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ABSTRACT

Major efforts have been made to detect the presence of retroviruses in normal and malignant human tissue. The organ which has been the most promising for detecting retroviruses in normal tissue has been the placenta. The major evidence for the presence of a retrovirus in placenta comes from electron microscopic studies which demonstrate retroviruses budding from the syncytiotrophoblast layer of the placenta. While these studies are highly suggestive of the existnce of a human retrovirus, further evidence was sought in an effort to detect the viral associated RNA-directed DNA polymerse (RDDP) in placental extracts. In the research presented, extracts from over 200 normal human placentas were examined for RDDP activity. In more than 80% of the placentas examined, this enzyme activity was found after isopycnic centrifugation in sucrose at 1.15-1.17 g/ml, the density characteristic of type-C retroviruses. The RDDP activity appeared to be viral rather than of cellular origin by a number of biochemica criteria. 1) The enzymatic reaction utilized the RNA template primers $(C)_n \cdot (dG)_{12-18}$

and $(Cm)_n \cdot (dG)_{12-18}$ more efficiently than the DNA template primer $(dC)_n \cdot (dG)_{12-18}$. 2) The enzyme also copied heterologous 70S RNA, a characteristic of viral RDDP. 3) The product of the endogenous reaction, which was sensitive to RNase digestion, had the characteristics of a small DNA associated with a large RNA by hydrogen bonding. After heat treatment, the enzymatic activity at 1.15 g/ml in sucrose was shifted in density to 1.22-1.24 g/ml. These observations suggest that normal human placentas contain a retrovirus-like RDDP.

Convincing proof that the placental RDDP is unique requires the purification of the enzyme and comparison of its antigenic properties with other cellular DNA polymerases. A result of this investigation has been the identification of a new high molecular weight β-like DNA polymerase in the nuclear fraction of human placental extracts. The DNA polymerase did not bind to DEAE-cellulose and eluted from phosphocellulose at 0.3 M KCl. The purified enzyme preferred the synthetic DNA template primer $(dC)_n \cdot (dG)_{12-18}$ and activated calf thymus DNA. The polymerase did not copy the synthetic RNA template-primers $(A)_n \cdot (dT)_{12-18}$, $(C)_n \cdot (dG)_{12-18}$, or heteropolymeric mRNA. During purification and storage, a shift in template preference from activated DNA to $(dC)_n \cdot (dG)$ was observed for the enzyme. The enzyme was active over a narrow pH range with an optimum at 7.8. The polymerase activity was dependent on low concentrations of $MgCl_2$ and was inhibited by $MnCl_2$. Enzyme activity was relatively insensitive to 0.1 M KCl, 5 mM N-ethylmaleimide, and 2 μg/ml aphidocolin. However, 50 mM phosphate inhibited the reaction. The DNA polymerase had a sedimentation coefficient of 5S in glycerol and appeared

as a single band at 78,000 daltons on SDS polyacrylamide gel electrophoresis.

Another result from the biochemical characterization of the placental polymerases was the identification of a specific inhibitor of RDDP. This factor copurified with the placental RDDP activity and could be separated from the enzyme complex by sequential salt extraction. The RDDPs of several mammalian retroviruses were found to be sensitive to the inhibitor. However, the RDDP of RSV and the normal cellular DNA polymerases α and γ were insensitive. Nucleases, proteases, and phosphatases were not detected in the inhibitory extract. The factor does inhibit specifically mammalian viral polymerases. Inhibition was not due to the nonspecific inactivation of substrate in the reaction mixture. A partial characterization of the inhibitory substance revealed that it was relatively stable to heat up to 85°C and pH from 2 to 12. The inhibitor was insensitive to ether extraction and resistant to trypsin and phospholipase C digestion. Extraction with chloroform/methanol did inactivate the inhibitory factor. The inhibitor is larger than 14,000 daltons since it is nondialyzable and sediments in the middle third of a glycerol gradient at 0.5 M NaCl. By radial immunodiffision tests, the inhibitor is not an immunoglobulin of the IgG or IgM type. A role for this inhibitor in the regulation of DNA synthesis during placental development is discussed.

RNA-Directed DNA Polymerase Activity in Human Placentas

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"The effort to understand the universe is one of the very few things that lifts human life a little above the level of farce and gives it some of the grace of tragedy."

Steve Weinberg, The First Three Minutes

Table of Contents

List of Illustrations

List of Illustrations (cont'd)

List of Illustrations (cont'd)

I. Background

A. Retroviruses

The viral etiology of leukemias and sacromas was first demonstrated by Ellerman and Bang (1908) and Rous (1911) in their experiments with avian species. They found that cell-free filtrates of chicken leukemia cells and solid tumors could induce the same diseases when injected into healthy chickens. The causative agents of those diseases were later identified as viruses from the Family Retroviridae. Since that time these RNA tumor viruses have been isolated from a variety of animal groups ranging from fish to primates. (For review see Tooze, 1973; Levy, 1978). Recently, the association of endogenous RNA tumor viruses with normal embryonic and reproductive tract tissue has suggested that these oncogenic agents may also play a role in developmental processes. (For review see Levy, 1978).

Electron Microscopy

The retroviridae was established as a family of viruses that contain a 60 to 70S single-stranded RNA associated with an RNA-dependent DNA polymerase, designated reverse transcriptase (Dalton et al., 1974). By electron microscopy the various members of the family have been classified according to their morphology. The various particles are described in the following table:

A-particles - Intracellular particles which are 60-90 nm in diameter, and exist in two forms, intracytoplasmic and intracisternal. Both forms of the particle have a unit membrane envelope and electron translucent center. The

role of the intracisternal particle is unknown, but the intracytoplasmic particle is considered to be a precursor of the type B particle.

B-particle - Extracellular particle which develops by budding at the cell membrane as a complete nucleoid. The mature type B particle, approximately 100 nm in diameter contains an eccentric electron dense nucleoid and prominent spikes on its outer envelope. The only well-characterized retrovirus with B-type morphology is the murine mammary tumor virus (MMTV).

C-particle - This extracellular particle buds with a crescent-shaped nucleoid at the cell membrane. The mature extracellular form of the virus, approximately 100 nm in diameter, contains a central electron-dense core. The envelope of the virion consists of a unit membrane with inconspicuous spikes on the surface. The envelope is closely associated with the core which is composed of a capsid outer layer surrounding a core ribonucleoprotein.

D-particle - This extracellular particle is derived from a ring-shaped intracellular particle, 90 nm in diameter, which buds from the cellular membrane. The extracellular particle measures 120 to 130 nm and contains an electron-dense core with knobs on the virion envelope. The D-type particle differs from the C-type form by intracellular

formation and extracellular size. The D-type particle is derived from primates and is classically represented by the Mason-Pfizer monkey virus (MPMV) (Fine and Schohetman, 1978).

Biochemical and Biological Characteristics

Mature retroviruses contain three classes of structural proteins 1) internal structural proteins, 2) envelope glycoproteins, and 3) RNA-dependent DNA polymerase (RDDP). The internal structural proteins comprising the icoschedral core are relatively small. These proteins range from 10,000 to 30,000 daltons and are designated with the prefix "p". The envelope of retroviruses contains glycosylated virus-specific proteins. These glycosylated proteins vary from 12,000 to 85,000 daltons and have the prefix "gp". RDDP purified from viral cores can be classified into three types based on molecular weight and subunit structure. Mammalian type-C viruses contain a reverse transcriptase that exists as a single polypeptide with a molecular weight of about 70,000 daltons. The second type of RDDP contains two subunits with a total molecular weight of 170,000 daltons. The large subunit, β, and small subunit, α, have molecular weights of 110,000 daltons and 70,000 daltons respectively. The α subunit results from proteolytic cleavage of the β subunit. All the avian leukosis and sarcoma viruses contain this type of enzyme. The third type of RDDP found in MMTV and MPMV has a single subunit with a molecular weight of 110,000 daltons.

Retroviral particles contain RNA of both low and high molecular weight. The low molecular weight RNA components range from 4-28S and

are composed of cellular tRNA, partially degraded 60-70S viral RNA and ribosomal 18S and 28S RNA (Bishop et al., 1970; Robinson et al., 1967). The high molecular weight RNA has a sedimentation value of 60-70S. Upon denaturation by heat the 60-70S RNA generates two 35S subunits and 4S host cell tRNA. One 35S molecule contains all the information necessary for virus production. The genetic order of the known genes on the 35S RNA from 5' to 3' is gag (coding for internal structural proteins), pol (coding for reverse transcriptase), env (coding for envelope glycoproteins), sarc (coding for the transforming protein) and c or common region. The viral 35S RNA also contains poly A at the 3' end and a 5' end cap-$m^7G^{5'}ppp^{5'}$GmpCp - properties which are characteristic of eucaryotic messenger RNA.

The replication of retroviruses involves several phases. After absorption to the cell surface and entrance into the cytoplasm, the virion capsid is uncoated, releasing the viral RNA. RNA-dependent DNA polymerase transcribes this RNA into a double-stranded DNA provirus which subsequently becomes integrated into the host's genome. The host's DNA-dependent RNA polymerase transcribes the integrated provirus into mRNA and virion RNA. New virions are produced by budding through the cellular membrane. (For review see Luria et al., 1978; Tooze, 1973).

Retrovirus transmission can occur via two possible modes. The first, horizontal transmission, involves infection of a host cell by an exogenous virus. The other mode of infection occurs when viral information already present in the host cell can be vertically transmitted in

the chromosomal DNA. Viruses transmitted in this fashion are called endogenous viruses. These endogenous retroviruses have been found in several species ranging from fish to chimpanzee (Levy, 1979). By definition the virus must be present in at least one copy per haploid genome of the host cell (Beemon, 1978). Endogenous viruses are hypothesized to have originated in species by horizontal infection of germ cells with exogenous virus (Benveniste and Todaro, 1974). Proviral sequences in these cells are then vertically transmitted to the host's progeny. The retroviral genes may remain latent or be expressed in productive (mature virus produced) or non-productive (no virus produced but viral markers present) modes (Jaenisch, 1976).

Murine endogenous C-type viruses have been classified into three different groups depending on their cell preference for infectivity. Ecotropic viruses are able to replicate efficiently in cells from which the virus was originally derived. Xenotropic viruses compose the second class of endogenous viruses which replicate in cells from animal species foreign to the host. Amphotropic viruses make up the last class. These viruses can efficiently replicate in cells from their own host species as well as cells from a heterologous species. All three of these classes have distinct type-specific antigens and nucleic acid sequences (Levy, 1978).

B. Retrovirus Expression During Development

1. EM Studies

The laboratory isolation of replicating retroviruses from some animal species has been difficult. In particular, retrovirus

isolation from guinea pig, rabbit, dog, and human tissue has not been done. Yet, there has been ample electron microscopic evidence for retroviruses in the reproductive tissue of these species. The first evidence for the association of C-type viruses with developmental processes was provided by Kajima and Polland (1968). They examined developing embryos of laboratory mice by electron microscopy and observed budding retroviruses. These particles have been found budding from the syncytiotrophoblast of guinea pig, rabbit, marmoset, baboon, rhesus monkey, Patas, cynomologus, and chimpanzee placentas (Hsuing et al., 1974; Bedigian et al., 1976; Kalter et al., 1973; Schidlovsky and Ahmed, 1973). In human tissue, particles resembling retroviruses have been detected by electron-microscopy in embryos (Chandra et al., 1970) and placentas (Kalter et al., 1973; Vernon et al., 1974; Dalton et al., 1976; Imamura et al., 1976; Dirksen and Levy, 1977)). The predominant particles observed in human placentas have envelopes which appear to be applied directly to the nucleoid without an intervening electron translucent space. However, particles with typical C-type morphology have been observed (Dirksen and Levy, 1977).

2. Presence of Retroviruses in Reproductive Tissue Using Viral Markers

For many species in which viral replication in vitro has not been demonstrated, retroviral markers have been useful in identifying endogenous viruses. The most commonly used markers to identify the presence of retroviruses in tissue are group specific antigens (p30 for murine, feline and primate viruses; p27-28 for avian; for review see Bishop, 1978) and RDDP activity. While viral group specific antigens

are easily identified; viral RDDP activity must be distinguished from cellular enzymes on the basis of template preference, monovalent and divalent cation preference, and antigenic specificity.

Although Daniels (1973) observed type C particles budding from rabbit blastocysts and uterine living cells, no infectious virus has been recovered from rabbit cells. The presence of a rabbit retrovirus was further verified by Meier and Fox (1973). They demonstrated interspecies reactivity for the group-specific antigen (p 30) in rabbit tissues using complement-fixing antisera against disrupted feline and mouse type C retroviruses. Bedigian, et al., 1976, detected RDDP activity associated with a particulate fraction banding at a density characteristic of retroviruses in extracts of normal rabbit placenta and uterine tissues. High levels of RDDP activity were detected early in gestation but decreased as the fetus reached term. The particle-associated enzyme was partially purified and shown to be distinct from the known cellular DNA polymerases by its elution pattern on DE52 cellulose and phosphocellulose, template primer preferences, molecular weight determination, and absolute requirement for divalent cations. Chilton and Daniel (1978), experimenting with pregnant and hormone induced pseudo-pregnant rabbits, also found that the endometrial tissue contained RDDP activity. This enzyme activity appeared 48 hours after coitus and reached a maximum 1-2 days later. Control samples from lung, liver, and spleen tissue were found to contain no detectable RDDP activity. Their experiments demonstrated that this enzyme activity appeared to be under hormonal control and not necessarily correlated with cell proliferation. When rabbits were treated with a combination of estrogen and progesterone a stimula-

tion of RDDP was obtained. However, the hormones administered separately had no effect. Infectious retrovirus has not been recovered from rabbit tissue that expresses viral RDDP activity. This phenomenon may be due to the fact that the rabbit virus is xenotropic and the appropriate cell has not been found for virus replication.

Hellman and Fowler (1977) examined the uterus, fetus, and placentas of pathogen-free, pregnant cats for expression of the cat endogenous virus RD114. When the tissues were sampled at various stages of gestation for the presence of the viral antigen p28, they found quantitative changes during the pregnancy. The uterine tissue was either negative or had low levels of viral expression throughout the pregnancy. However, the placental and fetal tissue expressed high levels of p28 antigen as early as 28 days into gestation, with a gradual decrease in expression as the fetus approached term.

The influence of hormones on endogenous type C viral expression was examined with NIH/Swiss and BALB/c mice (Fowler et al., 1975). Mice that were ovariectomized demonstrated low levels of the viral antigen p30 and RDDP activity in the uterine tissue. When these mice were treated with estrogen both viral markers increased dramatically within 24 hours after administration. This virus response to estrogen might be an enhancement effect due to the interaction of a hormone with its target tissue. The expression of p30 and RDDP was also examined during pregnancy (Fowler et al., 1977). NIH Swiss mice demonstrated a doubling of p30 antigen shortly after coitus which remained elevated throughout gestation. RDDP activity, however, increased 8-fold during the first

weeks of gestation. This elevation in activity was followed by a gradual decrease with an increase immediately before parturition.

3. Isolation of a Replicating Retrovirus from Placentas

The Endogenous Baboon Virus (BaEV or M7), a type C-type retrovirus, was recovered from primary baboon placental cells after 30-90 days of co-cultivation with human rhabdomyosarcoma, fetal canine thymus, and bat lung cells (Kalter and Heberling, 1974; Benveniste et al., 1974). By hybridization techniques with BaEv cDNA and DNA extracted from baboon liver and placenta, the virus isolated was shown to be derived from baboon cells. When hybridization studies were performed between BaEV cDNA and RNA extracted from the same tissues previously examined, only the placenta RNA contained sequences complementary to the viral probe. These experiments suggest that liver and placenta cells contain information necessary for viral production but the virus is preferentially expressed in placentas. BaEV replicates efficiently only in cells from heterologous species, a characteristic of xenotropic viruses. The virus is believed to be transmitted vertically through germ cells since baboon cells prevent replication of the virus.

Mason Pfizer Monkey Virus (MPMV), a type D retrovirus, was originally isolated from a spontaneous mammary tumor of rhesus monkey (Jensen et al., 1970). Subsequently, FTP-1, a retrovirus similar to MPMV, was isolated from rhesus monkey placenta and other fetal and postnatal tissue (Ahmed et al., 1974). There is supporting evidence that indicates that MPMV, unlike BaEV, is transmitted both horizontally and vertically, (Benveniste and Todaro, 1977).

4. Retroviral Markers in Human Tissue

Immunological and biochemical probes to detect the presence of retroviruses in humans have become important with the failure to isolate a replicating virus from these malignant or normal tissues (Gardner et al., 1977). Whether the unsuccessful attempt to isolate a human retrovirus is due to production of defective viruses, the use of inappropriate host cells or the failure of *in vitro* cultivation techniques is not known.

Using the technique of radioimmunoassay (RIA) the presence of the retroviral antigen p30 in human tissue extracts has yielded positive (Hebrink *et al.*, 1978; Mellors and Mellors, 1976) and negative results (Gardner *et al.*, 1977). Gardner and his colleagues, (1977) examined the sera from over 100 cancer patients and found no cross-reactivity with p30 antigen isolated from several primate and non-primate retroviruses. However, Mellors and Mellors (1976) examining antibody eluted off renal lesions of patients with systemic lupus erythematosis found that the eluted immunoglobulins cross-reacted with Rauscher Murine Leukemia Virus (R-MuLV) and Simian Sarcoma Virus (SSV). The presence of the major protein (p25) of MPMV was examined in the tissues of patients with myelogenous leukemia (Kim, 1977). The antigen was found to be present in the peripheral blood leukocytes and splenic tissue in 12 out of 12 patients with the disease. Gardner *et al.*, (1977), screened human sera for antibodies to viral RDDP with negative results. Jacquemin *et al.*, (1978), however, found that human leukocytes possess an immunoglobulin on the membrane surface which specifically neutralizes feline leukemia virus (FeLV), gibbon ape leukemia virus (GaLV), and SSV reverse tran-

scriptases. Humoral and cell mediated immune responses in pregnant and non-pregnant women against BaEV antigens were examined by Hirsch et al., 1978. Using microcytotoxicity assays and RIA, these invesitgators found that five out of six pregnant women tested developed selective cell-mediated reactivity to BaEV. This specific reactivity reached a maximum response at the end of gestation. Non-pregnant individuals were found to have no cellular immune response to BaEV.

The discovery that RDDP was found in virions of Rous Sarcoma Virus (Temin and Mitzutani, 1970) and MuLV (Baltimore, 1970) provided a useful tool for investigating retroviruses. The procedure for assay of the viral enzyme is relatively simple and specific. RDDP has been used for quantitating known virus, studying viral replication, and as an indicator for the presence of retrovirus in tissues. The screening of normal and malignant tissue for RDDP activity associated with a particulate fraction is a method which was employed early in the search for the human retrovirus (Gallo et al., 1970; Schlom and Spiegelman; 1971). The presence of an RDDP activity in cells was first reported by Gallo et al., (1970), with human acute leukemia. This enzyme activity has also been associated with human breast cancer (Schlom and Spiegelman, 1971; Michalides et al., 1975), malignant melanoma (Parsons et al., 1974; Balda et al., 1975), leukemic spleens (Witkim et al., 1975; Chandra and Steel, 1977), and prostatic cancer (Arya et al., 1976).

The first evidence for the presence of RDDP activity in embryonic tissue was reported in extracts of baboon placentas (Strickland et al., 1973). This enzyme was also isolated and purified from the microsomal pellets of two rhesus monkey placentas in the early stages of gestation

(Mayer et al., 1974). The rhesus monkey enzyme was found to be biochemically similar yet immunologically distinct from the RDDP of primate retroviruses with the exception of BaEV. A human DNA-synthesizing complex sharing many of the properties of cytoplasmic-derived particulate RDDP complexes in human tumors was identified in cell-free seminal fluid and in association with sperm nuclei (Witkin et al., 1975; Bendich et al., 1976; Witkin et al., 1977). This particulate-associated enzyme activity banded in sucrose at a bouyant density of 1.15-1.19 gm/ml. After treatment with detergent or phospholipase C, activity originally isolated at 1.15-1.19 g/ml was shifted to a density of 1.21-1.25 g/ml. An endogenous ^{3}H-labeled DNA was produced by this DNA-synthesizing complex. The DNA product had a bouyant density associated with RNA. When the RNA-DNA hybrid was examined by sedimentation velocity a high molecular weight product was found. This indicates that the newly synthesized DNA was associated with a high molecular weight RNA. When sperm preparations were observed by electron microscopy no virus-like components were detected. Extracts of normal term human placentas have also been reported to contain DNA-synthesizing complexes with the same characteristics as these mentioned above (Leong et al., 1978; Nelson et al., 1978). In these studies, virus-like particles were observed in those fractions containing RDDP activity.

The detection of RDDP activity was thought to be definitive proof for the presence of retroviruses in tissue. However, criteria for the existence of a viral RDDP in human tissue was redefined when it was demonstrated that γ polymerase, a normal cellular DNA polymerase, could copy the RNA strand of a synthetic RNA:DNA homopolymer (Spandari and

Weissbach, 1974a and b). Thus criteria were established for identifying a retrovirus by reverse transcriptase activity (Sarngadharan et al., 1976). The criteria are:

1) The enzyme should be present in a particulate fraction with a density of 1.15-1.18 g/ml and should shift to 1.24-1.25 g/ml upon treatment with nonionic detergent or phospholipase C. The polymerase should catalyze an endogenous synthesis of DNA which requires all four deoxyribonucleotide triphosphates and a divalent cation. The reaction should proceed in the presence of actinomycin D, and be at least partially sensitive to RNase digestion.

2) The endogenous labeled DNA product should be part of an RNA:DNA hybrid and the labeled DNA should move to the DNA density region after alkali or heat treatment in cesium sulfate equilibrium centrifugation.

3) The purified enzyme should show a preference for an RNA template with a DNA primer.

4) The enzyme should transcribe the ribostrand of the primer template $(rC)n \cdot (dG)_{12-18}$. A preferred template-primer is $(rC_{methyl}) \cdot (dG)_{12-18}$ which is more resistant to nuclease and is not copied by cellular DNA polymerase γ (Gerard, 1975). The best biochemical criterion for defining the RDDP according to its template-primer preference is the ability to copy heteropolymeric regions of high molecular weight RNA derived from RNA tumor viruses.

5) The enzyme should elute at a characteristic position from those of cellular DNA polymerases in column chromatography.

6) The molecular weight of the enzyme should be about 70,000 daltons for the mammalian type C viruses, 160,000 daltons for the avian type C, 110,000 daltons for the type B and D viruses.

7) The enzyme should be inhibited by antipolymerase sera prepared against a reverse transcriptase from an immunologically related system and not be inhibited by antisera prepared against known cellular polymerases.

Purification of RDDP from Tissue and Tissue Fluids

The purification of virus-related RDDP from extracellular virus is a relatively easy task (Gerard and Grandgenett, 1975; Gerwin et al., 1975; Lin and Papin, 1979; Sarin et al., 1979). However, the isolation of this polymerase from cells not producing virus is very difficult. This situation is due to the presence of normal cellular polymerases which interfere with RDDP detection, the distribution of RDDP in various subcellular compartments, the amount of RDDP per unit protein, and the presence of cellular nucleases, proteases, and phosphatases co-purifying with the polymerase. In order to identify an intracellular RDDP activity in whole cell or cytoplasmic homogenates, Lewis et al., (1974), developed a technique for isolating and separating DNA polymerases in human lymphoblastoid cells infected with simian sarcoma virus (Please see figure 1). Briefly, this method utilizes high concentrations of salt and detergent to disrupt the cells and cellular components. Nucleic acids, which alter the elution profile of the DNA polymerases, are removed by passing the extract through fibrous DEAE cellulose. In the second DEAE cellu-

lose chromatography, RDDP and β polymerase elute in the 50 mM KCl wash followed by α and γ polymerase which elute at 0.3 M KCl. Further chromatography on phosphocellulose and DNA cellulose separate the four DNA polymerases further. This method has been successfully used to purify RDDP from the high speed (100,000 x g) fraction of the postmitochondrial pellet of human acute leukemic cells (Mondal et al., 1975; Gallagher et al., 1974; Gallo et al., 1975), chronic lymphocytic leukemic spleens (Witkin et al., 1975), spleens with myelofibrotic syndrome (Chandra and Steele, 1977), and human milk (Kantor et al., 1979).

The RDDP activities purified from the cytoplasmic fraction of tissues and tissue fluids previously mentioned significantly prefer the synthetic primer template $(A)_n \cdot (dT)_{12-18}$ over $(dA) \cdot (dT)_{12-18}$. These polymerases have also been shown to copy $(C)_n \cdot (dG)_{12-18}$, $(Cm)_n \cdot (dG)_{12-18}$, $(dC)_n \cdot (dG)_{12-18}$ and activated DNA. Another important characteristic of these enzymes is the ability to transcribe heteropolymeric portions of both avian and mammalian viral high molecular weight RNA. The sizes of isolated intracellular RDDP have varied considerably. Mondal et al., (1975), recovered a high molecular weight (HMW) form of the enzyme 130,000 daltons - when the enzyme was solubilized in low salt buffer. However, a low molecular weight (LMW) form - 70,000 daltons - was recovered when the polymerase was solubilized in the presence of high salt buffer. The HMW form can be converted to the LMW form with high salt and non-ionic detergent, and the LMW form can be reaggregated to the HMW form by a dialysis against low salt. Both forms of the enzyme were found to exhibit some different immunological and

biochemical properties. The HMW RDDP was found to utilize synthetic primer-templates less efficiently but transcribe high molecular weight RNA more efficiently than the LMW polymerase. Immunologically the LMW form was strongly inhibited by antisera to the RDDP of SSV. The HMW form is an aggregate of the LMW form with modified function. This aggregation phenomenon of RDDP has been observed in polymerase isolated from leukemic spleen (Witkin et al., 1975), RD 114-infected cells (Gerwin et al., 1975), moloney sarcoma virus transformed cells (Rokutanda et al., 1977), and purified visna virus (Lin and Papini, 1979).

