

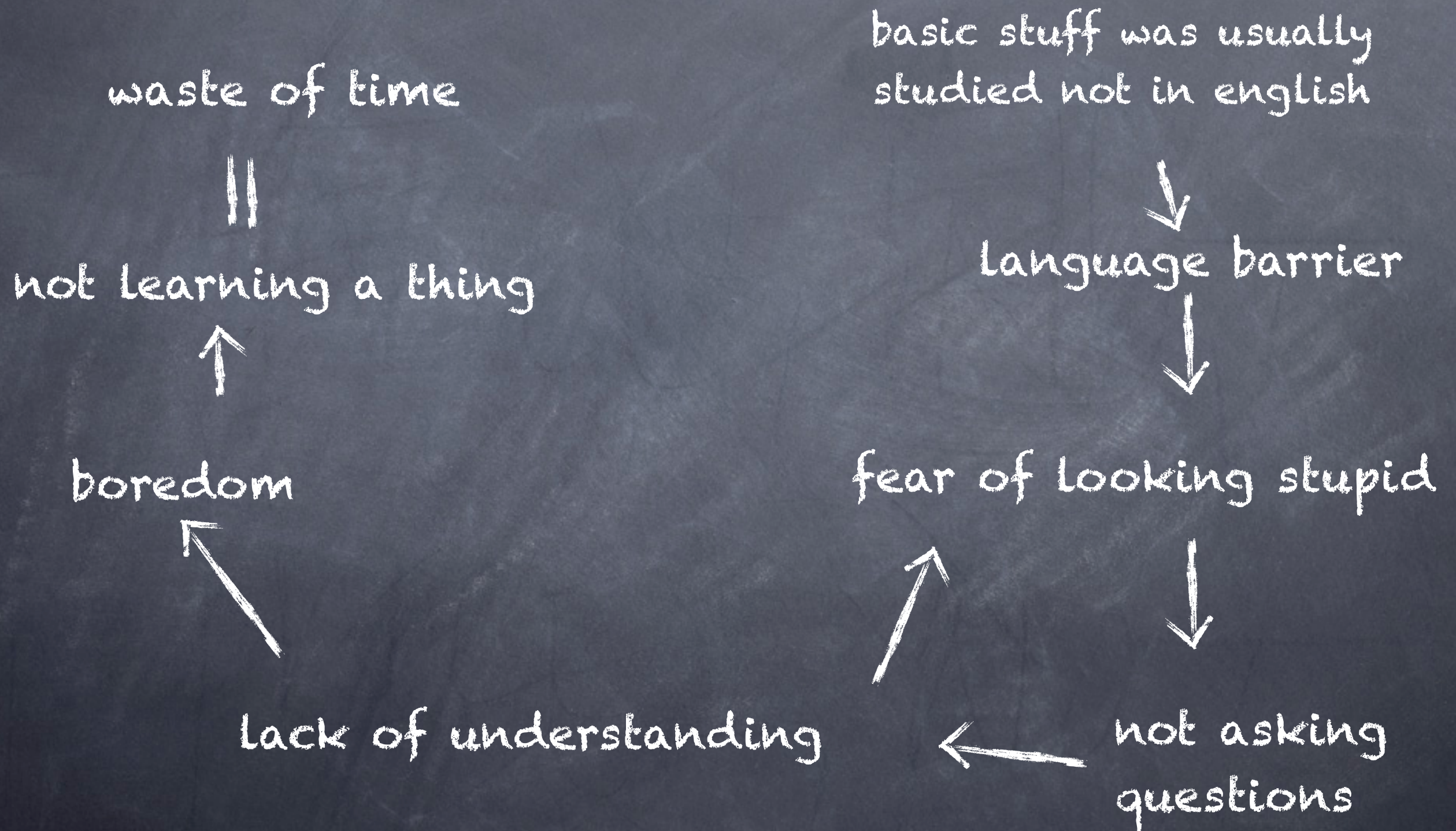


# qPCR course

Alyona Minina  
Department of Plant Biology  
SLU



# A scientific representation of challenges to be met during this course





- work together (you will learn stuff better while teaching each other)
- climb over your language barrier, there are no stupid questions
- tell me what do you want to know?



an example of a language barrier

$$x = a * b^c$$

$$\log_k L = m$$

Please interrupt and ask!!



# What do you want to know?

Data Analysis and interpretation

Statistical analysis of qPCR

Application of qPCR

Basics

How qPCR works

Normalization

Troubleshooting

Everything

More



- The ends of DNA
- Polymerase
- Concept of PCR

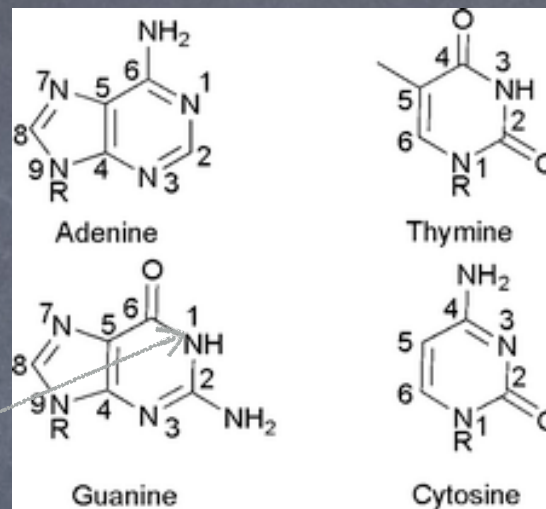
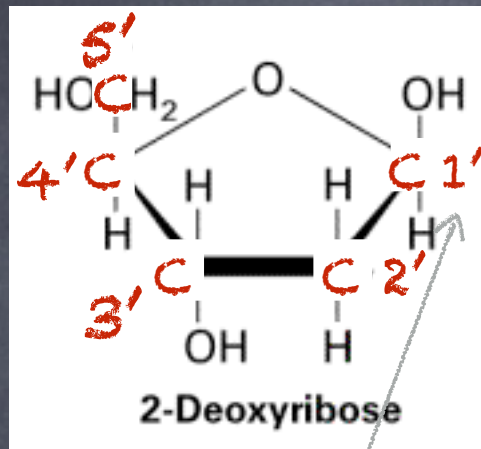
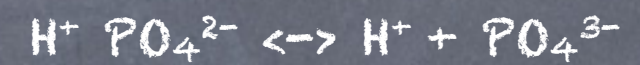
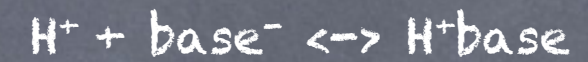


# DNA

Deoxyribonucleic Acid

5-Carbon sugar

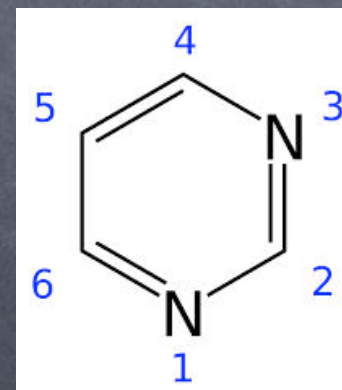
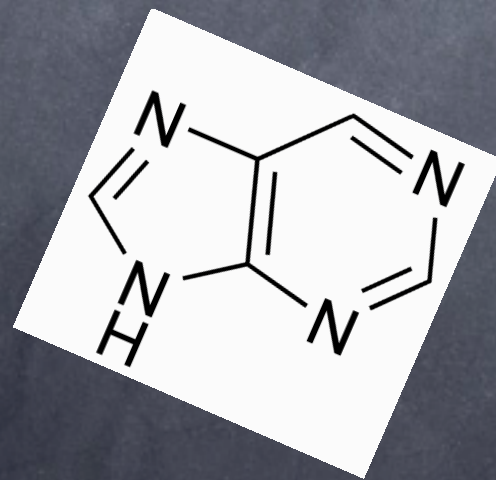
Nucleobase



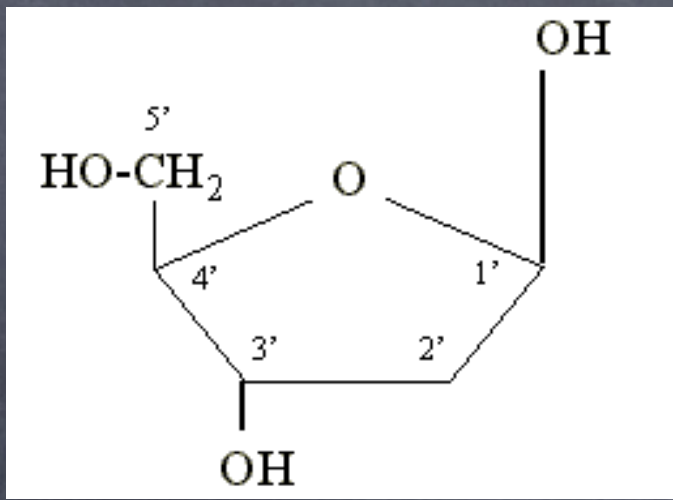
' = prime  
to distinguish  
numeration of atoms in  
nucleobases and sugar  
backbone

