AN ABSTRACT OF THE THESIS OF

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Title: <u>THE EFFECT OF DDT ON INFECTIVITY OF RNA TUMOR</u>

<u>VIRUSES</u>

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Chicken embryonic cells (CEC) cultured in vitro were able to withstand the toxicity of DDT at concentrations below 10 µg/ml culture medium. When the DDT concentration exceeded the toxic level, CEC appeared vacuolated and lost the spindle-like fibroblastic appearance. Gas chromatographic analysis had shown that the uptake of DDT (3µg/ ml culture medium) by about 10^7 CEC was 0.93 µg in 72 hours. At this DDT concentration, the infectivity of the RNA tumor virus MC29 in CEC was inhibited by approximately 80% in 72 hours when DDT was added to the cultures immediately after infection. Short terms of pretreatment, as low as two hours, before infection inhibited virus infectivity nearly as much as continuous DDT treatment after infection.

CEC received only six hours of DDT pretreatment before infection was found to be more susceptible to MC29 infection than CEC that received the six hour pretreatment before infection and continuous

treatment after infection. Susceptibility of virus infection was measured by cell transformation in terms of focus forming units. A virus-specified protein (reverse transcriptase enzyme) was reduced by about 50% in 72 hours under continuous DDT treatment. The enzyme from DDT treated cultures sedimented at about the characteristic sedimentation coefficient (8.2S). DNA synthesis of pretreated cells measured by ^{3}H-thymidine incorporation changed little. MC29 virus adsorption (60-70%) was found unaffected by DDT.

The Effect of DDT on Infectivity of RNA Tumor Viruses

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To Edwin

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INTRODUCTION

There are two types of RNA tumor viruses -- leukemia and sarcoma viruses. Tumors resulting from the infection of sarcoma viruses are of mesenchymal derivation and those malignancies of leukemia viruses are of blood cells. Both types of viruses produce progeny virus in fibroblast cultures; but fibroblast cell cultures infected by leukemia viruses are not altered morphologically, whereas those infected by sarcoma viruses undergo morphological transformation.

In 1908, Ellerman and Bang (14) reported that the transmission of erythro-myeloblastic leukemia of chicken by cell-free filtrate of leukemia cells. Three years later, Rous (42) described the transmission of a solid tissue tumor by cell free filtrate and isolated several filtrates capable of inducing a variety of sarcomas in chickens. Using inbred mice, Bittner (7) in 1936 discovered that a mouse mammary gland carcinoma is transmitted from mother to offspring by a factor in milk. This factor in milk was later proven to be a filterable virus by Bittner in 1942 (8) and Andervont and Bryan in 1944 (1). Attempts had been made to transmit murine leukemia by cell-free filtrate from leukemia cells, but failed, until finally Gross in 1951 (21) successfully recovered a virus in transmitted leukemia.

Following this discovery, several strains of murine leukemia viruses have been isolated including the Friend, Moloney, Rauscher and Graafi strains. The first murine sarcoma virus was discovered by Harvey in 1964 (24) by passing murine leukemia virus in rats and he obtained virus which induced sarcoma instead of leukemia in mice. Subsequently, Moloney in 1966 (39) and Kirsten and Mayer in 1967 (29) isolated murine sarcoma viruses. However, none of these sarcoma viruses was obtained from a naturally occurring sarcoma. Similar agents have also been isolated from guinea pigs, cats, cattle, dogs and hamsters (16, 55).

To describe the replication of an RNA sarcoma virus in host cells, a hypothetical life cycle was suggested by Temin (53), which consisted of the following steps in sequence.

Step 1. The virus is adsorbed onto the cell and enters the cell. Its outer coat is removed and the viral nucleoid is transported to the cell nucleus. Enzymes in the nucleoid are exposed.

Step 2. DNA is synthesized using the viral RNA as a template. The proviral DNA is integrated into the cell chromosome at a specific site. Specific enzymes in the virions control these processes.

Step 3. RNA is synthesized using the proviral DNA as template. The viral-specified RNA is transported to the cytoplasm and directs protein synthesis. Cellular machinery is not modified.

This activation of RNA and protein synthesis requires cell division.

Step 4.   A modified cellular polymerase transcribes RNA from the proviral DNA. The RNA is transported to the cytoplasm and translated into protein by cellular machinery.

Step 5.   Membranes of the infected cells are modified by viral envelope components. Virions are assembled and bud off from the cell membrane.

Step 6.   The integrated provirus replicates together with the cell chromosomes and is passed on to progeny cells in mitosis.

Step 7.   Neoplastic transformation of the infected cells is obtained. Cell surfaces are altered after infected with RNA tumor viruses.

All these steps can be studied experimentally except 2 and 6, which involve the formation of the proviral DNA. This provirus has not been isolated and chemically chracterized.

Transformation is an independent event and does not depend on virus production. Separation of viral information for transformation and virus replication is supported by the evidence of temperature sensitive mutants (35, 60, 62). Transformed cells of these mutants lose their transformed morphology when shifted from permissive to non-permissive temperature. But infectious viruses are produced by cells at the temperature non-permissive for transformation.

My problem was to investigate the effect of DDT on infectivity of RNA tumor viruses. There are several possible routes of looking into the problem.

1. Virus synthesis (step 5) is a consequence of virus infectivity and it seems to be the most logical place to start on the problem. DDT can be added to the cell cultures before virus infection, after virus infection, or before and after. DDT may have stimulatory or inhibitory effect on virus infectivity.

2. The extent of cell transformation (step 7) reveals the number of susceptible cells to virus infection, but the process may be independent of virus synthesis. It is necessary to know whether there is correlation between virus synthesis and cell transformation.

3. If virus synthesis is indeed affected by DDT, the very next step is to determine at which step in the life cycle of an RNA tumor virus is affected by DDT the most. Adsorption (early step 1) can be studied by following the the number of virus disappearing from the culture medium. Activation of RNA and protein synthesis (step 3) requires cell division (53). DNA synthesis is generally studied by incorporation of labelled thymidine into DNA. Synthesis of viral specified RNA presents some problems since it is difficult to distinguish viral RNA from host cell RNA. Virus-specified enzymes can be studied by following its activity and appearance in infected cells.

MC29

Strain MC29 (myelocytomatosis) avian leukosis virus was isolated in Sofia, Bulgaria (27), from a Rhode Island hen chicken with spontaneous disease. Transmission of the virus induces neoplastic response mainly of the myeloid hematopoietic tissue in the intact chicken, such as myelocytomatosis and myelocytomas (9, 27). Associated frequency with the myeloid neoplasm are endothelioma, erythroblastosis, and renal carcinoma.

Exposure of chicken embryo cells (CEC) to strain MC29 resulted in the induction of virus synthesis in parallel with morphological conversion of the culture, within 72 hours under suitable conditions (31), unlike other leukosis viruses. Virus was liberated into the culture medium in physically measurable amounts within 48-72 hours in cultures infected with MC29 virus (9, 31, 32). With low multiplicities of virus and an agar overlay, the transformed cells form foci (29) and grow in layers which are indicative of loss of contact inhibition. Other properties associated with cell alterations are indefinite proliferation in vitro, and altered mucopolysaccharide contents and immunological properties (43).