In general, the preferred divalent cation for mammalian type C retroviruses is Mn^{++} while Mg^{++} is preferred by type B, Type D, and avian type C viruses. The divalent cation required for optimal DNA synthesis has been shown to vary with respect to the primer-template (Marcus et al., 1978; Wu and Gallo, 1975), the monovalent cation concentration and purity of the enzyme (Colcher et al., 1977). RDDP activity is stimulated by inorganic phosphate (Marcus et al., 1978) and is not inhibited by aphidocolin, an inhibitor of α polymerase (M. S. Horowitz, personal communication).

At present, there is no definitive evidence that RDDP is expressed in adult tissues. The observations reviewed thus far demonstrate that this enzyme activity may be present in malignant as well as embryonic tissue.

C. Normal Cellular DNA Polymerases

In order to distinguish an endogenous RDDP from normal cellular polymerases the characteristics of all cellular DNA polymerase must be

well known. The existence of multiple species of DNA polymerase has been recognized in a wide variety of eucaryotic cells (for review see Weissbach, 1975). Three classes of these polymerases can be distinguished by cellular location, biochemical and immunological properties. These are DNA polymerase α, DNA polymerase β, and DNA polymerase γ. Some of the relevant biochemical and immunological properties of these enzymes are as follows: (see Table 1).

DNA Polymerase α

Although DNA polymerase α was the first eucaryotic enzyme to be identified (Bollum, 1960), purification of the polymerase to homogeneity has proven difficult due to aggregation and the association of other proteins in the replication complex. The enzyme is found in the cytoplasmic fraction of mammalian tissues and cultured cells. The amount of enzyme increases when cells are in a proliferative stage when the polymerase can represent 80-90 percent of the total cell DNA polymerase content (Holmes and Johnston, 1975). DNA polymerase α can be distinguished from other cellular polymerases by its high molecular weight. The purified enzyme from cultured human KB cells has a sedimentation coefficient of 7S (Fisher and Korn, 1977). By gel filtration the molecular weight was 140,000 daltons which by denaturing polyacrylamide gel analysis was shown to be a heterodimer comprised of two subunits each of 76,000 and 66,000. Similar results were obtained by Mechali et al., (1980), in regenerating rat liver. The reported molecular weights of α-polymerase range up to 250,000 daltons but these high molecular weight forms probably represent aggregates. The preferred divalent cation is Mg^{++} and the enzyme will efficiently copy activated DNA and

the primer template $(dA\text{-}dT)_n$. The polymerase does not utilize $(rC)_n \cdot (dG)_{12-18}$, natural RNA, or mitochondrial DNA effectively. The enzyme is sensitive to high ionic strength (>25 mM) and the sulfhydryl group inhibitor, N-ethylmaleimide (NEM) (Bollum, 1975). The polymerase is also sensitive to aphidicolin, a tetracyclic diterpene-tetraol, which inhibits mitotic division (Ikegami et al., 1978; Oguro et al., 1979). This sensitivity to inhibitors of mitotic events and the increase of the enzyme when the cells are in a proliferative stage suggests that the polymerase is involved with cellular DNA replication. Antibody prepared against DNA polymerase α from human lymphocytes was found not to inhibit DNA polymerases β, γ, or RDDP's isolated from known retroviruses (Smith et al., 1975).

DNA Polymerase β

DNA polymerase β is a small molecular weight enzyme normally found in the nucleus of eucaryotic cells. The enzyme purified from different sources has a sedimentation coefficient of 3-4S with a molecular weight ranging from 35,000 to 45,000 daltons (Chang and Bollum, 1971; Chang, 1974). The polymerase is composed of a single polypeptide which has a tendency to form large aggregates under low ionic strength conditions (Wang el al., 1975). Activated DNA and $(dA\text{-}dT)_n$ are the templates most efficiently transcribed with Mg^{++} the preferred divalent cation (Chang, 1974). The enzyme will not utilize high molecular weight RNA or the primer template $(rC)_n \cdot (dG)_{12-18}$ (Spadari and Weissbach, 1975). Unlike α DNA polymerase, β polymerase is stimulated by high ionic strength and is not inhibited by NEM or aphidocolin (Longiaru et al., 1979). The pH

optimum for the polymerase ranges from 8.5 to 9.0 and the enzyme is inhibited by phosphate concentrations above 25 mM (Knopf et al., 1976). β polymerase comprises 5-15 percent of the total DNA polymerase activity in growing mammalian cells and 50 percent in resting tissues (Weissbach, 1975). DNA polymerase β is considered the DNA repair enzyme (Bertazzoni et al., 1976). The enzyme is immunologically distinct from α and γ DNA polymerase as well as the RDDP's from several retroviruses (Holmes and Johnston, 1975).

DNA Polymerase γ

DNA polymerase γ was first isolated from Hela cells by Fridlender et al., (1972). Since then the enzyme has been isolated from NC-37 cell line (Lewis et al., 1974), calf thymus (Yoshida et al., 1974), WI38 cells, normal rat spleen (Spadari and Weissbach, 1974), and many other cells (Bolden et al., 1973). Unlike DNA polymerases α and β, γ polymerase has the ability to copy the synthetic ribohomopolymer $(rA)_n \cdot (dT)_{12-15}$ much more efficiently than $(dA)_n \cdot (dT)_{12-15}$ or activated DNA. However, the enzyme, unlike viral RDDP, utilizes $(rC)_n \cdot (dG)_{12-18}$ at low efficiency and will not use poly(2'-O-methylcytidylate)$_n \cdot (dG)_{12-18}$-$(rCm)_n \cdot (dG)_{12-18}$ or transcribe heteropolymeric portion of natural RNA templates (Gerard, 1975; Robert-Guroff et al., 1977). The enzyme is moderately sensitive to NEM, utilizes Mg^{++} or Mn^{++} as the divalent cation, exhibits a broad pH optimum, and is not inhibited by aphidicolin (Longiaru et al., 1979). The purified enzyme has a molecular weight which ranges from 100,000 to 120,000 daltons and has a sedimentation coefficient of 6S. The polymerase which represents one percent of the total cellular DNA polymerase

activity is mainly found in the cytoplasm. Functionally, the enzyme was found to play a major role in the replication of mitochondrial DNA (Hübscher et al., 1979). DNA polymerase γ was found to be distinct immunologically from known viral RDDP's (Lewis et al., 1974).

Another polymerase later found to be a contaminant, mitochondrial DNA polymerase, has been partially purified from rat (Meyer and Simpson, 1970) and mouse (Hecht, 1975) livers, and Hela cells (Tibbets and Vinograd, 1973; Fry and Weissbach, 1973). The enzyme is similar to DNA polymerase α in template preferences and size - 106,000-160,000 daltons. However, unlike α polymerase, mitochondrial DNA polymerase is stimulated by 0.15 M KCl and is not sensitive to NEM. The polymerase contains an associated endonuclease activity (Tibbets and Vinograd, 1973) and has the unique ability to use mitochondrial circular DNA with a free 3'-OH end as a template primer. Recent studies have suggested that there are two polymerases associated with the mitochondria. One polymerase is similar to or is cellular DNA polymerase γ; the other enzyme is a mycoplasma-derived contaminant (Bolden et al., 1977). When mitochondria are isolated from mycoplasma-free Hela cells or rat liver, only cellular DNA polymerase γ is obtained. These results suggest that mitochondrial DNA polymerase may be a contaminant.

The occurrence of multiple species of DNA polymerases in eucaryotic cells seems to be a general phenomenon. In addition to the DNA polymerases already described some unique polymerases have been identified. A terminal transferase activity has been described in calf thymus and human peripheral lymphocytes of patients with various myelogenous and

In eucaryotic systems where DNA-mutants are rare, indirect methods must be utilized. Most of the evidence for the function of a particular DNA polymerase has been obtained by comparing the levels of the different enzymes in resting versus actively dividing cells. The subcellular compartment from which the enzymes were isolated has also given clues to the respective roles of the different polymerases.

The levels of DNA polymerase α have been measured during stationary and growth phases of mouse L cells (Chang et al., 1973), Hela cells (Spadari and Weissbach, 1974b; Chin and Baril, 1975) and BHK 21 cells (Craig et al., 1975). These studies found that both nuclear and cytoplasmic α polymerase increased in correlation with DNA synthesis. A rise in α polymerase activity was also noted in regenerating rat liver (Baril et al., 1973) and in mouse spleens induced to undergo erythropoiesis (Roodinan et al., 1975; 1976). DNA polymerase α was also found to be the major polymerase associated with replicating simian virus 40 chromosomes (Oho and Fanning 1978; Edenberg et al., 1978). These observations strongly indicate that DNA polymerase α is the nuclear replicating enzyme. This idea was supported further by the finding that aphidicolin, a selective inhibitor of DNA polymerase α, prevents mitotic division in sea urchin embryos, which requires replicate DNA synthesis, but has no effect on meiotic division in starfish oocytes, which is not dependent on DNA replication (Ikegami et al., 1978).

The nuclear enzyme, DNA polymerase β, is considered to be the eucaryotic repair enzyme. Evidence that β polymerase has a DNA repair function comes from Bertazzoni et al, (1976), who found that the major increase in β polymerase activity coincided with a peak in DNA repair

lymphoblastic leukemias (Chang and Bollum, 1971; McCaffrey et al., 1973; Coleman et al., 1974; Sarin and Gallo, 1974). The enzyme does not require a template and utilizes any one of the four deoxyribonucleoside triphosphates to extend an initiator from the 3'-hydroxyl end. The calf thymus enzyme has a molecular weight of 33,000 daltons and consists of two subunits, 26,000 and 8,000 daltons respectively. Terminal transferase can be readily differentiated from the other cellular enzymes by its unique ability to extend single-stranded DNA or short oligonucleotides. The function of the enzyme is not known but it has been postulated to be involved in the immune response by diversifying the section of the genome coding for variable regions of the immunoglobulin chains (Baltimore, 1974).

Neither partially purified nor homogenous preparations of DNA polymerases α, β, and γ have been shown to possess an exonuclease activity (Loeb, 1974). However, a high molecular weight DNA polymerase-δ-associated with a 3' to 5' exonuclease, was isolated from the cytoplasm of erythroid hyperplastic bone marrow of rabbits (Byrnes et al., 1976). The exonuclease activity was found to be inseparable from the polymerase by chromatography or density gradient centrifugation. This phenomenon is similar to the association of RNase with RDDP. RNase H is a ribonuclease which hydrolyzes the RNA of an RNA-DNA hybrid.

D. Cellular Expression of DNA Polymerases

Designing experiments to investigate the roles of specific DNA polymerases in eukaryotic systems has been difficult. In the prokaryotic system, mutants defective in a particular stage of DNA replication have provided direct evidence for the function of different DNA polymerases.

capacity and minimally with DNA replication phytohemagglutinin-stimulated lymphocytes. This repair role of β polymerase was further substantiated by Hübscher et al., (1979), who demonstrated a 7 to 10-fold stimulation of DNA repair synthesis attributable to the enzyme in UV-irradiated neuronal nuclei of rats.

DNA polymerase γ, which is found in the cytoplasm, has been implicated in mitochondrial DNA replication (Hüscher et al., 1979). In synchronized Hela cells γ polymerase was found to show a unique rise in activity immediately preceding and during the onset of DNA synthesis (Spadari and Weissbach, 1974b). In a study designed to measure α and β polymerase activity in rapidly proliferating tumor tissue, these enzyme activities were monitored in the liver tissue of rats given oral doses of diethylnitrosamine (Craddock and Ansley, 1979). This potent carcinogen produces hepatocellular carcinoma in rats. The study found that α polymerase activity increased with tumor formation. Likewise, β polymerase activity increased during tumor growth but rapidly decreased when the carcinogen was discontinued. However, α polymerase levels remained high during rapid cellular growth even though direthylnitrosamine was not present in the diet. This data suggests that high levels of α polymerase activity correlates with rapid cellular growth. These studies did show that diethylnitrosamine must be present to stimulate the repair activity of β polymerase. In rat embryos, the activities of DNA polymerases α, β, and γ were determined in brain neurons, cardiac muscle, and spleen tissue (Hubscher et al., 1977). In brain neurons and cardiac tissue α-polymerase remained at a high level until the approach of term when

these cells stop dividing and the enzyme activity disappeared. However, in splenic tissue during the late gestation, when the rate of cell division is low, α-polymerase was almost absent. After birth, with the sudden onset of cell proliferation, the enzyme activity increased dramatically. The activity of DNA polymerases β and γ demonstrated no correlation with the rate of cell division. The enzyme levels of α and β polymerase were measured in midgestation mouse embryo, trophoblast, and decidual (Sherman and Kang, 1973). Both enzymes were found to be highest in the rapidly dividing embryonic tissue and lowest in the non-replicating decidual cells. These polymerase activities were also measured in mouse spermatogenic cells during early and later stages of spermatogenesis (Hecht et al., 1979). Both α and β polymerase were substantially higher during the early stages of spermatogenesis with a decrease in activity in later germ cells. This same fluctuation phenomenon of β polymerase was also noted in rabbit endometrial tissue during pregnancy (Chilton and Daniel, 1978). High levels of α and β polymerase have been detected in normal human term placentas (Krauss and Linn, 1980; Seal et al., 1979). However, changes in activity during placental development were not determined.

Fluctuations in RDDP, as described previously, were reported in rabbit endometrial and placental tissues (Chilton and Daniel, 1978; Bedigian et al., 1976; Yang et al., 1976). In human amniotic fluid, RDDP activity associated with particles characteristic of retroviruses was detected in the first trimester (Mondal, 1977). This polymerase

activity decreased or was absent in the amniotic fluid obtained from the second and third trimesters.

The observations reviewed so far, are highly suggestive that retrovirus expression appears to be augmented in human placental tissue. For this reason the human placenta is the ideal tissue to search for the presence of a retrovirus. Utilizing the viral biochemical markers proposed by Sarngadharan et al., (1976) we have attempted to provide evidence for the expression of a retrovirus during placental development.

Bibliography

1. Ahmed, M., G. Schidlovsky, W. Korol, G. Vidrine, and J. L. Cicmanec. 1974. Cancer Res. 34:3504-3508.
2. Arya, S. K., R. F. Zeigel, J. S. Horoszewicz, and W. A. Carter. 1976. J. Surg. Oncology 8:321-332.
3. Balda, B. -R., R. Hehlman, J. -R. Cho, and S. Spiegelman. 1975. PNAS 72:3697-3700.
4. Baltimore, D. 1970. Nature(London)226:1209.
5. Baltimore, D. 1974. Nature 248:409-411.
6. Bedigian, H. G., R. R. Fox, and H. Meier. 1976. Cancer Res. 36: 4687-4692.
7. Beemon, K. 1978. Current Topics in Microbiology and Immunology 79:73-110.
8. Bendrich, A., E. Borenfreund, S. Witkin, D. Beju, and P. J. Higgins. 1976. Prog. Nuc. Acid Res. Mol. Biol. 17:43.
9. Benveniste, R. E. and G. J. Todaro. 1974. Nature 252:456-459.
10. Benveniste, R., M. M. Lieber, D. M. Livingston, C. J. Sherr, G. J. Todaro, and S. S. Kalter. 1974. Nature 248:17-20.
11. Benveniste, R. E. and G. J. Todaro. 1977. PNAS 74:4557-4561.
12. Bertazzoni, U., M. Stefanini, G. C. F. Pedrali Noy, G. Guilotto, F. Nuzzo, A. Falschi, and S. Spardari. 1976. PNAS U.S.A. 73:785-789.
13. Bishop, J. M. 1978. Ann. Rev. Biochem. 47:35-88.
14. Bishop, J. M., W. E. Levinson, N. Quintrell, D. Sullivan, L. Fanshier, and J. Jackson. 1970. Virology 42:182-195.

15. Bolden, A., M. Fry, R. Muller, R. Citarella and A. Weissbach. 1972. Archs. Biochem. Biophys. 153:26-33.

16. Bolden, A. G., G. P. Noy, and A. Weissbach. 1977. J. Biol. Chem. 252:3351.

17. Bollum, F. J. 1960. J. Biol. Chem. 235:2399-2403.

18. Bollum, F. J. 1975. Progress in Nucleic Acid Research and Molecular Biology. 15:109-144. J. N. Davidson and W. E. Cohn (eds.). Academic Press, New York.

19. Byrnes, J. J., K. M. Downey, V. L. Black, and A. G. So. 1976. Biochem. 15:2817-2823.

20. Chandra, S., T. Liszczak, W. Korol, and E. M. Jensen. 1970. Int. J. Cancer 6:40-45.

21. Chandra, P. and L. K. Steel. 1977. Biochem. J. 167:513-524.

22. Chang, L. M. S. 1974. Methods in Enzymology 29:81-88. L. Grossman and K. Moldave (eds.). Academic Press, New York.

23. Chang, L. M. S. and F. J. Bollum. 1971. J. Biol. Chem. 246: 909-916.

24. Chang, L. M. S. and F. J. Bollum. 1971. J. Biol. Chem. 246: 5835-5837.

25. Chang, L. M. S., M. Brown and F. J. Bollum. 1973. J. Molec. Biol. 74:1-8.

26. Chilton, B. S., and J. C. Daniel. 1978. Bio. of Reprod. 18: 371-378.

27. Chiu, R. W. and E. F. Baril. 1975. J. Biol. Chem. 250: 7951-7957.

28. Colcher, D., R. L. Heberling, S. S. Kalter, and J. Schlom. 1977. J. Virol. 23:294-301.

29. Coleman, M. S., J. J. Hutton, and P. DeSimone, F. J. Bollum. 1974. PNAS U.S.A. 71:4404-4408.

30. Craddock, V. M. and C. M. Ansley. 1979. Biochem. Biophys. Acta 564:15-22.

31. Craig, R. K., P. A. Costello, and H. M. Keir. 1975. Biochem. J. 145:233-240.

32. Dalton, A. J., J. L. Melnick, H. Bauer, G. Beaudreau, P. Bentvelzen, D. Bolognesi, R. Gallo, A. Graffi, F. Haguenau, W. Heston, R. Heuber, G. Todaro, and U. I. Heine. 1974. Intervirology 4:201-206.

33. Daniels, J. C. Paper presented at meeting of American Society of Zoologists, December 1973, Houston, Texas.

34. Dirksen, E. R. and J. A. Levy. 1977. J. Natl. Cancer Inst. 59: 1187-1192.

35. Ellerman, V. and O. Bang. 1908. Zentra bakt parasit abt 1 orig. 46:595-597.

36. Fine, D. and G. Schocketman. 1978. Cancer Research 38:3123-3139.

37. Fisher, P. A. and D. Korn. 1977. J. Biol. Chem. 252:6528-6535.

38. Fowler, A. K., N. M. Koutlab, P. D. Kind, J. E. Strickland, and A. Hellman. 1975. Proc. Soc. Exp. Biol. Med. 148:14-18.

39. Fowler, A. K., J. E. Strickland, N. M. Loutlab, and A. Hellman. 1977. Biol. Reprod. 16:344-348.

40. Fridlender, B., M. Fry, A. Bolden, and A. Weissbach. 1972. PNAS U.S.A. 69:452-455.

41. Fry, M. and A. Weissbach. 1973. Biochem. 12:3602-3608.

42. Edenberg, H. J., S. Anderson, and M. L. DePamphilis. 1978. J. Biol. Chem. 253:3273-3280.

43. Gallagher, R. E., G. J. Todaro, R. G. Smith, D. M. Livingston, and R. C. Gallo. 1974. P.N.A.S. U.S.A. 71:1309.

44. Gallo, R. C., S. S. Yang, and R. C. Ting. 1970. Nature(London)228: 927.

45. Gallo, R. C., R. E. Gallagher, N. R. Miller, H. Mondal, W. C. Saxinger, R. G. Smith, and D. H. Gillespie. 1975. C.H.S.Q.B. 39:933.

46. Gardner, M. B., S. Rasheed, S. Shimizu, R. W. Rongey, B. E. Henderson, R. M. McAllister, V. Klement, H. P. Charman, R. V. Gilden, R. L. Heberling, and R. J. Huebner. 1977. Search for RNA Tumor Virus in Humans in Origins in Human Cancer, Vol. B., pp. 1235-1251. by H. Hiatt. J. D. Watson, and J. A. Winsten. CSHL 1977, N. Y.

47. Gerard, G. F. 1975. Biochem. Biophys. Res. Comm. 63:706.

48. Gerard, G. F. and D. P. Grandgenett. 1975. J. Virol. 15:785-797.

49. Gerwin, B. I., S. G. Smith, and P. T. Peebles. 1975. Cell 6:45-52.

50. Hecht, N. B. 1975. Biochem. Biophys. Acta 414:59-70.

51. Hecht, N. B., D. Farrell, and J. L. Williams. 1979. Biochem. Biophys. Acta 561:358-368.

52. Hellman, A., and A. K. Fowler. 1977. In: Microbiology-1977. Schlessinger, D. (ed.). Washington, D.C. American Society for Microbiology 1977, pp. 552-558.

53. Herbrink, P., G. N. P. vanMuyen, and S. O. Warnarr. 1978. Abstracts of CSHL meeting of RNA tumor viruses.

54. Hirsch, M. S., A. P. Kelley, D. S. Chapin, T. C. Fuller, P. Black, and R. Kurth. 1978. Science 199:1337-1340.

55. Hirschman, S. Z., M. Gerber, and E. Garfinkel. 1978. J. Med. Virol. 2:61-76.

56. Holmes, A. M. and I. R. Johnston. 1975. FEBS Letters 60:233-243.

57. Hsiung, G. G., C. K. Fong, and C. J. Evans. 1974. Intervirology 3:319-331.

58. Hübscher, U., C. C. Kuenzle, and S. Spadari. 1979. PNAS 76: 2316-2320.

59. Ikegami, S., T. Taguchi, M. Ohashi, M. Oguro, H. Nagano, and Y. Mano. 1978. Nature 275:458-460.

60. Imamura, M., P. E. Phillips, and R. C. Mellors. 1976. Amer. J. Pathol. 83:383-390.

61. Jackquemin, P. C., W. C. Saxinger, and R. C. Gallo. 1978. Abstract in CHSL meeting of RNA tumor viruses.

62. Jaenisch, R. 1976. PNAS 73:1260-1264.

63. Jensen, E. M., I. Zelljadt, H. C. Chopra, and M. M. Mason. 1970. Cancer Res. 30:2388-2393.

64. Kajima, M., and M. Pollard. 1968. Nature(London)218:188-189.

65. Kalter, S. S., R. J. Helmke, M. Panigel, P. J. Felsberg, and L. R. Axelrod. 1973. Science 179:1332-1333.

66. Kalter, S. S., R. J. Helmke, R. L. Heberling, M. Panigel, A. K. Fowler, J. E. Strickland, and A. Hellman. 1973. J. Natl. Cancer. Instit. 50:1081-1084.

67. Kalter, S. S., and R. L. Heberling. 1974. Abstracts of the Annual Meeting, American Society for Microiology 233.

68. Kalter, S. S., R. L. Heberling, R. J. Helmke, M. Panigel, G. C. Smith, D. C. Kraemer, A. Hellman, A. K. Fowler, and J. E. Strockland. 1975. Bibl. Haematol. 40:391-401.

69. Kantor, J. A., Y.-H. Lee, J. G. Chirikjian, and W. F. Feller. 1979. Science 204:511-513.

70. Kim. B. Y. 1977. Europ. J. Cancer 13:721-728.

71. Knopf, K. W., M. Yamada, and A. Weissbach. 1976. Biochem. 15: 4540-4548.

72. Krauss, S. W. and S. Linn. 1980. Biochem. 19:220-228.

73. Leong, J., J. Nelson, and J. A. Levy. 1978. Ann. Meeting Amer. Soc. Microbiol. 223.

74. Lewis, J. W. Abrell, R. G. Smith, and R. C. Gallo. 1974. Biochem. Biophys. Acta 349:148-160.

75. Levy, J. A. 1978. Current Topics in Microbiology and Immunology 79:111-213.

76. Lin, F. H. and M. Papini. 1979. Biochem. Biophys. Acta 561:383-395.

77. Loeb, L. A. 1974. The Enzymes 10:173-209. P. D. Boyer (ed.). Academic Press, New York.

78. Longiaru, M., J. -E. Ikeda, Z. Jarkovsky, and S. B. Horwitz, M. S. Horwitz. 1979. Mucl. Acids Res. 6:3369-3387.

79. Marcus, S. L., N. H. Karkar, and J. J. Modak. 1978. Biochem. Biophys. Acta. 519:317-330.

80. Mayer, R. J., R. G. Smith, and R. C. Gallo. 1974. Science 185: 864-866.

81. McCaffrey, R., D. F. Smoler, and D. Baltimore. 1973. PNAS 70: 521-525.

82. Mechali, M., J. Abadiedebat, and A. M. DeRecondo. 1980. J. Biol. Chem. 255:2114-2122.

83. Meier, H. and R. R. Fox. 1973. Bibliotheca Hematol. 39:79-92.

84. Meyer, R. R. and M. V. Simpson. 1970. J. Biol. Chem. 245:3426-3435.

85. Mellors, R. C. and J. W. Mellors. 1976. PNAS 73:233.

86. Michalides, R., S. Spiegelman, and J. Schlom. 1975. Cancer Res. 35:1003-1008.

87. Mondal, H. 1977. Biochem. Biophys. Res. Comm. 79:67-75.

88. Mondal, H., R. E. Gallagher, and R. C. Gallo. 1975. PNAS 72: 1194-1198.

89. Nelson, J. A., J. A. Leong, and J. A. Levy. 1978. PNAS 75:6263-6267.

90. Oguro, M., C. Suzuki-Hori, H. Nagano, Y. Maro, and S. Kiegami. 1979. Eur. J. Biochem. 97:603-607.

91. Otto, B. and E. Fanning. 1978. Nucl. Acids Res. 5:1715-1728.

92. Parsons, P. G., P. Goss, and J. H. Pope. 1974. Int. J. Cancer 13: 606-618.

93. Robert-Guroff, M., A. W. Shrecker, B. J. Brinkman, and R. C. Gallo. 1977. Biochem. 16:2866-2873.

94. Robinson, W. S., A. Pitkanen, and H. Ruben. 1965. PNAS 54:137-144.

95. Robinson, W. S., H. L. Robinson, and P. H. Duesberg. 1967. PNAS 58:825-834.

96. Rokutanda, M., Y. Maeda, N. Watanabe. 1977. Biochem. Biophys. Acta 479:317-337.

97. Roodinan, G. D., J. J. Hulton, and F. J. Bollum. 1975. Exp. Cell. Res. 91:269.

98. Roodinan, G. D., J. J. Hulton, and F. J. Bollum. 1976. Biochem. Biophys. Acta 425:478.

99. Rous, P. 1911. J. Am. Med. Assoc. 56:198-200.

100. Sarin, P. S. and R. C. Gallo. 1974. J. Biol. Chem. 249:8051-8053.

101. Sarin, P. S., B. Friedman, and R. C. Gallo. 1977. Biochem. Biophys. Acta 479-198-206.

102. Sarngadharan, M. G., H. S. Allaudeen, and R. C. Gallo. 1976. Methods in Cancer Research: Busch (editor) Vol. 12:3-48.