purines

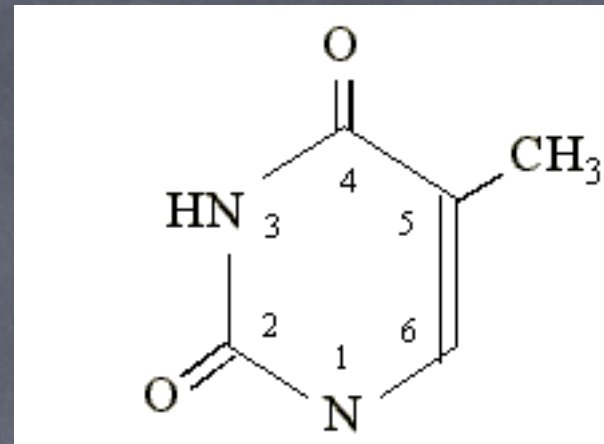
pyrimidines







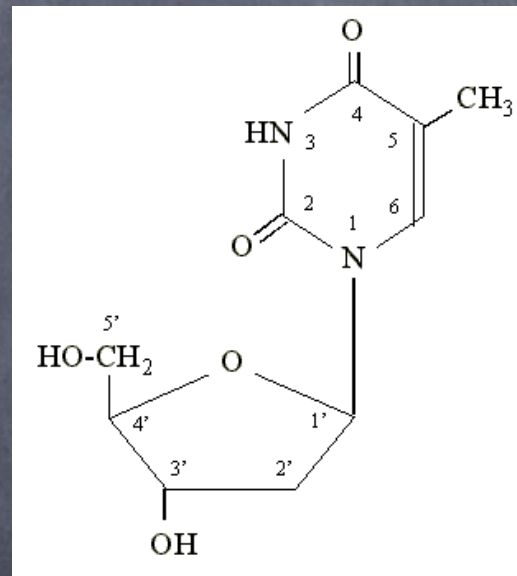
deoxyribo



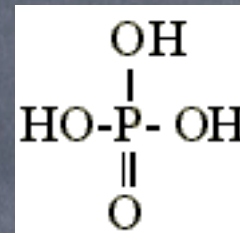
nucleic

deoxyribonucleic

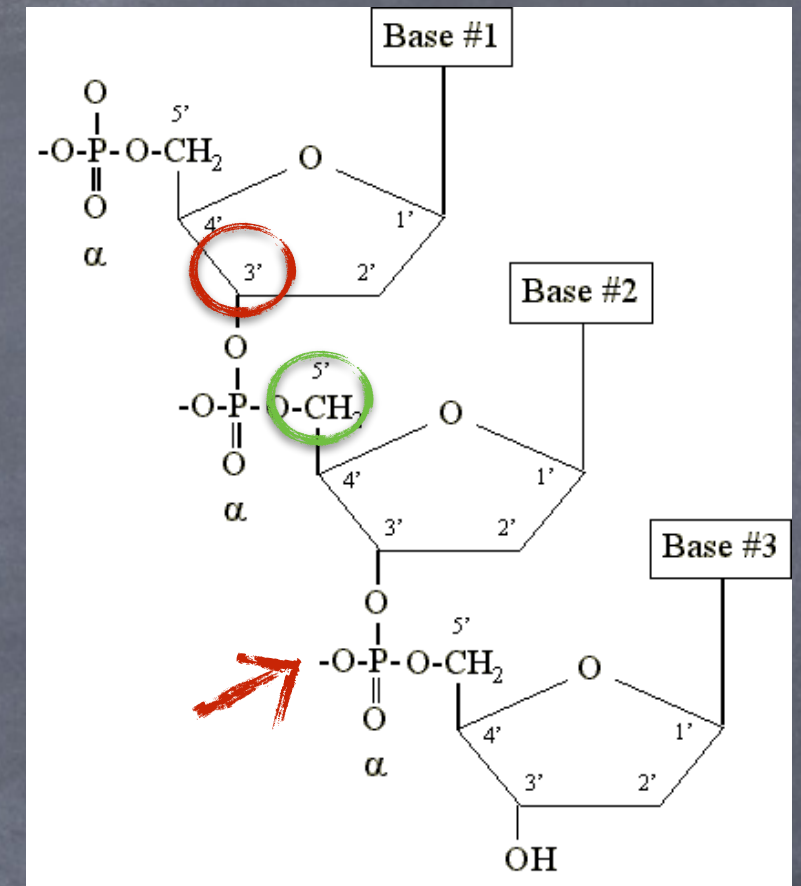
acid



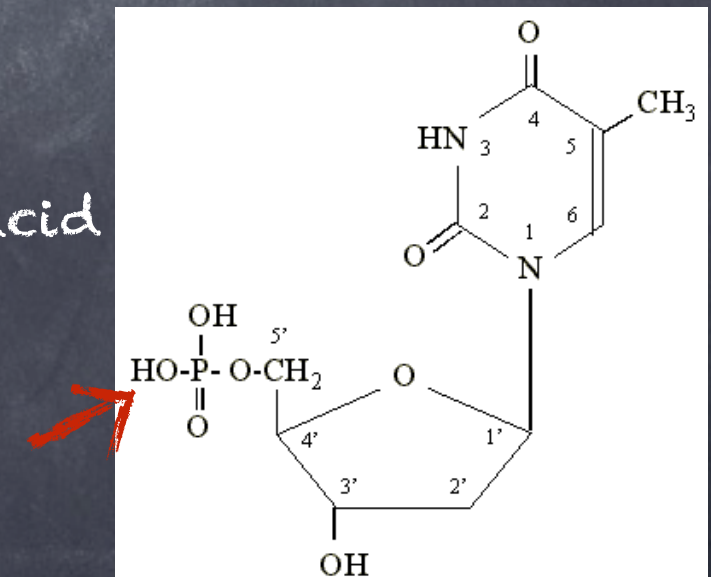
+



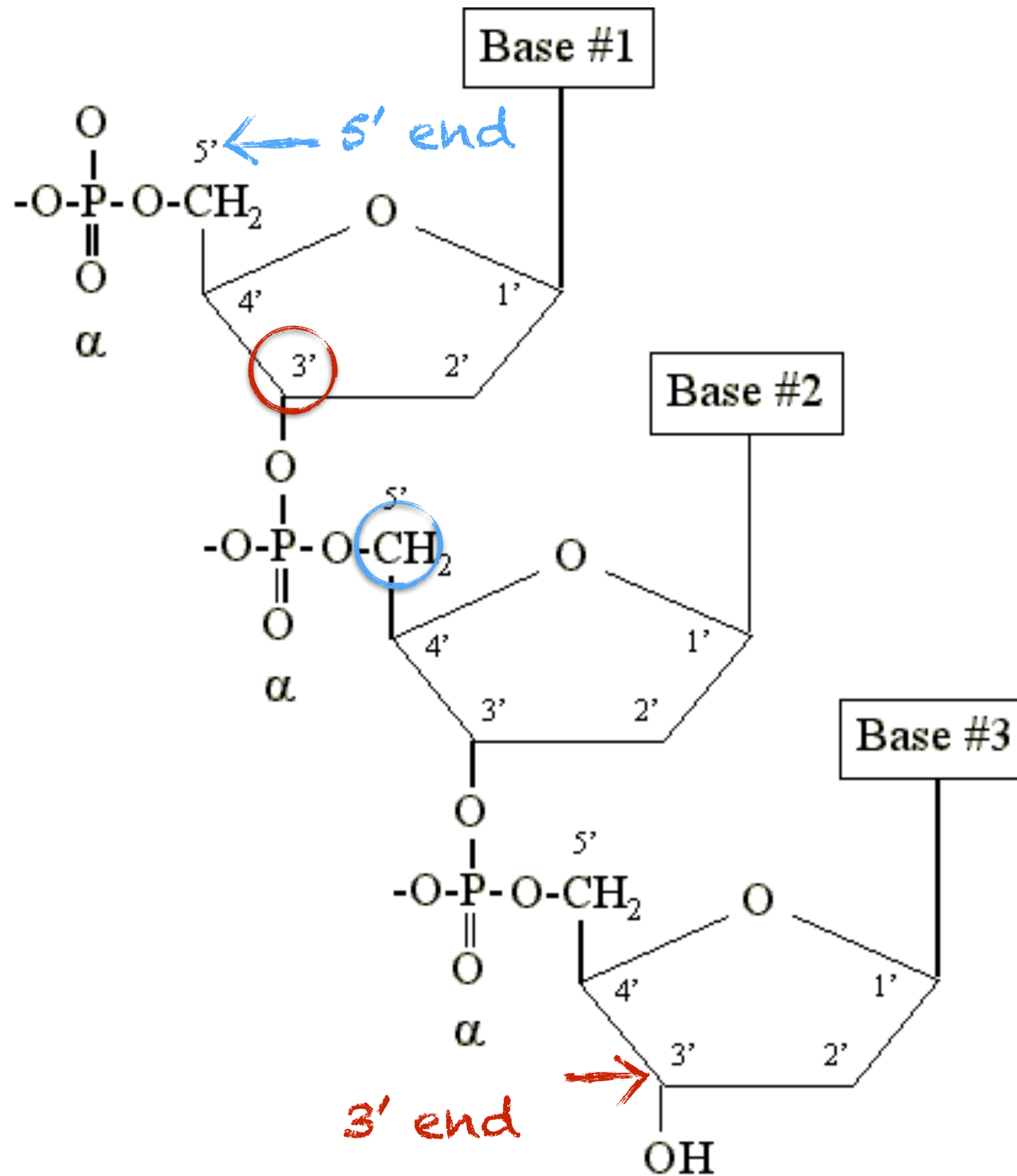
DNA is a polymer molecule



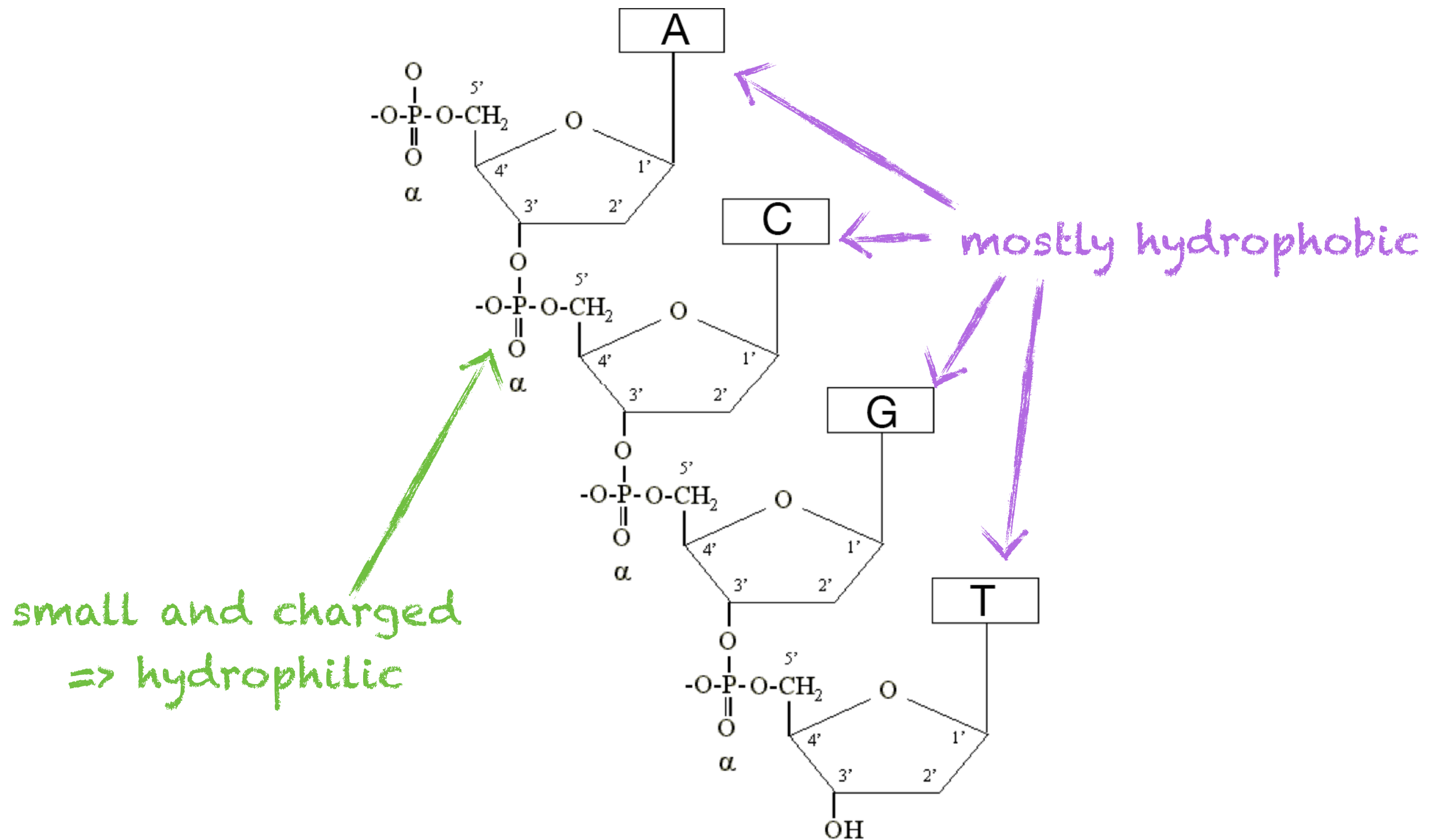
deoxyribonucleic acid









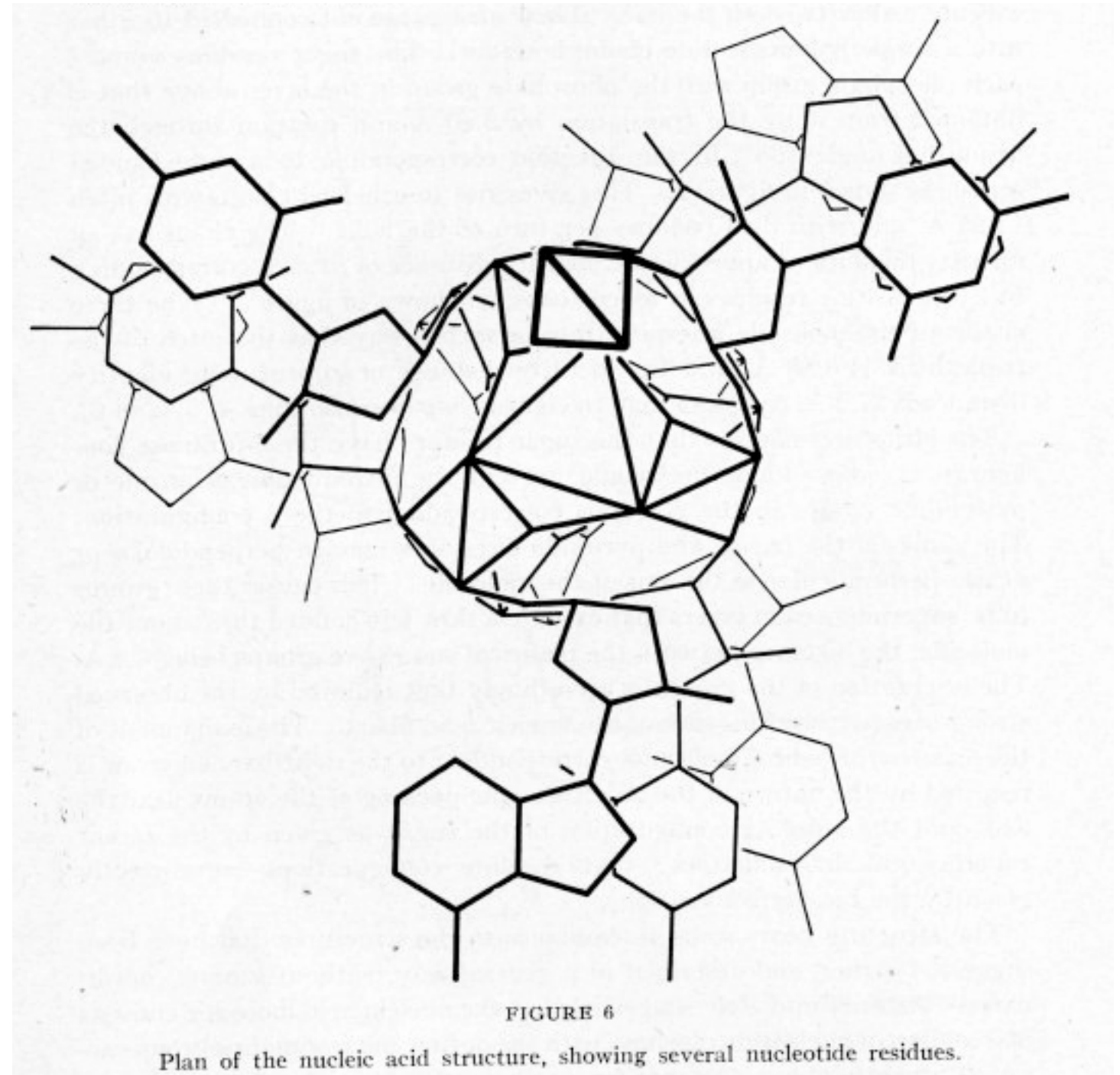
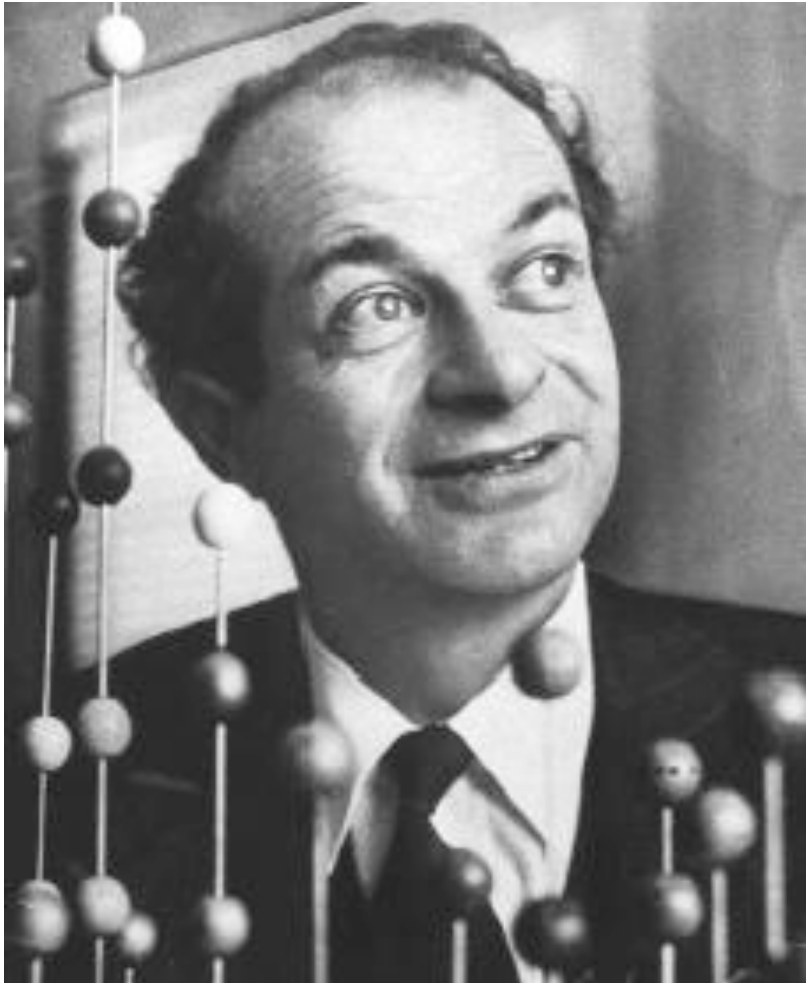


cell environment is mostly hydrophilic



# Triple helix model

Linus Pauling



14 pages long article in PNAS (February 1953) describing a model with phosphates looking inwards