There are five avian tumor virus subgroups determined by envelope antigens -- A, B, C, D, and E (12, 61). Strain MC29 virus has both subgroups A and B. When a virus of one subgroup infects a

chicken cell which is genetically resistant to infection by virus of this subgroup, such as MC29 virus subgroup A added to cells from 'C/A chicks, no progeny virus are produced and the cell is not transformed.

DNA Polymerases in RNA Tumor Viruses

Replication of RNA tumor viruses is inhibited by actinomycin D (4, 51), implying that a DNA intermediate was required for the replication of the viral RNA genome. In 1964, Temin proposed the provirus hypothesis (51) which required a DNA intermediate in the RNA replication of an RNA tumor virus. It was also noted that there was no early requirement for protein synthesis (52), therefore the enzyme which was able to transcribe the information from RNA to DNA was either present in the host cells or brought in by the virions. Two research groups (5, 54) independently found an enzyme in RNA tumor viruses capable of polymerizing DNA using an RNA template. Thus, the enzyme is known as RNA-dependent DNA polymerase, or reverse transcriptase. The activity of this enzyme is inhibited by treatment with ribonuclease A and T$_1$. It has a mandatory requirement for all four deoxyribonucleoside triphosphates and magnesium ions (6, 46, 65, 66). Manganese ions are, sometimes, used instead of magnesium ions (44). The enzyme fails to incorporate ribonucleoside triphosphates. The product formed is acid-insoluble and can be degraded by deoxyribonuclease, but not by ribonuclease or pronase. When the

product is banded in caesium sulphate density gradient, it is found in the DNA region and has a density of 1.45 g/cm^3. The density is 1.66 g/cm^3 for RNA and 1.57-1.65 g/cm^3 for RNA:DNA hybrid. When the DNA product is hybridized with RNA from the virion, it shifts from the DNA region in a caesium sulphate density gradient to the characteristic RNA region. This implies that the DNA synthesized is complementary to the virus RNA. Experiments by Fanshier, et al., and Verma, et al. (15, 59) show that the initial product of the reverse transcriptase is a covalently linked RNA-DNA molecule and the RNA moiety is degraded (38).

For cell transformation, this single-stranded DNA has to be converted to a double stranded DNA in order to integrate into the genome of the cell. This conversion requires the DNA - dependent DNA polymerase. Indeed, this enzyme activity has been found in numerous RNA oncogenic viruses (41, 47). Its activity requires the presence of all four deoxyribonucleoside triphosphates and magnesium ions.

Several synthetic DNA-RNA hybrids have been tested to serve as templates for the DNA polymerase system (20, 48, 63). They are not only functional, but also several-fold more effective than the natural template, such as dC:rG. DNA polymerases in the RNA oncogenic viruses resemble DNA polymerase I in that they require a primer and a template. To distinguish the reverse transcriptase from

cellular polymerase, the reverse transcriptase prefers initiator-primed synthesis with $(dT)_{10}$. poly(A) as template, whereas cellular polymerase gives reactions with $(dT)_{10}$. poly(dA). Immuned serum from rabbits immunized with purified reverse transcriptase from tumor virions inhibits only activity of viral-induced enzyme. Also, the sedimentation coefficient of the virus induced enzyme is different from that of cellular polymerase. The activity of the reverse tran-scriptase appears and is increased after infection with tumor virions, whereas cell polymerase activity remains unchanged.

Properties of DDT

DDT is chemically known as 1, 1, 1-trichloro-2, 2-bis(p-chloro-phenyl)ethane. The p, p'-isomer is the most potent component of the pesticide. For over a quarter of a century DDT has served well in combating insect-borne diseases, particularly malaria, plague and typhus, and in increasing the production of food. Recently the use of DDT was banned in the United States due to the questionable role of DDT in animal body tissue.

Owing to its high lipid solubility and chemical stability, DDT is stored mainly in the fat tissue. Placental transfer of DDT is known to occur in man and other mammals (3, 40, 64), and the presence of this pesticide and its metabolites has been demonstrated in human milk (13, 67). Rats subjected to DDT diet at a level of 400 ppm for a

long-term resulted in liver lesions and hepatic cell tumors, suggesting that DDT has some tumorigenic potential (17). Experimental results indicated that DDT causes greater incidences of leukemia in pure line mice (50).

Reports have shown that DDT induces drug-metabolizing enzymes (18, 19, 23). In addition, DDT produces changes in the endoplasmic reticulum in the microsomal fraction, but no pathological effects are associated with these changes (40). Toxicity of DDT in cultures HeLa S cells is evidenced by progressive inhibition of cell growth. Cellular protein synthesis is decreased with increasing quantity of DDT, whereas DNA synthesis changes little (10, 11).

The studies undertaken in the following report attempt to establish the nature of DDT on infectivity of MC29 avian leukosis virus in chick embryo cell cultures.

MATERIALS AND METHODS

Culture of Chicken Embryonic Cells

Primary Culture

The chicken embryos used in this work were from White Leghorn eggs obtained from Dr. R. E. Luginbuhl, University of Connecticut, Storrs, through the Special Virus Cancer Program of National Cancer Institute. Embryos 10-11 days old were decapitated, deviscerated and minced. The minced tissue fragments were washed several times with phosphate buffer saline (PBS) until the supernatant appeared free of red blood cells. 0.25% trypsin in PBS was added to the washed tissues for 30 minutes at 37°C. The cells were filtered through eight layers of sterile gauze and collected in a chilled Erlenmeyer flask containing 5 ml of newborn calf serum. They were then sedimented at 150 x g for five minutes and resuspended in growth medium, which consisted of 80% (by volume) medium 199, 8% newborn calf serum (Microbiological Associates, Albany, California), 10% tryptose phosphate broth (Difco, Detroit, Michigan), 0.56% sodium bicarbonate (100 mg/ml), 0.2% penicillin (50 unit/ml) and streptomycin (50 μg/ml) (Eli Lilly & Co., Indianapolis, Indiana) and 0.1% amphotericin B (0.5 μg/ml) (E. R. Squibb & Sons, New York, New York). A diluted portion of resuspended cells was stained with crystal violet and

counted in a hemocytometer. 8×10^6 cells per 10 ml growth medium were added to each Petri dish (100 x 20 mm, Flacon plastic culture dish, Los Angeles, California), and incubated at $38.5^{\circ}C$ in a humidified 5% CO_2 incubator.

Secondary Culture

Primary chicken embryonic cells (CEC) were usually subcultured three to four days. Growth medium was decanted and confluent cell sheet was washed with 4 ml of PBS. Four ml of 0.05% trypsin were added and cells were incubated at $38.5^{\circ}C$ for seven minutes. Cells were aspirated off the culture dish with a 5 ml pipette and were pooled in chilled conical centrifuge tubes containing 1 ml of newborn calf serum. Cells were sedimented at 150 x g for five minutes, resuspended in growth medium, and seeded at 5×10^6 cells per culture dish.