103. Seal, G., C. W. Sherman, and L. A. Loeb. 1979. J. Biol. Chem. 254:5229-5237.

104. Schidlovsky, G. and M. Ahmed. 1973. J. Natl. Cancer Inst. 51: 225-233.

105. Schlom, J., and S. Spiegelman. 1971. PNAS 68:1613.

106. Sherman, M. I., and H. S. Kang. 1973. Develop. Biol. 34:2000.

107. Smith, R. G., J. W. Abrell, B. J. Lewis, and R. C. Gallo. 1975. J. Biol. Chem. 250:1702.

108. Spadari, S. and A. Weissbach. 1974a. J. Biol. Chem. 249:5809-5815.

119. Spadari, S. and A. Weissbach. 1974b. J. Mol. Biol. 86:11.

110. Stephenson, J. R., S. Hino, E. Garrett, and S. Agronson. 1976. Nature 261:609-611.

111. Strickland, J. E., A. K. Fowler, P. P. Kind, A. Helman, S. S. Kalter, R. L. Heberling,. R. J. Helmke. 1973. Proc. Soc. Exp. Biol. Med. 144:256-258.

112. Temin, H. M., and S. Mizutani. 1970. Nature(London)226:1211.

113. Tibbets, J. B. C., and J. Vinograd. 1973. J. Biol. Chem. 248: 3367-3379.

114. Tooze, J. 1973. The Molecular Biology of Tumor Viruses. Cold Spring Harbor Laboratory.

115. Vernon, M. L., M. J. McMahon, and J. J. Hackett. 1974. J. Natl. Cancer Inst. 52:987-989.

116. Wang, T. S.-F., W. D. Sedwick, and D. Korn. 1975. J. Biol. Chem. 250:7040-7044.

117. Weissbach, A. 1975. Cell 5:101-108.

118. Witkin, S. S., G. C. Korngold, and A. Bendich. 1975. PNAS U.S.A. 72:3295-3299.

119. Witkin, S. S., T. Ohno, and S. Spiegelman. 1975. PNAS 72:4133-4136.

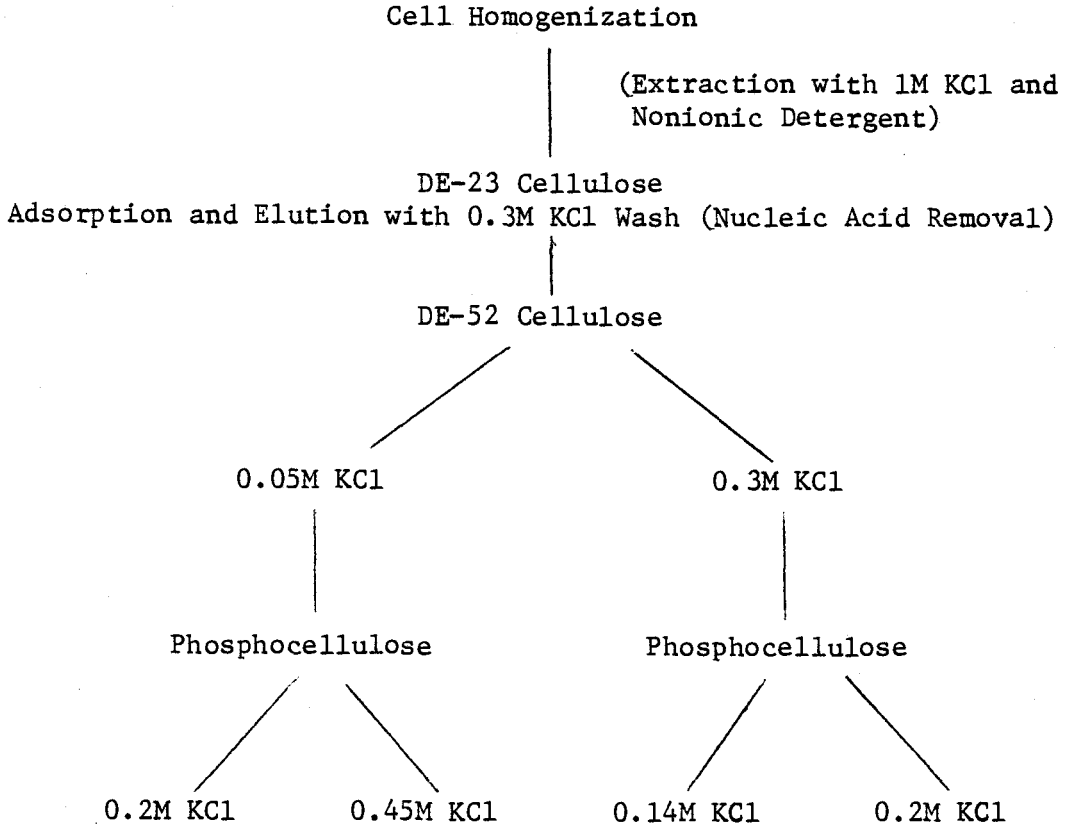
120. Witkin, S. S. and A. Bendich. 1977. Exper. Cell Res. 106:47-54.

121. Wu, A. M., and R. C. Gallo. 1975. C. R. C. Crit. Rev. Biochem. 289-347.

122. Yang, W. K., R. L. Tyndall, and J. C. Daniel. 1976. Biol. Reprod. 15:604-613.

123. Yoshida, S., T. Kondo, T. Ando. 1974. Biochem. Biophys. Acta 353:463-474.

Figure 1



SCHEMATIC OUTLINE OF THE TECHNIQUES OF DNA POLYMERASE SEPARATION*

*From Lewis et al., 1974

II. Normal Human Placentas Contain Virus-like RNA-Directed DNA Polymerase Activity

Introduction

Endogenous B, C, and D type viruses have been identified in tissues of many vertebrates including humans (cf ref. 1,2). They were first detected in solid tumors and leukemias (cf ref. 3) but have since been observed as well in normal tissues of several different animals (cf ref. 4). They have been described in synctinotrophoblast cells in normal rhesus monkey, marmoset, and baboon placentas (5 - 13).

Spiegelman and associates (14) were among the first to note the association of RNA-directed DNA polymerase with virus-like particles in human tissues. In their studies, the enzyme was only detected in human malignancies. We report here the presence of this RDDP activity in normal human placentas associated with particles possessing the density characteristics of endogenous RNA viruses. We believe this activity resides in the virus-like particles detected in the trophoblast layer of human placentas (5,10,13).

MATERIALS AND METHODS

Extraction of human placental tissues

Human placentas were obtained within one hour after delivery from Good Samaritan Hospital, Corvallis, Oregon. Tissues were used immediately or stored at -70°C. The trophoblast layer facing the maternal side of the placenta was excised and approximately 100g of this tissue was rinsed in iced STE buffer (0.01 M TRIS-HCl, 7.8; 0.1 M NaCl; 0.001 M EDTA). This tissue was then minced and the fragments were suspended in two volumes of the STE buffer and homogenized on ice in a Virtis homogenizer (maximum speed) in ten second episodes for 10-20 times. The suspension was centrifuged at 2,000 x g for 10 min. at 4° and the resulting supernatant was centrifuged at 10,000 x g for 10 min. at 4°. The supernatant was collected and then layered onto a 20 ml pad of 20% sucrose in STE and centrifuged at 95,000 x g for 60 min. at 4° in a Beckman Type 30 rotor. The resulting pellet was resuspended in STE and homogenized by forcing the suspension through an 18 gauge needle repeatedly and then a 23 gauge needle twice. The suspension was then layered onto a linear gradient of 20-60% sucrose (w/v) in STE with a 65% sucrose cushion and centrifuged for 13 hours at 90,000 x g, 4°, in a Beckman SW 41 rotor. The gradient was collected from below into 25 equal fractions. Unless indicated otherwise, each fraction was assayed for $(C_n)\cdot(dG)_{12-18}$ template-primer directed DNA polymerase activity. PNE buffer (0.1 M NaH_2PO_4, 7.8; 0.1 M NaCl; 0.001 M EDTA) was used in some experiments because it appeared to stabilize the enzyme activity.

Assays for DNA synthesis with homopolymeric-oligomeric template-Primers.

A 20-30 µl aliquot was taken from each fraction collected from a sucrose gradient after isopycnic centrifugation of the placenta extract. Each sample was disrupted by the addition of 10 µl of Buffer E (0.267 M TRIS-HCl, 8.3; 41.7% glycerol; 0.55 M KCl; 1.67% NP40; 0.03 M dithiotreitol; 0.4 M NaCl) for 15 min. at 0°. A 75 µl reaction mixture was then established that contained 5 µg of the appropriate template-primer (P & L Biochemicals, Milwaukee) 6 mM Mg (CH_3COO_2), and 0.18 µmoles [^{3}H-dGTP (3-4,000 cpm/pmole) (ICN Pharmaceuticals, California). After 30 min at 37°, the reaction was stopped by the addition of 10% trichloroacetic acid, 0.01 M Na pyrophosphate. The precipitate was collected on Millipore cellulose nitrate filters and counted in a Beckman LS-8000 liquid scintillation counter. The enzyme activity was generally stable at 4° in sucrose for 4-6 days. We were unable to preserve activity by storage at -20° in either 50% glycerol, 1 mg/ml bovine serum albumin, or 10% fetal calf serum.

Effect of RNase on the endogenous DNA polymerase activity.

Fractions obtained after equilibrium density gradient centrifugation of placental extracts were assayed for endogenous DNA polymerase activity. A 30 µl aliquot of the appropriate fractions was diluted to 78.5 µl with H_2O and pretreated with 10 µl of lysing buffer (0.5 M TRIS-HCl, pH 7.8; 0.1% NP40; 0.2 M dithiotreitol; 0.6 M NaCl; 33 mM $MgCH_3COO$) and 10 µl (^{3}H)-dTTP (New England Nuclear Corp) (0.02 mM, 3200 cpm/pmole) for 5 min at room temperature. The reaction was started by the addition

of 0.05 µg each of unlabeled dATP, dGTP, dCTP (1.5 µl). Duplicate samples were incubated with boiled pancreatic RNase A (320 µg/ml, Sigma) and T_1 RNase (60 µg/ml) for 15 to 30 min, 37°. All reactions were stopped by the addition of 1 ml 0.1 M sodium pyrophosphate and 1 ml 10% trichloroacetic acid. After 15 min at 0°, the acid-insoluble radioactivity was collected on Millipore filters and counted in a liquid scintillation counter.

Electron Microscopy.

Fractions corresponding to peaks of RDDP activity at 1.15 to 1.17 g/ml in sucrose were pooled, diluted with phosphate buffered saline and centrifuged at 100,000 x g for 60 min. The supernatant fluid was removed and the pellet was fixed with glutaraldehyde. Subsequently, the pellet was post-fixed with osmium tetroxide, dehydrated, and embedded in Epon-araldite (Electron Microscopy Sciences). After thin-sectioning, the material was stained with lead citrate and uranyl acetate and examined in a Phillips EM300 electron microscope by A. Soeldner.

Analysis of endogenous reaction product.

An endogenous DNA polymerase reaction mixture was incubated with (^{3}H)-dTTP (0.02 mM, 41,000 cpm/pmoles and (^{3}H)-dGTP (0.001 mM, 20,000 cpm/pmole) at 37° for 30 min and terminated by the addition of sodium dodecyl sulfate (SDS) to final concentations of 1% SDS and 0.4 M NaCl. After the addition of an equal volume of phenol:cresol:chloroform mixture (7:1:7), containing hydroxyquinoline (0.18 g/100 ml), the mixture was shaken at room temperature for 5 min and centrifuged at 2000 x g

for 5 min. The aqueous phase was reextracted once more and then analyzed by cesium sulfate equilibrium centrifugation in a Beckman SW 50.1 rotor, 110,000 x g for 66 hours, 15°.

RESULTS

Detection of RDDP activity in placental extracts.

The gradient fractions obtained from extracts of the trophoblast layer of human placentas, banded to equilibrium in sucrose, were assayed for RDDP activity with $(C)_n \cdot (dG)_{12-18}$. Most of the RDDP activity banded at 1.15 to 1.17 g/ml (Fig. 1). An additional peak of enzymatic activity at 1.22 to 1.25 g/ml was observed in some cases and in certain placental extracts, a distinct peak of enzymatic activity was detected at 1.12 g/ml. The distribution of RDDP activity in over 100 placentas is shown in Figure 2. The amount of enzyme activity detected did not correlate with the number of pregnancies nor the age of the woman. The absence of activity in some placentas appears to be related to the presence of an anti-enzyme inhibitor. This inhibitor also prevents the RNA-dependent DNA synthesis by Rauscher Murine Leukemia Virus (in preparation).

The gradient fractions were also analyzed with the template-primers, $(C_{methyl})_n \cdot (dG)_{12-18}$, $(dC)_n \cdot (dG)_{12-18}$, and $(dG)_{12-18}$ alone (Table 1). The $(C_{methyl})_n \cdot (dG)_{12-18}$ template primer is specific for viral RDDP (15) and enzymatic activity detected with this template-primer coincided with activity assayed with $(C)_n \cdot (dG)_{12-18}$. Assays done with $(dC)_n \cdot (dG)_{12-18}$ or $(dG)_{12-18}$ alone did not detect any enzymatic activity at 1.15 to 1.17 g/ml. Divalent cation was required for the reaction with $(C)_n \cdot (dG)_{12-18}$; Mg^{++} was preferred over Mn^{++} under the reaction conditions employed.

Incorporation of labeled dGTP into acid-insoluble material in the exogenous reaction depended on concentration of the enzyme preparation

(Fig. 3a); the kinetics of incorporation were linear for at least 30 min (Fig. 3b).

RNase sensitivity of the endogenous DNA polymerase activity.

The pooled fractions of 1.15 to 1.17 g/ml density region of the sucrose gradient had endogenous activity in the presence of (^{3}H)-dTTP and three other nonradioactive deoxyribonucleotide triphosphates. The peak of endogenous activity occurred with fractions from the density region 1.55 to 1.17 g/ml. The rate of incorporation was linear for the first 30 min and incorporation of radioactivity into acid-insoluble material was RNase sensitive (Fig. 4).

Conversion of activity to core density.

Upon heating at 37° the structures banding in sucrose at 1.15 to 1.17 g/ml were found to undergo a shift in density from 1.22 to 1.24 g/ml (Fig. 5). After heating, no loss in activity was observed although 50% of the activity originally present at 1.15 g/ml shifted in density to 1.24 g/ml. RDDP activity that banded primarily at 1.24 g/ml was also observed in extracts from placentas which had been frozen at -85° for more than 24h and in placentas frozen and thawed more than once. The freezing procedure appears to increase the activity banding at 1.24 g/ml at the expense of the activity present at 1.15 g/ml.

Characterization of Endogenous Reaction Product.

The material banding at 1.15 g/ml was used to catalyze an endogenous reaction in which radioactively-labeled DNA was synthesized. The (^{3}H)-TTP and (^{3}H)-dGTP labeled DNA product was subjected to Cs_2SO_4 gradient centrifugation. Most of the labeled DNA banded in the RNA density region, 1.63 g/ml, and some labeled DNA was bound in the equi-

molar DNA:RNA hybrid region of 1.54 g/ml. Labeled DNA was also detected in the DNA region (1.44 g/ml) (Fig. 6A). After heat treatment at 100° for 2 min, the majority of labeled material shifted in density to the DNA region (Fig. 6B).

Electron microscopic examination of fractions containing polymerase activity.

The material found at 1.15 g/ml was fixed, sectioned and stained with uranylacetate and lead citrate. Numerous virus-like particles of approximately 100 nm in diameter were observed. These structures contained central electron dense cores and double-unit membrane envelopes (Fig. 7).

DISCUSSION

Retrovirus-like particles have been detected by electron microscopy in nonhuman and human primate placentas. RNA-directed DNA polymerase (RDDP) activity has been described in normal placental tissue of rhesus monkeys (16) and rabbits (17) and in human amniotic fluid (18). The experiments described in this paper demonstrate that normal human placental tissue also possess RDDP activity associated with structures banding at densities characteristic of complete retroviruses and viral cores. The results indicate that virus-like RDDP, previously recognized only in human tumors, can be detected as well in normal human tissues.

This RDDP activity in human placentas was demonstrated by assays using the exogenous template-primers, $(C)_n \cdot (dG)_{12-18}$ and not $(dC_n) \cdot (dG)_{12-18}$. To date, only the methylated template-primer has been reported as specific for viral RDDP (15). No terminal nucleotidyl transferase activity was found in those placental fractions that had high RDDP activity although fractions at different density regions did show low levels of this enzymatic activity for some placentas. Although template specificities cannot be used to prove unambiguously the presence of viral enzyme, the results suggest that the enzyme is of viral rather than cellular origin.

The endogenous activity found at density 1.15 to 1.17 g/ml was sensitive to treatment with RNase and preliminary evidence suggests that the endogenous DNA product synthesized by placenta particles of density 1.15 to 1.17 g/ml sediments in a molecular weight region corresponding

to DNA associated with a 35S template RNA. We have been unable to obtain a labeled 70S RNA from these particles. Either nucleases are preventing its detection or the placental RDDP activity is associated with immature viral particles which contain 35S instead of 70S RNA. The shift in density of the material containing RDDP activity from 1.15 g/ml to that characteristic of viral cores would be expected of a viral enzyme. Further data in support of a viral enzyme in human placentas is the fact that the DNA synthesized in an endogenous reaction banded in cesium sulfate gradients either as RNA (presumably because the RNA is high molecular weight) or as an RNA:DNA hybrid (1:1). Moreover, after boiling, this labeled DNA banded as DNA. This latter result indicates that the labeled DNA which banded as RNA was indeed hybridized to a high molecular weight RNA.

As seen in the electron microscope, material banding at 1.15 g/ml had numerous particles of 100-150 nm. These structures have several common structural features, including a two unit membrane envelope and a centrally located electron dense core. In some cases, surface subunits were visible in the core and resembled the surfaces of murine leukemia virus cores (19).

The significance of these placental virus-like particles is not known. More than 80% of all placentas examined contained some RDDP activity. Whether these structures represent congenital transplacental infection by a virus or whether they are endogenous viruses involved in developmental processes in the host is not known.

The enzyme activity in crude preparations from isopycnic gradients was not inhibited by antisera prepared against RDDP of the primate-derived virus. The immunological relationship of purified human placenta RDDP to other primate, cellular and viral, polymerases will be the subject of a future report.

BIBLIOGRAPHY

1. Hehlmann, R. (1976) Current Topics in Micro. and Immunol. 73, 141-215.
2. Levy, J. (1976) Endogenous C type viruses:double agents. Biomedicine 24, 84-93.
3. Gross, L. (1970) Oncogenic Viruses, 2nd ed. Pergaman, Oxford.
4. Levy, A. (1978) Current Topics in Microbiology and Immunology 79, 111-213.
5. Kalter, S. S., Helmke, R. J., Heberling, R. L. (1973) J. Nat. Cancer Inst. 50, 1081-1084.
6. Schidlovsky, G., Ahmed, M. (1973) J. Natl. Cancer Inst. 51, 255-
7. Benveniste, R. E., Lieber, M. M., Livingston, D. M., Sher,, C. J., Todaro, G. J., and Kalter, S. S. (1974) Nature 28, 17-20.
8. Feldman, D. G. (1973) Pro. Nat. Acad. Sci. 70, 1976-1980.
9. Chandra, S., Liezczack, T., Korol, W., Jensen, E. M. (1970) Int. J. Cancer 6, 40-45.
10. Vernon, M. L., McMahon, J. M., and Hackett, J. J. J. Natl. Cancer Inst. 52, 987-989.
11. Dalton, A. J., Hellman, A., Kalter, S. S. (1974) J. Nat. Cancer Inst. 52, 1379-1381.
12. Imamura, M., Philips, P. E., and Mellors, R. C. (1976) Amer. J. Path. 83, 383-390.
13. Dirksen, E. R., and J. Levy (1977) J. Nat. Cancer Inst. 59, 251-256.
14. Schlom, J., and S. Spiegelman (1971) Science 174, 849-843.
15. Gerard, G. (1975) Biochem. Biophys. Res. Commun. 63, 706-711.
16. Mayer, R., Smith, R. G., and Gallo, R. (1974) Science 185, 864.

17. Bedigian, H. G., Fox, R. R., and Meier, H. (1976) Cancer Res. 36, 4687-4692.

18. Mondal, H. (1977) Biochem. Biophys. Res. Commun. 79, 67-75.

19. Nermut, M. V., Frank, H., and Shaefer, W. (1972) Virology 49, 345-358.

LEGEND TO FIGURES

Figure 1: RNA-directed DNA polymerase (RDDP) activity in the microsomal pellet from the extracts of a human placenta. The microsomal pellet was prepared as described in Materials and Methods and fractionated by sedimentation to equilibrium in a 20-65% sucrose gradient. 30 µl aliquots of each fraction were assayed for RDDP activity with (0) $(rC)_n \cdot (dG)_{12-18}$, as described in Materials and Methods. Density (0) was determined by refractometry.

Figure 2: RDDP activity in human placentas correlated with number of pregnancies. Each point represents the $(C)_n \cdot (dG)_{12-18}$ directed activity for a different placenta. Activity is measured by (^{3}H)-dGTP incorporation into acid precipitable cpm under standard assay condition (See Methods), for fractions at 1.21-1.24 g/ml (A), 1.15-1.17 g/ml (B), and 1.12-1.13 g/ml (C).

Figure 3: RDDP activity in placental extract banding at 1.15 to 1.17 g/ml in the presence of $(rC_{methyl})_n \cdot (dG)_{12-18}$. (A) Enzyme dependency of the reaction. The reaction mixture with varying amounts of placental extract was incubated at 37°C for 30 min. (B) Kinetics of the DNA synthesis. DNA polymerase activity was assayed with 30 µl of the pooled fractions at 1.15 to 1.17 g/ml as enzyme for the indicated lengths of time.

Figure 4: The effect of Ribonuclease treatment on the Endogenous Polymerase Reaction. Material obtained at 1.15 g/ml was used to catalyze an endogenous DNA polymerase reaction. The kinetics of DNA synthesis with (O) and without (O) pretreatment with ribonuclease A and T are shown. Pretreatment with ribonuclease is described in Methods.

Figure 5: Production of "cores" from human placenta extracts. The microsomal pellet was obtained from 140g of placental tissue from a woman with 11 previous preganancies. The preparation was layered on a 20-65% linear sucrose gradient and centrifuged at 150,000 x g for 12 hours, 4° (Spinco SW 50.1 rotor). A) Each fraction was assayed for exogenous DNA polymerase activity with $(rC)_n \cdot (dG)_{12-18}$. Material banding at 1.15 g/ml was pooled, pelleted at 190,000 x g and resuspended in STE buffer. B) After heating for 15 min at 37°, the material was layered on a 20-65% linear sucrose gradient and centrifuged for 3 hours, 190,000 x g, 4° (Spinco SW 50.1 rotor). The gradient was fractionated and each fraction was then analyzed for exogenous DNA polymerase activity with $(rC)_n \cdot (dG)_{12-18}$. Density was measured by refractometry.

Figure 6: Cesium sulfate bouyant density analysis of the endogenous reaction product of placenta extract banding at 1.15 g/ml in sucrose density gradients. The labeled material was isolated and centrifuged in cesium sulfate as described in Methods. (A) [^{3}H] DNA product without treatments. (B) [^{3}H] DNA product denatured at 100° for 2 min. DNA and RNA markers banded at 1.44 g/ml and 1.64 g/ml respectively.

Figure 7: Electron micrographs of material from placenta extracts banding at 1.15 g/ml in sucrose density gradients. Particles were fixed and negatively stained as described in Methods. Bar represents 100 nm.

