A STUDY OF POLYNUCLEAR AROMATIC HYDROCARBON CARCINOGEN
TRANSPORT AND DEOXYRIBONUCLEIC ACID REPAIR

DISSERTATION

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BY

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This investigation addresses the interrelated problems of A) uptake and vascular transport of lipophilic chemical carcinogens, and intracellular interactions between lipoproteins and carcinogens; B) biochemical mechanisms by which polynuclear aromatic hydrocarbon carcinogens inhibit the replicative and repair DNA synthesis in cells.

The results observed in this study suggest that ingested benzo(a)pyrene (BaP) enters the gastrointestinal lymphatic drainage sequestered within lymphatic lipoproteins, and that low-density lipoproteins (LDL) play a major role in the vascular transport of BaP. BaP is taken up into cells by adsorptive endocytosis mediated by an interaction between apolipoprotein-specific receptors on the cell membrane and the specific apolipoproteins on LDL. Having entered peripheral cells sequestered within the lipid core of LDL, an electrophilic metabolite of BaP covalently binds to cellular DNA, and may interact with other cellular macromolecules. Data presented here suggest that LDL is also
absolutely required for the activation of DNA polymerase-a, which is the major enzyme of DNA excision repair necessary to correct the DNA damage caused by BaP.

This study concludes that an active metabolite of the polynuclear aromatic hydrocarbon carcinogen, benzo(a)pyrene, suppresses DNA polymerase-a activity by inhibiting the binding of 2'-deoxyguanosine 5'-triphosphate to an acceptor site on the DNA polymerase-a complex with the DNA substrate, thereby competitively inhibiting interaction of 2'-deoxyguanosine 5'-triphosphate in the DNA synthetic process.
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CHAPTER I
AN INTRODUCTION TO POLYNUCLEAR AROMATIC HYDROCARBON CARCINOGENS

In the last two decades enormous progress has been made toward understanding the modes of action of polynuclear aromatic hydrocarbon (PAH) carcinogens and the molecular strategies employed by cells to prevent or repair carcinogen damage to cellular genetic material. Early works by Miller and Miller (1952, 1966, 1968) marked the beginning of general acceptance of the concept that most carcinogenic chemicals, and particularly the PAH, require metabolic activation by enzymes in the host tissues to forms which are ultimately mutagenic and carcinogenic. These ultimate carcinogens are, in general, highly reactive. Those which are not direct-acting are readily converted, nonenzymatically, to electrophilic forms which can covalently bind to negatively charged oxygen or nitrogen atoms in the nucleic acids, or to nitrogen or sulfur atoms in proteins (Miller, 1978).

How such interactions lead to the initiation of cancer has been, and continues to be, a major thrust of contemporary cancer research. Early investigators in the field of carcinogenesis proposed that transformation of normal cells into neoplastic cells involves a permanent, heritable,
alteration in the cell phenotype. This hypothesis suggested that the initial step of cell transformation involved the generation of a mutation, and that all neoplastic states resulting from treatment of cells with chemical carcinogens arise as the result of mutations. Accordingly, a great deal of research attention has been focused on the binding of, and subsequent chemical interactions of, carcinogen metabolites with DNA. This research has resulted in the current concept that formation of carcinogen adducts with DNA produces mutations which may function as initial critical steps in the process of cellular transformation. Although the concept of mutation as the initiation step in chemical carcinogenesis is generally accepted, a clear relationship between formation of carcinogen adducts with DNA bases and mutagenesis, stable changes in gene regulation, dedifferentiation of cells, cell transformation, and subsequent formation of a neoplastic state have not been unequivocally established. Thus, to understand the molecular mechanisms involved in chemical carcinogenesis, detailed studies of interactions of chemical carcinogens, such as the PAH, with all classes of cellular macromolecules are needed.

Benzo(a)pyrene (BaP) is widely recognized as the prototype PAH carcinogen. This compound is formed by the incomplete pyrolysis of vegetal materials and is ubiquitously distributed as a major environmental pollutant. BaP
serves researchers as a model PAH carcinogen which is known to induce neoplastic states in animals, including humans (MacLeod et al, 1980; Brookes and Osborne, 1982). BaP, in the parental form, is not a reactive compound, and is neither mutagenic nor carcinogenic. It is, however, readily metabolized by microsomal mixed function oxygenase enzymes to a variety of electrophilic forms with varying reactivity (Figure 1-1). The most mutagenic of the BaP metabolites is generated by enzymatic attack at the double bonds between the 7 and 8 carbons and between the 9 and 10 carbons, with initial formation of a trans 7,8-diol, and subsequent formation of an epoxide between the 9 and 10 carbons on the molecule (Brookes and Osborne, 1982). The epoxide is quite labile, and degrades resulting in formation of an electrophilic site at the number 10 carbon. Reaction of this electrophile with nucleophilic sites on cellular macromolecules results in formation of covalent bonds between BaP and the macromolecules (Kouri et al, 1980) (Figure 1-2).

Benzo(a)pyrene is characteristic of the PAH carcinogens in that it is extremely insoluble in water. Shu and Nichols (1980) reported that BaP in blood is found almost exclusively in the serum, and that greater than 90% of BaP in the serum is associated with serum lipoproteins (LP). Chen et al (1979) corroborated the report of Shu and Nichols with data suggesting that the majority of BaP in lipoproteins is found
FIGURE 1-1. Benzo(a)pyrene metabolism. Structures shown are metabolites that have been isolated and characterized, with the exception of the 9,10- and 7,8-epoxides, whose presence is confirmed by enzymatic diol products and nonenzymatic phenol formation. Bracketed figures are possible or probable metabolites whose existence has not been confirmed.
FIGURE 1-2. Benzo(a)pyrene diol epoxide structures and major DNA adduct. The (anti) (+/-) benzo(a)pyrene diol epoxides are shown as A and B, respectively. The (anti) form of BaP diol epoxides covalently bind to DNA forming adducts. The major adduct is formed between the diol epoxide and the C-2 exocyclic amino group of guanine.
in low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL), but that BaP can partition freely among the classes of LP. Busbee et al (1984) reported that BaP in the diet is taken into the vascular circulation sequestered within chylomicrons absorbed from the gastrointestinal system via the lacteals, that the quantity of BaP transported by serum LP is dictated by the concentration of triglycerides within the apolar core of LP, and that serum VLDL concentrations are particularly well correlated with BaP uptake and transport by serum (Yoo et al, 1984). At serum concentrations of BaP above about 100 ng/ml, the carcinogen enters cells by partitioning across the membrane (Remsen and Shireman, 1980). Movement of BaP and related apolar compounds from the vascular circulation into cells of peripheral tissues is also apparently a function of LP entry into cells by receptor mediated adsorptive endocytosis. Busbee et al (1982) reported that LDL facilitated the transport of an active BaP metabolite into human lymphocytes, resulting in the formation of BaP-DNA adducts. In this instance, the serum BaP concentration was less than 15 ng/ml, suggesting that LDL facilitation of carcinogen entry into cells may be particularly important when the carcinogen concentrations are very low.

While the role of LP in vascular transport and intracellular uptake of lipophilic compounds is beginning to be
understood, there are other activities of LP related to
carcinogenesis that are not understood. Busbee et al (1982)
reported that the electrophilic BaP metabolite, (+/-)
-t-7,\_t-8-dihydroxy-t-9,10-7,8,9,10-tetrahydrobenzo(a)pyrene
(BPDE), forms DNA adducts which are not removed by DNA
excision repair in cells which have been depleted of LP, and
that the repair capacity can be restored by adding as little
as 0.02 mg/ml of human LDL back to the cell medium. At this
time there are no coherent data to show the basis for this
phenomenon.

Although it is generally accepted that binding of
active carcinogen metabolites with DNA, and the subsequent
intervention of repair mechanisms in the removal of those
carcinogen-DNA adducts, play a central role in the carcino-
genic sequence, interactions of carcinogens with
intracellular macromolecules other than DNA does occur
resulting in inactivation of key physiological process such
as glycolysis (Wheeler, 1962), DNA replication (Detke et al,
1980), DNA repair (Cleaver, 1982), transcription and trans-
lation (Wheeler, 1967), and immune cell proliferation
(Schnizlein et al, 1982). Most, if not all, of the carcin-
gen interruption of cellular activities are apparently
associated with decreasing the activity of key enzymes.
Among these key enzymes are the enzymes associated with DNA
synthesis in eukaryotic cells, DNA polymerase-a, -b, and -g.
The DNA polymerases-a, -b, and -g can be differentiated by their size, their subcellular location, and their substrate specific inhibitors (Weissbach, 1979). DNA polymerase-a, is a tetrameric high molecular weight enzyme, (Mr=100,000), found in both the nucleus and the cytoplasm which functions as the major enzyme of DNA elongation and of long patch excision repair (Ciarrocchi et al, 1979). DNA polymerase-b has been identified as a low molecular weight enzyme, (Mr=40,000), found in the nuclei of mammalian cells. This enzyme is now considered to function in short patch, X-ray type, DNA excision repair, commonly repolymerizing from 1-5 nucleotides. DNA polymerase-g is the sole mitochondrial DNA polymerase. It is apparently found as a number of differentially active isozymes with molecular weights ranging between Mr=120,000 and Mr=320,000. The polymerization rate of these enzymes has been reported to be 30, 2.5, and 4 nucleotides/second respectively (Weissbach, 1979). The eukaryotic DNA polymerases are characteristically inhibited by different types of compounds. Polymerase-a is inhibited by aphidicolin, cytosine arabinose 5'-triphosphate (ara C), and N-ethylmaleimide (NEM). Polymerase-b is inhibited by 2',3'-dideoxythymidine 5'-triphosphate (ddTTP). Polymerase-g is inhibited by ddTTP and NEM. Of these three polymerases, only a and b are involved in repair of nuclear DNA.
Repair of chemical carcinogen-induced damage to the DNA in eukaryotic cells is a complex process involving the recognition of damage and the function of endonucleases, exonucleases, polymerases, and polynucleotide ligases, together with other factors necessary for associated changes in the higher order of structure of DNA and chromatin. Many of these steps, particularly those involving the polymerases, can be blocked by drugs that limit the nucleotide pool size, that block chain termination (ddTTP), or that directly inhibit function of the polymerases (ara C, aphidicolin, NEM). Some of the alkylating agents, including methyl methanesulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoamine are known to inhibit semiconservative DNA synthesis (Painter, 1977) and DNA repair synthesis (Freeman and Larcom, 1983), resulting in the accumulation of single strand breaks.

In this study, a series of investigations have been carried out to analyze (A) the effects of lipoproteins on the transport of polynuclear aromatic hydrocarbon carcinogens within the vascular system, (B) the requirement of lipoproteins for repair of DNA damage caused by an active metabolite of benzo(a)pyrene, and (C) the biochemical mechanism by which DNA excision repair and DNA synthesis are inhibited by an electrophilic metabolite of benzo(a)pyrene.
CHAPTER II
BENZO(a)PYRENE UPTAKE BY LYMPH: A POSSIBLE TRANSPORT MECHANISM FOR LIPOPHILIC XENOBIOTICS

INTRODUCTION

In many animals, including humans, lymphocytes circulate from tissues to lymph, through the thoracic duct to the peripheral blood vascular system, and back to the nodes of origin (Scollay et al., 1976; Hall et al., 1976). Although it is widely recognized that diet-derived lipophilic compounds are absorbed into the lymphatic circulation via the lacteals, transiting the thoracic duct to enter the blood vascular circulation (Guyten, 1976), and that a number of the lipophilic polynuclear aromatic hydrocarbons are known to adversely affect immune function in laboratory animals and in humans (Street and Sharma, 1975; Bekesi et al., 1979), the mode of exposure to lymphocytes or other immune system cells has not been carefully delineated for the majority of lipophilic xenobiotics. Mesenteric lymph node cells and circulating lymphocytes in the thoracic duct could be exposed to transiently high concentrations of xenobiotic compounds which enter the lymphatic circulation and are transported by lipoproteins.

Lipoproteins (LP) found in serum and lymph (Reichel et al., 1977), are reported to bind and transport a variety of
lipophilic compounds including the carcinogens, p-dimethyl-aminoazobenzene (Chen et al, 1979), and benzo(a)pyrene (BaP), (Busbee et al, 1982). Shu and Nichols (1979) report that 90% of BaP in blood is in the serum, and that 70% of BaP in serum is associated with serum LP. Interaction of lipophilic xenobiotics with human LP is inversely correlated to water solubility of the compounds, suggesting that lipophilic compounds do not, in fact, bind to LP, but partition into LP from the aqueous phase of serum (Maliwal and Guthrie, 1981). Busbee et al (1982) reported that benzo(a)-pyrene partitions into low density lipoproteins (LDL), and that it may be carried into lymphocytes in vitro by adsorptive endocytosis of LDL. If LP in lymph take up and transport lipophilic hydrocarbons, which may include mutagenic or cytotoxic agents, then cells of the immune system resident in mesenteric lymph nodes or circulating through the thoracic duct could be periodically subject to internalization of lymphatic LP carrying deleterious lipophilic xenobiotics. Although microsomal monooxygenase enzymes which may activate exogenous hydrocarbon compounds to highly reactive forms are induced both in vitro and in vivo in lymphocytes and phagocytic cells exposed to polynuclear aromatic hydrocarbons (McLemore et al, 1978), responses of these cells to elevated concentrations of xenobiotics carried by circulating lipo-
proteins are not documented at this time.

