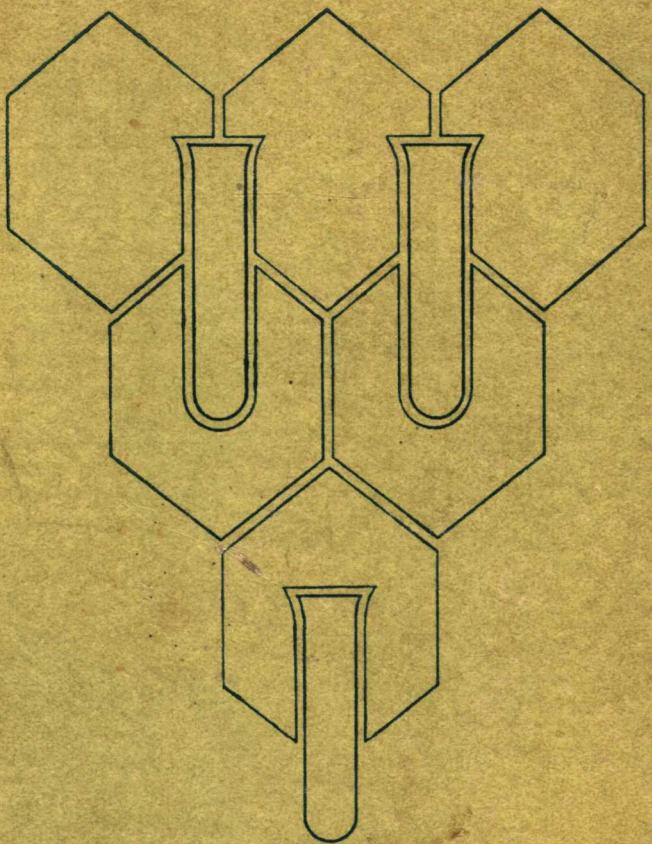

Volume III

Methods in Cancer Research

Edited by HARRIS BUSCH

METHODS IN CANCER RESEARCH

Edited by

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VOLUME III

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Preface

Volume III of this treatise, "Methods in Cancer Research," was designed to review primarily two major groups of topics which are of very great importance in cancer research, namely, the molecular biology of cancer and approaches to cancer chemotherapy. In the chapters on molecular biology, DNA and its synthesis and RNA and its synthesis and degradation are reviewed in detail. Methods for study of nuclear proteins and other macromolecules are also presented in this volume.

The problems in design of anticancer agents are discussed with particular reference to organic synthesis and aspects of inhibition of enzymes and cellular biosynthetic reactions. Since valuable drugs for cancer chemotherapy have been obtained from biological sources, this volume concludes with a discussion of natural products in cancer chemotherapy.

Volume III supplements Volume I, which dealt with morphology of tumor cells, transplantation and metastases, and carcinogenesis, and Volume II, which dealt with immunology and special products of cancer cells, cell fractionation, and enzymes. The final volume of this treatise will contain chapters on the important topics of human carcinogenesis, the "Morris hepatomas," which represent the most differentiated experimental cancer cells, evaluation of cancer chemotherapeutic agents, and viral components including infective viral RNA and the lactate dehydrogenase virus.

It is a pleasure to express appreciation to the many authors who have labored diligently to produce this work and to the staff of Academic Press for their splendid cooperation.

HARRIS BUSCH

Houston, Texas
August, 1967

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CHAPTER I

DEOXYRIBONUCLEIC ACIDS AND CANCER

K. S. KIRBY

I. Introduction

The concept of DNA as the repository of information required for the manufacture of proteins in the cell has, particularly in microorganisms, led to an enormously increased understanding into the mechanism of transformation and transduction in bacteria, the nature of the induction of enzymes, and the transfer of information via mRNA. Virus infection is now susceptible to molecular studies and it is clear that while the ultimate information resides in the DNA, in certain viruses sufficient information may be carried in a molecule of RNA, which may be single or double stranded. The molecular mechanism of mutation by addition, deletion, or replacement of bases in the "wild type" nucleic acid together with the solution of the problem of coding sequences of bases in the nucleic acids required for each amino acid in the protein, is beginning to unfold the importance of primary, secondary, tertiary, and quaternary structures in the biological functioning of proteins.