Figure 1

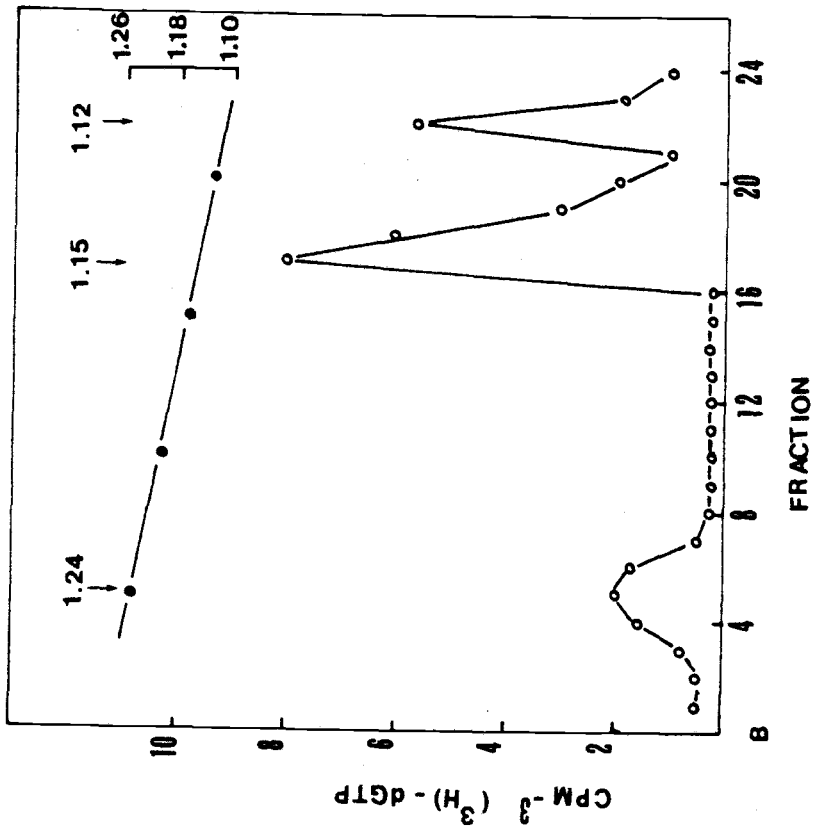
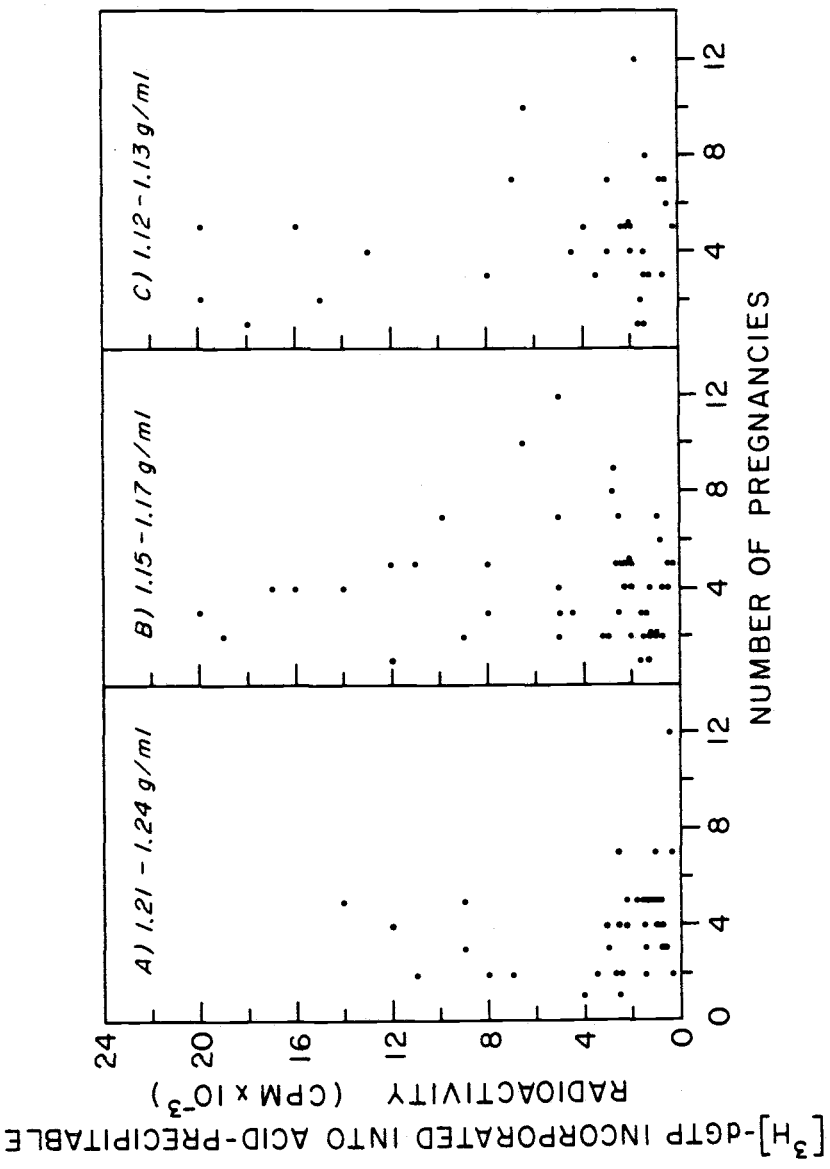

1.26
1.18
1.10
1.12
1.15
1.24
10
8
6
4
2
CPM -3 (3H) - dGTP
8
4
8
12
16
20
24
FRACTION

Figure 2


A) 1.21 - 1.24 g/ml
B) 1.15 - 1.17 g/ml
C) 1.12 - 1.13 g/ml
NUMBER OF PREGNANCIES
0
4
8
12
[3H]-dGTP INCORPORATED INTO ACID-PRECIPITABLE
RADIOACTIVITY (CPM x 10-3)
24
20
16
12
8
4
0

TABLE

SUMMARY OF TEMPLATE-PRIMER REQUIREMENTS OF PARTICLES OBTAINED FROM HUMAN PLACENTAL EXTRACTS*

Template Primer	Labelled Precursor	Activity[a]
$(rC)_n \cdot (dG)_{12-18}$	dTTP	1.83
$(rC_m)_n \cdot (dG)_{12-18}$	dGTP	1.17
$(A)_n \cdot (dT)_{12-18}$	dTTP	2.56
$(dG)_{12-18}$	dGTP	0.05
$(dC)_n \cdot (dG)_{12-18}$	dGTP	0.23
Endogenous	dTTP	1.87
70S RNA (Avian Myelobastosis Virus)	dGTP	3.29

*Reactions were performed under standard reaction conditions as described in Methods.

[a]Enzyme activity is expressed as pmoles of ^{3}H-labelled deoxyribonucleotide triphosphate incorporated per 100 µl of reaction mixture/30 min reaction.

Figure 3

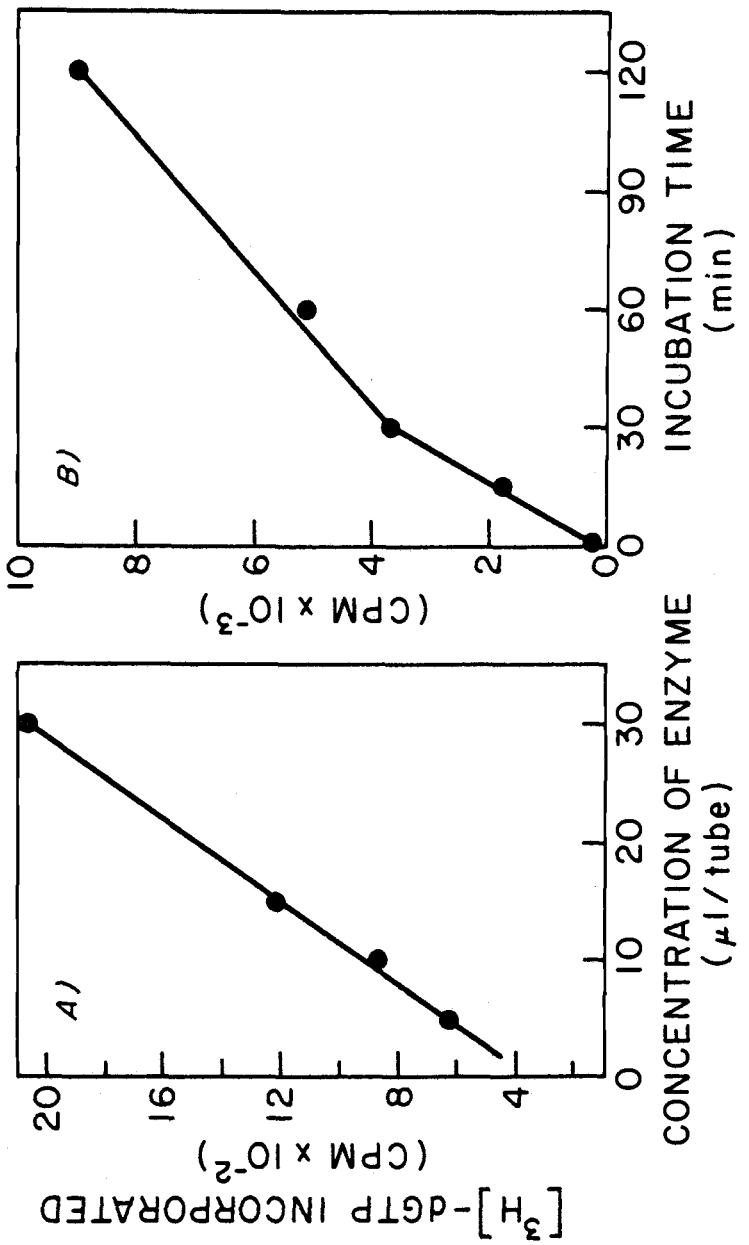

[³H]-dGTP INCORPORATED
(CPM x 10⁻²)
A)
20
16
12
8
4
0
10
20
30
CONCENTRATION OF ENZYME
(µl/tube)
(CPM x 10⁻³)
B)
10
8
6
4
2
0
0
30
60
90
120
INCUBATION TIME
(min)

Figure 4

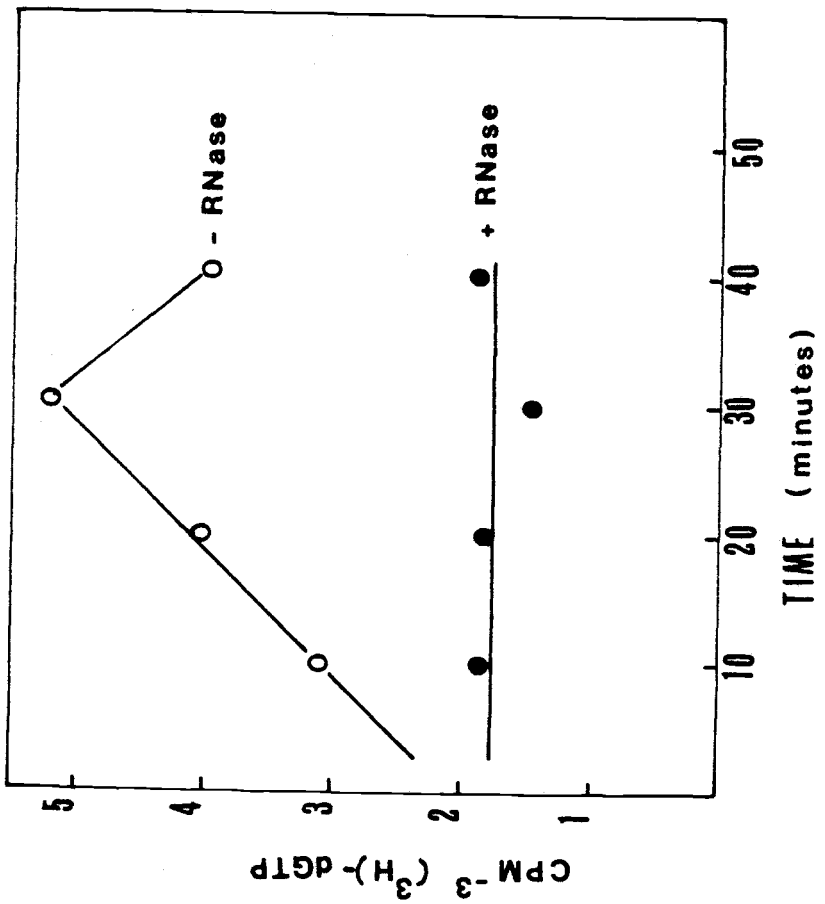


Figure 5

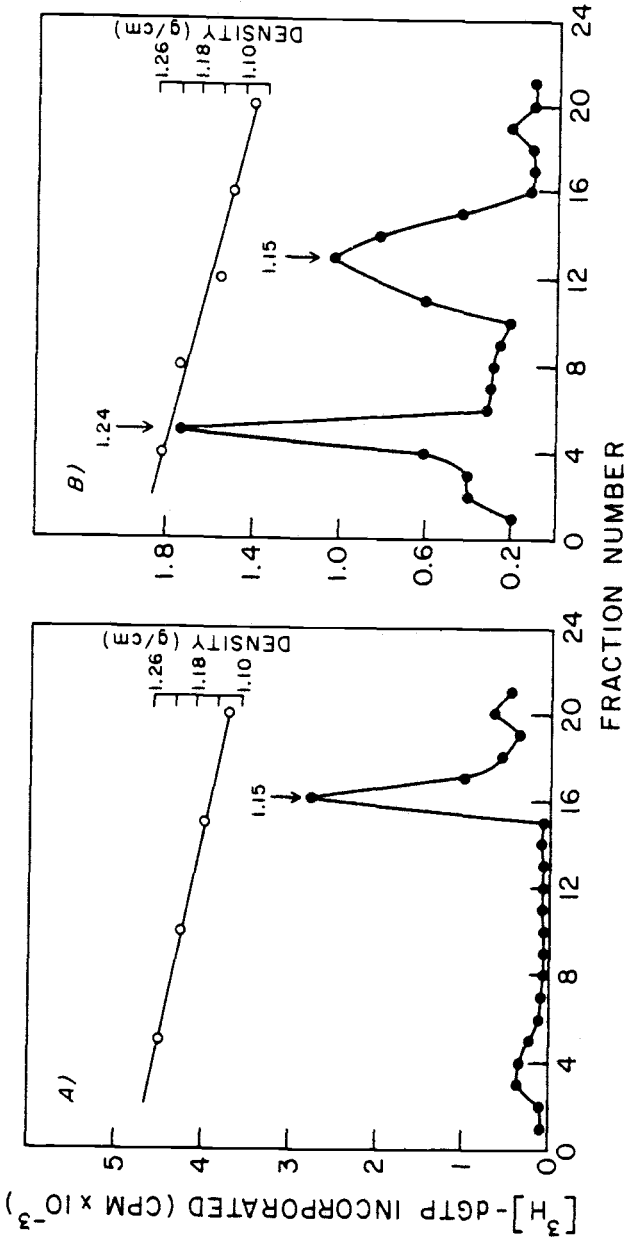
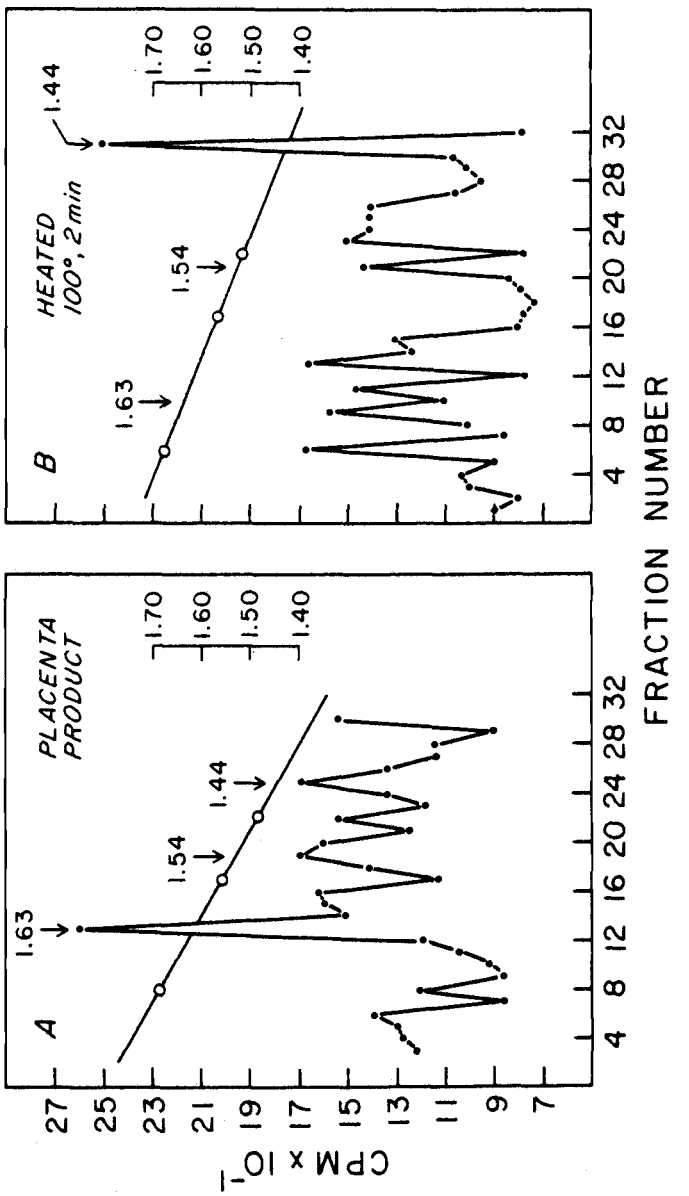
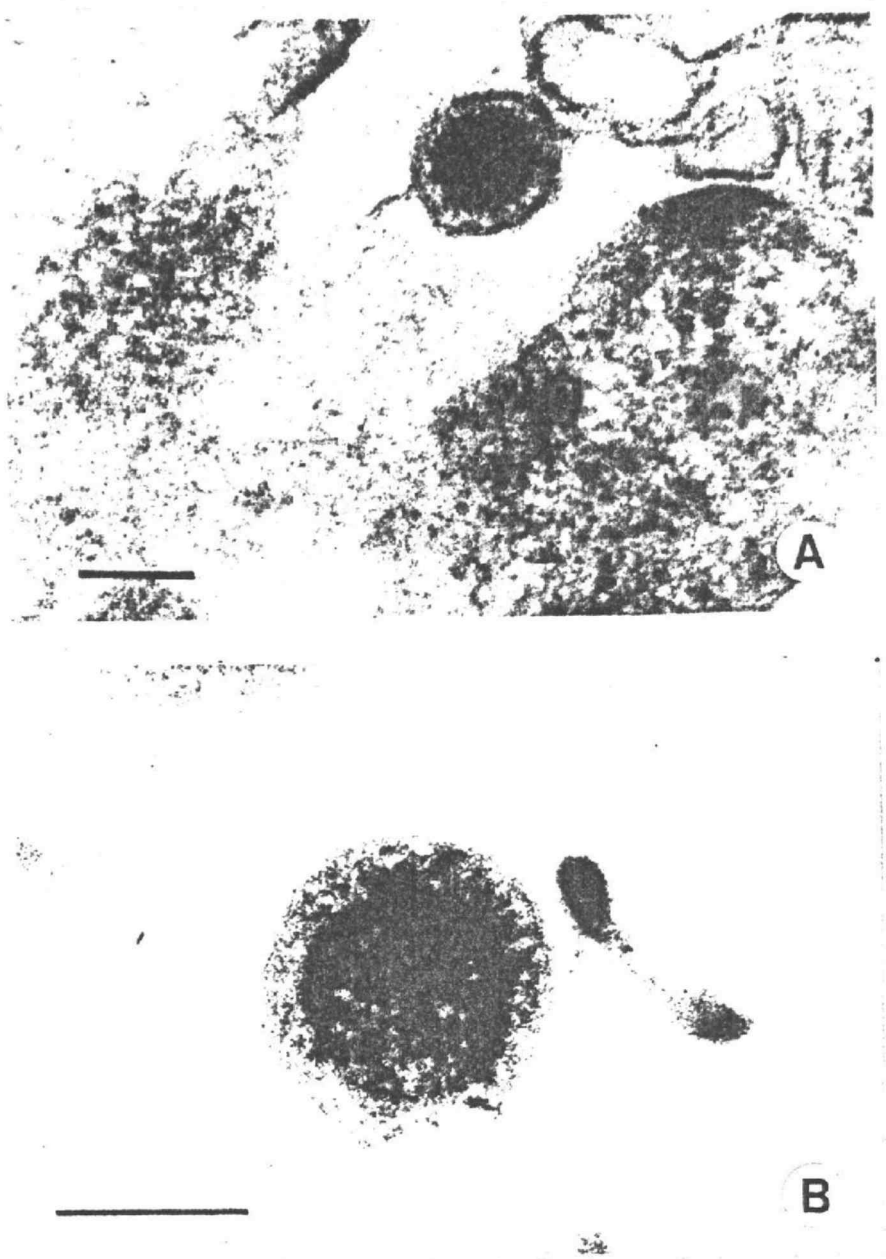

A)
B)
DENSITY (g/cm)
1.26
1.18
1.10
1.15
1.24
[3H]-dGTP INCORPORATED (CPM x 10-3)
FRACTION NUMBER

Figure 6


A
PLACENTA PRODUCT
1.63
1.54
1.44
1.70
1.60
1.50
1.40
B
HEATED 100°, 2 min
1.63
1.54
1.44
1.70
1.60
1.50
1.40
CPM x 10⁻¹
27
25
23
21
19
17
15
13
11
9
7
4 8 12 16 20 24 28 32
4 8 12 16 20 24 28 32
FRACTION NUMBER


A
B

III. Characterization of a Large Beta DNA Polymerase in Human Placenta

Characterization of a Large Beta DNA Polymerase in Human Placenta

Introduction:

At least three different classes of DNA polymerase, designated α, β, γ, have been identified commonly in human tissue (for review see Weissbach, 1979). These enzymes have been classified by primer-template specificity, size, intracellular location, elution properties on ion exchange chromatography, and sensitivity to inhibitors such as N-ethyl-maleimide and aphidocolin. DNA polymerase α is the major (80-90%) DNA polymerase in mammalian cells and is believed to be involved in the process of replication. It is large (200-155,000) when analyzed by velocity sedimentation and these are four to five species of the enzyme (Holmes et al., 1977; Krauss and Linn, 1979). DNA polymerase β is found in the nucleus where it is thought to participate in DNA repair. All reports on this enzyme indicate that it is small (30-40,000) with a sedimentation coefficient of 3.4S in glycerol. Multiple species of β polymerase have been observed for human KB cells (Wang et al., 1975), calf thumus (Chang, 1973), Novikoff hepatoma cells (Mosbaugh et al., 1977), and human placenta tissue (Krauss and Linn, 1979). DNA polymerase γ id distinguished by the ability to copy efficiently the ribostrand of $(A)_n \cdot (dT)_{12-18}$ (Spadari and Weissbach, 1974). This DNA polymerase is associated with mitochondria in the cell (Bolden, Noy, and Weissbach, 1977) and has been purified from Hela cells. Its molecular weight is 150,000 daltons (Spadari and Weissbach, 1974).

In addition to these enzymes, there are reports of several new species of DNA polymerases: polymerase N3 in human KB cells (Wang et

al., 1975) and polymerase Cm (Gerard, Lowestein, and Green, 1980). These enzymes are distinct from polymerase α, β, and γ. Other reports of unique DNA polymerase in human cells include those of cytoplasmic, particle-associated DNA polymerases with biochemical (Gallo et al., 1974; Witkin et al., 1974; Ohno and Spiegelman, 1977; Chandra and Steel, 1977) and immunological properties similar to primate retrovirus RNA-dependent DNA polymerase. It is clear from these studies that there are a large number of DNA polymerases in mammalian cells.

The observation that each class of DNA polymerase has a number of different forms suggests that each form of polymerase might function differently at different times in the cell cycle or in tissue differentiation. Since human placenta are a type of rapidly proliferating tissue which can be easily obtained, we undertook a study of these DNA polymerases in the trophoblastic layer of the placenta. During all operations, trasolyl, a potent protease inhibitor (Werle, 1972) was present in the buffer solutions. Purification of the polymerase was completed in less than 3 days. We report here on the isolation and characterization of a β-like DNA polymerase activity which is located in the nuclear fraction of human placental extracts. This enzyme differs from β polymerase by its larger size and reactivity to changes in pH and monovalent cation concentration.

MATERIALS AND METHODS

Reagents:

Unlabeled deoxyribonucleoside triphosphates were obtained from P-L Biochemicals, Inc. and pretreated with Chelex 100 (BioRad Laboratories) before use. All radioactive deoxyribonucleoside triphosphates were obtained from ICN radiochemicals division. Activated Calf Thymus DNA (CalBiochem Co.) was prepared as described by Aposhian and Kornberg (1962). Rabbit globin messenger RNA was the generous gift of Dr. Dallice Mills, Oregon State University. The template-primers $(C)_n \cdot (dG)_{12-18}$, $(dC)_n \cdot (dG)_{12-18}$, and $(A)_n \cdot (dT)_{12-18}$ were purchased from P-L Biochemicals, Inc. or Collaborative Research, Inc. Aphidocolin was obtained through the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute and the assistance of Dr. John Douros. Stock solutions of aphidocolin were prepared in dimethyl sulfoxide to 1 mg/ml. N-ethylmaleimide (NEM), phenylmethylsulfonly flouride (PMSF), and ethylene glycol-bis-(β-amino-ethylether) N,N'-tetra-acetic acid (EGTA) were purchased from Sigma Chemical Co. Trasolyl was obtained from FBA Pharmaceuticals.

Buffers:

All buffers contained 4 mM 2-mercaptoethanol, 100 KIU/ml Trasolyl, and 1 mM EGTA. Buffer A contained 20 mM Tris-HCl (pH 7.4 at 22° C), 5 mM $MgCl_2$, 25% glycerol, and indicated concentrations of KCl and NP40. Buffer B contained 20% glycerol, 20 mM Tris-HCl, pH 7.4, and 50 mM KCl unless indicated otherwise. Buffer C contained 20% glycerol, 0.5 M KCl, and potassium phosphate (pH 7.5) at indicated concentrations.

Column Chromatography Materials:

DEAE-cellulose (Type DE-23, Whatman Biochemicals, Ltd.) was equilibrated in Buffer A with 0.3 M KCl. Preswollen DEAE-cellulose (Type DE-52, Whatman Biochemicals, Ltd.) was equilibrated in Buffer B and used within two days. Phosphocellulose (Sigma Chemical Co.) was prepared by the method of Burgess (1969) and equilibrated in Buffer B. Hydroxyapatite (Bio-gel HT, BioRad Laboratories) was defined and equilibrated in Buffer B with 0.5 M KCl. The protein loading capacity of each batch of resin was predetermined with ovalbumin.