In this study I present data from an examination of gastrointestinal BaP uptake into sheep lymph lipoproteins collected from the thoracic duct. This system may serve as a potential model for investigating the exposure of immune system cells to high concentrations of ingested xenobiotic compounds. I have examined both absorption of BaP into the lymphatic circulation from the gastrointestinal system, and BaP partitioning into lymph lipoproteins in vitro.

MATERIALS AND METHODS

High Performance Liquid Chromatographic Separation of Lipoproteins: A high performance liquid chromatographic (HPLC) separation of lipoproteins from sheep lymph was completed using the procedures of Busbee et al (1981). The TSK-4000SW columns employed are modified silica gel size exclusion columns. Lymph samples were eluted through the HPLC column system using sodium phosphate buffer (0.2 mol/L, pH 6.8), containing 0.02% NaN₃, at 1 mL/min. Lymph components in the column effluent were detected by spectral analysis at 254 nm.

³H-BaP Association with Lipoproteins: I used the method of Busbee et al (1982), to determine partitioning of
$^3$H-benzo(a)pyrene ($^3$H-BaP) into components of sheep lymph. To measure total associated $^3$H-BaP, 500 μL of sheep lymph was added to 690 μL of phosphate buffered saline, pH 7.4, containing bovine serum albumin, 435 mg/L (BSA/PBS). To this was added 10 μL of an ethanol solution containing $^3$H-BaP (G-$^3$H benzo(a)pyrene, $5.3 \times 10^{16}$ dpm/mol, 0.375 mol/L; New England Nuclear). The preparation was vigorously vortex-mixed and was incubated for 30 min at 37° C in the dark. After incubation, the tubes were placed on ice and 200 μL of the reaction mixture was removed to measure total $^3$H-BaP (see below). To the remaining solution was added 200 μL of a charcoal/dextran suspension (6% activated, washed NORIT A and 0.06% dextran, Mr = 70,000, in PBS). The preparation was vortex-mixed, placed on ice for 30 min, centrifuged at 3000 X g for 10 min, and 200 μL of the supernatant (equivalent to 167 μL of the original incubation mixture) was carefully removed and counted. The 200 μL aliquots were added to 5 mL of Beckman Ready-Solv EP and radioactivity was determined in a liquid scintillation counter. Quenching was corrected by automatic external standardization (H-number). Counting efficiency was routinely 38-42%. The amount of $^3$H-BaP associated with a sample is expressed as the percentage of the $^3$H-BaP remaining in the supernatant after treatment with charcoal (i.e., the percentage of $^3$H-BaP bound to lymph
components and thus unavailable for adsorption to charcoal). Background levels of $^3$H-BaP in the supernatant obtained by performing the assay only in the presence of BSA/PBS were subtracted from the values determined for each sample.

Collection of Sheep Lymph: A 33 kg female sheep was surgically prepared for lymph collection by placing a cannula in the thoracic duct. The sheep was fasted for 36 hours prior to surgery, given atropine as a pre-anesthetic, and the surgical procedure performed as described in detail (Ziprin et al, 1980; Wilson et al, 1982). The basic procedure involved a thoracotomy on the right side, from which the 7th rib was removed. The thoracic duct was ligated proximally and silastic tubing was inserted 3 cm into the thoracic duct in a caudal direction. The tube was exteriorized between the 5th and 6th rib and returned to the vena cava. The animal had normal lymphatic flow. Drs. Wilson and Ziprin completed these procedures for this research.

The sheep was allowed to recover from surgery in a metabolism cart with feed and water available ad libitum. Seven days later the exteriorized portion of tubing was cut, lymph was allowed to flow continuously from the thoracic duct cannula, and equal volumes of lactated Ringer's solution were replaced via the vena cava.

To determine BaP uptake from the gastrointestinal
system into lymph, 100 uCi of $^{3}$H-BaP (New England Nuclear, 24 Ci/mmol) and 25 mg of unlabeled BaP were dissolved in 2 mL of corn oil and introduced directly into the stomach of the sheep through an abomasal cannula. Accumulated lymph was collected every 15 min. Blood samples were collected from the vena cava catheter every 30 min. Urine was collected at 1 hr intervals via a Foley urinary catheter. $^{3}$H-BaP in lymph and serum was determined radiometrically.

**Enzymatic Cholesterol Determination:** A colorimetric determination of total cholesterol in lymph samples, and in separate fractions collected from the HPLC separation of whole lymph, was completed using a Cholesterol Reagent Kit (Sigma, No. 350-A).

**RESULTS**

Lipoprotein cholesterol concentrations in fractions from an HPLC size exclusion separation of sheep lymph components may be determined colorimetrically, and are presented in Figure 2-1. This separation procedure has been characterized (Busbee et al, 1981), showing chylomicrons and very low density lipoproteins (VLDL) to elute at 6.8 minutes in a sharp peak, and low density lipoproteins (LDL) to elute in a sharp peak at 9 minutes, while the high density lipo-
FIGURE 2-1. An analysis of total cholesterol in fractions collected during the HPLC separation of lymph components. Sheep lymph, 500 uL, was separated into components by HPLC size exclusion chromatography. Multiple separations were pooled, and a colorimetric cholesterol analysis was completed on pooled fractions. Cholesterol concentrations are given in ug/mL of sample. Recovery from the column was calculated to be approximately 95%.
proteins, (HDL) elute in a broad peak between 10 and 13 minutes. The initial peak shown in Figure 2-1 is observed at the void volume, and contains both chylomicrons and VLDL. An analysis of BaP partitioning into lymph lipoproteins separated by HPLC is shown in Figure 2-2. These data were collected in a manner designed to allow initial partitioning of BaP into LP at 37° C, but to impede subsequent dynamic loss of BaP from LP by holding the preparation on ice at the time of removal of excess BaP from the aqueous environment. The profile of partitioning into lymph components shows BaP associating with components at the VLDL, LDL, and HDL elution positions. A comparison of the relative quantities of LP-cholesterol in these chromatographic fractions (Figure 2-1) with the levels of BaP which associate with LP in the fractions (Figure 2-2) suggests that while VLDL, LDL, and HDL all associate with BaP, quantitative partitioning of BaP is not equivalent for each LP class. For sheep lymph, VLDL totaled 22.85 ug of cholesterol with 15,478 dpm of BaP for an average of 34.08 pmoles of BaP/ug cholesterol; LDL totaled 6.15 ug of cholesterol with 34,136 dpm of BaP for an average of 277.54 pmoles/ug; HDL totaled 20.7 ug of cholesterol with 32,586 dpm of BaP for an average of 78.72 pmoles/ug. These figures were derived by summing total cholesterol and total BaP dpm for the HPLC elution profile frac-
FIGURE 2-2. An assessment of $^3$H-benzo(a)pyrene ($^3$H-BaP) taken up by lipoproteins in fractions of sheep lymph separated by size exclusion chromatography. Fractions of sheep lymph collected from the HPLC procedure were pooled (8 isolation procedures) and were analyzed for $^3$H-BaP uptake as given in Materials and Methods. Data are presented as $^3$H-BaP non-covalently associated with lymph components in 500 uL of each fraction.
tions of lymph known to contain either VLDL, LDL, or HDL. Although LDL comprised only 12.4% of the total LP in this lymph sample, it took up 41.5% of the BaP as shown in Figure 2-2. Efficiency of BaP uptake into either lymph or serum LP is shown in Figure 2-3. Total cholesterol in the preparation was 81.5 ug/mL. Mean BaP associated with LP was linear at 26% of the compound available over a range of BaP concentrations. BaP partitioning into LP was not, however, linear when the LP-cholesterol concentration varied. Data presented in Figure 2-4 indicate that increases in cholesterol concentration increased the efficiency of partitioning of BaP into LP from the aqueous phase. Partitioning efficiency appeared to be linear at low cholesterol concentrations, showing a plateau effect starting at a cholesterol concentration of about 25 ug/mL and increasing to about 40% partitioning efficiency at 150 ug of cholesterol per mL.

Although it is widely recognized that lipids present in ingested materials are absorbed into the mesenteric lymphatic drainage, absorption of BaP, and many other known lipophilic xenobiotics, has not been specifically delineated. I wished to know whether BaP entering the gastrointestinal tract is absorbed into the mesenteric venous system from which it enters the hepatic portal vein, or if it is absorbed into the mesenteric lymphatic drainage from
FIGURE 2-3. An examination of $^3$H-benzo(a)pyrene ($^3$H-BaP) partitioning into sheep lymph lipoproteins. $^3$H-BaP, at final concentrations between 0.5 and 12 pmoles/mL was added to sheep lymph containing 81.5 ug/mL of total cholesterol. Efficiency of binding (this is not a binding process but represents partitioning in which the dynamic loss of BaP from the lipoproteins is inhibited by decreasing the temperature of the reaction solution prior to removal of excess BaP with activated charcoal) of $^3$H-BaP to lymph lipoproteins is expressed as pmoles bound plotted against pmoles added. The average binding efficiency was calculated to be 26%.
FIGURE 2-4. An examination of $^3$H-benzo(a)pyrene ($^3$H-BaP) partitioning into sheep lymph lipoproteins as a function of cholesterol concentration of the lymph. Lymph was examined for total cholesterol concentration, was aliquoted into samples containing specified amounts of cholesterol and was analyzed for $^3$H-BaP partitioning efficiency as given in Materials and Methods. The percent of $^3$H-BaP added to each sample which remained in the lipoprotein fraction after charcoal-dextran removal of free BaP was plotted against the cholesterol concentration in each sample.
which it enters the thoracic duct. Data presented in Figure 2-5 show maximal concentrations of BaP in lymph obtained by cannulation of the thoracic duct approximately 105 min after instillation directly into the stomach. Examinations of serum samples suggest a consistent absence of BaP in blood for 6 hr after instillation of BaP into the stomach.

DISCUSSION

Lipophilic hydrocarbon transport by serum components, specifically by LP, was initially hypothesized by Chalmer (1955). Kotin et al (1959) subsequently demonstrated rapid transport of BaP to the liver by LP. Initial data suggesting that lipophilic xenobiotics may be transported by LP was supported by studies concluding that blood serves as the major transport system for carcinogens effecting cellular changes at sites other than the portal of entry and/or metabolism (Weisberger et al, 1969). A mechanism for internalization of LP-transported lipophilic compounds into cells was suggested by studies showing that LDL and VLDL, but not HDL, are bound to receptors specific for apoprotein B. HDL, lacking apoprotein B, would not be expected to bind cells with receptor sites specific for lysine/arginine rich apoproteins. Following apoprotein-specified binding of
FIGURE 2-5. An examination of BaP absorption into lymph from the gastrointestinal system. BaP (100 uCi of G-\(^3\)H-BaP, 5.3 x \(10^{16}\) dpm/mol, 0.375 mol/L; New England Nuclear; and 25 mg of unlabeled) dissolved in 2 mL of corn oil was instilled into the stomach of a sheep through an abomasal cannula. Analyses for BaP and cholesterol were completed on lymph samples collected every 15 min. Bap was determined on blood samples collected every 30 min.
lipoproteins to the coated-pit regions of cells, LP are internalized by endocytosis and are hydrolyzed releasing free and esterified cholesterol, phospholipids, and amino acids into the cell (Brown et al, 1975; Shireman et al, 1977; Goldstein and Brown, 1977; Alam et al, 1980).

Lipoprotein uptake into human lymphocytes has been reported to require this type of apoprotein-specified binding followed by endocytosis of membrane-bound lipoproteins (Strazzullo et al, 1978).