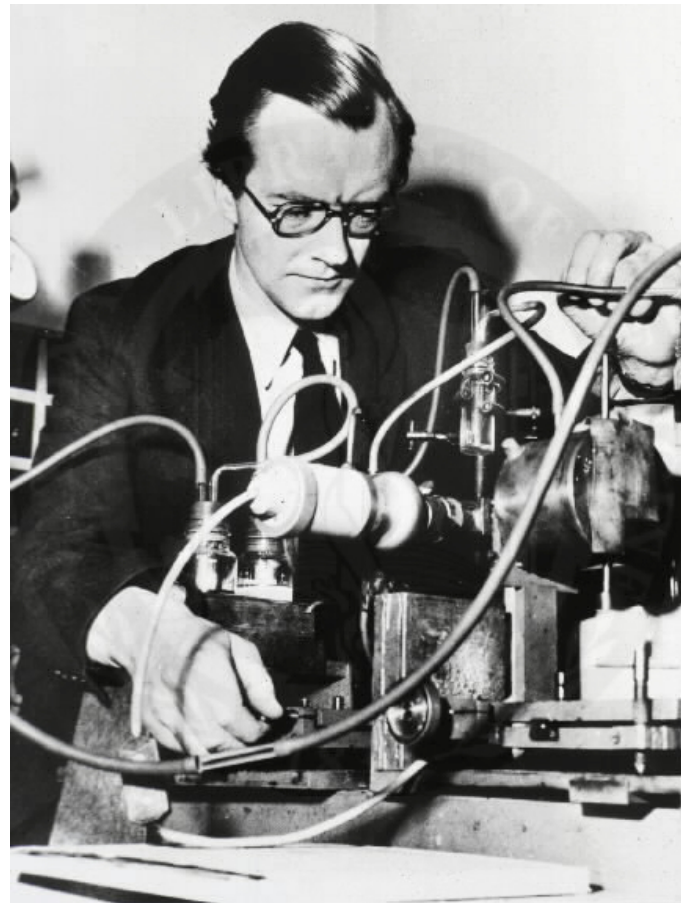


# Double helix model

Rosalind Franklin



Maurice Wilkins



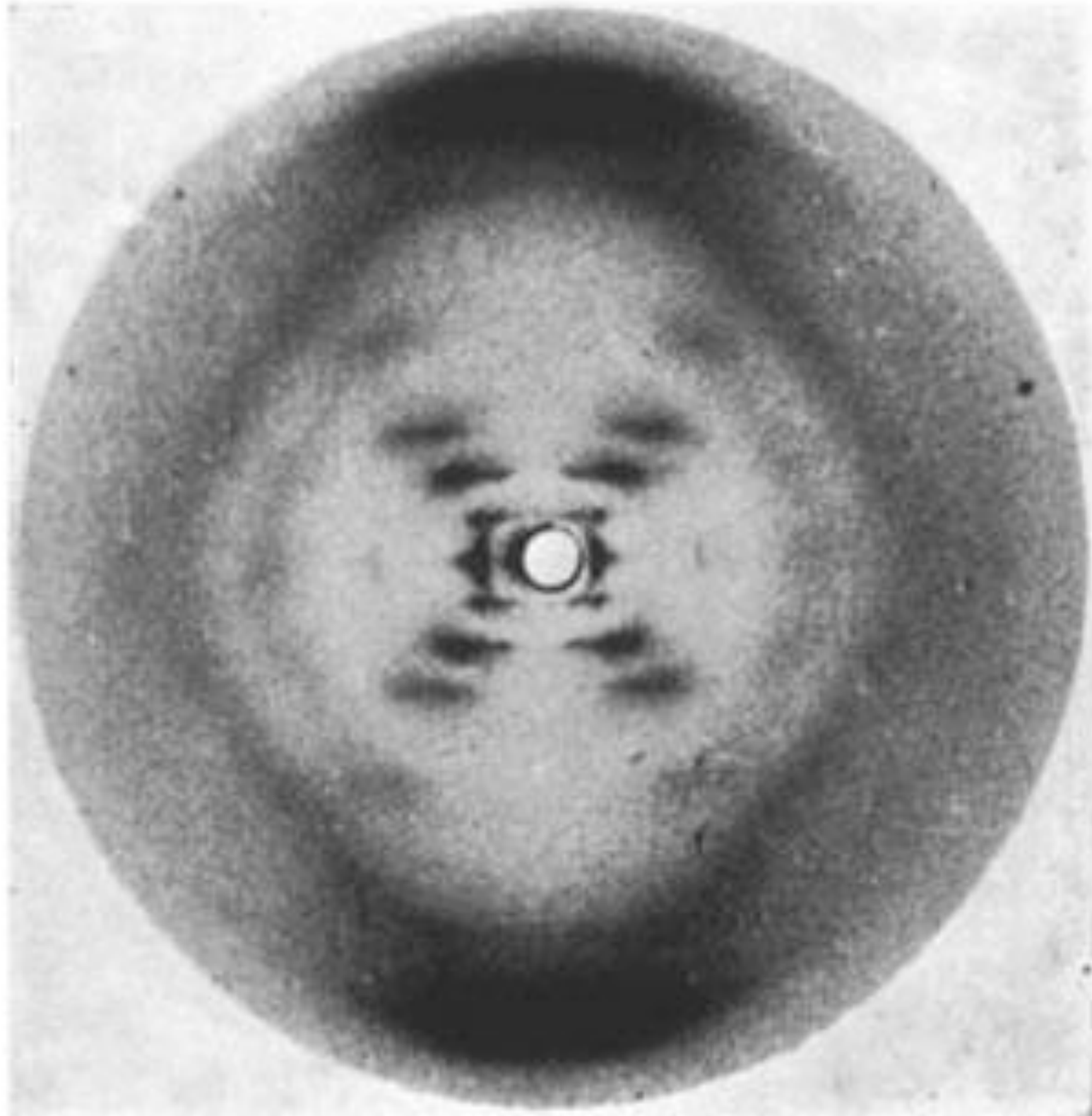
Ray Gosling



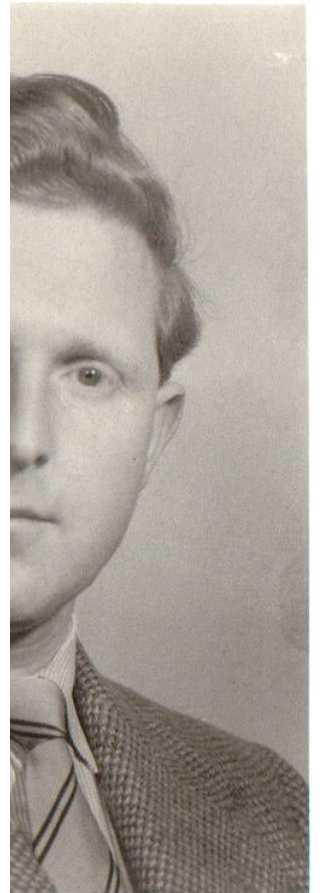


# Double helix model

Rosalind

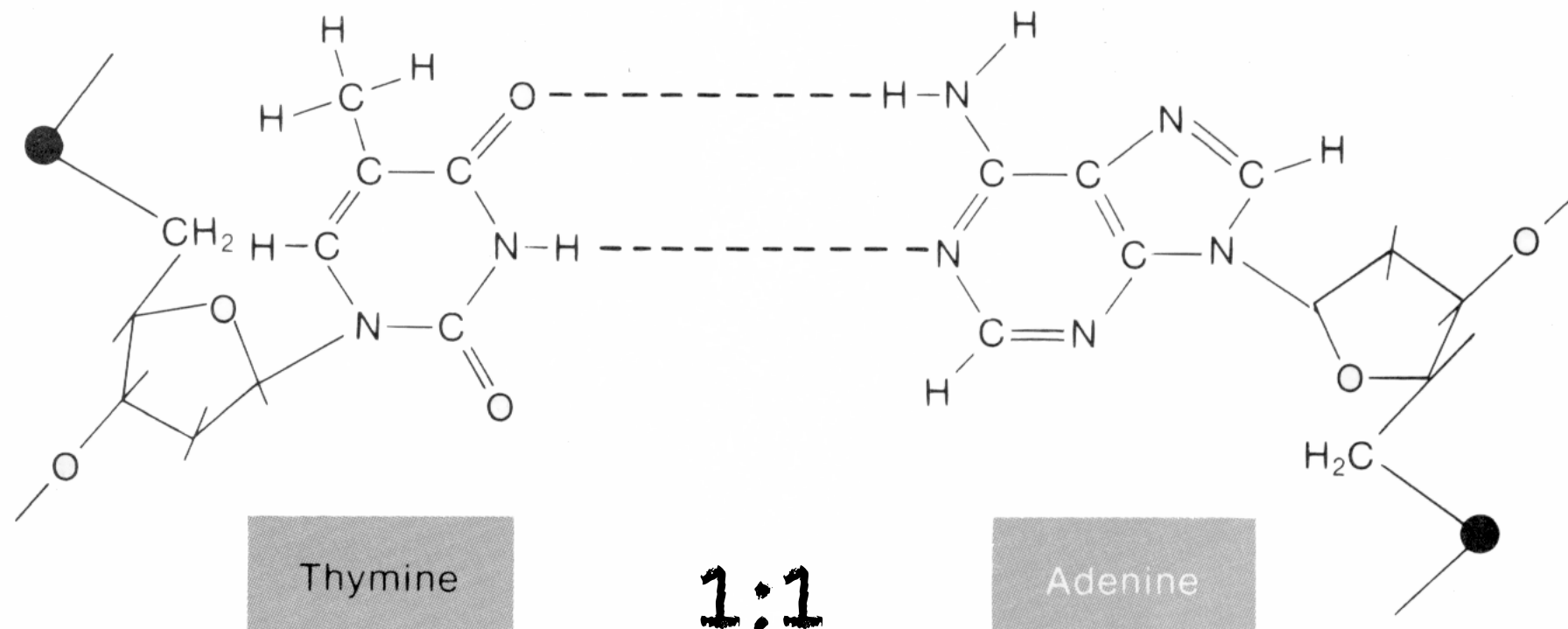
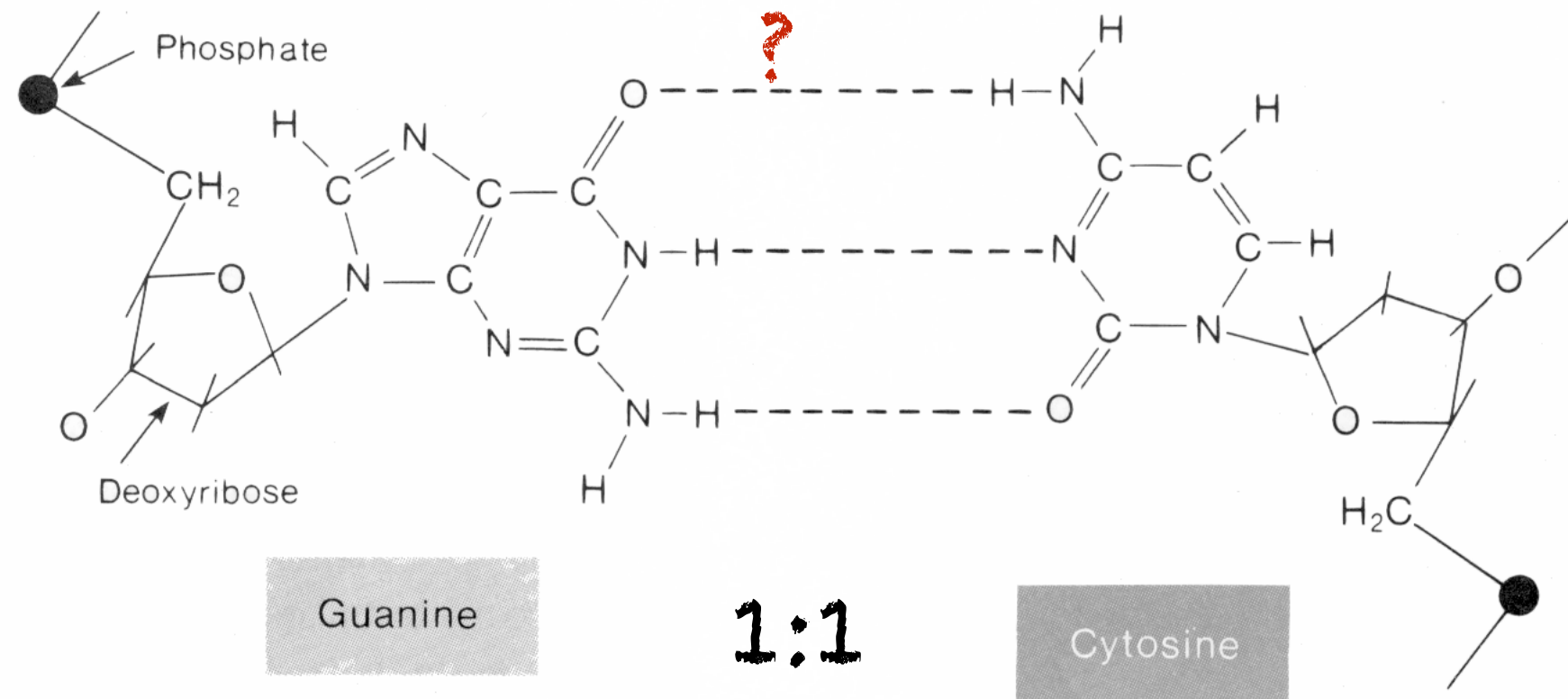