Preparation of MC29 Virus Stock

Primary cells at 8×10^6 per culture were plated in plastic culture dishes. Twenty-four hours later, growth medium was decanted. The cells were washed with 4 ml of PBS, and were infected with 0.2 ml of MC29 virus (the original MC29 stock was kindly supplied by Dr. J. W. Beard). After 30 minutes of adsorption at $38.5^{\circ}C$, 10 ml of growth medium were added and the cells were returned to

the incubator. After three days, the cells were subcultured and were

plated at 5 x 10^6 cells. Growth medium was changed on the following

two days. Culture fluid was collected at 72 hours and centrifuged at

6,000 x g for ten minutes to remove cell debris. The clarified culture

fluid was flash frozen and stored at -70°C. The virus stock prepared

in this manner contained about 1 x 10^6 focus forming units (FFU) per

ml of culture fluid. In some instances, culture fluid was collected at

six hour intervals.

Titration of MC29 Virus

Confluent primary CEC were subcultured and filtered through

eight layers of sterile gauze and seeded at 1 x 10^5 cells per culture

dish (60 x 15 mm). After 10 to 12 hours of incubation, growth medium

was decanted. The non-confluent cell layer was washed with 4 ml of

PBS and infected with 0.1 ml of MC29 virus dilution. The virus

was adsorbed for 30 minutes. Five ml of growth medium were added

and CEC were returned to the incubator. The following day, CEC were

washed with 4 ml of PBS and 4 ml of growth medium containing 0.45%

Nobels Agar (Difco, Detroit, Michigan) were added and allowed to

solidify. Three ml of liquid medium were added on the third day after

infection. On the seventh day, both fluid and agar were removed. The

cells were washed with PBS, and fixed with 3 ml of cold, absolute

methanol for five minutes. The alcohol was decanted and the cells

were stained with 5 ml of cold May-Grünwald stain (Allied Chemical, Morriston, New Jersey). The stain was removed in ten minutes and the cells were counterstained with 5 ml of cold Giemsa stain (Allied Chemical, Morriston, New Jersey) for ten minutes. The cultures were rinsed with distilled water and allowed to air dry. If necessary, the cultures were destained with 4 ml of 0.001 N HCl followed by 5 ml of PBS. Foci were counted under low power of magnification.

Infection of CEC with MC29 Virus

CEC after 12-24 hours seeding were washed with PBS and infected with 0.2 ml of MC29 stock, at a multiplicity of infection about 0.04-0.06 FFU per cell. Control cultures were mock-infected with 0.2 ml medium. The virus was allowed to adsorb at 38.5°C for 30 minutes. Virions not adsorbed were washed off with 4 ml of PBS. Ten ml of medium were added and the cells were returned to the incubator. Culture fluids and CEC were collected at the designated time.

Collection of MC29 Infected and Uninfected Culture Fluids

Fluids from MC29 infected and uninfected cultures were collected, pooled, and clarified by adding 0.5 g of Kieselguhr and centrifuged at 150 x g for ten minutes to remove cell debris. The supernatant fluid was centrifuged at 5,000 rpm for another ten minutes.

The clarified culture fluids were flash frozen in liquid nitrogen and stored at -70°C.

Collection of MC29 Infected and Uninfected Cells

Medium was decanted from MC29 infected and uninfected cells, which were washed with 4 ml of PBS. One ml of fresh medium was added and the cells were removed with a rubber policeman. Cells from four plates were pooled and pelleted at 1,640 x g for ten minutes and the cell pellets were stored at -70°C.

DNA Polymerase Procedures

Preparation of DNA Polymerase from MC29 Infected and Uninfected Culture Fluids

The culture fluids were rapidly thawed and centrifuged in a Spinco SW25 rotor at 25,000 rpm for 60 minutes at 2°C. The pellet was resuspended in 0.2 ml of 2X Buffer 0 (0.05M Tris, pH 8.3; 0.02M MgCl$_2$; 0.01M reduced glutathione; 0.001M EDTA; 0.30M KCl; and 20% glycerol) and 0.2 ml of 2X Nonidet stock (0.5% Nonidet, Shell P40, plus 0.13M dithiothreitol). The nondiet was added to lyse the virions. This mixture was incubated at ice temperature for overnight before assayed for enzyme activity.

Preparation of DNA Polymerase from MC29 Infected and Uninfected Cell Homogenates

Washed cells from four plates were thawed, resuspended in 0.6 ml Buffer 1 (0.01M Tris, pH 8.3; 0.01M KCl; 0.005M MgCl$_2$; 0.005M EDTA; 0.001M reduced glutathione; and 10% glycerol) and kept in an ice bath for about two hours with occasional mixing. The cells were disrupted using a tightly-fitted glass Dounce homogenizer (about 50 strokes). The homogenates were centrifuged at 1,640 x g for ten minutes. Five-hundredths of a ml supernatant was removed for protein determination. To the remaining supernatant, one-tenth its volume of 10X Nonidet stock (1.25% Nonidet, Shell P40, plus 0.325M dithiothreitol) was added and allowed to stand in ice for 15-30 minutes, after which it is ready for DNA polymerase assay.

DNA Polymerase Assay (Standard Enzyme Assay)

A standard reaction mixture (45) contained in a volume of 0.1 ml: Tris-hydrochloride (pH 8.3), 4 μmoles; MgCl$_2$, 0.8 μmoles; NaCl, 6 μmoles; reduced glutathione, 0.37 μmoles; unlabeled dATP, dCTP, TTP (PL Biochemical, Inc.), 0.02 μmoles; ^{3}H-labeled dGTP (Amersham Searle; specific activity nine curies per mmole), 1 μCi. One pmole of ^{3}H-dGTP was equivalent to 3000 counts per minute under the counting efficiency of the liquid scintillation spectrometer. Avian myeloblast DNA (nicked, about 4 μg per assay) was used. The amount

of enzyme added was indicated in each experiment. All assays were

performed at 37°C for 60 minutes and the reactions were terminated

by chilling in ice and adding 0. 5 ml of cold trichloroacetic acid mix-

ture (equal parts of 100% trichloroacetic acid, saturated $Na_4P_2O_7$ and

saturated NaH_2PO_4). The precipitate was collected on nitrocellulose

membrane filter (B6, Scheicher and Schuel, Keene, New Hampshire),

washed 12 times with 4 ml of 10% TCA (w/v), and dried at 80°C for at

least ten minutes. The filter was put in a counting vial containing

10 ml of liquid scintillation fluid (0. 4% w/v BBOT, 2, 5-bis(5'-tert-

butylbenzoazolyl)-thiophene; Packard Instrument Company, Downers

Grove, Illinois) in toluene. Radioactivity was measured in a liquid

scintillation spectrometer (Packard Instrument Company, Downers

Grove, Illinois).

Preparation of Nicked DNA

DNA was extracted from chicken myeloblasts, using the method

described by Marmur (34), and dissolved in 0. 1X SSC (0. 015M NaCl;

0. 0015M sodium citrate; pH 7. 0) at approximately 500 μg/ml.