Data presented here suggest that the major categories of LP found in lymph partition lipophilic compounds from an aqueous environment. The data further suggest that LDL partitions a disproportionate ratio of available BaP when compared to the uptake by VLDL and HDL. These data readily explain a logical mechanism for transport of solubilized lipophilic compounds from the gastrointestinal system through the mesenteric lymph channels, and into the thoracic duct. Lipophilic xenobiotics which have entered the intestinal lymphatic drainage and have partitioned into lipoproteins are available to be internalized with lipoproteins into exposed cells by apoprotein-specified binding to membrane LDL receptors and absorptive endocytosis of membrane-bound LDL into cells. Busbee et al (1982) reported that LDL carrying associated $^{14}$C-BaP metabolites will enter
human lymphocytes in vitro, with subsequent binding of labeled BaP metabolites to nucleic acids. Binding to nucleic acids of a lipophilic mutagen transported into lymphocytes as a function of LDL internalization resulted in the initiation of unscheduled DNA synthesis in treated cells.

Under the conditions of this experiment, lymph containing BaP is diverted from the animal and does not enter the blood vascular circulation. The relative absence of BaP in venous blood under these conditions suggests that lipophilic xenobiotics do not enter the mesenteric venous drainage; rather, they enter the lymphatic drainage of the intestine, transit the thoracic duct, enter the blood vascular system, and are distributed to peripheral tissues prior to hepatic detoxification. The data suggest that lipoproteins are the components of lymph that take up and transport BaP. The very high concentrations of BaP in lymph would be expected to expose cells of the mesenteric lymph nodes and T-cells circulating through the thoracic duct to significantly higher concentrations of BaP than would be expected for other tissues of the body.
CHAPTER III.

HUMAN LYMPHOCYTES TREATED WITH \textit{r}-7,\textit{t}-8-DIHYDROXY-
\textit{t}-9,10-EPOXY-7,8,9,10-TETRAHYDR0BENZ0(a)PYRENE REQUIRE
LOW-DENSITY LIPOPROTEINS FOR DNA EXCISION REPAIR

INTRODUCTION

Hydrophobic xenobiotics partition into lipoproteins from an aqueous medium dependent on the relative lipid content of the lipoproteins, and on the partition coefficients of the xenobiotic compounds (Shu and Nichols, 1979; Maliwal and Guthrie, 1981). Lipoproteins in the vascular and lymphatic circulation have been implicated in the transport of lipophilic agents such as dolichol and \textit{a}-tocopherol (Kennan et al, 1977), \textit{p}-dimethylaminoazobenzene (Chen et al, 1979), and benzo(a)pyrene (Maliwal and Guthrie, 1981; Busbee et al, 1982). Busbee et al, (1982) suggested that low-density lipoproteins (LDL) may facilitate the uptake of a carcinogenic benzo(a)pyrene metabolite into human lymphocytes in vitro, ostensibly through the mechanism of adsorptive endocytosis. Internalization of LDL into cells via adsorptive endocytosis has been characterized, with LDL uptake followed by lysosomal lipoprotein hydrolysis releasing amino acids, free and esterified cholesterol, and phospholipids (Goldstein and Brown, 1974; Brown et al, 1975;
Lipid-soluble compounds, including the polynuclear aromatic hydrocarbon carcinogens, partitioned into LDL would be expected to be released into cells along with other LDL components at the time of lysosomal hydrolysis. The lack of available data concerning this proposed phenomenon is characteristic of the general absence of understanding of the interactions between LDL and cellular functions.

Serum has long been considered a necessary additive to medium used for the short-term culture of human lymphocytes, and serum lipoprotein components are known to be vital to membrane synthesis and to the mitogenic activation of lymphocytes. However, precise cellular requirements for LDL components are not fully understood. While there is a relative absence of data directly applicable to an understanding of lipoprotein interactions in DNA synthesis, this investigation has provided data to suggest that LDL, or a component(s) of LDL such as phospholipid, is necessary for the initiation of unscheduled DNA synthesis in non-mitogen-stimulated lymphocytes. This study presents the results of an investigation of lipoprotein interaction in repair of DNA in human lymphocytes treated with a carcinogenic agent. The formation of carcinogen-DNA adducts was examined in the presence and absence of LDL, and DNA excision repair, measured as $^{3}H$-thymidine ($^{3}H$-dTdr) incorporation, was
assessed in carcinogen-treated lymphocytes either depleted of, or supplemented with, lipoproteins.

MATERIALS AND METHODS

Lipoprotein Separation Using High Performance Liquid Chromatography: High performance liquid chromatographic (HPLC) separation of serum lipoproteins was completed using the procedures of Busbee et al, (1981), which employed TSK-4000SW modified silica gel size exclusion columns. Serum samples were eluted through the HPLC system using pH 6.8 phosphate buffered saline (PBS). Detection of serum components in the column effluent utilized spectral analysis at 254 nm.

Lymphocyte Isolation: Human lymphocytes were prepared from fresh venous blood by layering a mixture of heparinized 0.9% NaCl solution and blood (1:2) onto a 6% Ficoll, 10% Hypaque solution with centrifugation at 400 x g for 15 min (Rankin et al, 1980). The cell layer at the serum-Ficoll interface contained mononuclear cells which were removed by aspiration and resuspended in RPMI-1640 (Gibco H-18) medium containing an antibiotic-antimycotic (Gibco 524; penicillin, streptomycin, mycostatin). The resuspended cell preparation was centrifuged at 175 x g for 19 min, leaving most of the platelets in the supernatant, and the cell pellet was resus-
pended in RPMI-1640 containing 0.02 mg/ml of LDL cholesterol. Lymphocyte isolations were completed at room temperature. Cell counts were determined using a Neubauer bright line hemocytometer. Cells were diluted to a concentration of 1-2 x 10^6 cells/ml for treatment with mutagen dissolved in dimethylsulfoxide (0.5% maximum final DMSO concentration).

**Mutagen Binding to Cellular Components:** Binding of the mutagen, (+/-)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenz o(a)pyrene (BPDE) to cellular macromolecules was measured using human lymphocytes. Lymphocytes isolated and prepared as given above were treated with ^3^H-BPDE under specified conditions. At the end of specified time intervals cells in each 1-ml experimental sample were counted and examined for Trypan Blue exclusion and for total adenylate charge as a measure of viability. Cell samples were centrifuged at 700 x g for 5 min, supernatants were removed by aspiration, and cell pellets were resuspended in 1 ml of RPMI-1640. Cell pellets were frozen in 1-ml aliquots in RPMI-1640 at -20°C until assayed. Within 48 hr cell samples were thawed and incubated with 0.1 mg of protease (Sigma, type VI) at 37°C for 1 hr. Digested cell samples were poured onto glass fiber filters (Whatman, 934 AH presoaked in 20 mmol/L sodium pyrophosphate containing 1 mM thymidine) held in a Millipore 1225 sampling manifold. Each sample was washed twice with 5 ml of cold 10% trichloroacetic acid. The
wash from each tube was poured over its corresponding filter. Samples on the filters were washed twice with 5 ml of cold 95% ethanol and air-dried. After drying, the samples were counted in a liquid scintillation counter using Beckman Ready-Solv EP as the cocktail. DNA, with a calculated RNA contamination of about 15%, was retained on the filters (Busbee et al, 1982). The technique does not distinguish between labeled mutagen bound to DNA or to RNA. Counting efficiency was 85% for $^{14}$C and 10-12% for $^{3}$H on the filters.

$^{3}$H-Thymidine Incorporation: $^{3}$H-Thymidine (methyl-$^{3}$H, 1.22 x $10^{14}$ dpm/mmol, 0.0182 mmol/L; ICN Chemical and Radioisotope Division), was added to mutagen-treated cells in suspension to give a final concentration of $10^{-3}$ mCi/mL. The cell suspension was divided into 1 mL aliquots and incubated at 37°C under 5% CO$_2$. Each sample group was examined in triplicate. Although the lymphocytes were not mitogen activated, hydroxyurea, 5 mmol/L, was added to preclude scheduled DNA synthesis. After 2 hr of incubation, cell aliquots were removed from each sample and examined for Trypan Blue exclusion and total adenylate charge as measures of viability. Cell preparations with Trypan Blue exclusion above 95%, and with a total adenylate charge between 0.5 and 0.8, were considered to show no cytotoxicity. Cell samples were centrifuged at 700 x g for 3 min, the supernatants were removed by aspiration, and cell pellets were resuspended in
RPMI-1640 and stored frozen in 1 mL aliquots until assayed. Within 48 hr cell samples were thawed, incubated with protease (0.1 mg/mL of Sigma Type VI), and radiometrically assessed as described above.

Preparation of Carcinogen-Modified Lymphocyte DNA:
Lymphocytes treated with \( ^3 \text{H-}-\text{BPDE} \) (10^{-6} g/mL for 1 h), were washed x 3 with cold PBS. A 6 mL aliquot of cells, 1-2 x 10^6 cells/mL, was treated with sodium dodecyl sulfate (0.06 mL of a 20% stock solution), and protease (0.1 mg/mL Sigma Type VI), and incubated overnight at 37°C. Solubilized cells were extracted five times with water-saturated phenol containing 0.1% sodium dodecyl sulfate and 0.1% 8-hydroxyquinoline, and one time with chloroform:isoamyl alcohol (3:1) before the addition of 0.03 volumes of 2 mmol/L sodium acetate (pH 5.0) and precipitation x 2 with 2 volumes of cold ethanol. RNA was hydrolyzed by incubating overnight in 0.3 N NaOH at 37°C. Nucleic acid preparations were neutralized with acetic acid and chromatographed on a 2 x 19 cm Sephadex LH-20 column at 0.3 mL/min using 0.2 mmol/L NH\(_4\)CO\(_3\) as the eluent. Mutagen modified, single stranded, deoxyribonucleotides eluted in the column void volume without contamination by modified ribonucleotides, which were eluted from the column with 45% methanol (mutagen bound to RNA and mutagen bound to DNA were determined and used to calculate the mutagen-RNA contamination of DNA on glass fiber filters). Short frag-
ments of single stranded DNA which did not contain bound mutagen were recovered from the included volume of the LH 20 column. BPDE-modified DNA fractions eluting at the column void volume were pooled and dialyzed overnight against 2 L of 10 mmol/L Tris-HCl (pH 7.0) containing 1 mM MgCl₂. The dialysate was concentrated using an ultramembrane (Millipore CX-10) and recovered. Material passing through the membrane contained no ^3H-BPDE. DNA, modified by bound ^3H-BPDE, was hydrolyzed overnight at 37° C using 20 units of DNase I (Sigma) in 10 mM Tris-HCl containing 1 mM MgCl₂ (pH 7.0). A second DNA hydrolysis was completed using 1 unit of alkaline phosphatase (Sigma, type III-S) incubated overnight at 37° C in 10 mM Tris-HCl (pH 8.5). The digest of deoxyribonucleic acid was concentrated over flowing N₂ gas from a volume of 2.5 mL to a volume of 0.6 mL, and was applied to an LH-20 column. Nucleosides were eluted from the column with 80% methanol. Elution fractions containing ^3H-BPDE modified nucleosides were combined and concentrated under flowing N₂ gas to a total volume of 0.1 mL, and were examined by HPLC using a system modified from that of Jeffrey et al.,(1977), which employed an ALTEX Ultrasphere 4.6 x 250 mm ODS 0.005 mm column eluted with 45% methanol at 50°C and 2,000 psi. Elution of modified nucleosides was radiometrically determined.
RESULTS

Human lymphocytes, held 24 hr in RPMI-1640 to deplete lipoproteins, bound slightly greater levels of $^{14}$C-BPDE to cellular DNA than did lymphocytes held in RPMI-1640 supplemented with human LDL at 0.02 mg/ml (Figure 3-1). These carcinogen-treated cells were assessed for $^3$H-dThd incorporation, as a measure of DNA excision repair, in PBS without LDL. Lymphocytes depleted of lipoproteins showed no $^3$H-dThd incorporation, while cells held 24 hr in the presence of LDL exhibited incorporation of $^3$H-dThd into DNA concomitant with a reduction in $^{14}$C-BPDE bound to DNA (Figure 3-1). Each point represents the average of three values from each of two separate experiments (n=6).

