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Interactions Of Dimethylbenz(c)acridines And Polycyclic Hydrocarbons With Dna And Their Effects On Its Template Activity

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INTERACTIONS OF DIMETHYLBENZ(c)ACRIDINES AND POLYCYCLIC AROMATIC HYDROCARBONS WITH DNA AND THEIR EFFECTS ON ITS TEMPLATE ACTIVITY

by

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To My Wife

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ABSTRACT

The direct in vitro interaction of polycyclic hydrocarbons and dimethyl derivatives of benz(c)acridine was studied. The possibility that such compounds might have an effect on transcription was investigated in an in vitro DNA dependent RNA synthesizing system.

Of the 3 dimethylbenz(c)acridines chosen for study, the carcinogenic 7,9- and 7,10- derivatives could form physical complexes with DNA readily under conditions of low ionic strength and pH, whereas the non-carcinogenic 7,11- could not. Studies of the characteristics of the physical interactions strongly indicated that they bind to the DNA double helix internally, slipping in between 2 adjacent bases.

It was not possible to assay the template activities of these physical complexes of DNA:dimethylbenz(c)acridines or DNA:polycyclic hydrocarbons, as they were dissociated even by the minimum ionic strength and pH required by the RNA polymerase of E. coli to function. However, covalent complexes between the polycyclic hydrocarbons and DNA are formed in vitro by γ-irradiation and are not dissociated in the enzyme assay system. An improved technique for efficient binding of pyrene and benz(a)-pyrene to DNA by γ-irradiation was developed. Benz(a)pyrene

bound three times as well as pyrene, whereas none of the dimethylbenz(c)acridines could covalently bind to DNA under the influence of γ-irradiation. Preliminary experiments with the covalent polycyclic hydrocarbon:DNA complexes indicated that benz(a)pyrene inhibits RNA synthesis, and likely its effect is on polymerization rather than on the initiation step.

I. REVIEW OF LITERATURE

A. Development of the Study of Chemical Carcinogenesis

1. Historical introduction

Cancer may be defined as a tumour, the growth of which exceeds and is uncoordinated with that of the normal tissue and which can invade adjacent tissues and spread to other parts of the body.

The existence of cancer has been evident in palaeo-pathological remains in various parts of the globe (Bett, 1957). Ancient Egyptians recorded diseases not unlike breast cancer which was treated by cauterization. Hippoerates and his school described the external manifestations of cancer. Celsus distinguished between benign and malignant neoplasms. Galen's contention that people in whom black bile persisted were susceptible to cancer was unchallenged for more than a millenium. The idea was attacked in 1757 by LeDran who also described cancer as a local lesion, which spread along lymphatic vessels and tended to recur.

Cancer, which has afflicted mankind from pre-historic times, had been shrouded by mystical concepts. The first realization that cancer might be due to external factors and not to the "wrath

of the gods" followed the publication by Sir Percival Pott of "Surgical Observations" (1772), in which he reported a high incidence of scrotal cancer in chimney sweeps. This indicated the presence of some cancer-producing factor(s) in soot. Sir James Paget introduced the term "carcinogen" to describe the "material" responsible for the initiation of cancer in 1853. However, the search for such carcinogenic factors had to wait until 1915 when the Japanese workers, Yamagiva & Ichikawa, first succeeded in producing cancer at will by repeated applications of tar to the skin of rabbits. Tsutsui confirmed the carcinogenic effects of coal tar using mice in 1918.

2. Isolation and identification of natural chemical carcinogens

The isolation and identification of the carcinogenic agents in tar was difficult. The composition of tar is complex and varied considerably with source and manner of preparation. Block & Dreifuss (1921) obtained 100% incidence of very malignant tumours in mice painted with a fraction of high-boiling coal-tar. They reported that the carcinogenic factor(s) contained no low-boiling hydrocarbons, phenols or bases and was free from sulfur and nitrogen. This carcinogenic fraction was able to form picrates, probably of the aromatic hydrocarbon type.

Kennaway (1925) was able to produce carcinogenic tars from organic materials such as hair, human tissues, cholesterol, etc., and also from acetylene or isoprene exposed to high temperature with hydrogen. He initiated the search for the carcinogenic

constituents of coal-tar pitch.

The exciting discovery of the first carcinogenic constituent of coal-tar, 3,4-benzopyrene, came in 1933. The fractions of tar which were most carcinogenic showed a striking fluorescence which led Mayneord to examine their fluorescence spectra. Cook, Hewett & Hieger (1933), using the characteristic fluorescence spectrum as a guide, obtained from 2 tons of coal-tar pitch, 7 g. of a crystalline fraction from which, among other substances, 3,4-benzopyrene was isolated. Cook & Hewett (1933) established its structure by synthesis. The exciting story of the isolation and identification of 3,4-benzopyrene from coal-tar has been recorded in detail by Cook (1943) and Kennaway (1955).

A number of other carcinogenic substances have subsequently been shown to be present in coal-tar and perhaps some others have not yet been identified. Although 3,4-benzopyrene is one of the most potent carcinogens for the mouse skin, it is less effective than some of the other tar constituents when tested by subcutaneous injections or on the rabbit's skin (Berenblum & Schoental, 1947). In view of the tissue and species differences in response to carcinogenic agents, it is not yet certain which, if any, of the various tar constituents are carcinogenic for the human skin.

More than 450 compounds have been found to be carcinogenic up to 1953 and are entered in Hartwell's "Survey of compounds which have been tested for carcinogenic activity" (1951)

and in its supplement (1957). Of these compounds, more than 200 were polycyclic aromatic hydrocarbons, their derivatives and analogues. Azo-compounds and aromatic amines formed the other two large groups of carcinogenic substances. Most studied of the hydrocarbons are 3,4-benzopyrene, 1,2, 5,6-dibenzanthracene, 20-methylcholanthrene and 9,10-dimethyl-1,2-benzanthracene, all of which are available commercially.

Although for routine testing of polycyclic aromatic hydrocarbons, mice are most frequently used, these carcinogens have been found to induce tumours in almost every tissue and animal species in which they have been tested. As these compounds are insoluble in water, their application involves the use of lipids or lipid solvents in which the hydrocarbons are soluble to varying degrees. Application to the skin of mouse results in benign warts, benign or malignant papilloma and epithelioma on the treated area with a latent period of several months. The latent period is inversely proportional to the activity of the substance tested. For subcutaneous injections, the substance is dissolved or suspended in vegetable oil or tricapryline and injected subcutaneously. The appearance of a tumour can usually be detected by palpation after a latent period of several weeks or months.

The effective dose of the potent carcinogenic polycyclic hydrocarbons is minute, of the order of micrograms. In contrast, many other types of carcinogens, such as azo-dyes, aromatic amines, urethane, pyrrolizidine alkaloids, aliphatic nitrosamines etc., require higher doses (by a factor of 10^2 - 10^5)

and produce tumours in organs remote from the site of application.

From early studies on the experimental induction of skin cancer resulting from cutaneous administration of pure chemical carcinogens, it was evident that many factors influenced the carcinogenic process. The number, size, location, type of tumour and the latent period were found to be dependent on the particular carcinogen used, its route of administration, the amount given, the solvent employed as well as the strain, sex, age and dietary conditions of the experimental animals. Also involved are genetic and hormonal factors.

It has been observed that certain solvents can themselves induce cancer (Dickens, 1947; Peacock, Beck & Anderson, 1945; Riska, 1956). Some solvents may act as co-carcinogens which, although not carcinogenic, enhance tumour production and shorten the latent period when applied together with or shortly after the carcinogen. On the other hand, there are agents that act as anti-carcinogenic agents which inhibit carcinogenesis by decreasing the tumour production and lengthening the latent period (Crabtree, 1947; Berenblum, 1947; Berenblum & Shubik, 1947; Gelboin, 1965).

The carcinogenic power of a given compound is usually defined by a numerical index which is evaluated experimentally according to Iball (1939):

$$\text{Iball Index} = \frac{\text{No. of Animals bearing tumors}}{\text{No. of animals living at the appearance of the first tumor}} \times \frac{100}{\text{Latent Period (days)}}$$

As various important experimental factors have great influence on this numerical value, it is necessary to associate with each carcinogenic substance, not a series of numbers but a series of functions each of which can be written:

$$\text{Iball Index}_{(S,T)} = F\ (A,M)$$

where the subscripts S indicates the substance used, T the type of cancer observed, A the animal studied and M the means of application of the substance.

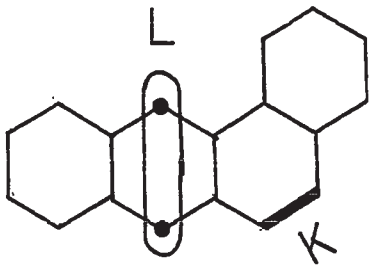
3. Structure and metabolism of alternant hydrocarbons in relation to carcinogenicity

As has been suggested by Daudel & Daudel (1966), the development of chemical carcinogenesis can be divided into four periods. The first period was mainly concerned with the discovery of chemicals able to induce tumors (as has been discussed in the previous section). During the second period, the main interest of research workers was to establish a relationship between the carcinogenic power of a compound and its molecular structure, and also to isolate some of the metabolites produced when an animal was treated with a carcinogen.

Carcinogenic activity has been found mainly in certain appropriately substituted tri- and tetra-cyclic hydrocarbons and also in some penta-, hexa- and higher cyclic ones. Among aromatic hydrocarbons a certain degree of molecular complexity and chemical reactivity is required for carcinogenic action. This aspect has been reviewed by Badger (1948).

Schmidt (1939) first attempted to correlate carcinogenic potency with the electronic configuration of polycyclic aromatic hydrocarbons. The attempt was extended by theoretical chemists Pullman & Pullman (1955) and Coulson (1953).

Cook & Schoental (1957) found that osmium tetroxide added to the 9,10-phenanthrenoid bond (I) which became known as the K-region (K for Krebs). This finding was taken



I. Benzanthracene.

as a model for the attachment of cell constituents to the hydrocarbon. Badger (1948) demonstrated that in a series of polycyclic hydrocarbons and some of their derivatives, there was a good correlation between the calculated values for the "bond order" of the K-region and the rate of addition of osmium tetroxide. However, there was no correlation between the bond order of the K-region and carcinogenic activity of the respective compounds.

Pullman & Pullman (1955) showed a direct correlation between the reactivity of the K-regions and carcinogenicity in a

series of hydrocarbons. However, 1,2-benzonaphthacene which has a very active K-region, is unable to produce tumours. A great number of analogous cases is known. The conclusions from these studies of aromatic hydrocarbons are that the presence of a bond which is active for addition reaction (K-region) is favourable for the appearance of carcinogenic activity and that the presence of two reactive para positions (L-region) is unfavourable for carcinogenic activity.

The attempts to correlate carcinogenicity of the alternant hydrocarbons with their electronic configuration and chemical reactivities have met with only limited success. This is not surprising in view of our ignorance of the mechanism of carcinogenesis and of the essential role of the tissue of the recipient animal.

The metabolism of carcinogens has received a great deal of attention in the hope of learning something about the mechanism of carcinogenesis. Using fluorescence spectroscopy it was possible to follow metabolites retaining the intact ring structure. Berenblum & Schoental (1946) and Cook, Ludwiczak & Schoental (1950) showed the metabolic hydroxylation at the 8- and 10-positions of the angular benzene ring of 3,4-benzopyrene. This pattern of hydroxylation was also found in metabolites of related polycyclic hydrocarbons (Berenblum & Schoental, 1949; Harper, 1959; LaBudde & Heidelberger, 1958; Boyland & Sims, 1962).

With the use of compounds labelled with radioactive

carbon it was possible to follow ring degradation products. The detailed study of LaBudde & Heidelberger (1958) showed that not only the M-region was oxidized during metabolic transformation but that the chemically more reactive centres - the K- and L-regions - were also attacked with subsequent ring opening.

Hydroxylation of polycyclic aromatic hydrocarbons *in vitro* by liver microsomal preparations has been observed by Conney, Miller & Miller (1957) and by Booth & Boyland (1958). The products formed were the same as those formed *in vivo*.

4. Interactions of alternant hydrocarbons with cellular constituents

The discovery of a significant interaction between carcinogenic hydrocarbons and cellular constituents (proteins, RNA, DNA, lipids etc.) was the major development in the third period of

chemical carcinogenesis.

The possibility of a direct interaction between a carcinogen and a protein was suggested in the pioneering work of Schmidt (1941). Using mice dosed with 3,4-benzopyrene she was able to observe fluorescence of substances "which appeared to be derived from the carcinogen and combined through chemical bonds with the protein." Wiest & Heidelberger (1953) using labelled carcinogens showed the covalent binding of label to the soluble and particulate fractions of mouse skin proteins.

Various attempts have been made to determine the precise structure of the hydrocarbons bound to mouse skin proteins. However this has not been possible to date. Daudel, Muel, Lacroix & Prodi (1962) examined the fluorescence spectrum of protein-bound 3,4-benzopyrene, and the hydrazides of the hydrocarbon recovered from the protein. They came to the conclusion that binding of the hydrocarbon to protein involved addition reactions to the K-regions and most likely through amide bonds.

The nature of the proteins in mouse skin involved in the binding of labelled hydrocarbons was studied by Davenport, Abell & Heidelberger (1962). A fraction of the soluble proteins, proteins I, separable by starch gel electrophoresis, was found to have a relatively high specific activity and this fraction was absent in the case of non-carcinogenic hydrocarbons. Furthermore there was a fairly good correlation between the carcinogenic activity of the compound and its binding to the protein fraction I.

Heidelberger & Davenport (1961) provided the first experimental evidence that hydrocarbons, 1,2:3,4- and 1,2:5,6-dibenzanthracene, did bind firmly in vivo to the DNA and RNA of mouse skin following local application of the hydrocarbons. Similar binding has also been shown for 3,4-benzopyrene (Brookes & Lawley, 1962; Jacquier & Daudel, 1964). Brookes & Lawley (1964) found a fair relationship between the binding of a hydrocarbon to DNA in vivo and the carcinogenic power of the hydrocarbon.

Some evidence has been obtained suggesting that alternant hydrocarbons can covalently bind to DNA in vitro. Grover & Sims (1968) demonstrated the chemical linkage of tritium from tritiated hydrocarbons to DNA and to protein, catalyzed by a rat-liver microsomal enzyme system in vitro. Gelboin (1969) also demonstrated the binding of H^3-3,4-benz(a)pyrene to DNA in a similar system. Chemical binding of alternant hydrocarbons to DNA can also be induced in vitro by photoirradiation (Ts'o & Lu, 1964), x-ray irradiation (Rapaport & Ts'o, 1965) or by treatment with hydrogen peroxide (Morreal, Dao, Eskins, King & Dienstag, 1968), or with hydrogen peroxide or iodine (Umans, Lesko & Ts'o, 1969). However labelled hydrocarbons of very high specific activity were used in all these studies, and the extents of binding to DNA observed were very low, of the order of 1 molecule of hydrocarbon to 50,000 - 500,000 DNA nucleotides. Because of such a low binding ratio, it has not been possible to establish the exact nature of the DNA-bound radioactivity. Therefore, it is still questionable whether the hydrocarbons do, in fact, bind to DNA. The nature and significance of the binding will

be discussed in a later section.

Physical binding of alternant hydrocarbons to DNA has also been observed *in vitro*. Booth, Boyland, Manson & Wiltshire (1951) discovered that 3,4-benzpyrene is more soluble in an aqueous solution of DNA than in water. The solubilization of the alternant hydrocarbons by DNA was studied in greater detail by Boyland & Green (1964), Liquori, Lerma *et al* (1962) and by Ball, McCarter & Smith (1965). The nature and significance of such binding will be discussed in a later section.

The physical binding both *in vivo* and *in vitro* of aromatic hydrocarbons, 3,4-benzpyrene and 20-methylcholanthrene, to the chromatin of rats has been observed by O'Brien, Stanton & Craig (1969). The same hydrocarbons were able to bind to the deoxyribonucleoprotein of calf thymus (Kodama, Tagashira & Nagata, 1968).

Vescia, Giordano & Hermann (1968) demonstrated that transfer RNA isolated from rat liver could solubilize 3,4-benzpyrene *in vitro*, whereas ribosomal and other kinds of RNA were not able to form physical complexes with the hydrocarbon under the same conditions. Transfer RNA from liver tumours showed a higher solubilizing power than transfer RNA from normal liver.

The studies made in the third period of development of chemical carcinogenesis on the interactions of the alternant hydrocarbons with cellular constituents are still being pursued. These findings suggested that these compounds might have effects on the biosynthesis of many important molecules in the cell. Thus the

fourth period began with the study of the effect of chemical carcinogens on the biosynthesis of proteins and nucleic acids.

5. Effects of alternant hydrocarbons on protein and nucleic acid synthesis

Von der Decken & Hultin (1960) found that microsomes isolated from rats treated with methylcholanthrene had a higher capacity for incorporating L-leucine into protein and also a higher RNA to protein ratio than microsomes from untreated rats.

Loeb & Gelboin (1963) showed that nuclei isolated from livers of rats 16 hours after injection of methylcholanthrene, had a higher than normal content of RNA. The DNA content was not altered by the treatment. RNA extracted from liver nuclei of treated rats had a higher messenger RNA activity than that from normal rats when tested in an *in vitro* protein synthesizing system of Nirenberg (1963). When methylcholanthrene and actinomycin D were given simultaneously, there was an inhibition of the methylcholanthrene stimulation (Gelboin & Blackburn, 1963). As actinomycin D blocks the synthesis of RNA, the authors believed that methylcholanthrene induction of enzyme activity depends on the synthesis of RNA under the experimental conditions used.

An increase in the RNA to DNA ratio in *E. coli* as a result of growth in the presence of methylcholanthrene has been observed by Bond & Gilleland (1955). The increase in ratio paralleled that of the carcinogenic potency of the hydrocarbon tested.

The in vivo effects of carcinogenic hydrocarbons on DNA synthesis have not been studied in detail. Iversen & Evensen (1962) found that a single application of a small amount of 1% solution of methylcholanthrene provoked two successive changes in the rate of DNA synthesis in the epidermal cells of mice. A few hours after the application the synthesis of DNA was strongly depressed, but two days after application the rate of DNA synthesis was then increased. The authors proposed that the depression might have been caused by a temporary injury of the enzymes responsible for the synthesis, and that the succeeding increasing rate of DNA synthesis might have been caused by loss of some growth inhibitor due to the increased number of dying cells. McCarter & Quastler (1962) injected tritiated thymidine intraperitoneally into mice at various times after the mice had received dimethylbenzanthracene on the skin. The mice were killed forty-five minutes afterwards. The incorporation of thymidine into the treated skin tissue was estimated by means of an autoradiographic technique. These authors found a marked decrease in thymidine incorporation within minutes after treatment with the carcinogen. Analogous results were found for the incorporation of cytidine into RNA (Sinclair & McCarter, 1964). Jensen, Ford & Huggins (1963) showed that the incorporation of thymidine into the DNA of the testis, ileum and adrenal tissues of rat was inhibited by the action of 9,10-dimethylbenzanthracene..

B. Theories on the Mechanism of Carcinogenesis for Alternant Hydrocarbons

The following discussion is limited to the consideration of a single major class of chemical carcinogens, namely the polycyclic aromatic or alternant hydrocarbons. The studies on the mechanisms of action of the other classes of carcinogens such as the aromatic amines, azo-compounds and alkylating agents, have been reviewed by Daudel & Daudel (1966).

The identification of 3,4-benzpyrene as a tumour producing agent in coal tar in 1933 signaled the beginning of an exciting and fruitful period of study of chemical carcinogenesis. Although massive information has been gathered in the longer interval since, regarding the relationship between physico-chemical structures of the alternant hydrocarbons and carcinogenicity (2nd period), their interaction with cellular constituents (3rd period) and their effects on protein, RNA and DNA synthesis (4th period), little actual advancement has been made toward the understanding of the mechanism of chemical carcinogenesis either at the biological or molecular level.

Many studies on the mechanism of action of alternant hydrocarbons have been made by chemists and molecular biologists based on the assumption that the carcinogens somehow directly transform a normal cell into a cancerous cell. Early attempts to correlate physico-chemical properties of these carcinogens with their carcinogenic potencies were met with little success. In view of

the complexities of the cancer problem it is not surprising that no simple correlation has been found for the carcinogenicity of an alternant hydrocarbon and its number and arrangement of benzene rings, or its electronic configuration. For instance, it is not known whether it is the carcinogen per se or its reactive metabolites that initiate the cancer process. Furthermore, the target tissue or macromolecule which is being attacked in the initial carcinogenic step is not known. It has been shown that the carcinogens bind to lipids, proteins, RNA and DNA and that these compounds influence protein and nucleic acid synthesis in vivo. At the present time it is impossible to determine which, if any, of these interactions is of primary causal significance in the carcinogenic process. These interactions might be competitive and/or complementary.

1. Theories at the molecular level

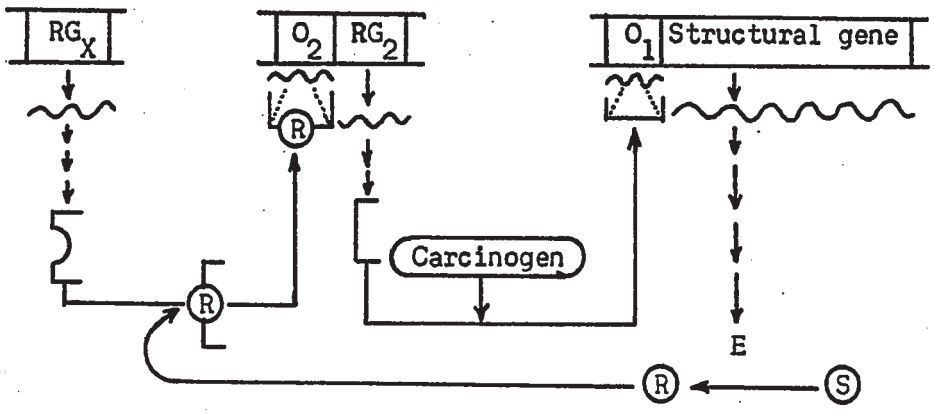
At the molecular level, the theories on the mechanism of chemical carcinogenesis can be grouped into two general categories: (1) the carcinogen reacts directly with DNA to produce a somatic mutation, (2) the carcinogen reacts with RNA or protein exerting its effect at the level of gene expression to produce a perpetuated change.

The somatic mutation theory of cancer was first postulated by Boveri (1929), based on the observation that tumour cells had an abnormal chromatin complex. He proposed that the carcinogen interacts with DNA of a somatic cell producing a mutation which

gives rise to a cancerous cell. Burnet (1959) restated the somatic mutation theory as: (1) normal cells are transformed into cancer cells by a series of mutational events, (2) descendants of cancer cells form a clone which will develop into a tumor if they have a proliferative advantage over other cells in the organism. These postulates are at best descriptive of the disease of cancer. The theory is rather vague and cannot be pushed too far, because mutation followed by selection can be used to explain any biological phenomenon (Clayson, 1962). The theory has suffered as experimental proof is hard to obtain. Many attempts have been made to determine whether carcinogens are mutagens. There has been no experimental technique available to study somatic mutation in mammalian cells. One has to resort to studying mutation of germ cells or of "lower" organisms. Then one extrapolates the results to somatic cells wherein lies the uncertainty. Burdette (1955) reviewed the large amount of work done in this area (mainly of work done with molds and bacteria) and came to the conclusion that there is no evidence for an association between carcinogenesis and mutagenesis. The experiments failed to show that mutagens are carcinogens or vice versa. However, the lack of supporting experimental evidence, which might only reflect the inadequacy of techniques used, does not warrant the rejection of the hypothesis.

An alternative molecular mechanism based on specific protein-binding has been proposed by Heidelberger (1959) and Abell & Heidelberger (1962) which became known as the protein-deletion theory. This theory states that the chemical carcinogen reacts

with some protein(s) that are enzyme systems essential for the control of growth, and that as a result of such an interaction, these protein(s) become deleted from some cells which, as a consequence of the loss of these growth controlling enzymes become cancer cells. To explain how such a cytoplasmic deletion could be perpetuated in daughter cells, Pitot & Heidelberger (1963) proposed the following metabolic circuit based on the theories of biological control mechanisms of Jacob & Monod (1961) designed for bacterial systems:



Modified product perpetuation carcinogenesis circuit.
(Pitot & Heidelberger, 1963).

The enzyme which is postulated to be a cancer specific enzyme or antigen is made by structural gene 1 whose operator gene (O_1) is controlled by a repressor made by regulator gene 2 (RG_2). The operator of RG_2 is in turn controlled by an apo-repressor made by the regulator gene X (RGx). The apo-repressor by itself is not active. Its activation requires the product R made by the cancer specific enzyme. The carcinogen is postulated to bind to the repressor made by RG_2 and inactivates the repressor. As a result, the cancer specific enzyme is made. The enzyme makes the product R which then activates the apo-repressor and no further repressor of RG_2 is made. Thus, the synthesis of the enzyme continues indefinitely as long as the substrate S is present. This situation will be perpetuated in the daughter cells because R is always present in the cytoplasm which is shared during cell division.

This theoretical metabolic circuit serves to explain how a single application of a carcinogen can bring about a perpetual change. Although the theory is based on the biological control mechanisms which are known to operate in microorganisms, there has been some evidence for similar control mechanisms in the more complicated mammalian system. The experimental evidence supporting the protein-deletion theory is only circumstantial. The exact nature of the cancer specific enzyme or antigen has not been defined. The appearance of tumor antigens following neoplastic transformation is taken as indicative of the presence of the cancer specific gene product. The protein fraction I to which carcinogenic hydrocarbons preferentially bind, is taken to represent the repressor

molecule which acts on the operator of the structural gene for the cancer specific protein. The presence of this protein fraction in normal tissue and its absence in tumor tissue (Abell & Heidelberger, 1962) are in keeping with the specific-protein deletion theory.

No formal theory based on the interaction of alternant hydrocarbons with RNA has been proposed. 3,4-benzpyrene can form physical complexes in vitro with transfer RNA but not ribosomal RNA isolated from rat liver (Vescia et. al., 1968). Such binding could well interfere with the normal control mechanism at the level of gene translation.

On the whole, there has been no lack of theories on chemical carcinogenesis. However, they are largely premature and are based on some limited correlations, lacking unequivocal experimental proof. A great deal more knowledge should be gained on the action of these compounds at the biological level before the significance of any of these hypotheses can be assessed.

2. Theories at the biological level

At the present time there are three different proposed mechanisms of chemical carcinogenesis at the biological level: (1) the carcinogen transforms normal cells into cancer cells, (2) the carcinogen selects pre-existing cancer cells, (3) the carcinogen activates a latent tumor virus. It is impossible at the moment to decide among these alternatives. The first of these has been assumed by almost all of those working in the field. The assumption is the basis of all the theories discussed in the above sections. The

second has been proposed by Prehn (1964) as a clonal selection theory, based on the much greater toxicity of carcinogens to normal cells than to cancer cells. The third alternative which has received much attention lately, has come from the demonstration of cell-free transmission of some hydrocarbon-induced lymphomas in mice (Irino, Ota, Suzaki, Suzaki & Kiraki, 1963; Toth, 1963; Haran-Ghera, 1967). However, some of these observations could not be repeated (Shubik, 1963) and there have been several reports of negative results (Salaman, 1959; Millon, 1961; Domochowski, 1966).

More recently, Igel, Huebner, Turner, Kotin & Falk (1969) were able to show the appearance of murine leukemia viral antigen in most of the lymphoid tumors in mice treated with methylcholanthrene, or other types of carcinogens such as urethan and dimethylnitrosamine. During the process of cell-free transmission of these chemically-induced lymphomas, they found that antibodies to murine leukemia virus developed in the recipient mice. The involvement of a virus whose presence was demonstrable even by the sensitive and specific immunological technique, strongly suggested that the "unmasking of a latent leukemia virus" was "an indigenous actuating cause of the lymphomas."

The above finding may represent a very significant turning point in the development of chemical carcinogenesis. In the light of this elucidation, efforts must be made to understand the mechanism of induction of a latent tumor virus by chemical carcinogens. A number of questions are immediately raised. How did the different agents activate the virus(es)? Do human cancers have a

viral etiology? Will it be possible to control such viral induced tumours with anti-viral agents or vaccines?

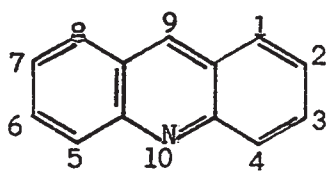
C. Studies on the Binding of Chemical Carcinogens to DNA

The binding of chemical carcinogens to DNA has been extensively studied. Both physical and chemical binding of alternant hydrocarbons has been observed. This section is a review on these studies.

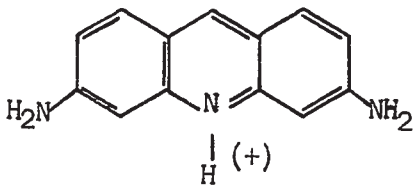
1. Physical binding

a. Acridines and derivatives

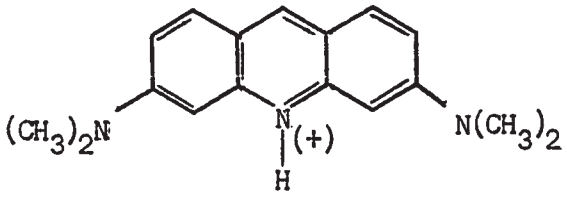
Some of the derivatives of acridine such as the dimethyl derivatives of benz(c)acridine have been found to be carcinogenic (Lacassagne, Buu-Hoi & Zajdela, 1956). Little is known about the interaction of these carcinogens with DNA, except for the demonstration by Lerman (1964a) that 7,9-dimethylbenz(c)acridine enhanced the viscosity of DNA solutions. However the interaction of various amino acridines with DNA has been extensively studied (Lerman, 1963 & 1964a; Peacocke & Skerrett, 1956; Weill & Calvin, 1963; Cairns, 1962; Houssier & Fredericq, 1966; Neville & Davies, 1966; Walker, 1965; Gersch & Jordan, 1965; Chambron & Sadron, 1966a & b; Blake & Peacocke, 1968). It is useful to review these studies here.



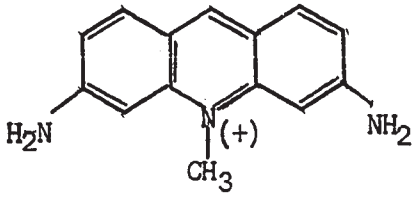
IV Acridine



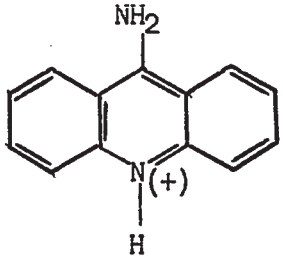
V. Proflavine cation



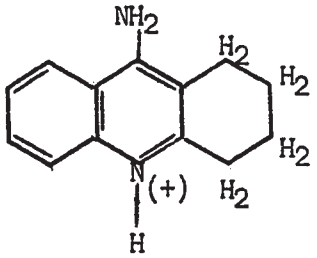
Vl. Acridine orange cation.



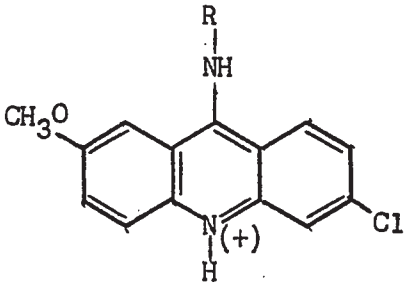
Vll. Acriflavine cation.



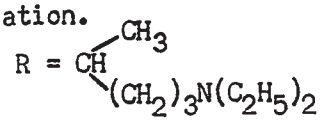
Vll. 9-Aminoacridine cation.

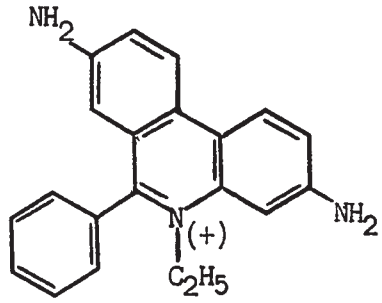


lX 9-Amino-1,2,3,4-tetrahydroacridine cation.



X. Atebrin cation.





Xll. Ethidium cation.

Xl. Acranil cation.

$R = CH_2CH(OH)CH_2N(C_2H_5)_2$

A number of aminoacridines have been found to be able to bind directly to DNA dissolved in an aqueous solution (Peacocke et. al., 1956; Lerman, 1961). Such binding has been shown to occur by both a strong (I) and a weak (II) binding process (Peacocke et. al., 1956). At low concentrations of the acridines, the strong binding process I predominates. At high concentrations of the dyes, the weak binding, process II becomes significant.

The weak binding is known to be due to the self-stacking of the dye molecules in solution and is strongly dependent on the concentration of the dye. The mode of the strong binding of process I has been studied in detail by Lerman who first proposed that the acridine molecules intercalate between normally neighbouring base pairs of the DNA in a plane perpendicular to the helix axis; the space is provided by an extension and local untwisting of the helix. He and others obtained overwhelming evidence supporting the intercalation model.

The extension of the DNA double helix as a result of the binding of proflavine was evident from low angle x-ray diffraction data of a proflavine-DNA complex. There was a drop in the number of electrons per unit length (hence, a decrease in mass per unit length) in the complex as compared with the control DNA (Luzzati, Masson & Lerman, 1961). A decrease in sedimentation rate (Lerman, 1961; Lloyd, Prutton & Peacocke, 1968) and an increase in viscosity (Lerman, 1961; Drummond, Pritchard, Simpson-Gildemeister & Peacocke, 1966) of the DNA on binding aminoacridines are also

indicative of an extension of the DNA double helix. Cairns (1962) confirmed the elongation of the DNA chain upon binding of proflavine by means of autoradiography.

The elongation of the DNA chain is the result of local untwisting of the double helix, without affecting the perpendicular orientation of the plane of the DNA base pairs to the axis of the helix.

The helical rotation between the affected base pairs must be reduced in order to provide the space into which the aminoacridine molecule fits. This is because the phosphodiester backbones linking the base pairs are already fairly well stretched in the normal helix. The angle of back-rotation which occurs has been estimated at 36° (Lerman, 1964a). The minimum needed is 12° (Fuller & Waring, 1964).

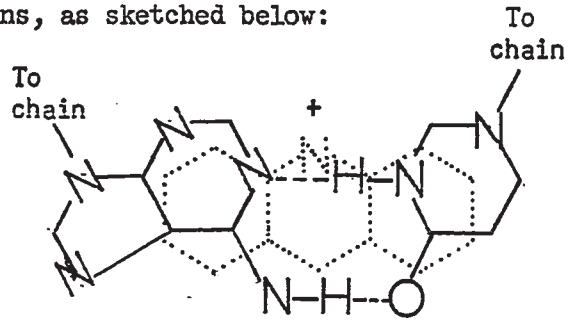
The plane of the intercalated aminoacridines is thought to be perpendicular to the helix axis and this has been verified by the ingenious studies of Lerman (1963) who measured the changes in the intensity of polarized fluorescence of the DNA-bound dye under conditions of stream-flow. He was able to conclude that the plane of the aminoacridine was perpendicular to the axis of the DNA helix within broad limits ($\pm$ 34°). This rather large variation was partly due to the incomplete alignment of all the aminoacridine:DNA helices parallel to the direction of flow.

The intercalation model proposes that the orientation of the planes of the DNA base pairs in a DNA:acridine complex remains

perpendicular to the helix axis. This was shown to be true by carrying out studies of the dichroism of a DNA-quinacrine complex under conditions of stream-flow (Lerman, 1963). The complex was found to be more, rather than less, dichroic than the control DNA, indicating there was no tilting of the DNA bases in the complex.