osling

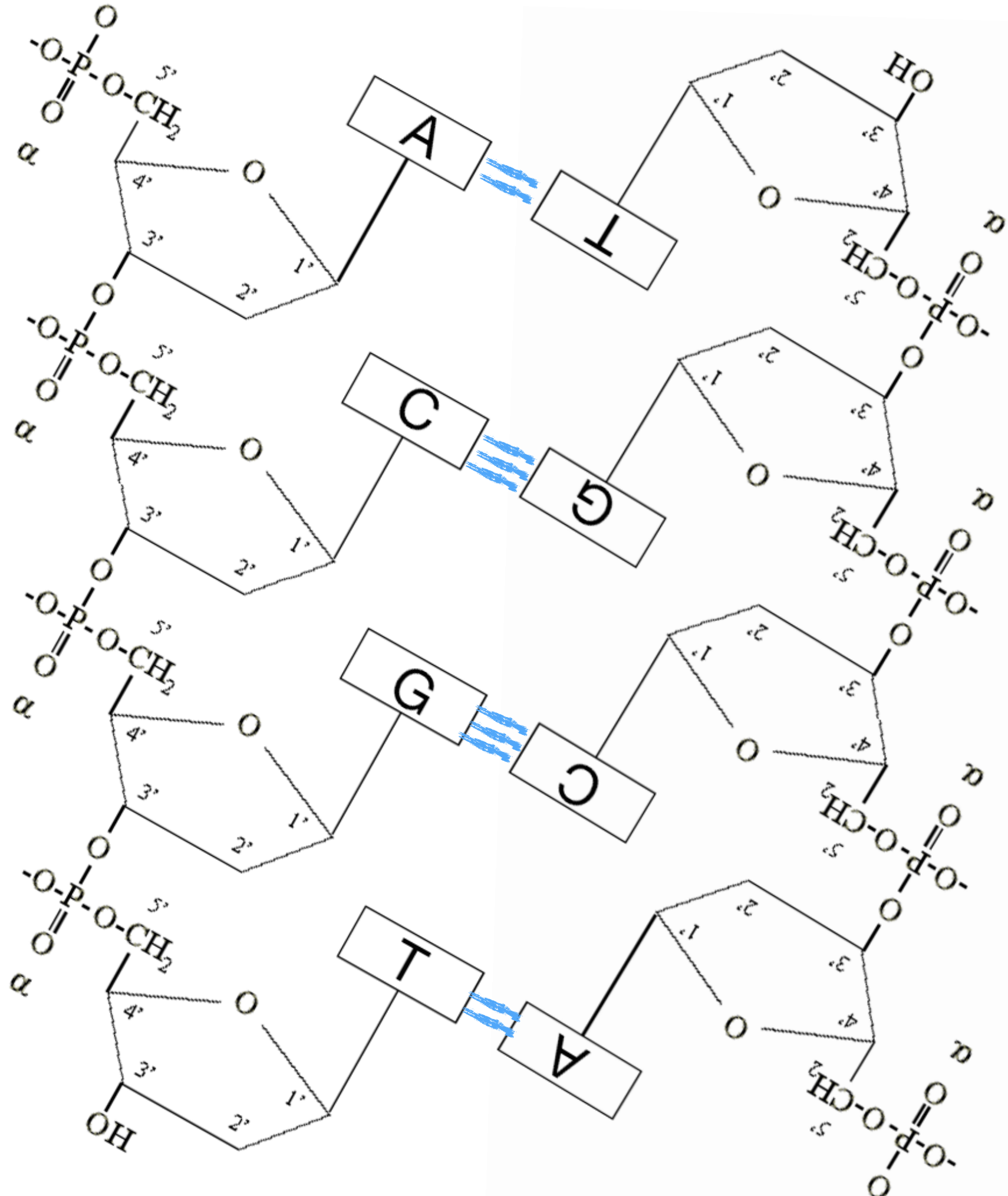




# What will hold two strands together?









But Francis, do you think we were lucky to have solved it?



Francis Crick

James Watson



equipment, and to Dr. G. E. R. Deacon and the captain and officers of R.R.S. *Discovery II* for their part in making the observations.

<sup>1</sup> Young, F. B., Gerrard, H., and Jevons, W., *Phil. Mag.*, **40**, 149 (1920).

<sup>2</sup> Longuet-Higgins, M. S., *Mon. Not. Roy. Astro. Soc., Geophys. Supp.*, **5**, 285 (1949).

<sup>3</sup> Von Arx, W. S., Woods Hole Papers in Phys. Oceanog. Meteor., **1** (3) (1950).

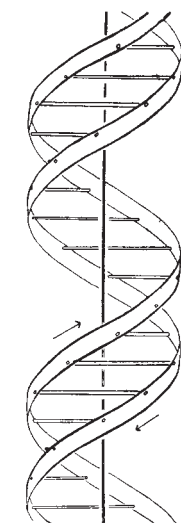
<sup>4</sup> Ekman, V. W., *Arkiv. Mat. Astron. Fysik. (Stockholm)*, **2** (11) (1900).

## MOLECULAR STRUCTURE OF NUCLEIC ACIDS

### A Structure for Deoxyribose Nucleic Acid

**WE** wish to suggest a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biological interest.

As a first approximation, the structure suggested by Fraser (in the press). In his model the phosphates are on the outside and the bases on the inside, linked together by hydrogen bonds. This structure as described is rather ill-defined, and for this reason we shall not comment on it.



This figure is purely diagrammatic. The two ribbons symbolize the two phosphate-sugar chains, and the horizontal rods the pairs of bases holding the chains together. The vertical line marks the fibre axis.

We wish to put forward a radically different structure for the salt of deoxyribose nucleic acid. This structure has two helical chains each coiled round the same axis (see diagram). We have made the usual chemical assumptions, namely, that each chain consists of phosphate diester groups joining  $\beta$ -D-deoxy-ribofuranose residues with 3',5' linkages. The two chains (but not their bases) are related by a dyad perpendicular to the fibre axis. Both chains follow right-handed helices, but owing to the dyad the sequences of the atoms in the two chains run in opposite directions. Each chain loosely resembles Furberg's<sup>2</sup> model No. 1; that is, the bases are on the inside of the helix and the phosphates on the outside. The configuration of the atoms near it is close to Furberg's 'standard configuration', the sugar being roughly perpendicular to the attached base. There

is a residue on each chain every 3.4 Å. in the z-direction. We have assumed an angle of 36° between adjacent residues in the same chain, so that the structure repeats after 10 residues on each chain, that is, after 34 Å. The distance of a phosphorus atom from the fibre axis is 10 Å. As the phosphates are on the outside, cations have easy access to them.

The structure is an open one, and its water content is rather high. At lower water contents we would expect the bases to tilt so that the structure could become more compact.

The novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases. The planes of the bases are perpendicular to the fibre axis. They are joined together in pairs, a single base from one chain being hydrogen-bonded to a single base from the other

chain. If the sequence of bases on one chain is given, then the sequence on the other chain is automatically determined.

It has been found experimentally<sup>3,4</sup> that the ratio of the amounts of adenine to thymine, and the ratio of guanine to cytosine, are always very close to unity for deoxyribose nucleic acid.

It is probably impossible to build this structure with a ribose sugar in place of the deoxyribose, as the extra oxygen atom would make too close a van der Waals contact.

The previously published X-ray data<sup>5,6</sup> on deoxyribose nucleic acid are insufficient for a rigorous test of our structure. So far as we can tell, it is roughly compatible with the experimental data, but it must be regarded as unproved until it has been checked against more exact results. Some of these are given in the following communications. We were not aware of the details of the results presented there when we devised our structure, which rests mainly though not entirely on published experimental data and stereochemical arguments.

It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.

Full details of the structure, including the conditions assumed in building it, together with a set of co-ordinates for the atoms, will be published elsewhere.

We are much indebted to Dr. Jerry Donohue for constant advice and criticism, especially on interatomic distances. We have also been stimulated by a knowledge of the general nature of the unpublished experimental results and ideas of Dr. M. H. F. Wilkins, Dr. R. E. Franklin and their co-workers at

King's College, London. One of us (J. D. W.) has been aided by a fellowship from the National Foundation for Infantile Paralysis.

J. D. WATSON

F. H. C. CRICK

Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, Cambridge. April 2.

<sup>1</sup> Pauling, L., and Corey, R. B., *Nature*, **171**, 346 (1953); *Proc. U.S. Nat. Acad. Sci.*, **39**, 84 (1953).

<sup>2</sup> Furberg, S., *Acta Chem. Scand.*, **6**, 634 (1952).

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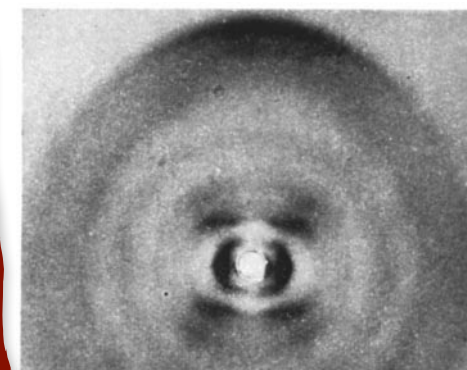
<sup>5</sup> Astbury, W. T., *Symp. Soc. Exp. Biol.*, **1**, Nucleic Acid, 66 (Camb.

alter considerably) in nucleoprotein, extracted or in cells, and in purified nucleate. The same linear group of polynucleotide chains may pack together parallel in different ways to give crystalline<sup>1-3</sup>, semi-crystalline or paracrystalline material. In all cases the X-ray diffraction photograph consists of two regions, one determined largely by the regular spacing of nucleotides along the chain, and the other by the longer spacings of the chain configuration. The sequence of different nitrogen bases along the chain is not made visible.

Oriented paracrystalline deoxypentose nucleic acid ('structure B' in the following communication by Franklin and Gosling) gives a fibre diagram as shown in Fig. 1 (cf. ref. 4). Astbury suggested that the strong 3.4-Å. reflexion corresponded to the internucleotide repeat along the fibre axis. The ~34 Å. layer lines, however, are not due to a repeat of a polynucleotide composition, but to the chain configuration repeat, which causes strong diffraction as the nucleotide chains have higher density than the interstitial water. The absence of reflexions on or near the meridian immediately suggests a helical structure with axis parallel to fibre length.

### Diffraction by Helices

It may be shown<sup>5</sup> (also Stokes, unpublished) that the intensity distribution in the diffraction pattern of a series of points equally spaced along a helix is given by the squares of Bessel functions. A uniform continuous helix gives a series of layer lines of spacing corresponding to the helix pitch, the intensity distribution along the  $n$ th layer line being proportional to the square of  $J_n$ , the  $n$ th order Bessel function. A straight line may be drawn approximately through



layer line, corresponding to  $C$  in Fig. 2.