It was necessary to determine if decreased $^3$H-dThd incorporation into cells depleted of lipoprotein and treated with BPDE was a function of differing DNA damage in the presence or absence of lipoproteins. Human lymphocytes were held in RPMI-1640 for 24 hr in the absence of lipoproteins or in the presence of LDL at 0.02 mg/ml. These cells were treated with 0.01 mg/ml of $^3$H-BPDE, a carcinogen concentration which was determined to inhibit DNA repair in human lymphocytes (Figure 5-1). DNA isolated from BPDE-treated cells was chromatographed through a Sephadex LH-20 column. The major peak of single-stranded DNA eluted at the void volume, with detection showing coincidence of the spectral
FIGURE 3-1. Binding of BPDE to DNA, and incorporation of $^3$H-thymidine ($^3$H-Tdr) as a measure of DNA repair, in lymphocytes. Lymphocytes were held in medium with or without 0.02 mg/ml of human low density lipoprotein for 24 hr prior to exposure of the cells to carcinogen, at a final concentration of 400 ng/ml, in phosphate buffered saline (PBS). Cells were held in the presence of carcinogen for 1 hr, washed in PBS, and resuspended in PBS containing $^3$H-Tdr. Cell aliquots were examined at 1 hr intervals for the presence of carcinogen bound to DNA, and for $^3$H-Tdr incorporated into DNA as a function of time. $^{14}$C-Carcinogen bound to DNA is given as (●) for lipoprotein-depleted cells, and as (○) for LDL-supplemented cells. $^3$H-Thymidine incorporation is given as (▲) for lipoprotein-depleted cells, and as (△) for LDL-supplemented cells.
FIGURE 3-2. The elution of BPDE-modified human lymphocyte DNA from an LH-20 column. Non-mitogen-activated human lymphocytes held 24 hr in RPMI-1640 in the presence or absence of autologous LDL were treated with BPDE at a final concentration of 0.01 mg/ml. Carcinogen-modified DNA was isolated, applied to an LH-20 column, and eluted with 0.02 M NH₄CO₃. The eluate was spectrally monitored at 260 nm and 0.25 ml elution fractions were collected. Carcinogen present in each fraction was radiometrically determined by counting 0.01 ml of each 0.25 ml fraction.
and radiometric values (Figure 3-2). When cells were treated with 400 ng/ml BPDE, DNA from LDL-depleted cells bound slightly more carcinogen than did DNA from LDL-supplemented cells (Figure 3-1). DNA isolated from lipoprotein-depleted or lipoprotein-supplemented cells showed similar 260 nm absorbance unit/$^3$H-BPDE cpm ratios when treated with BPDE at 0.01 mg/ml, suggestive of similar carcinogen binding to DNA at high carcinogen concentrations in the presence or absence of lipoproteins. In each instance a second peak of short DNA strands present in the included column volume showed no $^3$H-BPDE binding. DNA isolated from cells not treated with carcinogen exhibited only the void volume peak. Protein could not be detected by Bradford assay (Bradford, 1976) in either the void volume or included volume peaks seen in Figure 3-2. RNA, which was hydrolyzed during the procedure, eluted as ribonucleotides and contained approximately 15% of the total bound $^3$H-BPDE. Modified deoxynucleosides from lipoprotein-depleted or lipoprotein-supplemented lymphocytes contained similar concentrations of $^3$H-BPDE in fractions obtained by the hydrolysis of equivalent DNA quantities. The two samples of carcinogen modified deoxynucleosides were compared by high performance liquid chromatography, showing elution profiles with no significantly distinguishable differences (Figure 3-3).
FIGURE 3-3. The high performance liquid chromatographic elution profile of carcinogen-modified deoxyribonucleosides prepared from human lymphocyte DNA treated with BPDE. Human lymphocytes were held 24 hr in RPMI-1640 medium with or without serum supplementation, and were treated with carcinogen at 0.01 mg/ml. Carcinogen-modified DNA was isolated, applied to an LH-20 column (see Figure 3-2), collected from the void volume of the column, and digested using DNase I and alkaline phosphatase. Deoxynucleosides were rechromatographed on an LH-20 column with collection of the carcinogen-modified nucleosides. Fractions containing radioactivity were evaporated under flowing N₂ and analyzed by HPLC. Nucleosides from LDL-supplemented or lipoprotein-depleted cells were chromatographed independently. Each sample showed a minor peak at fraction 27 and a major peak at fraction 31, the peaks being of equivalent amplitude.
I wished to determine whether lipoprotein-depleted lymphocytes, having lost the capacity to repair DNA, could regain DNA excision repair capacity if they were resupplemented with LDL. LDL-depleted cells were treated with BPDE and supplemented with LDL at 0.02 mg/ml for 1 hr prior to the addition of $^3$H-dThd. Cells received 5 mM hydroxyurea to preclude scheduled DNA synthesis. Lipoprotein-depleted lymphocytes which did not receive LDL showed $^3$H-dThd incorporation which was not significantly different from control cells with or without hydroxyurea, and not distinguishable from control cells treated with carcinogen at a concentration which precludes DNA excision repair. Lipoprotein-depleted lymphocytes treated with BPDE at 500 ng/ml and supplemented with 0.02 mg/ml of LDL showed an 8-fold $^3$H-dThd incorporation above the control level (Figure 3-4). Each point represents the average of three values from two experiments ($n=6$).

DISCUSSION

In these in vitro experiments, lipoprotein-depleted human lymphocytes were not found to engage in $^3$H-dThd incorporation following treatment with an electrophilic, highly reactive metabolite of benzo(a)pyrene (BPDE), while lipoprotein-supplemented lymphocytes incorporated $^3$H-dThd under
FIGURE 3-4. Effects of lipoprotein depletion on $^3$H-thymidine incorporation into DNA of carcinogen-treated lymphocytes. Lymphocytes isolated and held 24 hr in medium without serum supplementation were treated with BPDE and $^3$H-thymidine incorporation into cellular DNA was assessed. Control cells remained lipoprotein-depleted, while test cells received human LDL at 0.02 mg/ml final concentration. Hydroxyurea, 5 mM, was added to the cell preparations 1 hr prior to $^3$H-thymidine addition to preclude any possible scheduled DNA synthesis in these non-mitogen-stimulated cells.

- (●) Hydroxyurea, BPDE, and LDL.
- (○) Hydroxyurea and BPDE, no LDL.
- (□) BPDE and LDL, no hydroxyurea.
- (■) Hydroxyurea, 10 mM, and BPDE, no LDL.
the same conditions. Several possible explanations for this phenomenon include: (A) the active carcinogen binds the cellular DNA in a qualitatively or quantitatively different manner in the presence or absence of lipoproteins; (B) human lymphocytes require lipoproteins for the internalization of exogenously available thymidine; or (C) human lymphocytes require lipoproteins in order to engage in excision repair of DNA damaged by carcinogens.

Results presented here suggest that $^3$H-BPDE reacts similarly with human lymphocytes in the presence or absence of lipoproteins, resulting in binding of the carcinogen to lymphocyte DNA. Enzymatic hydrolysis of the modified DNAs resulting from carcinogen treatment of lymphocytes in the presence or absence of lipoproteins produced major $^3$H-BPDE-modified deoxynucleosides which were chromatographically indistinguishable. The nucleoside adducts were not specifically identified; however, comparison of their retention times with published HPLC profiles of DNA adducts formed by cells incubated with $^3$H-BaP suggests the major adduct in each instance to be a deoxyguanosine designated dG1-3 by Jeffrey et al (1977). This adduct is formed by covalent binding between the BaP C-10 and the C-2 exocyclic amine of guanosine.

Busbee et al, (1982) determined that both lipoprotein-depleted and undepleted lymphocytes treated with BPDE take
up $^3$H-dThd from the medium. In that study cells depleted of lipoproteins exhibited a cytoplasmic $^3$H-dThd pool slightly greater than that of undepleted cells. Discounting differences in BPDE-DNA adduct formation and in differential cellular $^3$H-dThd uptake as explanations for the absence of $^3$H-dThd incorporation into DNA in lipoprotein-depleted cells treated with carcinogen, I assessed the potential requirement for LDL in cells engaged in excision repair. Human lymphocytes depleted of lipoproteins did not incorporate $^3$H-dThd into DNA in response to treatment with a known carcinogenic agent. Lipoprotein-depleted lymphocytes which were treated with carcinogen and then supplemented with 0.02 mg/ml LDL did incorporate $^3$H-dThd into cellular DNA. Scheduled DNA synthesis occurring in response to LDL supplementation or to the cell-holding procedure was not a factor, since the cells were treated with hydroxyurea to preclude scheduled DNA synthesis.

Data presented in Figure 3-2 do not resolve the question of the included volume DNA peak which was not labeled with $^3$H-BPDE and which was not present in control DNA. DNA shown in this figure was isolated from lymphocytes treated with 0.01 mg/ml BPDE, a carcinogen concentration which inhibits unscheduled DNA synthesis. The absence of $^3$H-BPDE in the peak of short DNA strands could be explained by incomplete DNA repair. If the DNA were nicked by endonuclease and
an oligonucleotide were removed by exonuclease without subsequent resynthesis of the excised segment of nucleotides, and if the DNA were then converted from the double stranded to the single stranded state, the fragmented pieces of DNA would be expected to be unlabeled with $^3$H-BPDE. This explanation would be valid if the polymerase which accomplishes repolymerization were inactive (in this instance the conditions of the experiment cause inhibition of this enzyme).

These results indicate that lipoproteins may be required by human lymphocytes in order to initiate $^3$H-dThd incorporation via excision repair of carcinogen-damaged DNA. Only human low-density-lipoprotein was examined in this study. The nature of LDL interaction in DNA excision repair is not addressed, neither is the question of whether the requirement is for intact LDL or for an LDL component such as phospholipid. These questions will be addressed in continuing investigations.
CHAPTER IV
DNA POLYMERASE ALPHA FROM HUMAN LYMPHOCYTES REQUIRES LOW DENSITY LIPOPROTEINS FOR ACTIVATION

INTRODUCTION

Investigations of the mechanisms of chemical carcinogenesis have shown clear evidence that cell transformation may be correlated with chronic exposure to coal tar and related products (Hammond et al, 1976; Fysh et al, 1980), and is observed in persons who habitually ingest smoked foods (Falk and Kotin, 1965), and who smoke tobacco products (Hammond et al, 1975; Steinfeld, 1971; Sterling, 1975). These xenobiotic sources have in common the presence of complex mixtures of polynuclear aromatic hydrocarbons (PAH), such as benzo(a)-pyrene (BaP). A number of the PAH, including BaP, are enzymatically metabolized to mutagenic forms known to bind DNA, forming mutagen-DNA adducts which have been well characterized (Thakker et al, 1976; Varanasi and Gmur, 1980). BaP-DNA adducts are removed by excision, with resynthesis of excised oligonucleotides by DNA polymerases-a and -b (Miller and Chinault, 1982).

Busbee et al (1982) and Joe et al (1984) reported that lipoprotein (LP) depleted human lymphocytes do not incorporate $^3$H-thymidine ($^3$H-Tdr) as a function of DNA excision repair following treatment with the electrophilic benzo(a)-
pyrene metabolite, (+/-) r-7, t-8-dihydroxy- t-9,10-
epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE). The absence
of $^3$H-Tdr incorporation in mutagen treated cells could be
due to any number of phenomena, including (A) LP-depleted
cells do not make the same DNA-mutagen adducts found in
non-LP-depleted cells, (B) LP-depleted cells do not take up
$^3$H-Tdr from the medium, (C) BPDE is more toxic to lympho-
cytes in the absence of LP than in their presence, or (D)
enzymatic DNA repair processes do not function in LP-de-
pleted cells. In this paper I present data showing that DNA
polymerase-a does not function under the physiological
condition of lipoprotein depletion.

MATERIALS AND METHODS

Lipoprotein Separation Using High Performance Liquid
Chromatography: High performance liquid chromatographic
(HPLC) separation of serum lipoproteins was completed using
the procedures of Busbee et al (1981), which employed TSK
modified silica gel size exclusion columns. Serum samples
were eluted through a TSK125 guard column (BioRad) coupled
in series with a TSK4000SW (Beckman) analytical column using
pH 6.8 phosphate buffered saline (PBS) at 1 ml/min at a
column pressure which did not exceed 100 psi. Detection of
serum components in the column effluent utilized spectral
analysis at 254 nm.

**Lymphocyte Isolation:** Human lymphocytes were prepared from fresh venous blood using the ficoll-Hypaque centrifugation technique of Busbee et al (1980). Fresh venous blood was mixed (2:1) with sterile normal saline containing 50 units of heparin/ml (sodium salt, grade II, Sigma), and was layered carefully onto the surface of Hypaque solution (sodium diatrizoate, saturated solution, Winthrop Laboratories) containing 6% Ficoll in flat bottom 9 dram vials. The discontinuous gradient of blood and Ficoll-Hypaque was centrifuged at 1,000 x g for 15 min, and mononuclear cells were aseptically removed from the serum/Ficoll-Hypaque interface. Cells were washed once in sterile saline with heparin, and were gently sedimented in a conical plastic centrifuge tube at 175 x g for 20 min. Cells were diluted to a concentration of 1-2x10^6 cells/mL in phosphate buffered saline (PBS) for treatment with mutagen dissolved in dimethylsulfoxide (0.5% maximum final DMSO concentration).

