18), tea (22), tomato leaves (62), wheat (70) fruits e.g. tomato (25), and apple pollen (35).

Sugar nonspecific nucleases have been studied in mung bean (11), pea seedling (13) corn roots (36,10), oat (12), potato (14) and wheat leaves (20).

There are, however, no reports on the isolation and partial purification of DNases in plants (8). It is possible that the function of DNases is performed by non-specific nucleases in plants. Plant nucleases, thus may substitute DNases and help the plants to effect economy in protein synthesis or it is also possible that specific DNases are present in plants but could not be detected due to lack of sensitive detection methods.

1.6 Methods for Purification

Essential requirement for all the fundamental studies, is a highly purified and homogeneous preparation of the enzyme. For this purpose, many techniques, ranging from simple pH adjustment or isoelectric precipitation, to the highly developed, sophisticated techniques such as use of cellulosic ion exchangers and gel filtration are employed. Prominent amongst them are isoelectric precipitation of inert proteins, heat treatment to denature contaminating proteins, fractional precipitation of the enzyme with organic solvents, fractional precipitation with salts, use of adsorbents, use of certain cation and anion exchangers and gel filtration through sephadex etc.

1.6.1 : Heat treatment to remove contaminating proteins by coagulation

Enzymes are very sensitive to heat treatment and get inactivated when heated. RNase more particularly pancreatic RNase, are exception to this behaviour. Heat treatment can result in the activation of some of the RNases, nucleases due to removal of heat labile inhibitor (80,81) e.g. mung bean nuclease (66).

Heat treatment is usually recommended for crude extracts before precipitation with organic solvents or with salts. The heat treatment can, however, be extended to purified fractions.

In general, the enzyme solution is heated just below its stability temperature for a required period and then immediately cooled

13

in ice bath. Proteins, other than the enzyme of interest, get denatured and are precipitated. After removing the precipitate of denatured proteins, the supernatant is further purified by other methods.

1.6.2 : Use of organic solvents as a precipitant for enzymes

Organic solvents are recommended for the purification of enzymes, especially when the enzyme cannot be precipitated with salts or when the lengthy dialyzing procedure to remove electrolytes are undersirable.

The most commonly used organic solvents are acetone and ethanol.

Precipitation with solvents is carried out generally, in initial stages of purification. However, in case of mung bean nuclease (66), the enzyme was initially precipitated with ammonium sulfate followed by ethanol treatment.

Solubility of the proteins in organic solvents varies greatly with pH, most of them being least soluble at their isoelectric points. The pH, therefore is carefully controlled. Mung bean nuclease was precipitated with 55% ethanol at pH 5.9 (66).

When acetone is used in the purification, the precipitate of enzyme proteins can be stored for several days without lass of activity.

The concentration of organic solvents employed is critical, different proteins being precipitated at different concentration. Mung bean nuclease was precipitated between 35% to 55% ethanol concentration (66).

Chloroform and phenol are also used as solvent. Use of organic solvents is limited by the fact that exposure of the enzymes to organic solvents results in denaturation of the enzymes. Low temperatures are used to minimize denaturation.

1.6.3 : Fractional precipitation with salts

Proteins vary markedly in their solubilities in concentrated salt solutions. Consequently, purification of the protein of interest can be achieved by discarding the precipitate of contaiminating proteins obtained at finite salt concentration, leaving more soluble proteins in the supernatant.

Most commonly used salts are ammonium sulfate, magnesium sulfate, sodium sulfate and sodium chloride. Ammonium sulfate is preferably used as it can give very concentrated salt solutions which can also be buffered. Enzymes are, therefore, not exposed to extremes of pHs and denaturation and inactivation of enzymes due to unfavourable conditions is avoided.

Precipitation with ammonium sulfate is the essential step in the purification of most of the nuclease like mung bean nuclease (66)

wheat nucleases (70) corn root nuclease (77).

1.6.4 : Use of adsorbents

Proteins are selectively adsorbed on and eluted from several solid adsorbents. Ion exchange, ionic binding,binding through hydrogen bonds. Van der waals forces, electrostatic bonds, hydrophilic or hydrophobic interactions, partition and molecular adsorption are the principal forces that operates and these forces are sensitive to pH and ionic strength.

Adsorption chromatography may be performed either on a column or with slurries of the adsorbent, i.e. batchwise way. Commonly used adsorbents are alumina Cγgel, calcium phosphate gel, hydroxyapatite, celite and starch.

Alumina Cγgel was used in the purification of RNase from tobacco leaves (19). RNase from rye grass (15) was purified by calcium phosphate gel chromatography.

1.6.5 : Use of Cellulose Ion Exchangers

Modified cellulose in which ionizable groups like DEAE,CM, phospho etc. are attached to the cellulose matrix were first introduced by Peterson and Sober (82,83) and have found wide applicability.

DEAE-Cellulose : Diethylaminoethyl - cellulose (DEAE-Cellulose) which dissociates over the pH range 3.0 to 8.0 is a weak anion exchanger.

16

It is quite useful for chromatography of acidic or slightly basic proteins are stable and anionic in character. Gradient of pH and ionic strength is used for elution purpose.

Nuclease from corn (77), sugarcane leaves RNase (18) and RNase from spinach leaves (17) have been purified on DEAE - Cellulose.