Assays of DNA Polymerase Activities:

General Polymerase Assay - This assay was designed to screen for all DNA polymerase activities. Assays were carried out in 100-µl reaction mixtures containing 5 mM $MgCl_2$; 20% ethylene glycol; 50 µM each of dATP, dCTP, and dGTP; 5 µM dTTP; 1.485 µM [^{3}H]-dTTP (67.3 Ci/mmole), 150 µM/ml activated calf thymus DNA. Routinely, 20 µl aliquots of sample were assayed in each 100 µl reaction at 37 C.

Synthetic Template-Primer Assay - Samples of 20 µl were tested in 100 µl reaction mixtures containing 40 mM Tris-HCl (pH 7.8 at 22 C), 0.12 mM EGTA, 3.9 mM dithiothreitol, 3.7 mM glutathione (reduced form), 5% glycerol, 6 mM NaCl, 60 mM KCl, 0.1% NP40, indicated concentrations of either $MgCl_2$ or $MnCl_2$, 20% ethylene glycol, 2 µg of the appropriate template-primer, and either 12.5 µM ^{3}H-dGTP (8 Ci/mmole) or 1.48 µM [^{3}H]-dTTP (63.7 Ci/mmole).

Heteropolymeric mRNA Assay - The buffer conditions for these assays were the same as those used for the synthetic template-primer assays. However, the reaction mixture contained 0.1 µg calf thymus DNA primer (prepared as described by Taylor et al., 1976); 1 µg globin messenger RNA; 5 mM dTTP, and 1.485 µM [^{3}H]-dTTP (67.3 Ci/mmole).

All enzyme reactions were conducted at 37°C and terminated by the addition of an equal volume of 0.01 M sodium pyrophosphate and then trichloroacetic acid to a final concentration of 5%. After 10 minutes in an ice bath, the acid precipitable material was collected onto glass fiber filters (GF/C, Whatman Biochemicals, Ltd.) by vacuum filtration and washed with 15 ml of 0.1 N HCl, 0.01 M sodium pyrophosphate. After a wash with 95% ethanol, the filters were dried and the radioactivity was measured in a Beckman liquid scintillation spectrometer using toluene Omifluor (New England Nuclear).

One unit of enzyme activity is defined as one nmole of deoxyribonucleotide triphosphate incorporated in one hr at 37°C.

Determination of Salt and Protein Concentration:

Protein concentration was determined by A260/A280 ratios and the BioRad protein assay. The salt concentration was determined by conductivity.

Sedimentation Analysis:

$S_{20,w}$ value relative to those of standard proteins was estimated using 5-25% (v/v) glycerol density gradients containing 0.5 M KCl, 0.05 M Tris-HCl, pH 7.4 at 22°C, 0.1% NP40, 4 mM dithiothreitol, and 20% ethylene glycol. A 0.5 ml aliquot of the enzyme preparation was layered onto

10.5 ml of glycerol gradient and centrifuged in the SW41 Ti rotor at 4°C for 17 h at 17,000 rev/min in the Beckman L5-65B centrifuge. Standard markers used were bovine serum albumin (4.4S) and ovalbumin (3.6S). The gradients were collected in 0.3 ml fractions and assayed for DNA polymerase activity.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis:

SDS-gel electrophoresis was conducted according to the methods of Laemmli (1970) and Weber and Osborn (1969) using a slab gel apparatus (model 220, Bio-Rad Laboratories, Inc.). Protein samples, in 30 µl of sample buffer containing 20% glycerol, 0.0625 M Tris-HCl, pH 7.8, and 50 mM dithiothreitol were immersed in a boiling water bath for 5 min. The sample was loaded onto an SDS-polyacrylamide gel consisting of a 4.5% stacking gel and a 10% separating gel. Electrophoresis was performed with initial settings of 10-14 mA current under constant current and run for 9 h. A set of protein standards was run simultaneously. The protein standards were obtained from Bio-Rad Laboratories and were composed of lysozyme 14,300, soybean trypsin inhibitor 21,000, carbonic anhydrase 30,000, ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B 94,000, and myosin (200,000). Gels were stained in a solution of 50% TCA and 0.1% Commassie brilliant blue R-250 destained in 7.5% acetic acid.

Purification of DNA polymerase:

Placenta Extraction - Human placentas were obtained after caesarian section from Albany General Hospital, Albany, Oregon and processed

within 1 h. All procedures were done at 0-4°C. Approximately 100 g of tissue from the trophoblast layer facing the maternal side of the placenta was excised, minced, and rinsed in STEg buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.001 M EGTA and 1 x 10^{-5} M PMSF). The minced tissue was drained, resuspended in 300 ml Buffer A, and homogenized on ice in the VirTis homogenizer (maximum speed) in 30 sec episodes for 4 times. The homogenate was clarified by centrifugation in a Sorvall HS-4 rotor, 5,000 x g for 5 min. The supernatant fluid was removed and 3 M KCl was added to this fluid to a final concentration of 0.3 M KCl. Microscopic examination of the pellet material showed nuclei and cell debris. This pellet was resuspended in 200 ml of Buffer A (0.005 M KCl and 3% NP40) or Buffer A-high salt (0.3 M KCl and 3% NP40) and homogenized as above. The pellet homogenate were then centrifuged at 5000 x g for 5 min (Sorvall HS-4 rotor). The supernatant fluid was combined with the first supernatant. Only after homogenization in 0.3 M KCl and 3% NP40 Buffer A did microscopic examination of the pellet show lysed nuclei. The combined supernatant fluids were centrifuged at 20,000 x g (Sorvall GSA rotor) for 60 min. To remove the nucleic acid from the mixture, the high speed supernatant fluid was batch adsorbed onto DEAE cellulose (DE-23, fibrous form) which had been equilibrated with Buffer A containing 0.3 M KCl. The resin was used at 1 ml of wet packed cellulose per 30 mg of protein. This procedure removed 90 to 100% of the nucleic acid as determined by A_{260}/A_{280} ratios of input and effluent sample. The eluted material was concentrated with the slow addition of solid ammonium sulfate (Schwartz/Mann Ultrapure) to 75% (47.6 g/100 ml).

After the mixture was stirred for 30 min at 0 C, the precipitated protein was pelleted at 10,000 x g for 15 min and the supernatant was removed by suction. The pellet was resuspended in and dialyzed against Buffer B.

DEAE-Cellulose Chromatography - The nucleic acid free extract was adsorbed in a DEAE-cellulose (DE-52) column (2.5 x 46 cm) previously equilibrated in Buffer B. After the column was washed with 120 ml of Buffer B, a 400 ml linear gradient of KCl from 0.05 to 1 M in Buffer B was passed through the column at 30 ml per hour. Fractions of 6 ml were collected and assayed by the general polymerase assay. Fractions containing polymerase activity which eluted in the void volume (DE-peak I) were pooled, concentrated two- to three-fold by dialysis against 30% polyethylene glycol (w/v) in Buffer B, and then dialyzed extensively against Buffer B before adsorption to phosphocellulose.

Phosphocellulose Chromatography - Approximately 35 ml of DE-peak I was loaded onto a phosphocellulose column (2.5 x 20 cm, 6 mg protein per ml packed volume). The column was washed with Buffer B followed by linear gradient of 0.05-1.0 M KCl in Buffer B. Fractions of 3 ml were collected and every other fraction was assayed for DNA polymerase activity with activated calf thymus DNA. The fractions containing DNA polymerase were pooled and dialyzed against 2 l of Buffer B in 30% polyethylene glycol (w/v) for 5 hr with two changes of buffer.

Hydroxyapatite chromatography - Before hydroxyapatite chromatography, the concentrated samples were dialyzed extensively against Buffer B with 0.5 M KCl. A 13 ml hydroxyapatite columm previously equilibrated in Buffer B with 0.5 M KCl was loaded with 30 ml of eluant. The column was washed with Buffer B-0.5 M KCl and developed in with a step gradient from 0.02 M to 0.2 M potassium phosphate, pH 7.5.

Glycerol Gradient Centrifugation - Gradients of 5-25% glycerol containing 50 mM Tris-HCl, pH 7.4, 0.5 M KCl, 2 mM dithiothreitol, 20% ethylene glycol, and 0.1% NP40 were prepared. To the top of each 12 ml gradient, 0.5 ml of sample was applied and centrifugation carried out for 17 h at 40,000 rpm in a Spinco SW40 Ti rotor at 4°C. Parallel gradients were run with bovine serum albumin (4.4S) and ovalbumin (3.6S) as sedimentation markers.

RESULTS

Purification of DNA Polymerase Activity.

Cellular extracts of the trophoblast layer of human placentas were examined for soluble DNA polymerase activity detected with activated calf thymus DNA as template-primer. Crude homogenates from 100 g of tissue yielded total activities of 50-100 units (Table 1) where 1 unit of enzyme is defined as the activity required to incorporate 1 nmole of total ^{3}H-dTTP into polydeoxynucleotide per h at 37°C. The DNA polymerase activity was further purified by DEAE-cellulose chromatography where two peaks of activity were found in the eluant fractions. The first peak of enzyme activity which eluted in the 0.05 M KCl wash was only observed in extracts from cells homogenized in the presence of high salt (0.5 M KCl) and 3% NP40 (Figure 1B). This procedure produced a total cell extract. Detergent treatment in low salt (0.05 M KCl) did not produce nuclear lysis and resulted in a cytosol extract. DNA polymerase activity was observed only at 0.15 M KCl in the cytosol extract (Figure 1A). Further processing of the 0.15 M KCl eluant by phosphocellulose and hydroxyapatite chromatography led to the isolation of α and γ polymerases. These enzymes were identified by elution properties on phosphocellulose, sedimentation in glycerol, and sensitivity to phosphate, monovalent cation concentration, N-ethylmaleimide, and aphidocolin.

The polymerase activity eluting at 0.05 M KCl was processed further through phosphocellulose where a major peak of activity eluted at 0.3 M KCl, PCI-0.3 (Fig. 2). A minor peak of activity was observed in the wash eluant. Preliminary characterization of the 0.05 M eluting activity

demonstrated a DNA polymerase which utilized $(C)_n \cdot (dG)_{12-18}$ as template-primer. However, this enzyme activity was extremely unstable despite numerous efforts to preserve it.

The final step in the purification of PCI-0.3 was chromatography on hydroxyapatite where a large increase in specific activity was observed (Table 1). In a step gradient, the enzyme activity was eluted between 0.1 and 0.15 M KPi (Fig. 3).

Enzyme activity was preserved by the addition of 20% ethylene glycol, 2 mg/ml ovalbumin, and storage at 4°C. The DNA polymerase activity has remained stable for over 6 months under these conditions.

Properties of the DNA Polymerase

The template specificity of the DNA polymerase (PCI-0.3) was determined for different synthetic template-primers, activated calf thumus DNA, and globin mRNA (Table 2). The enzyme was found to copy the synthetic template-primer $(dC)_n \cdot (dG)_{12-18}$ 3.5-fold better than activated calf thymus DNA when assayed in the presence of $MgCl_2$. In the presence of $MnCl_2$, neither activated DNA nor $(dC)_n \cdot (dG)_{12-18}$ was active for DNA synthesis (Fig. 4). The optimum $MgCl_2$ concentration was centered around 0.8 mM. With the synthetic template-primers $(rC)_n \cdot (dG)_{12-18}$, $(RC_m)_n \cdot (dG)_{12-18}$, $(rA)_n \cdot (dT)_{12-18}$, and globin mRNA, little or no DNA synthesizing activity was observed in the presence of $MgCl_2$ or $MnCl_2$. The enzyme exhibited no terminal transferase activity with $(dG)_{12-18}$.

The optimal pH for DNA polymerase activity with $(dC)_n \cdot (dG)_{12-18}$ was determined over a range of pH 6 to 9. The enzymatic activity was found to exhibit maximum activity over a narrow pH range with an optimum at pH

7.8 (Fig. 5). When enzyme activity was measured at pH 7.8 in the presence of 0.8 mM Mg^{++}, incorporation of radioactively-labeled deoxyribonucleotide was linear for at least 60 min with $(dC)_n \cdot (dG)_{12-18}$ and activated DNA (Fig. 6).

The DNA synthesizing capacity of the enzyme in the presence of inorganic phosphate, aphidocolin, NEM, and high monovalent cation concentration was studied to characterize the DNA polymerase. The enzyme was resistant to 5 mM NEM and 2 mg/ml aphidocolin (Fig 6). However 50 mM potassium phosphate (KPi) inhibited the reaction by 89%. The enzyme was unaffected by low salt (36 mM and 100 mM KCl) but did show decreased activity in 180 mM KCl. Thus, this enzymatic activity can be classifeid as a β-like form by its resistance to NEM and aphidocolin, sensitivity to KP_i, and nuclear location. Unlike β polymerases, this enzyme exhibited optimal activity at pH 7.8 whereas β-like enzymes prefer more alkaline pH levels near pH 9.2 (Weissbach, 1979). The enzyme also differs from other β-like enzymes by its sensitivity to 180 mM KCl (Table 2). Other β DNA polymerases are stimulated by this monovalent cation concentration.

Change in Template Specificity with Purification and Storage:

During enzyme purification, column eluants were routinely assayed for DNA synthesizing activity in reactions with activated DNA or $(dC)_n \cdot (dG)_{12-18}$. A predictable decline in the ability of the enzyme to utilize activated DNA as template-primer in a DNA synthesizing reaction was detected during purification and upon storage (Table 3). The activity for the synthetic template-primer remained high even after 6

months of storage. After 90 days in storage, no DNA synthesis was observed with activated DNA as the template-primer.

Molecular Weight of the Placental DNA-polymerase:

Another criterion to distinguish β DNA polymerase species is the small size of the enzyme which usually sediments at 3.4S (Weissbach, 1979) in high ionic strength glycerol gradients. Eucaryotic DNA polymerases subjected to velocity sedimentation analysis in solutions of low ionic strength tend to aggregate (Mosbaugh et al., 1977; Hecht, 1975; Wang et al., 1975; Chang, 1973). To prevent this phenomenon the purified polymerase was sedimented in a 5-25% linear glycerol gradient in 0.5 M KCl (Fig. 7). The enzyme activity sedimented as a single peak of activity at 5S. Assuming a globular shape of the protein, the estimated molecular weight of the protein is 80,000 daltons.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified DNA polymerase from phosphocellulose and the glycerol gradient was carried out in a 10% polyacrylamide gel. For both samples, a single band corresponding to a molecular weight of 78,000 daltons was observed (Fig. 8).

DISCUSSION

A DNA polymerase resembling the β species has been purified over 9,000 fold from total cell extracts of the trophoblast layer of human placenta. The polymerase is similar to previously described mammalian DNA β polymerases in its sensitivity to inorganic phosphate and insensitivity to aphidocolin and NEM. Like the β polymerases, this enzyme also does not bind to DEAE cellulose and elutes at 0.3 M KCl on phosphocellulose (Krauss and Linn, 1979; Chang and Bollum, 1971). However, it does differ from reported mammalian β polymerase species by its large size, 5S in glycerol gradients at 0.5 M KCl and 78,000 daltons by SDS gel electrophoresis, optimal pH at pH 7.8, preference for Mg^{++} and sensitivity to 200 mM KCl.

Krauss and Linn reported at least two species of β polymerase from fresh human placentas delivered by Caesarian section. In their study, the two species sedimented at 4.6 and 4.8S in glycerol gradients at 0.5 M KCl and as aggregates at 6.3, 8.6S, and 8.0S in glycerol without added KCl. This difference may be due to differences in the method of enzyme purification and tissue extraction. Even more importantly, the difference may be due to the type of tissue selected for extraction. In our study, only 100 grams of tissue was removed specifically from the trophoblast layer of each placenta. The trophoblast layer is that region of the placenta that abuts on the maternal uterine wall and participates directly in the exchange of nutrients and fluids between mother and fetus. Thus, it is possible that a particular β polymerase was selected for in this tissue. Also, every precaution was taken to

inhibit protease activity and preserve the size of the enzyme in this study. Trasolyl and PMSF was present during the purification process. Multiple forms of β polymerase have been observed for calf thumus (Chang, 1973), human KB cells (Wang et. al., 1975), murine tissues (Hecht, 1975), and Novikoff hepatoma cells (Mosbaugh et al., 1977). These studies demonstrated salt-dependent disaggregation of β polymerase and regulatory proteins which stimulate or inhibit enzyme activity, confer stability or enzyme specificity.

It is interesting to note that with enzyme purification the template preference of the large placental beta polymerase changed from activated DNA to $(dC)_n \cdot (dG)_{12-18}$. A similar report was made for placental β polymerases by Krauss and Linn (1979). In their study, the 8.8S (β_2) enzyme exhibited increased misincorporation of nucleotides with $(A)_n \cdot (dT)_{12-18}$ as template-primer in 0.5M KCl when the enzyme exists as a 4.8S unit.

The function of this enzyme in placental trophoblast cells is unknown. We are trying to determine whether it represents a unique embryonic enzyme by processing adult tissue through a similar extraction procedure. The relationship between this large molecular weight enzyme and the known cellular DNA polymerases remains to be determined.

REFERENCES

1. Aposhian, H. V., and A. Kornberg. 1962. J. Biol. Chem. 237:519-525.
2. Bolden, A. G., G. P. Noy, and A. Weissbach. 1977. J. Biol. Chem. 252:3351.
3. Byrnes, J. J., K. M. Downey, V. L. Black, and A. G. So. 1976. Biochem. 15:2817-2823.
4. Chandra, P., and L. K. Steel. 1977. Biochem. J. 167:513-524.
5. Chang, L. M. S. 1973. J. Biol. Chem. 248:6983-6992.
6. Chang, L. M. S. and F. J. Bollum. 1971. J. Biol. Chem. 246:909-916.
7. Gallo, R. C., R. E. Gallagher, N. R. Miller, H. Mondal, W. C. Saxinger, R. J. Mayer, R. G. Smith, and D. H. Gillespie. 1975. Cold Spring Harbor Symp. Quant. Biology 39:933-961.
8. Gerard, G. F., P. M. Loewenstein, and M. Green. 1980. J. Biol. Chem. 255:1015-1022.
9. Hecht, N. B. 1975. Biochem. Biophys. Acta 414:59-70.
10. Holmes, A. M., I. P. Hesslewood, and I. R. Johnston. 1974. Eur. J. Biochem. 43:487-499.
11. Krauss, S. W., and S. Linn. 1980. Biochem. 19:220-228.
12. Mosbaugh, D. W., D. M. Stalker, G. B. Probst, and R. R. Meyer. 1977. Biochem. 16:1512-1518.
13. Ohno, T., and S. Spiegelman. 1977. PNAS 74:2144-2148.
14. Spadari, S., and A. Weissbach. 1974. J. Biol. Chem. 249:5809-5815.

15. Taylor, J. M., R. Illmensee, and J. Summers. 1976. Biochem. Biophys. Acta. 442:324-330.

16. Wang, T. S-F., P. A. Fisher, W. D. Sedwick, and D. Korn. 1975. J. Biol. Chem. 250:5270-5272.

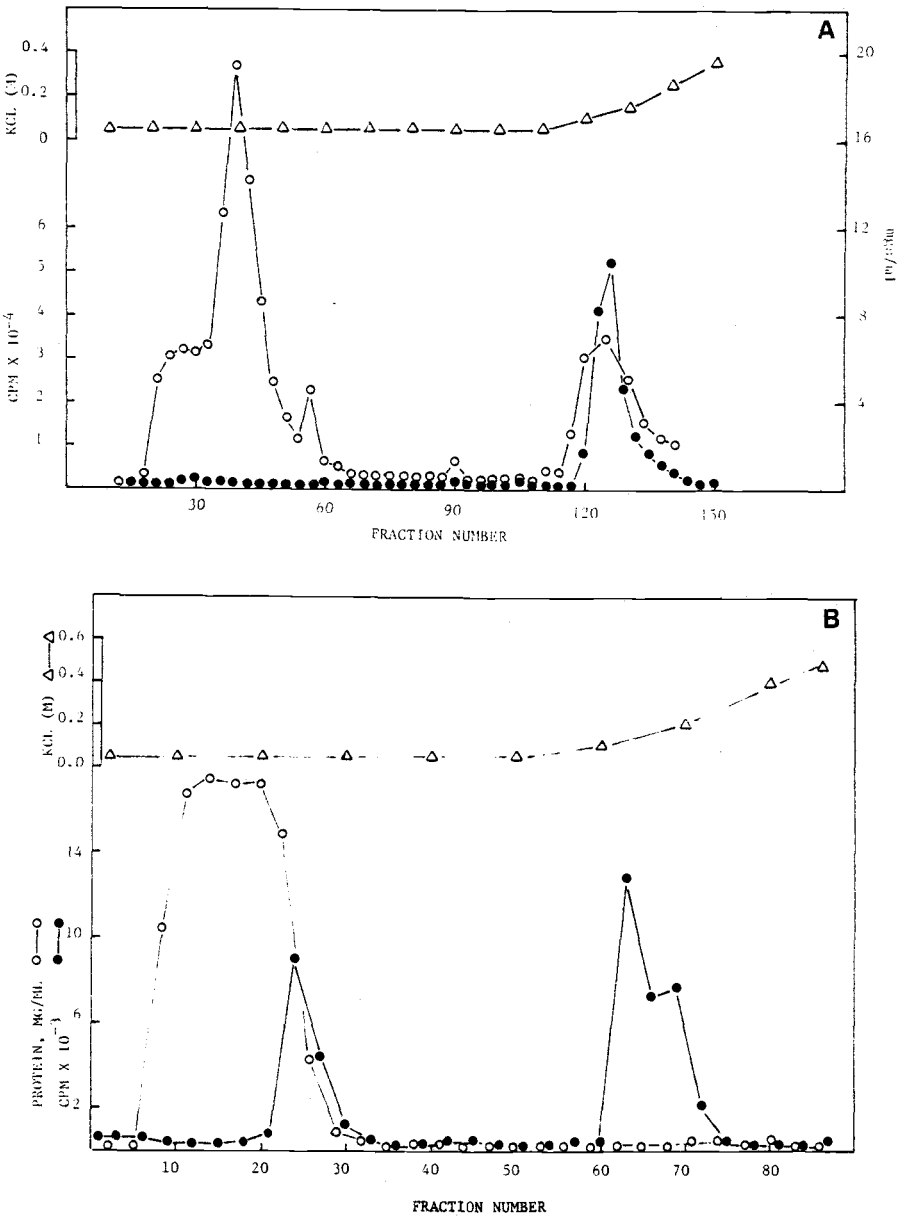
17. Weissbach, A. 1979. Arch. Biochem. Biophys. 198:386-396.

18. Werle, E. 1972. New Aspects of Trasylol Therapy. Vol. 5.

19. Witkin, S. S., G. C. Korngold, and A. Bendich. 1975. PNAS 72: 3295-3299.

Fig. 1. DEAE-cellulose chromatogram of DNA polymerase activities from cytoplasmic fraction and total cell extract of human placental tissue. The trophoblast layer of human placentas were homogenized and extracted with 3% NP40 in low salt (0.005 M KCl) to yield a cytoplasmic fraction (Figure 1A) or high salt (0.3 M KCl) to yield a total cell extract (Figure 1B). The nucleic acid was removed by adsorption and elution to DE-23 cellulose. The DE23 eluant was dialyzed and concentrated as described under "Materials and Methods". The extracts were loaded onto a DEAE-cellulose column (2.5 x 46 cm) equilibrated in Buffer B, washed with 100 ml of Buffer, and then the column was developed with 400 ml linear gradient of KCl from 0.05 M to 1 M in Buffer B. Fractions of 6 ml were collected from the column with a flow rate of 30 ml/hr. Aliquots (20 µl) from every third fraction were assayed for DNA polymerase activity with activated DNA in 20 min incubations. Reaction mixtures contained [^{3}H]-dTTP (10,130 cpm/pmol) in the general polymerase reaction (O—O). Protein was determined by A_{280} (O—O) and the concentration of KCl was measured by conductivity (Δ—Δ).

Figure 1


A
KCL (M)
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0.2
0
CPM X 10⁻⁴
6
5
4
3
2
1
20
16
12
8
4
30
60
90
120
150
FRACTION NUMBER
B
KCL (M)
0.6
0.4
0.2
0.0
PROTEIN, MG/ML
CPM X 10⁻³
14
10
6
2
10
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 2. Phosphocellulose chromatogram of DNA polymerase activity. The flow through material containing DNA polymerase activity at 0.05 M KCl was adsorbed to phosphocellulose in a 2.5 x 20 cm column in Buffer B (0.05 M KCl) at 6 mg protein per ml of packed cellulose. The column was washed with 100 ml of Buffer B (0.05 M KCl) and developed with 200 ml linear gradient from 0.05 M to 1.0 M KCl in Buffer B at a flow rate of 15 ml/h. Fractions of 3 mls were collected and assayed for [^{3}H]-dTTP (10,130 cpm/pmole) using the general polymerase reaction (O—O). Protein (O—O) and salt (Δ—Δ) concentrations were determined by OD_{280} and conductivity respectively.