**Mutagen Binding to Cellular Components:** Mutagen binding to cellular macromolecules was measured using human lymphocytes isolated and prepared as described above, and treated with BPDE. At the end of appropriate time intervals cells in each 1 mL experimental sample were counted and examined for Trypan Blue exclusion and for total adenylate charge (Chapman et al, 1971) as a measure of viability. Cells were
centrifuged at 175 x g for 10 min and supernatants were removed by aspiration. Cell pellets were resuspended in RPMI 1640 and were frozen in aliquots of 1 mL at -20° C until assayed. Within 48 h cell samples were thawed and incubated with 0.1 mg of protease (Sigma, type XI) at 37° C for 1 h. Digested cell samples were poured onto glass fiber filters (Whatman grade 934 AH, presoaked in 20 mmol/L sodium pyrophosphate containing 1 mmol/L thymidine) held in a Millipore 1225 sampling manifold. Each sample was washed twice with 5 mL of cold 10% trichloroacetic acid and twice with 5 mL of cold 95% ethanol. After drying, samples were counted in a liquid scintillation counter using Beckman Ready-Solv EP as the cocktail. DNA, with a calculated RNA contamination of less than 15%, was retained on the filters. Counting efficiency was approximately 85% for 14C and 10-12% for 3H on the filters.

3H-Thymidine Incorporation: 3H-Thymidine (methyl-3H, 1.22 x 10^{14} dpm/mmol, 0.0182 mmol/L; ICN Chemical and Radioisotope Division), was added to mutagen treated cells in suspension to give a final concentration of 10^{-3} mCi/mL. The cell suspension was divided into 1 mL aliquots and incubated at 37° C under 5% CO2. Each sample group was examined in triplicate. Hydroxyurea, 2 mmol/L, was added to preclude scheduled DNA synthesis. After 2 hr of incubation, cell aliquots were removed from each sample and examined for
Trypan Blue exclusion and total energy charge as measures of viability. Cell preparations with Trypan Blue exclusion above 95%, and with a total adenylate charge between 0.5 and 0.8, were considered to show no cytotoxicity. Cell samples were centrifuged at 700 x g for 3 min, the supernatants were aspirated, cell pellets were resuspended in PBS and stored frozen in 1 mL aliquots until assayed. Within 48 hr cell samples were thawed, incubated with protease (0.1 mg/mL of Sigma Type XI, Clostridium protease mixture), and radiometrically assessed as previously described.

Preparation of Carcinogen-Modified Lymphocyte DNA:
Lymphocytes treated with $^3$H-BPDE ($10^{-6}$ g/mL for 1 h), were washed x 3 with cold PBS. A 6 mL aliquot of cells, 1-2 x $10^6$ cells/mL, was treated with sodium dodecyl sulfate (0.06 mL of a 20% stock solution), and protease (0.1 mg/mL Sigma Type XI), and incubated overnight at 37° C. Solubilized cells were extracted five times with water-saturated phenol containing 0.1% sodium dodecyl sulfate and 0.1% 8-hydroxyquinoline, and one time with chloroform:isoamyl alcohol (3:1) before the addition of 0.03 volumes of 2 mmol/L sodium acetate (pH 5.0) and precipitation x 2 with 2 volumes of cold ethanol. RNA was hydrolyzed by incubating overnight in 0.3 N NaOH at 37° C. Nucleic acid preparations were neutralized with acetic acid and chromatographed on a 2 x 19 cm Sephadex LH 20 column at 0.3 mL/min using 0.2 mmol/L NH$_4$CO$_3$
as the eluent. Mutagen modified, single stranded, DNA eluted in the column void volume without contamination by modified ribonucleotides, which were eluted from the column with 45% methanol. Short fragments of single stranded DNA which did not contain bound mutagen were recovered from the included volume of the LH 20 column. BPDE-modified DNA fractions eluting at the column void volume were pooled and dialyzed overnight against 2 L of 10 mmol/L Tris-HCl (pH 7.0) containing 1 mmol/L MgCl₂. The dialysate was concentrated using an ultramembrane (Millipore CX-10) and recovered. Material passing through the membrane contained no ³H-BPDE. DNA, modified by bound ³H-BPDE, was hydrolyzed overnight at 37°C using 20 units of DNase I (Sigma) in 10 mmol/L Tris-HCl containing 1 mmol/L MgCl₂ (pH 7.0). A second DNA hydrolysis was completed using 1 unit of alkaline phosphatase (Sigma, type III-S) incubated overnight at 37°C in 10 mmol/L Tris-HCl (pH 8.5). The digest of deoxynucleic acid was concentrated over flowing N₂ gas from a volume of 2.5 mL to a volume of 0.6 mL, and was applied to an LH 20 column. Nucleosides were eluted from the column with 80% methanol. Elution fractions containing ³H-BPDE modified nucleosides were combined and concentrated under flowing N₂ gas to a total volume of 0.1 mL, and were examined by HPLC using a system modified from that of Jeffrey et al (1977), which employed an ALTEX Ultrasphere 4.6 x 250 mm ODS 5 um column
eluted with 45% methanol at 50°C under 2,000 psi pressure.
Elution of modified nucleosides was determined radio-
metrically.

**Sucrose Gradient Analysis of Lymphocyte DNA:** Prepara-
tions of lymphocyte DNA were resuspended in 0.3 N NaOH
containing 0.7 mmol/L NaCl and 1 mmol/L EDTA, and were held
overnight to hydrolyze RNA. The DNA preparation was layered
onto a 5% to 20% exponential gradient of sucrose prepared in
the same buffer. One h after layering DNA onto the gradient
surface, preparations were centrifuged at 11,000 rpm for 16
hr using a Beckman SW-41 rotor. Gradients were eluted using
a gradient eluting device of designed and built in this
laboratory which gave fractions beginning with the surface
(5% sucrose). SV40 DNA, forms I and II, were used as
molecular size markers.

**DNA Polymerase Determinations:** To a sample of packed
lymphocytes, about $10^7$ cells, was added 1 mL of 0.25 mmol/L
sucrose, 50 mmol/L Tris-HCl (pH 8.0), 25 mmol/L KCl, and 7.5
mmol/L MgCl$_2$. Cells were disrupted by 35 passes of a Teflon
pestle in a glass homogenizer. To the cell homogenate we
added 1 mL of 50 mmol/L Tris-HCl (pH 8.0) containing 7.5
mmol/L MgCl$_2$. The suspension was centrifuged for 1 hr at
35,000 rpm using a Beckman Ti-50 rotor to precipitate DNA.
After this step the polymerase assays were completed indep-
endently. DNA polymerase-a was assayed using incorporation
of $^3$H-dTTP (20 Ci/mmol, ICN) into activated DNA. To make activated polymerase substrate salmon sperm DNA was digested using pancreatic DNase I (Sigma) until 25% of the DNA was acid soluble. The remaining DNA was repurified by phenolic extraction and dialysis against 50 mmol/L Tris-HCl containing 7.5 mmol/L MgCl$_2$ (Schlabach et al, 1971). I added 0.02 mL of the centrifuged cell homogenate to a reaction mixture containing 50 mmol/L Tris-HCl (pH 8.2), 0.5 mmol/L dithiothreitol (DTT), 100 ug of activated DNA, 70 ug BSA, and 0.05 mmol/L each of all 4 dNTP, only one of which, dTTP, was labeled (Thymidine-5'-triphosphate, tetrasodium salt; methyl-$^3$H, 45 Ci/mmol; ICN). The reaction mixture was incubated at 37°C for 1 h and 5 mL of cold TCA was added. The mixture was poured onto Whatman glass fiber filters (934 AH), and was washed with 5 mL x 2 of 10% TCA and 5 mL x 2 of cold 95% ethanol. Filters were counted in a liquid scintillation counter with a counting efficiency of 10-12% for tritium. The DNA polymerase-b assay was essentially the same as the polymerase-a assay. The differences were that the polymerase-b assay used 10 mmol/L N-ethylmaleimide instead of DTT, and the pH was 8.0 instead of 8.2. Both polymerase assays were completed at 0°C (Edenberg et al, 1978).
RESULTS

Human lymphocytes held 24 hr in medium without serum supplementation remain viable, but do not respond to mitogen stimulation and do not initiate DNA excision repair when treated with a direct acting carcinogen (Busbee et al, 1982). Although Busbee et al (1982) showed that DNA synthesis was not initiated in LP-depleted lymphocytes, they did not address whether or not DNA polymerase-a was involved in these processes. In this study a series of experiments were designed to determine if the initiation of DNA synthesis in carcinogen treated lymphocytes was correlated with de novo synthesis of DNA polymerase-a, or with DNA polymerase-a activation.

When LP-depleted lymphocytes were exposed to BPDE, the level of mutagen binding to DNA was linearly correlated to the log BPDE concentration in the medium (Figure 4-1, right panel), but the cells did not incorporate $^3$H-Tdr as a function of unscheduled DNA synthesis. Human lymphocytes held 24 hr in low density lipoprotein (LDL) supplemented medium did not show linear binding of BPDE to DNA correlated to the mutagen concentration (Figure 4-1, left panel); rather, at a BPDE concentration of $4 \times 10^{-7}$g/mL they exhibited binding to DNA at about 35% of the level seen for BPDE binding to DNA in LP-depleted cells. Incorporation of $^3$H-Tdr into DNA was observed in human lymphocytes treated with BPDE if the cells
FIGURE 4-1. An examination of BPDE binding to DNA with resultant $^3$H-thymidine ($^3$H-Tdr) incorporation as a measure of excision repair in lipoprotein-depleted or non-depleted human lymphocytes. Lymphocytes were held 24 h in RPMI 1640 with or without 10% serum supplement, were treated with BPDE for 1 h, and were allowed to repair for 3 h in the presence of $^3$H-Tdr. The left panel shows serum supplemented cells, the right panel shows LP-depleted cells. DNA excision repair was assessed by $^3$H-Tdr incorporation in mutagen treated cells (○). Mutagen-DNA adduct formation was determined as a measure of $^{14}$C-$(+\text{-})$-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) which was bound to DNA from the treated cells (●).
were supplemented with LDL during the 24 hr holding period, with maximal incorporation occurring at a BPDE concentration of $4 \times 10^{-7}$ g/mL.

The absence of $^3$H-Tdr incorporation into DNA in LP-depleted cells treated with BPDE could be a function of differences in mutagen binding to DNA in the presence or absence of LP. To assess this, lymphocytes were treated with $^3$H-BPDE at $10^{-5}$ g/mL, a concentration of mutagen which totally suppressed DNA repair. Mutagen modified DNA from these cells was purified and hydrolyzed, and the $^3$H-BPDE labeled deoxynucleoside fractions were collected and analyzed. HPLC profiles of modified deoxynucleosides from LP-depleted or LP-supplemented lymphocytes are shown in Figure 4-2. The profiles show no chromatographically detectable differences in BPDE-DNA adducts correlated with the presence or absence of LP, and are consistent with profiles reported for BPDE-modification of guanosine at the #2-carbon exocyclic amino group (Jeffrey et al, 1977).

Having determined that BPDE-DNA adducts from lymphocytes are not chromatographically distinguishable without respect to their state of lipoprotein depletion, and considering the literature report showing that $^3$H-Tdr is taken up into the thymidine pool in both LP-depleted and LP-supplemented lymphocytes (Busbee et al, 1982), and considering data showing that LP-depleted lymphocytes treated with
FIGURE 4-2. A comparison of the mutagen-DNA adducts formed in LP-depleted or non-LP-depleted human lymphocytes treated with $^3$H-BPDE. Cells were treated with $^3$H-BPDE, DNA was hydrolyzed, mutagen modified nucleosides were recovered and compared using high performance liquid chromatographic elution from an ODS column as given in Materials and Methods.
mutagen are viable, it was assumed that the differences in DNA repair between LP-depleted and LP-supplemented cells are unexplained. Studies were initiated to examine processes of excision repair affected by LP depletion. Since mutagen treated DNA is known to be fragmented as a function of the number of mutagen adducts removed when the excised oligonucleotide is not repolymerized, an initial examination was completed to determine the fragmentation of mutagen treated DNA in an alkaline sucrose gradient.

To assure that shearing during the isolation of lymphocyte DNA did not result in fragmented DNA, an alkaline sucrose gradient analysis of DNA strand length was completed showing that the technique utilized here did not decrease the DNA strand length below that of control DNA. Cells grown in the presence of $^3$H-Tdr were lysed on the gradient surface and sedimentation was determined showing undamaged DNA to peak at fraction 15. DNA obtained by a phenolic extraction of proteinase-lysed lymphocytes was also applied to a gradient and sedimented with the peak at fraction 15. A comparison of the two profiles suggested that mechanical fragmentation of lymphocyte DNA was minimal during our DNA isolation procedure.