As a further test on the correctness of the intercalation model, Lerman (1963, 1964) compared the rates of diazotization of the amino groups of proflavine and other acridines when bound to DNA and free in solution. DNA strongly inhibited the reaction. This is consistent with the intercalation model. The aminoacridine molecules being 'sandwiched' in between and in direct van der Waals contact with two DNA base pairs, are out of contact with the solvent. As a result, the attack on the amino groups of the acridine by a reagent that requires an out-of-plane transition state (for example, through direct interaction with the π electrons) should be severely restricted. Such a severe restriction was not found when the aminoacridines were bound to other anionic polymers, such as polyphosphate, polyvinyl sulfate, polystyrene sulfonate, etc.

Lerman assumed that the aminoacridine was located over a base pair with the acridinium ion at equal distances from the sugar-phosphate chains, as sketched below:



Shown is an arrangement of an acridine molecule across a hydrogen bonded base pair of DNA as proposed by Lerman. Dotted lines represent the acridine.

However, it has become apparent recently that the above model cannot adequately explain some of the experimental observations. In view of these difficulties, Pritchard, Blake & Peacocke (1966) proposed a modification of the intercalation model. The modification is that the acridine, rather than interacting with a hydrogen bonded base pair, interacts with two adjacent bases on the same polynucleotide chain. That is, the acridine in the figure is sitting to one side instead of at the centre of the base pair. A negatively charged oxygen atom on the phosphate group between two bases can swing in to the inside of the chain, where it directly interacts with the charged ring nitrogen of the aminoacridine. This can explain the marked effect which ionic strength has on the binding of these compounds to DNA. Since this modified intercalation model proposes that binding occurs between an aminoacridine molecule and adjacent bases on the same polynucleotide chain, the complementary bases on the other chain will not be involved directly in the binding. As a result strand separation would not be expected to affect greatly the overall extent of the strong binding process. This has in fact been demonstrated by Drummond et. al. (1965) and Liersch & Hartmann (1965) using heat-denatured DNA.

One advantage of this modified intercalation model is that it does not require as much local unwinding of the helix to increase the spacing between two adjacent base pairs (to approximately

6.8 A°) to allow sufficient room for the acridine. Neville & Davies (1966) compared optical transformations based on Lerman's model with x-ray diffraction patterns of a proflavine:DNA complex and found quantitative differences. These authors pointed out that these discrepancies could be resolved if the untwisting of the base pair were much less than that proposed by Lerman and if the bound dye were not situated wholly over a base pair. These structural requirements are satisfied by the modified intercalation model.

The modified model can also explain why DNA can accommodate intercalators such as the acridines and their derivatives having such a range of structural differences. Acridines with long side chains on the 9 position, such as atebrin (X) and acranil (XI), can bind as strongly to DNA as proflavine (Drummond et. al., 1965). The fact that 9-amino-tetrahydroacridine (IX) does bind, although not as strongly as proflavine (Drummond et. al., 1965), favours this partial intercalation model. If the binding were across a base pair, it is difficult to see why this acridine with a 'buckled' ring should bind at all. But in the modified model, the two bases directly adjacent to the tetrahydroacridine can still interact with the two remaining aromatic rings of the tetrahydroacridine.

The modified intercalation model of Pritchard et. al. (1966) has been generally accepted. It explains a number of experimental observations which are incompatible with the previous intercalation model.

The energetics of binding of aminoacridines by intercalation to DNA are not fully understood. The interactions between the heterocyclic rings of the acridines and those of the DNA bases have been recognized and emphasized as being an important force involved in the stabilization of an intercalation complex (Tubbs, Ditmars & van Winkle, 1964; Gersch & Jordan, 1965). The energy associated with these interactions has been calculated by Gersch & Jordan. However, it was the contention of Gilbert & Claverie (1968) that no firm conclusion could be drawn from the calculations of "stacking" energies alone. The authors doubted that these short range and relatively ionic strength independent interactions, could account for the observed strong dependence of binding on ionic strength, in particular, the steepness of the decrease in binding even at low ionic strength (Drummond et. al., 1965). Furthermore, the weak binding of aromatic hydrocarbons (as opposed to the strong binding of the aminoacridines) for which intercalation also seems to be the likely mode of binding also indicates the dominant role of the electrostatic interactions between the positively charged dye molecules and the DNA phosphates in stabilizing the complex. Three types of electrostatic interactions are believed to exist: (1) the attraction between the intercalated cations and the DNA phosphates, (2) the repulsion between the cations, (3) the reaction of the solution to the electric field of the phosphates and the cations. The first interaction contributes to stabilization. The second destabilizes. When the binding ratio of acridine to DNA nucleotide is high, such repulsive forces are not negligible even at infinite ionic strength, and are believed to play a decisive role in limiting

the extent of binding. The third type of interaction tends to reduce all electrostatic interactions. The reduction is more effective for interactions involving charges nearly in contact with the solution. With increasing ionic strength, the interactions between the phosphates and the acridines are more effectively reduced than the interaction between the acridines themselves. This unequal reduction results in a net destabilization of the complex with increasing ionic strength.

Tubbs et. al. (1964) from measurements of fluorescence quenching of DNA-acriflavine solutions, obtained experimental data showing the relative affinity of acriflavine for binding sites in DNA as: AT:AT > AT:CG > GC:GC. These results are in agreement with those of Kleinwachter & Koudelka (1964) who studied the relationship between base composition of DNA and the increase in Tm of DNA-acridine orange complexes over that of the control DNA. They showed that the extent of the increase in Tm of the complex increased with the rise in AT content. Jordan & Ellerton (1967) calculated the free energy change involved in the intercalation of proflavine between different base pairs arranged in different sequences. Their data showed that the order of increased stabilization of the complex is AT:TA > AT:AT > TA:AT > TA:GC > AT:GC > AT:CG > GC:CG > TA:CG > GC:GC > CG:GC. Thus, the base sequences with the highest free energy in native DNA are stabilized the greatest in the DNA-proflavine complex. However conflicting evidence has been published by Chan & van Winkle (1969). Their results which were based on fluoreseence and absorption data, indicated that the binding constant (k_1) of acriflavine is independent of the G + C content of the various DNA's tested.

b. Alternant hydrocarbons

Various carcinogenic and non-carcinogenic alternant hydrocarbons have been shown to be able to bind directly to and form physical complexes with DNA dissolved in an aqueous solution (Boyland & Green, 1962; Liquori et. al., 1962). This finding has been confirmed in other laboratories (Lerman, 1964b; Ts'o & Lu, 1964; Ball et. al., 1965).

Evidence for physical complex formation has been obtained by means of sucrose gradient electrophoresis, ultracentrifugation and radio-chemical assay. The binding was found to be strongly dependent on the pH and ionic strength of the medium. Different extents of binding for the same hydrocarbon have been achieved by different workers using different techniques. When complexes were made by shaking aqueous DNA solutions with solid benz(a)pyrene, the maximum binding ratio obtained was of the order of 1 mole of hydrocarbon to 1000 - 3000 bases (Boyland & Green, 1964; Lesko, Smith, T'so & Umans, 1968; Kodama, Tagashira, Imamura & Nagata, 1966; Liquori et. al., 1962). Ball et. al. (1965) by mixing an aqueous solution of DNA with 3,4-benzpyrene dissolved in a small volume of methanol (final concentration about 3%) obtained a binding ratio of 1 mole of the hydrocarbon to 60 - 80 nucleotides. The solubility of 3,4-benzpyrene in the binding buffer containing 3% methanol was negligible (unpublished observations). Thus, the latter binding technique is more efficient.

The precise nature of the interaction is still not fully known. From considerations of the extremely low solubilities of the hydrocarbons in aqueous medium and the similarities of their

structures to the acridines, an intercalative mode of binding has been suspected by all those working in the field.

Lerman (1964b) observed an increase in the viscosity of DNA on binding hydrocarbons, suggesting an increase in contour length of the DNA helix. Nagata, Kodama, Tagashira & Imamura (1966) obtained evidence from flow dichroism studies, indicating that pyrene and benz(a)pyrene, when solubilized in aqueous solutions of DNA, have a specific orientation which was believed to be perpendicular to the DNA helix axis. Green & McCarter (1967) studied the effect of flow-orientation on the polarized fluorescence of polycyclic hydrocarbons dissolved in aqueous DNA solutions and observed an increase with both excitation and emission polarizers transmitting light polarized in a plane perpendicular to the flow axis and a corresponding decrease when parallel to the flow axis. These results strongly support the intercalation model.

The spectral studies carried out by Lesko et. al. (1968) provided an indirect but strongly suggestive evidence for the intercalation model. A correlation was found between the bathochromic shift (shift of absorption maxima to higher wavelength) of the benz(a)pyrene absorption spectrum which is characteristic of the binding of the hydrocarbon to DNA and the face-to-face interaction of the hydrocarbon with compounds having a system of unsaturated bonds, such as benzene, pyridine and various purines. Ethanol which inhibited the "stacking" forces, also eliminated the bathochromic effect of the purines.

The above studies strongly suggest that hydrocarbons bind to DNA also by intercalation. The binding forces are considered to be mainly hydrophobic, van der Waals, London, π - electron dispersion forces, etc.

The hydrocarbons have been found to bind slightly better to heat-denatured DNA than to native DNA (Lesko et. al., 1968). The binding to denatured DNA has not been studied in detail. It is not unlikely that the modified intercalation model of Pritchard et. al. (1966) is also applicable here.

2. Chemical interactions

The in vivo and in vitro chemical-binding of polycyclic aromatic hydrocarbons to DNA has been briefly reviewed in an earlier section. The exact nature of the interaction is still not known.

The time course of the binding observed in vivo suggested to Brookes & Lawley (1964) that metabolism of the hydrocarbons was necessary to enable reaction with cellular constituents. The involvement of some reactive metabolites of the hydrocarbons was implied.

The microsomal enzyme-catalyzed binding in vitro was believed to be mediated by similar intermediates (Grover & Sims, 1968). The level of binding was quite low, of the order of one hydrocarbon per 50,000 to 500,000 DNA nucleotides. The low binding might reflect the specificity of the reaction, such as the requirement of a unique sequence of nucleotides in the calf thymus DNA

(Gelboin, 1969).

A large number of metabolites of benz(a)pyrene have been observed in vivo including various free and conjugated hydroxy and dihydroxy derivatives (Sims, 1967). The conversion of benz(a)-pyrene to hydroxylated products has also been observed to be catalyzed by the microsomal enzyme system (Conney, Miller & Miller, 1957). The enzyme, Aryl hydrocarbon hydroxylase, was found to be quite ubiquitous by Wattenberg & Leong (1962) using histochemical techniques. Furthermore the enzyme was highly inducible. The inducibility of this enzyme varied between different species and in different tissues (Nebert & Gelboin, 1968). The cell that contained the enzyme was generally sensitive to the toxic action of the polycyclic hydrocarbons. The enzyme was lacking in five cell lines grown in culture. These lines were resistant to the toxic action of the hydrocarbon. Thus there seemed to be a positive relationship between the toxic action of the polycyclic hydrocarbons and the presence of the enzyme system. This may be true for the carcinogenic activity as well.

The above system was studied further by Gelboin, Huberman & Sachs (1969). These authors demonstrated the induction of the microsomal enzyme, aryl hydrocarbon hydroxylase in cultures of normal and transformed hamster, mouse and human cells by pretreatment with benz(a)anthracene. They found a correlation between the level of the enzyme activity and the susceptibility of the cells to cytotoxicity produced by treatment with benz(a)pyrene. It was apparent that the enzyme was responsible for the susceptibility of the cells to the toxic effects of benz(a)pyrene and the toxic

effect of benz(a)pyrene was due to its enzymatic conversion to a cytotoxic metabolite. One of the metabolites, 3-hydroxybenz(a)-pyrene, was demonstrated to be cytotoxic.

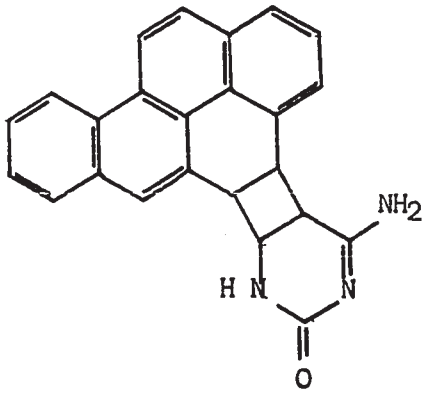
Chemical linkage of the carcinogen benz(a)pyrene to DNA in vitro could be induced by either photoirradiation (Ts'o & Lu, 1964) or x-ray irradiation (Rapaport & Ts'o, 1966). The mechanism of chemical linkage induced by x-rays was believed to be most likely due to the formation of free radicals by direct or indirect action, while the mechanism of photoirradiation at 350 - 390 mu was most likely due to the formation of excited states and perhaps biradicals. The energy transfer process of x-ray irradiation is far less selective and involves much more energy than photoirradiation.

Significant destruction of the DNA was observed as a result of irradiation. A dose of 20 kilorads of x-ray reduced the sedimentation rate of the DNA from 13.5S to 5.6S (Rapaport & Ts'o, 1966). The effects of irradiation on DNA have been extensively reviewed by Muller (1967) and Shooter (1967).

Chemical binding to denatured DNA induced by x-ray irradiation was found to be significantly better than to native DNA (Rapaport et. al., 1966). The extent of binding to native DNA was of the order of 1 molecule of the hydrocarbon to 25,000 - 50,000 DNA bases, and to denatured DNA 1 molecule to about 7,000 bases.

The exact nature of the binding is not known. Rice (1964) found that benz(a)pyrene could bind directly to the natural pyrimidines, uracil, thymine, cytosine and 5-methylcytosine. Based on

spectroscopic evidence and assuming that the K-region of benz(a)-pyrene took part in the reaction, the following structure for the addition product was proposed.



Proposed structure of benz(a)pyrene:pyrimidine complex.

The mechanisms of binding of hydrocarbons to DNA induced by weak free-radical initiators such as hydrogen peroxide (Morreal et. al., 1968) are not known. The reactions could have proceeded through the formation of hydrocarbon epoxides, through free radical fragments of the nucleic acids or through an alternate mechanism.

D. In Vitro RNA Synthesizing System

The polymerization of ribonucleoside triphosphates into RNA is catalyzed by the enzyme, RNA polymerase (systematic name: nucleosidetriphosphate: RNA nucleotidyltransferase (DNA dependent); EC 2.7.7.6). RNA polymerase plays a key role in the functioning of a cell and is responsible for the synthesis of all types of cellular

RNA. It is present in all cells in which RNA synthesis occurs (Elson, 1965). The best preparations of the enzyme have been obtained from bacterial sources and much of our knowledge about the mechanism of RNA synthesis has come from the studies made in an *in vitro* RNA synthesizing system catalyzed by bacterial RNA polymerase. A detailed review on RNA polymerase and control of RNA synthesis has been written recently by Richardson (1969). The following discussion is restricted to the *in vitro* RNA synthesizing system involving highly purified DNA templates and bacterial RNA polymerase.

1. RNA polymerase

Methods for the isolation and purification of the enzyme, RNA polymerase, from *E. coli* have been developed independently by Chamberlin & Berg (1962); Furth, Hurwitz & Anders (1962) and Maitra & Hurwitz (1967). These methods were similar, involving the fractionation of a cell extract with streptomycin sulfate, protamine sulfate, ammonium sulfate and DEAE-cellulose column chromatography. Physically homogeneous fractions were obtained by centrifuging the above preparations through a sucrose density gradient (Jones, Dieckmann & Berg, 1968; Ishihama & Kameyama, 1967).

Richardson (1966a) and Maitra & Hurwitz (1967) both reported an optical density ratio for the enzyme at 280 mu and 260 mμ of 1.7 which suggested the enzyme preparations contained less than 0.1% nucleic acid (Warburg & Christian, 1942).

These preparations were virtually free of other contaminating enzyme activities, such as DNase, RNase, DNA polymerase,

inorganic pyrophosphatase, nucleoside diphosphokinase, nucleoside triphosphatase etc.

The purified enzymes had very high specific activities. In ten minutes at 37° under optimal conditions the amount of nucleotides polymerized by one mg. of the enzyme protein was of the order of μ moles.

RNA polymerase has a tendency to aggregate at low ionic strengths (approximately 0.1 M) giving dimers having a sedimentation rate of 21S, which corresponds to a molecular weight of $8.8 \pm 1.6 \times 10^5$ daltons (Richardson, 1966a). Aggregation can be prevented by raising the ionic strength of the enzyme solution (0.5 M KCl). Under these conditions of high ionic strength, only monomers having a sedimentation rate of 13S (molecular weight = $4.4 \pm 0.8 \times 10^5$ daltons) were observed. Similar observations and sedimentation values have been obtained by other workers (Preiss & Zillig, 1967; Maitra & Hurwitz, 1967; Stevens, Emery, Jr. & Sternberger, 1966; Pettijohn & Kamiya, 1967).

The amino acid compositions of the RNA polymerases isolated from three different strains of E. coli, B, K12 and W, have been determined (Maitra & Hurwitz, 1967; Preiss & Zillig, 1967; Ratliff, Smith & Trujillo, 1967) and were found to differ. The amino acid sequences and secondary and tertiary structures of the enzyme have not been studied. The enzyme protein from E. coli B is acidic. At pH 8 (which is optimal for enzyme activity), it migrates to the anode (Richardson, 1966a).

The enzyme has been found to consist of several different polypeptides (Zillig, Fuchs & Millete, 1966; Burgess, 1968). Burgess, Travers, Dunn & Bautz (1969) electrophoresed a highly purified preparation of RNA polymerase in polyacrylamide gel in the presence of either 8 M urea or 0.1% sodium dodecyl sulfate and demonstrated the presence of four different polypeptides. The main components α and β polypeptides made up the "core" enzyme, having molecular weights of approximately 40,000 and 160,000 respectively. Two other polypeptides σ and τ were observed, having sedimentation rates of 5S and 8S respectively. The σ polypeptide was found to be an important factor responsible for specific initiation of transcription. This will be discussed in Section D. - 8. The function of the τ polypeptide was not known. The normal enzyme was believed to consist of $\alpha:\beta:\sigma$ polypeptides in a ratio of 2:2:1. These estimates were consistent with the large molecular weight determined for the enzyme.

2. Template role of DNA

The activity of RNA polymerase was strictly dependent on the addition of a DNA template (Chamberlin & Berg, 1962; Furth, Hurwitz & Anders, 1962).

The template function of DNA was elucidated by Geiduschek, Nakamoto & Weiss (1961), who showed that the RNA product made by a T_2 phage DNA-directed RNA polymerase reaction could form RNA-DNA hybrids very efficiently with heat-denatured T_2 phage DNA, but not with DNA from any other source. Richardson (1966b) confirmed the

above observation using phage T_7 DNA as template.

Several reports have appeared indicating that only one of the strands of a double helical DNA serves as template for transcription. Such asymmetric transcription has been demonstrated unequivocally employing ϕX174 RF-DNA as template (Hayashi, Hayashi & Spiegelman, 1964). The DNA of the mature bacteriophage ϕX174 is single-stranded. But upon infection of a host cell, this single stranded form is converted by partial replication to a cyclic double-stranded form known as ϕX174 RF-DNA (Elson, 1965), which can be isolated intact. When the ϕX174 RF-DNA was used as template for E. coli RNA polymerase, the RNA product had a base composition identical to that of the DNA strand found in the mature phage. Furthermore, this RNA could not form an RNA-DNA hybrid with the phage DNA strand but could with denatured RF-DNA. Thus, RNA polymerase must have transcribed only the complementary strand of the phage DNA. Comparison of these results with the hybridization properties of ϕX174 RNA synthesized in vivo indicated that the same DNA strand is transcribed in vivo as in vitro.

Another clear case of asymmetric transcription has been demonstrated by Geiduschek, Tocchini-Valentini & Sarnat (1964) involving the DNA of Bacillus megaterium phage α . The two strands of the linear phage DNA molecule can be separated on the basis of a difference in their densities. Therefore, α -specific RNA can be analyzed by determination of how well it hybridizes with the two different DNA strands. Such an analysis shows that α RNA made in vivo or in vitro is complementary to only one and the same DNA

strand.

The use of ϕX174 RF-DNA and ∝ DNA as template was fortunate because in both cases the one strand of the DNA is transcribed in all regions. However, for other DNA's such as phage λ DNA, one strand is transcribed in some regions and the other strand in other regions.

The double helical structure of the template is essential for asymmetric transcription. Denatured DNA's are transcribed symmetrically, as are DNA's that have been degraded to such an extent that many single-stranded regions are exposed. Further, the exact ionic conditions of the reaction mixture may be important. Also, the conditions required for proper strand selection with Micrococcal lysodeikticus RNA polymerase are very different from those found to be appropriate for the E. coli enzyme (Richardson, 1969).

3. The binding of RNA polymerase to DNA and nature of binding sites

A necessary first step in the initiation process is the interaction between RNA polymerase and DNA. The direct binding of the enzyme to DNA in the absence of the four nucleoside triphosphates has been demonstrated in a number of laboratories (Crawford, Crawford, Richardson & Slayter, 1965; Jones & Berg, 1966; Fox, Gumport & Weiss, 1965; Sternberger & Stevens, 1966). Such enzyme:DNA complexes could be isolated by zone sedimentation, zone electrophoresis and the membrane filter technique (Jones & Berg, 1966).

This binding process is very rapid, taking place in a matter of seconds. The association is strongly dependent on the ionic strength and pH of the medium but is not dependent on magnesium ions.

The binding reaction is by itself specific under proper conditions (Crawford et. al., 1965; Jones & Berg, 1966; Pettijohn & Kamiya, 1967; Richardson, 1966a; Sternberger & Stevens, 1966). In general, low ionic strength (0.05) allows secondary and weaker binding, but such weaker non-specific binding is eliminated by high ionic strength. Pettijohn & Kamiya (1966) under conditions of high ionic strength (0.18) found a saturation value of 0.6 - 0.9 ug. of RNA polymerase bound per microgram of polyoma DNA and from these values estimated that there are between 5 - 9 binding sites per polyoma DNA molecule. Such a value is consistent with an estimate of one binding site per cistron for polyoma DNA.

The enzyme binds to complete cyclic polyoma and papilloma DNA's (Crawford et. al., 1965). While attachment of the enzyme to DNA does not require free ends, the enzyme does bind better to denatured DNA. There are more sites per unit weight of a single-stranded DNA than on a double-helical DNA (Richardson, 1966a). It is possible that transcription of a denatured DNA template is less specific, as there are more attachment sites for the RNA polymerase.

Recently, attempts have been made to elucidate the nature of the binding sites on DNA and the mechanism whereby the enzyme recognizes these sites. It is unlikely that free ends in the double helical DNA normally provide attachment sites for the enzyme and

initiation points for transcription. Enzyme binding with efficient RNA synthesis can be demonstrated using circular ϕX174 DNA which has no free ends. The rate of transcription of the fully circular DNA was significantly faster than that of the linear DNA (Ishihama & Kameyama, 1967).

An alternative and more attractive model has been proposed (Berg, Kornberg, Fancher & Dieckmann, 1965; Jones & Berg, 1966). This model assumes that a local unwound region in the double helical DNA serves as the signal for RNA polymerase attachment. Further, some difference in the nucleotide sequences between the two unwound strands enables the enzyme to "recognize" the proper strand to be copied (i.e. the codogenic strand). The model is based on the observation that denatured or single-stranded DNA and even dAT bind considerably more RNA polymerase than does native helical DNA (Jones & Berg, 1966). The better binding capacity of dAT for RNA polymerase may be due to the generally weaker interstrand interaction characteristic of the A-T base pair (Sueoka, Marmur & Doty, 1959). Since the double helical structure of DNA is dynamic (von Hippel & Printz, 1965), there might be sufficient unwinding and rewinding of the helix at certain loci to allow the enzyme to bind.

Matsukage, Murakami & Kameyama (1969) digested an RNA polymerase-DNA complex with deoxyribonuclease and found that the DNA regions complexed with the polymerase, were resistant to the deoxyribonuclease. The DNA recovered from these polymerase-bound regions, after deproteinization with phenol, had sedimentation rates of 2.4S and 2.7S which were estimated to be about the length of an RNA

polymerase molecule. The shorter DNA fragments had a significantly higher GC content. However it is not known whether these regions represent a true binding site or a true initiation site.

It is quite possible that the recognition of the codogenic strand is based on the specificity of a special nucleotide sequence. Studies of RNA synthesis with certain synthetically prepared DNA homo and copolymer pairs such as (dG:dC), (dA:dT) and d(A-G):d(T-C), have indicated that RNA polymerase has a strong preference for the use of the pyrimidine strand of these pairs as the template (Chamberlin & Berg, 1962; Nishimura, Jones & Khorana, 1965). This preference is also reflected in the binding studies with single-stranded components of the homopolymer pairs. Jones & Berg (1966) showed that RNA polymerase binds more strongly to poly dT than to poly dA. Thus, it is possible that the polymerase could make its choice by binding to the strand with the greater pyrimidine content in the unwound region.

A number of cases have been reported where there are stretches of pyrimidine nucleotides and purine nucleotides on opposite strands of double-helical DNA. In the case of the bacteriophage T_7 DNA, only one of the two strands form complexes with poly G and it is this strand that is complementary to all the natural T_7-specific RNA (Summers & Szybalski, 1968). With phage λ DNA, both strands form complexes with poly G, but the strand that binds the greater amount of this homopolymer is also the strand that is complementary to a greater number of different λ -specific mRNA's (Taylor et. al., 1967).

4. Formation of initiation complex

The initiation of polymerization begins with the condensation of the second nucleoside triphosphate to the first one. This takes place after they become bound in their proper sites in the polymerase-DNA complex.

In the absence of RNA synthesis, the binding of the enzyme to DNA is reversible. However when an RNA chain is synthesized, the binding is irreversible, at least until the chain has reached some minimum length (Bremer & Conrad, 1964; Berg et al., 1965; Richardson, 1966a; Jones & Berg, 1966). It seems that the binding of the first two nucleotides alone is sufficient to cause significant stabilization of the polymerase-DNA complex (Anthony, Zeszotek & Goldthwait, 1966).

The first nucleotide incorporated has been found to be predominantly a purine. Maitra & Hurwitz (1965) examined the product transcribed from several DNA templates in vitro and found that less than 15% of the RNA chains made were initiated with a pyrimidine nucleotide. In general, there is a preference for initiation with GTP. The higher the GC content of the template, the greater is this preference. Only in some of the cases in which the AT content of the template is greater than the GC content, are more of the RNA chains initiated with ATP, for example, DNA's of phage T_2, T_4, T_5, SP3.

The binding of the first nucleotide is different from the binding of the rest, because there is no 3' hydroxyl group present

to which the first nucleotide can add. It has been suggested by Anthony et. al. (1966) that the rate-limiting step may be the binding of the first nucleotide and/or the condensation of the first two nucleotides, if the initiation step is a bimolecular reaction. Anthony, Wu & Goldthwait (1969) found that the apparent K_m for nucleotides for the formation of the first phosphodiester bond (0.15 mM) was very much higher than the Km for nucleotides for the formation of subsequent phosphodiester bonds (0.015 mM). Therefore, these authors proposed that the initiation step involving the formation of the first phosphodiester bond, may be the rate limiting step of RNA synthesis.

The binding of the first two nucleotides to a polymerase-DNA complex completes what has been referred to as an initiation complex.

5. Growth of RNA chain

Following the formation of an initiation complex, nucleoside triphosphates are polymerized in a sequence complementary to that of the codogenic strand of the double helical DNA template. The polymerization step involves a nucleophilic attack on the 3'-hydroxyl of the preceding nucleotide by the 5' phosphate of the incoming nucleoside triphosphate with a stoichiometric release of inorganic pyrophosphate. Thus, the 5' terminal nucleotide of the RNA chain ends with a triphosphate and the 3' terminal with a free 3'-hydroxyl group.

The growth of the RNA chain has been shown to proceed

in a 5' to 3' direction (Bremer, Konrad, Gaines & Stent, 1965; Maitra & Hurwitz, 1965). This conclusion is confirmed by the results of Shigeura & Boxer (1964) who studied the effects of an analog of ATP on the RNA polymerase-catalyzed reaction. They showed that 3'-deoxyadenosine 5'-triphosphate was never incorporated into the 5' end of an RNA chain, as it does not have a hydroxyl group at its 3' position. For the same reason, the analog, when incorporated into an RNA chain, stopped further chain growth and was always found at the 3' terminal end of a chain.

The growth rates of the RNA chains have been estimated by various workers. Bremer & Konrad (1964) found the average growth rate of T_4 RNA chains is 2.5 nucleotides per second under their conditions of *in vitro* synthesis. This rate was later extrapolated to 4 nucleotides per sec. at saturating concentration of nucleoside triphosphates (Bremer, 1967). However, So, Davie, Epstein & Tissieres (1967), using 0.2M KCl in their assay conditions, obtained a chain growth rate for T_4 RNA of 16 nucleotides per second. As there is a possibility that chain initiation may not have been synchronous, this value is believed to be an underestimation. The *in vivo* growth rate of T_4 RNA has been measured by Bremer & Yuan (1968) to be 28 nucleotides per second. Therefore the *in vitro* rate determined by So *et. al.* is comparable to the *in vivo* rate.

6. Termination of RNA chains

In vivo, RNA's synthesized, whether transfer, ribosomal or messenger, have discrete sizes. There must be some kind of termination signal to stop the polymerization process and

to release the RNA chain.

Early studies of chain termination in vitro were complicated by the use of low salt concentrations in the reaction mixture. If RNA synthesis is carried out under conditions of low ionic strength (eg. u = 0.05), there is no release of the RNA chains. These nascent chains remain bound to the template and stop polymerization completely after about 1 hour at 37°. Such product inhibition can be eliminated by increasing the salt concentration of the reaction mixture (eg. 0.2 M KCl). Under these conditions RNA synthesis continues for several hours with little decrease in rate. These studies will be discussed further in a later section (Discussion, Section C - 1).

Under conditions of high salt concentration, the bulk of the T_4 RNA synthesized in vitro after 20 minutes of synthesis, was found to have a sedimentation rate of 25 S (Bremer & Konrad, 1964). However the T_4 RNA synthesized in vivo has a value of about 14 S (Asano, 1965). These differences could result from degradation of the in vivo RNA during isolation, or from a preferential synthesis in vitro of the longer transcriptive units, or from a loss of proper termination signals in the purified in vitro systems of synthesis.

To isolate the termination regions of Bacillus subtilis DNA template, Novak (1969) added deoxyribonuclease to an RNA synthesizing mixture containing the bacterial DNA, at or near the end of RNA synthesis. He obtained nuclease-resistant DNA fragments which were still complexed to the RNA polymerase. These fragments

were isolated after deproteinization and were found to have a higher than average GC content for the bacterial DNA. A GC-rich termination signal for transcription in Bacillus subtilis DNA was therefore proposed. However the relevance of this finding to the in vivo situation is uncertain.

7. Regulation of RNA synthesis

It is currently conceived that in the cell there are a number of controlling factors which determine the 'switching-on' and 'switching-off' of genes. Various aspects of control of transcription have been reviewed by Richardson (1969). In mammalian cells, the picture is more complicated. All the natural components that associate with DNA in chromatin can potentially regulate transcription, such as histones, non-histone proteins, RNA's, polyamines, etc. RNA synthesis in higher organisms is also strongly influenced by hormones and antibiotics.

More significant advancement has been made in the studies of bacterial systems. Considerable evidence has been obtained by Jacob & Monod (1961) in support of their analysis of the factors that control the synthesis of enzymes for lactose metabolism in E. coli. A regulator gene makes a product called a repressor, which blocks the synthesis of all the enzymes for lactose metabolism. The lactose repressor has been isolated (Gilbert & Muller-Hill, 1966) and has been shown to bind specifically to the DNA region it is supposed to affect (Gilbert & Muller-Hill, 1967). Positive control factors probably are important in the regulation of viral genes. It is known, for example, that products of "early" genes of bacteriophages

T_4 and λ are required for the expression of the "late" genes.

Recently, Burgess, Travers, Dunn & Bautz (1969) discovered a sigma factor which is a normal component of E. coli RNA polymerase. By itself the factor has no polymerase activity. The "core" enzyme which has the sigma factor removed, is able to transcribe E. coli DNA normally, but cannot transcribe T_4 DNA unless the factor is added back. Thus the sigma factor stimulates transcription specifically of T_4 DNA. Several minutes after infection of E. coli by T_4 phage, the sigma factor could no longer be detected. Five to fifteen minutes post-infection, a new analogous factor appeared in the infected cell. This factor is called T_4 factor, as it is required to initiate the transcription of the second portion of the T_4 genome.

Most probably more controlling factors will be identified. It is therefore reasonable to assume that there is a hierarchy of controlling factors, some positive, some negative, involved in the regulation of RNA synthesis in bacteria and in higher organisms.

8. Effects of irradiation on template activity of DNA

The damaging effects of ionizing irradiation on DNA have been reviewed by Shooter (1967) and Muller (1967). Relatively little work has been carried out to study the synthesis of RNA directed by radiation-damaged DNA templates.

Harrington (1963) showed that x-irradiation strongly reduced the ability of purified calf thymus DNA to serve as template

for RNA synthesis in an *in vitro* system employing *E. coli* RNA polymerase. The irradiation was found to be effective whether delivered *in vivo* or *in vitro*.

Very recently, Hagen *et. al.* (1970) confirmed the above observation, employing ultraviolet light and γ -irradiation. One of the reasons for the decreased template activity of radiation-damaged DNA was the presence of single-stranded breaks which stop polymerization and create new binding sites for the enzyme that may be ineffective for transcription. Similar conclusions were drawn from an independent study of Goddard *et. al.* (1970).

E. Introduction to Present Work

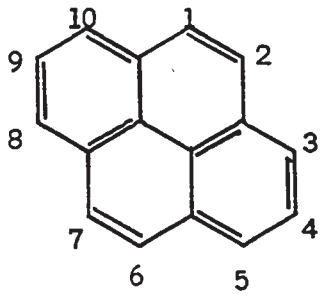
While the mechanism of action of cancer-producing chemical agents has not been elucidated, the interaction of these compounds with DNA has been recognized and considered to be of likely importance in the genesis of cancer (see Review of Literature, Section B).

Rather extensive studies have been carried out on the physical interaction of DNA with polycyclic hydrocarbons, which constitute a major class of chemical carcinogens(see Review of Literature, Section C - 1,b). However, although several dimethyl derivatives of benz(c)acridine have been known to be potent carcinogens for some time (Lacassagne *et. al.*, 1956), little is known about their interaction with DNA (see Review of Literature, Section C - 1,a). The first objective of the present work was therefore to study the extent and mode of *physical binding* of these acridine derivatives to DNA, in a purified *in vitro* system. The carcinogenic 7,9-, 7,10-

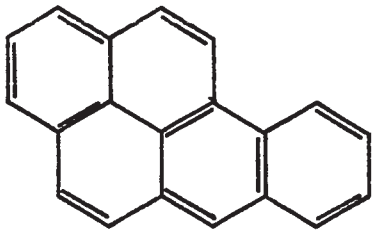
and the non-carcinogenic 7,11-dimethyl derivatives of benz(c)-acridine are selected for study because of their varied carcinogenic potencies.

Evidence has been obtained which suggests that polycyclic hydrocarbons can bind covalently to DNA in vivo. Such covalent binding has also been observed to be catalyzed by a microsomal enzyme system in vitro, or to be inducible under the influence of ionizing agents, such as hydrogen peroxide, iodine and x-irradiation (see Review of Literature, Section C - 2). However, because of the very low levels of binding observed in all these cases, it was not possible to characterize the nature of the binding. An attempt was made, as a second objective, to induce high levels of covalent binding of the dimethylbenz(c)acridines and polycyclic hydrocarbons (pyrene and benz(a)pyrene) to DNA by γ -irradiation in vitro.