$MgCl_2$ was added to give a final concentration of 0. 006 M. DNase

(RNase-free; Worthington, Freehold, New Jersey) was added (0. 08

μg/ml) and the mixture was incubated for one hour at 37°C. The

mixture was quickly cooled and NaCl was added to yield a final concen-

tration of 0. 5 M. DNA was then heated for ten minutes at 80°C. The

denatured DNA was dialyzed in 20X volume of 0.1X SSC for overnight against two to three changes of buffer. Absorbancy of the nicked DNA at 260 nm was measured. A standard enzyme assay was performed to determine the template activity of the nicked DNA.

Calibration Curve between FFU of MC29 Virus and DNA Polymerase Activity

Several known volumes of titrated MC29 virus suspension were diluted to a total of 5 ml and centrifuged in a Spinco SW50 rotor at 50,000 rpm for 30 minutes. The pellet was resuspended in 0.1 ml 2X Buffer and mixed in 0.01 ml 10X Nonidet stock and allowed to stand in ice for overnight before assayed for DNA polymerase activity. Under standard assay condition, 1 pmole of ^{3}H-dGTP was equivalent to 3000 counts per minute.

Fractionation of DNA Polymerase with a Sucrose Density Gradient

Preformed 5-20% (w/v) sucrose density gradients in Buffer 0 and 0.065 M dithiothreitol were used. Forty-five hundredths of a ml of homogenate and 0.05 ml of human gamma-globulin (14 mg/ml) for fractionation were carefully layered on top of the gradient which was centrifuged in a Spinco SW50 rotor at 50,000 rpm for 15 hours at 2°C. Human gamma-globulin was added to the homogenates to give a standard protein in the gradient. A hole was punctured in the

bottom of the tube and 0.2 ml fractions were collected. Portions from each fraction were assayed for protein by the TCA precipitation method and enzyme activity.

TCA Precipitation Protein Determination

Samples of 0.05 ml were diluted to 0.4 ml with distilled water and precipitated with 0.6 ml 5% TCA. Precipitation was allowed to completion in ten minutes and absorbancy was measured at 400 nm in a spectrometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Standardization was done with bovine serum albumin.

Lowry Protein Determination

Samples were precipitated with 2 ml 10% TCA (cold), allowed to stand in ice for ten minutes and centrifuged at 12,000 rpm for ten minutes. This precipitation was repeated. Protein was then dissolved in 0.2 ml of NaOH (0.4 N) for 60 minutes at 37°C. The protein assay followed the Lowry determination of protein (33). Three ml of solution C (50:1 v/v of solution A to solution B; solution A, 2% Na_2CO_3 in 0.1 M NaOH; solution B, 0.5% $CuSO_4 \cdot 5H_2O$ in 1% Na or K tartrate) were added and allowed to stand at room temperature for at least ten minutes. 0.3 ml of solution E (diluted Folin reagent, 1 N in acid) was slowly added and allowed to stand for 30-35 minutes. Absorbancy at 750 nm was read against a reagent blank (water or buffer).

Comparison of DNA Synthesizing Rate in
DDT Treated and Untreated Cells

The medium was removed from control cells and cells pretreated

six hours with DDT solution (p, p-isomer, 3 μg/ml) and 5 ml of fresh

medium were added. Two-tenths ml of ^{3}H-thymidine at about 30 μCi/

ml growth medium was mixed in. After one or two hours, as indicated

in the experiment, the medium was decanted. The cultures were

washed one time with 5 ml of cold PBS, and three times with 5 ml of

cold 10% TCA. One ml of NaOH (0. 2N) was added and spread over the

cultures for 15 minutes. The extract was transferred to a counting

vial and was neutralized with 0. 1 ml of HCl (2N). Fifteen ml of

liquid scintillation fluid (Aquasol, New England Nuclear, Boston,

Massachusetts) were added and the radioactivity incorporated into

acid insoluble polymer was measured in a liquid scintillation spectro-

meter.

RESULTS

Calibration of Enzyme Activity and Virus Particles

Detection of RNA tumor virus can be done either by assaying the infectivity (or focus forming units, FFU) of the virus particles, or by determining the reverse transcriptase activity in the virus particles. In general, the reverse transcriptase activity assay is most often used because of convenience and time. This enzyme activity is measured by the ^{3}H-dGTP incorporated into DNA polymer. Under the standard assay condition for the reverse transcriptase, the calibration (Figure 1) showed that one count per minute (cpm) of ^{3}H-dGTP was incorporated into DNA for every four infectious virus particles. This relationship holds when cpm of ^{3}H-dGTP incorporated is below 1×10^6.

Throughout this report, measurement of virus particles released in the culture fluids is given in terms of cpm of ^{3}H-dGTP incorporated. The infectious particle number can be estimated, using the above relationship.

Effects of DDT on CEC Cells

DDT has extremely low solubility in water, approximately 1.2 parts per billion (58). Addition of DDT to the culture medium is accomplished by dissolving DDT in ethanol, giving a stock solution of 1 mg DDT/ml ethanol. Virus infected chick embryo cells (CEC) were
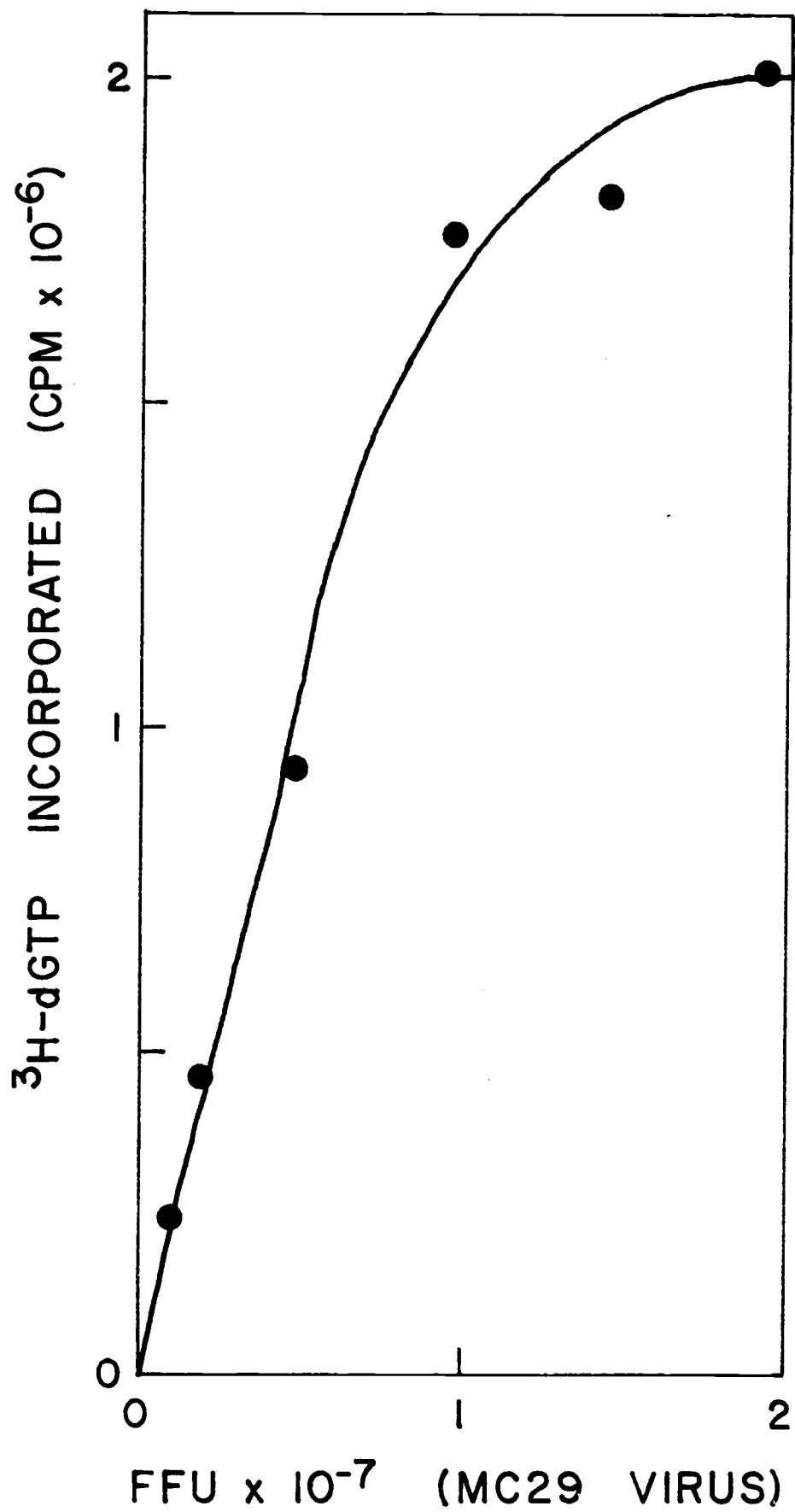
treated with several concentrations of ethanol. It was found that