Figure 2

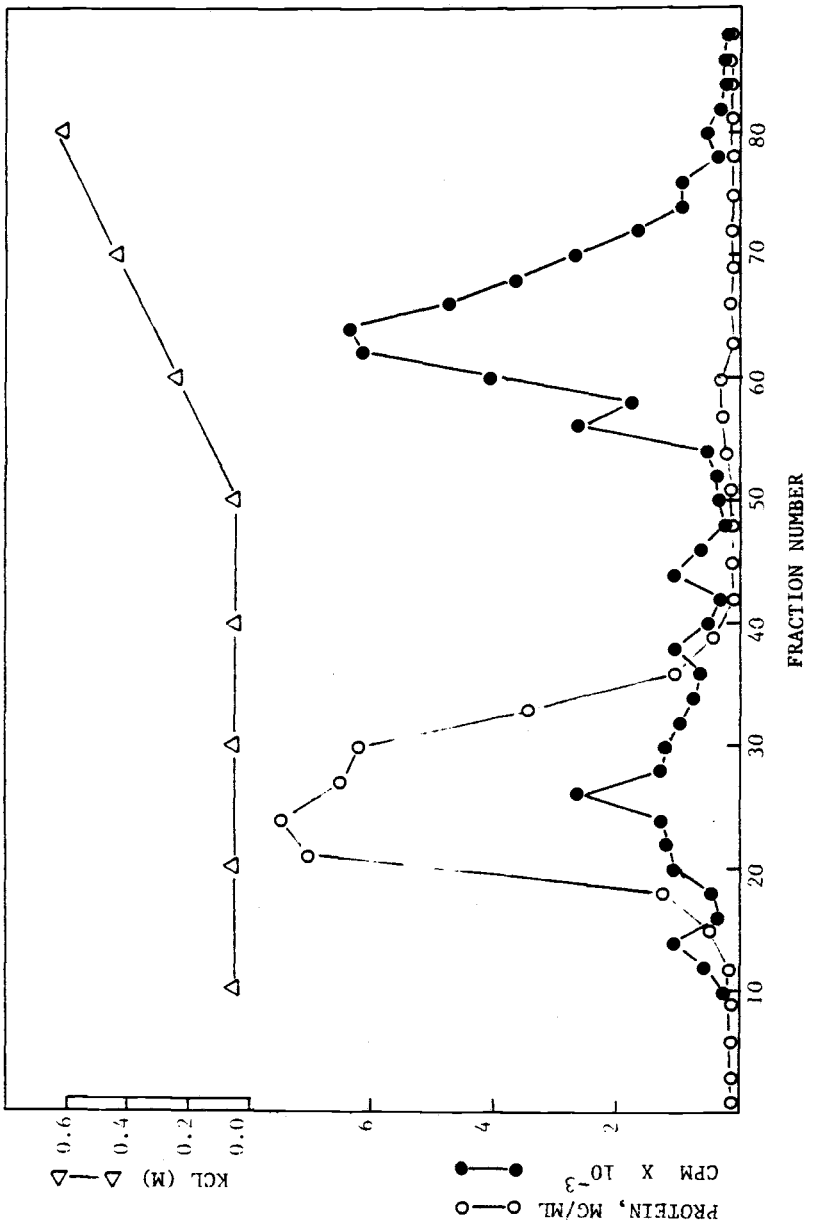

KCL (M) Δ—Δ
0.0
0.2
0.4
0.6
PROTEIN, MG/ML O—O
CPM X 10^-3
2
4
6
10
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 3. Hydroxyapatite chromatography of DNA polymerase activity. Fractions with DNA polymerase activity eluting at 0.3 M KCl on phosphocellulose were pooled and dialyzed against Buffer B with 0.5 M KCl. A hydroxyapatite column pre-equilibrated in the same buffer was loaded with the dialyzed material at 1.8 mgs/ml pack column volume. The column was washed with 10 mls starting buffer followed by a step gradient from 0.02 M-0.2 M KPO_4 (pH 7.5). Fractions with a volume of 1.5 mls were collected and assayed for [^{3}H]-dGTP incorporation (5280 cpm/pmole) with synthetic template primer $(dC)_n \cdot (dG)_{12-18}$. The protein concentration was determined by the BioRad protein assay with BSA as a standard.

Figure 3

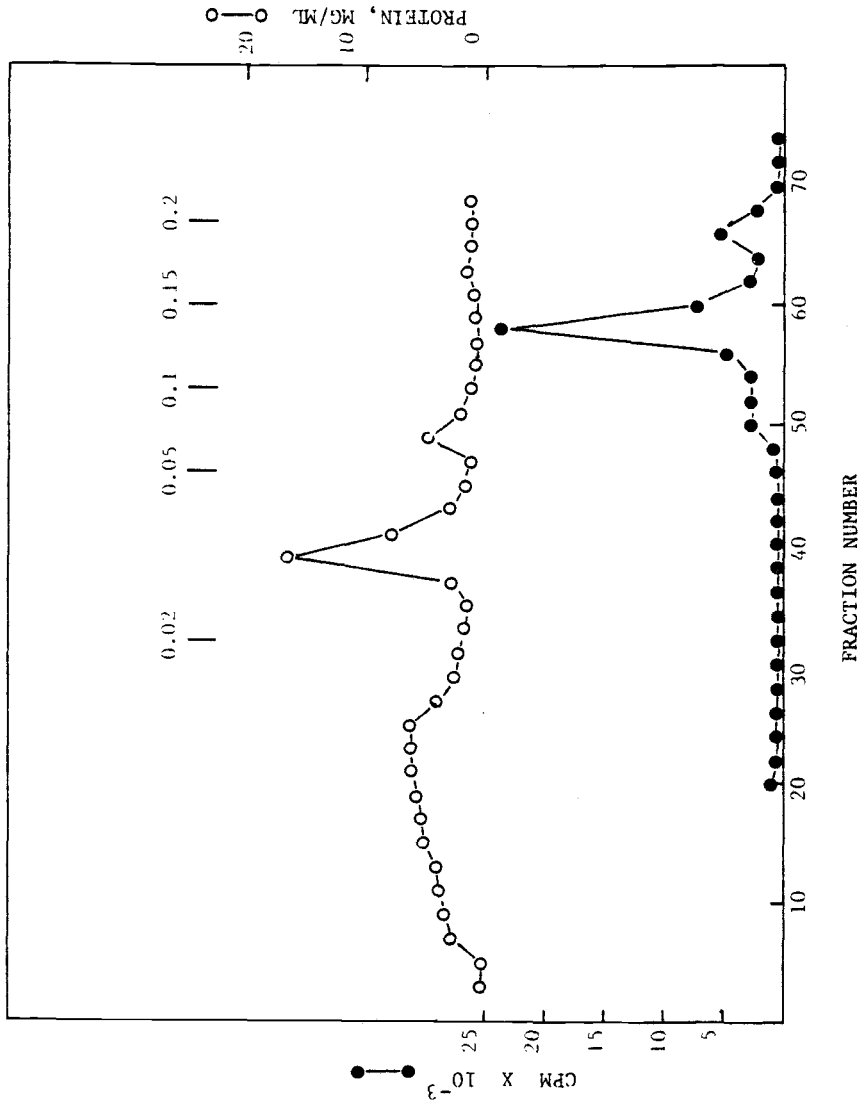

PROTEIN, MG/ML
0
10
20
0.02
0.05
0.1
0.15
0.2
CPM X 10^-3
5
10
15
20
25
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 4. Divalent cation preference of the DNA polymerase. The divalent cation preference of the DNA polymerase activity from the phosphocellulose column was determined by using the synthetic template-primer reaction mix with $(dC)_n \cdot (dG)_{12-18}$ as the template. Divalent cations were added individually to each reaction mix and incubated at 37°C for 30 min. Solid and open bars represent $MgCl_2$ and $MnCl_2$, respectively.

Figure 4

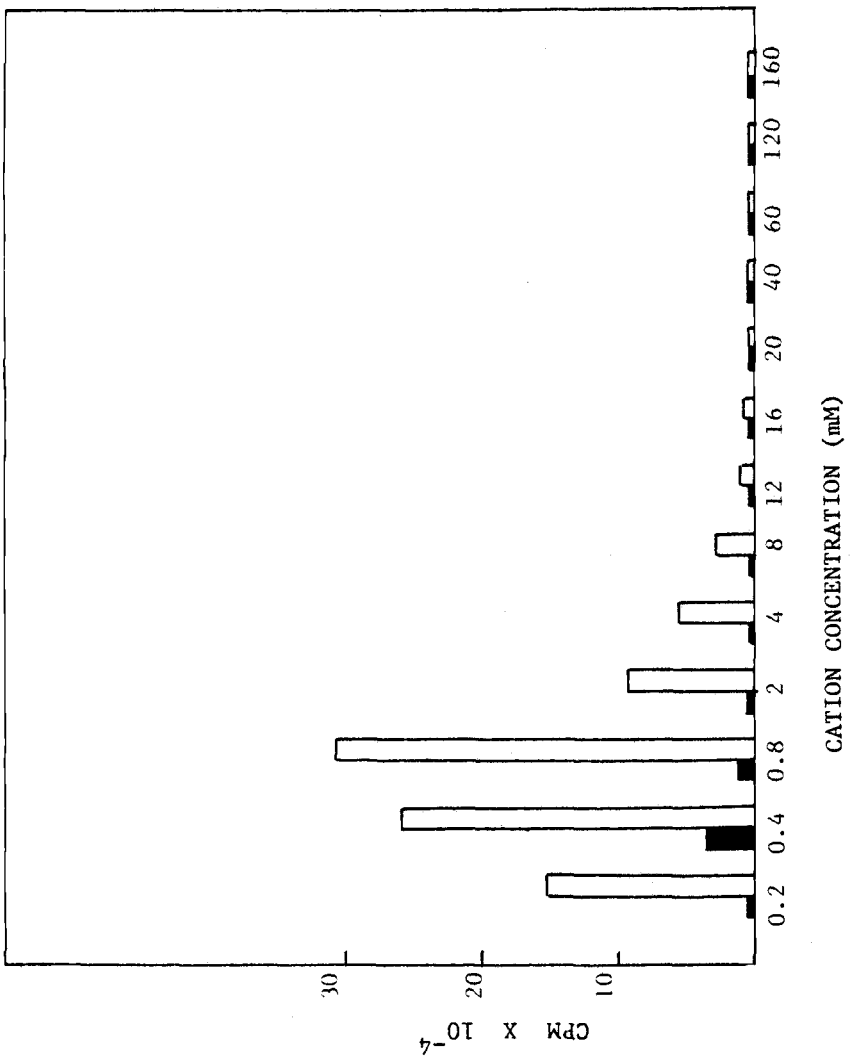

CPM X 10^{-4}
30
20
10
0.2
0.4
0.8
2
4
8
12
16
20
40
60
120
160
CATION CONCENTRATION (mM)

Figure 5. DNA polymerase activity as a function of pH. The effect of pH on DNA polymerase activity was examined utilizing the template-primer $(dC)_n \cdot (dG)_{12-18}$. Enzyme purified from the phosphocellulose column was tested in the standard reaction mix. To each sample a final concentration of 40 mM Tris HCl (in range of pH 6.5 to 9.5 at 22°C) was added and incubated 30 minutes at 37°C.

Figure 5

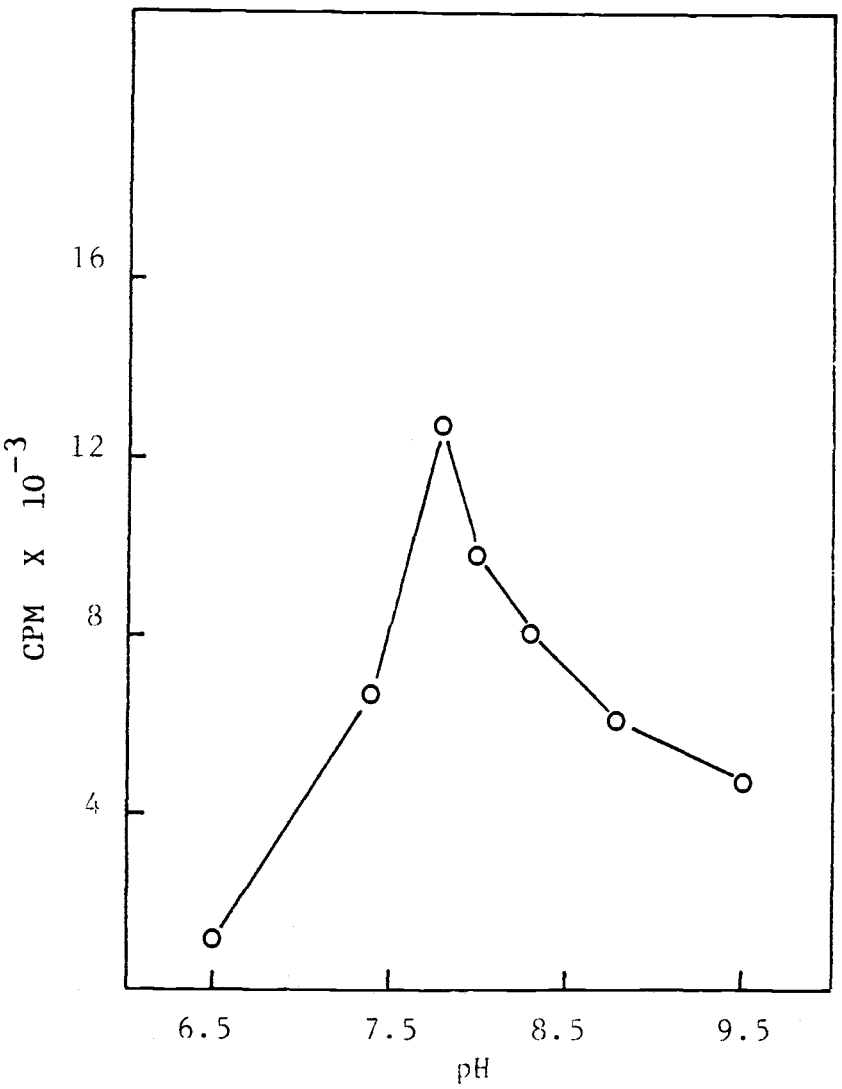


Fig. 6. Effect of inhibitors on DNA polymerase. The effect of various inhibitors of DNA polymerases was tested against the enzyme using the template primer, $(dC)_n \cdot (dG)_{12-18}$. The final concentration of the various inhibitors in reaction mixed of 400 µl were: 50 mM KPO_4 (0—0), 0.1 M KCl (□—□), 5 mM N-ethylmaleimide (△—△), 2 mg/ml aphidocolin (○— ○), and a control (0—0). At the indicated intervals, the reaction was incubated at 37°. 80 ml aliquots were removed and trichloroacetic acid was added to a final concentration of 5%. Radioactive precipitates were collected and assayed for radioactivity as described in Materials and Methods.

Figure 6

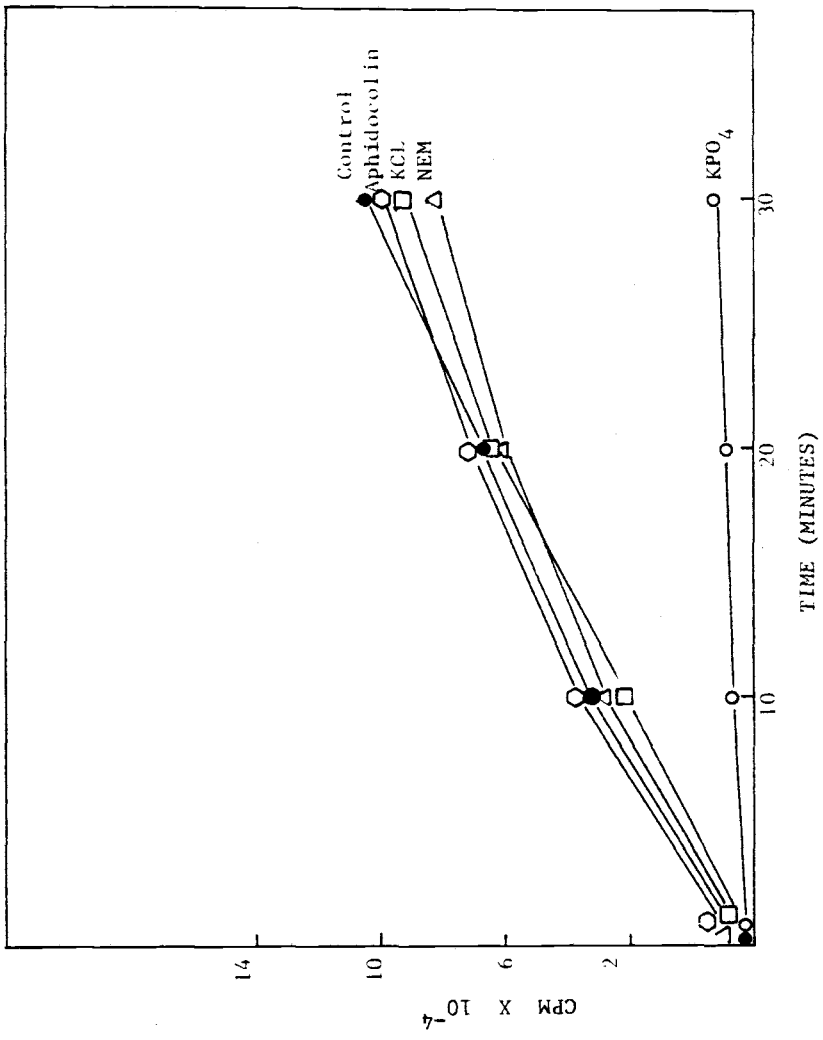


Figure 7. Glycerol gradient sedimentation analysis. A 0.5 ml aliquot of purified DNA polymerase was layered onto a 10.5 ml 5-25% (v/v) linear glycerol gradient buffered with 0.05 M Tris-HCl (pH 7.4), 0.5 M KCl, 2 mM dithiothreitol, 20% ethylene glycol, and 0.1% NP40. Centrifugation was in the SW41 rotor at 4° for 17 hrs at 40,000 RPM. Fractions of 0.3 ml were collected from the top and 20 µl of each was assayed for DNA polymerase activity utilizing the synthetic template-primer, $(dC)_n \cdot (dG)_{12-18}$. Sedimentation in parallel gradients was performed with bovine serum albumin (4.4S) and ovalbumin (3.6S) as size markers.

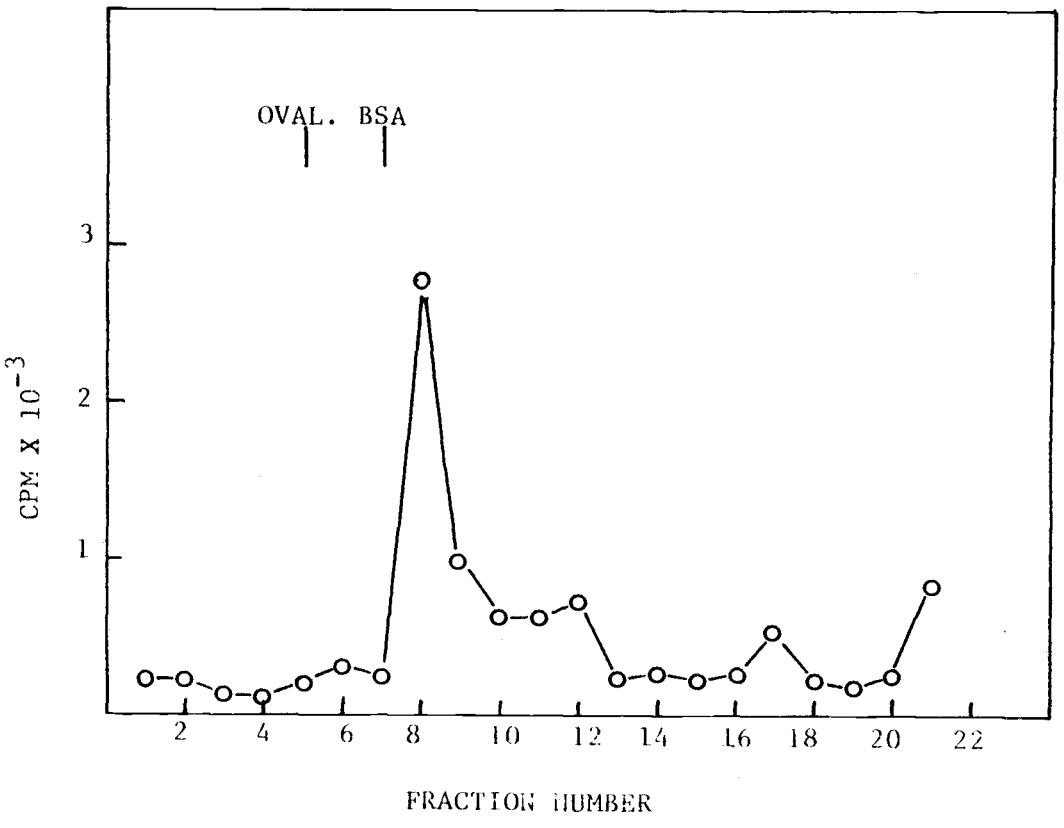


Figure 7

Fig. 8. SDS-polyacrylamide gel electrophoresis of purified β DNA polymerase. Purified DNA polymerase was prepared and electrophoresed as described in materials and methods. Lanes 1 and 2 contain the high and low molecular weight markers, respectively. Lane 3 contains the phosphocellulose purified enzyme. Lane 4 contains the proteins eluting at 0.05 M KCl in phosphocellulose. Lane 5 contains DNA polymerase active fractions from the glycerol gradient shown in Fig. 7. The protein standards were composed of lysozyme (14,300), soybean trypsin inhibitor (21,000), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (96,000), and myosin (200,000).

Figure 8

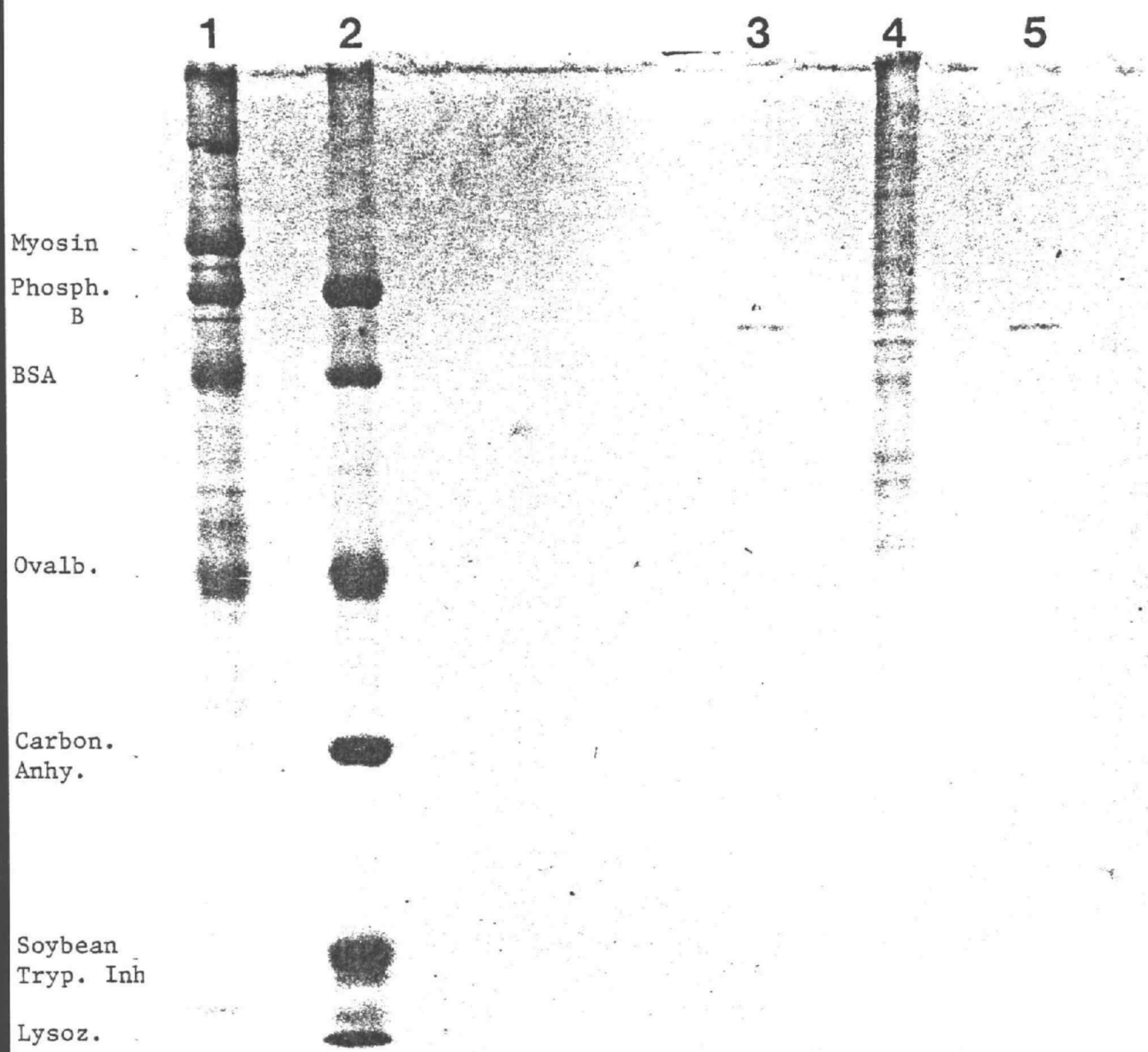

1
2
3
4
5
Myosin
Phosph. B
BSA
Ovalb.
Carbon. Anhy.
Soybean Tryp. Inh
Lysoz.

Table I. Purification the DNA polymerase from human placenta. DNA polymerase activity was determined for each step during the purification of the enzyme. Aliquots of 20 µl were assayed for (^{3}H)-dTTP incorporation (10,130 cpm/pmole) using the general polymerase reactions. Protein was determined by OD_{280} and the BioRad protein assay. One unit of enzyme activity catalyzed the polymerization of 1 nmole of dTMP into an acid-precipitable product in 1 hr at 37°C. The activity is represented in nanomoles of [^{3}H]-dTTP incorporated per hour per total volume.

PURIFICATION OF PLACENTAL β DNA POLYMERASE

Fraction	Total Volume (ml)	Total (mg) Protein	Total Activity (units/mg)	Specific Activity (units/mg)	Purification	Yield
Low speed	500	13,317	50.5	0.004	-	100%
Hi-speed	490	9,196	43.6	.005	1	87%
De-23	540	4,694	23.1	.01	1	46%
De-52	35	483	19.7	0.04	4	39%
Phospho-cellulose	40	8	19	2.37	237	38%
Hydroxy-apatite	3	0.45	14	31.80	3,180	28%
Glycerol gradient	24	0.12	11	94.67	9,467	23%

Table II. Template specificity shift during purification. Aliquots from the indicated fractions during the purification and storage of the DNA polymerase were tested for incorporation of (^{3}H)-dGTP using the $(dC)_n \cdot (dG)_{12-18}$ template-primer reaction and (^{3}H)-dTTP in the general polymerase reaction. Figures in column 1 represent pmoles of (^{3}H)-dGTP or (^{3}H)-dTTP incorporated in 30 mins. Figures in column 2 represent the ratio of pmoles of (^{3}H)-dGTP incorporated versus pmoles of (^{3}H)-dTTP incorporated.