We will now briefly analyse in physical terms some of the effects of the shape and size of the repeat unit or nucleotide on the diffraction pattern. First, if the nucleotide consists of a unit having circular symmetry about an axis parallel to the helix axis, the whole diffraction pattern is modified by the form factor of the nucleotide. Second, if the nucleotide consists of a series of points on a radius at right-angles to the helix axis, the phases of radiation scattered by the helices of different diameter passing through each point are the same. Summation of the corresponding Bessel functions gives reinforcement for the inner-

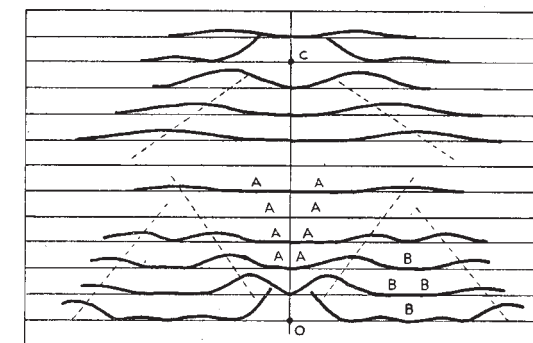


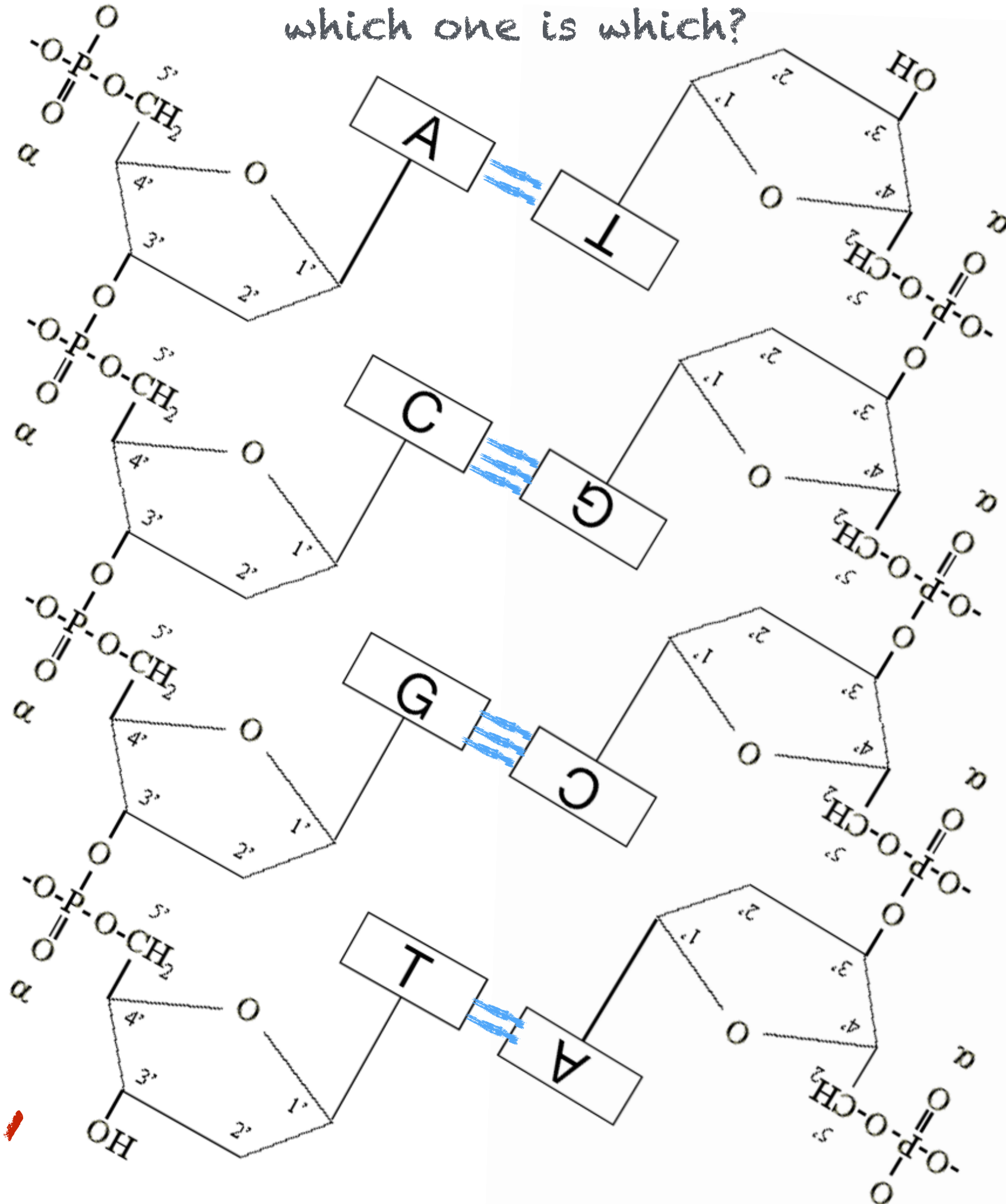
Fig. 2. Diffraction pattern of system of helices corresponding to structure of deoxypentose nucleic acid. The squares of Bessel functions are plotted about 0 on the equator and on the first, second, third and fifth layer lines for half of the nucleotide mass at 20 Å. diameter and remainder distributed along a radius, the mass at a given radius being proportional to the radius. About  $C$  on the tenth layer line similar functions are plotted for an outer diameter of 12 Å.



which one is which?

5'

3'



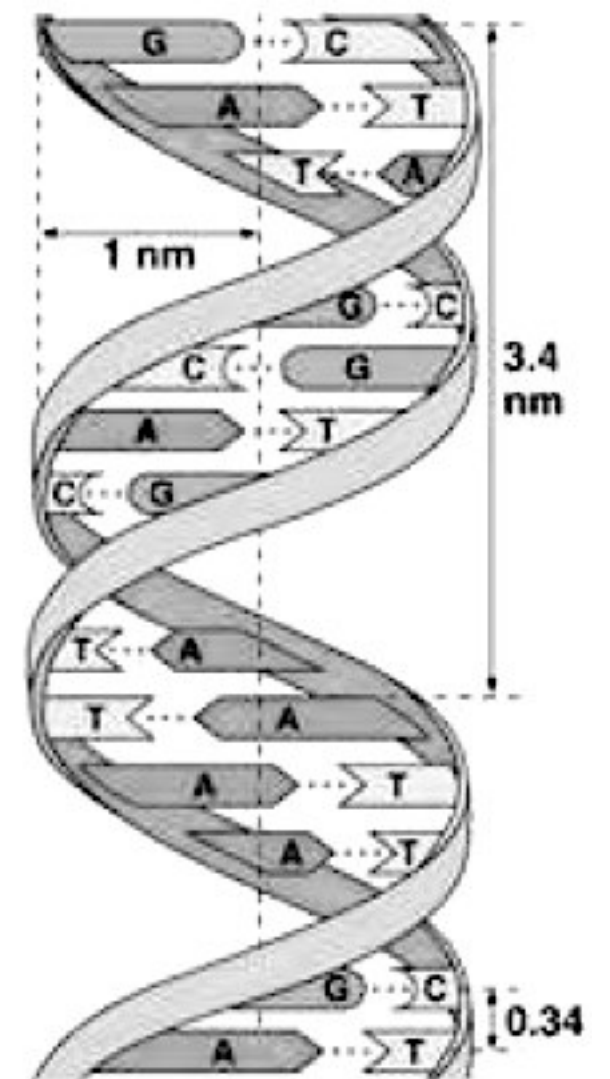
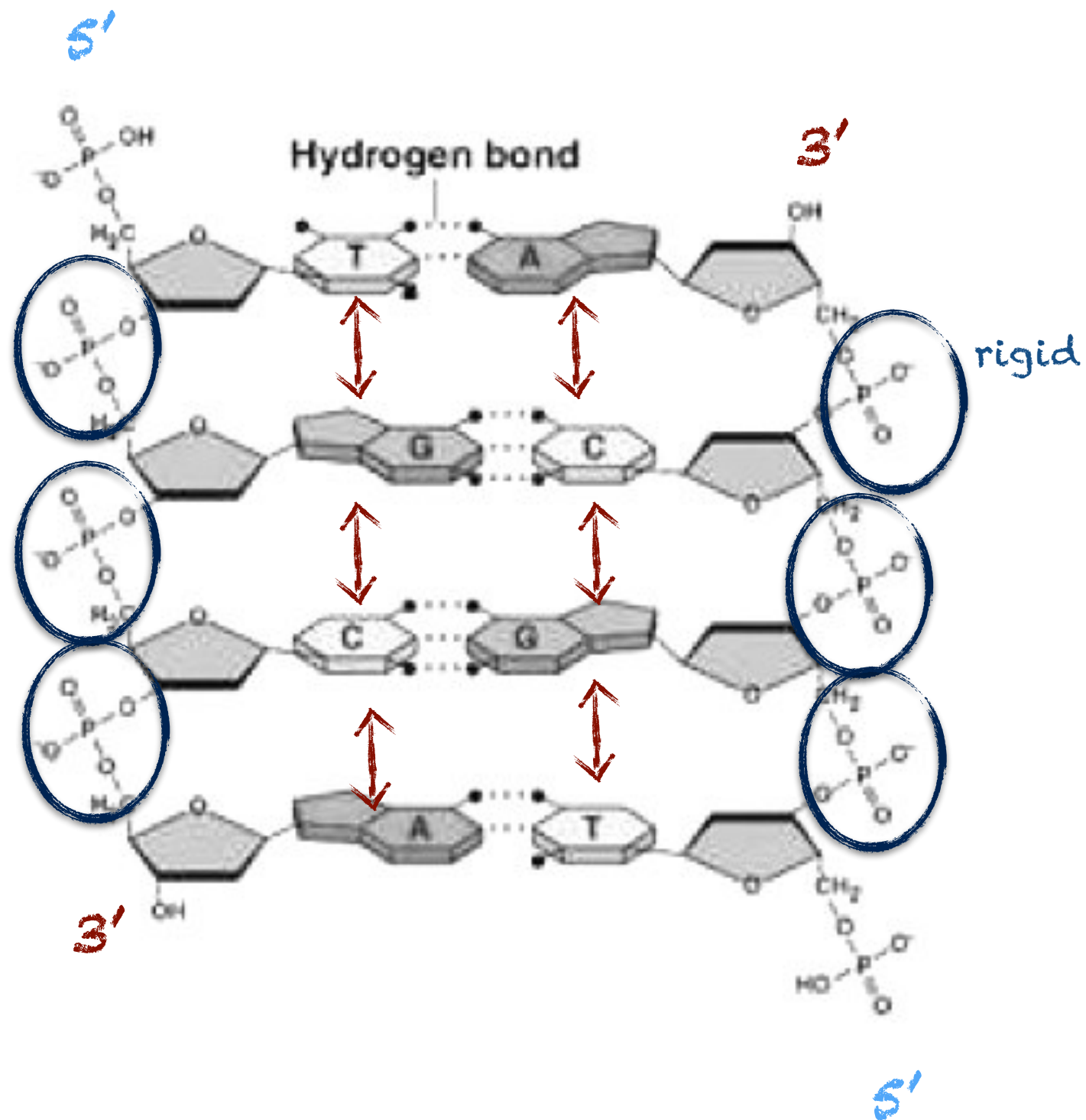
Reverse complementary

3'

5'



hydrophobicity effect



equipment, and to Dr. G. E. R. Deacon and the captain and officers of R.R.S. *Discovery II* for their part in making the observations.

- <sup>1</sup> Young, F. B., Gerrard, H., and Jevons, W., *Phil. Mag.*, **40**, 149 (1920).  
<sup>2</sup> Longuet-Higgins, M. S., *Mon. Not. Roy. Astro. Soc., Geophys. Supp.*, **5**, 285 (1949).  
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WE wish to suggest a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biological interest.

A structure for nucleic acid has already been proposed by Pauling and Corey<sup>1</sup>. They kindly made their manuscript available to us in advance of publication. Their model consists of three intertwined chains, with the phosphates near the fibre axis, and the bases on the outside. In our opinion, this structure is unsatisfactory for two reasons: (1) We believe that the material which gives the X-ray diagrams is the salt, not the free acid. Without the acidic hydrogen atoms it is not clear what forces would hold the structure together, especially as the negatively charged phosphates near the axis will repel each other. (2) Some of the van der Waals distances appear to be too small.

Another three-chain structure has also been suggested by Fraser (in the press). In his model the phosphates are on the outside and the bases on the inside, linked together by hydrogen bonds. This structure as described is rather ill-defined, and for this reason we shall not comment on it.



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is a residue on each chain every 3.4 Å. in the z-direction. We have assumed an angle of 36° between adjacent residues in the same chain, so that the structure repeats after 10 residues on each chain, that is, after 34 Å. The distance of a phosphorus atom from the fibre axis is 10 Å. As the phosphates are on the outside, cations have easy access to them.

The structure is an open one, and its water content is rather high. At lower water contents we would expect the bases to tilt so that the structure could become more compact.

The novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases. The planes of the bases are perpendicular to the fibre axis. They are joined together in pairs, a single base from one chain being hydrogen-bonded to a single base from the other chain, so that the two lie side by side with identical z-co-ordinates. One of the pair must be a purine and the other a pyrimidine for bonding to occur. The hydrogen bonds are made as follows: purine position 1 to pyrimidine position 1; purine position 6 to pyrimidine position 6.

If it is assumed that the bases only occur in the structure in the most plausible tautomeric forms (that is, with the keto rather than the enol configurations) it is found that only specific pairs of bases can bond together. These pairs are: adenine (purine) with thymine (pyrimidine), and guanine (purine) with cytosine (pyrimidine).

In other words, if an adenine forms one member of a pair, on either chain, then on these assumptions the other member must be thymine; similarly for guanine and cytosine. The sequence of bases on a single chain does not appear to be restricted in any way. However, if only specific pairs of bases can be formed, it follows that if the sequence of bases on one chain is given, then the sequence on the other chain is automatically determined.

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<sup>4</sup> Wyatt, G. R., *J. Gen. Physiol.*, **36**, 201 (1952).  
<sup>5</sup> Astbury, W. T., *Symp. Soc. Exp. Biol.*, **1**, Nucleic Acid, 66 (Camb. Univ. Press, 1947).  
<sup>6</sup> Wilkins, M. H. F., and Randall, J. T., *Biochim. et Biophys. Acta*, **10**, 192 (1953).

### Molecular Structure of Deoxypentose Nucleic Acids

WHILE the biological properties of deoxypentose nucleic acid suggest a molecular structure containing great complexity, X-ray diffraction studies described here (cf. Astbury<sup>1</sup>) show the basic molecular configuration has great simplicity. The purpose of this communication is to describe, in a preliminary way, some of the experimental evidence for the polynucleotide chain configuration being helical, and existing in this form when in the natural state. A fuller account of the work will be published shortly.