ethanol was not toxic to cells at very low concentrations. At 0.3%

concentration in culture medium, there was no significant difference

in virus production (Table 1). The ethanol concentration in all DDT

treatments of cells did not exceed this concentration.

Table 1. Effect of ethanol on virus production.

Condition*	^{3}H-dGTP incorporated** (cpm)
- ethanol	36,600
+ ethanol	35,300

* Culture medium containing 0.3% ethanol was added to
the cells immediately after infection and continued for
72 hours after which the culture fluids were assayed for
virus.
** Four ml of tissue culture fluid were assayed for reverse
transcriptase activity. The cpm of ^{3}H-dGTP incorporated
into DNA was the mean cpm of three identical determinations.

At higher concentrations of ethanol, the inhibition of virus pro-

duction was observed (Figure 2). The production of virus in CEC

was inhibited about 50% by 1% of ethanol in culture medium and was

completely suppressed when the concentration of ethanol was increased

to 3% and 5%.

The toxic level of DDT in culture medium was determined in

order to estimate the range of DDT concentrations to be used in these

experiments. In medium containing DDT concentration below 10 μg/ml,

the cells appeared normal; however, they became vacuolated when the
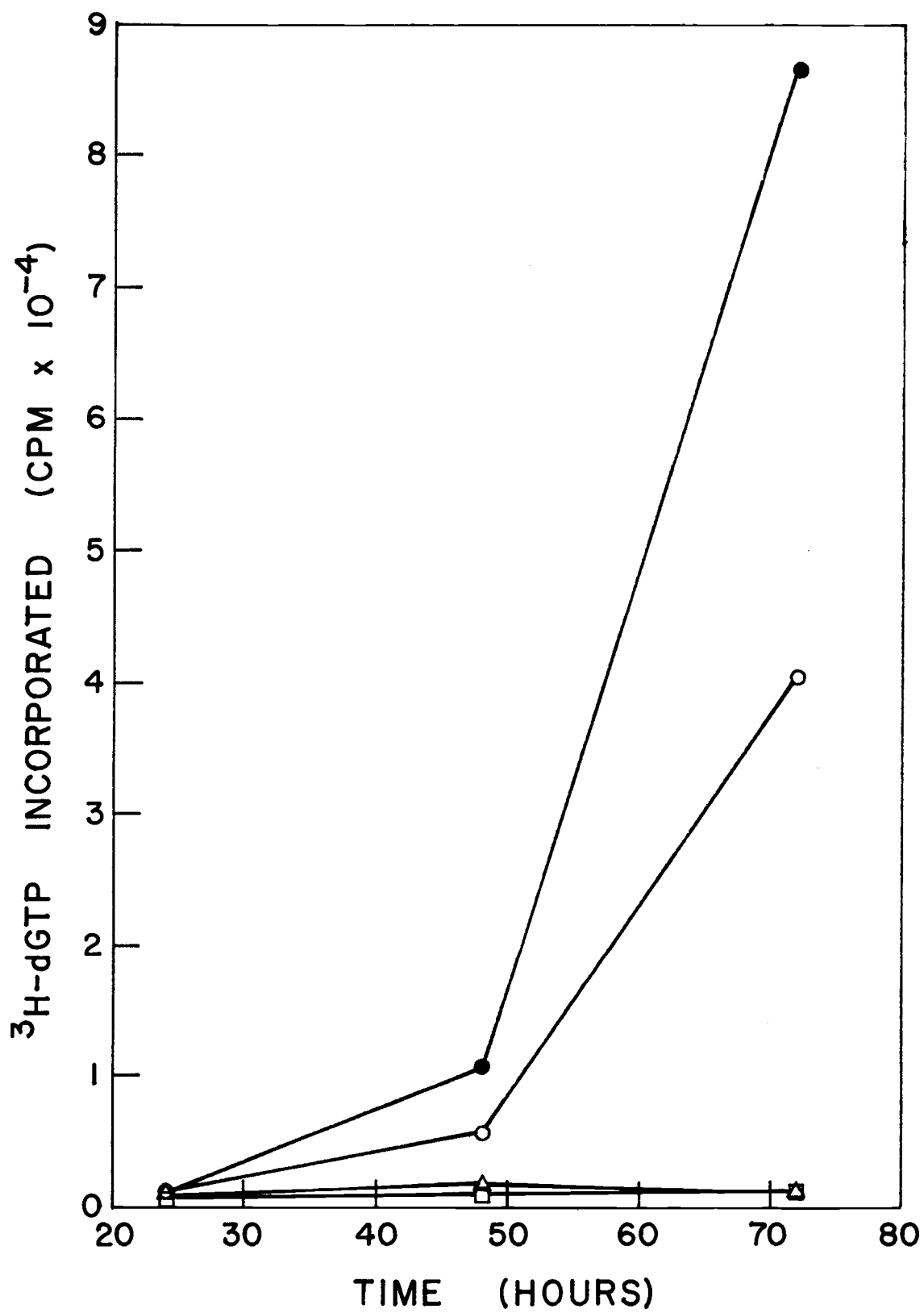
DDT concentration exceeded 10 µg/ml. The concentration of DDT at 3 µg/ml was chosen as the concentration of DDT used throughout this research since it was well below the toxic level. Chromatographic analysis recovered 0.93 µg of DDT in about 10^7 cells after treated with DDT 3 µg/ml for 72 hours.