CHANGE IN TEMPLATE SPECIFICITY

Fraction	[^{3}H]-dGTP/[^{3}H]-dTTP[a]	Ratio
DE-52	0.50/1.84	0.27
Phosphocellulose	5.69/2.17	2.62
Hydroxyapatite	4.50/1.52	2.96
Storage after phosphocellulose:		
Day 4	4.50/0.48	9.38
Day 90	4.50/0.10	45.00

[a]Ratio of pmoles incorporated into acid precipitable radioactivity. [^{3}H]-dGTP incorporated in enzyme assay with template-primer, $(dC)_n \cdot (dG)_{12-18}$. [^{3}H]-dTTP incorporated in enzyme assay with activated calf thumus DNA.

Table III. Template preference of the DNA polymerase. The template preference of the purified DNA polymerase was examined in freshly prepared enzyme. Activities with synthetic template-primers, rabbit globin mRNA, and activated DNA were examined in the presence of 0.8 m$\underline{M}$ $MgCl_2$ or 0.4 m$\underline{M}$ $MnCl_2$. The figures represent pmoles incorporated in 30 min.

TEMPLATE PREFERENCE OF PLACENTAL DNA POLYMERASE β-78

Template	0.8 mM Mg^{++} pmoles incorp/30 min	0.4 mM Mn^{++} pmoles incorp/30 min
Act. DNA	1.75	0.07
rCdG	0.08	0.08
dCdG	6.08	0.05
dG	0.05	0.09
rAdT	0.08	0.03
rCmdG	0.05	NI*
mRNA	0.09	-

*NI - No incorporation.

IV. Specific Inhibitor of RNA-Directed DNA Polymerase in Human Placenta Extracts.

Introduction

Major efforts have been made to detect retroviruses in normal and malignant human tissue (Garder et al., 1977). In normal tissue, evidence for retroviruses has been found most frequently in the placenta (Panem, 1979). The major evidence comes from electron microscopic studies of placental tissue which demonstrate retroviruses budding from the syncytiotrophoblast layer (Kalter et al., 1973; Vernon et al., 1974; Dalton et al., 1976; Imamura et al., 1976; and Dirksen and Levy, 1977). While these studies are highly suggestive of the presence of a human retrovirus, the detection of the virus-associated RNA-directed DNA polymerase (RDDP) have been inconsistent (Nelson, Leong, and Levy, 1978; Kiessling and Goulian, 1979; Krauss and Linn, 1980). These discrepancies may be due to the presence of an inhibitor of RDDP which must be removed before enzyme activity is detectable.

Inhibitors of DNA polymerases have been found in many tissue extracts. A DNA molecule from the sera of tumor-bearing mice was found to inhibit ribopolymer-transcribing cellular DNA polymerases in a murine myeloma cell line (Gottlieb et al., 1973; Gottlieb et al., 1980). This inhibitor had no effect of the RDDP activity of murine leukemia virus (MuLV) or avian myeloblastosis virus (AMV). A small protein from tissue extracts of bovine mammary glands was found to decrease DNA synthesis in HeLa and Ehrlich cells (Gonzalez and Verly, 1978). In human milk, nonspecific inhibitors of the RDDP reaction have been found to co-purify with the enzyme. These inhibitors have been identified tentatively as RNases (McCormick et al., 1974) or phosphatases

(Sanner, 1976) which can be removed by sedimentation of the milk extract through Metrizamide.

Immunoglobulins bound to the trophoblast layer of human placentas have been implicated as inhibitors of RDDP activity (Falk et al., 1974; Kouttab et al., 1976; Jurjus et al., 1979). A partially purified gamma globulin fraction was found to neutralize nonspecifically the activity of RDDP from several retroviruses (Jurjus et al., 1979). We report here the isolation and characterization of a specific inhibitor of mammalian retroviral RDDP. This inhibitor substance co-purified with an RDDP activity in human placentas and can be removed from the particle-associated enzyme by salt extraction. Upon removal of the inhibitor, RDDP activity is regained.

Materials and Methods

Reagents and Materials

Trypsin, recrystallized from bovine pancreas, and trypsin inhibitor, prepared from soybean, were purchased from Sigma Chemical Co. Phospholipase C was obtained from the Worthington Biochemical Corp. The deoxyguanosine-5'-triphosphate tetrasodium salt (8-^{3}H) was purchased from ICN radiochemicals division. The template-primers $(C)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ were obtained from P-L Biochemicals, Inc. Trasolyl was purchased from FBA pharmaceuticals. Azocoll was obtained from the Calbiochem-Behring Corp. Sepctrapor 2 dialysis membrane was purchased from VWR Scientific Inc. Immunodiffusion plates containing antisera to human gamma globulins, IgG and IgM, were purchased from Hyland Laboratories, a division of Traverol Laboratories, Inc.

Viruses and Enzymes

Calf thumus DNA polymerases α and γ from fetal calf liver were purchased from Worthington Biochemical Corp. Mouse mammary tumor virus (MMTV), Rauscher murine leukemia virus (R-MuLV), Simian sarcoma virus (SSV), Mason-Pfizer Virus (MPV), Baboon Endogenous Virus (BaEV), Prague B Rous sarcoma virus (RSV), and RD114 were obtained through the assistance of Jack Gruber, Biological Carcinogenesis Branch, National Cancer Institute, the Frederick Cancer Research Center, and the John L. Smith Memorial for Cancer Research.

Virus and Polymerase Assays

DNA synthesizing activity in retroviruses, purified DNA polymerases, and samples from placental preparations were assayed in 100 µl reactions containing 40 mM Tris-HCl (pH 7.8 at 22 C), 0.12 mM ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA), 3.9 mM dithiothreitol, 3.7 mM glutathione (reduced form), 5% glycerol, 6 mM NaCl, 0.1% NP40, 6 mM $MgCl_2$, 1 µg of either $(C)_n \cdot (dG)_{12-18}$ or $(dC)_n \cdot (dG)_{12-18}$, and 12.5 µM (^{3}H)-dGTP (8 Ci/mmole). Viruses and placental preparations were lysed for 30 min at 0°C before the addition of template-primer and (^{3}H)-dGTP. Reactions were incubated 30 min at 37°C, unless otherwise indicated, and terminated by the addition of an equal volume of 0.01 M sodium pyrophosphate and then trichloroacetic acid to a final concentration of 5%. After 10 min in an ice bath, the acid precipitable material was collected onto glass fiber filters (GF/C, Whatman Biochemicals, Ltd.) by vacuum filtration and washed with 15 ml of 0.1 N HCl 0.01 M sodium pyrophosphate. After a wash of 95% ethanol, the filters were dried and suspended in toluene-Ominfluor (New England Nuclear Corp).

Extraction of Placental RDDP and Inhibitor

Full term human placentas were obtained from Good Samaritan Hospital, Corvallis, Oregon and Albany General Hospital, Albany, Oregon. The placentas were processed within 1 h after birth. All procedures were done at 4°C. Approximately 100 g of tissue from the trophoblast layer facing the maternal side of the placenta was excised, minced, and rinsed in STEg buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.001 M EGTA). The minced tissue was drained and resuspended in 300 ml Buffer F (25%

glycerol , 4 mM EGTA, 5 mM KCl, 24 mM Tris-HCl, pH 7.4, 200 µ/ml Trasolyl, 1 mM dithiothreitol). The mixture was homogenized on ice in the VirTis homogenizer (maximum speed) in 30 sec episodes 4 times. Then the crude extract was centrifuged in Sorvall HS-4 rotor, 5,000 x g for 5 min at 4°C. The supernatant was decanted to separate the fluid from the cell debris on the bottom and a lipid pellicle on top. After centrifugation at 9500 x g for 10 min, the resulting supernatant was centrifuged at 95,000 x g for 60 min at 4°C in the Beckman Type 35 rotor, Beckman L5-65 centrifuge. The resulting pellet was resuspended in STEg and dispersed by repeated passage through an 18 gauge and then 23 gauge needle. The suspension was then layered onto a 10 ml cushion of 17.5% Metrizamide (analytical grade) (Accurate Chemicals Co.) and centrifuged at 125,000 x g for 30 min in the Beckman SW27 rotor. The band at the interphase of the Metrizamide and the placental suspension was removed and resuspended in STEg. A volume of 2 ml of this suspension was then layered onto a gradient consisting of 2 ml of 15% sucrose on a 7 ml linear gradient of 20-65% sucrose (w/v) in STEg. After centrifugation in the Beckman SW41 rotor at 150,000 x g at 4°C for 12 h, 4 ml were removed and then the gradient was fractionated into 25 equal aliquots. These fractions were assayed for RNA-directed DNA polymerase (RDDP) activity with the template-primer $(C)_n \cdot (dG)_{12-18}$ as described.

If RDDP activity was not detected, the fractions between the densities 1.10 g/ml and 1.19 g/ml were pooled for sequential extraction with NaCl. This process involved the addition of NaCl to 0.5 M with gentle agitation followed by centrifugation in the Beckman SW41 rotor at 200,000 x g,

4°C, 30 min. The supernatant was saved for further analysis and the pellet resuspended in 1.0 M NaCl, 0.01 M Tris-HCl, pH 7.4. The above procedures were repeated with a 1.5 M NaCl extraction. The supernatants from the extraction process were dialyzed against three changes of 500 ml of either STEg or a buffer containing 0.01 M NaCl. 0.01 M Tris-HCl, pH 7.4. The pellet fractions from the 0.5 M and 1.5 M NaCl extractions were resuspended in STEg and layered onto a linear gradient of 20-65% sucrose (w/v) in STEg. After centrifugation in the Beckman SW50.1 rotor at 234,000 x g for 90 min, the gradient was fractionated and assayed for RDDP activity as described above.

Protease and Nuclease Assays

The general proteolytic substrate Azocoll was utilized to determine protease activity in the inhibitor fraction. The assay consisted of incubating the sample in 0.1 M potassium phosphate, pH 7.0, with 50 mg Azocoll at 37°C for 15 min. The sample was filtered and the OD_{520} was determined for the filtrate on a Perkin-Elmer spectrophotometer. Trypsin was used as a positive control.

Assays for the presence of RNase and DNase were performed with (^{3}H)-RNA (5×10^4 cpm/µg) isolated from BHK/21 cells and (^{3}H)-DNA (6×10^3 cpm/µg) extracted from Bdellovibrio bacteriovorus. After incubation with varying concentrations of inhibitor at 37°C in the buffer used to assay inhibitor activity with MuLV (40 mM Tris-HCl, pH 7.8 at 22°C, 0.12 mM EGTA, 3.9 mM dithiothreitol, 37 mM glutathione (reduced form), 5% glycerol, and 6 mM NaCl), the acid precipitable radioactivity was determined at the indicated intervals. DNase I and RNase A (Worthington

Biochemical Corp.) at a concentration of 1 µg per 100 µl were utilized as positive controls.

Treatment with Trypsin and Phospholipase C

A volume of 100 µl inhibitor (0.4 mg/ml) was incubated with 10 µl of 10 mg/ml trypsin at 37°C for 15 min. The trypsin was inactivated by the addition of 10 µl of a 2 mg/ml soybean trypsin inhibitor. Phospholipase C treatment was conducted in the presence of 6 mM $CaCl_2$ at 22°C. Controls for these experiments utilized STEg instead of inhibitor in the reactions. After each treatment, the inhibitor or control STEg was tested for activity against MuLV as described.

Treatment with Acid and Base

The pH of the inhibitor was adjusted to 2 or 12 by the addition of 1 M HCl or 1 M NaOH, respectively. Samples were incubated for 1 h at 4 C and neutralized to pH 7. Samples with STEg were treated as controls.

Extraction with Ether, Ethanol/Ether, and Chloroform/Methanol

A volume of 200 µl of the placental inhibitor was extracted with an equal volume of peroxide-free diethyl ether buffered in STEg for 10 min at 0°C with shaking. After removal of the ether phase, both phases were evaporated and resuspended in 200 µl STEg. The inhibitor was also extracted with 20 volumes ethanol/ether (3:1) at 4°C for 1 h. The precipitate was recovered by centrifugation at 8,900 x g for 3 min in a Brinkman Microfuge B centrifuge. The aqueous and organic phases were pooled, evaporated and resuspended in 200 µl STEg. The pellet was washed with ether, evaporated to dryness, resuspended in 200 µl and dispersed

through a 23 gauge needle. Extraction with 20 volumes of chloroform/methanol (2:1) over 1 h in an ice bath was used as another method for examining the physical-chemical properties of the inhibitor. The mixture was centrifuged as above and the band at the interphase of the solvent and aqueous layers was removed and resuspended in 200 µl STEg. The solvent and aqueous phases were pooled, evaporated, and resuspended in the original sample volume of STEg. A volume of 200 µl go STEg was treated similarly and used for all extractions.

Immunodiffusion Tests

Radial immunodiffision tests were performed with prepared immunodiffusion plates containing antisera to either human or IgM. Wells were loaded with 4 µg and 8 µg (by protein assay) of placental inhibitor and incubated at 4°C. Plates were checked for precipitin lines every day for 7 days. These plates detect up to 0.1 µg of immunoglobulin.

Sedimentation Analyses

Linear gradients for estimation of the sedimentation coefficient of the placental inhibitor were composed of 10-30% (v/v) glycerol containing 0.5 M KCl and 0.05 M Tris-HCl, pH 7.4 at 22 C. A 0.5 ml aliquot of the inhibitor was layered onto 10.5 ml of glycerol gradient and centrifuged in the SW41 Ti rotor at 4 C for 18 h at 40,000 rev/min in the Beckman L5-65B centrifuge. Standard markers were bovine serum albumin (4.4 S), ovalbumin (3.6S) and purified human IgG (7S). The gradients were collected in 0.3 ml fractions and the position of the inhibitor determined in an MuLV-RDDP inhibition assay.

Determination of Protein Concentration

Protein concentration was determined by the BioRad protein assay.

Results

Extraction of Inhibitor from Placental Extracts

We have reported previously the presence of an RDDP activity in the microsomal fraction of extracts of the trophoblast layer of human placentas (Nelson, Leong, and Levy, 1978). This activity was found at a density of 1.15 g/ml after equilibrium centrifugation in sucrose (Fig. 1). An additional peak of enzymatic activity was observed at 1.12 g/ml. In some placental extracts after equilibrium centrifugation, RDDP activity was very low or absent throughout the gradient. The apparent absence of RDDP activity may be related to a specific inhibitor of mammalian retrovirus RDDP which has been isolated from placental extracts.

A full term placenta was obtained after normal delivery from a woman who had 4 previous spontaneous abortions. The placenta was extracted for RDDP activity as described in Materials and Methods. Initial screening of the placental extracts after equilibrium centrifugation revealed no apparent activity at 1.15 g or 1.12 g/ml (Fig. 2A). Gradients were pooled and sequentially extracted with 0.5 M, 1.0 M, and 1.5 M NaCl in 0.01 M Tris-HCl, pH 7.4. After centrifugation, the pelleted materials from the 0.5 M and 1.5 M NaCl extraction were banded to equilibrium on sucrose. The supernatants from the salt concentrations were dialyzed against either STEg or 0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4 and stored at -30°C. After extraction with 0.5 M NaCl, no change in the RDDP activity profile was observed in the material after centrifugation to equilibrium in sucrose (Fig. 2B). However, the pellet remaining after the 1.5 M NaCl extraction did produce RDDP activity at densities of 1.15 g/ml and 1.19 g/ml (Fig. 2C).

Placental extracts which exhibit normal RDDP activity profiles on equilibrium centrifugation in sucrose were extracted with 1.5 M NaCl as described above. Analysis of the extracted material in sucrose gradients by the RDDP assay revealed no significant change in density or activity of the particle-associated RDDP. Some placentas which were initially negative for RDDP activity were sequentially extracted with NaCl to concentrations of 3 M NaCl before enzymatic activity appeared.

Specificity of the inhibitory Factor

The dialyzed supernatants from salt extractions of the placenta were tested for inhibitory activity against Rauscher murine leukemia virus RDDP activity (Table 1). The 1.5 M NaCl extraction released an inhibitor into the supernatant which decreased the MuLV reaction by 88%. The 0.5 M and 1.0 M NaCl extracts did not remove the inhibitor factor. Instead, the supernatant fluids from these extractions contained factors that stimulated rather than inhibited the RDDP reaction. These observations suggested that the inhibitory factor was bound tightly to the placental extract and high salt concentrations were necessary for its removal. These studies also demonstrated that a stimulatory factor for the RDDP reaction is released at the lower salt concentrations.

The effect of increasing concentrations of inhibitor on the RDDP reactions of the placental particle-assocaited enzyme, MuLV, BaEV, and SSV was examined (Fig. 3). All enzymes tested were sensitive to the inhibitory factor. After the addition of apparently saturating levels of inhibitor, a resistant DNA synthesizing activity was noted for placental enzyme (14.4%), SSV (13.6%), and MuLV (20.3%). However, saturating

concentrations of inhibitor for BaEV were not attained with even 10 µg of inhibitor. The concentration of VaEV per reaction mixture was 4.5 µg of viral protein which is approximately a third of the protein present in other viral enzyme reactions. These observations suggest that a larger portion of the placental enzyme, MuLV and SSV RDDPs are more sensitive to the placental inhibitor.

The effect of the placental DNA polymerase inhibitor on several mammalian retroviruses, an avian retrovirus, and normal cellular DNA polymerases was examined for inhibitor specificity. Saturating amounts of the inhibitor were mixed with each virus or enzyme and incubated at 0°C for 30 min. The RDDP reaction was begun by the addition of (^{3}H)-dGTP and samples were taken at 0, 10, 20, and 30 min after the start of the reaction. The mammalian retroviruses RD114 (Type C virus), Mason-Pfizer virus (Type D virus) and MMTV (Type B virus) were all sensitive to the inhibitor (Fig. 3A, 3B, and 3D). However, the RDDP activity of RSV, α DNA polymerase and γ DNA polymerase (Fig. 3C, 3E, 3F) were all resistant to the inhibitory effect. These observations suggest that the placental inhibitor specifically blocks mammalian retrovirus RDDP activity. Again, a resistant level of DNA synthesis was observed in the RDDP reactions after treatment with the inhibitor. The reaction kinetics exhibited by the RSV, α and γ DNA polymerase reactions with and without inhibitor also indicate that an RNase, DNase, phosphatase, or proteases are not present in the inhibitory extract.

Mode of Action of the RDDP Inhibitor

The inhibitory factor was tested further for the presence of nucleases and proteases. A mixture containing 80 µg of inhibitor with either 10 µg of (^{3}H)-RNA or 11.4 µg of (^{3}H)-DNA was incubated for 30 min at 37 C. Aliquots were taken at 10 min intervals and measured for acid precipitable radioactivity. No decrease in radioactivity was observed with either the labeled DNA or RNA. These observations indicate that nucleases were not present in the inhibitory extract in sufficient quantity to account for RDDP inhibition.

The presence of proteases in the extract was measured with Azocoll at 37° and 45°C. After 15 min with 2.5, 5.0, and 12.5 µg of inhibitor, no protease activity was detected for any inhibitor concentration. In control reactions, less than 1 µg of trypsin was detected by this method.

Although the negative reactions with RSV or α and γ DNA polymerases do suggest that nucleases and phosphatases are not present in the inhibitor, further support for this hypothesis was sought in the following experiment. A saturating concentration of inhibitor was added to a predetermined amount of MuLV. Then increasing concentrations of virus were added to this reaction. If the inhibition were due to the loss of a necessary substrate in the reaction mixture, increased virus should not result in increased RDDP activity. Figure 4 illustrates data that shows that an increase in virus paralleled an increase in enzyme activity until limiting substrate concentrations were reached. These results suggest that the inhibitor affects the viral polymerase directly.

Characterization of the RDDP Inhibitor

A summary of the characteristics of the placental RDDP inhibitor is listed in Tables 2 and 3. The inhibitor was treated to heat, pH 2 or 12, enzymatic digestions, and solvent extraction. After each particular treatment the activity of the inhibitor was tested against MuLV RDDP. Control samples of STEg were treated in the same manner.

Heating and pH Treatment

The placental inhibitor was stable at -30 C for more than a year. When the inhibitor was heated for 10 min at 37°, 56°, or 85° no change in inhibitory activity was found. However, the factor was unstable to heating at 100°C for 10 min.

The activity of the inhibitor was tested after treatment with high and low pH. The factor was found to be stable for 1 h at pH 2 or pH 12 at 4°C. The control samples containing STEg instead of the factor had no effect on the RDDP reaction.

Enzymatic Digestion

The inhibitory factor was treated for 30 min at 37°C with trypsin as described in Materials and Methods. Soybean trypsin inhibitor was added and then tested for remaining RDDP inhibitory activity. The factor was stable to the trypsin treatment. The inhibitory factor was also resistant to treatment with 10 μg/ml phospholipase C.

Solvent Extractions

Treatment of the inhibitor with ether had no effect on the RDDP inhibition which was present in the aqueous phase. When the factor

was treated with ethanol/ether the inhibitory activity was found in the precipitate and not the residue of the organic phase. Treatment with chloroform/methanol inactivated the inhibitor.

No inhibitor activity was lost after dialysis with membrane having a molecular weight cut-off of 12,000-14,000 daltons. Repeated attempts were made to determine the molecular weight of the substance by sedimentation in a linear glycerol gradient. The inhibitor activity was found in a diffuse band in the middle third of the gradient. The gradients were sedimented in 0.5 M KCl to prevent protein aggregation.

Radial immunodiffusion tests were performed for the presence of human IgG and IgM in the inhibitor extract. No IgG or IgM was detected by this test that had a limit of detection of 0.1 μg of gamma globulin protein.

Discussion

The results of these experiments indicate that an inhibitory factor which specifically interferes with the RDDP of mammalian retroviruses is present in some human placentas. This factor co-purifies with the RDDP-synthesizing complex which has been isolated from human trophoblast extracts (Nelson, Leong, and Levy, 1978). The removal of this RDDP inhibitor from the microsomal fraction requires high concentrations of salt. The most dramatic effect of salt extraction on placental RDDP activity was observed in the extracts obtained from a full term placenta from a woman with a history of 4 previous spontaneous abortions. Salt extraction did not lead to a shift in density of the DNA-synthesizing complex which banded at 1.15 g/ml after equilibrium centrifugation in sucrose. However, an RDDP-like activity banding at 1.19 g/ml in sucrose was observed. Activity at this density may represent viral cores or aggregates. The presence of this inhibitor may explain the absence of soluble RDDP activity in human placentas reported by others (Kiessling and Goulian, 1979; Krauss and Linn, 1980).

The placental RDDP inhibitor appears to be selective for mammalian RDDPs since it does not inhibit the avian retrovirus polymerases or the normal cellular DNA polymerases α and γ. Although strong inhibition of the enzyme reaction was observed for the mammalian retorviral enzymes, a resistant fraction of DNA synthesis was found for RD114, MPV, MMTV, SSV, and Rauscher MuLV. Despite reduced virus protein concentration in each reaction mixture, saturating concentrations of inhibitor were never reached for BaEV. This result does suggest that the inhibitor can

distinguish between mammalian viral enzymes. Further study is required to determine the specificity of the inhibitor with purified viral polymerases.

Nonspecific inhibition of the enzyme reaction by nucleases, proteases, or phosphatases was ruled out by observations that avian retrovirus polymerase and cellular α and γ polymerases do catalyze DNA synthesis in the presence of the inhibitor. The absence of nucleases and proteases in the inhibitor extract was substantiated further in specific tests for these enzymes. More importantly, the specificity of the RDDP inhibition was indicated by an experiment which showed that at saturating concentrations of inhibitor, the inhibition can be removed by the addition of more virus. Thus, inhibition is due to the direct effect of the extract on a virus component.

The specificity of inhibition by the placental extract suggests that the active factor may be an immunoglobulin or neutralizing lipoprotein. Both substances have been shown to specifically inactivate retroviruses (Hirsh et al., 1978; Leong et al., 1977). However, immunodiffusion tests for IgG or IgM were negative. Ether-resistance of the factor does rule out lipoproteins as active components. The inhibitor does not appear to be an RNA or DNA molecule by its resistance to alkaline pH and sensitivity to extraction by chloroform/methanol. At this time, the exact chemical nature of the inhibitor remains uncharacterized.

The presence of a specific mammalian RDDP inhibitor in human placental extracts suggests that the inhibitor has a role in placental development. Further studies must be carried out to determine whether levels of

the factor fluctuate during gestation. If abnormal amounts of this polymerase inhibitor manifests itself at critical times during placental development, it is possible that this may result in abnormal fetal development and subsequent spontaneous abortion. Further investigation into the identity and mode of action of the RDDP inhibitor is currently in progress.

REFERENCES

1. Dalton, A. J., A. Hellman, S. S. Kalter, and R. J. Helmke. 1974. J. Natl. Cancer Inst. 52: 1379-1381.

2. Dirksen, E. R. and J. A. Levy. 1977. J. Natl. Cancer Inst. 59: 251.

3. Faulk, W. P., M. Jeannet, W. D. Creighton, A. Carbonara, and F. Hay. 1974. J. Reprod. Fert. (Suppl.) 21: 43-57.

4. Gardner, M. B., S. Rasheed, S. Shimizu, R. W. Rongey, B. E. Henderson, R. M. McAllister, V. Klement, H. P. Charman, R. V. Bilden, R. L. Heberling, and R. J. Huebner. 1977. Search for RNA Tumor Virus in humans in origins of human cancer, Vol. B, p.p. 1235-1251. by H. Hiatt, J. D. Watson, and J. A. Winsten. C.S.H.L., New York.