The structure of deoxypentose nucleic acid is the same in all species (although the nitrogen base ratios alter considerably) in nucleoprotein, extracted or in cells, and in purified nucleate. The same linear group of polynucleotide chains may pack together parallel in different ways to give crystalline<sup>1-3</sup>, semi-crystalline or paracrystalline material. In all cases the X-ray diffraction photograph consists of two regions, one determined largely by the regular spacing of nucleotides along the chain, and the other by the longer spacings of the chain configuration. The sequence of different nitrogen bases along the chain is not made visible.

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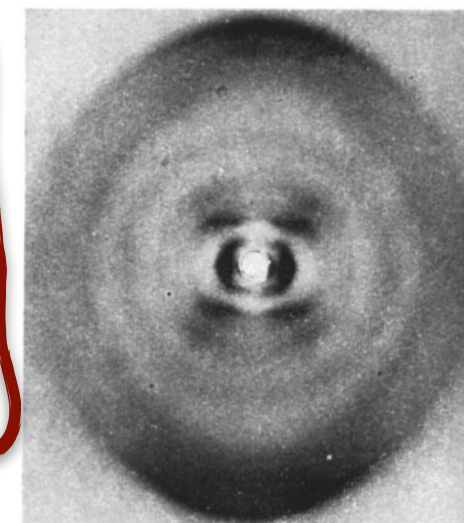


Fig. 1. Fibre diagram of deoxypentose nucleic acid from *B. coli*. Fibre axis vertical

the innermost maxima of each Bessel function and the origin. The angle this line makes with the equator is roughly equal to the angle between an element of the helix and the helix axis. If a unit repeats  $n$  times along the helix there will be a meridional reflexion ( $J_0^2$ ) on the  $n$ th layer line. The helical configuration produces side-bands on this fundamental frequency, the effect<sup>2</sup> being to reproduce the intensity distribution about the origin around the new origin, on the  $n$ th layer line, corresponding to  $C$  in Fig. 2.

We will now briefly analyse in physical terms some of the effects of the shape and size of the repeat unit or nucleotide on the diffraction pattern. First, if the nucleotide consists of a unit having circular symmetry about an axis parallel to the helix axis, the whole diffraction pattern is modified by the form factor of the nucleotide. Second, if the nucleotide consists of a series of points on a radius at right-angles to the helix axis, the phases of radiation scattered by the helices of different diameter passing through each point are the same. Summation of the corresponding Bessel functions gives reinforcement for the inner-



Fig. 2. Diffraction pattern of system of helices corresponding to structure of deoxypentose nucleic acid. The squares of Bessel functions are plotted about 0 on the equator and on the first, second, third and fifth layer lines for half of the nucleotide mass at 20 Å. diameter and remainder distributed along a radius, the mass at a given radius being proportional to the radius. About  $C$  on the tenth layer line similar functions are plotted for an outer diameter of 12 Å.

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# Arthur Kornberg



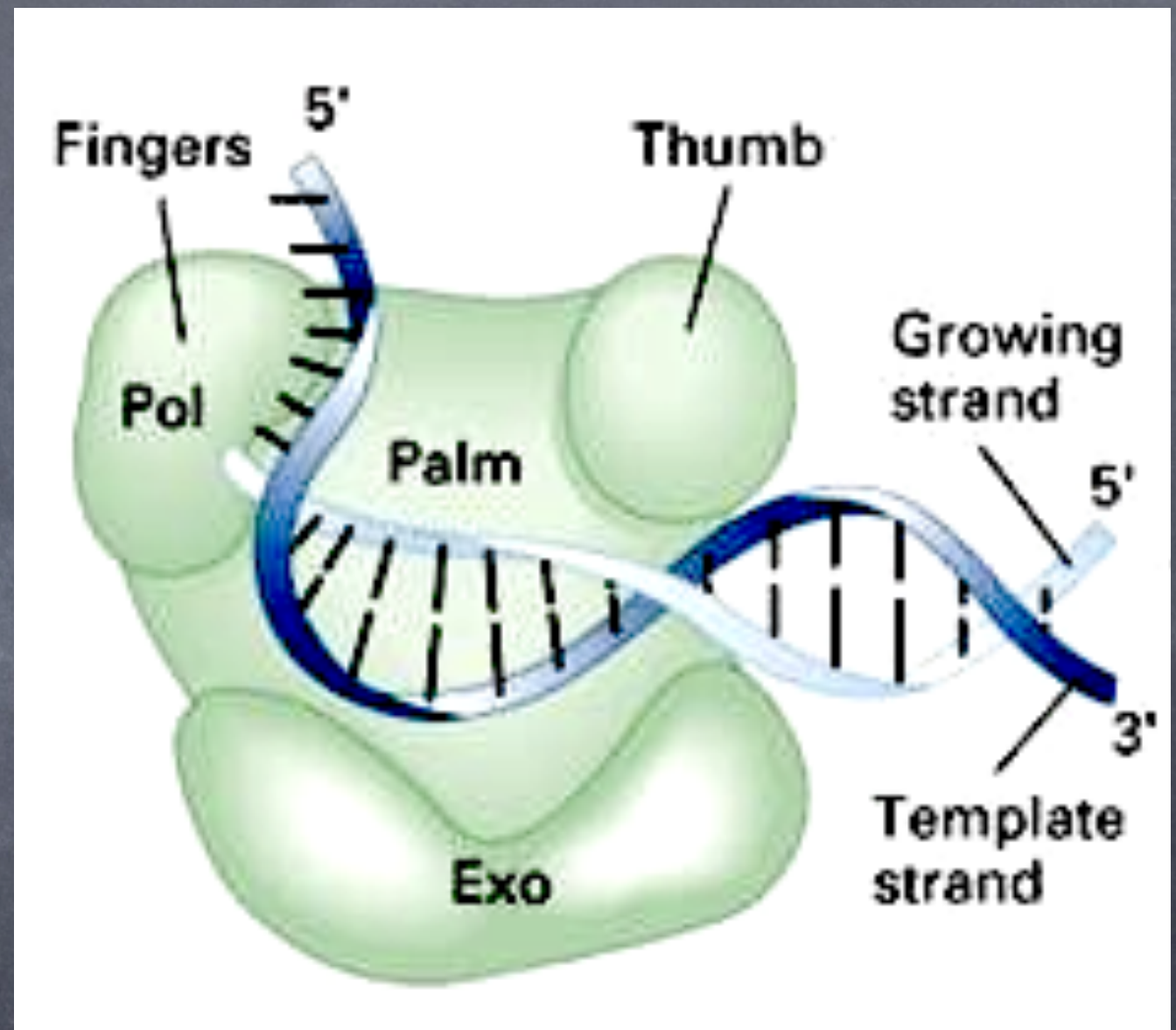
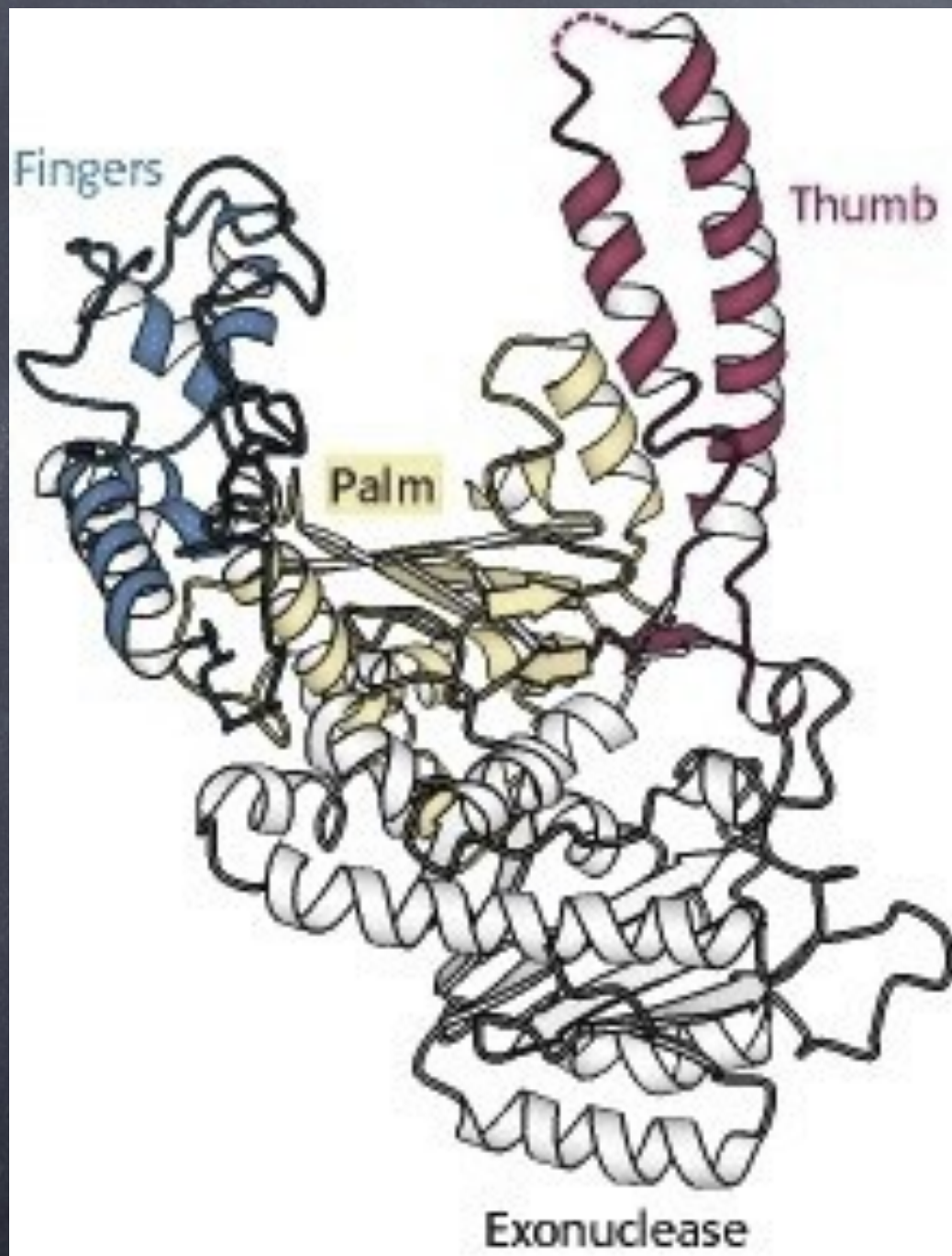
"Enzymic synthesis of  
deoxyribonucleic acid"

Kornberg et al, 1956

Biochimica et Biophysica Acta

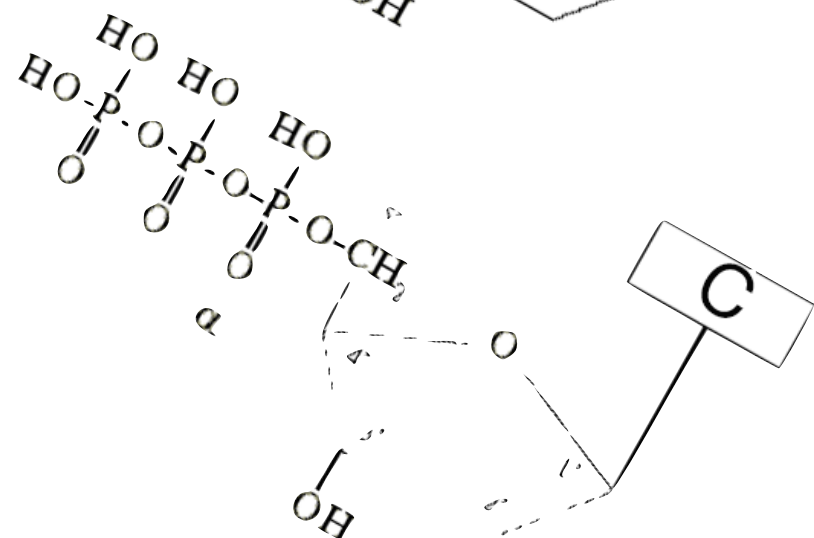
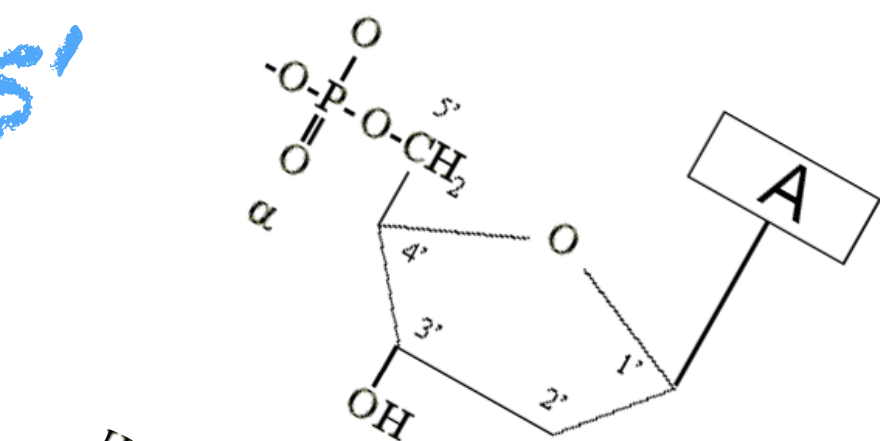