Inhibition of Virus Production by DDT

Normally, release of virus particles occur in cell cultures between 12-24 hours after infection with MC29 virus and the release was measured by reverse transcriptase activity. In these experiments, one set of cultures was infected with MC29 virus and DDT 3µg/ml was added immediately. Another set of culture was infected without receiving any DDT treatment. Mock-infected cultures were set up in the same manner. Figure 3 showed a steady release of virus particles by the untreated cultures from 24-72 hours post-infection. The release of virus particles was inhibited by about 80% at 72 hours in DDT-treated cultures. This inhibition suggested that DDT blocked inclusively the steps between the events of adsorption (early step 1) and virus release (step 5).

It was necessary to find out whether addition of DDT had any effect on infected cells which had surpassed step 5. In order to determine this, the cells used were chronically infected and were releasing virus into culture medium. The data in Table 2 showed that the virus
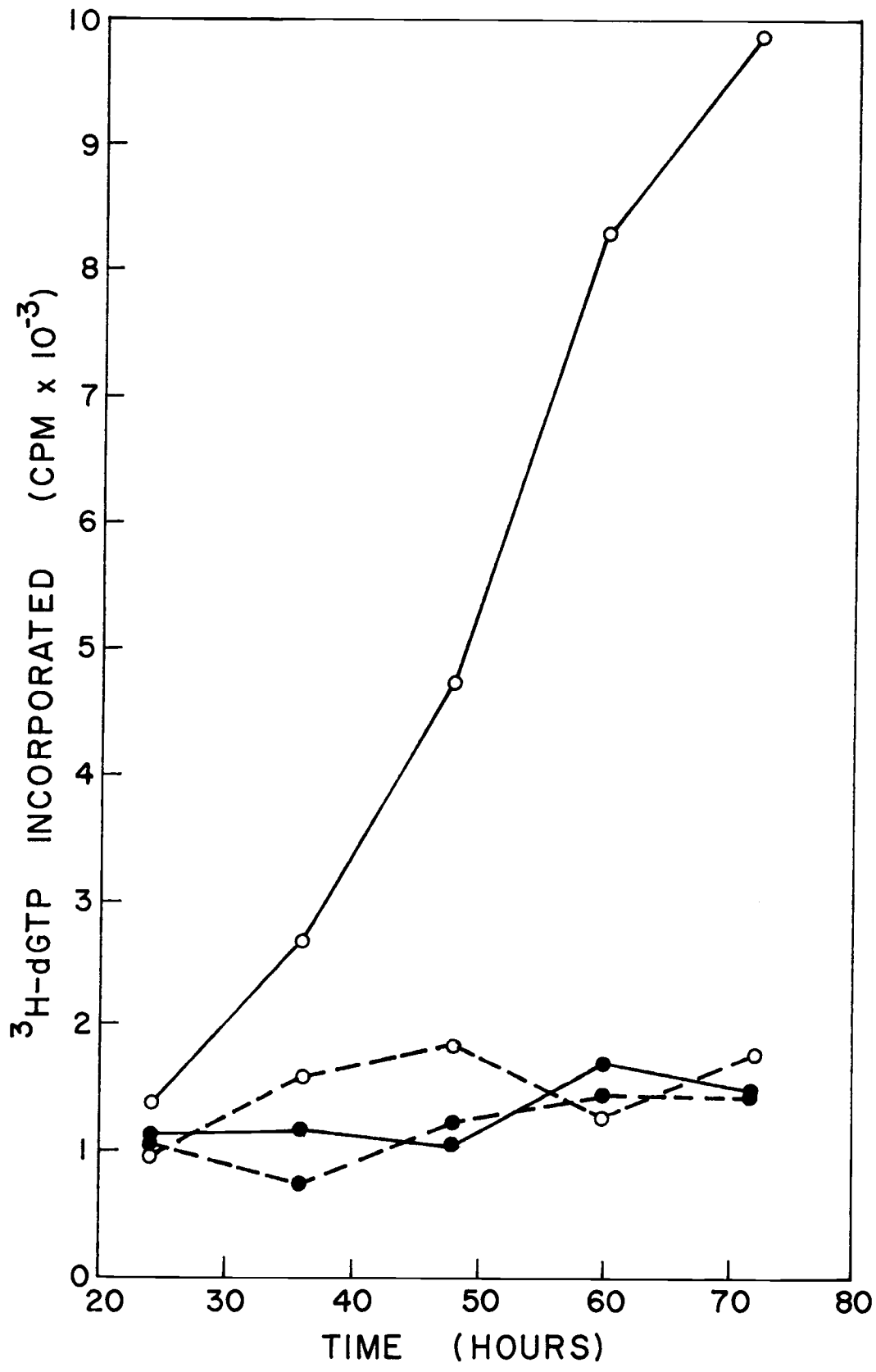
production as measured by incorporation of ^{3}H-dGTP in DNA by viral enzyme was only slightly reduced in infected cells receiving DDT treatment 72 hours post-infection, relative to the virus released by cells that received no treatment. In comparison to the results obtained in Figure 3, the inhibition of virus production by chronically infected cells was insignificant.

Table 2. Effect of DDT on chronically MC29 virus infected cells.

Hours post-infection*	^{3}H-dGTP incorporated (cpm x 10^{-3})***	
	-DDT	+DDT**
96	116	111
120	204	158
144	193	181

  * Culture fluids were collected at the indicated time post-infection.
 ** DDT 3μg/ml culture medium was added 72 hours post-infection.
*** ^{3}H-dGTP incorporated into DNA by the reverse transcriptase activity was determined in the virus pellet from 4 ml of culture medium.

The data in Table 2 suggested that once the cells were stably infected by MC29 virus, addition of DDT only slightly inhibited the synthesis of the virus. Perhaps there is an early function, cellular or viral in origin, that is sensitive to the action of DDT, but once that function is fully established, DDT exerts little effect on virus release.

Production of virus had been examined only in cultures receiving DDT continuously after infection. It was found that short terms of pretreatment (before virus infection) gave nearly as much inhibition

as continuous treatment, and pretreatment for only two hours before infection showed considerable inhibition of virus production. Comparison was done on cultures receiving the various short term pretreatments to those that had no DDT treatment and continuous DDT treatment. The results were summarized in Table 3.

The data was in complete agreement with that of chronically infected cells. Both experiments indicated that an early function in the infectious cycle was blocked by DDT. But, this early function could be a cell specified function necessary for virus formation or a virus-specified expression in the cell that was blocked by DDT accumulated in the lipid layer of the cell membrane and slowly released into the cell. Since short term pretreatment and continuous treatment gave about the same percent of inhibition, virus adsorption to the cells (early step 1) was most likely not responsible for the inhibition.