Batchwise treatment with DEAE-Cellulose was given to squid RNase extract to remove large amounts of protein (84)

CM-Cellulose

Carboxymethyl-cellulose (CM-Cellulose) is a weak cation exchanger and is more useful for weakly basic or neutral species of proteins, as well as those proteins having low isoelectric points (85). CM-Cellulose has been used to purify RNases from corn (77).

Phosphocellulose

Phosphorylated cellulose (P-cellulose) serves as a cationic exchanger at relatively low pH, particularly below pH 4.0, Where CM-Cellulose is ineffective.

For the removal of coloured impurities and for the separation of phosphomonoeste-rase, P-cellulose is more effective (86).

1.6.6 : Gel filtration on sephadex columns

Mung bean nuclease (11), Wheat leaves nuclease (70) and corn nuclease (77), etc. are purified by gel filtration on sephadex columns.

17

Gel filtration through polyacrylamide columns is used for separation of RNase activity from a nuclease fraction (87).

1.7 Applications of Nucleases (88)

1) Preparation of mono-and dinucleotides with sufficient enzyme S1 and P1 nucleases completely degrade RNA to produce 5'-mononucleotides. The enzymes are quite thermostable. Therefore they have been used for the industrial production of 5'-mononucleotides from yeast RNA.

2) Estimation of double-helical content and Isolation of rapidly reannelaing regions in nucleic acids.

Single-stranded nucleic acids tend to form regions with double-stranded structure. S1-nuclease is used to estimate the double helicle content of various single-stranded nucleic acids and to isolate the double stranded regions of single stranded nucleic acids.

3) Specific cleavage of tRNA and rRNA

At pH 4.5 in high salt at 20°C, S1 nuclease specifically hydrolyzes tRNAs in the anticodon loop and at the 3'-end.

The specific cleavage in the anticodon loop is very useful for sequence anadysis of tRNA.

4) Elimination of short single-stranded Ends

To construct a recombinant DNA molecule between different restriction endonuclease-derived fragments or an in vitro deletion mutant by exonuclease digestion, it is necessary to remove single stranded tails before T4-DNA ligase treatment, S1 nuclease is used for this purpose.

5) Location of Nonbase-paired Regions in Superhelical DNA.

Negative superhelicity introduces localized unwinding of helicle base pairs can recognize such regions and cleave both strands of the DNA to gene unit-length linear duplex molecules with no nicks.

e.g. Mung bean nuclease

6) Specific Fragmentation of Double stranded DNA.

S1 nuclease can cleave the opposite strand at the preexisting interruptions to generate specific fragments.

Mung bean nuclease I at high concentration introduces double-strand cleavages in linear duplex DNA.

7) Recovery of Inserts from Hybrid Plasmids constructed by the Poly (dA) : poly (dT) Method.

S1 nuclease was successfully used for the specific and efficient excision of the inserted DNA segment from a hybrid plasmid constructed by the poly (dA) : poly (dT) tailing method.

19

8) Detection of Locally Altered Structures in Double-stranded DNA.

S1 nucleases can recognize structural alterations induced by uv-irradiation and introduce single-and double-stranded breaks into DNA.

1.8 : Physiological Role of RNase

RNases must play an imp. role in the mechanism controlling nucleic acid metabolism. Taking into account the essential function of RNA in living matter, a knowledge of the reactions catalysed by RNase may give some clues as to mechanisms and controls of all the reactions in organisms.

1.9 : Meta bolic Functions of Plant Nucleases

The major metabolic function for the nucleases is presumably the degradation of nucleic acids, but little is known about the in vivo rates of reaction or the control mechanisms that prevent the nucleases from rapidly degrading all cellular nucleic acid. Given the variety of enzymes known, we may speculate that each has a certain function, is active on specific substrates at the appropriate time in the life cycle, and is kept inactive at other times by physical separation, natural inhibitors, lack of suitable intracellular conditions, binding of substrates to proteins, or substrates in resistant conformational states. Without knowledge of these things, we cannot with certainty place the nucleases in an overall picture of cellular metabolism.

The potential degradation reactions of the known nucleases an associated enzymes are summarized by seven reactions:

RNA (DNA) $\xrightarrow{\text{nuclease I, exonuclease}}$ pN (pdN) 1.

RNA $\xrightarrow{\text{RNase I,RNase II}}$ N>p (R>p + Y>p) 2.

R>p $\xrightarrow{\text{RNase I, RNase II, cyclic PDase}}$ Rp 3.

Y>p $\xrightarrow{\text{RNase II, cyclic PDase}}$ Yp 4.

Np $\xrightarrow{\text{nucleoside phosphotransferase}}$ pN 5.

Np $\xrightarrow{\text{3'-nucleotidase, nuclease I}}$ N + P_i 6.

N + ATP $\xrightarrow{\text{nucleoside kinase}}$ pN 7.

Once a nucleic acid has lost its biological value, its constituent parts may be recycled into a new nucleic acid if the breakdown products can be converted into 5'-nucleotides. Nuclease I and exonuclease yield the desired products in one step (reaction I), but several enzymes and two pathways (reactions 2,3 and 4, followed by 5 or 6 and 7) may be used when the RNases degrade RNA. No data exist that allow us to decide which pathway predominates in vivo.