# DNA polymerase I E. coli



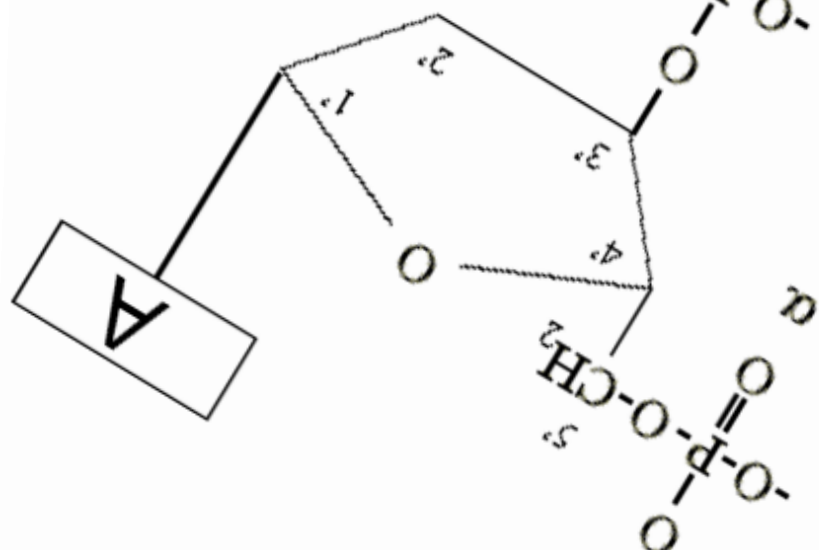
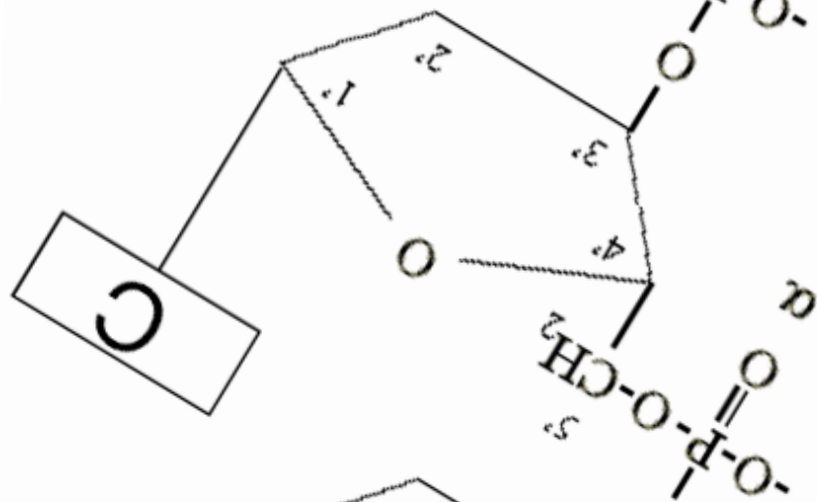
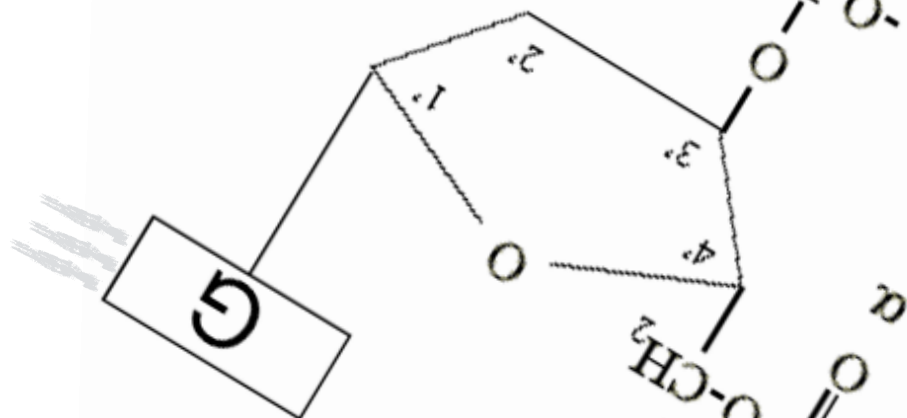
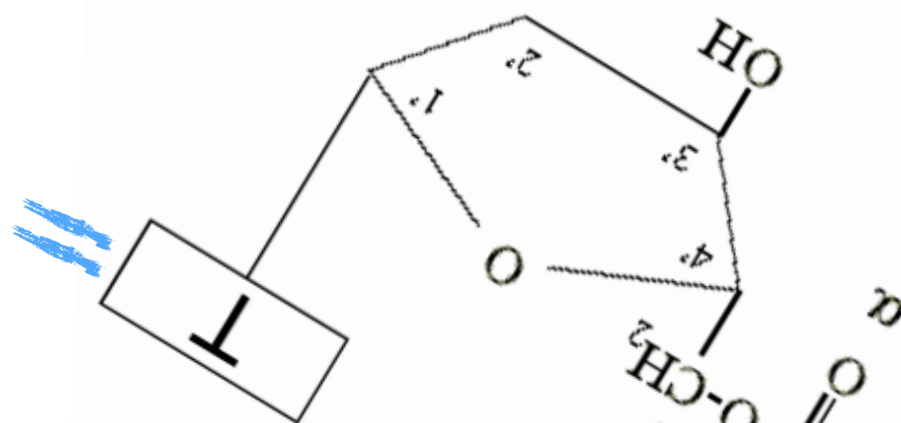


5'



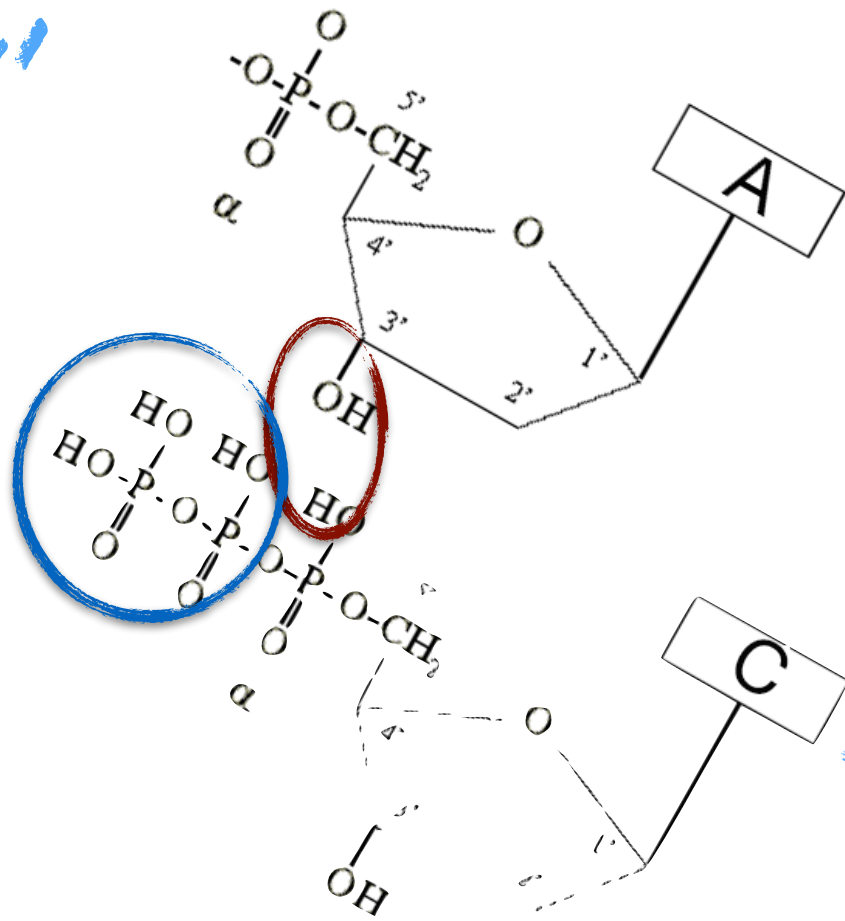
3'

3'

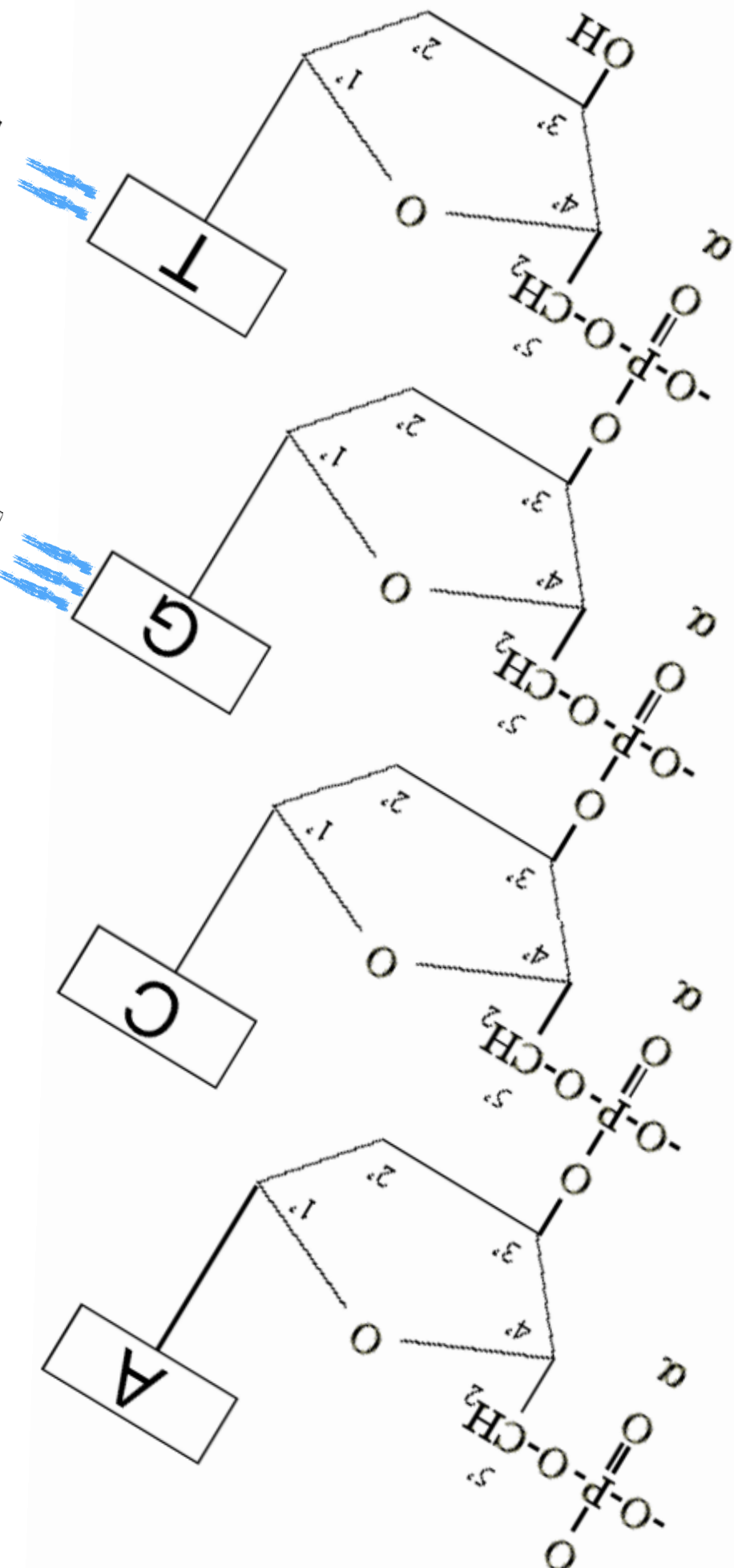


5'

5'



3'



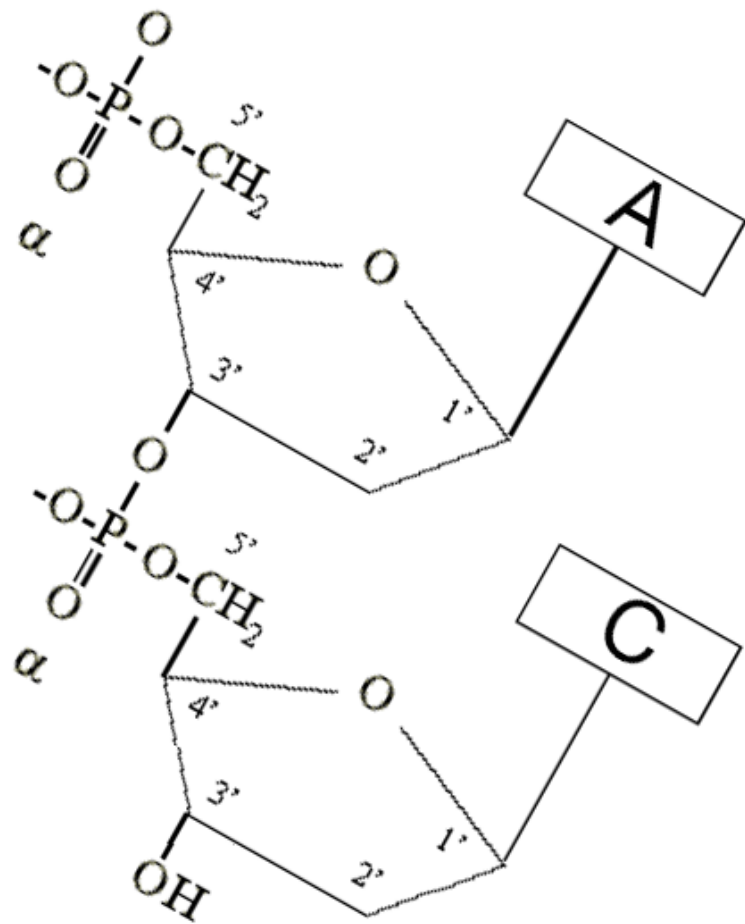
PPi = pyrophosphate

3'

5'