Effect of DDT on MC29 Adsorption in CEC

Short term of DDT pretreatment had nearly as much of inhibition in virus production as in continuous DDT treatment throughout the incubation. This implied that virus adsorption onto the cells (early step 1) was not responsible for the inhibition.

Confluent cells were subcultured and seeded at 5×10^6 cells per culture. One set of cultures was treated with DDT solution (3 μg/ml) at the time of seeding; the other set acted as control. Six hours after

Table 3.   DDT pretreatment effect on MC29 virus production in CEC.

Conditions**	% inhibition by DDT treatment***	
	72 hours*	96 hours*
Cells treated with DDT only after infection.	84	72
Cells treated with DDT six hours before infection and remained after infection.	87	76
Cells treated with DDT six hours before infection only.	63	58
Cells treated with DDT four hours before infection only.	57	62
Cells treated with DDT two hours before infection only.	73	60

* The indicated times were hours post-infection.

** DDT concentration was 3 μg/ml of growth medium.

*** Percent inhibition was based on reverse transcriptase activity in culture fluids of DDT-treated and infected cells relative to that of untreated and infected cells at the indicated time of harvest. The untreated cell culture had 36 x 10^3 cpm of ^{3}H-dGTP incorporated into DNA at 72 hours and 103 x 10^3 cpm of ^{3}H-dGTP incorporated into DNA at 96 hours.

plating, culture medium was decanted and washed with 4 ml of PBS. The cells were infected with 0.2 ml of MC29 virus stock (9.6 x 10^5 FFU/ml). Two and one half ml of medium were added after 30 minutes of adsorption at 38.5°C. Culture fluid was collected and pooled from two plates every hour after infection for six hours. Enzyme activity in the culture fluids was determined.

Figure 4 showed adsorption was indeed unaffected by DDT. Virus adsorption of 60-70% was observed in both untreated and DDT pretreated (six hours) cultures.

Inhibition of Cell Transformation by DDT

According to Temin (53), cell transformation can be an independent event as a consequence of tumor virus infection. Usually, infection with a leukosis virus does not result in cell transformation, but MC29 avian leukosis virus is an exception. Neoplastic transformation of cells (step 7) occurs in about 72 hours after infection with MC29 virus. These malignant cells lose their contact inhibitory properties and pile up on top of each other resulting in heaps or foci. Langlois et al. (30) developed an assay to determine foci formed by MC29 virus in CEC cultures.

The various short term pretreatment did not show significant difference in inhibiting virus production (Table 3). The six hour pretreatment period was chosen for the study of cell transformation.
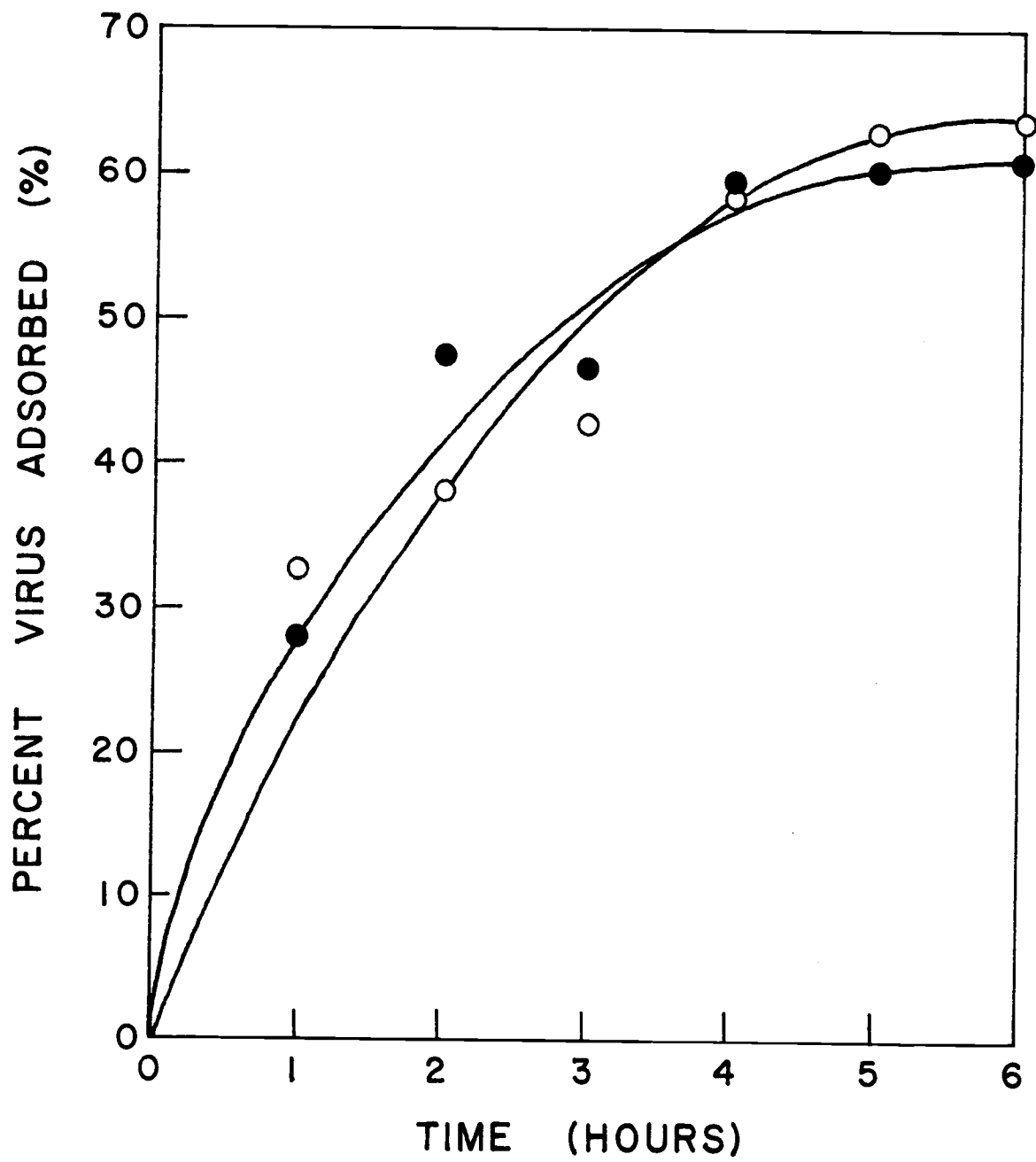
The data in Table 4 showed that a reduced number of cells were susceptible to virus infection after they received DDT 3 μg/ml six hours before infection and DDT 3 μg/ml immediately after infection. The number of susceptible cells to infection increased when the cells were given DDT 3 μg/ml only six hours before infection. The inhibition of cell transformation due to DDT treatment and pretreatment was similar to the inhibition of virus production. Perhaps reduction in the number of virus particles released was a direct consequence of the reduced number of susceptible cells.

Table 4. Effect of DDT on cell transformation.