5. Gonzalez, R., and W. G. VERly. 1978. Europ. J. Cancer 14: 689-697.

6. Gottlieb, A. A., A. H. Smith, F. J. Persico, and D. E. Nicholson. 1973. Nature 246: 480-482.

7. Gottlieb, A. A., J. Chang, D. P. Buhrer, and B. A. Brennessel. 1980. Cancer Res. 40: 758-770.

8. Hirsch, M. S., A. P. Kelly, D. S. Chapin, T. C. Fuller, P. H. Black, and R. Kurth. 1978. Science 199: 1337-1340.

9. Imamura, M., P. E. Philips, and R. C. Mellors. 1976. Amer. J. Path. 83: 383.

10. Jurjus, A., D. A. Wheeler, R. C. Gallo, and I. P. Witz. 1979. Arthritis and Rheumatism 22: 1308-1313.

11. Kalter, S. S., R. J. Helmke, R. L. Heberling, M. Panigel, A. K. Fowler, J. E. Strickland, and A. Hellman. 1973. J. Natl. Cancer Inst. 50: 1081-1084.

12. Kiessling, A. A. and M. Goulian. 1979. Cancer Res. 39: 2062-2069.

13. Kouttab, N. M., A. K. Fowler, J. E. Strickland, A. Hellman. 1976. J. Immunol. 117: 1644-1650.

14. Krauss, S. W. and S. Linn. 1980. Biochem. 19: 220-228.

15. Leong, J. C., J. P. Kane, O. Oleszko, and J. A. Levy. 1977. P.N.A.S. 74: 276-280.

16. McCormick, J. J., L. J. Larson, and M. A. Rich. 1974. Nature 251: 737-740.

17. Nelson, J. A., J. C. Leong, and J. A. Levy. 1978. P.N.A.S. 75: 6263-6267.

18. Panem, S. 1979. Current Topics in Pathology 66: 175-189.

19. Sanner, T. 1976. Cancer Res. 36: 405-408.

20. Vernon, M. L., J. M. McMahon, and J. J. Hackett. 1974. J. Natl. Cancer Inst. 52: 987-989.

Figure 1. Normal RDDP activity in Human Placenta Extracts.

The trophoblast layer of a human placenta was excised and processed as described in Materials and Methods. The microsomal fraction was layered onto a 20-65% sucrose gradient in STEg and centrifuged to equilibrium, SW Ti 41 rotor, 150,000 x g, 12 h, 4 C. The gradient was then fractionated and 20 ul samples from each fraction was assayed for RDDP activity with the template-primer $(C)_n \cdot (dG)_{12-18}$ (●—●). Density was determined by refractometry (O—O).

Figure 1

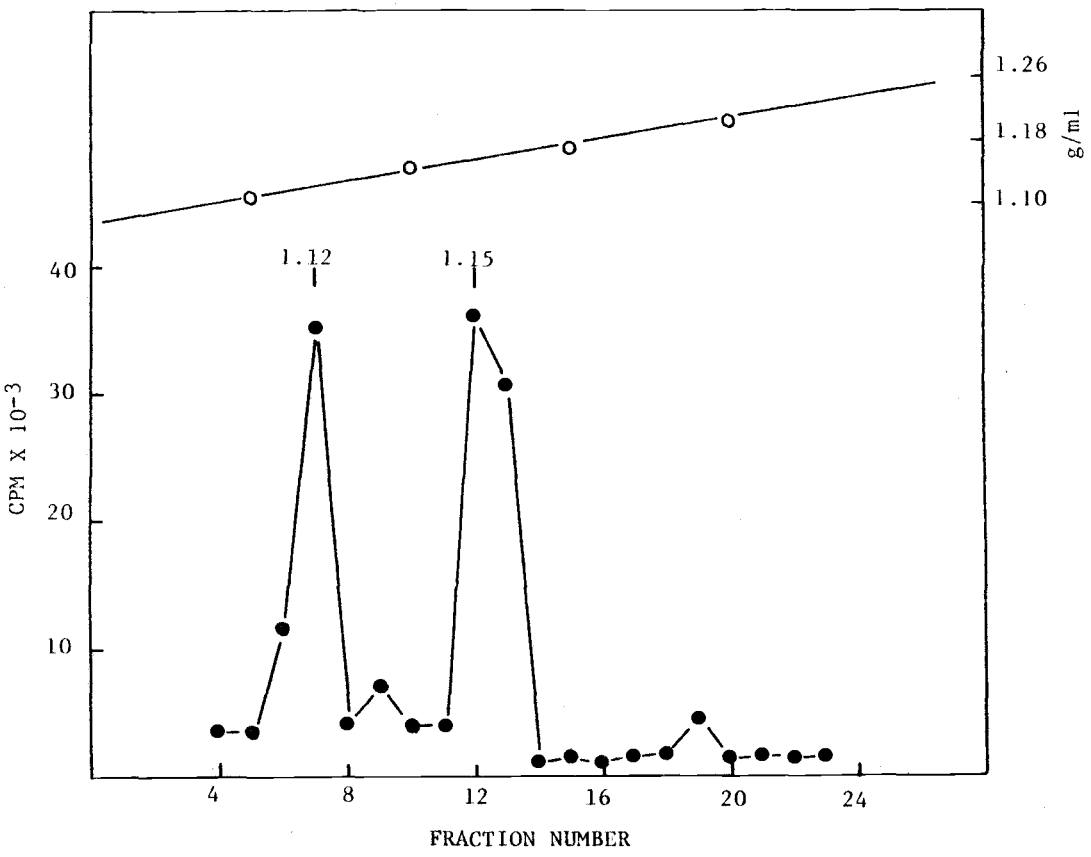

1.26
1.18
1.10
g/ml
1.12
1.15
40
30
20
10
CPM X 10-3
4
8
12
16
20
24
FRACTION NUMBER

Figure 2. The RDDP Activity Before and After Salt Extraction.

The placental extracts from a woman with a history of 4 previous spontaneous abortions were prepared as described in Fig. 1. After equilibrium centrifugation in sucrose, the gradient was aliquoted into 25 equal fractions and 20 µl samples from each fraction was assayed for RDDP activity (●—●) (Fig. 2A). The gradients were pooled and sequentially extracted with 0.5 M NaCl (Fig. 2B) and 1.5 M NaCl (Fig. 2C). After pelleting the NaCl extracted material, the 0.5 M and 1.5 M pellets were resuspended in STEg, homogenized through an 18 gauge needle, and sedimented to equilibrium in a 20-65% sucrose gradient in a Beckman SW50.1 rotor. The gradients were fractionated and 20 µl samples from each fraction were assayed for RDDP activity. The densities were determined by refractometry (O—O).

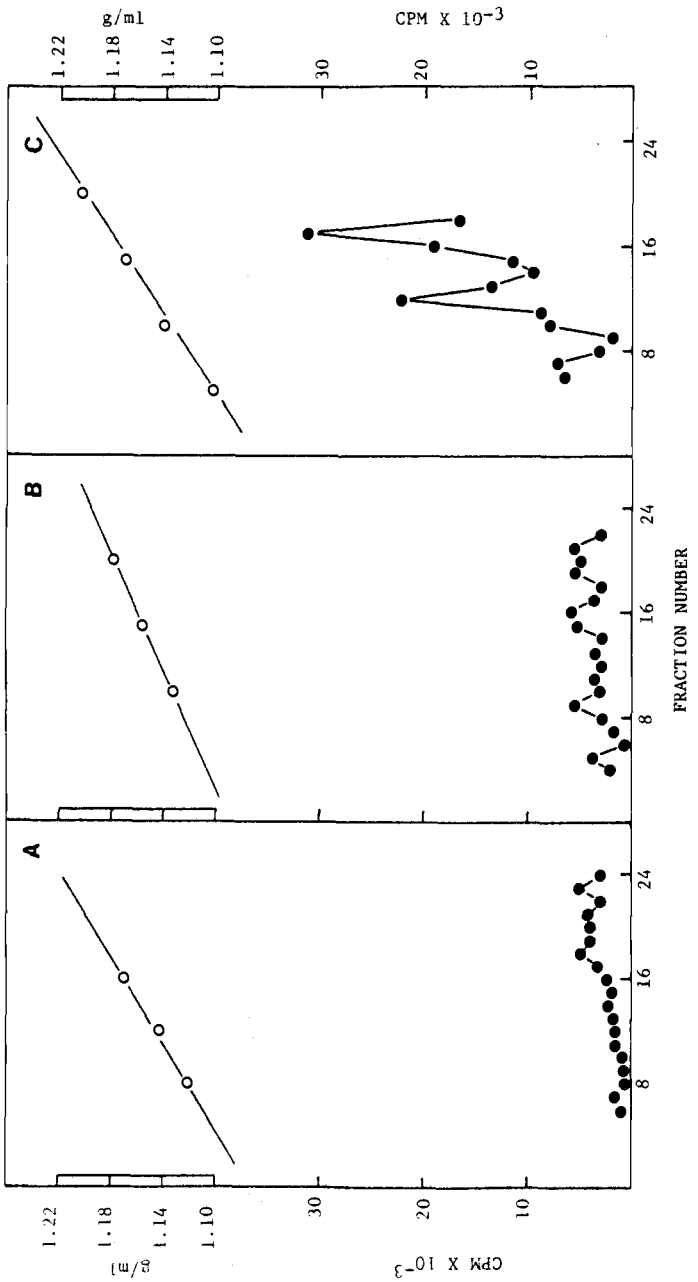
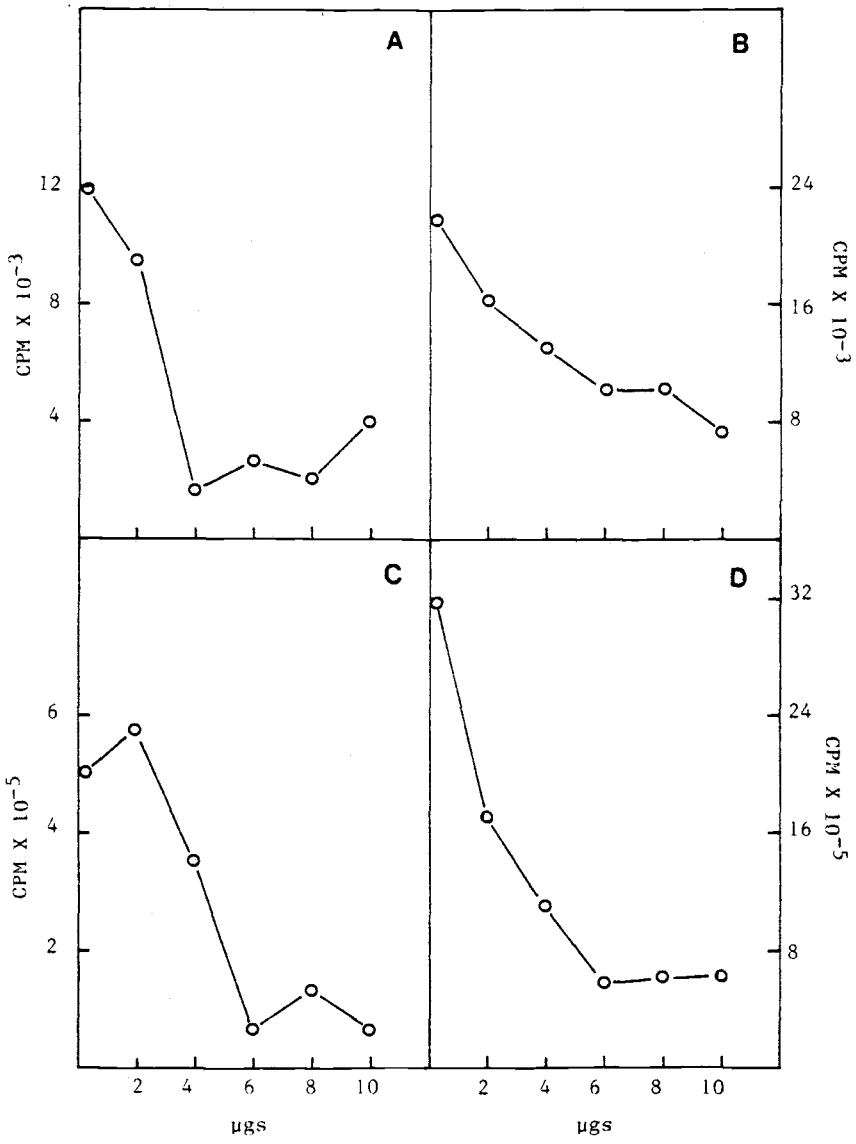

g/ml
CPM X 10^{-3}
1.22
1.18
1.14
1.10
30
20
10
A
B
C
8
16
24
FRACTION NUMBER

Figure 3. Effect of Increasing Concentrations of Inhibitor.

The effect of increasing concentrations of inhibitor on RDDP activity was examined with placental particle-associated enzyme, BaEV, SSV, and MuLV. Each 100 µl reaction contained 135 µg, 4.5 µg, 14.8 µg, or 12.4 µg of placental enzyme (3A), BaEV (3B), SSV (3C), and MuLV (3D) respectively. The mixture also contained the indicated amount of inhibitor and standard reaction components as described in Materials and Methods. The reactions were preincubated with inhibitor on ice for 30 min before the addition of (^{3}H)-dGTP and $(C)_n \cdot (dG)_{12-18}$. After incubation for 30 min at 37 C, the reactions were terminated by the addition of an equal volume of 10% trichloroacetic acid and 0.01 M sodium pyrophosphate. The specific activity of the (^{3}H)-dGTP was 5,280 cpm/pmole.

Figure 3


A
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CPM X 10^{-3}
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CPM X 10^{-5}
CPM X 10^{-5}
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µgs
2 4 6 8 10
µgs

Figure 4. Effect of Inhibitor on Rate of DNA Synthesis by Avian and Mammalian RDDP and Normal Cellular DNA Polymerases.

Standard RDDP reaction conditions described in Materials and Methods were employed. Reactions of 400 µl contained 8.0 µg inhibitor and 22.8 µg RD114 (4A), 37.6 µg MMTV (4B), 24 µg MPV (4D), or 49.6 µg RSV (4C). For DNA polymerases α (4E) and γ, 1 µg $(dC)_n \cdot (dG)_{12-18}$. The inhibitor used for the α polymerase reaction was dialyzed against 0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4 since polymerase is sensitive to high concentrations of salt. Reaction mixes were preincubated for 30 min on ice before the addition of (^{3}H)-dGTP and template. A volume of 80 µl was taken at the time intervals indicated and the radioactivity incorporated into DNA analyzed as described in Materials and Methods. The specific activity of the (^{3}H)-dGTP was 5,280 cpm/pmole. Reactions contained either STE_g (●---●) or inhibitor in STE_g (O---O).

Figure 4

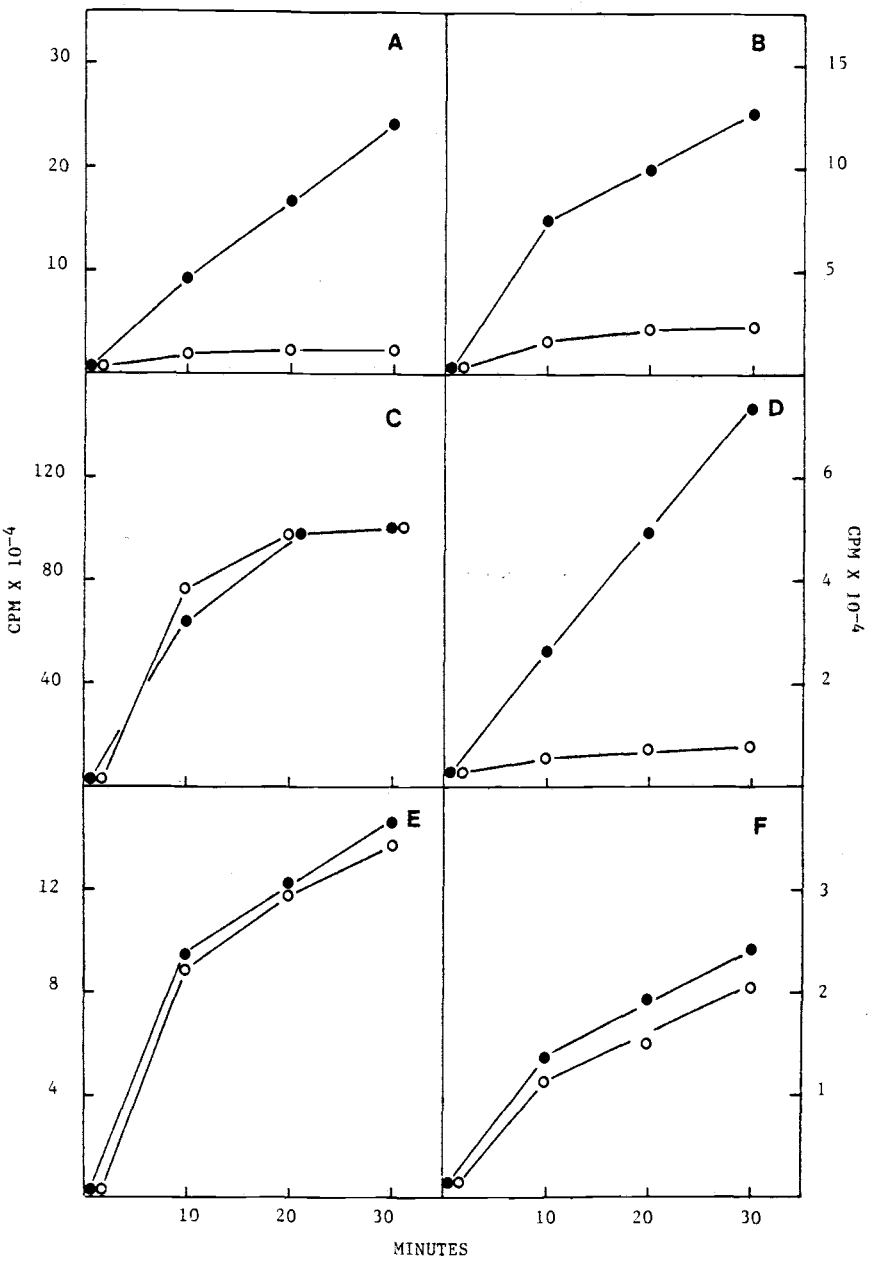
A
B
C
D
E
F
30
20
10
15
10
5
120
80
40
6
4
2
12
8
4
3
2
1
CPM X 10^{-4}
CPM X 10^{-4}
10
20
30
MINUTES

Figure 5. The Effect of Increasing Virus Concentration on the Inhibition of MuLV.

Each 100 µl reaction mixture contained 16 µg of inhibitor and the indicated amounts of MuLV. Standard RDDP reaction conditions were employed as described in Materials and Methods. Reaction mixes were preincubated with inhibitor for 30 min on ice before the addition of (^{3}H)-dGTP and $(C)_n \cdot (dG)_{12-18}$. Reactions were terminated after 30 min at 37 C.

Figure 5

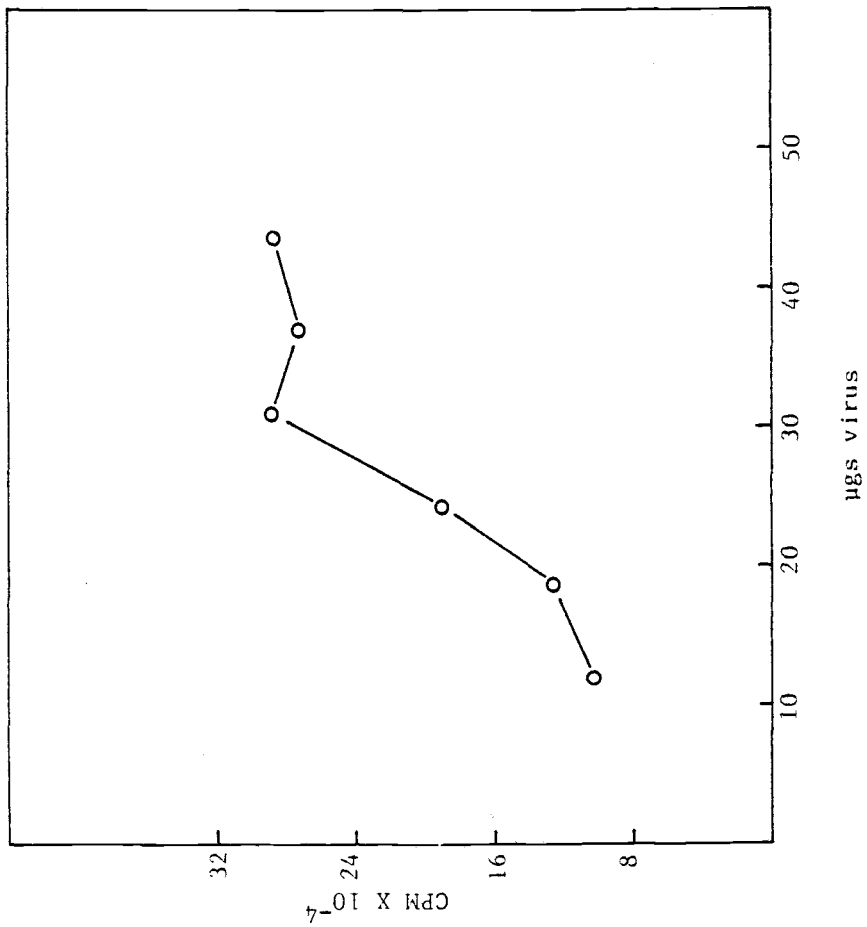

Table 1. Test for Inhibitor Activity in Salt Extracter Material.

The supernatant material remaining after salt extraction of the placental microsomal fraction were dialyzed against two changes of STEg and tested for inhibition of MuLV RDDP activity. A 100 µl standard reaction contained 7.5 µg of each extract and 12.4 µg of MuLV. The buffer STEg was used as a control. Reactions were preincubated for 30 min on ice before the addition of labeled dGTP and template-primer. The activity was determined by the amount of (^{3}H)-dGTP incorporation into acid insoluble radioactivity in 30 min at 37 C with $(C)_n \cdot (dG)_{12-18}$ as template-primer. The specific activity of the (^{3}H)-dGTP was 5,280 cpm/pmole.

Table 1. Test for Inhibitor Activity in Sequential Salt Extracts

Fraction	CPM	Percent Activity	Inhibition
Control STEg	565,822	100%	-
0.5 M NaCl Extract	781,685	138%	-
1.0 M NaCl Extract	1,063,560	188%	-
1.5 M NaCl Extract	67,393	12%	88%

Each figure represents the average of duplicate assays.

Table 2. Characterization of Inhibitor by Heat, pH, and Enzyme Treatment

All treatments were conducted as described in Materials and Methods. Assays for inhibitor activity were conducted with 12.4 µg of MuLV and 20 µg of treated and non-treated inhibitor using standard RDDP assay conditions. The figures represent pmoles of (^{3}H)-dGTP incorporated in 30 min at 37°C. The specific activity was 5,280 cpm/pmole.

*As an enzyme control, a volume of STEg was treated similarly.

+This column represents the percent inhibition of the MuLV RDDP reaction with untreated inhibitor.

‡This column represents the percent inhibition of the MuLV RDDP reaction with the treated inhibitor.

Table 2. Characterization of Inhibitor by Heat, pH, and Enzymatic Digestion

Treatment	Inhibitor Treated	Inhibitor Not Treated	Virus w/o* Inhibitor	Percent Inhib.+ Activity	Percent inhib.‡ after treatment	Sensitivity
Heat stability						
10', 37 C	6.32	6.06	39.94	85%	84%	Resistant
10', 56 C	10.96	6.06	39.94	85%	73%	Resistant
10', 85 C	3.64	6.06	39.94	85%	93%	Resistant
10', 100 C	45.48	23.37	48.11	51%	5%	Sensitive
pH Stability						
pH 2, 1 h	13.31	6.06	40.20	85%	67%	Resistant
pH 12, 1 h	11.43	6.06	55.15	89%	79%	Resistant
Enzymatic Digestion						
Trypsin	8.86	6.06	41.95	86%	79%	Resistant
Phospholipase	9.47	4.99	24.92	80%	62%	Resistant

Table 3. Characterization of Inhibitor by Solvent Extraction.

All treatments were conducted as described in Materials and Methods. Assays for inhibitor activity were conducted with 12.4 µg of MuLV and 50 µl of the corresponding resuspended phases and precipitates. Standard RDDP assay conditions were used. Each number represents pmoles of (^{3}H)-dGTP incorporated in 30 min at 37 C. The specific activity of the labeled nucleotide triphosphates was 5,280 cpm/pmole.

*As an enzyme control, a volume of STEg similar to the inhibitor volume was treated similarly and assayed for RDDP inhibitory activity as described.

+This column represents the percent virus RDDP activity in the extraction phases and precipitates of the treated inhibitor versus the treated STEg control.

Table 3. Characterization of Inhibitor by Solvent Extraction

Treatment	Extracted Inhibitor	Nonextracted Inhibitor	Control* STEg	Percent + Virus Activity
Ether Extraction				
Aqueous Phase	57.85	34.17	117.49	49%
Residue of Ether Phase	123.49	34.17	117.49	105%
Ethanol/Ether				
Residue or organic Phase	33.27	6.06	42.55	78%
Resuspended ppt.	2.01	6.06	51.35	4%
Chloroform/Methanol				
Residue or organic and aqueous phase	32.45	6.06	ND	81%
Resuspended ppt.	46.79	6.06	ND	117%

ND, not done.

Table 4. Summary of the Properties of the Placental Inhibitor

No Protease Activity
No RNase, DNase, Phosphatase Activity
Nondialyzable
Trypsin insensitive
Phospholipase C insensitive
Ether insensitive
Stable to Ph 2 and 12
Stable of Heat, 85°C, 10 min
Chloroform/Methanol sensitive