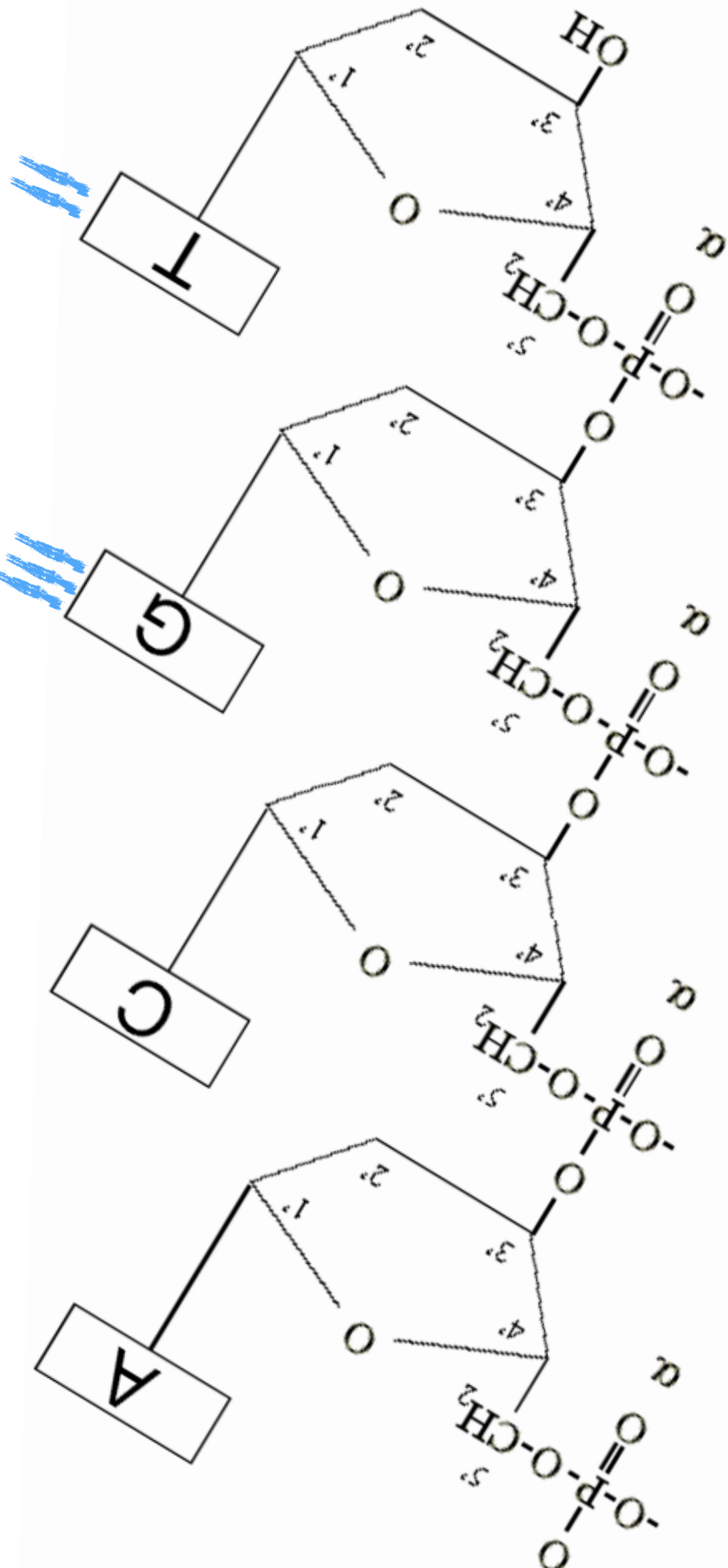
5



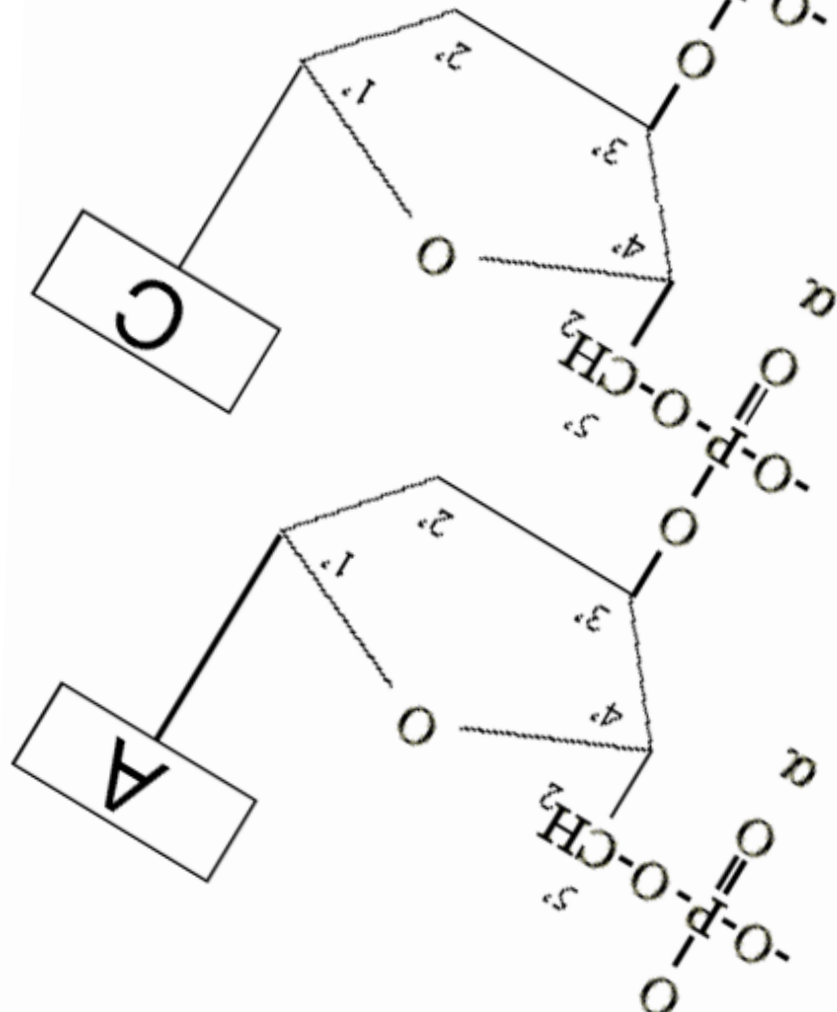
+

PPi = pyrophosphate

3'

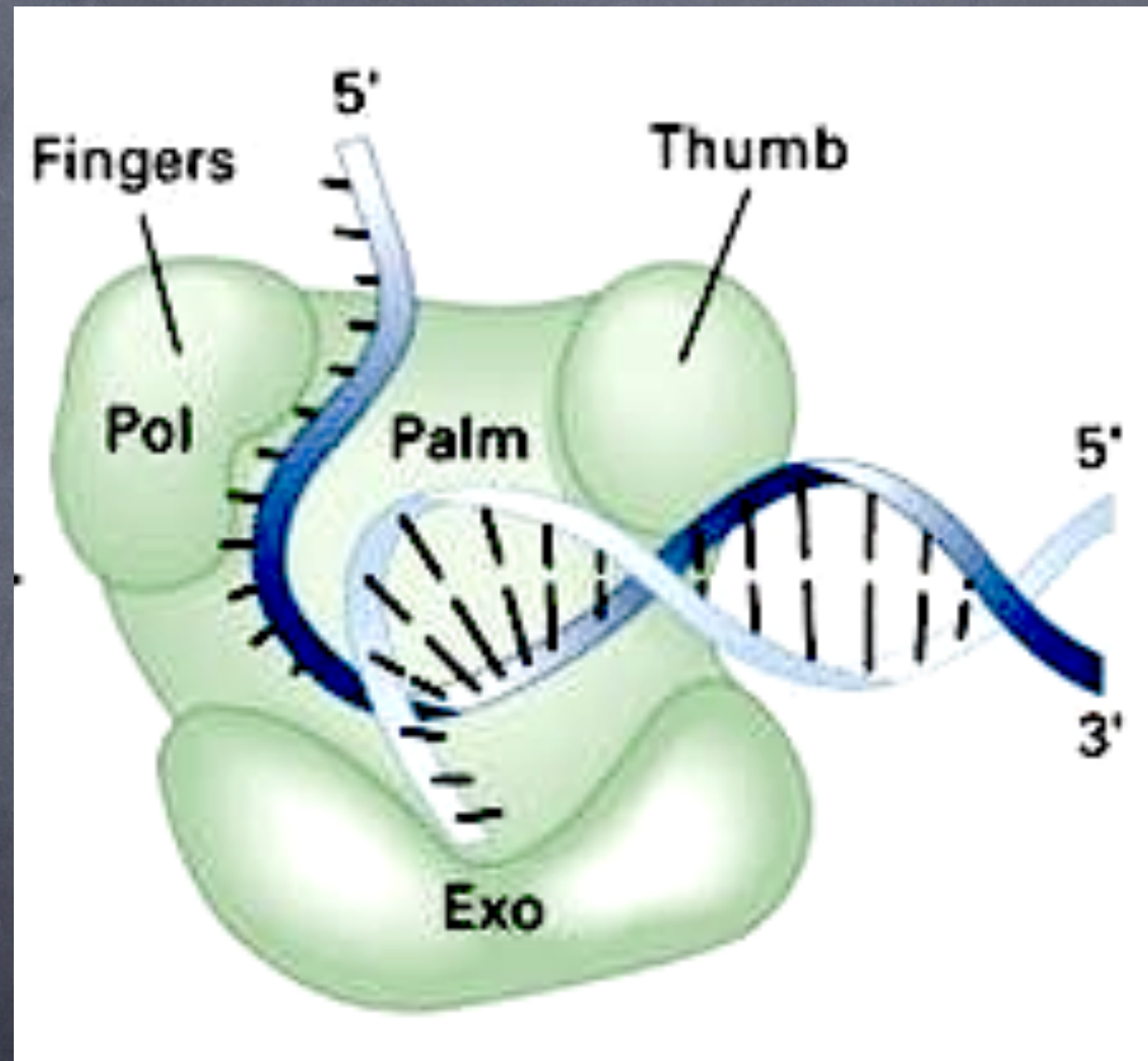


3'



5'

# DNA polymerase I exonuclease activity





# DNA polymerase

- Can synthesise only in one direction
- Needs (among other things):
  1. ssDNA (single strand) as a template
  2. 3'-OH group to add new nucleotides to



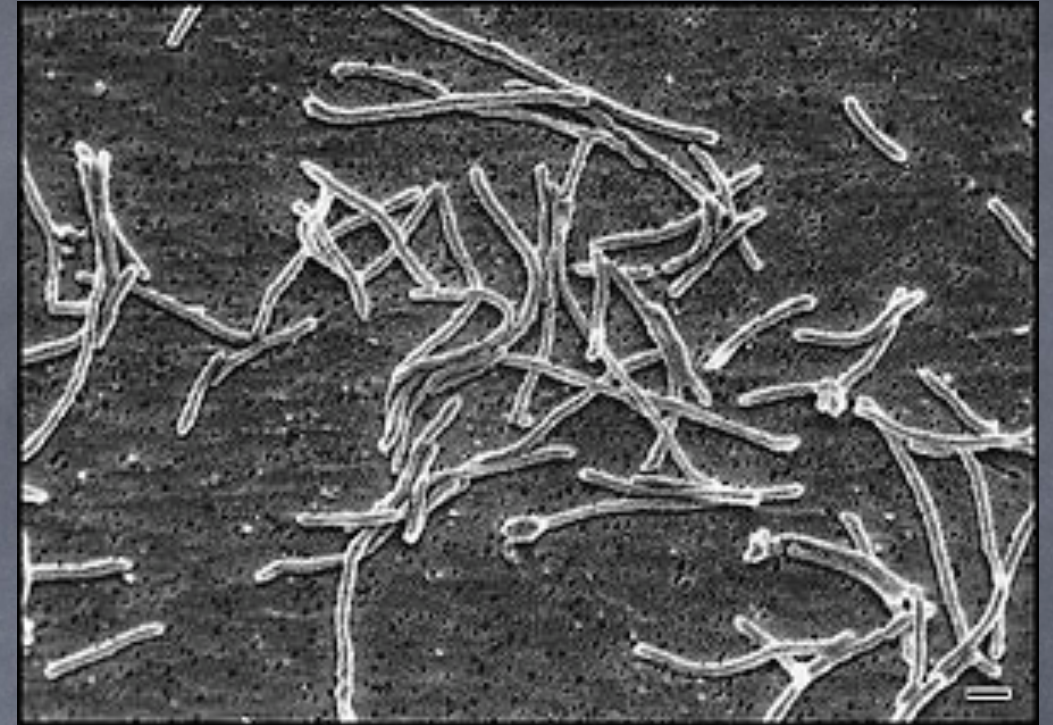
Draw replication of one circular DNA molecule  
how you think it works in an E. coli cell



# Concept of PCR



Yellowstone National Park



Thermus aquaticus  
50-80°C



Thomas D. Brock

Taq DNA polymerase





# Concept of PCR

dsDNA



why are the strands so easy to separate without breaking them?

95°C



melting = denaturation

ssDNA



+



60°C



primer annealing

Taq pol

does this process require energy?



72°C



synthesis = extension

Look up this:

Paul Rothemund: The astonishing promise of DNA folding





# Inventor of PCR Kary Mullis

## 4 Nobel Prize Winners Who Were Clearly Insane

### #3. Kary Mullis

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Prize: Chemistry

Talks Out Of His Ass About: Everything Else

On the other hand, Kary Mullis, 1993 chemistry Nobel Prize winner, is by all accounts a dick.

# Concept of PCR



1 cycle





1. why  $Mg^{2+}$  is important?

what will happen if you add too much/too little?

2. why concentration of dNTP is important?

what will happen if you add too much/too little?

3. why concentration of primers is important?

what will happen if you add too much/too little?

4. why GC content of the template is important?

what to do if it is too high/too low?

5. What is two step and what is three step PCR?