Sucrose gradient analyses were completed on DNA isolated from LP-depleted or LDL supplemented lymphocytes treated with BPDE and allowed to reamir in the presence of
$^3$H-Tdr. DNA from LDL supplemented cells showed $^3$H-Tdr peaks at both fraction 15, where one would expect to find unfragmented DNA, and at fraction 6, where one would find short fragments of DNA. There was no $^3$H-Tdr peak in the LP-depleted lymphocyte DNA at fraction 15, suggesting an absence of intact, repaired, DNA. DNA from LP-depleted lymphocytes showed two small peaks of $^3$H-Tdr at fractions 4 and 10 (Figure 4-3, upper panel). The small $^3$H-Tdr labeled peak of DNA from LP-depleted cells seen at fraction 4 (Figure 4-3, upper panel) corresponds to major peaks of fragmented DNA isolated from lymphocytes exposed to BPDE and treated with aphidicolin, a known DNA polymerase-a inhibitor (Figure 4-3, lower panel). DNA isolated from LP-depleted lymphocytes treated with BPDE and aphidicolin showed relatively little $^3$H-Tdr incorporation (Figure 4-3, middle panel), presented a 260 nm absorption profile showing a major peak at fraction 4 corresponding to fragmented DNA, and exhibited no undamaged DNA at fraction 15 (Figure 4-3, lower panel). A comparison between $^3$H-Tdr incorporation into DNA of BPDE-treated lymphocytes either depleted of lipoproteins or treated with aphidicolin (Figure 4-3, middle panel) shows the suggestion of some DNA repair in aphidicolin-treated cells, but virtually no suggestion of repair in LP-depleted cells. Aphidicolin treatment (inhibition of DNA polymerase-a) essentially eliminated any differences between profiles of DNA sedimentation
FIGURE 4-3. An alkaline sucrose gradient analysis of strand length and $^3$H-thymidine ($^3$H-Tdr) incorporation in DNA from human lymphocytes.

Upper panel: A comparison of $^3$H-Tdr incorporation into DNA from BPDE treated lymphocytes which were depleted of lipoproteins (▲), or LDL supplemented (●), prior to mutagen treatment.

Center panel: A comparison of $^3$H-Tdr incorporation into DNA of BPDE-treated lymphocytes when one sample was lipoprotein-depleted (▲), and one sample was LDL supplemented but was treated with aphidicolin at 10 ug/mL (●).

Lower panel: A comparison of the A 260 nm profiles of sedimentation of DNA from BPDE-treated lymphocytes. One lymphocyte sample was lipoprotein-depleted (▲), the other was LDL supplemented and treated with aphidicolin (●).
or of $^3$H-Tdr incorporation due to lipoprotein depletion. When LDL supplemented, BPDE treated, lymphocytes were allowed to repair in the presence of $^3$H-Tdr, the fragmented DNA peak at fraction 4 was shifted to the right, indicative of increasing strand length, and showed an increase in $^3$H-Tdr incorporation, as seen in the peak at fraction 6 in the upper panel of figure 4-3.

The accumulation of fragmented DNA in LP-depleted lymphocytes suggested an alkaline sucrose gradient distribution of nucleic acid similar to that obtained for DNA isolated from cells in which DNA polymerase-a had been inhibited by aphidicolin. To assess this we completed direct assays of a and b polymerase in LP-depleted and LDL supplemented cells treated with BPDE (Figure 3-4). When lymphocytes were depleted of LP they showed no increase in polymerase-a activity during the 8 hr incubation interval after BPDE treatment, while LDL supplemented lymphocytes showed an increase in polymerase-a activity peaking at 3 hr after BPDE treatment. DNA polymerase-b did not appear to be influenced by LP-depletion prior to BPDE treatment. Data in figure 4-4 suggested the possibility that polymerase-a activity was induced in cells treated with BPDE. In order to determine whether the enzyme was induced by BPDE treatment, or was present in an inactive form and was in some way activated by BPDE treatment, lymphocytes were treated with BPDE, BPDE
FIGURE 4-4. An examination of DNA polymerases-a and -b in human lymphocytes which had been depleted of lipoproteins or supplemented with LDL prior to BPDE treatment. A unit of polymerase is defined as the amount necessary to give 1 n mole of dTTP incorporation in one hour of incubation.
and cycloheximide, or cycloheximide alone, and assays for both polymerases were completed after 3 hr (Figure 4-5). Polymerase-a activity was slightly higher in the presence of both BPDE and cycloheximide than in the presence of BPDE alone, suggesting that inhibition of protein synthesis did not interfere with the initiation of polymerase-a activity following mutagen treatment. DNA polymerase-b data were inconclusive and were not consistent between experiments, a factor attributable to the insensitivity of the polymerase-b assay; however, the data did suggest that inhibition of protein synthesis in cells treated with BPDE decreased the measureable DNA polymerase-b levels.

DISCUSSION

The apparent inhibition of unscheduled DNA synthesis (UDS) in lipoprotein depleted human cells was originally reported as a preliminary observation in lymphocytes treated with a mutagenic BaP metabolite (Busbee et al, 1982; Joe et al, 1984). Several mechanisms could be postulated to explain the inhibition of UDS in LP-depleted cells. The absence of LP could inhibit the entry of lipophilic mutagens into cells, or could interfere in some way with mutagen binding to DNA. Cells depleted of LP could be deficient in thymidine pools or in $^{3}$H-Tdr uptake from the medium, or BPDE
FIGURE 4-5. An examination of the requirement for protein synthesis in the induction of DNA polymerases-a and -b activity. Human lymphocytes were treated with (+/-)-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE), BPDE+cycloheximide, or cycloheximide alone, and examined for enzyme activity over a three hr period following treatment. A unit of polymerase is defined as the amount necessary to give 1 nmole of dTTP incorporation in one hour of incubation.
could be more toxic to LP-depleted cells, killing the cells. Lastly, there could be an effect of LP depletion on excision repair of DNA.

Busbee et al (1982) reported that $^{3}\text{H-Tdr}$ uptake and total thymidine pools in lymphocytes depleted of LP were similar to those of non-LP-depleted cells. Even though intracellular thymidine was available for repair, no $^{3}\text{H-Tdr}$ incorporation would be expected if BPDE-DNA adducts were not formed in LP-depleted cells; however, the formation of BPDE-DNA adducts was found to be greater in LP-depleted lymphocytes than in non-depleted lymphocytes. Even though BPDE-DNA adducts were chromatographically indistinguishable between the two differing physiological conditions in lymphocytes, LP-depleted cells showed no $^{3}\text{H-Tdr}$ incorporation while non-depleted cells showed $^{3}\text{H-Tdr}$ incorporation dependent on the concentration of BPDE to which they were exposed. BPDE at $4 \times 10^{-7}$ g/mL initiated optimal $^{3}\text{H-Tdr}$ incorporation in LDL supplemented lymphocytes while $^{3}\text{H-Tdr}$ incorporation in LP-depleted cells treated with the same concentration of BPDE was below control levels. LP-depleted cells were not killed by BPDE concentrations as high as $10^{-5}$ g/mL, evidenced by an adenylate charge between 0.7 and 0.8, and by the exclusion of Trypan Blue.

Having resolved the questions of carcinogen entry into cells with subsequent binding to DNA, of cell death due to
BPDE toxicity, of $^3$H-Tdr uptake from the medium, and of differential BPDE-DNA adduct formation in LP-depleted or non-depleted lymphocytes, it was decided that the processes of $^3$H-Tdr incorporation in lymphocytes during repair of DNA damage should be examined. Joe et al. (1984) reported that inhibition of UDS in LP-depleted cells was reversible upon addition of LDL to the cell medium. This suggested the possibility that LP depletion interferes either directly or indirectly with some enzymatic process during excision repair of DNA. When DNA was isolated from LP-depleted or LDL supplemented cells incubated in the presence of $^3$H-Tdr following BPDE treatment the DNAs showed different sucrose gradient sedimentation profiles. LP-depleted cells exhibited fragmented DNA with very little $^3$H-Tdr incorporation. This suggested that DNA had been damaged and that repair had not been completed subsequent to the damage. Joe et al (1984) suggested that cells depleted of LP prior to BPDE treatment remove $^3$H-BPDE-DNA adducts from DNA as a function of repair endonuclease and exonuclease, but that resynthesis of excised oligonucleotides did not occur, with resultant fragmentation of the DNA.

DNA from lymphocytes, either LP-depleted or not, showed increased fragmentation and no $^3$H-Tdr incorporation when treated with both BPDE and aphidicolin. This was expected, since aphidicolin is a specific DNA polymerase-a inhibitor.
which does not inhibit the excision removal of DNA adducts. DNA isolated from LDL supplemented cells treated with BPDE was still somewhat fragmented after 12 hr of repair, but the strand length was greater than was typical for DNA isolated from LP-depleted or aphidicolin treated cells, and the DNA had significant $^3$H-Tdr incorporation, suggesting that repair had occurred in LDL supplemented cells.

Direct analyses of DNA polymerase-a activity were completed on BPDE treated lymphocytes. LP-depleted cells exhibited no increase in polymerase-a activity following BPDE treatment, while LDL supplemented cells showed a characteristic increase in polymerase-a activity, reaching maximal levels at about 3 hr after BPDE treatment. Increased DNA polymerase-a activity could have been due either to de novo synthesis of the enzyme, or to activation of existing enzyme by mutagen treatment. When cycloheximide was added to lymphocyte preparations, to prevent protein synthesis, prior to treatment with BPDE, the increase in polymerase-a activity was not diminished. This suggests that increased levels of enzyme activity in lymphocytes treated with BPDE represents an activation of existing enzyme rather than enzyme induction. When one considers these findings in light of the report by Danse et al (1981), of the in vitro activation of DNA polymerase-a by a nuclear protein kinase in chick embryo, they appear to suggest a requirement for lipopro-
teins in a protein kinase step for activation of DNA polymerase-b. Relative insensitivity of the polymerase-b assay renders an assessment of LP-depletion on polymerase-b function inconclusive.

Data presented here indicate that LP-depleted human lymphocytes exhibit a distinct inhibition of excision repair of damaged DNA. The data further suggest that cellular mechanisms for the activation of DNA polymerase-a do not function in LP-depleted cells, or that cellular processes requisite to the function of DNA polymerase-a do not occur in the absence of LP. The depletion of LP as a mechanism of inhibiting DNA repair in mutagen treated cells may provide a useful tool in the investigation of mutagen interactions in cellular functions.
CHAPTER V

INHIBITION OF DNA SYNTHESIS BY AN ELECTROPHILIC METABOLITE OF BENZO(a)PYRENE

INTRODUCTION

Exposure to toxic compounds encountered as environmental pollutants constitutes a major hazard to human health (Kraybill, 1978; Tomatis, 1976), with estimates of annual marine pollution due to polynuclear aromatic hydrocarbons (PAH) alone ranging between 0.2 and $6 \times 10^6$ metric tons (2). This level of environmental contamination is particularly alarming when one considers the number of PAH known to be mutagenic, carcinogenic, teratogenic, and cytotoxic (Brookes and Osborne, 1982; MacLeod et al, 1980; Bridbord et al, 1976). Cytotoxicity, which may be evidenced as impairment of either replicative DNA synthesis or DNA excision repair, is reported to occur in humans and animals exposed either experimentally or occupationally to PAH (Freeman and Larcom, 1983; Peterson, 1980; Rasmussen, 1975; Poirier et al, 1975). Iatrogenic exposure to toxic compounds presents an additional health hazard, since many therapeutic drugs have secondary effects similar to those reported for known cytotoxic and genotoxic compounds. Examples of therapeutic drug toxic effects include impaired DNA synthesis, decreased immune competence, and increased occurrence of cancer among
patients treated with chemotherapeutic agents (Schechtman et al., 1980; Gaudin et al., 1974; Kraybill, 1978). Chemotherapeutic agents and chemical carcinogens such as the PAH may be either direct alkylating agents or may be enzymatically activated to electrophilic forms capable of binding to cellular macromolecules. Benzo(a)pyrene (BaP) has been widely investigated and is considered to be a prototype compound which exhibits many of the mutagenic, carcinogenic, and cytotoxic effects common to both PAH and chemotherapeutic agents.

Benzo(a)pyrene is ubiquitously distributed as an environmental pollutant derived from the incomplete pyrolysis of combustible materials (Shinohara and Cerutti, 1977). The highly reactive electrophilic metabolite of BaP, (+/−) antipr-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE), is reported to cause mutations in both prokaryotic and eukaryotic cells, to initiate DNA excision repair, to be cytotoxic, to inhibit DNA synthesis, and to be carcinogenic. Although BPDE is reported to form covalent bonds with nucleophilic sites on any cellular macromolecules, cell damage resulting from BPDE is generally thought to result from the formation of adducts with DNA. While DNA alkylation and adduct formation result in changed cellular activities, the relationship between alkylation of DNA and alkylation-associated changes in cell characteristics are
not well understood. Brookes and Osborne (1982) suggested that differences in DNA repair in cells treated with reactive BPDE stereoisomers are due to the differences in spatial configuration between (+) and (-) BPDE-DNA adducts affecting the template properties of DNA during repair replication. However, other studies of carcinogen interactions with cells suggest the possibility that a mechanism other than the formation of DNA adducts may explain both cytotoxicity and the inhibition of DNA synthesis by some mutagenic compounds.