However, it is certain that molecular processes in multichromosomal and multicellular organisms are relatively much more complex than those in bacteria and while investigations have proceeded on adjacent lines these have not necessarily been parallel, and indeed many differences are to be expected. Cells in a multicellular organism are interdependent requiring distribution of nutrients, transmission of information by nervous, hormonal, and other means, and the cells of any particular organ tend to be specialized, performing a limited number of functions compared with those carried out by the whole individual. If therefore, as seems probable, the amount of DNA in each somatic cell is twice that of the sperm cell (Leslie, 1955) [cases of polyploidy being neglected] then it is clear that all of the DNA in each somatic cell cannot be used for the transmission of information. While it is possible then that some common segments of DNA are used by all cells, it is likely that certain segments of the DNA which are used for the transmission of information (i.e., on which mRNA is biosynthesized) differ in one organ of a multicellular organism from those used by another. One point should be made clear: in a mammalian cell the amount of DNA which carries information for the biosynthesis of enzymes is not necessarily the larger proportion of the DNA utilized by the cell for, indeed, the amount required for operating the control mechanisms may well be greater. It has, for example, been estimated that 80% of the DNA of interphase lymphocytes can be recovered in the repressed chromatin fraction (Frenster *et al.*, 1963) in which RNA precursors are not used and mRNA is evidently not biosynthesized.

The steps which lead to the conversion of a normal into a cancer cell are unknown and it is at this point, in the light of the previous knowledge, that we must consider the possible mechanisms which may lead to this change. Since a cancer line, once established, maintains its genetic type over a number of generations it is reasonable to assume that some modification to the genetic material has taken place. This could be through a mutation acting directly on the DNA or through a series of changes analogous to differentation. Foulds (1964) has discussed the series of changes cells undergo in the development of a cancer line and has used the term "progression" for this. A series of mutations, rather than a single event, is likely to be required for the carcinogenic process and in this respect the discussion by Brues (1958) criticizing the linear dose-effect relationship with respect to the effects of radiation is relevant. The replacement, addition, or deletion of bases in DNA or RNA leading to an altered sequence of amino acids in the protein coded by the particular sequence concerned is fairly well understood. Among the best examples is the replacement of amino acids

in the coat protein of tobacco mosaic virus following the mutagenic action of nitrous acid on the RNA (Wittman and Wittman-Liebold, 1963). However, the result of a series of mutations cannot always be forecast. Yanofsky (1963) has found that, in the enzyme tryptophan synthetase of *Escherichia coli*, replacement of glycine at position 8 in the CP2 peptide by arginine or glutamic acid resulted in a loss of enzyme activity. This activity could be recovered by revertants in which arginine was replaced by threonine, serine, or glycine or in which glutamic acid was replaced by glycine, alanine, or valine. In four of these cases then the original protein was not recovered. However, a second type of recovery was found by isolation of a slower growing type in which a second mutation had taken place, tyrosine being replaced by cystein, in which type the original mutation remained. Changes in the primary structure of a protein (i.e., amino acid sequence) may affect the enzyme activity by a change in the active site, by altering the secondary structures (H-bonding or α-helical regions), the tertiary structure (regions associated through van der Waals and London forces, also called hydrophobic regions), or the quaternary structure (association of different polypeptides into the "active" protein, as in the association of the α-and β-polypeptides with hemin to form hemoglobin). The effect of changes in the amino acid sequences in the polypeptide chains of hemoglobin on the biological properties of the protein are among the best examples, and demonstrate the clear results that can be obtained from studies in human genetics (Baglioni, 1962).

The process of differentiation requires a progressive modification of the information available from the DNA, but at present no molecular mechanism is available for this. Illuminating examples of the effect of ecdysone, a steroid hormone, which induces molting in *Chironomus thummi* have been described by Becker (1964). This hormone produces a sequence of puffs on the chromosome, from each of these mRNA is made and evidence has been presented by Karlson (1963) that one of these will code for the enzyme dopa-oxidase, required for the molting procedure. Although this sequence makes clear what may happen after the hormone is produced, it is as yet unknown why one cell type at a particular stage produces the particular hormone.

If DNA could be isolated from normal and cancer cells and compared directly, it would be possible to decide whether the carcinogenic process simulated mutagenesis or differentiation. Isolation of the chromatin material or comparison of the mRNA from this would be required to investigate the nature of the change. However, direct comparisons of DNA from mammalian sources is extremely difficult and while hybridization techniques have been used as an approach to bacterial taxon-

omy (Marmur *et al.*, 1963) these are not so satisfactory with mammalian material.

DNA of some cancer tissues is certainly not the same as that of the average somatic cell, since the number of chromosomes is different. However these cases could be analogous to polyploidy where the DNA is the same but in double the amount. The number of chromosomes is not always different in cancerous tissues (Bayreuther, 1960; Sandritter *et al.*, 1965a,b,) and hence a change in the number of chromosomes may be a result of rather than a cause of the malignancy.