At the present time, the significance of the observed in vivo or in vitro binding of polycyclic hydrocarbons to DNA in relation to the mechanism of chemical carcinogenesis is difficult to assess (see Review of Literature, Section B). No effect on the function of the DNA as a result of such binding has been demonstrated in a well-characterized in vitro system. Therefore, the third objective of this work was to investigate the effects, if any, of the DNA-bound dimethylbenz(c)acridines and polycyclic hydrocarbons on the ability of the DNA to function as a template for RNA synthesis using a highly purified in vitro system, catalyzed by E. coli RNA polymerase.

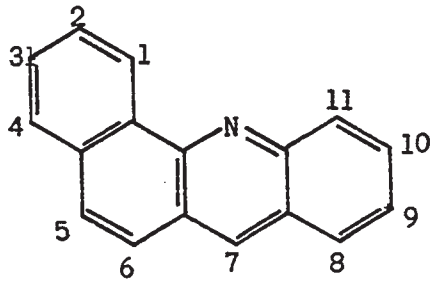


Pyrene



3,4-Benzpyrene

(Benz(a)pyrene)



Benz(c)acridine

II. MATERIALS AND METHODS

A. Physical Interactions of Dimethylbenz(c)acridines with DNA

1. Preparation of DNA solutions

Highly polymerized calf thymus DNA (Nutritional Biochemicals Company, Cleveland, Ohio) was dissolved, unless specified otherwise, in a standard binding buffer (SBB = 1×10^{-4}M Tris HCl, pH = 7.0, in 5×10^{-4}M NaCl), centrifuged in a Sorvall RC-2B centrifuge at 13,000 rpm for 20 min. and then dialysed. The concentration was then adjusted to 1 mg./ml., assuming an extinction coefficient $E_{260}^{1\%} = 200$, and was checked by phosphate analysis (Fiske and SubbaRow, 1957). Denatured DNA solutions were prepared by pouring heated DNA solutions (30 min. in boiling water) over ice prepared from SBB and dialyzing to obtain the desired ionic strength and recentrifuging.

2. Preparation of solutions of dimethylbenz(c)acridines

Saturated solutions of 7,9- and 7,10-dimethyl derivatives of benz(c)acridine (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and of the 7,11-dimethyl derivative (Koch-Light Laboratory Ltd., Colnbrook, Buckinghamshire, England) were prepared in redistilled 100% ethanol and kept in the dark. Chemical Abstracts

system of numbering is used throughout. The concentration of each solution was checked using the following extinction coefficients $E_{278}^{1M} = 57.3 \times 10^3$ for 7,9; $E_{277}^{1M} = 61.44 \times 10^3$ for 7,10 and $E_{280}^{1M} = 71.03 \times 10^2$ for 7,11-dimethylbenz(c)acridine. Purity of these compounds was checked and found to be $\sim$ 95%, by means of chromatography on thin layer silica gel plates (Brinkmann Instruments Ltd., 50 Galaxy Blvd., Rexdale, Ontario) developed in n-butanol:5N Acetic acid (7:3, v/v).

3. Preparation of dimethylbenz(c)acridine-DNA complexes

To 13 ml. of a DNA solution was added dropwise and with continuous mixing 0.8 ml. of one of the dimethylbenz(c)acridine solutions. Uncomplexed dimethylbenz(c)acridine which precipitated out of solution was removed by centrifugation at 4° either in a Spinco SW40 or SW50 rotor at 40,000 rpm for 3 hrs. The top 5/6 of the clear supernatant was carefully withdrawn. The control DNA sample to which alcohol only had been added was treated in an identical manner.

4. Determination of sedimentation rate

Sedimentation data were obtained in the Beckman Model E Analytical Ultracentrifuge equipped with U-V optics. Diluted samples of control DNA and of dimethylbenz(c)acridine:DNA complexes in SBB were centrifuged in 30 mm cells in a two-cell AnE rotor at 39,460 rpm. Pictures were taken at 8 min. intervals. The boundary positions of the sedimenting material at various times were determined from tracings of the U-V negatives using a

Kipp and Zonen densitometer. Sedimentation rates were calculated for each sample at 4 to 5 DNA concentrations within the range of 4-16 µg./ml. The S^{o}_{20} values of control DNA and dimethylbenz(c)-acridine:DNA complexes were compared.

5. Calculation of the binding ratio

The binding or molar ratio (M.R.) was calculated from the concentrations of DNA nucleotide phosphate and dimethylbenz(c)-acridine present in a given complex and is expressed as the number of moles of dimethylbenz(c)acridine bound per mole nucleotide phosphate. The concentration of dimethylbenz(c)acridine was determined by exhaustive extraction of the complex with cyclo-hexane. The completeness of extraction was confirmed in control experiments where a known amount of each of the dimethylbenz(c)-acridines was added to a DNA solution and recovery was 100%.

6. Sucrose density gradients

Sucrose solutions for density gradient experiments were prepared by dissolving sucrose in SBB. Linear gradients of 5 - 20% or 5 - 40% in 4.7 ml. or 12.6 ml. volumes respectively were prepared. Centrifugations were carried out in a Spinco Model L or L2, or L2-65B ultracentrifuge in an SW40 or SW50 rotor at 4°. Linearity of the gradients was confirmed by measuring the refractive index of each of the collected fractions following centrifugation.

7. Thermal denaturation studies

Thermal denaturation studies of dimethylbenz(c)-

acridine:DNA complexes were carried out in a Gilford Multiple Sample Absorbance Recording Spectrophotometer. The samples, dissolved in SBB at concentrations specified in the experiments, were heated at a rate of 1° per 85 sec. The optical density changes at 260 mμ or at 380 mμ in a 10 mm quartz cuvette were recorded.

To determine the spectrum at any given time during the denaturation process, heating was stopped for about 5 min. to allow time for the optical densities at various wavelengths to be measured. Heating was resumed immediately after the spectrum was recorded. During this 5-min. interval, the temperature remained relatively constant (±0.5°).

B. γ ray-induced Covalent Linkage of Polycyclic Aromatic Hydrocarbons to DNA

1. Preparation of DNA solutions

DNA solutions were prepared as described in Part A except the solvent used throughout was 2×10^{-4}M cacodylate buffer (pH 5.2).

2. Preparation of solutions of polycyclic aromatic hydrocarbons

Pyrene was obtained from Matheson, Coleman and Bell, Norwood, Ohio, and benz(a)pyrene from Eastman Organic Chemicals, Rochester 3, N.Y. The hydrocarbons were dissolved in redistilled methanol. Purity was checked by chromatography on thin-layer silica gel plates with hexane as the developing solvent. Pyrene

yielded a major fluorescent spot and a minor one at about 1/4 of the R_f of the major spot. Benz(a)pyrene yielded a major spot and several slower-moving minor ones. Elution of the materials in these spots with cyclohexane and analysis of their absorption spectra revealed that the major spots were pyrene and benz(a)-pyrene respectively. None of the minor spots gave any distinct absorption spectra. Based on the peak optical densities at 336 mμ for pyrene and at 297 mμ for benz(a)pyrene, they were estimated to be approximately 95% and 90% pure respectively.

The concentrations of hydrocarbon solutions in cyclo-hexane were determined spectrophotometrically, using extinction coefficients calculated by Smith (1965): $E^{M}_{336} = 5.012 \times 10^4$ for pyrene and $E^{M}_{297} = 7.065 \times 10^4$ for benz(a)pyrene.

3. Preparation of covalent complexes

Physical complexes of DNA made with pyrene, benz(a)-pyrene, 7,9- or 7,10-dimethylbenz(c)acridine were prepared as described in Part A, except that the DNA was dissolved in 2×10^{-5}M cacodylate buffer (pH 5.2). The physical complexes were irradiated in unsealed glass tubes with ^{60}Co γ -rays in a Gammacell 220 (Atomic Energy of Canada Ltd., Ottawa, Canada). Lead shields were used to reduce the dose rate to 290 rads per min. Following γ -irradiation, the DNA and complexes were precipitated by addition of 0.5M ammonium acetate and three volumes of cold ethanol. The precipitates were washed three times with 100% ethanol and two times with ether, and redissolved in one-third of the original

volume of 10^{-2}M NaCl in 10^{-2}M Tris-HCl pH 7.9. The temperature during irradiation was not controlled. Samples, initially at about 5°, were allowed to warm in the Gammacell 200. The rise in temperature was at a rate of about 6° for every 10 minutes of continuous exposure to the γ-rays.

4. Determination of molar ratio of covalent complexes

The amount of hydrocarbon covalently bound to DNA was determined indirectly by measuring the amount unrecoverable by extraction with cyclohexane. The binding ratio was computed from the concentrations of the unextractable hydrocarbons and of the DNA and is expressed as the moles of hydrocarbon per mole of DNA nucleotide-phosphate.

5. Thermal denaturation studies of covalent complexes

Thermal denaturation studies were carried out as described in Part A, except that the solvent used was 1×10^{-2}M NaCl in 1×10^{-2}M Tris-HCl pH 7.9.

6. Sucrose density gradient analysis of covalent complexes

Sucrose density gradient experiments were carried out as described in Part A, except that the solvent used was 1×10^{-2}M NaCl in 1×10^{-2}M Tris-HCl (pH 7.9).

C. Effects of Polycyclic Carcinogens on Transcription

1. Preparation of E. coli RNA polymerase

DNA-dependent RNA polymerase was prepared from frozen

E. coli B cells which were either purchased from General Biochemicals, Laboratory Park, Chagrin Falls, Ohio 44022, or harvested from a late log phase culture, grown in 15 l. fermenting jars containing 140 g. K_2HPO_4, 60 g. KH_2PO_4, 100 g. dextrose and 100 g. Difco yeast extract in 10 l. of distilled water. The procedure of Chamberlin and Berg (1962) for the extraction and purification of RNA polymerase was followed, except that the 0.002M phosphate buffer was replaced by the same concentration of tris (pH 7.0) in the buffer systems used for the final DEAE-cellulose column chromatography step. Unless otherwise noted, all operations were carried out at 4° and all centrifugations were performed at 30,000 x g for 15 min. in a Sorvall RC-2B refrigerated centrifuge.

Frozen cells (140 g.) were allowed to thaw and were suspended in 150 ml. buffer A (0.01M $MgCl_2$ and 0.0001M EDTA in 0.01M Tris pH 7.9). The cell suspension was mixed with 420 g. of acid-washed glass beads (0.20MM Schwartz BioResearch Inc., Orangeburg, N.Y.) in a Waring Blendor equipped with a cooling coil (coolant at -15°) for 25 minutes at 1/3 maximum speed. Maximum temperature during mixing was below 15°. A further 75 ml. of buffer A was added and the glass beads were allowed to settle. The supernatant was decanted and the residue was washed with 75 ml. of buffer A. The supernatant and wash were pooled and centrifuged for 30 minutes. The supernatant (Fraction I) was centrifuged in a Spinco Model L preparative ultracentrifuge for 3 hours at 30,000 rpm in No. 30 retor. The protein concentration in the supernatant was determined by the protein assay of Lowry, et. al. (1951) and adjusted to a

concentration of 12 mg. per ml. with buffer A. Mercaptoethanol was added to a final concentration of 0.01M. To 350 ml. diluted supernatant was added, dropwise and with continuous mixing, 17.5 ml. of 10% (w/v) solution of streptomycin sulfate (Mann Research Laboratories, Division of Becton, Dickinson and Co., N.Y.). After 15 minutes, the solution was centrifuged and to 350 ml. of the supernatant was added, dropwise and with continuous mixing, 14.0 ml. of a 1% (w/v) solution of protamine sulfate (Eli Lilly Laboratories, Indianapolis, U.S.A.). The precipitate collected by centrifugation was washed by suspension in 175 ml. of buffer A containing 0.01M β-mercaptoethanol. The washed precipitate was then suspended in 35 ml. of buffer A containing 0.01M mercaptoethanol and 0.10M ammonium sulfate, centrifuged 30 min. and the supernatant was collected (Fraction II).

To 37 ml. of Fraction II was added 15.8 ml. of ammonium sulfate solution (saturated at 25° and adjusted to pH 7 with ammonium hydroxide). The mixture was stirred for 15 min., and the precipitate removed by centrifugation. To the supernatant was added an additional 16.2 ml. of saturated ammonium sulfate, and after 15 min. the precipitate was collected by centrifugation for 30 min. and dissolved in buffer B (0.01M $MgCl_2$, 0.01M B-mercapto-ethanol and 0.0001M EDTA in 0.002M Tris, pH 7.9) to a final volume of 5.0 ml. (Fraction III).

DEAE-cellulose (Matheson, Coleman and Bell, Norwood, Cincinnati, Ohio) was pre-washed with 1N HCl, 1N NaOH and water, and then blended briefly in a Waring Blendor. A column (10 cm x

1 cm^2) of the DEAE-cellulose was packed and washed with at least 150 ml. of buffer B prior to use. Fraction III was diluted to a protein concentration of 3 mg. per ml. with buffer B and adsorbed onto the column at a rate of about 0.8 ml. per min. The column was washed with 10 ml. of buffer B and then with 20 ml. of the same buffer containing 0.16M KCl and finally with buffer B containing 0.23M KCl. The enzyme was eluted at both salt concentrations.

The fractions with peak activities were pooled and stored at about 0.2 mg. protein per ml. in 30% glycerol at -20°. The enzyme was stable for several months under these storage conditions with little loss of activity.

2. Standard assay of RNA polymerase activity

The standard conditions of assay for RNA polymerase activity of Chamberlin and Berg (1962) were used except where otherwise specified. The assay measured the conversion of P^{32} from labelled ribonucleoside triphosphate into an acid-precipitable form. The reaction mixture (0.25 ml.) contained: 10 umoles of Tris buffer, pH 7.9, 0.25 umole of $MnCl_2$, 1.0 umole of $MgCl_2$, 100 mmoles each of ATP, CTP, GTP and UTP, 80 ug. calf thymus DNA, 3.0 umoles of B-mercaptoethanol and 3 - 20 ug. of enzyme protein. One of the nucleoside triphosphates was labelled with approximately 300 - 600 cpm per mumole. After incubation for 10 min. at 37°, 0.03 ml. of bovine serum albumin (40 mg. per ml.) and 3 ml of cold 3.5% perchloric acid were quickly added to the reaction mixture,

mixed and kept in the cold for 15 min. or longer. The precipitate was dispersed, centrifuged 15 min. in an International clinical centrifuge (Model CL) at top speed, and washed twice with 3 ml. portions of cold perchloric acid. The residue was suspended in 0.5 ml. of 2N ammonium hydroxide, transferred to an aluminum planchet, and after drying under an infrared lamp was counted in a Nuclear-Chicago windowless gas-flow counter.

One unit of enzyme activity corresponded to an incorporation of 1 mumole of CMP^{32} per hr. under conditions described above.

III RESULTS

A. Physical interactions of dimethylbenz(c)acridines with DNA

1. Formation of physical complexes with native DNA

The mixing of a DNA solution in low ionic strength and at neutral pH, with the dimethylbenz(c)acridines dissolved in a small volume of redistilled alcohol, allowed efficient binding of the dimethylbenz(c)acridines to DNA. These compounds thus solubilized by DNA gave a bright yellow color, having characteristic absorption spectra distinct from those of their free solutions in neutral alcohol (unionized form) or in acid (cationic form)(Fig. 1). When solubilized by DNA the spectra of 7,9- and 7,10-dimethylbenz(c)acridines closely resembled their respective spectra in acid, except for bathochromic shifts and depression of the absorption maxima. The complete disappearance of absorbance after centrifugation of the dimethylbenz(c)acridines in the binding buffer, demonstrated the insolubility of the dimethylbenz(c)acridines in the buffer and the role of DNA in solubilizing the benz(c)acridines.

When the three dimethylbenz(c)acridine:DNA complexes were centrifuged in a sucrose density gradient, the dimethylbenz(c)acridines absorbance co-sedimented with the DNA (Fig. 2). The

FIGURE 1.

Spectral comparisons of 7,9-, 7,10- and 7,11-dimethylbenz(c)-acridines in various reagents.

The absorption spectra of each of the 3 dimethylbenz(c)-acridines ((a) 7,9 (b) 7,10 (c) 7,11) dissolved in DNA (------) after high speed centrifugation are compared with their respective spectra when dissolved at equal concentrations in ethanol (_____), in 0.04N HCl (•-•-•-•) or in standard binding buffer after centrifugation (••-••-••-).

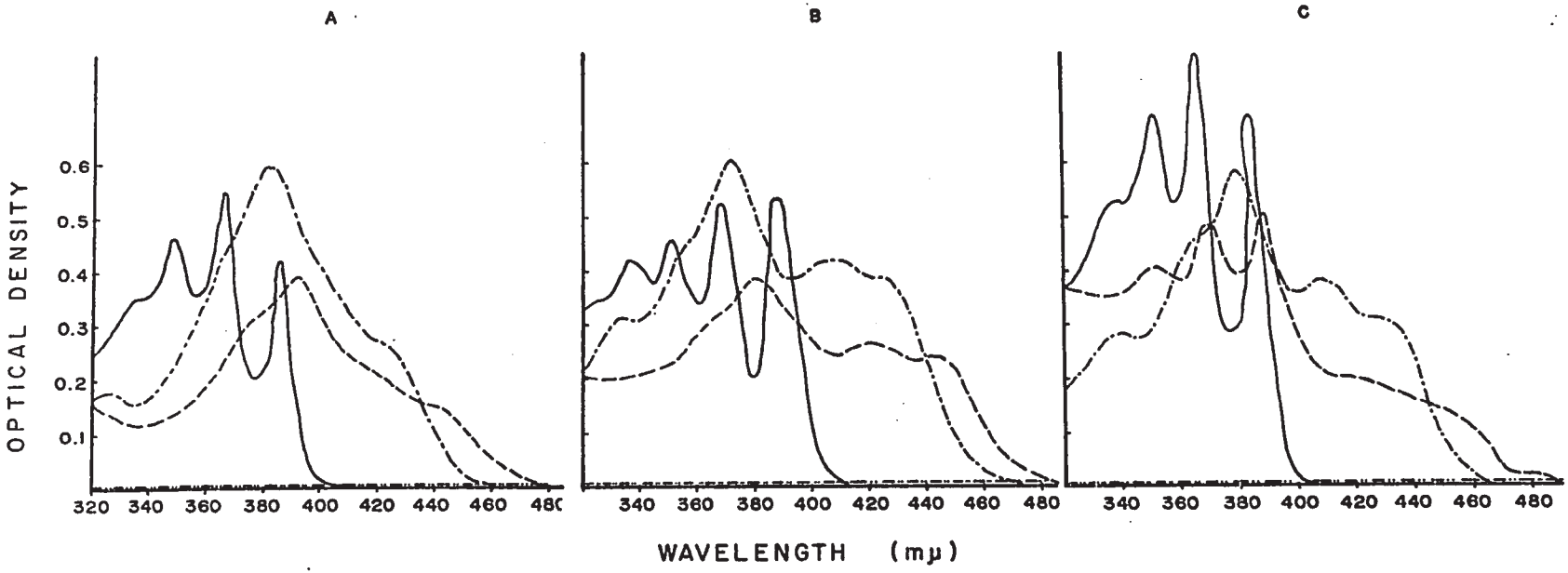

A
B
C
OPTICAL DENSITY
0.6
0.5
0.4
0.3
0.2
0.1
320 340 360 380 400 420 440 460 480
340 360 380 400 420 440 460 480
340 360 380 400 420 440 460 480
WAVELENGTH (mμ)

FIGURE 2.

Sedimentation of 7,9- (a), 7,10- (b) and 7,11- (c) dimethylbenz(c)-acridine:DNA complexes in sucrose density gradients.

On to each of 4.7 ml. of the premixed gradients was layered 0.4 ml. of one of each of the DNA:dimethylbenz(c)acridine complexes. The molar ratio for each complex was 0.043 for 7.9; 0.058 for 7,10 and 0.02 for 7,11. The concentration of DNA in the 7,9 complex was 0.5 mg./ml. and for the 7,10 and 7,11 complexes was 1 mg./ml. All complexes were centrifuged in an SW50 rotor at 40,000 rpm at 4°C for 12 hr. Fractions were collected by puncturing the bottom of the centrifuge tube. The optical density at 260 mμ and at the peak of absorption for the particular complex was determined for each fraction.

● ——— ● O.D. at 260 mu

o ——— o O.D. at 380 mu or 390 mu

A
OPTICAL DENSITY AT 260 mμ
3.0
2.0
1.0
OPTICAL DENSITY AT 390 mμ
0.04
0.03
0.02
0.01
BOTTOM FRACTION NUMBER TOP
1 2 3 4 5 6 7 8 9 10 11 12
B
OPTICAL DENSITY AT 260 mμ
7.0
6.0
5.0
4.0
3.0
2.0
1.0
OPTICAL DENSITY AT 380 mμ
0.03
0.02
0.01
BOTTOM FRACTION NUMBER TOP
1 2 3 4 5 6 7 8 9 10 11 12
C
OPTICAL DENSITY AT 260 mμ
7.0
6.0
5.0
4.0
3.0
2.0
1.0
OPTICAL DENSITY AT 390 mμ
0.05
0.04
0.03
0.02
0.01
BOTTOM FRACTION NUMBER TOP
1 2 3 4 5 6 7 8 9 10 11

recovery of both DNA and dimethylbenz(c)acridine for the 7,9-and 7,10-derivatives was 100%. These results provided direct evidence of complex formation between these dimethylbenz(c)acridines and DNA.

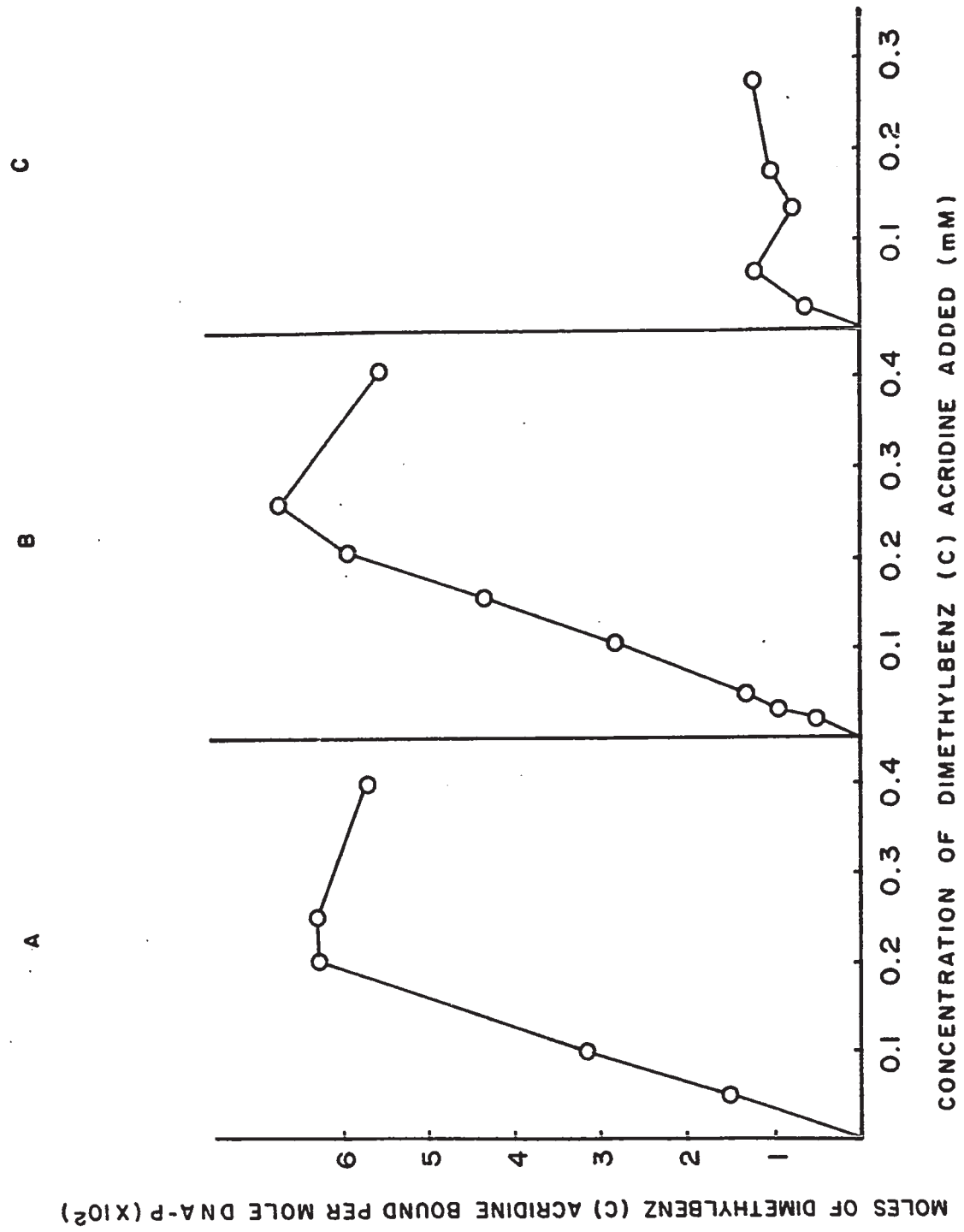
To determine the maximum extent of binding of the dimethylbenz(c)acridines to DNA, increasing amounts of each of the 3 dimethylbenz(c)acridines were added to a given amount of DNA. Fig. 3(a) shows that the extent of binding reached a saturation level, which for the 7,9-derivative corresponded to a maximum binding ratio of 1 mole to 16 moles of DNA nucleotide phosphate. Results of Fig. 3,b show a similar maximum binding ratio for the 7,10-derivative.

The interaction of the 7,11-derivative with DNA differed from that of the 7,9- and 7,10-derivatives in several respects: (1) The absorption spectrum of the 7,11-derivative in DNA resembled more closely its spectrum in alcohol (unionized form) than in acid (ionized form) as can be seen in Fig. 1. (2) The 7,11- derivative was bound to a much smaller extent to DNA (Fig. 3), the saturation level of binding being only 1 mole to 82 moles of DNA nucleotide. (3) The 7,11-derivative could not form stable complexes with DNA, and dissociated from DNA gradually and steadily on standing at 4°, whereas the 7,9- and 7,10-compounds remained fully complexed with DNA over long periods of time (6 months or longer). (4) The 7,11-derivative did not quantitatively remain associated with DNA during sedimentation through a sucrose density gradient. Although all of the DNA (O.D. 260) layered onto the

FIGURE 3.

Binding curves for 7,9- (a); 7,10- (b) and 7,11 (c) dimethylbenz(c)acridines to DNA.

Complexes of DNA with each of the 3 dimethylbenz(c)acridines were made under standard conditions, except with varying amounts of the dimethylbenz(c)acridines. The binding ratio of each of the complexes was determined as described in Materials and Methods.


A
B
C
0.1
0.2
0.3
0.4
0.1
0.2
0.3
0.4
0.1
0.2
0.3
CONCENTRATION OF DIMETHYLBENZ (C) ACRIDINE ADDED (mM)
1
2
3
4
5
6
MOLES OF DIMETHYLBENZ (C) ACRIDINE BOUND PER MOLE DNA-P (X10²)

gradient appeared in the optical density band (Fig. 2), only about 40% of the original 7,11-dimethylbenz(c)acridine (O.D. 390) remained associated with the DNA. The other 60% was found as a precipitate at the bottom of the centrifuge tube.

The binding of the 7,10-derivative to DNA was strongly dependent on the pH of the medium. Fig. 4 shows that a low pH greatly enhanced complex formation, which was increased by about 4-fold when the pH was lowered from 8 to 6.

The binding of 7,10-dimethylbenz(c)acridine was also strongly dependent on the ionic strength of the medium. Fig. 5 shows that high ionic strength inhibited the interaction. Complex formation dropped almost 4-fold when the ionic strength was raised from 10^{-3} to 10^{-2}M.

As shown in Fig. 1, binding to DNA resulted in a bathochromic shift in the absorption peaks of the dimethylbenz(c)-acridines. Results of Table I show that no such shifts were observable when these compounds were dissolved in the group A solvents which had a large range of dielectric constants and hydrogen-bonding properties. However, bathochromic shifts were observed when they were dissolved in the group B reagents all of which had a system of unsaturated bonds. The bathochromic effect of caffeine on the absorption spectrum of the dimethylbenz(c)-acridines was eliminated by the addition of ethanol.

FIGURE 4.

Effect of pH on the binding of 7,10-dimethylbenz(c)acridine to DNA.

A series of complexes was made under conditions of excess 7,10-dimethylbenz(c)acridine (0.25 mM) with DNA at a concentration of about 0.6 mg./ml. in 10^{-3}M of either cacodylate (●) or Tris-HCl (X) buffer at various pH's, as described in Materials and Methods. Ratio of bound acridine in each of the complexes was determined.

FIGURE 5.

Effect of ionic strength on the binding of 7,10-dimethylbenz(c)-acridine to DNA.

A series of complexes was made under conditions of excess 7,10-dimethylbenz(c)acridine (0.25 mM) with DNA at a concentration of about 1 mg./ml. dissolved in 10^{-4}M Tris-HCl (pH 7.0) with varying concentrations of NaCl, as described in Materials and Methods. The binding ratio of each of the complexes was determined.

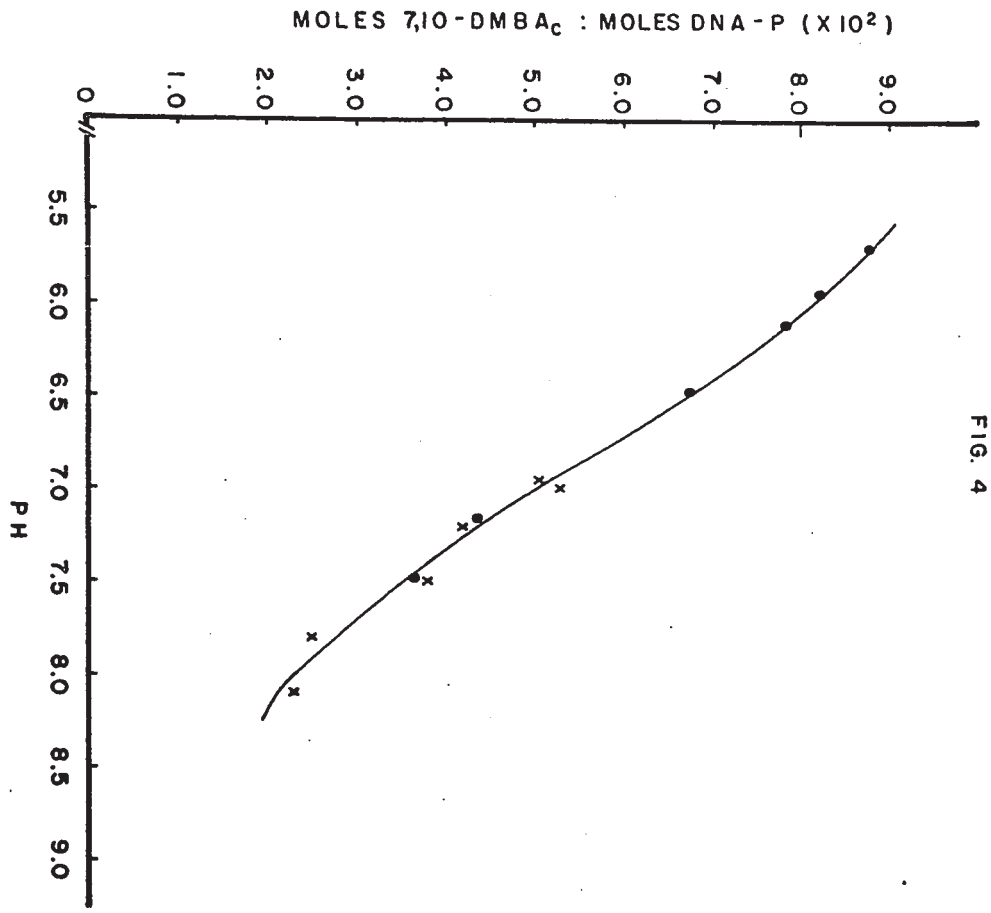

MOLES 7,10-DMBA$_C$: MOLES DNA-P (X10^2)
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
PH
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0

FIG. 4

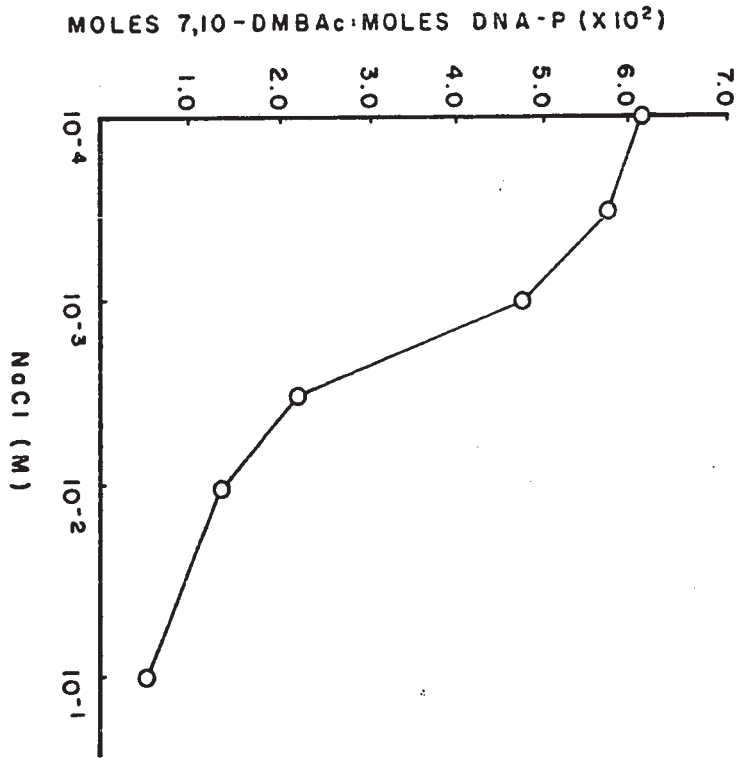

MOLES 7,10-DMBAc:MOLES DNA-P (X10^2)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
NaCl (M)
10^{-4}
10^{-3}
10^{-2}
10^{-1}

FIG. 5

TABLE I. Spectral maxima of dimethylbenz(c)acridines in various solvents and solutions

The dimethylbenz(c)acridines were each dissolved in various solvents and solutions. Their spectra were recorded in a Cary 15 spectrophotometer and the positions of the absorption peaks (mμ) are given below. Numbers in brackets represent the extent of the bathochromic shift in millimicrons.

Dimethylbenz(c)acridine	A					B			C
	50% EtOH	100% EtOH	100% MeOH	100% CH	100% DMF	I	II	III	IV
7,9	387	385	385	386	386	386 (1)	390 (5)	389 (4)	386
	367	365	365	366	367	366 (1)	370 (5)	369 (4)	367
	349	347	348	348	349	348 (1)	352 (5)	352 (5)	349
	-	334	334	333	335	-	-	-	335
7,10	389	388	388	388	389	392 (4)	397 (9)	-	389
	369	368	368	368	369	372 (4)	375 (7)	-	369
	352	351	351	350	352	354 (3)	357 (6)	-	352
	337	336	335	334	336	339 (3)	343 (7)	-	337
7,11	386	386	385	386	387	387 (1)	390 (4)	-	386
	366	366	365	366	367	367 (1)	370 (4)	-	366
	349	348	348	349	350	350 (1)	-	-	349
	-	337	335	335	338	338 (1)	-	-	336

Abbreviations used: EtOH - ethanol; MeOH - methanol; CH - cyclohexane; DMF - dimethylformamide

I - benzene

II - saturated caffeine in water

III - saturated tetramethyluric acid in water

IV - saturated caffeine in ethanol

2. Physical properties of complexes

The binding of 7,10-dimethylbenz(c)acridine to DNA stabilized the DNA against denaturation by heat. Fig. 6 shows that at a binding ratio of 1 mole per 12 DNA base pairs, the 7,10-derivative raised the thermal denaturation temperature (Tm) by 6.7°. Fig. 7(a) shows that the increment in Tm was directly proportional to the binding ratio. The 7,9-derivative was able to exert the same effects qualitatively and quantitatively (Fig. 7b).