Freeman and Larcom (1983) reported irreversible inhibition of unscheduled DNA synthesis (UDS) unrelated to cytotoxicity in human lymphocytes treated with the alkylating agents methyl methanesulfonate (MMS) and 2-propiolactone (BPL) but did not suggest a mechanism for the inhibition. Peterson (1980) demonstrated that MMS and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) form cellular lesions associated with either cytotoxicity or the inhibition of DNA excision repair which differ from lesions associated with increased levels of alkali-labile DNA or with mutations, but suggested that both types of lesions were due to the formation of DNA alkylation products. Park et al. (1981) reported that MMS inhibits excision repair of UV-initiated pyrimidine dimers, and concluded that the inhibition was not due to DNA methylation by MMS; they proposed that the inhibition was
due to a direct effect of MMS on the activity of repair enzymes. In a continuation of the same study Cleaver (1982) suggested that inhibition of DNA synthesis by MMS was due to the direct inactivation, by alkylation, of a repair enzyme complex.

BPDE inhibition of both scheduled DNA synthesis in mitogen-stimulated lymphocytes and unscheduled DNA synthesis (UDS) occurring as a function of excision repair was examined in non-mitogen-stimulated lymphocytes. I also evaluated the LBPDE inhibition of DNA polymerase-a activity in a cell-free system. The data show that BPDE inhibits both replicative DNA synthesis and DNA excision repair in human lymphocytes, and that this inhibition does not result from BPDE-DNA adduct formation; rather, the data indicate that LBPDE inhibits DNA synthesis by directly inhibiting the function of DNA polymerase-a.

MATERIALS AND METHODS

Materials: Unlabeled 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxyguanosine 5'-triphosphate (dGTP), 2'-deoxycytidine 5'-triphosphate (dTTP), and 2'-deoxycytidine 5'-triphosphate (dCTP), were purchased from Sigma. Tritiated 2'-deoxynucleoside 5'-triphosphates were obtained from ICN Chemical and Radioisotope Division and are shown as follows.
$^3$H-dTTP (20 Ci/m mole); $^3$H-dCTP (30 Ci/m mole); $^3$H-dATP (9 Ci/m mole); and $^3$H-dGTP (5 Ci/m mole). r-7,1-8-
Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (+/-)(anti) was obtained from the Division of Cancer Cause
and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, MD, as either the unlabeled
(BPDE), or the tritiated (403 mCi/m mole)($^3$H-BPDE) form.
DEAE-52 cellulose was obtained from Serva Corp., Heidelberg.
Aphidicolin was obtained as a gift from Imperial Chemical
Industries, London.

DNA Preparation and Analysis: Salmon sperm DNA (Sigma)
was converted to the active (template/primer) form using the
procedure of Schlabach et al, (1971), and was repurified by
phenolic extraction (Joe et al, 1984). BPDE-DNA adducts were
quantitated using the techniques described in detail by Joe

Thymidine Incorporation: $^3$H-Thymidine ($^3$H-Tdr) incorp-
oration was used as an indicator of unscheduled DNA
synthesis in mutagen treated cells, and of scheduled DNA
synthesis in mitogen-activated cells. Human lymphocytes were
isolated as described by Busbee et al, (1982). Incorporation
of $^3$H-Tdr as a function of DNA excision repair in BPDE-
treated lymphocytes was completed as previously described.
The initiation of blastogenesis was assessed by $^3$H-Tdr
incorporation during scheduled DNA synthesis in mitogen
stimulated lymphocytes. The techniques employed for UDS were used, except that hydroxyurea was not added.

**Cell Culture:** Mouse tumor cells, C3HMT, were maintained in a monolayer in Dulbeccos modified Eagles Minimal Essential Medium supplemented with 10% fetal calf serum. Logarithmically growing cells were synchronized with 1 mM hydroxyurea (HU) for 16 hr, and were harvested in S phase 3 hr after removal of HU.

**DNA Polymerase Isolation:** DNA polymerase-a was purified using the sequential steps of Mechali et al., (1980), with modifications for the use of either human lymphocytes or C3HMT cells as follows. To each sample of approximately $10^8$ packed lymphocytes or mouse tumor cells, 1 ml of buffer containing 0.25 M sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, and 7.5 mM MgCl$_2$ was added. Cells were disrupted by 35 strokes of a Teflon pestle in a glass homogenizer. The homogenate and homogenizer wash were pooled and centrifuged at 35,000 rpm in a Beckman 56 rotor for 1 hr. The supernatant was subjected to ammonium sulfate fractionation as specified by Mechali et al., (1980), was adjusted to 0.4 M KCl, 10 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.5), 0.5 mM dithiothreitol, and was adsorbed onto a DEAE-52 cellulose column (1 ml) equilibrated with the same buffer. The eluate was dialized against 50 mM Tris-HCl (pH 8.0) containing 0.5 mM dithiothreitol and 7.5 mM MgCl$_2$. This enzyme solution was either used immediately,
or 10% glycerol was added and the preparation was stored at -70°C. Additional purification beyond DEAE-Cellulose chromatography was as specified by Mechali et al (25). Due to the loss of both specific activity and total activity beyond the DEAE-Cellulose step, and to the relatively high degree of purity at this step, we completed all analyses except those requiring essentially single band SDS-PAGE purity without further purification.

**DNA Polymerase Assay:** Each 0.2 ml of reaction buffer for DNA polymerase-a assays contained 50 mM Tris-HCl (pH 8.0), 7.5 mM MgCl₂, 0.5 mM dithiothreitol, 70 ug of bovine serum albumin (A grade Sigma), 20 ul of enzyme preparation, and all four deoxynucleoside triphosphates (dNTP) as specified, of which only one was labeled. The reaction mixture was incubated with or without BPDE (added in 2 ul of DMSO), at 37⁰ for 1 hr. The reaction was stopped by adding 5 ml of cold trichloroacetic acid, and ³H-2'-deoxynucleoside 5'-monophosphate incorporation into acid insoluble material was radiometrically determined.

**Adenylate Energy Charge Determination:** The adenylate energy charge was used as a measure of cellular viability in human lymphocytes and was determined using the methods of Chapman et al, (1971).
RESULTS

In studies using human lymphocytes a correlation was noted between the concentration of BPDE with which cells were treated and three separate cellular phenomena. These included a dramatic decrease in mitogen-stimulated blastogenesis, and both the inhibition of DNA excision repair and the rapid increase in accumulation of BPDE-DNA adducts in non-mitogen-stimulated cells treated with BPDE at concentrations above 500 ng/ml (Figure 5-1). Analysis of the total adenylate energy charge in these cells showed them to be viable (adenylate charge between 0.5 and 0.8) when treated with BPDE concentrations as high as 5 ug/ml. These phenomena were not reversed upon removal of the cells from contact with BPDE. Treatment of lymphocytes with both BPDE and the DNA polymerase-a inhibitor, aphidicolin, suggest a possible cumulative or synergistic interactions between BPDE and aphidicolin acting on DNA synthesis (Figure 5-2). Although aphidicolin is known to inhibit DNA polymerase-a, no data are available on interactions between BPDE and DNA polymerases. An examination was completed to determine the effect of BPDE concentration on, (A) DNA polymerase-a activity, (B) DNA excision repair, or (C) mitogen stimulated blastogenesis in three identical preparations of human
Figure 5-1. Effects of r-7, t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) on lymphocytes. Lymphocytes were treated with BPDE at varying concentrations. Blastogenesis was measured in cells treated with PHA (●). DNA excision repair (△), and formation of BPDE-DNA adducts (■), were measured in non-mitogen-stimulated lymphocytes. Both blastogenesis and excision repair were measured as a function of 3H-Tdr incorporated into the DNA extracted from 10^6 cells. Adducts were measured as BPDE modified nucleosides recovered from 10^6 cells.
Figure 5-2. A comparison of the inhibitory effects of BPDE alone on lymphocyte blastogenesis (\(\sigma\)) or DNA excision repair (\(\triangle\)), with the inhibitory effects of BPDE combined with aphidicolin on lymphocyte blastogenesis (\(\bullet\)) or DNA excision repair (\(\Delta\)).
lymphocytes, showing a coincidence between decreased blastogenesis, decreased DNA excision repair, and inhibition of DNA polymerase-a activity in BPDE treated cells (Figure 5-3).

Inhibition of DNA synthesis by BPDE has been reported to be a function of the formation of BPDE-DNA adducts (1982). An experiment was designed to assess the effects of different BPDE-DNA adduct concentrations on DNA polymerase function in a cell-free system. DNA was treated with $^3$H-BPDE at either 500 or 1000 ng/ml and was recovered with detectable adducts (Figure 5-4, panel A). DNA template preparations produced as above, but using unlabeled BPDE, were reacted with polymerase-a in the presence of a mixture of dNTP. Levels of $^3$H-dTMP incorporated are shown (Figure 5-4, panel B) to be similar between control template and template containing two different adduct concentrations, suggesting that BPDE-DNA adducts did not inhibit DNA polymerase-a activity under these conditions.

To determine whether DNA polymerase-a was directly inhibited by BPDE binding to the enzyme, we treated purified enzyme with an inhibitory concentration of $^3$H-BPDE (1 ug/ml), and recovered enzyme containing 13.2 pmoles of BPDE/unit of enzyme activity. When the enzyme-BPDE preparation was reacted with template DNA containing no BPDE adducts, $^3$H-dTMP incorporation in vitro (0.68 pmoles dTMP
Figure 5-3. A comparison of the BPDE concentrations required to inhibit either blastogenesis ( ■ ) or DNA polymerase activity ( ● ) in mitogen-stimulated lymphocytes, or to inhibit DNA excision repair in non-mitogen-stimulated lymphocytes ( ▲ ).
Figure 5-4. An examination of $^3$H-dTMP incorporation in a cell-free system employing isolated DNA polymerase-a and template DNA containing different concentrations of BPDE adducts. Activated DNA was treated with two concentrations of BPDE and repurified resulting in template DNA preparations containing different levels of adducts. These DNA preparations were then used as template DNA for dTMP incorporation as a function of DNA polymerase-a activity.

Panel A. BPDE concentrations in DNA (in nmoles /mg of DNA) treated with BPDE at 500 or 1000 ng/ml.

Panel B. dTTP incorporation into template DNA by DNA polymerase-a when the template has varying concentrations of BPDE adducts. The concentrations of dTTP incorporation are seen to be equivalent between the three test groups.
incorporated/hr) did not differ from that observed for control enzyme preparations (0.70 pmoles dTMP incorporated/hr).

When DNA polymerase-α and template DNA were mixed together prior to the addition of BPDE, inhibition of \(^3\)H-dTMP incorporation occurred. When the template DNA concentration was varied, the resulting double reciprocal plot indicated uncompetitive inhibition of enzyme activity by BPDE (Figure 5-5). Lineweaver Burk plots of deoxynucleoside incorporation in enzyme-template preparations exhibit noncompetitive inhibition of DNA polymerase-α activity by BPDE when dATP, dCTP, or dTTP were varied, and competitive inhibition when dGTP was varied (Figure 5-6).

**DISCUSSION**

Investigators have assumed that DNA is the specific target macromolecule for BPDE initiated changes in cell function and that BPDE-DNA adduct formation was the direct cause of these phenomena. There have, however, been reports suggesting that formation of DNA adducts may not be associated with either cytotoxicity or the inhibition of DNA synthesis cause by BPDE. Peterson (1980) reported that MMS- and MNNG-initiated DNA lesions which caused cytotoxicity and inhibition of DNA synthesis differed from lesions associated
Figure 5-5. An examination of the inhibition of $^3$H-dTTP incorporation as a function of DNA polymerase-a activity in a cell-free system. The double reciprocal plot show BPDE inhibition of $^3$H-dTTP incorporation when the concentration of template DNA is varied between 2.5 ug/ml and 50 ug/ml. Uninhibited incorporation of $^3$H-dTTP (without BPDE) (△). Inhibitory effect of 0.5 ug/ml BPDE (●). Inhibitory effect of 1 ug/ml BPDE (■). The $K_i$ was approximately 4.0 uM.
Figure 5-6. An examination of BPDE-initiated inhibition of incorporation of specific 2'-deoxynucleoside 5'-triphosphates as a function of DNA polymerase-a activity in a cell-free system. The double reciprocal plots show BPDE inhibition of $^3$H-dNMP incorporation when the 2'-deoxynucleoside 5'-triphosphate (dGTP, dATP, dCTP, dTTP) concentrations were varied. Uninhibited $^3$H-dNMP incorporation (without BPDE) (■). Inhibitory effect of 1 ug/ml BPDE (●). The apparent $K_i$ values are as follows: dATP, 5.9 uM; dGTP, 4.0 uM; dCTP, 6.0 uM; dTTP, 6.6 uM.
with increases in either mutations or in alkali lability of DNA. Park et al. (1981) and Cleaver (1982) concluded that MMS alkylation of cellular macromolecules other than DNA had a direct effect on repair enzymes caused by alkylation of a DNA repair enzyme complex. Cleaver further suggested that MMS inhibition of the DNA repair process occurred subsequent to the initial endonuclease nicking step.