To make comparisons at all it is first necessary to isolate DNA in as quantitative a manner as possible and as free as is practicable from other associated macromolecules, the principal groups being RNA, proteins, and polysaccharides. Any of these remaining groups associated with the DNA may give rise to alterations in the physical properties which then make the extract behave differently from DNA extracted from a different cellular type.

II. Isolation of DNA

The binding of DNA to the protein in the chromosome varies with the tissue. The DNA in salmon sperm is bound to the protamine (in which about half of the amino acid residues are arginine) by salt links and these can be split by concentrated sodium chloride, after which the protein may be removed by the action of detergent and dialysis. Mammalian sperms are more refractory, partly because of the keratin-type protein in the coat, and require treatment with mercaptoethanol since the simple reaction with sodium chloride does not release DNA free from protein (Borenfreund *et al.*, 1961).

Detergents remove protein and inhibit enzymes, but the amount of residual protein varies with the tissue. Kay *et al.* (1952) have described a method, which is widely used, based on the action of dodecylsulfate (Marko and Butler, 1951). Simmons *et al.* (1952) preferred to use sodium xylene sulfonate. The methods require the separation of cell nuclei, rupturing the DNA-protein links with concentrated sodium chloride and a chelating agent (citrate or ethylenediaminetetraacetate), and denaturing the protein with the detergent. Final deproteinization requires several treatments with chloroform containing 1-pentanol or octanol. The yield may not be quantitative as DNA bound to residual protein may be trapped in the interfacial gel and discarded. The procedure has been described in detail by Zamenhof (1958). Colter *et al.* (1957) compared the two detergents and decided that xylene sulfonate

gave the better product from Ehrlich ascites cells as judged by the viscosity of the DNA. Woernley *et al.* (1959) were unable to release DNA free from protein from Ehrlich ascites cells by either method. Zahn *et al.* (1962) have carefully listed the points at which degradation may occur during the isolation and warmed their extracts at $(T_m - 10)$°C [usually ~62°C, cf. Marmur and Doty, 1959] for 15 minutes to inactivate nucleases and removed added ribonuclease by treatment with bentonite (Brownhill *et al.*, 1959). The DNA prepared from Ehrlich ascites cells by this method was judged free from protein after nine extractions with chloroform and a sample of DNA from *Holothuria tubulosa* maintained its viscosity for 2 years (Zahn *et al.*, 1962).

Phenol is an alternative to detergent for removing protein (Kirby, 1957, 1959). Nucleases are inactivated and it is not necessary to separate nuclei. The yield of DNA and the amount of residual protein depend upon the nature of the salt used in association with the phenol. At 0.3 *M* concentration, hydrophilic salts release very little DNA from mammalian tissues while a good yield of DNA results if the tissue is first broken down in the salt of a lipophilic anion. In the latter case some residual protein remains with the DNA, and this can be reduced to a minimum by using an anion which combines lipophilic and chelating properties; empirically the best results have been obtained with 4-aminosalicylate. However, combinations of salts are equally effective and naphthalene-2-sulfonate and ethylenediaminetetraacetate were more effective in releasing DNA with a smaller amount of residual protein from mouse liver than 4-aminosalicylate (Kirby, 1958). Addition of dodecylsulfate to the salt mixture had no effect on the amount of residual protein.

Addition of 8-hydroxyquinoline and *m*-cresol to the phenol results in an improved deproteinizing mixture (Kirby, 1962, 1965), and the original extraction can be carried out in the cold since this mixture does not crystallize as easily as phenol alone. With this mixture several salts that were originally found to be ineffective with phenol release DNA from the tissues, particularly if ethylenediaminetetraacetate is present. The results emphasize that some of the DNA is probably bound to the protein through bivalent metal linkages (Kirby, 1957). Generally it has been found useful after the first extraction to make the aqueous phase 0.5 *M* with respect to NaCl and reextract with the phenol-cresol mixture as this will reduce residual protein to extremely low values.

Protein may be removed from DNA of rat spleen by this method, and in this particular case diethyldithiocarbonate (0.3 *M*) with phenol-

phthalein diphosphate (0.015 *M*) is a very useful combination for the first extraction (Kirby, 1961a, 1962, and unpublished results), giving a higher yield of DNA.