The binding of the 7,10-derivative to DNA lowered the sedimentation rate of the DNA, analyzed by means of sucrose density gradient centrifugation (Fig. 8). The decrease in the sedimentation rate was confirmed (Table 2a) from data obtained by studying the sedimentation of the complexes in the Spinco Model E ultracentrifuge. When a 7,10:DNA complex was dissociated and the ligand removed, the dissociated DNA had the same sedimentation rate as the control DNA (Table 2b).

3. Thermal dissociation of complexes

Fig. 9(a) shows the relative absorbance of DNA at 260 mu and of the 7,10-dimethylbenz(c)acridine at 380 mu during the course of heat denaturation of a 7,10-dimethylbenz(c)acridine: DNA complex. Throughout the period of most pronounced change in hyperchromicity of the DNA (up to a temperature of 67°), the relative change in absorbance at 380 mu (Fig. 9b) dropped only slightly (approximately 9%). The major drop in optical density

FIGURE 6.

Effect of 7,10-dimethylbenz(c)acridine on the thermal denaturation of DNA.

Solutions of control DNA and of 7,10-dimethylbenz(c)acridine complex (Molar ratio = 0.043), prepared under standard conditions, were diluted with the standard binding buffer to a concentration of about 20 µg./ml. with respect to DNA. The changes in optical density at 260 mu* with increasing temperature were recorded as described in Materials and Methods. ●_____● Control DNA (Tm = 53.1°); x_____x Complex (Tm = 59.8°)

*Contribution of 7,10-DMBA to O.D. at 260 mu was ~ 6%.

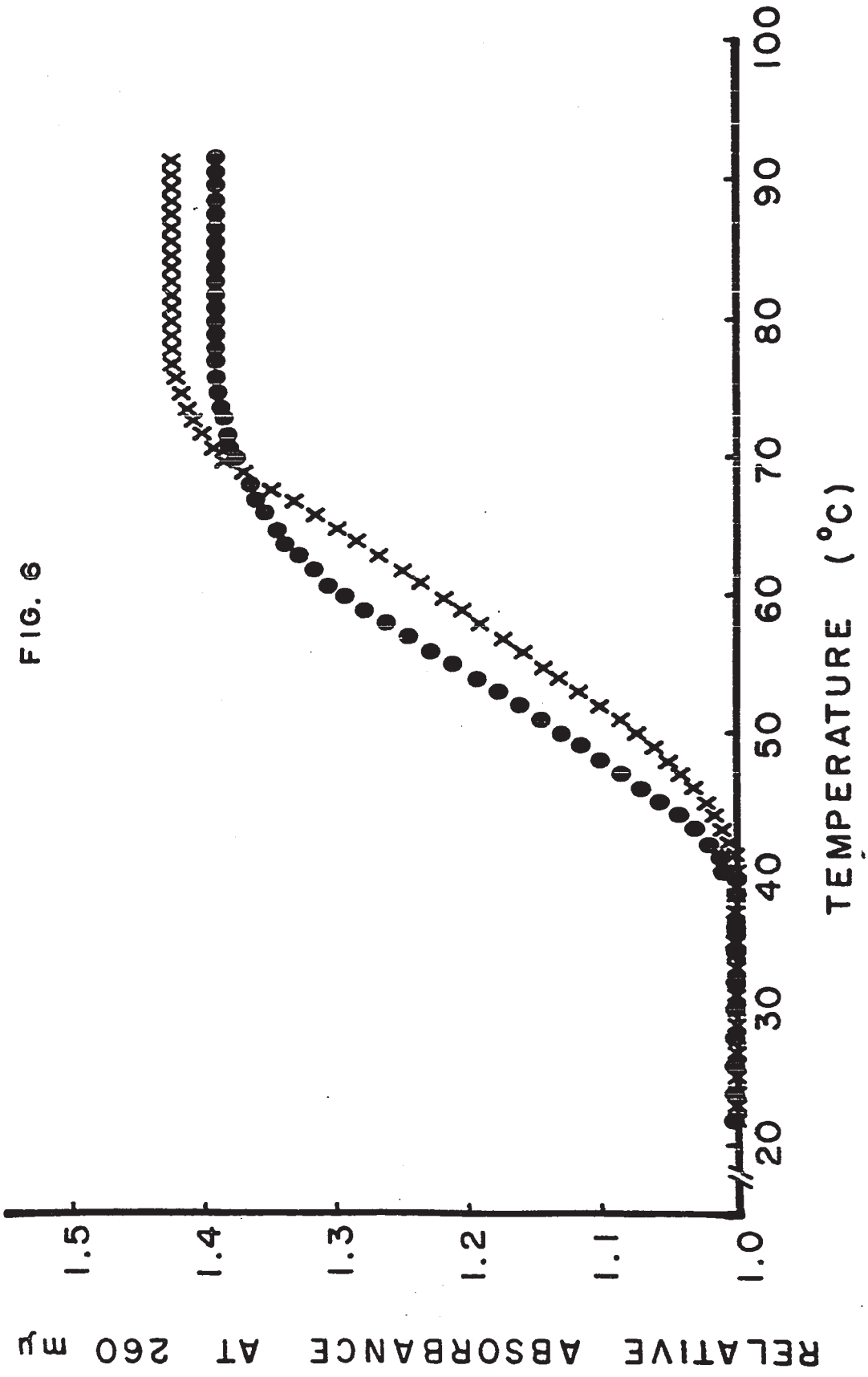

RELATIVE ABSORBANCE AT 260 mμ
1.0
1.1
1.2
1.3
1.4
1.5
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)

FIG. 6

FIGURE 7

Relationship between increase in denaturation temperature and the molar ratio of 7,9- and 7,10-dimethylbenz(c)acridine:DNA complexes.

The denaturation temperatures (Tm) of control DNA and of DNA complexes containing different amounts of 7,10- (a) or of 7,9- (b) dimethylbenz(c)acridine were determined as described in Materials and Methods. The increase in Tm of the complex over that of the control DNA is plotted against the binding ratio. Each point represents the average of at least three determinations and the bars indicate the range of variation.

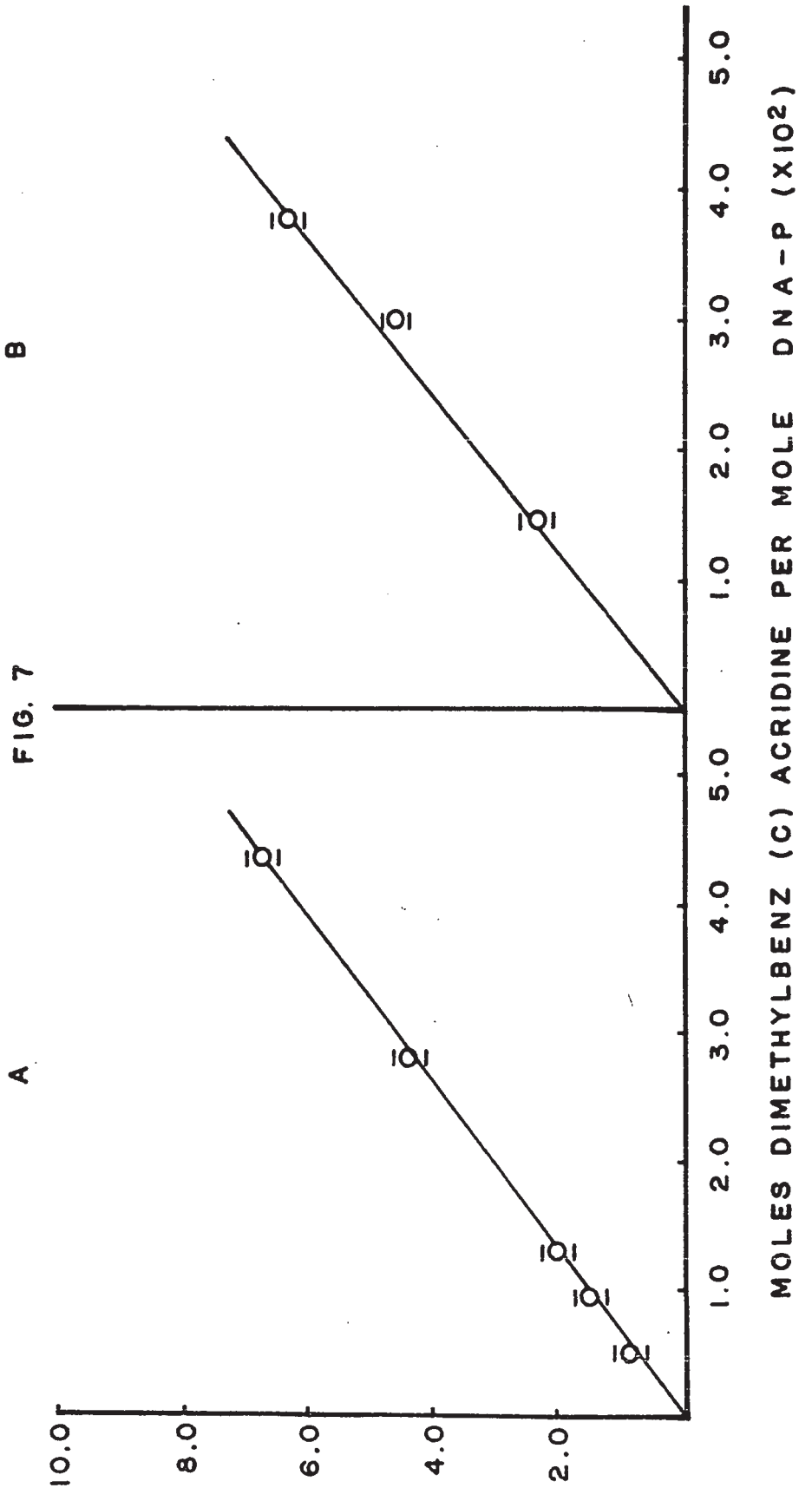

INCREASE IN Tm (C°)
10.0
8.0
6.0
4.0
2.0
A
B
1.0
2.0
3.0
4.0
5.0
MOLES DIMETHYLBENZ (C) ACRIDINE PER MOLE DNA-P (X10²)

FIG. 7

FIGURE 8.

Effect of 7,10-dimethylbenz(c)acridine on the sedimentation rate of DNA in a sucrose density gradient.

A sample of 0.2 ml. of control DNA (●____●) or of a 7,10-dimethylbenz(c)acridine:DNA complex (Molar Ratio = 0.033) (O____O) prepared under standard conditions, was layered on a 12.4 ml. premixed 5-40% linear sucrose density gradient. The tubes were centrifuged in an SW40 rotor in the Spinco Model L2-65B ultracentrifuge at 40,000 rpm for 38 hrs. at 4°C. Fractions were collected by puncturing the bottom of the tube and the optical density at 260 mμ for each fraction was determined.

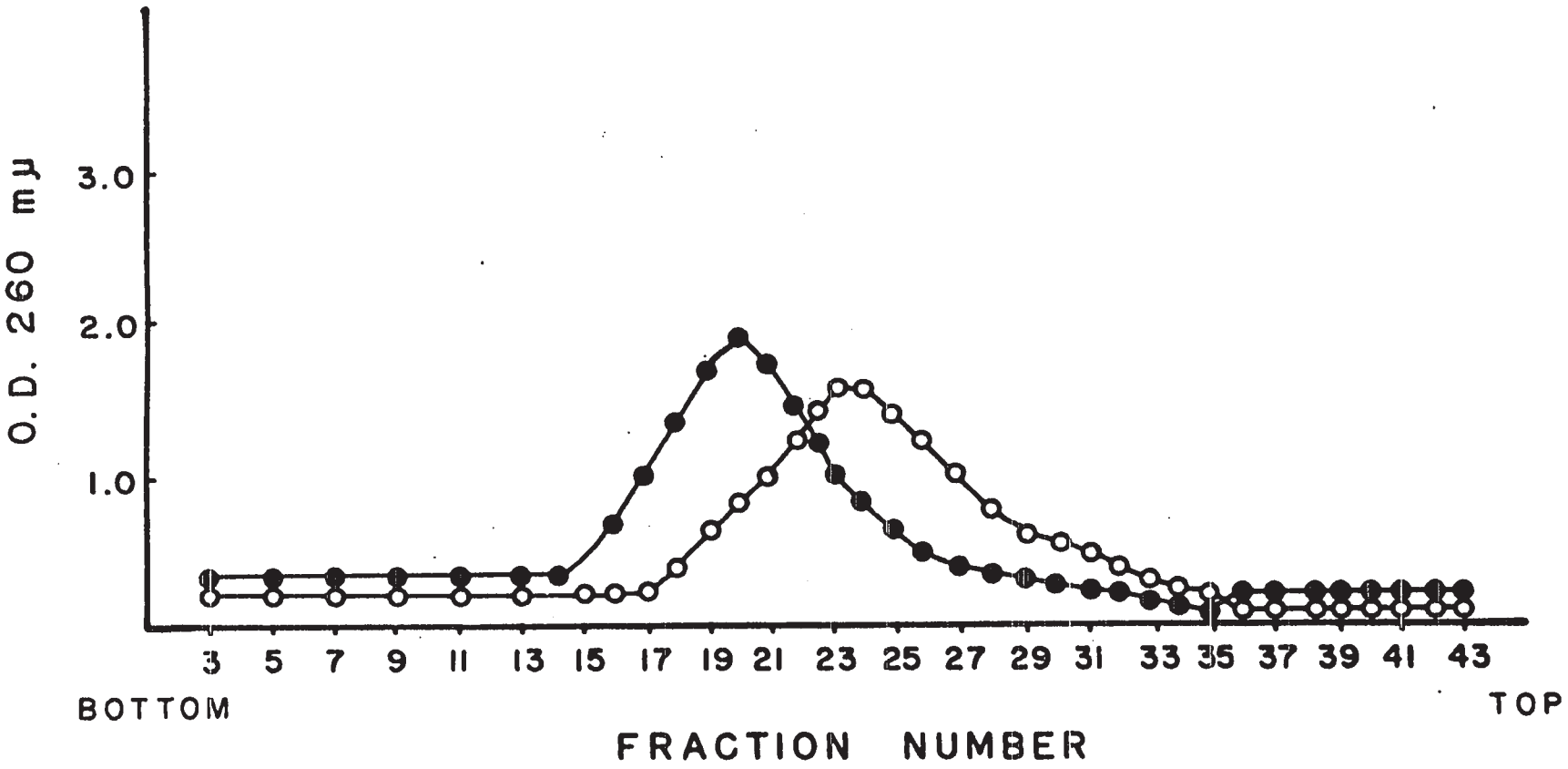

O.D. 260 mμ
3.0
2.0
1.0
3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43
BOTTOM
TOP
FRACTION NUMBER

TABLE 2. (a) The Effect of Binding of 7,10-dimethylbenz(c)acridine on Sedimentation Rate of DNA.

	Molar Ratio ($x10^2$)	S^0_{20}
DNA control	0	10
7,9:DNA complex	5.6	5
DNA control	0	22
7,10:DNA complex	5.6	6

The sedimentation rates were determined as described in Materials and Methods.

(b) The Effect of "dissociation" of a 7,10-dimethylbenz(c) acridine:DNA Complex on Sedimentation Rate of DNA

	Observed Sedimentation Rate (S)
Control DNA	20.0
"Uncomplexed" DNA	19.7

A 7,10:DNA complex in SBB at 1 mg/ml (M.R. = 0.059) was dissociated by adding 0.2 M NaCl. The dissociated and insoluble 7,10 was removed by centrifugation for 3 hours at 100,000 g (99% completion as judged by drop in absorption spectrum of the complex). The "uncomplexed" DNA was diluted to 16 ug/ml in SBB containing 0.1 M NaCl. Sedimentation rate was determined as described in Materials and Methods. The control DNA was treated and analyzed in the same manner.

FIGURE 9.

Melting profiles of 7,10-dimethylbenz(c)acridine:DNA complexes.

The effect of temperature on optical density changes at 260 mu ((a) top curve) and 380 mμ((b) bottom curve) of a 7,10-dimethylbenz(c)acridine:DNA complex. The complex (Molar Ratio = 0.0278) prepared under standard conditions was diluted to 84 μg. DNA per ml. in the standard binding buffer and heated in a Gilford Absorbance Recording Spectrophotometer as described in Materials and Methods. Changes in optical density at 260 and 380 mu were recorded in 2 separate experiments and are plotted on the same temperature axis.

FIGURE 10.

Effect of temperature on the absorption spectrum of 7,10-dimethylbenz(c)acridine:DNA complex.

The absorption spectrum of the 7,10-dimethylbenz(c)acridine: DNA complex at each of the 4 stages of thermal dissociation as marked in Fig. 9 (lower curve) was recorded as described in Materials and Methods.

I. 25° (———)

II. 55° (------)

III. 70° (·-·-·-)

IV. 92° (··-··-)

On standing overnight at room temperature at end of the experiment (-x-x-x-)

FIG. 9

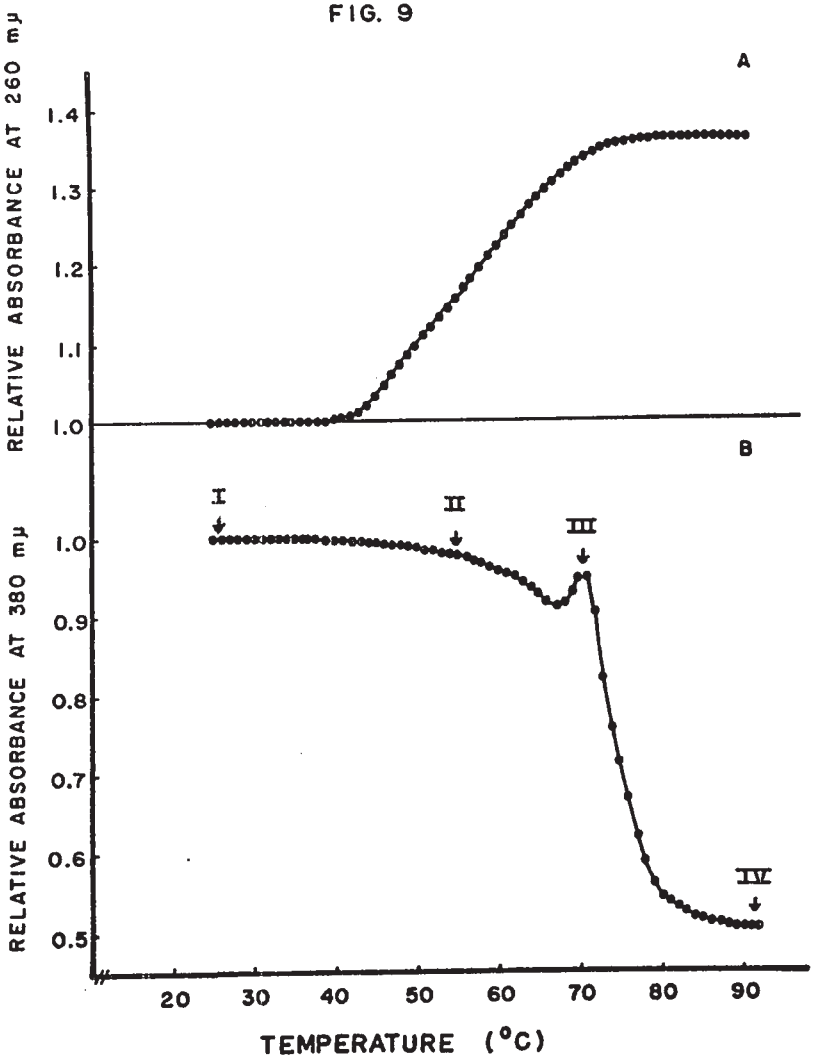
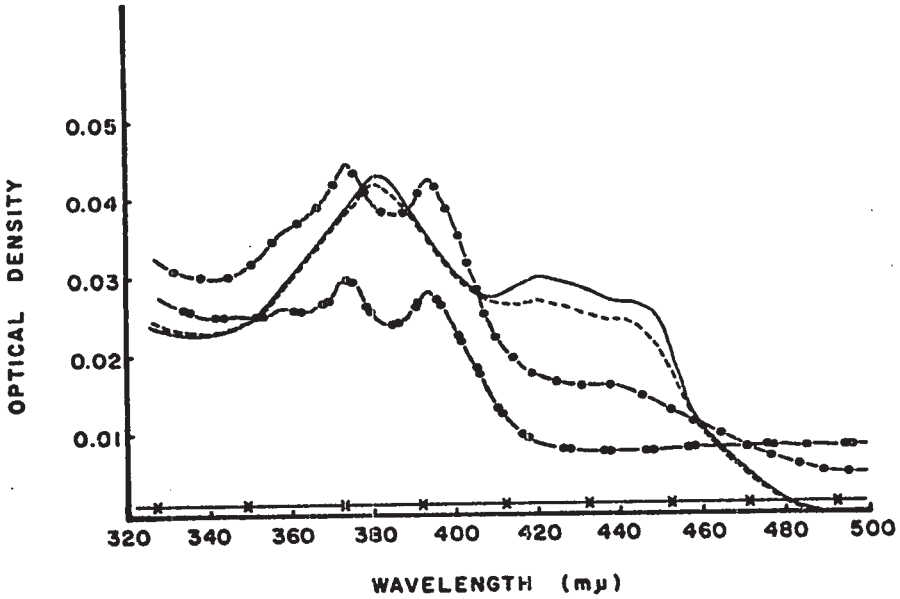

A
B
RELATIVE ABSORBANCE AT 260 mμ
RELATIVE ABSORBANCE AT 380 mμ
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
I
II
III
IV
20
30
40
50
60
70
80
90
TEMPERATURE (°C)

FIG. 10


OPTICAL DENSITY
0.05
0.04
0.03
0.02
0.01
320
340
360
380
400
420
440
460
480
500
WAVELENGTH (mμ)

at 380 mμ, which was characteristically preceded by a brief increase in the relative absorbance (optical density peak), did not occur until the temperature reached 70°, at which time the denaturation of the DNA had progressed to 93% completion. In a separate experiment the denaturation process was arrested briefly at the temperatures indicated by the arrows (Fig. 9b) to allow the absorption spectrum to be recorded. The results, shown in Fig. 10, revealed that the spectrum of the 7,10-dimethylbenz(c)-acridine:DNA complex remained relatively unchanged up to 70°, at which temperature the spectrum changed markedly. The spectrum at 70° and at 92° had the characteristics of a dissociated complex. Following heating to 92° precipitates of free dimethylbenz(c)-acridine were visible. Upon standing at room temperature, the precipitates settled and the absorption spectrum of the supernatant indicated that no dimethylbenz(c)acridine remained associated with the DNA (Fig. 10). Therefore, the optical density peak on the O.D. 380 profile indicated the point at which the dimethylbenz(c)acridine left the DNA. The temperature at which the optical density peak occurred was taken as the dissociation temperature of the complex.

The dissociation temperature was found to be dependent on the molar ratio of the complex as shown in Fig. 11. The higher the molar ratio, the lower was the dissociation temperature.

Fig. 12 shows that when a complex having the maximum molar ratio was heated, the dissociation temperature was 63.2° and the Tm of the complex was 57.5°. Fig. 13 shows that if the

FIGURE 11.

Effect of molar ratio on the dissociation temperature of 7,10-dimethylbenz(c)acridine:DNA complexes.

Complexes of various molar ratios were heated as described for Fig. 9. The temperature at which optical density peak appeared on the melting profile of each complex recorded at 380 mμ is plotted against its molar ratio. The variation between repeated determinations was less than $\pm 0.2^{\circ}$.

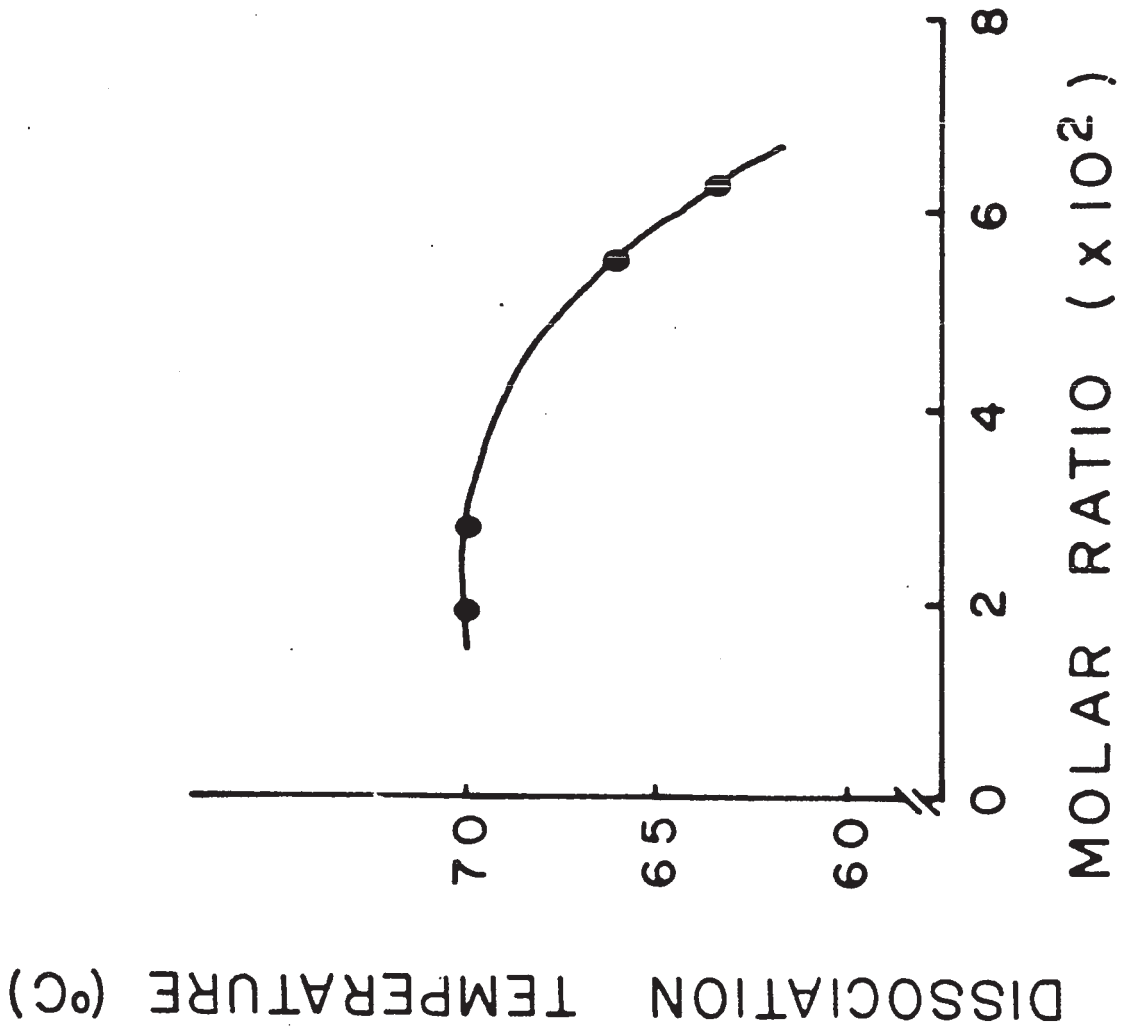

DISSOCIATION TEMPERATURE (°C)
70
65
60
0
2
4
6
8
MOLAR RATIO (x10²)

FIGURE 12.

Continuous thermal dissociation of 7,10-dimethylbenz(c)acridine:DNA complex.

A complex having a high molar ratio of 0.0625 was heated at a concentration of 50 µg. DNA per ml. as described in Fig. 9. Changes in OD_{260} and OD_{380} and in temperature are plotted against time.

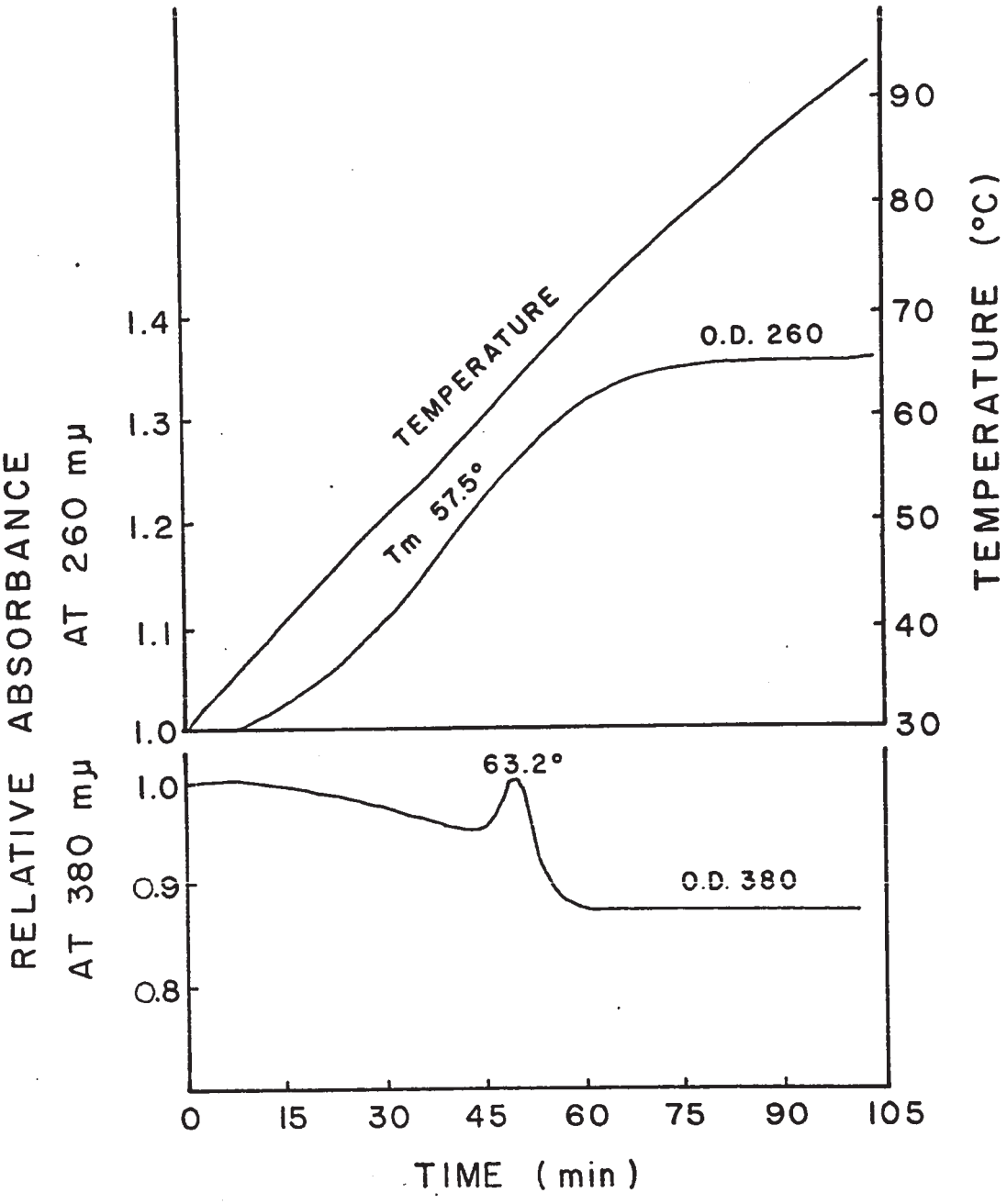
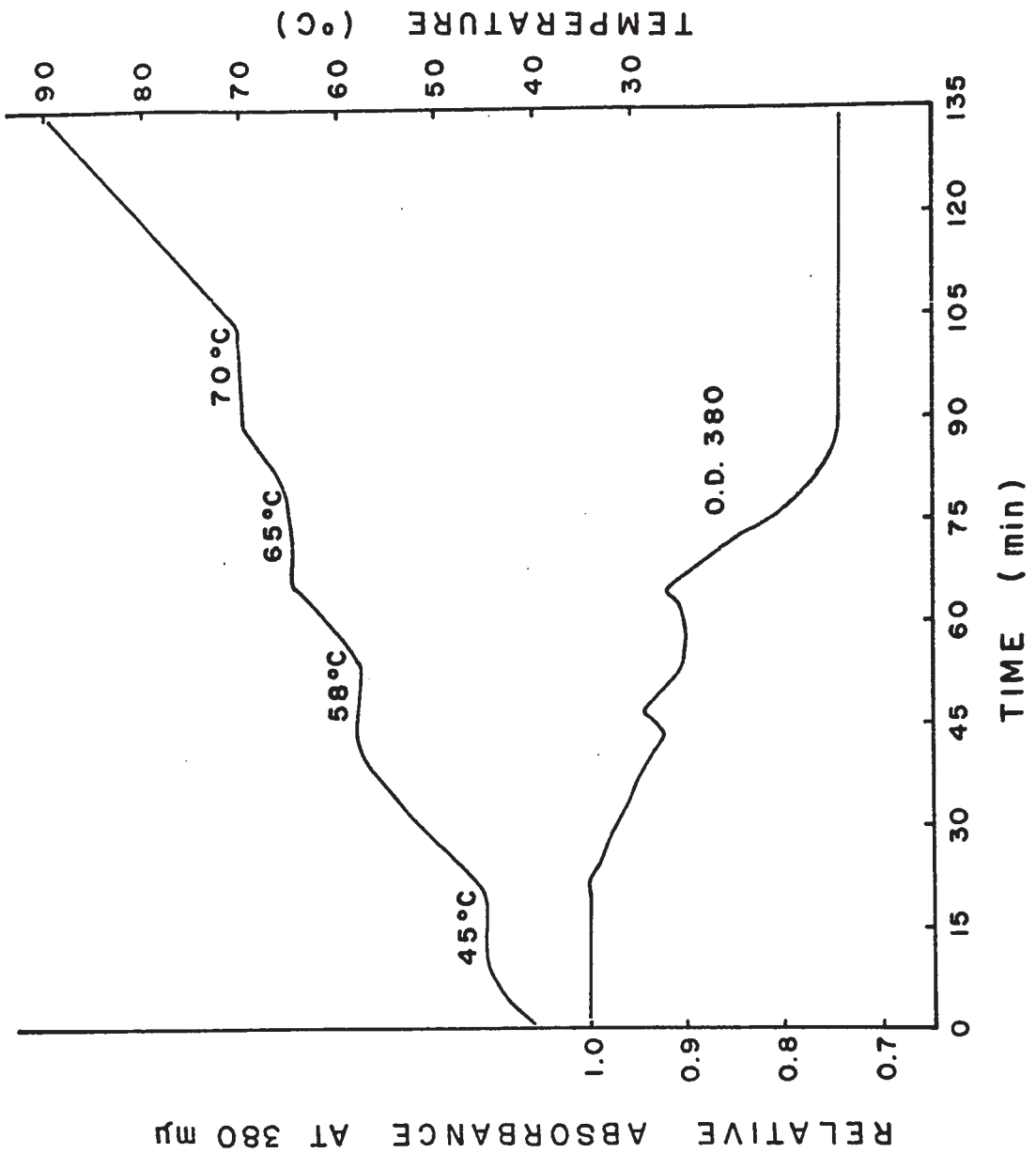

RELATIVE ABSORBANCE
AT 260 mμ
AT 380 mμ
TEMPERATURE (°C)
TEMPERATURE
O.D. 260
Tm 57.5°
63.2°
O.D. 380
1.4
1.3
1.2
1.1
1.0
0.9
0.8
90
80
70
60
50
40
30
0
15
30
45
60
75
90
105
TIME (min)

FIGURE 13.

Stepwise thermal dissociation of 7,10-dimethylbenz(c)acridine:DNA complexes.