Data presented here demonstrate that human lymphocytes treated with BPDE at concentrations between 500 and 800 ng/ml showed inhibition of DNA synthesis. Since lymphocytes were viable at BPDE concentrations 10-fold higher than those which inhibited DNA synthesis, inhibition of DNA synthesis by BPDE in human lymphocytes must not have occurred as a result of generalized cytotoxicity. BPDE treatment of lymphocytes inhibited both replicative DNA synthesis (in mitogen-stimulated cells) and UDS (as a function of DNA excision repair) at about the same concentration which inhibited DNA polymerase-a activity. DNA polymerase-a is known to be active in both replicative and repair DNA synthesis (Hanaoka et al, 1979; Cleaver, 1983; Oguro et al, 1979). Therefore, inhibition of both types of DNA synthesis could be expected to result from inhibition of polymerase-a activity. In order to determine if either BPDE-DNA adduct formation or direct BPDE-initiated inactivation of DNA polymerase-a could be disregarded as a possible factor in the
inhibition of DNA synthesis, we completed a series of in
vitro experiments measuring polymerase-a activity under
defined conditions.

When BPDE-treated template DNA was used to promote dTMP
incorporation by polymerase-a in a cell-free system, equi-
valent incorporation was observed using DNA with no adducts,
template with (A) 0.44 adducts/kb (0.96 pmoles BPDE/ug DNA),
or template with (B) 0.89 adducts/kb (1.90 pmoles BPDE/ug
DNA). The adduct preparations were produced by treating DNA
with BPDE at concentrations which (A) elicit near maximal
UDS (500 ng/ml), or (B) inhibit both UDS and replicative DNA
synthesis (1 ug/ml). This indicates that dTMP incorporation
into template DNA by DNA polymerase-a in a cell-free system
was not affected by the concentration of BPDE adducts in the
template DNA.

Repolymerization of the excised DNA oligonucleotide
during excision repair occurs subsequent to the function of
endonuclease and exonuclease. It is initiated by DNA poly-
merase-a for UV-type (long patch) repair, with polymerase-b
resynthesis of the last few nucleotides (gap filling), and
reformation of the last phosphodiester bond by polynucleo-
tide ligase. Cleaver (1982) reported that MMS inhibition of
excision repair was due to alkylation of the repair enzyme,
resulting in loss of activity at a step subsequent to the
initial nicking of DNA by endonuclease. We found that MMS
modification of purified polymerase-a inhibited enzymatic activity, but that BPDE binding to purified polymerase-a failed to inhibit enzymatic activity. However, when polymerase-a and template DNA were mixed together prior to the addition of BPDE, inhibition of enzyme activity occurred. The proposal by Cleaver (1983) that MMS inhibits DNA excision repair by alteration of a repair enzyme, and our finding that BPDE inhibits DNA polymerase-a activity in vitro, are reinforced by unpublished data from our laboratory showing that ecdysone, and 11-deoxycorticosterone inhibit DNA synthesis and DNA polymerase-a function, but are not interactive with template DNA. The kinetics of BPDE-associated inhibition of polymerase-a activity were assessed in a cell-free system resulting in Lineweaver-Burk plots which indicate uncompetitive inhibition of enzyme activity when the template DNA concentration was varied. These data indicate that polymerase-a activity was not decreased by BPDE binding to either the DNA template or the purified enzyme, and that BPDE-associated decreases in enzyme function required polymerase-a and template DNA to have formed a complex prior to BPDE exposure.

A parallel appears to exist between the inhibition of DNA synthesis by BPDE and by aphidicolin. In vitro studies of aphidicolin interaction with polymerase-a during DNA synthesis suggest the following sequence of events.
Polymerase-a binds to template DNA, after which the different dNTP bind to an acceptor site(s) on the enzyme-template complex. This binding site(s) is not available prior to formation of that complex. Aphidicolin binds polymerase-a only after the enzyme-template complex is formed, and competitively inhibits interaction of dCTP with the complex (Oguro et al., 1979; Ikegama et al., 1978). Data presented here suggest that BPDE may inhibit DNA synthesis in a manner similar to aphidicolin, but that BPDE competitively inhibits DNA polymerase-a function by blocking interaction of dGTP with the enzyme-template complex.

Data presented here are consistent with reports showing that known inhibitors of DNA polymerase-a activity cause a reduction in unscheduled DNA synthesis in UV-treated mammalian cells, and inhibit mitosis-associated replicative DNA synthesis. Data from this study demonstrate that BPDE inhibition of DNA polymerase-a activity in a cell-free system is not a function of the concentration of adducts in the template DNA, neither is it a function of BPDE binding to polymerase-a in the absence of template DNA. Rather, the inhibition of DNA synthesis is dependent on binding of BPDE to the template-enzyme complex, competitively blocking interaction of 2'-deoxyguanosine 5'-triphosphate with the enzyme. These findings suggest that chemicals, whether or not they are DNA interactive, may be potentially hazardous or indir-
ectly carcinogenic by inhibiting normal DNA excision repair or by decreasing the potential for lymphocyte proliferation associated with a normal immune response.
CHAPTER VI.
GENERAL CONCLUSIONS

This study presents an assessment of experimental data collected in a coordinated research project designed to study the relationships between lipoproteins, carcinogens, the production of DNA damage by carcinogens, and the repair of carcinogen-initiated DNA damage, and to enhance understanding of the biochemical mechanisms of DNA synthesis and DNA excision repair inhibition by chemical carcinogens.

Data presented in Chapter II demonstrate that the lipophilic chemical carcinogen, benzo(a)pyrene, is absorbed into the intestinal lymphatic drainage, and enters the vascular system in association with lymphatic lipoproteins. These data show that virtually none of the intra-gastrically instilled BaP may be recovered from the non-LP fractions of plasma. The data also show that BaP partitioning into sheep plasma lipoproteins increases in efficiency up to a lipoprotein cholesterol concentration of about 0.025 mg/ml, after which the efficiency of uptake begins to plateau, reaching a maximum of about 40% of available BaP. Shu and Nichols (1980) reported that BaP partitions into LDL more efficiently than it partitions into VLDL or HDL; however, Yoo et al (1984) recently reported the uptake of BaP into LP to be directly correlated with the total concentration of
triglycerides present in LP rather than to specific LP
classes. The data in Chapter II show three peaks of BaP
uptake into LP, but do not distinguish a particular LP comp-
oneent as being largely responsible for uptake of BaP from
the intestine. Unpublished data recently obtained in this
laboratory suggest that BaP originally enters the intestinal
lymphatic drainage sequestered within chylomicrons, and
partitions from chylomicrons into other of the LP types.

The major thrust of this research centered not on the
partitioning and vascular transport of LP, but on the
biochemical mechanisms of BaP inhibition of DNA synthesis
and DNA excision repair, and on the requirement of cells
for LP in order to repair BaP-initiated DNA damage. In
Chapter III data are presented that show a requirement of
human lymphocytes for LP, or LP components, in order to
carry out DNA excision repair. This line of investigation
was carried further in Chapter IV, where data were presented
to show a requirement for LP in order for DNA polymerase-a
to be activated following BPDE-initiated damage to cellular
DNA. These data suggest that DNA polymerase-a is present in
both LP-depleted and non-depleted cells after treatment with
BPDE, that protein synthesis is not necessary after DNA
damage occurs and prior to the onset of DNA polymerase-a
function, but that DNA polymerase-a activity can not be
measured in cells depleted of LP. The data also show that
LP-depleted cells are readily restored to their functional state by LP supplementation with as little as 0.02 mg/ml of LDL. The data do not address the point of whether the reversal of LP-depletion can be accomplished using purified LP components.

This study has not resolved the question of the requirement for LP in DNA excision repair, but has suggested a potential answer. One possible answer for this question may be that LP components are required for activation of a critical enzyme in DNA repair. The data suggest that DNA polymerase-a is inactive in cells depleted of LP, and that LDL can reverse that inactivation. Data supporting the role of LP as a critical factor in DNA excision repair show that cells accumulate fragmented DNA as a function of incomplete repair both when BPDE-damaged cells are LP-depleted and when DNA polymerase-a is inhibited in non-depleted cells treated with a specific DNA polymerase-a inhibitor. In concert, these results suggest that LP are required for DNA repair steps occurring after the endonuclease and exonuclease steps are completed in long patch excision repair initiated by an electrophilic metabolite of BaP.

These data are consistent with the literature in that both DNA polymerases-a and -b play important roles in DNA excision repair. Polymerase-a is now considered to be the major enzyme of oligonucleotide repolymerization in long
patch (UV-type) repair. This concept is due to the greater amount of long patch repair inhibition by the specific polymerase-a inhibitor, aphidicolin (Cozzarelli, 1977; Snyder and Reagan, 1981; Cleaver, 1982), and the relatively smaller degree of repair inhibition by the DNA polymerase-a inhibitor, dideoxythymidine (Seki and Oda, 1980; Miller et al, 1982). There are several indicators that DNA polymerase-a might require LDL for activation following DNA damage. Serum has been used as an additive in cell culture systems examining DNA repair, and repair does not occur in cells that are deprived of serum and not supplemented with lipoproteins. Cells in a quiescent state ($G_0$) can, by the addition of serum to the medium, be stimulated to grow and to enter a synchronous wave of DNA synthesis (Burk, 1970; Craig et al, 1975). Craig et al (1975) reported a specific increase in the activity of DNA polymerase-a of BHK-21/C3H cells which were stimulated to grow by the addition of serum to the medium. Lastly, Busbee et al (1984) reported that depletion of serum lipoproteins in human lymphocytes results in the inhibition of DNA excision repair, and data presented here show that depletion of lipoproteins results in both inhibition of DNA excision repair and in decreased DNA polymerase-a activity in human lymphocytes treated with an active carcinogen.
The majority of chemical carcinogens for which reliable information is available appear to initiate cell transformation via the formation of DNA-adducts resulting in mutations. Some carcinogens not only initiate mutations, but appear to be cytostatic and DNA synthesis inhibitory as well. In the past it was thought that these characteristics were associated with the formation of DNA adducts resulting in infidelity of DNA repair or DNA synthesis, or in the overt inhibition of DNA synthesis. Data now available suggest that it is possible for a carcinogen to interact only with proteins and still to cause mutations in DNA. Loeb et al (1977) reported that beryllium, a well known carcinogen which is not DNA binding, interacts directly with DNA polymerase, increasing the rate of errors in normal DNA replication, with increases in the mutation rate. Similar results were obtained in the prokaryote E. coli with alkylating agents (Miyaki et al, 1983).

In Chapter V data are presented suggesting that the electrophilic diol epoxide of BaP directly inhibits DNA polymerase-α activity. BPDE, or the converted form of BPDE (anti) (+/-) 7,8,9,10-tetrahydroxy-7,8,9,10-benzo(a)pyrene tetrold (Meehan and Bond, 1984) appears to inhibit DNA chain elongation by competitively inhibiting the incorporation of 2'-deoxyguanosine 5'-triphosphate into template DNA. Sterechemical considerations argue against BPDE or its tetrold
binding directly to dGTP acceptor sites on DNA polymerase-a. Instead, this carcinogen must prevent binding of dGTP to the enzyme by means of overlapping attraction sites, or by causing a conformational change(s) in the enzyme that distorts or masks the dGTP binding site.

Data presented in this study allow the conclusion that inactivation of DNA polymerase-a is involved in the inhibition or regulation of long patch DNA excision repair, and that lipoproteins are required for the function of DNA polymerase-a in carcinogen treated cells. If these conclusions are correct, then inactivation of DNA polymerase-a may offer explanations for many of the cytotoxic effects, such as reduced rate of growth (Graham and Fox, 1983), depression of lymphocyte proliferation (Ikegami et al, 1978), and generalized suppression of immune responses coupled with an increased rate of tumorigenesis (Urso and Gengozian, 1980) typically seen in cells exposed to chemical carcinogens.
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