Phenolphthalein diphosphate (Kirby, 1961) is of particular interest as, in combination with phenol, it is possible to separate DNA from RNA without the use of enzymes. If the tissues are broken down in this salt (pH 6.0, 0.15 *M*) and extracted with phenol, the DNA sedimented by high-speed centrifugation is completely free from RNA. However about five or six extractions with sodium benzoate (1.5 *M*) are required to solubilize DNA from the sediment, and it is usually recovered in about 75% of the yield obtained with the 4-aminosalicylate. It should be noted that this is not a general method for separating RNA and DNA as, if these are mixed and extracted, both DNA and RNA are precipitated. The method is only satisfactory if carried out on whole tissues or on the insoluble interfacial material after removing ribosomal and transfer RNA by a preliminary phenol extraction (Hastings, 1964).

Borenfreund *et al.* (1961) failed to extract DNA from bull or human sperm by detergent or phenol methods, but were successful after allowing sperm heads to react first with mercaptoethanol, then with trypsin, and finally removing protein by the method of Kay *et al.* (1952). DNA was released from rat sperm by sedimenting in the presence of cupferron or salicylate and then extracting with the same salt and phenol (Kirby, 1957).

Application of the phenol method to vaccinia virus gave a poor yield of single-stranded DNA, but after drying the virus at 40°C and treating with mercaptoethanol essentially as Borenfreund *et al.* (1961) had done for sperm, Pfau and McCrea (1962) isolated 90% of the DNA after three treatments with pronase. The double-stranded DNA was sedimented by centrifuging at 35,000 rpm for 5 hours and therefore must have been of very high molecular weight. Subsequent treatment with phenol did not split the DNA strands and it was assumed that both single- and double-stranded DNA is present in the virus.

An example of the importance in the choice of salt is demonstrated by the release of DNA from polyoma virus by the action of trichloroacetate and phenol. Weil (1961) found that with this particular salt DNA with the highest infectivity could be obtained.

III. Separation of DNA from RNA

Most procedures that liberate DNA from tissues also liberate RNA and the ease of separation depends very much on the relative amount of the two nucleic acids in the tissue. Separation of nuclei will give a sepa-

ration from cytoplasmic RNA, but nuclear RNA has still to be separated. A general solution, if the RNA is not required, is to add ribonuclease and then oligonucleotides can be removed by precipitating the DNA with cold 2-ethoxyethanol, redissolving and dialyzing. Precipitation with 2-ethoxyethanol will give a partial separation as DNA precipitates quickly as a jelly, whereas RNA precipitates flocculently and more slowly. Phenolphthalein diphosphate and phenol (Kirby, 1961a) will give a complete separation and though difficulty was first experienced in recovering RNA, precipitation of this from the aqueous phase with *m*-cresol affords a good recovery.

Since ribosomal RNA is insoluble in strong salt solutions a fractionation can be achieved in this manner. Colter *et al.* (1962) have used 1 *M* salt to prevent rRNA from going into solution. Cells were broken down in the presence of deoxycholate (which improves the yield of DNA from Ehrlich ascites cells or mouse liver which had not been powdered prior to extraction) and extracted with 1.0 *M* NaCl–0.22 *M* potassium phosphate, pH 7.3 and phenol. They found that DNA prepared in this way from ascites cells was more homogeneous in the ultracentrifuge and less contaminated by RNA than DNA prepared by the dodecylsulfate method (Borenfreund *et al.*, 1961). Pneumococcal DNA prepared by either method had the same transforming activity.

Apart from the phenolphthalein diphosphate method two other methods have been used in the author's laboratory for the separation of DNA from RNA without using enzymes. If the size of the DNA is unimportant, all the nucleic acids can be separated by extraction with 4-aminosalicylate and a phenol-cresol mixture, and after precipitation from the aqueous layer, DNA together with sRNA and glycogen can be extracted with 3 *M* sodium acetate, pH 6.0. This method is successful only if the DNA has a $s_{20,w}$ of less than 20, in which case the DNA dissolved readily in the sodium acetate (Kirby, 1965) DNA of higher molecular weight is not so easily dissolved and a good separation is rarely achieved. The separation therefore depends upon release of DNA while the tissue is being broken down in the blender.

The separation is successful if the tissue is first broken down in a mixture of a hydrophilic salt and phenol. In this case, nuclear and chromosomal material is not released (cf. preparation of phenolic nuclei, Georgiev *et al.*, 1960) and cytoplasmic RNA is found in the aqueous phase. The insoluble material may then be collected and reextracted with 4-aminosalicylate and phenol, which will release the DNA. Good yields of DNA can be obtained in this way only if the phenol contains 8-hydroxyquinoline (Hastings, 1964; Kirby, 1964b). It appears that the hydrophilic salt used in the first extraction and the 8-hydroxyquinoline