This experiment repeated that described in Fig. 12 with the exception that the linear increase in temperature was halted for 10 minutes at 45°, 58°, 65° and 70°.


TEMPERATURE (°C)
90
80
70
60
50
40
30
70°C
65°C
58°C
45°C
O.D. 380
1.0
0.9
0.8
0.7
RELATIVE ABSORBANCE AT 380 mμ
0
15
30
45
60
75
90
105
120
135
TIME (min)

temperature increase was stopped and maintained at 58° for about 10 minutes, an optical density peak of approximately half the original magnitude (of Fig. 12) was observed. When the temperature was again raised, a second optical density peak of again approximately half the original magnitude was seen at 64°. These results indicated that the dissociation of the complex occurred in at least two steps when the temperature was increased in a step-wise fashion.

4. Formation of physical complexes with denatured DNA

Fig. 14 shows that 7,10-dimethylbenz(c)acridine could bind well to denatured DNA and that the absorption spectrum of the complex was similar to that of a complex made using native DNA (Fig. 15). At the saturation level the maximum binding ratio for 7,10-was 0.05 for denatured DNA compared to 0.06 for native DNA. The molar extinction coefficient ($E_{380}^{1M} = 6.13 \times 10^3$) of the denatured DNA:7,10-dimethylbenz(c)acridine complex was found to be the same as that for the native DNA:7,10-dimethylbenz(c)-acridine ($E_{380}^{1M} = 6.10 \times 10^3$).

The effects of increasing temperature on the relative absorbance at 260 mu and at 380 mu for a complex of denatured DNA:7,10-dimethylbenz(c)acridine are shown in Figs. 16 & 17. The shape of the curve relating the change in relative absorbance at 380 mu on heating (Fig. 17) was very similar to that obtained using native DNA to prepare the complex (Fig. 9b). Furthermore, the absorption spectrum of the native DNA:7,10-dimethylbenz(c)acridine

FIGURE 14.

Binding curve for 7,10-dimethylbenz(c)acridine to heat-denatured DNA.

Complexes of denatured DNA with varying amounts of the acridine were prepared under standard conditions. Molar ratios were determined as described in Materials and Methods.

FIGURE 15.

Comparison of the absorption spectrum at stage II (Fig. 9, lower curve) of the thermal dissociation process (-----) with those of 7,10-dimethylbenz(c)acridine complexed to native (_____) and denatured (-·-·-·-) DNA.

FIG. 14

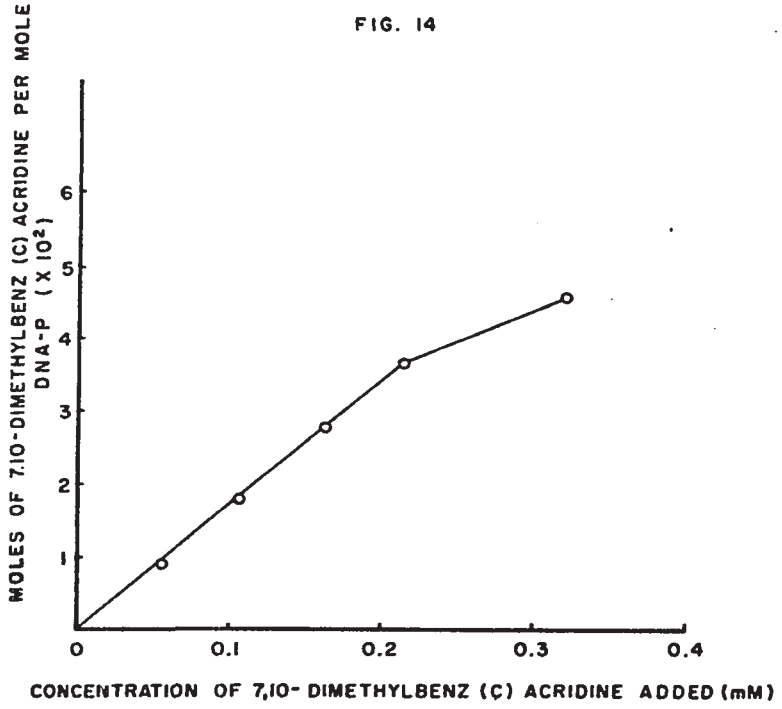
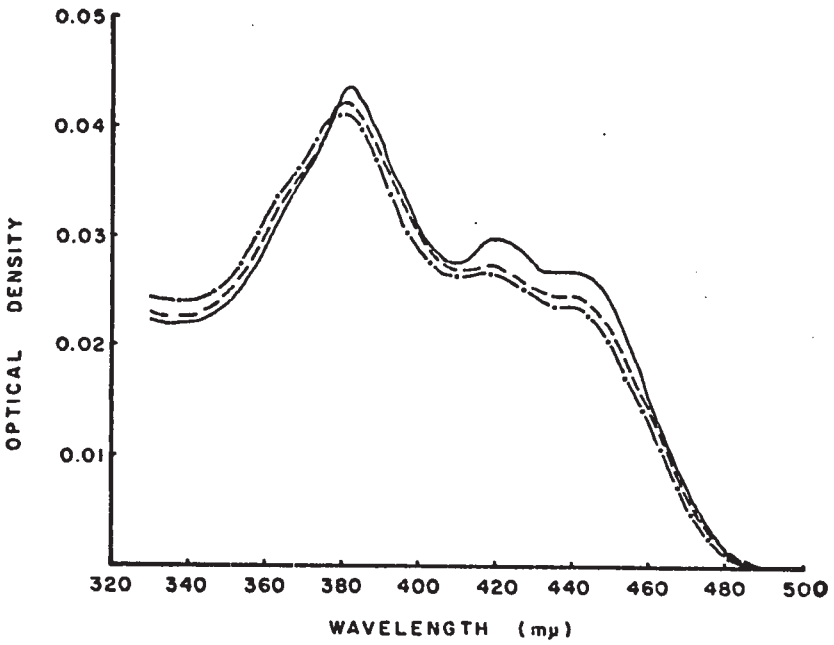

MOLES OF 7.10-DIMETHYLBENZ (C) ACRIDINE PER MOLE DNA-P (X 10²)
1
2
3
4
5
6
0
0.1
0.2
0.3
0.4
CONCENTRATION OF 7,10- DIMETHYLBENZ (C) ACRIDINE ADDED (mM)

FIG. 15


OPTICAL DENSITY
0.05
0.04
0.03
0.02
0.01
320
340
360
380
400
420
440
460
480
500
WAVELENGTH (mμ)

FIGURES 16 & 17.

Melting profiles of 7,10-dimethylbenz(c)acridine:DNA (denatured) complex.

The effect of temperature on optical densities at 260 mμ (Fig. 16) and 380 mμ (Fig.17) of a complex made with 7,10-dimethylbenz(c)acridine and denatured DNA is shown. The complex (molar ratio = 0.018) prepared as described in Materials and Methods, was diluted to 50 ug. DNA per ml. The experiment was carried out as described in Fig. 9.

FIG. 16.

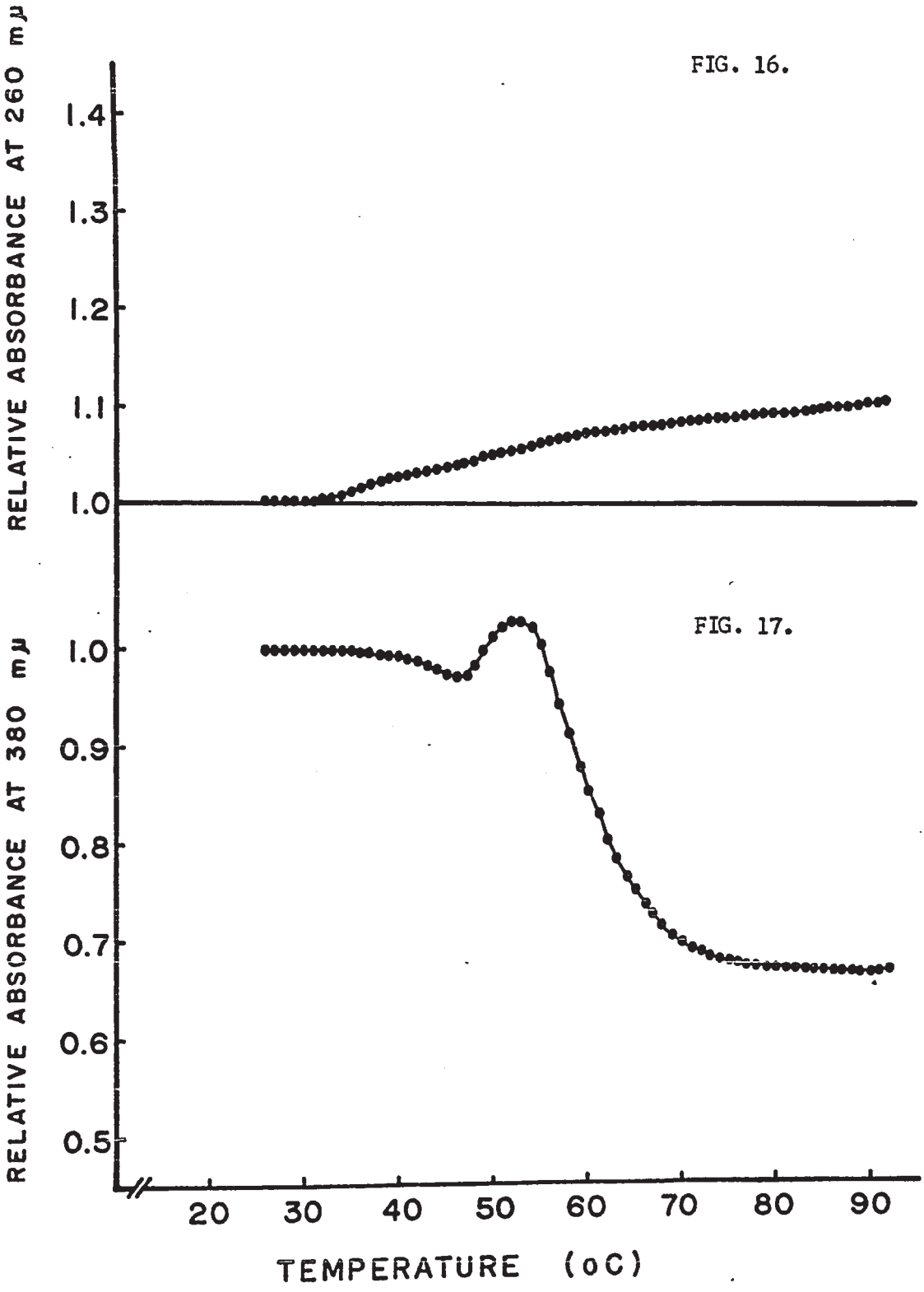

RELATIVE ABSORBANCE AT 260 mμ
1.4
1.3
1.2
1.1
1.0
RELATIVE ABSORBANCE AT 380 mμ
1.0
0.9
0.8
0.7
0.6
0.5
20
30
40
50
60
70
80
90
TEMPERATURE (oC)

FIG. 17.

complex as determined at 55° (Fig. 10) had spectral characteristics very similar to those of the 7,10-dimethylbenz(c)acridine complexes made using either native or denatured DNA (Fig. 15).

B. γ ray-induced covalent linkage of polycyclic aromatic hydrocarbons to DNA

1. Induction of covalent linkages

When physical complexes of DNA made with benz(a)pyrene or pyrene (prepared as described in Materials and Methods) were γ-irradiated, a fraction of the benz(a)pyrene or pyrene became covalently-linked to the DNA. This fraction could not be extracted by either cyclohexane or alcohol. Virtually all of the hydrocarbon (~99.9%) in the un-irradiated complexes could be removed by either extractant.

No such covalent linkage was observed when physical complexes of DNA made with either 7,9- or 7,10-dimethylbenz(c)-acridine were γ-irradiated.

Fig. 18 shows the dose dependence of the γ-ray-induced covalent-binding of benz(a)pyrene (a) and pyrene (b) to DNA. Binding rose sharply at radiation doses higher than 8 Kr. The binding ratio at 17 Kr was 1 = 100 DNA nucleotides for benz(a)-pyrene and 1 = 270 for pyrene.

Fig. 19 shows that covalent linkage induced a further bathochromic shift of the absorption peaks of the hydrocarbons in addition to the shifts which had taken place when the hydro-

FIGURE 18.

Effects of γ-irradiation on the covalent binding of hydrocarbons to DNA.

Physical complexes of DNA with benz(a)pyrene and pyrene (initial molar ratios were 0.0208 and 0.037, respectively) were exposed to various doses of γ-rays. Covalent complexes were isolated and their absorption spectra recorded as described in Materials and Methods. The peak absorptions of the complexes are plotted against dose.

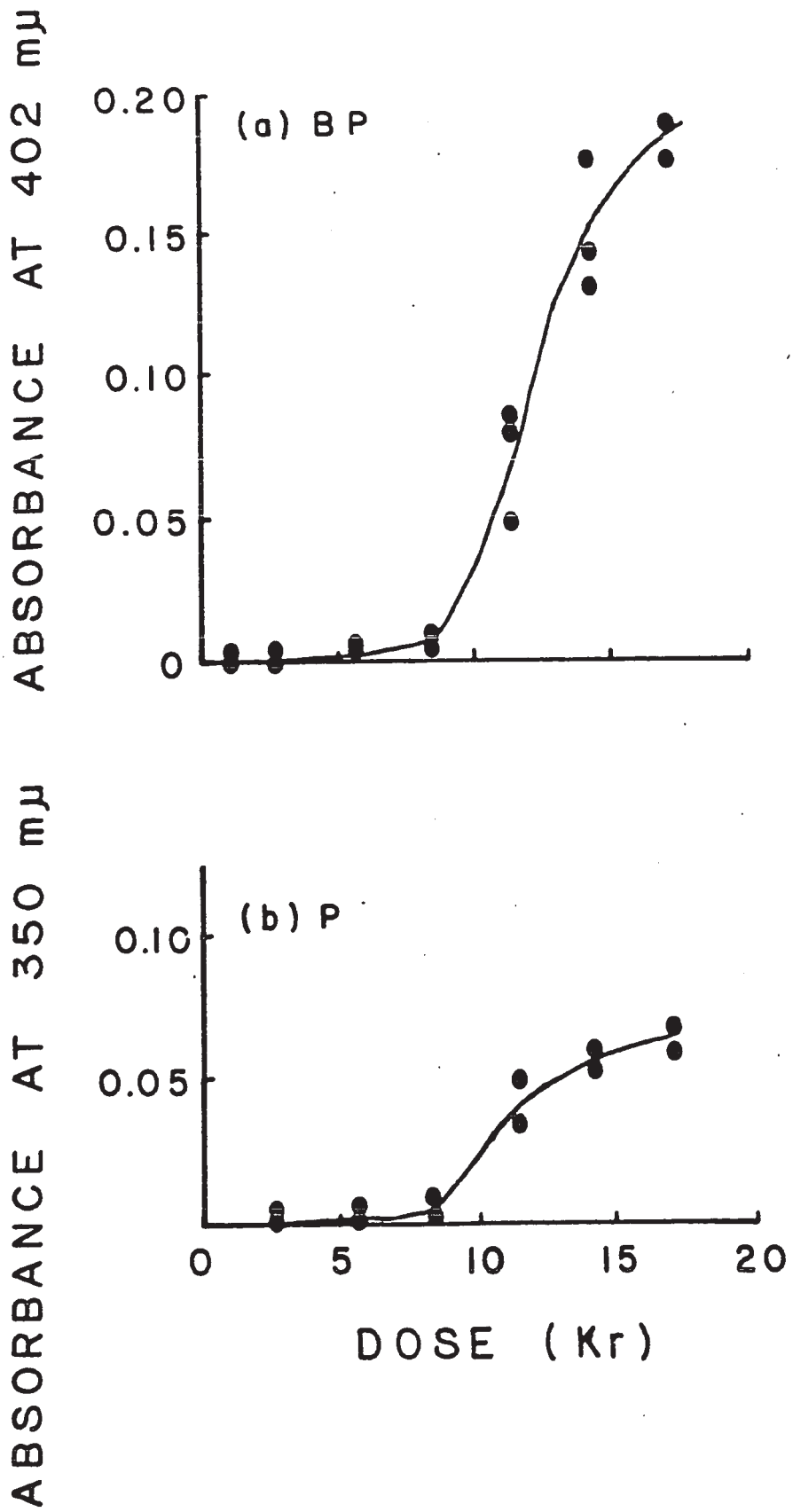
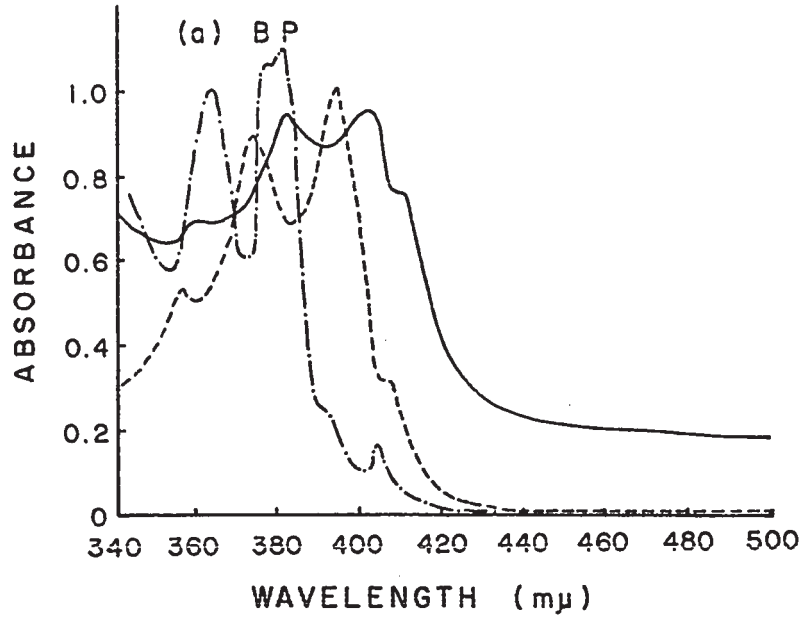
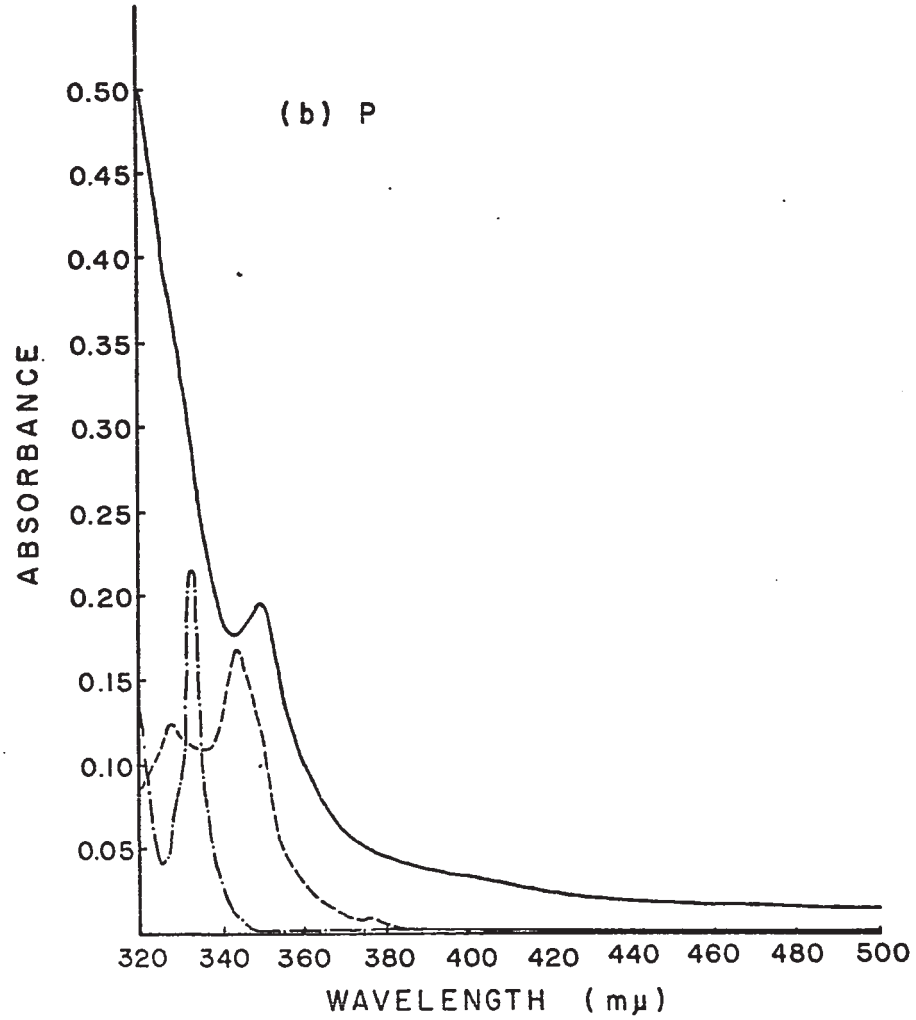

ABSORBANCE AT 402 mμ
0.20
0.15
0.10
0.05
0
(a) B P
ABSORBANCE AT 350 mμ
0.10
0.05
(b) P
0
5
10
15
20
DOSE (Kr)

FIGURE 19.

Absorption spectra of pyrene and benz(a)pyrene in methanolic solution and in DNA complexes.

The absorption spectra of benz(a)pyrene (A) and pyrene (B) in covalent complex with DNA (———) were compared at equal concentrations with those in physical complex with DNA (-----) and those in methanol (-·-·-).


(a) B P
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0
340 360 380 400 420 440 460 480 500
WAVELENGTH (mμ)


(b) P
ABSORBANCE
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
320 340 360 380 400 420 440 460 480 500
WAVELENGTH (mμ)

carbons became physically complexed to DNA.

2. Physical properties of covalent complexes and of γ-irradiated DNA

The effects of γ-irradiation on the thermal melting profile of the DNA are shown in Fig. 20. There was a dose-dependent increase in shallowness of the melting profile (a), a drop in hypochromicity (b) and Tm (c). The covalent DNA:benz(a)-pyrene and DNA:pyrene complexes were found to have the same thermal denaturation characteristics as the control-irradiated DNA. Fig. 20, b and c, show that these complexes had approximately the same decreases in hyperchromicity and Tm as the control-irradiated DNA.

Fig. 21 shows the effects of γ-irradiation and covalently-bound benz(a)pyrene on the sedimentation pattern of DNA in a sucrose density gradient. It was evident that irradiation caused a significant (about 40%) proportion of the DNA to sediment at a much slower rate. The covalent benz(a)pyrene DNA complex had essentially the same sedimentation profile as the γ-irradiated DNA.

C. Effects of polycyclic aromatic hydrocarbons on template activity of DNA

1. System for assay of template activity

Highly purified DNA-dependent RNA polymerase was isolated from E. coli B according to the procedure of Chamberlin

FIGURE 20.

The effects of γ -irradiation on thermal denaturation characteristics of DNA and covalent complexes of pyrene:DNA and benz(a)-pyrene:DNA.

Control DNA and physical complexes of P : DNA and BP : DNA (initial molar ratios of 0.0208 and 0.037, respectively) were exposed to various doses of γ -rays. The control DNA and covalent complexes were isolated, diluted to about 15 µg./ml. with respect to DNA and heat-denatured in the Gilford Multiple Sample Recording Spectrophotometer as described in Materials and Methods. A). Effect of γ -irradiation on melting profile of DNA only (——— 0Kr; ····· 6Kr; ------ 12Kr; -·-·- 18Kr). B). Comparing final hyperchromicity (● DNA; x BP-DNA; ▲ P-DNA). C). Comparing Tm (● DNA; x BP-DNA; ▲ P-DNA).

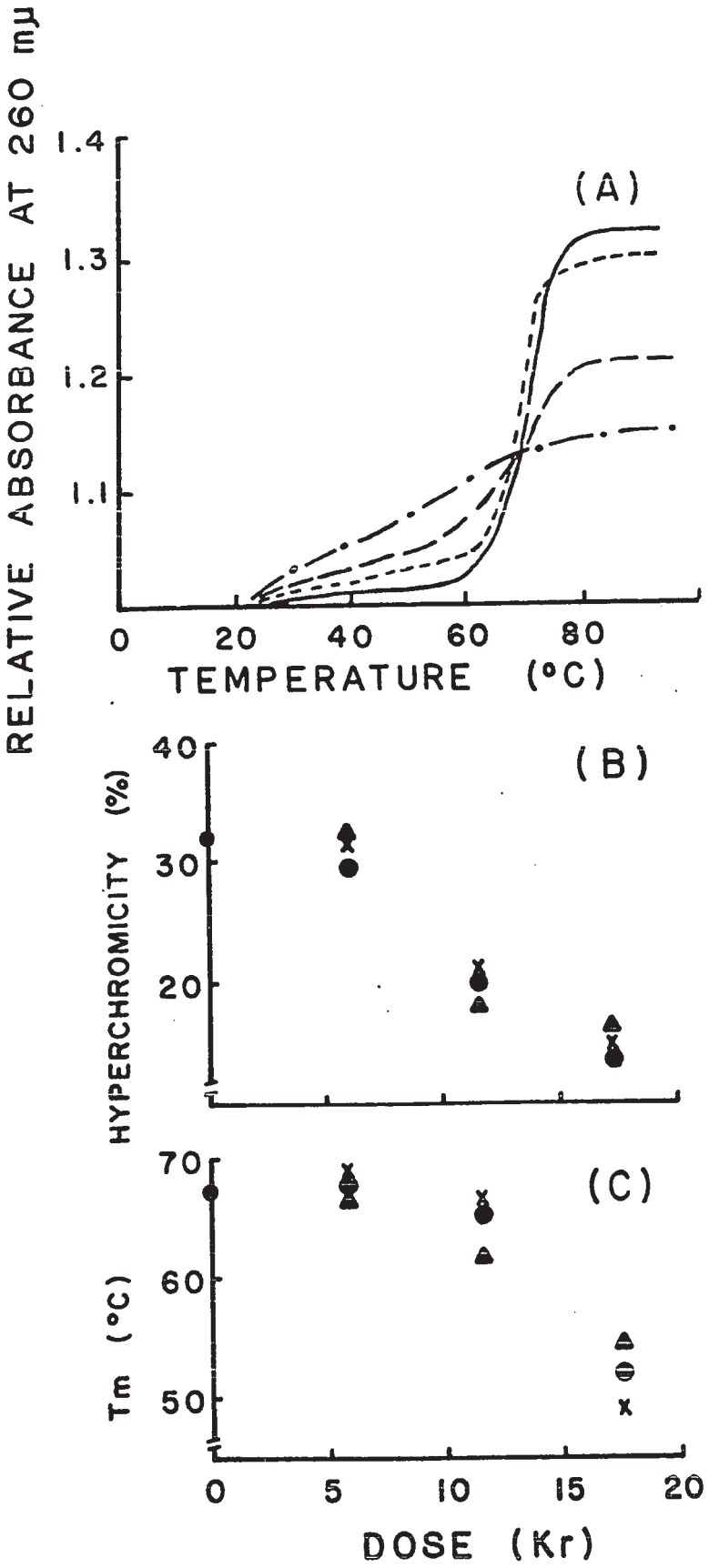
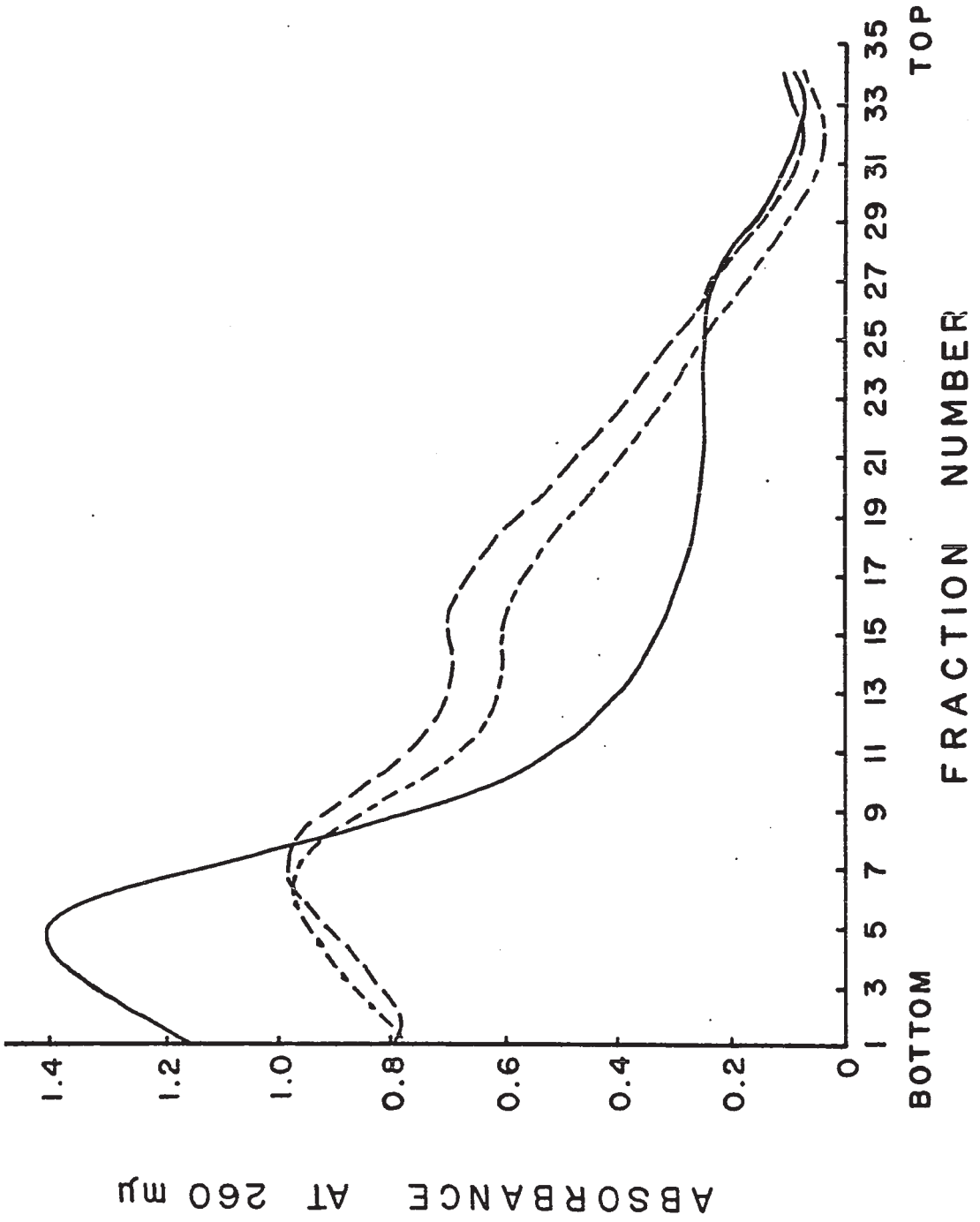

RELATIVE ABSORBANCE AT 260 mμ
1.4
1.3
1.2
1.1
(A)
0
20
40
60
80
TEMPERATURE (°C)
HYPERCHROMICITY (%)
40
30
20
(B)
Tm (°C)
70
60
50
(C)
0
5
10
15
20
DOSE (Kr)

FIGURE 21.

The effect of γ -irradiation on the sedimentation profile of DNA and benz(a)pyrene:DNA complex.

A volume of 0.3 ml. DNA solution, at a concentration of 1 mg./ml., was layered onto a 12.4 ml. premixed sucrose density gradient (5-40%) and spun for 37 hrs. at 40,000 rpm in a Spinco SW 40 rotor. ——— unirradiated DNA; ------ irradiated DNA (12 Kr);—·—·—·—BP:DNA irradiated complex (12 Kr).


ABSORBANCE AT 260 mμ
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
BOTTOM
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
TOP
FRACTION NUMBER

and Berg (1962). The results of a representative preparation of the enzyme are shown in Table 3. Template activity of calf thymus DNA was assayed using the standard conditions described by these authors. As shown in Table 4, the RNA synthesizing system was confirmed to be dependent upon added DNA, the divalent cations Mg^{++} and Mn^{++}, and all 4 nucleoside triphosphates. The system was completely inhibited by ribonuclease. The broad pH optimum of the system around pH8 was confirmed (Fig. 22). The reaction was linear for about 15 min. (Fig. 23). Under conditions of excess of enzyme, 40 ug DNA per tube or less was limiting (Fig. 24). The rate of synthesis was directly dependent on the amount of enzyme added (Fig. 25).

2. Assay of physical complexes

The physical complexes of DNA made with the 3 dimethylbenz(c)acridines and with pyrene and benz(a)pyrene were assayed for their template activities. Table 5 shows there was no significant difference in the activities between those of the control DNA and of the complexes even though in the case of the acridine derivatives a wide range of molar ratios was tested.

The absence of any effects of the dimethylbenz(c) acridines on the template activity of DNA was found to be due to the dissociation of these complexes under the enzyme assay conditions used. There was an immediate disappearance of the bright yellow colors of the complexes and the appearance of precipitates when the complexes were added to the rest of the incubation mixture.

TABLE 3. Purification of RNA Polymerase from E. coli

		Specific Activity (U*/Mg./hr.)	Enrichment of Specific Activity	Total Activity (U)
I.	Crude extract	27	1	85,500 (100)+
II.	Protamine eluate	527	20	41,000 (48)
III.	Ammonium sulfate	1724	64	40,900 (48)
IV.	Peak DEAE fraction	3993	148	25,300 (30)

The purification procedure and standard assay of Chamberlin and Berg (1962) were followed. (See Materials and Methods.)

* U = units of enzyme activity as defined in Materials and Methods.

+ Numbers in brackets represent percentages of recovery of activity at the various purification steps.

TABLE 4. Requirements for *in vitro* RNA Synthesis

Components	Incorporation of P^{32} CMP (mμmoles)
Complete system	2.65 ± 0.05
Minus DNA	0.03 ± 0.01
Minus enzyme	0.03 ± 0
Minus Mg^{++} and Mn^{++}	0.45 ± 0.05
Minus ATP	0.10 ± 0
Minus GTP	0.15 ± 0
Minus UTP	0.13 ± 0
Minus ATP, GTP & UTP	0.07 ± 0.02
Add pancreatic RNase (10 ug.)	0.17 ± 0.02

Standard assay conditions of Chamberlin and Berg (1962) were used. Each tube contained 5 ug. of Fraction IV protein and 80 ug. DNA. Results were averages of 2 determinations.

FIGURE 22.

The effect of pH on RNA polymerase activity.

Fraction IV enzyme (3 µg. per tube) was assayed in duplicate in Tris-HCl buffers of various pH's under otherwise standard conditions.

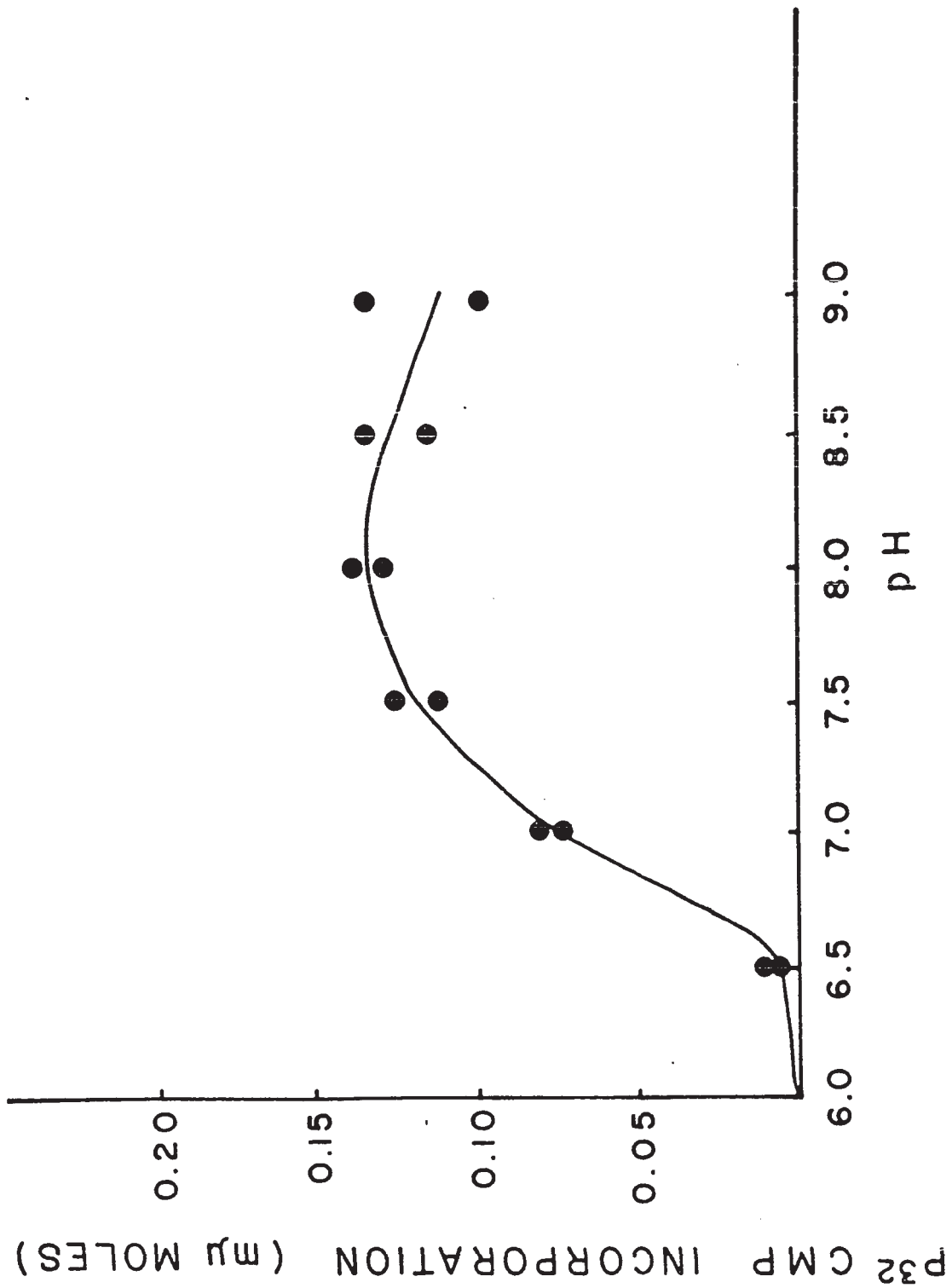

P32 CMP INCORPORATION (mμ MOLES)
0.20
0.15
0.10
0.05
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

FIGURE 23.

Time course of RNA polymerase activity.

Fraction IV enzyme (6 µg. per tube) was assayed in duplicate under standard conditions except for variation of incubation time.

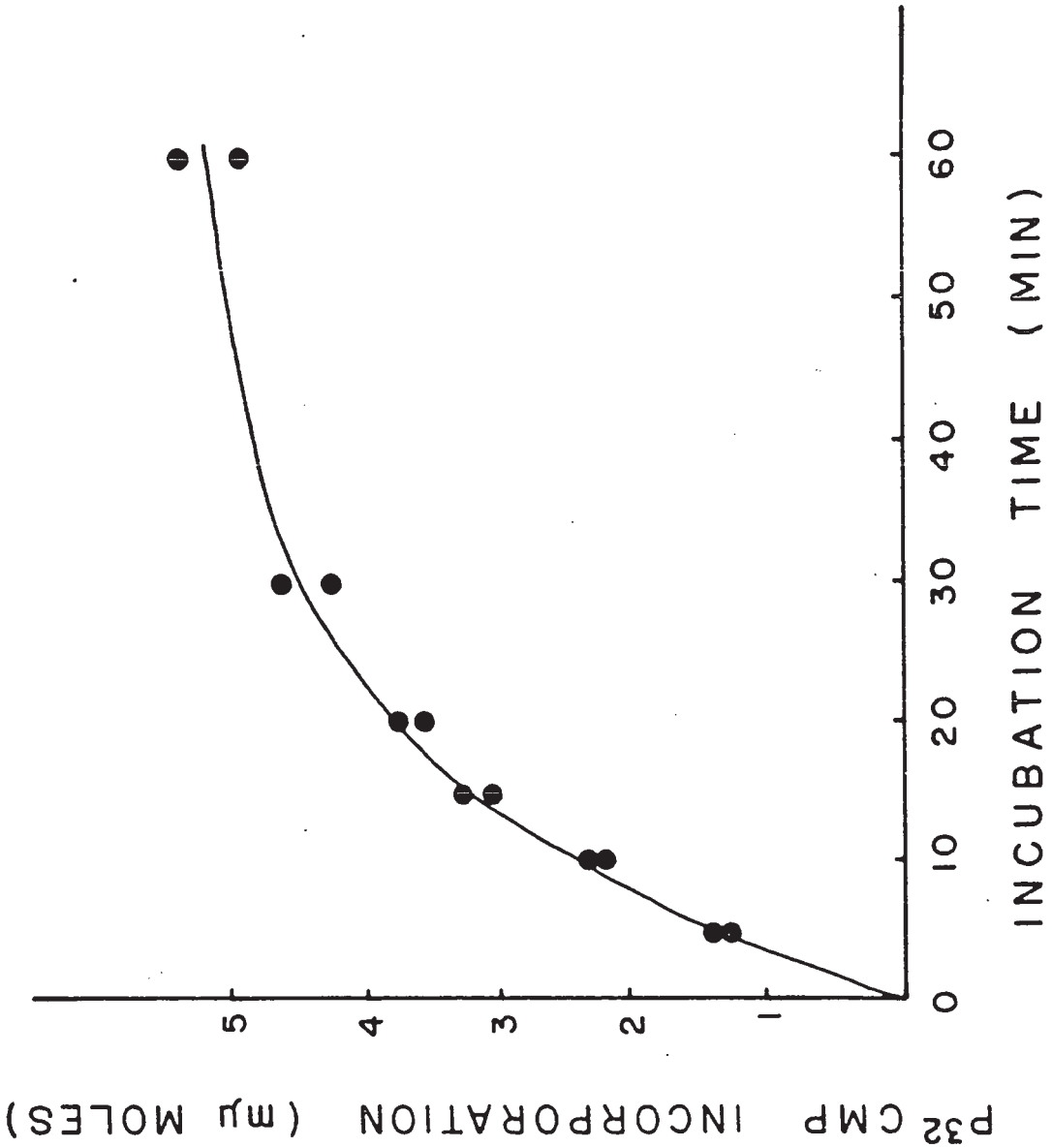

P32 CMP INCORPORATION (mμ MOLES)
0
1
2
3
4
5
10
20
30
40
50
60
INCUBATION TIME (MIN)

FIGURE 24.

Dependence of RNA polymerase activity on DNA template concentration.

The amount of DNA in a standard incubation mixture was varied. Duplicate assays using 6 µg. Fraction IV enzyme protein were carried out according to the standard procedure.

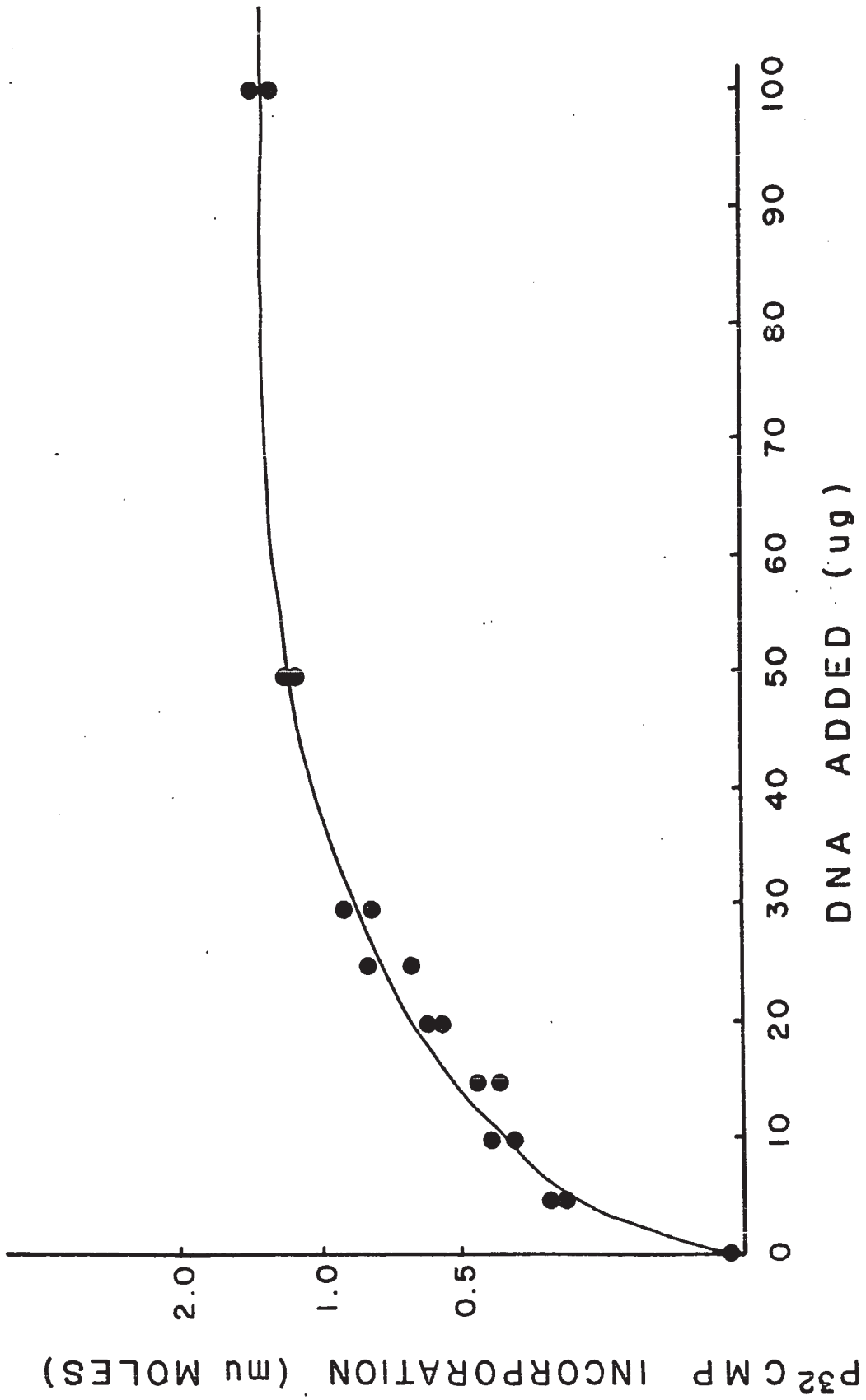

P32 CMP INCORPORATION (mu MOLES)
2.0
1.0
0.5
0
10
20
30
40
50
60
70
80
90
100
DNA ADDED (ug)

FIGURE 25.

Dependence of RNA synthesis on amount of Fraction IV enzyme added.

Various amounts of the enzyme were assayed in duplicate under standard conditions.

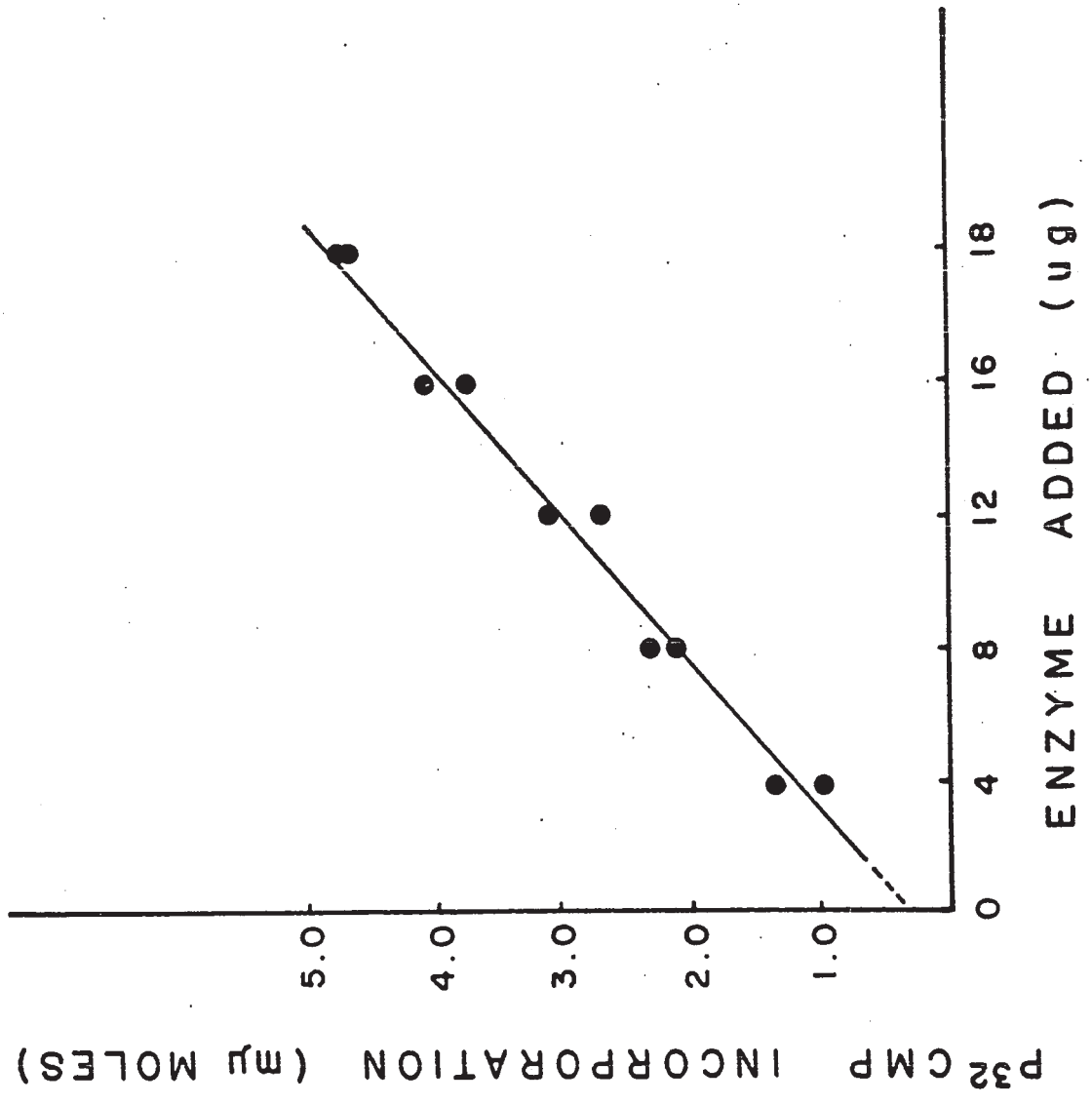

P^{32} CMP INCORPORATION (mμ MOLES)
5.0
4.0
3.0
2.0
1.0
0
4
8
12
16
18
ENZYME ADDED (μg)

TABLE 5. Template Activities of Physical Complexes of DNA Made With Various Polycyclic Carcinogens

DNA:Carcinogen Complexes		Molar Ratio ($x10^2$)	Incorporation of P^{32} CMP (mμmoles)
Dimethyl-benz(c)-acridine	7,9-	0	0.63 ± 0.01
		1.4	0.65 ± 0.02
		4.4	0.70 ± 0.08
		5.3	0.60 ± 0.03
	7,10-	0	0.72 ± 0.02
		1.7	0.72 ± 0.07
		6.3	0.67 ± 0.04
	7,11-	0	0.71 ± 0.03
		1.1	0.67 ± 0.01
		2.0	0.67 ± 0
		2.9	0.66 ± 0.03
Polycyclic hydrocarbon	P	0	0.84 ± 0.02
		0.4	0.82 ± 0.05
	BP	0	1.01 ± 0.06
		1.0	0.96 ± 0.01

Standard assay conditions of Chamberlin and Berg (1962) were used. Each tube contained 3 μg. of Fraction IV protein and 80 μg. of either control DNA or DNA:carcinogen complex. Results were averages of duplicate assays.

Analysis of the absorption spectra of the total reaction mixture (Fig. 26) and the sedimentation profiles in sucrose density gradients (Fig. 27) indicated that the complexes had dissociated. By monitoring the absorption spectra after addition of each of the components of the reaction mixture to the complexes individually, it was found that the addition of tris, divalent cations and of the enzyme solution (which contained tris and KCl) were responsible for the changes in absorption spectra and the dissociations of the complexes. By means of titration (for example, see Fig. 28 for effect of NaCl concentration upon the absorption spectrum of 7,9-dimethylbenz(c)acridine:DNA complex), the maximum concentrations of these components which the complexes could tolerate without undergoing dissociation, were determined. The results are summarized in Table 6. The optimal concentrations of the reaction components are compared in Table 7 with a set of concentrations based on the results shown in Table 6. Although this set of conditions allowed the complex to remain intact they were inadequate to permit the enzyme to function. Figure 29 shows that when the concentration of the tris buffer (pH7.9) was reduced to 5 mM, the RNA polymerase was virtually completely inhibited. A tris concentration of 9 mM gave 85% normal activity. When the system contained only 9 mM tris (pH7.9) and Mn^{++} was omitted, the enzyme activity was greatly reduced as the Mg^{++} concentration was reduced (Fig. 30). At 3 mM Mg^{++} (thirty times the maximum tolerable concentration), the activity was reduced to about 50%. When the optimal pH of 7.9 of this suboptimal system (9 mM tris, 3 mM Mg^{++}) was changed to 7.0, a further inhibition by 50% was observed (Fig. 31).

FIGURE 26.

The effect of RNA-synthesizing reaction mixture upon the absorption spectra of the dimethylbenz(c)acridine:DNA complexes.

The absorption spectra of the complexes in standard binding buffer (———) were compared at equal concentrations with those of the same complexes in the RNA synthesizing reaction mixture (------)

(a) 7,9:DNA, M.R. = 0.043

(b) 7,10:DNA, M.R. = 0.059

(c) 7,11:DNA, M.R. = 0.020.

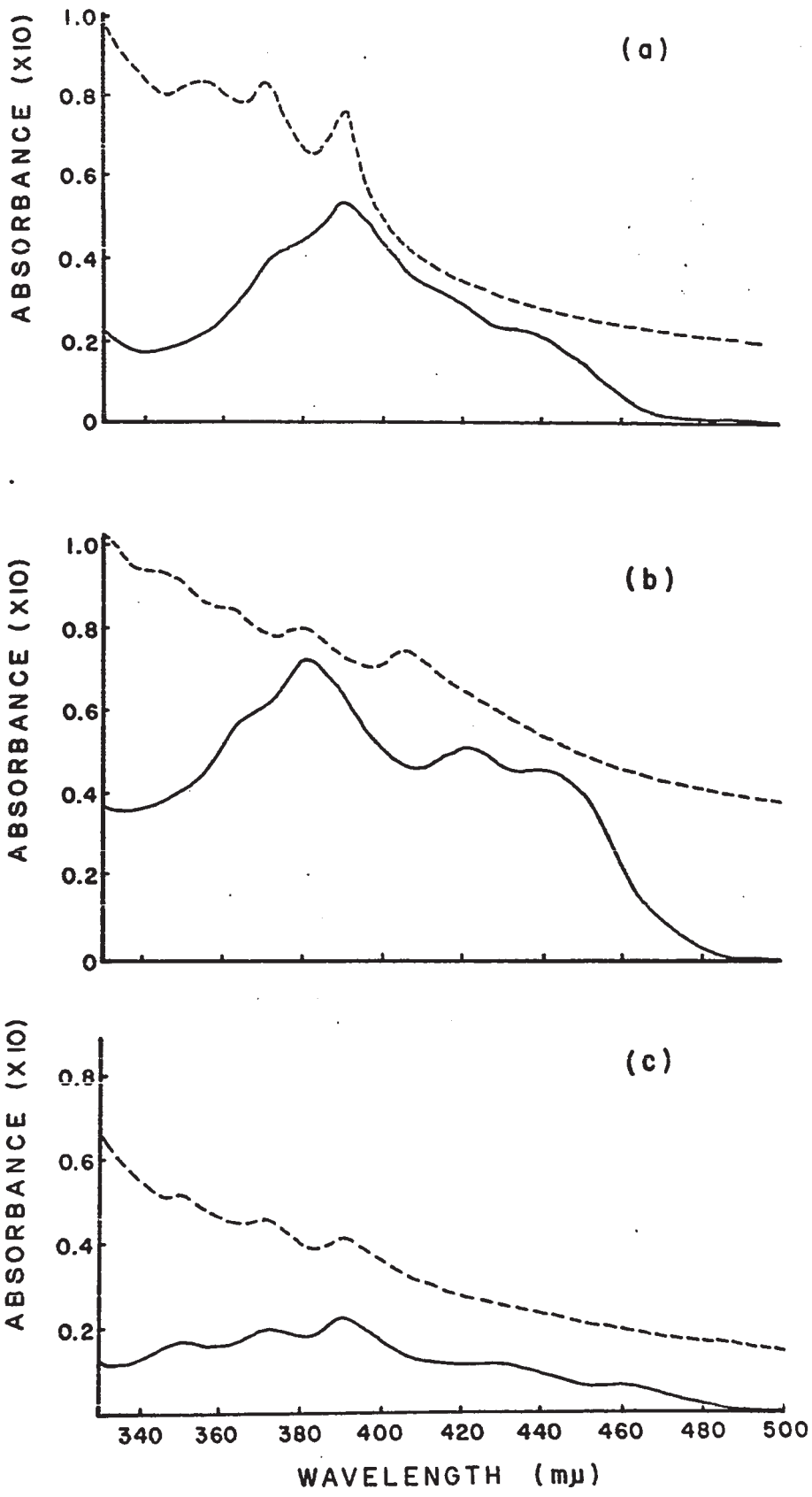

(a)
(b)
(c)
ABSORBANCE (X10)
1.0
0.8
0.6
0.4
0.2
0
340
360
380
400
420
440
460
480
500
WAVELENGTH (mμ)

FIGURE 27.

Effects of Mn^{++} and Mg^{++} on the sedimentation profiles of the dimethylbenz(c)acridine:DNA complexes.

A volume of 0.44 ml. of each of the complexes at a concentration of 1 mg. DNA per ml. was diluted to 0.50 ml. with water containing a final concentration of 1 mM $MnCl_2$ and 4mM $MgCl_2$. These mixtures were each layered onto 4.5 ml. of pre-mixed sucrose density gradient (5-20%), centrifuged in an SW50 rotor at 40,000 rpm at 4° for 13 hrs. Fractions were collected by puncturing the bottom of the centrifuge tube. The optical density at 260 mμ and at the peak of maximum absorption for the particular complex were determined for each fraction. (a) 7,9:DNA, M.R. = 0.043; (b) 7,10:DNA, M.R. = 0.059; (c) 7,11:DNA, M.R. = 0.020.

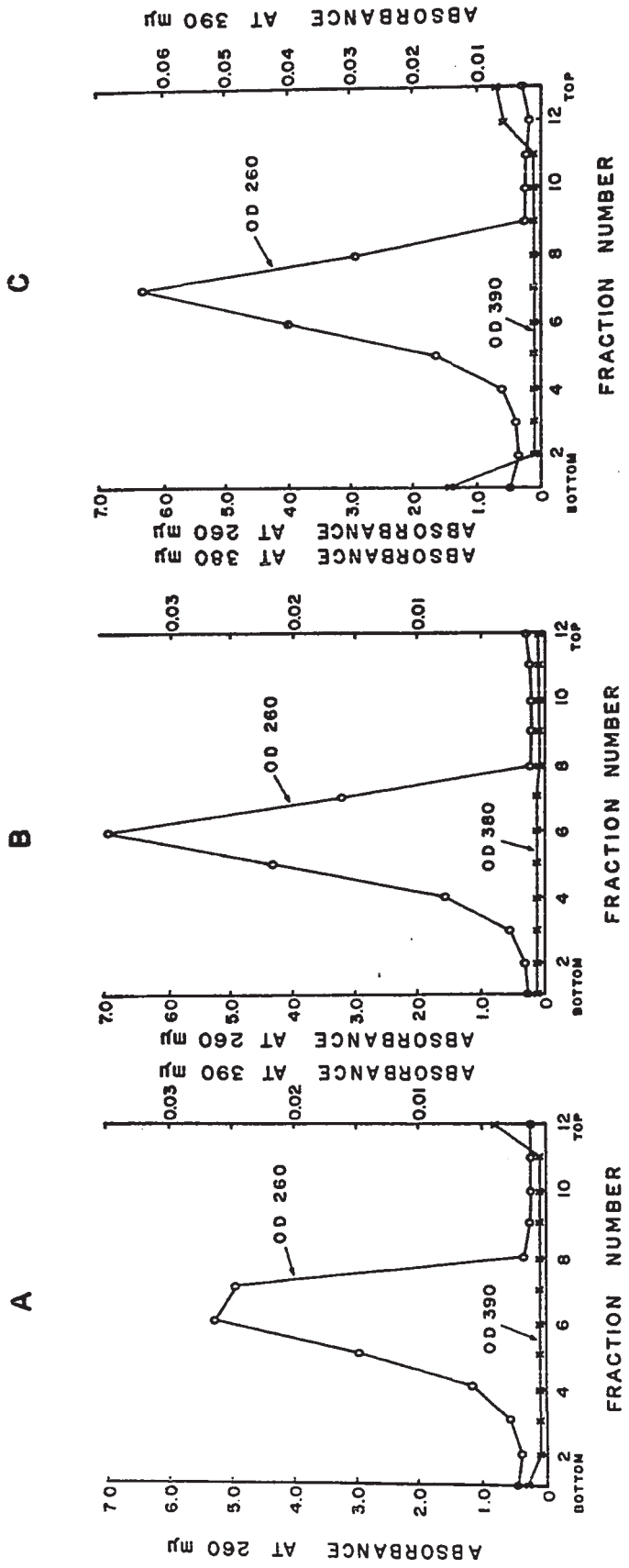

A
B
C
OD 260
OD 390
OD 380
ABSORBANCE AT 260 mμ
ABSORBANCE AT 390 mμ
ABSORBANCE AT 380 mμ
FRACTION NUMBER
BOTTOM
TOP

FIGURE 28.

Effect of NaCl concentration on the absorption spectrum of 7,9-dimethylbenz(c)acridine:DNA complex.

Aliquots of 0.3 ml. of 7,9:DNA at 1 mg./ml. (M.R. = 0.056) were each diluted to 0.5 ml. with water containing various amounts of NaCl. The absorption spectra were recorded in a Cary 15 Recording Spectrophotometer.

——— 0.5 mM NaCl

------ 5.0 mM NaCl

—·—·— 10.0 mM NaCl

-o-o-o 25.0 mM NaCl

-x-x-x 100.0 mM NaCl

-Δ-Δ-Δ 200.0 mM NaCl

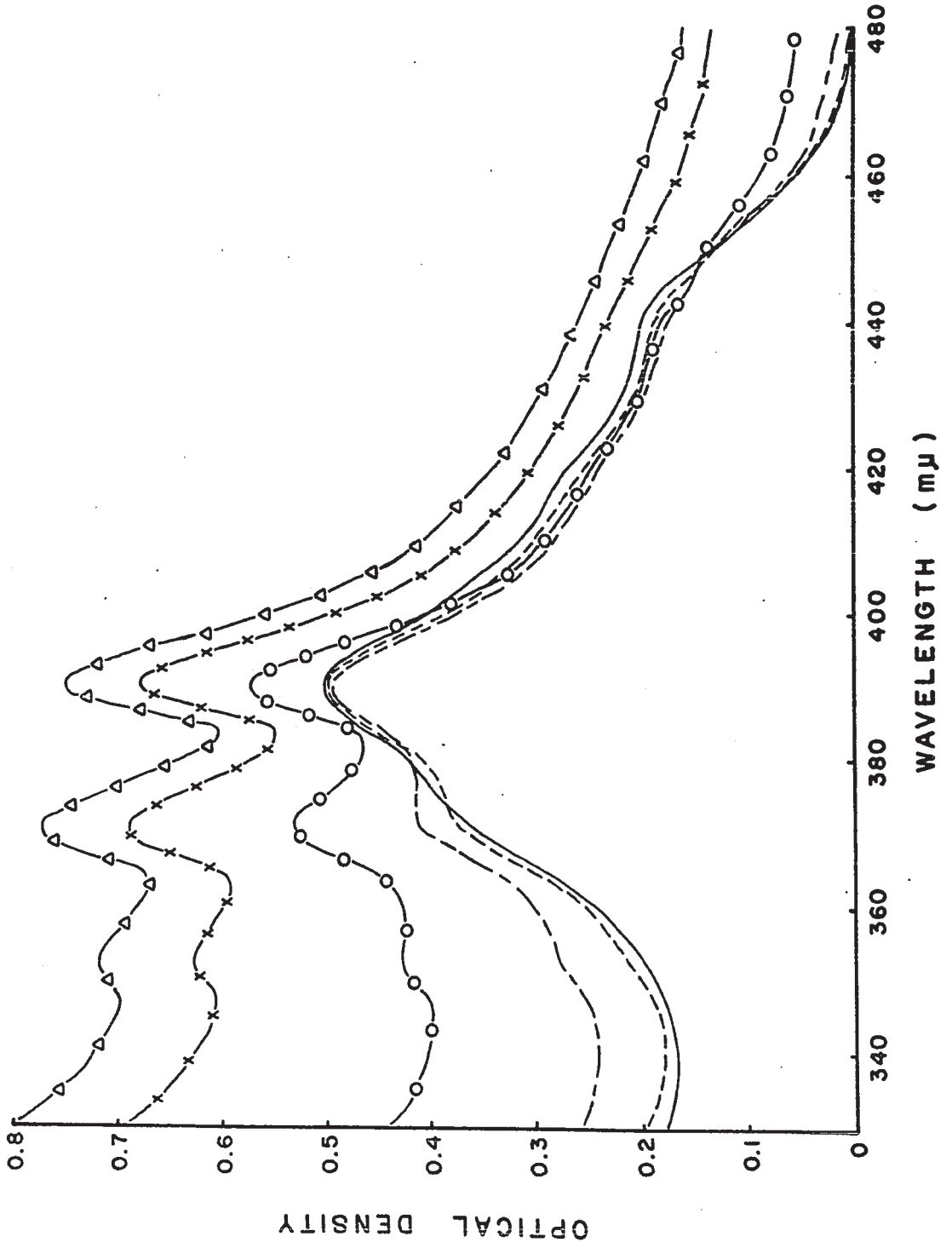

OPTICAL DENSITY
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
340
360
380
400
420
440
460
480
WAVELENGTH (mμ)

TABLE 6. Critical Concentrations of Various Cations and Buffers for the Physical Binding of Dimethylbenz(c)acridines to DNA

		Tolerable Concentration (mM)		Dissociating Concentration (mM)	
		7,9-	7,10-	7,9-	7,10-
Cations	Na^+	5.00	50.00	10.00	100.00
	K^+	10.00	100.00	50.00	200.00
	Mn^{++}	0.10	0.05	0.30	0.07
	Mg^{++}	0.06	0.10	0.08	0.20
Buffers at pH 7.0	Tris	1.00	4.00	2.00	6.00
	Cacodylate	1.00	-	5.00	-
	Phosphate	2.00	10.00	3.00	15.00
	Triethanolamine	1.00	-	5.00	-

Various concentrations of the cations and buffers at pH 7.0 were added to a complex at about 0.4 mg./ml. with respect to DNA in a final volume of 0.50 ml. The maximum concentration without causing a change in the absorption spectrum of the complex was taken to be the maximum tolerable concentration. The concentration which did cause a change was defined as the dissociating concentration.

FIGURE 29.

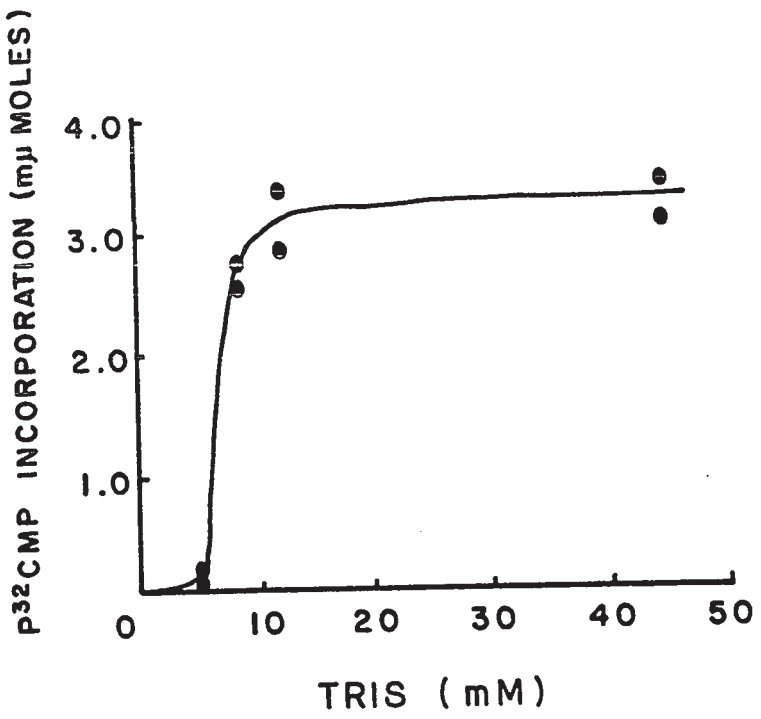
Dependence of RNA polymerase activity on concentration of Tris-HCl buffer (pH 7.9).

Fraction IV enzyme (10 µg. per tube) was assayed in duplicate at various concentrations of the Tris-HCl buffer (pH 7.9) under otherwise standard conditions.

FIGURE 30.

Dependence of RNA polymerase activity upon Mg^{++} concentration at a suboptimal concentration of the Tris-HCl buffer (pH 7.9) and in absence of Mn^{++}.

Aliquots of Fraction IV enzyme (10 µg. per tube) were assayed in duplicate at various Mg^{++} concentrations with two modifications of the standard assay: (1) concentration of the Tris-HCl buffer (pH 7.9) was reduced from 40 mM to 9 mM. (2) Mn^{++} was omitted from the reaction mixture.


P32CMP INCORPORATION (mμ MOLES)
4.0
3.0
2.0
1.0
0
10
20
30
40
50
TRIS (mM)

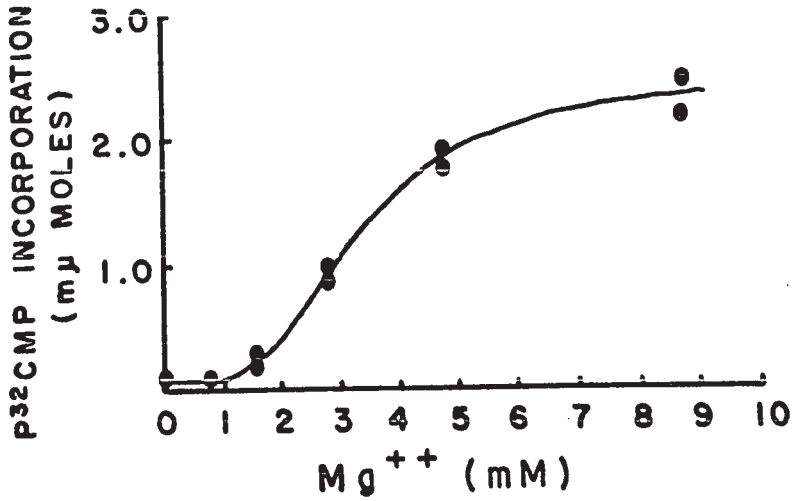

P32CMP INCORPORATION (mμ MOLES)
3.0
2.0
1.0
0
1
2
3
4
5
6
7
8
9
10
Mg++ (mM)

FIGURE 31.

Dependence of RNA polymerase activity upon Mg^{++} concentration and upon pH at a suboptimal concentration of Tris-HCl buffer (pH 7.9) and in absence of Mn^{++}.

Aliquots of Fraction IV enzyme (5 µg. per tube) were assayed in duplicate at various Mg^{++} concentrations and at various pH's of 9 mM Tris-HCl buffer. $MnCl_2$ was omitted from this modified incubation mixture. Assay procedure was otherwise standard.

● pH 8.0

x pH 7.5

▲ pH 7.0

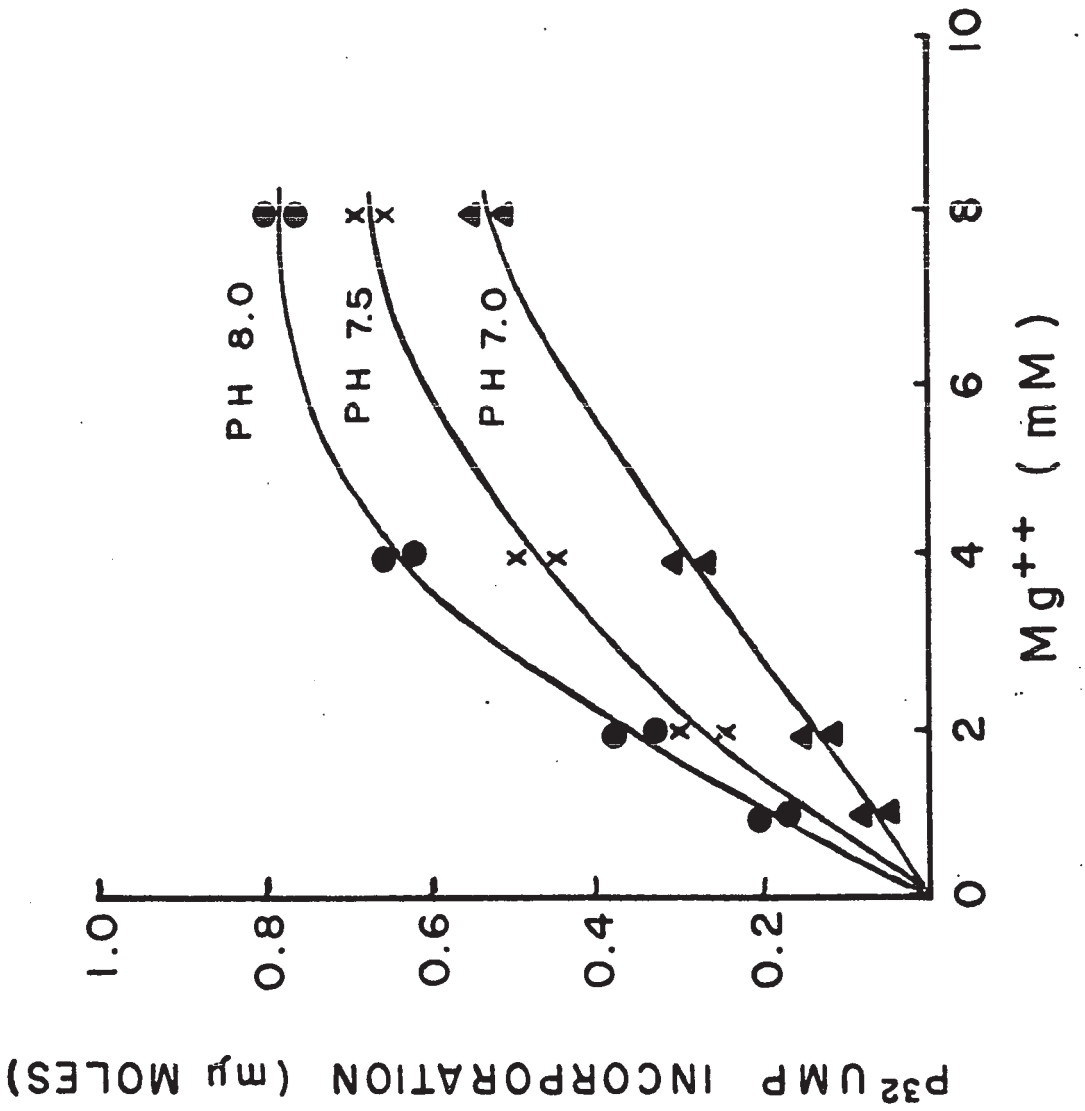

PH 8.0
PH 7.5
PH 7.0
0
2
4
6
8
10
Mg++ (mM)
0.2
0.4
0.6
0.8
1.0
P32 UMP INCORPORATION (mμ MOLES)

TABLE 7. Comparison of Conditions for Optimal RNA Polymerase Activity with Conditions Favouring Continued Binding of 7,10-dimethylbenz(c)acridine to DNA

	Optimal Enzyme Conditions (mM)	Complex Conditions (mM)
Tris	40.0 (pH 7.9)	1.0 (pH 7.0)
$MnCl_2$	1.0	0
$MgCl_2$	4.0	0.1
KCl (in enzyme)	50.0	50.0
ATP	0.4	0.4
CTP	0.4	0.4
GTP	0.4	0.4
UTP	0.4	0.4
Mercaptoethanol	12.0	12.0
DNA	0.2	0.2

"Optimal enzyme conditions" were the conditions of the standard assay. "Complex conditions" were a set of conditions based on the results in Table 6 and which did not dissociate the complex.

Despite the systematic search, it was not possible to find a set of suboptimal conditions which did not dissociate the complex and which, at the same time allowed the DNA-dependent RNA polymerase of E. coli B. to function.

Physical complexes of DNA with polycyclic aromatic hydrocarbons were also dissociated when added to the standard incubation mixture. These complexes had the same template activities as their control DNA under standard conditions of assay (Table 5).

3. Assay of covalent complexes

The effect of γ-irradiation on the template activity of DNA is shown in Figure 32. A covalent benz(a)pyrene:DNA complex containing 1 mole benz(a)pyrene per 370 moles of nucleotide is shown to have a lower template activity than the control γ-irradiated DNA (which had 46% activity of unirradiated DNA) (Fig. 33). The decreased RNA synthesis became evident at later incubation times (20 min. or longer). After 45 minutes of incubation, the template activity of the complex was 20% lower than that of the control-irradiated DNA. These results were confirmed with a different preparation of the complex (Fig. 34). The absorption spectrum of the complex in the reaction mixture remained unchanged during incubation at 37° for at least 2 hours. Subsequent deproteinization of the reaction mixture with phenol did not alter the absorption spectrum either. These results indicated that the benz(a)pyrene remained bound to the DNA template throughout RNA synthesis.

FIGURE 32.

Effect of γ -irradiation upon template activity of DNA.

Samples of DNA exposed to various doses of γ -rays were used as template (30 µg. per tube) in a standard RNA polymerase assay containing 20 µg. Fraction IV enzymes. Incorporation for control unirradiated DNA was 0.53 ± 0.02 mumoles of P^{32} GMP. Results of duplicate assays are given.

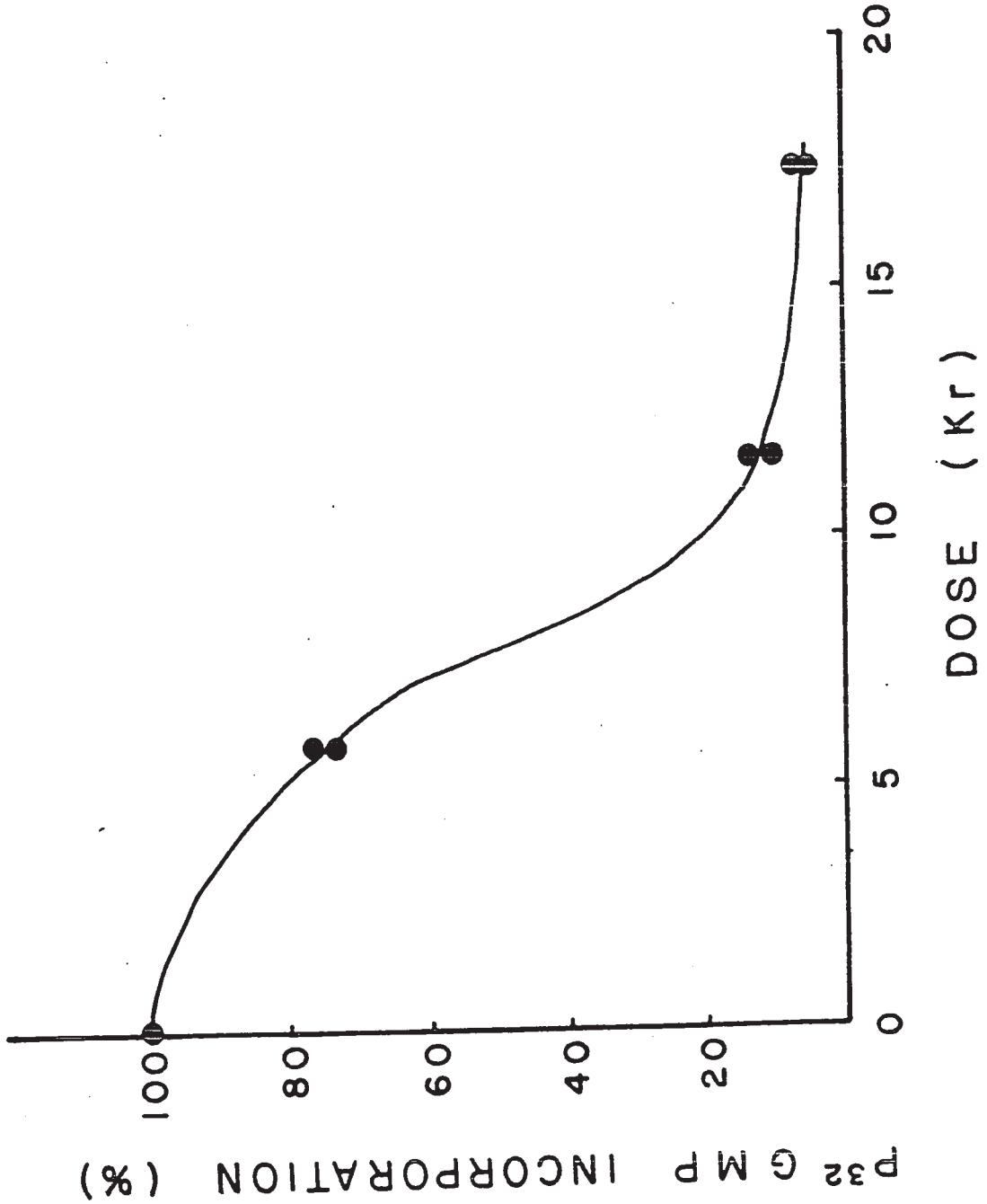

P^{32} G M P INCORPORATION (%)
100
80
60
40
20
0
5
10
15
20
DOSE (Kr)

FIGURE 33.

Time courses of template activity of control irradiated DNA and irradiated covalent benz(a)pyrene:DNA complex.

Control DNA and a covalent benz(a)pyrene:DNA complex were assayed in duplicate for template activity in a standard RNA synthesis assay using 20 µg. Fraction IV enzyme and 80 µg. DNA per tube. Both control DNA and the complex had been exposed to the same dose of γ -rays (10 Kr). The time courses of the reactions were followed.

● _____ ● Control irradiated DNA

x ----- x Irradiated BP = DNA (Molar ratio = 0.0027).

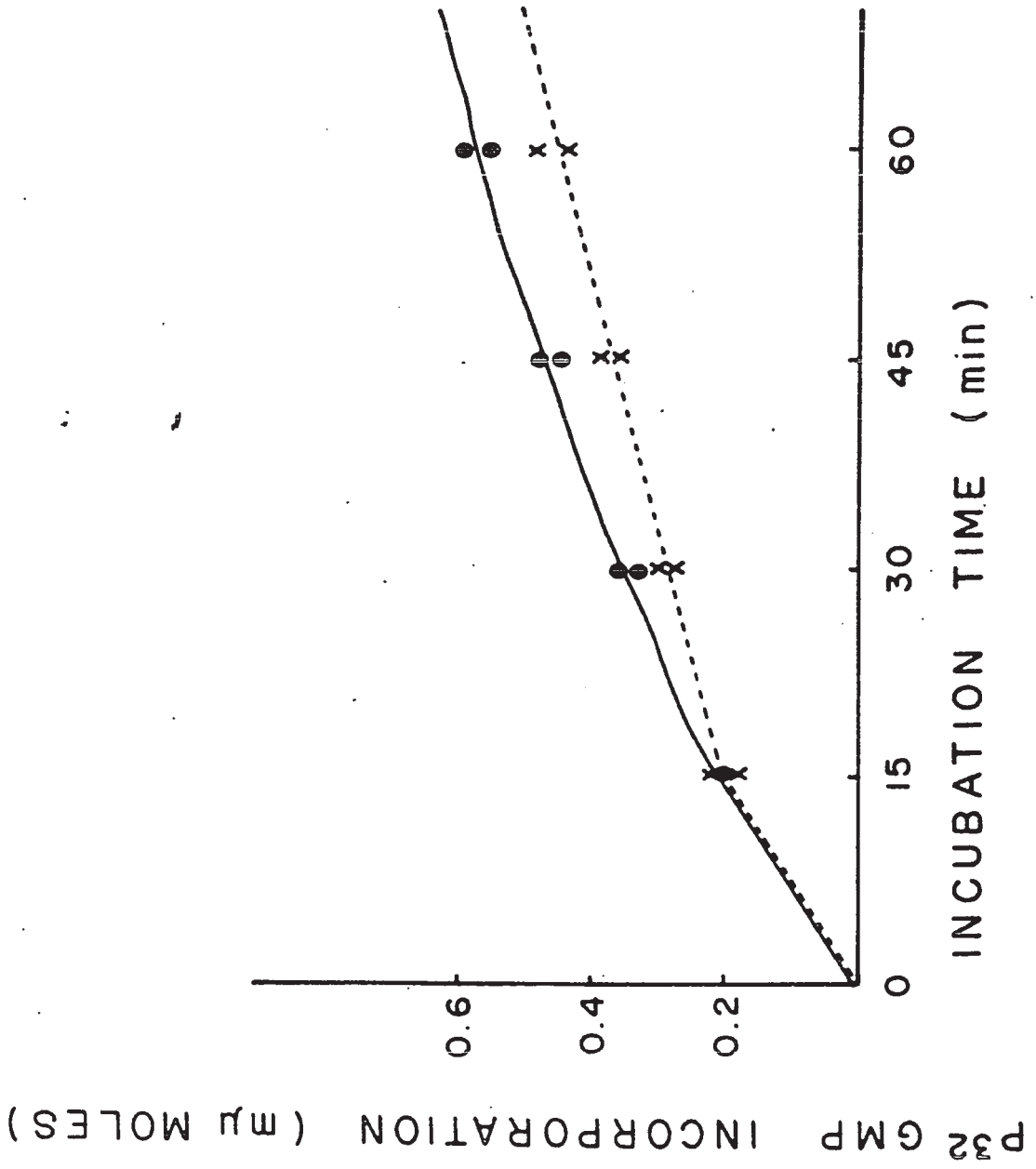

P32 GMP INCORPORATION (mμ MOLES)
0.6
0.4
0.2
0
15
30
45
60
INCUBATION TIME (min)

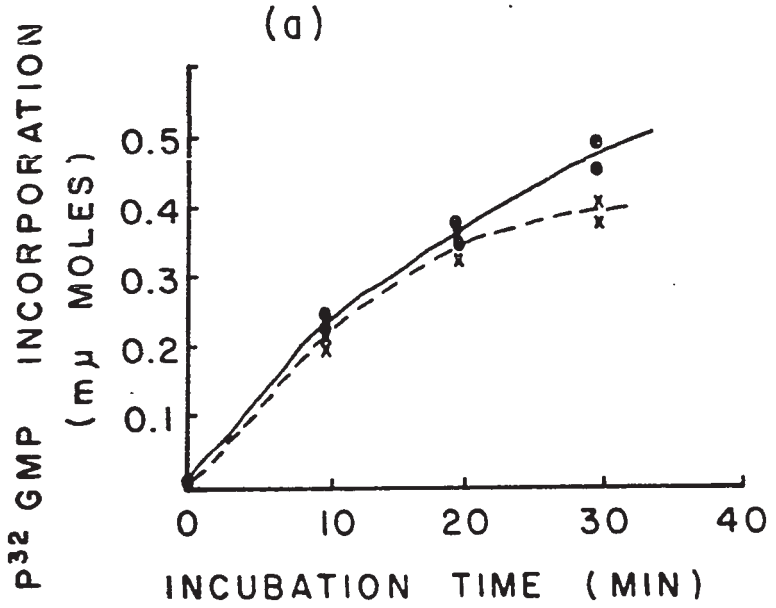
FIGURE 34.

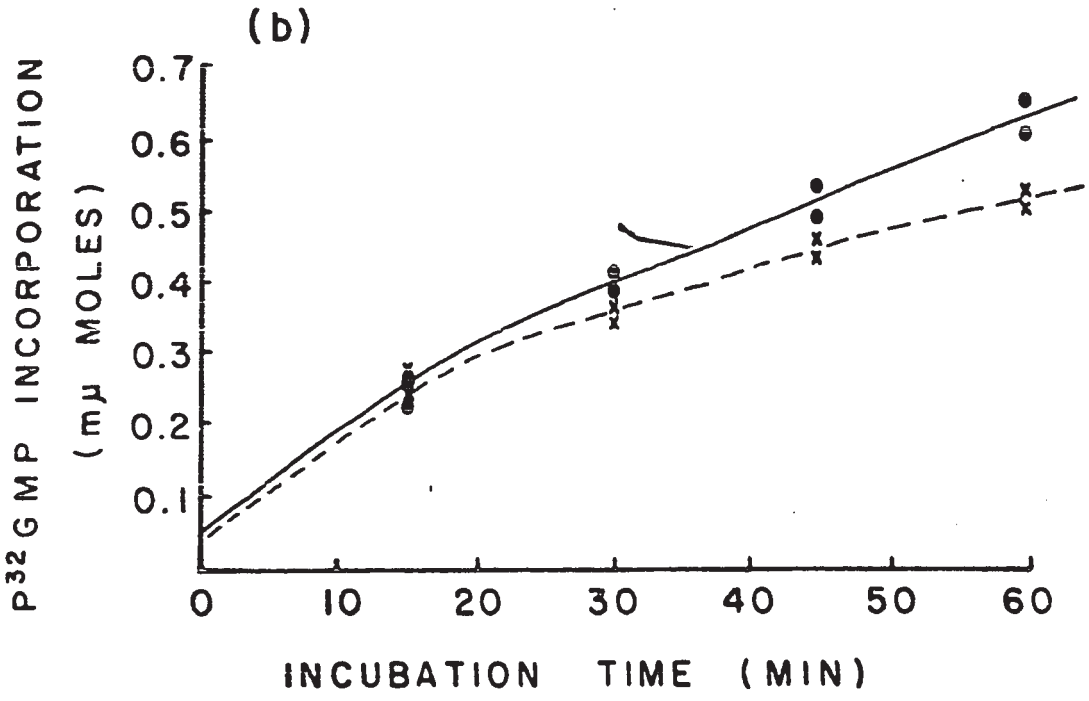
Time courses of RNA synthesis directed by control irradiated DNA and irradiated covalent benz(a)pyrene:DNA complex.

The experiment was carried out as described in Fig. 33, except the complex used had a lower molar ratio. The time courses were followed up to 30 minutes in (a) and up to 60 minutes in (b). Results of duplicate assays are given.

o ——— o Control irradiated DNA

x ----- x Irradiated BP:DNA (molar ratio = 0.0016)


(a)
P32 GMP INCORPORATION
(mμ MOLES)
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
INCUBATION TIME (MIN)


(b)
P32 GMP INCORPORATION
(mμ MOLES)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
INCUBATION TIME (MIN)

IV. DISCUSSION

A. Physical Interactions of Dimethylbenz(c)acridines with DNA

1. Formation of physical complexes with DNA

The binding technique used in these studies allows efficient binding of the dimethylbenz(c)acridines to DNA. The technique is essentially the same as that used by Ball *et. al.*, (1965) in studying the binding of polycyclic aromatic hydrocarbons to DNA. The procedure involves mixing an aqueous solution of DNA in low ionic strength and neutral pH with each of the dimethylbenz(c)acridines dissolved in a small volume of alcohol. The low ionic strength and the alcohol (which amounts to about 6%) do not denature the DNA since the DNA still yields a hyperchromicity of 39.1% ± 1.3% (S.D. between preparations) and since it has a low molar extinction coefficient per phosphate group, E(P), of 6558 ± 103 (S.D. between preparations).

Determination of the exact amount of the dimethylbenz(c)-acridines bound to DNA is relatively simple because of the fact that the dimethylbenz(c)acridines are highly insoluble in the aqueous buffer (10^{-8}M). In contrast, the amount solubilized by DNA is of the order of 10^{-4}M. Thus, we can consider in effect that all the dimethylbenz(c)-acridine present in the complexes as prepared, is bound to DNA. This assumption is strengthened by the fact that the binding ratio is unaltered

after sedimentation through a sucrose density gradient.

Of the three dimethylbenz(c)acridines studied, the carcinogenic 7,9- and 7,10-derivatives bind well to DNA (Fig. 3). They can both bind to the extent of 1 mole to every 16 moles of DNA nucleotide-phosphate. Their complexes are quite stable either following repeated centrifugation at high speeds or when stored at 4° for long periods of time (6 months or longer). However, the non-carcinogenic 7,11-derivative interacts poorly with DNA (Fig. 3). The maximum binding ratio of its complex with DNA when freshly prepared is 1 mole to 82 moles of DNA nucleotide. In contrast to 7,9- and 7,10- the 7,11-dimethylbenz(c)-acridine:DNA complex is unstable. This 7,11-derivative begins to dissociate from the DNA as soon as the complex is made. Repeated centrifugation or prolonged storage at 4° dissociated the complex completely.

These quantitative binding studies may suggest a correlation between the ability of these dimethyl derivatives of benz(c)acridine to produce tumors in mice (Lacassagne et. al., 1956) and their ability to bind to DNA. However, because of the small series of compounds studied this correlation may only be a fortuitous one.

Apart from the fact that the 7,9- and 7,10-derivatives bind equally well to DNA, they also have the same effects on the thermal stability and sedimentation rate of DNA. Their mode of interaction with DNA, for lack of contrary evidence, is considered at present to be similar if not identical.

2. Evidence for intercalation

The results of the spectral studies presented in Table I provide an explanation for the bathochromic shifts characteristic of the binding of the dimethylbenz(c)acridines to DNA. No such shifts were observed when these compounds were dissolved in the group A solvents which have a wide range of dielectric constant values and hydrogen-bonding properties. However, group B agents which have a system of unsaturated bonds were able to produce such a bathochromic effect. Most likely the bathochromic shifts were the result of interactions between the π-electron systems of these agents and those of the dimethylbenz(c)acridine. Such has been shown in the case of the interaction between aromatic hydrocarbons and these agents (Lesko et. al., 1968). Therefore, the bathochromic shifts characteristic of the interaction of the dimethylbenz(c)acridines with DNA probably reflect the interaction between the π-electron systems of the dimethylbenz(c)-acridines and those of the DNA bases. Conceivably, the best situation for such an interaction is when the dimethylbenz(c)acridine molecules and the DNA bases are in face-to-face contact. This evidence therefore, strongly favors the intercalative mode of binding because when so sandwiched inside a stack of DNA bases, the dimethylbenz(c)acridine molecules and bases are in direct van der Waals contact, allowing maximum interaction of the π-electron systems.

It is known (Boyland & Green 1962) that purines (such as caffeine or, tetramethyluric acid) in aqueous solutions have a tendency to self-stack and when polycyclic aromatic hydrocarbons are added, they form complexes with the hydrocarbons, sandwiching them in a 1:1 and 2:1

ratio (purine:hydrocarbon). We believe the purines in our system are also sandwiching the dimethylbenz(c)acridines (hence, the bathochromic effect), because Table I shows if the stacking forces are absent (by doing the experiment in 95% alcohol), the bathochromic effects of the caffeine on the absorption spectrum of the dimethylbenz(c)acridines are no longer evident.

The ability of the dimethylbenz(c)acridines to stabilize DNA against thermal denaturation is in keeping with the intercalative mode of binding because the dimethylbenz(c)acridine molecules contribute to the hydrophobic bonding which is the major force holding the two strands of the double helix together (DeVoe & Tinoco, 1962). This evidence by itself is no proof for such an internal binding model, because binding of any cations externally to the phosphate groups would also raise the denaturation temperature due to the neutralization of the negatively charged phosphate groups that normally repel one another.

However, if binding is external one would expect the sedimentation rate of the complex to be higher than that of free DNA. However, the sucrose density gradient and the Spinco Model E ultracentrifuge data show the reverse is true. Thus, these two effects (higher Tm and lower sedimentation rate) of the dimethylbenz(c)acridines on DNA, when considered together, would strongly support the internal binding model. The sedimentation rate of such a complex would be expected to drop because the DNA has to extend quite appreciably in order to accommodate these intercalating ligands especially when the molar ratio can be as high as 1 ligand to every 8 pairs of bases (Lloyd *et. al.*, 1968).

Consideration of the extremely low solubility of these dimethylbenz(c)acridines in SBB would cause one to suspect that they bind internally. It is very unlikely that they can bind externally to the negatively charged phosphate groups and remain soluble in the aqueous environment. Rather, it is more likely that they will go into the stack of DNA bases which provide the only hydrophobic environment in the system.

Sterically, these dimethylbenz(c)acridines pose no problem to the intercalation model. These molecules though more complex, have a planar structure like that of the acridines and polycyclic aromatic hydrocarbons which are the best known intercalating ligands.

3. Evidence supporting the modified intercalation model of Pritchard et. al., (1966)

Assuming that the dimethylbenz(c)acridines do intercalate in the stack of DNA bases, the question of how they are situated within the stack was studied.

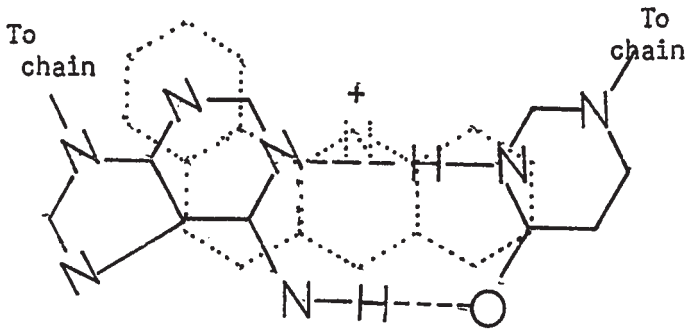
We have some idea, from the data obtained, whether the dimethylbenz(c)acridine molecules sit in the centre of the helix (equidistant from the two sugar phosphates) as in Lerman's intercalation model, (Fig. 35a) or whether they sit to one side (with the acridinium cation interacting with the negative phosphate group) as in the modified intercalation model of Pritchard et. al., (1966) (Fig. 35b). We believe the modified intercalation model is more correct for the following reasons: Firstly, the dimethylbenz(c)acridine molecules when bound to DNA are in the cationic form and therefore, one would expect

FIGURE 35.

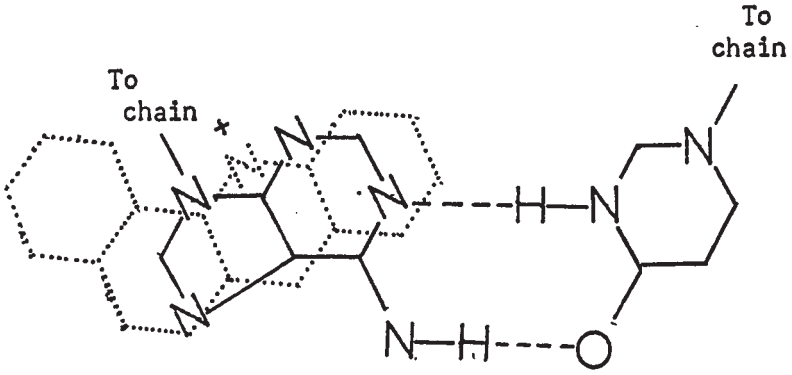
Schmatic representation of two possible arrangements of a benz(c)-acridine molecule intercalated in DNA.

Different orientations of a benz(c)acridine molecule with respect to a hydrogen bonded base pair are shown. Dotted lines represent the benz(c)acridine. (a) Orientation based on the intercalation model of Lerman (1964) - acridinium ion equidistant from the negative phosphate groups in the two chains. (b) Orientation based on the modified intercalation model of Pritchard et. al. (1966) - acridinium ion approaching and interacting with the negative phosphate group of one of the chains. Both surfaces of the molecule are shown (i) and (ii).

(a)

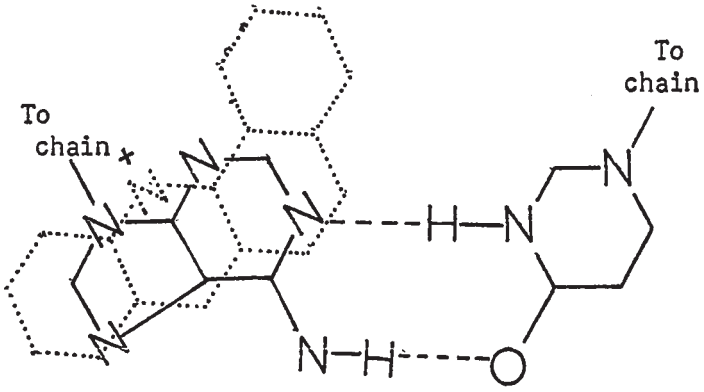

To chain
To chain
+
N—H---O

i.


To chain
To chain
+
N—H---O

(b)

ii.


To chain
To chain
+
N—H---O

electrostatic interaction between the acridinium cation and the negative phosphate to occur. If they do interact, the dimethylbenz(c)acridines could not be sitting at the centre of the helix. The evidence that they bind to DNA in the cationic form comes from spectral studies (Fig. 1). The spectra of their unionized form in neutral alcohol solutions are quite different from those of their ionized forms in acidic alcohol solutions. (The former solutions are colorless and the latter have a bright yellow color). The spectra of these dimethylbenz(c)acridines when dissolved in solutions of DNA, closely resemble their corresponding spectra in acidic alcohol. (These dimethylbenz(c)acridine:DNA solutions also have bright yellow colors). Therefore, it is the cationic form that binds to DNA. Thus, the DNA must have raised the pK's of the dimethylbenz(c)acridines, as has been observed by Booth & Boyland (1953) in the case of 3:4 - 6:7 - dibenzacridine.

Secondly, the strong dependence of binding on ionic strength is indicative of the existence of such an electrostatic interaction and hence, favors the (b) model. The counter cations presumably compete with the dimethylbenz(c)acridines for the phosphate groups.

Thirdly, the strong dependence of binding on the pH of the medium also points to the importance of electrostatic interaction. The higher pH presumably inhibits the ionization of the acridinium ion.

Fourthly, and most important of all, is the demonstration that the dimethylbenz(c)acridines can bind to single stranded DNA. Here, the only possible intercalation model is the (b) model. The heated and quick-cooled DNA used in these studies is no doubt quite

extensively denatured. The molar extinction coefficient per mole phosphate group, E(P), of the denatured DNA rises from the native state of 6560 to 8815, that is, 34% hyperchromicity. The average hyperchromicity of native DNA upon heat-denaturation is 39%. Thus, the DNA is at least 87% denatured. When the denatured DNA was heated in the Gilford the hyperchromicity observed is only 6% which means the preparation is at least 85% denatured. The 13-15% residual hypochromicity may represent the presence of an undenaturable core or, more likely represent a random reassociation of short hydrogen-bonded regions. The absence of a sharp "melting profile" of the denatured DNA favors the latter interpretation. Since there is a definite and fixed upper limit on the number of moles of dimethylbenz(c)acridine a double stranded region can bind, and since the denatured DNA having only 15% or less double stranded regions, can bind as much dimethylbenz(c)acridine as native DNA (in terms of molar ratios) there is little doubt that the dimethylbenz(c)-acridines do bind to single stranded DNA.

Since the spectral characteristics of the 7,10-dimethylbenz(c)acridines in denatured DNA and in native DNA (hypochromicity, bathochromicity, molar extinction coefficient) are qualitatively and quantitatively the same, it seems that the modes of binding of this dimethylbenz(c)acridine to native and denatured DNA's are identical. This would mean that even in a double helix, the dimethylbenz(c)acridine is sitting on one side of the stack of bases as shown in Fig. 35b. Our results therefore, strongly support the modified intercalation model as proposed by Pritchard et. al., (1966). Figure 35b shows schematically how the dimethylbenz(c)acridine molecule may be oriented to one side of

Model building from space-filling (Corey-Pauling-Koltun) atomic models

I. Structure of 7,10-dimethylbenz(c)acridine.

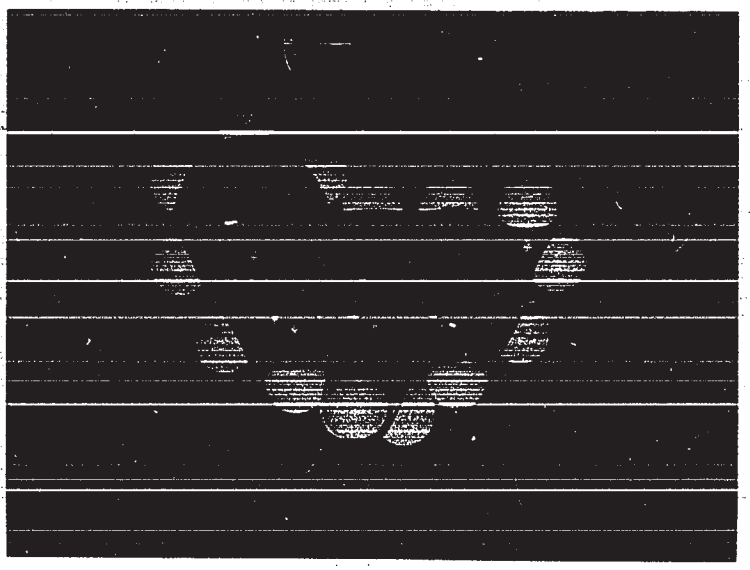
II. Intercalation of 7,10-dimethylbenz(c)acridine between two base-pairs of a double DNA helix, based on the modified intercalation model of Pritchard *et al* (1966).

a. Oblique view of the complex.

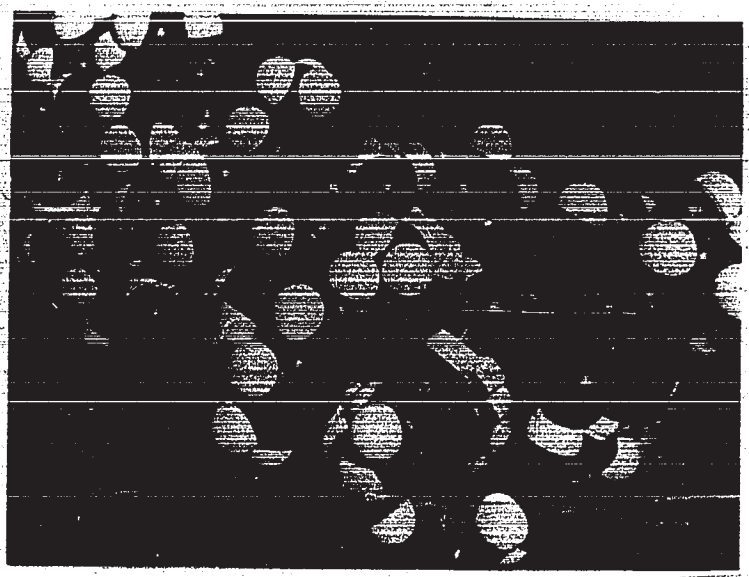
b. Edge-on view of the complex.

Note: White atoms are hydrogen, dark blue atoms carbon, light blue atoms nitrogen, red atoms oxygen, and yellow atoms phosphorus.

I.



II, a.



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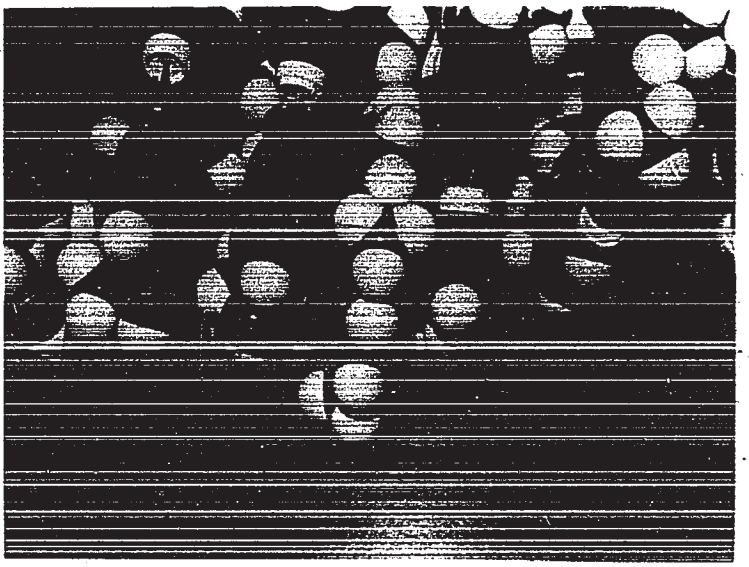
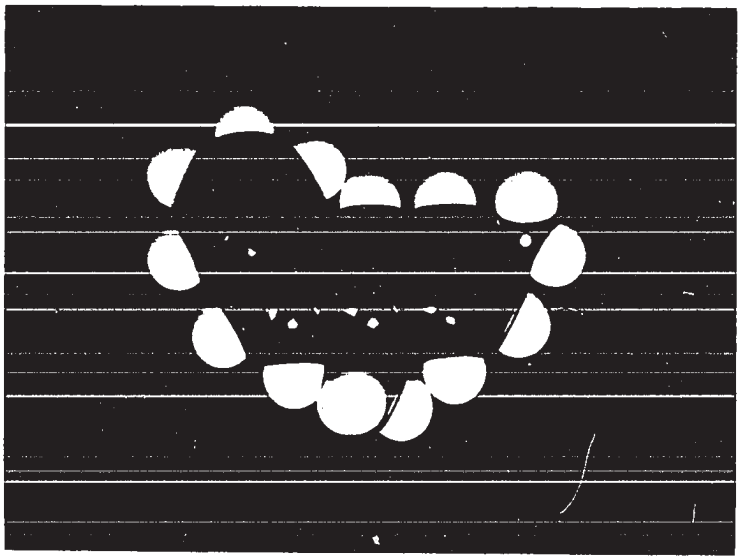
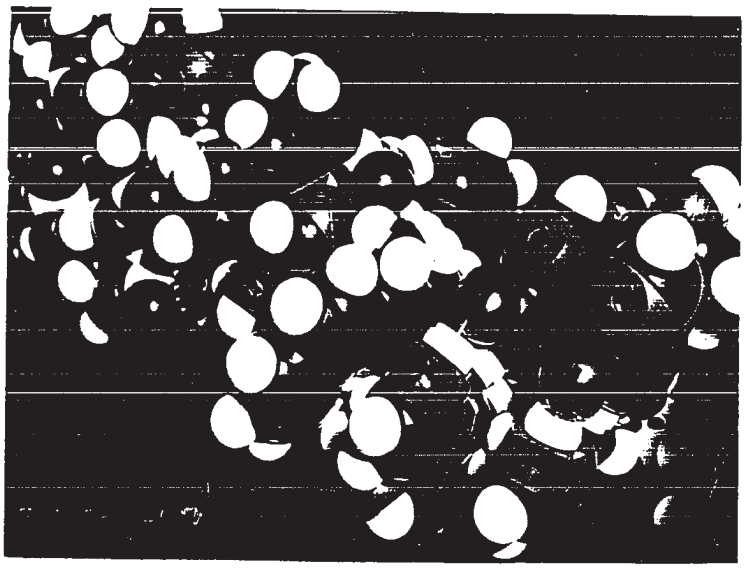


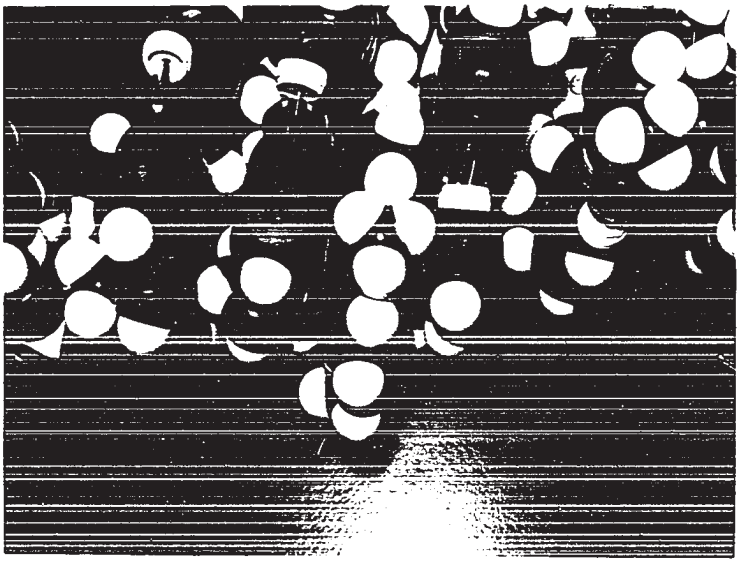
I.



II, a.



II, b.



the hydrogen-bonded base pair with the charged N facing the sugar phosphate back bone. This arrangement makes it necessary for the extremities of the dimethylbenz(c)acridine molecule to stick out of the base stack, into the major and(or) minor grooves. The presence of a methyl group at position 11 of the molecule hinders the interaction of this molecule with DNA as shown by the lesser and unstable binding of the 7,11-derivative to DNA (Fig. 3). The 11-methyl may block the electrostatic interaction by steric hindrance, and(or) by suppression of ionization of the acridinium ion. Work with atomic space-filling model (Corey-Pauling-Koltun) of DNA and of the ligands, indicated that the 11-methyl does not sterically block the interaction between the charged N and the phosphate group. Therefore, the suppression of the ionization of the acridinium ion by the 11-methyl is one possible explanation for the poorer binding of the 7,11-derivative to DNA.

The molecular model-building studies afforded an estimate of the total area of contact between the ligand and the two adjacent bases. This area of contact is a function of the sum of the areas of the two bases between which the ligand intercalates and therefore it decreases in the order: purine-purine, pyrimidine-purine, pyrimidine-pyrimidine, depending on the two bases involved in the interaction. If stacking forces are directly related to contact areas, it would seem that binding between pyrimidine-pyrimidine bases is less stable. Molecular model building also indicated that the angular benzene ring of the benz-(c)acridines can appear on either side of the sugar-phosphate chain i.e. in either the major or minor grooves of the double helix.

The binding between the dimethylbenz(c)acridines and DNA

is strictly physical, in that neither the DNA nor the ligands undergo any chemical or physical changes. The ligands can be recovered from a complex by means of extraction with cyclohexane and they retain their original absorption spectrum. The DNA recovered from a dissociated 7,10:DNA complex also has its original sedimentation rate (Table 2b).

The heat dissociation studies of a 7,10- complex with native DNA (Fig. 9) show that quite extensive denaturation of the DNA can take place before the ligand is dissociated from the DNA. These results can be explained by two hypothetical models and these have not been resolved, since the details of the heat-dissociation process have not been elucidated. One can postulate (Model I) that as the DNA strands are separating during heat denaturation, the ligands in the affected areas can migrate and become bound to more hydrophobic regions which are still double stranded. This re-location will cause localized over-packing. When over-saturation of the binding sites within a given region is reached, the ligands are released from the DNA. The rapid re-equilibration process proposed in this model can account for the absence of detectable dissociation of ligand between 52° and 67° (Fig. 9 & 10). It can also explain the sudden co-operative release of most or all of the ligands when 70° is reached. This temperature (70°) marks the over-saturation point. One might also explain, on the basis of this model, the effect of molar ratio on the dissociation temperature (Fig. 11). If one heat-denatures a complex having a rather low initial molar ratio (for example, 1 ligand to every 52 nucleotides, Fig. 9), the relatively few ligands present have a chance to migrate

to the more stable regions as the double-helix uncoils. Figure 9 shows that when the temperature reaches 67°, there is still 17% double-stranded regions in the DNA as judged by the extent of hyperchromicity. The molar ratio in this remaining double-stranded region would then be 1 mole to 6 nucleotides. When the initial molar ratio of a complex is high, then some of the ligands would have to bind to less stable regions of the DNA. Hence if such a complex is heat-denatured the over-saturation and dissociation would be expected to take place at a lower temperature.

If the dissociation temperature is held at 58° (Fig. 13) a temperature at which the DNA is about half denatured, approximately half of the ligand becomes dissociated, as evidenced by the optical density peak (at 380 mμ) which is half the original magnitude (Compare Figs. 12 & 13). The fact that one can so divide the dissociation process into 2 steps indicates that some of the binding sites are less stable than others.

The absence of dissociation at 58° when the temperature is continually increasing (Fig. 12) may be explained on the basis of a time-lag. When the temperature is held at 58° (Fig. 13), there is a lag of 3 min. before the first optical density peak begins to appear. If the first optical density peak were also to appear 3 min. after 58° is reached, in the experiment when the temperature is increasing continuously (Fig. 12), the temperature would have risen to 60° during the 3 min. lag. Thus the first optical density peak actually appears at 60°. But half way before the first peak is over (about 5 min.), the temperature would have reached 63° which is high

enough for the second peak to occur. Therefore, as the two optical density peaks overlap on the time scale, one would expect to see only one large peak when the temperature is increased continuously.

However, there is no need to postulate that there must be re-location of the ligand molecules. One may only need to propose (Model II) that the ligands bind preferentially to the most heat-stable regions of the DNA. Since the bound ligands further stabilize the DNA against heat-denaturation, those regions containing bound ligands could have an appreciably higher melting temperature. Thus, extensive internal and external melting of ligand-free regions may be taking place while the complexed regions are not disrupted. With a low binding ratio (1 mole:52 nucleotides), the DNA is denatured 83% before the ligand molecules begin to be released (Fig. 9). The binding to less stable sites when the molar ratio is increased can explain the reverse relationship between binding ratio and dissociation temperature as observed (Fig. 11). The postulate for the existence of stronger and weaker binding sites can also explain why the dissociation process can be split into two steps by holding the temperature at 58° (Fig. 13).

Either one of these two models may be correct and both may be applicable. To understand the dissociation events better one should know the binding constants at various sites and at various temperatures. One should know whether they bind better to G-C rich regions as compared to A-T rich regions, and whether binding between two purines is stronger than between a purine and a pyrimidine, or between two pyrimidines. If there is re-location of the ligands

from A-T rich regions to G-C rich regions of DNA as the complex is heated, one might be able to detect a change in the fluorescence-quenching of the ligand. Chan & McCarter (1970) have shown that the quantum yield of proflavine when bound next to a G:C base pair is reduced to zero, but when bound between two A:T base pairs, its fluorescence is not affected. By measuring the quantum yields of the DNA-bound ligands at various elevated temperatures, it might be possible to resolve the question as to whether there is re-location of the ligands or not.

B. Covalent Binding of Pyrene and Benz(a)pyrene to DNA

Our technique of inducing covalent linkage by γ-irradiation allows for very efficient and extensive binding of pyrene and benz(a)-pyrene to calf thymus DNA. At a dose of 18 Kr, the binding ratio is 1 mole to 270 DNA nucleotides. The binding ratio at an approximately equivalent dose of x-ray (20 Kr), calculated from the results published by Rapaport & Ts'o (1966) is of the order of 1 molecule of benz(a)pyrene to 24,000 - 30,000 nucleotides. Therefore the binding is about 100 fold better under our conditions. The higher binding efficiency may be partly due to the use of a different source of radiation and/or different conditions of irradiation. The major factor probably lies in the method we employed for the preparation of the physical complexes for irradiation. Our method gives physical complexes with a much higher initial binding ratio (1 molecule of benz(a)pyrene: 100 nucleotides, compared to 1 molecule: 1,000 nucleotides of Rapaport et. al., (1966)). As ionizing radiations have strong damaging effects on DNA (Muller, 1967; Shooter, 1967), the increased binding at lower doses

of irradiation would be desirable.

The extensive binding achieved by our technique makes it possible to carry out the binding studies with unlabelled hydrocarbons. Because of the high binding ratio it is possible to detect the absorption spectra of the covalently bound pyrene and benz(a)pyrene. The spectra (Fig. 19) are shown to be different from those of their physically-complexed forms in DNA, in that there are further bathochromic shifts of the absorption peaks. This is the first time that it has been possible to obtain the actual absorption spectra of polycyclic hydrocarbons covalently-bound to DNA. These results serve to indicate that the tritium count observed to be covalently-linked to DNA by Rapaport & Ts'o (1966) may represent covalently-linked benz(a)-pyrene.

The conditions of irradiation employed do not produce significant changes in the absorption spectral characteristics of the DNA. Neither does the treatment cause any loss of material absorbing at 260 mu upon alcohol precipitation. However, γ-irradiation does cause significant denaturation of the DNA. When the irradiated DNA's were heat-denatured, the extents of hyperchromicity observed decreased progressively as the dose of γ-irradiation was increased (Fig. 20, b & c). The results of the analysis of the irradiated DNA's in sucrose density gradients (Fig. 21) also indicated that rather extensive degradation of the DNA has taken place. Although the gradients are not calibrated with marker DNA, and the exact sedimentation rates of the DNA peaks are not known, the trailing optical density peak indicates that as much as one-half to one-third of the DNA has been broken into

fragments perhaps less than half their average original lengths. The fact that the sedimentation profiles of the irradiated DNA and the covalently-bound benz(a)pyrene:DNA complex are essentially the same, indicates that there is no preferential degradation (in terms of double stranded breaks) of the DNA in the presence of the bound benz(a)pyrene.

The results of Figure 20, b & c show that the dose-dependent denaturation is quantitatively the same for the control-irradiated DNA and for the irradiated benz(a)pyrene:DNA or pyrene:DNA complexes. These results therefore, indicate that there is no preferential denaturation of the DNA when the hydrocarbons are present during irradiation.

Interestingly, the denaturation of the DNA becomes apparent at doses higher than about 8 Kr (Fig. 20) and it is at this dose level that the covalent-binding of the hydrocarbons to DNA is first detectable (Fig. 18). This observation is true for both the binding of pyrene and benz(a)pyrene. Perhaps covalent-binding takes place only when the DNA becomes denatured. The orientation of the hydrocarbon molecules with respect to the DNA bases may then favor covalent-linkage. This assumption may explain why in the present study the binding curve is sigmoidal in shape rather than hyperbolic. Such an interpretation is supported by the finding of Rapaport & Ts'o (1966). By exposing a physical complex made of benz(a)pyrene and heat-denatured DNA to various doses of x-ray, these workers obtained a dose-dependent binding curve that is linear at low dose levels rather than sigmoidal.

The mechanism or the mode of covalent binding of these hydrocarbons to DNA has not been elucidated. As has been proposed by Rapaport and Ts'o (1966) the covalent linkage most likely takes place as a result of the formation of free radicals induced by the irradiation on the hydrocarbon and(or) on the DNA bases. The radiation-sensitive sites on the DNA bases are the C_4 - C_5 and C_8 - C_9 double bonds of pyrimidines (Shooter, 1967; Muller, 1967). The reactive centres on the benz(a)pyrene molecule are C_5, C_8 and C_{10}, and the C_6 - C_7 double bond (K-region) (Badger, 1954). Presumably free radicals at these sites induced either by the direct ionizing effect of γ-irradiation or indirect radiolysis products of water could result in covalent linkage between the hydrocarbon and DNA bases.

For unknown reasons, neither 7,9- nor 7,10-dimethylbenz(c)-acridine can covalently bind to DNA under conditions identical to those used for the binding of the polycyclic hydrocarbons to DNA.

C. Effects of Chemical Carcinogens on Transcription

1. System for assay of template activity

To compare the template activities of control DNA and of various dimethylbenz(c)acridine:DNA and polycyclic hydrocarbon:DNA complexes, the standard *in vitro* RNA synthesizing system of Chamberlin & Berg (1962) was used. The enzyme, DNA-dependent RNA polymerase from *E. coli* was isolated and purified, and the results of a representative preparation are shown in Table 3. The enzyme was assayed according to the standard conditions of Chamberlin & Berg (1962). To demonstrate

that the RNA synthesizing system prepared in the present study was analogous to that described by the above authors, the characteristics of the reaction catalyzed by the RNA polymerase were studied. It was shown (Table 4) that the system required the addition of a DNA template, divalent cations and all four of the ribonucleoside triphosphates. The enzyme showed a broad pH optimum (Fig. 22) and synthesis of RNA was linear for about 15 minutes (Fig. 23). The effect of DNA concentration on incorporation is shown in Figure 24. Limiting DNA concentrations (approximately 30 ug per 0.25 ml of incubation mixture) were used for assays of template activity. Figure 25 shows that the initial reaction rates were directly proportional to the amount of enzyme added.

The effects of salt concentration on RNA synthesis in this in vitro system have been studied in several laboratories. Under conditions of low salt concentration (e.g. 0.05 M KCl+ 5mM Mg Cl_2 or such as in the standard reaction mixture of Chamberlin & Berg, 1962), RNA synthesis stops completely after 1 hour at 37° (Bremer & Konrad, 1964; Fuchs, Millete, Zillig & Walter, 1967). The RNA product is not released from the DNA template. As a result of product inhibition, the enzyme can neither continue transcription nor initiate the synthesis of new RNA chains. However, in reaction mixtures with a much higher salt concentration (0.2M KCl+ 12 mM Mg Cl_2), RNA synthesis continues for several hours with only a slight decrease in rate (So, Davie, Epstein & Tissieres, 1967; Fuchs et. al., 1967). Under such conditions the RNA product is released and each enzyme can catalyze the synthesis of several RNA molecules.

It was recognized that the use of low rather than high salt conditions for RNA synthesis would be of advantage for the purposes of the present investigation. The interpretation of the results of transcription of control DNA and carcinogen:DNA complexes would be expected to be simplified, as each enzyme molecule would make only one RNA chain.

It must be noted that the in vitro RNA synthesizing system employed here is quite remote from the in vivo conditions of transcription. The calf thymus DNA template is transcribed with a bacterial enzyme in the absence of a number of control factors which are likely to play a part in transcription in the cell. These factors may be RNA, histone, non-histone acidic proteins, hormones, polyamines, specific protein factors etc. that determine the specificities of initiation and termination. It is unlikely that the E. coli polymerase can recognize the proper initiation base sequences of purified calf thymus DNA. Therefore, initiation sites observed in the present system are no doubt random. However, this purified and well-characterized in vitro system serves to provide a ready model system in which the effects, if any, of the DNA-bound chemical carcinogens might be elucidated.

2. Assay of physical complexes between DNA and various chemical carcinogens.

Table 5 shows that all of the complexes tested, irrespective of binding ratio, have the same template activities as their corresponding control DNA.

The inability of the carcinogens to exert any effect on

RNA synthesis was due to the fact that they did not remain associated with DNA under the conditions of the high pH and salt concentrations used in the standard enzyme assay reaction mixture of Chamberlin & Berg (1962). In the case of the dimethylbenz(c)acridines:DNA complexes, the incubation mixture turned turbid and precipitates appeared. The characteristic absorption spectra of the complexes disappeared when the complexes were added to the reaction mixture (Fig. 26). The addition of the divalent cations (Mn^{++}, Mg^{++}) only to the dimethylbenz(c)-acridine:DNA complexes, was enough to elicit the above changes. The concentrations of the $MnCl_2$ (1 mM) and $MgCl_2$ (4 mM) used in these studies were the same as those of the standard reaction mixture (Table 7). Analysis of the $MnCl_2$ and $MgCl_2$ treated complexes by means of sucrose density gradient centrifugation revealed that the complexes were indeed dissociated (Fig. 27).

The various components of the incubation mixture were tested individually for their ability to dissociate the complex. The addition of $MnCl_2$, KCl or Tris buffer resulted in the dissociation of the dimethylbenz(c)acridine:DNA complexes. The other components of the reaction mixture, β-mercaptoethanol, nucleoside triphosphates and glycerol had no effect on the complexes. The maximum tolerable concentration for each of the individual components was determined (Table 6), by adding increasing amounts of each of the test compounds to the complexes until a change in absorption spectrum of the complex was observed. Then a suboptimal set of reaction mixture conditions with much reduced concentrations of $MgCl_2$, Tris and KCl (based on the maximal tolerable concentrations) was set up,

as shown in Table 7. Such a reaction mixture (Table 7) did not dissociate a complex of the 7,10-dimethylbenz(c)acridine with DNA.

To determine the minimum requirements of pH and ionic strength for RNA polymerase activity, the concentrations of various components of the reaction mixture were reduced systematically. Figure 29 shows that the concentration of the Tris buffer at pH 7.9 in the standard reaction mixture can be reduced from 40 mM to 12 mM without inhibiting the reaction. But a further reduction of the concentration of the buffer resulted in a marked inhibition of the enzyme activity. A concentration of 9 mM of Tris reduced the reaction by only about 15% (Fig. 29) and was chosen for further studies. Figure 30 shows the strong dependence of the enzyme activity on $MgCl_2$ concentration when only 9 mM Tris was used. Figure 31 shows the extent of inhibition on enzyme activity, as the pH and the $MgCl_2$ concentrations of the reaction mixture are reduced, (but keeping the Tris buffer concentration at 9 mM).

It is quite evident from the above studies that the minimum pH and salt concentration required for enzyme function, far exceed the maximum levels which the 7,10-dimethylbenz(c)acridine:DNA complex can tolerate (Table 7). Therefore, it is concluded that it is not possible to assay the effect of this physically-complexed chemical carcinogen on the template activity of DNA. This conclusion is extended to the other dimethylbenz(c)acridines and polycyclic hydrocarbons, as their physical complexes with DNA are also dissociated under conditions of high pH and ionic strength.

These findings provide an explanation as to why there have been no reports of any biochemical effects of these polycyclic hydrocarbons when physically-complexed to DNA, in a well defined *in vitro* system, although these carcinogens have been known to interact physically with DNA for some time (Booth *et. al.*, 1951). The biological significance of such physical associations between the hydrocarbons and DNA, is therefore questionable, as these interactions are demonstrable *in vitro* only under non-physiological conditions.

3. Assay of covalent complexes

The *in vitro* RNA synthesizing system described above was used to compare the template activities of irradiated covalent benz(a)-pyrene:DNA complex and of control irradiated DNA. As the doses of irradiation employed to induce the formation of the covalent complexes caused significant denaturation (Fig. 20) and degradation (Fig. 21) of the DNA, the effects of γ-irradiation on the template activity of DNA was first investigated. Figure 32 shows that irradiation has a strong inhibitory effect on the template activity of the DNA. These results confirm the observation of Harrington (1964) and those of Hagen *et. al.*, (1970) and Goddard *et. al.*, (1970).

The results shown in figures 20 and 21 indicated that there was no preferential denaturation or degradation of the DNA whether benz(a)pyrene was present or absent during irradiation. Assuming that the extents of denaturation and degradation of the DNA were identical for the control irradiated DNA and for the covalent benz(a)pyrene :DNA complex, the template activities of the DNA and complex were compared.

It is evident from the time course of the reactions shown in Figures 33 and 34, there were essentially no differences in the template activities of the control irradiated DNA and of the irradiated complex during the early incubation times (approximately the first 15 min.). However, at later incubation times, the template activity of the complex became significantly lower than that of the control DNA. After 30 min. of incubation, the activity of the complex was only 80% of that of the control DNA.

The decreased template activity of the complex was most likely due to an inhibitory effect of the covalently-bound benz(a)-pyrene. There was no doubt that the benz(a)pyrene remained bound to the DNA template throughout the incubation period of 1 hour. The distinct absorption spectrum of the complex was not altered even after 2 hours of incubation in the enzyme reaction mixture. Removal of protein by treatment with phenol did not eliminate the spectrum of the complex. Therefore, the benz(a)pyrene did not become dissociated from the DNA nor did it become bound to RNA polymerase during RNA synthesis. These results indicate that benz(a)pyrene exerts its inhibitory effect on RNA synthesis while it is covalently-bound to the DNA template.

The absence of any difference in the template activities of the control DNA and of the complex during early times suggests that the benz(a)pyrene molecules in the complex neither block the binding of the RNA polymerase to the DNA template, nor interfere with the initiation of transcription. Presumably RNA polymerase attaches to and starts transcription at sites on the DNA not blocked by the

benz(a)pyrene molecules. As transcription proceeds along the template, some of the growing RNA chains encounter benz(a)pyrene molecules, and it is at this point that transcription is retarded or stopped.

The above interpretation suggests that the same number of RNA chains are initiated whether the control DNA or the complex was used as template. Since under the condition of low salt concentration used in the enzyme assay system (See Discussion, Section C, 1), each enzyme molecule only makes one RNA chain, the inhibition observed (after 20 min. incubation) most likely reflects the fact that RNA chains made on the complex are significantly shorter than those made with the control DNA as template. Whether these RNA chains are in fact shorter or not could be verified by analyzing the RNA products made by the control DNA and the complex in a sucrose density gradient (Ishihama & Kameyama, 1967).

Another aspect of the above interpretation could also be verified. By comparing the incorporation of purine nucleoside triphosphates labelled with P^{32} in the β or γ position into RNA directed by either the control-irradiated DNA or a covalent benz(a)-pyrene:DNA complex (Maitra & Hurwitz, 1965), one might be able to prove or disprove the suggestion that DNA-bound benz(a)pyrene has no effect on the initiation of transcription.

The enzyme most likely remains bound to the DNA template at the end of transcription, because of the conditions of low salt used in the RNA synthesizing system (Discussion, Section C, 1). At

or near the end of transcription of B. subtilis DNA in a similar in vitro system, Novak (1969) digested the DNA template using pancreatic DNase. Some regions were found to be resistant to the nuclease and were believed to be covered by the micrococcal RNA polymerase used to transcribe the DNA. These DNase-resistant regions of DNA had a higher than average GC content for B. subs. DNA and were postulated to be GC-rich termination signals for transcription. Parallel experiments could be carried out for the benz(a)pyrene:DNA complex. If polymerization of RNA chains end as the polymerase encounters benz(a)pyrene molecules, then the DNase resistant DNA fragments ought to show an enrichment of the hydrocarbon (a higher hydrocarbon:nucleoside ratio).

There are a number of difficulties inherent in the use of the present model RNA synthesizing system. The damage to the template due to γ-irradiation has not been fully determined. Although there were no major differences in the extent of denaturation (Fig. 20) and degradation between control irradiated DNA and irradiated complex, finer differences in base modification and base elimination have not been ruled out. In fact these fine differences would be hard to assess. Another difficulty is that it is not known whether it is the double-stranded or the single-stranded regions of the template that are being transcribed. However, in either case, the above interpretation of the inhibition studies remain unaltered, if one assumes that the modes of transcription of the γ-irradiated DNA and complex are identical. Since benz(a)pyrene can bind well to heat-denatured DNA covalently (Rapaport et. al., 1966), it might be simpler to use

complexes of the hydrocarbon made with DNA fully denatured by heat.

On account of the above difficulties inherent in the use of the present model RNA synthesizing system, and of the preliminary nature of the results, the evidence obtained for the inhibitory effect of DNA-bound benz(a)pyrene on RNA synthesis, should only be taken as suggestive. Nevertheless these results confirm the inhibitory effects of carcinogenic polycyclic hydrocarbons on RNA synthesis observed *in vivo* (Iversen *et. al.*, 1962; McCarter *et. al.*, 1962). It should be pointed out that this is the first time evidence of such an effect has been obtained in an *in vitro* system.

The significance of these results in relation to the mechanism of chemical carcinogenesis is hard to assess, because the experimental conditions employed have no resemblance of an *in vivo* situation. The artificiality of the RNA synthesizing system has been discussed (See Discussion, Section C, 1). The induction of covalent-binding of benz(a)pyrene to DNA by γ-irradiation is also quite artificial. The nature and mode of covalent binding of these hydrocarbons to DNA (whether induced by irradiation or enzymes) are not known (See Literature Review, Section A-4 and C-2). Therefore, it is not possible to equate or correlate the hydrocarbons bound to DNA by enzyme and by irradiation. Furthermore, only very low levels of binding are observed by enzyme systems. The *in vivo* binding of 9,10-dimethyl-1:2-benzanthracene to DNA was of the order of 1 molecule of the hydrocarbon to 20,000 DNA nucleotides (Brookes et. al., 1964). Similar low binding ratios (1 benz(a)pyrene molecule to 50,000 - 500,000 DNA nucleotides) were obtained for the microsomal enzyme-catalyzed binding of benz(a)-

pyrene to DNA (Gelboin, 1969). The low levels of binding make it difficult to characterize the nature of the binding. But very extensive binding was induced by γ-irradiation in the present study. The binding ratio obtained for benz(a)pyrene can be as high as one molecule of the hydrocarbon to 110 DNA nucleotides (Fig. 18). Such extensive binding induced by irradiation must lack the specificity of an enzyme-catalyzed reaction. However, it should be remembered that the enzyme-catalyzed binding in relation to the carcinogenic event is not known.

Recognizing the above deficiencies, one has to conclude that the present results are artificial and non-specific. However, the finding serves to indicate that if a hydrocarbon becomes bound to a DNA gene, the hydrocarbon might inhibit the expression of that gene. If one postulates that the hydrocarbon binds either to the operator gene (O_2) or the regulatory gene (RG_2) of the 'modified product perpetuation carcinogenesis circuit' of Pitot & Heideberger (1963), then one could have started a carcinogenesis circuit (See Review of Literature, Section B-1). However, such a mechanism will be difficult to establish.

A very important lead concerning the mechanism of action of chemical carcinogens has been provided by Igel et. al., (1969) in their recent finding which indicated that the "unmasking of a latent leukemia virus" is an "indigenous actuating cause" of chemically-induced lymphoma in mice. In view of this correlation, the immediate and most relevant approach to study the mechanism of chemical carcinogenesis is to find out if and how these chemical carcinogens activate

a latent leukemia virus. Such an investigation requires a knowledge of the molecular biology of the virus, the location of the viral genome in the host cells, whether it is in a lysogenic or autonomous state, and how it is propagated in a latent form to "normal" daughter cells of the host. The actual mechanism of induction of the latent virus by chemical carcinogens and the replication of the virus are of immediate concern. The present study points to some of the difficulties that will be encountered in any attempt to study, at the molecular or macromolecular level, the interaction of chemical carcinogens with those cellular constituents suspected to be involved in such a "viral unmasking process".

SUMMARY

The present work can be conveniently summarized in three parts:

A. Physical-binding Studies

Of the 3 dimethyl derivatives of benz(c)acridine studied, the carcinogenic 7,9- and 7,10-derivatives were found to be able to bind readily to DNA under conditions of low ionic strength and neutral pH, forming physical complexes with DNA which were stable during centrifugation through a sucrose density gradient. Under these conditions, the maximum binding ratio obtained for both the 7,9- and 7,10-derivatives was 1 mole of the acridine to 16 moles of DNA nucleotide-phosphate. However, the non-carcinogenic 7,11-derivative could not form stable complexes with DNA, dissociating from the DNA upon storage, or during sedimentation through a sucrose density gradient. The maximum binding ratio for the 7,11-derivative was 1 mole to 82 moles of DNA nucleotide-phosphate.

The formation of physical complexes between the dimethylbenz(c)acridines and DNA was characterized by a bathochromic shift (shift to longer wavelengths) of the absorption peaks of the dimethylbenz(c)acridines. Spectral studies were carried out and the results indicate that the bathochromic shift most likely reflects

the interaction of the π electron systems of the dimethylbenz(c)-acridines with those of the DNA bases.

The physical binding of the dimethylbenz(c)acridines to DNA decreased the sedimentation rate of the DNA, and increased the stability of the DNA against heat-denaturation. Studies on the thermal-dissociation of such physical complexes indicate that little or no dissociation of the ligands from the DNA occurs, even when the DNA is more than 50% denatured.

The 7,10-dimethyl derivative was found to be able to bind to heat-denatured DNA almost as well as to native DNA.

B. Covalent-binding Studies

Considerably higher levels of covalent-binding than those reported in the literature for the carcinogenic hydrocarbon, benz(a)pyrene, have been achieved by γ -irradiating solutions of its physical complex with DNA. The maximum amount of benz(a)pyrene that could be covalently bound to DNA by irradiating with a dose of 17 Kr was 1 mole of the hydrocarbon to 100 DNA nucleotides. Under the same conditions of irradiation, the non-carcinogen pyrene bound to the extent of 1 mole to 270 moles of DNA nucleotides.

Covalent-binding of the polycyclic hydrocarbons to DNA induced a bathochromic shift in the absorption peaks of the hydrocarbons. This shift was in addition to that observed when the hydrocarbons became physically-bound to the DNA.

Under the conditions employed for γ -irradiation,

covalent-binding of the hydrocarbons to DNA occurred only at doses high enough to produce detectable denaturation and degradation of the DNA.

It was not possible, however, to induce covalent-binding of either the 7,9- or the 7,10-dimethyl derivative of benz(c)acridine to DNA under conditions identical to those used for the polycyclic hydrocarbons.

C. Effects on Transcription

The template activity of the physical complexes of DNA, made with the hydrocarbons or the various dimethylbenz(c)acridines was assayed in an *in vitro* RNA synthesizing system, catalyzed by *E. coli* RNA polymerase. These attempts were not successful, because the physical complexes were dissociated under the pH and ionic strength conditions of the RNA synthesizing mixture. The ligands dissociated from DNA and because they were insoluble in the reaction mixture, had no effect on the enzyme reaction. Furthermore, it was found that the maximum levels of ionic strength and pH which the complexes could tolerate without undergoing dissociation, were far too suboptimal for the RNA polymerase to function.

The template activity of a covalent benz(a)pyrene:DNA complex (irradiated) was compared with that of a control-irradiated DNA, employing the *in vitro* RNA synthesizing system mentioned above. Preliminary results of these assays indicated that the DNA-bound benz(a)pyrene molecules had no effect on the template during the first 15 minutes of RNA synthesis. However, their inhibitory effect

on the template became apparent after about 20 minutes of synthesis, and at 30 minutes of incubation time, an inhibition of 20% on template activity was observed.

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ABBREVIATIONS

Nucleic acids, bases and nucleotides:

A - adenine

C - cytosine

G - guanine

T - thymine

XTP - nucleoside-5'-triphosphate (x = A,C,G,U, or T)

RNA - ribonucleic acid

DNA - deoxyribonucleic acid

Nucleases:

RNase - ribonuclease

DNase - deoxyribonuclease

Chemical compounds:

Tris - Tris(hydroxymethyl)aminomethane

DEAE-cellulose - diethylaminoethyl-cellulose

P - pyrene

BP - benz(a)pyrene

DMBA - dimethylbenz(c)acridine

Measurements and units:

m - milli (10^{-3})

μ - micro (10^{-6})

M - molar

U - unit of RNA polymerase activity

S - sedimentation coefficient

E - extinction coefficient

O.D. - optical density

M.R. - molar ratio

Kr - kilorads

Bacteria:

E. coli - Escherichia coli