	FFU observed*		
Conditions***	20 FFU added	40 FFU added	68 FFU** added
DDT untreated cells.	6	17	31
Cells treated with DDT six hours before infection only.	7	13	13
Cells treated with DDT six hours before infection and remained after infection.	3	2	4

* The FFU observed was the average of triplicate samples. FFU = focus forming units.
** Dilutions of 20, 40, 68 FFU/0.1 ml MC29 virus stock were used. 0.1 ml was added to each culture.
*** DDT concentration was 3 μg/ml growth medium.

Effect of DDT on the Reverse Transcriptase in
MC29 Virus Infected Cells

A new DNA polymerase appears in the virus infected cells at the time of virus released. Unlike other DNA polymerases in the cells,

this new polymerase is able to use RNA as a template for DNA synthesis (5, 54).

Since the reverse transcriptase is a virus-specified protein in infected cells, its activity in DDT-treated, but MC29 virus infected cells would expect to be reduced. Figure 5 showed that indeed the activity was reduced by about 50% in the infected cells receiving DDT 3 μg/ml immediately after infection for 72 hours. DDT had no inhibition of reverse transcriptase activity when it was added directly to the reaction mixture. Total protein in the fractions collected from the gradient was determined by Lowry determination (Table 5). Enzyme activity across the sucrose gradients was normalized as the activity that would be present in 100 μg of protein to make comparison possible.

The protein content determined in 0. 45 ml of cell homogenates (Table 5) showed that DDT treated cells, whether infected or uninfected, had a decrease in protein content compared to the untreated cells. DNA synthesis (described in the following section) was found to be inhibited by about 27%. Therefore, this decrease in protein content was probably due in part to a reduction of cells, rather than a loss of protein per cell.
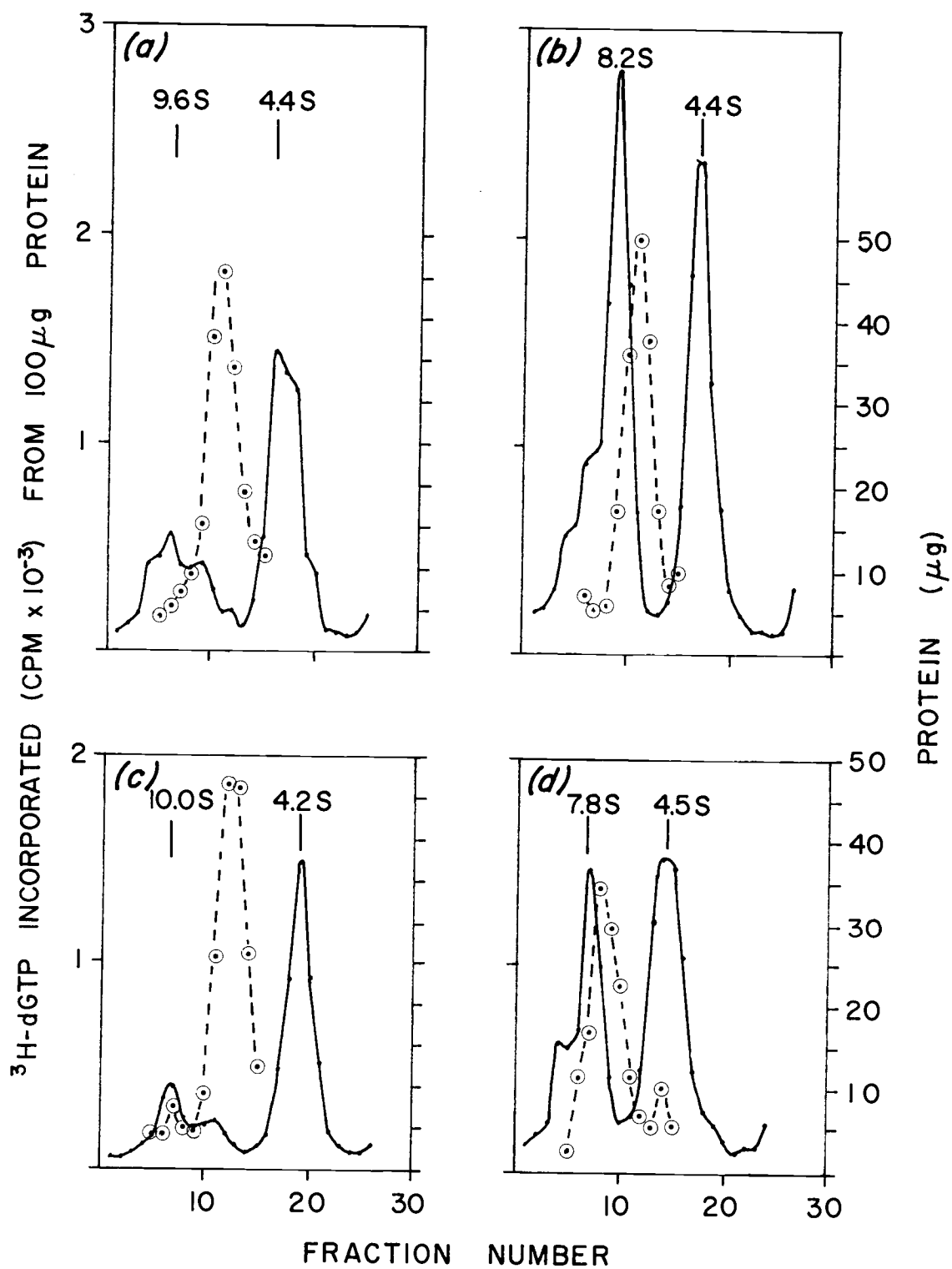
Table 5.   Protein content in the cell homogenates.

Condition*	Protein (μg)**
Untreated and uninfected cells.	105.88
Untreated and infected cells.	95.22
DDT treated and uninfected cells.	91.73
DDT treated and infected cells.	70.59

* DDT 3 μg/ml was added immediately after infection.
** The amount recorded represented protein in the total volume of
   fractions assayed (0.04 ml/fraction) and was based on the protein
   determined in 0.45 ml of cell homogenates.

The virus-specified reverse transcriptase was absent in unin-

fected cells.   The sedimentation profile of uninfected cells was given

in Figure 5a.   DDT-treated, but uninfected cells (Figure 5c) gave

similar profile of sedimentation and enzyme activity.   They both had

DNA polymerase activity in 10S and 4-5S regions.   The new DNA

polymerase of infected cells (Figure 5b) exhibited a large increase in

activity, and sedimented at a characteristic coefficient (8.2S) of virus-

specified polymerase.   Infected cells receiving DDT treatment

(Figure 5d) had a virus-specified enzyme slightly shifted in sedimen-

tation coefficient (7.8S) and reduced in activity.   However, this shift

in coefficient was not sufficient to prove that the virus-specified

polymerase was altered in its property.   The results in Figure 5

suggested that the synthesis of virus-specified protein was greatly

suppressed by DDT.   This came in reasonable agreement to the

inhibition of virus production and cell transformation.

Effect of DDT on DNA Synthesis

Virus production was inhibited both by short period of pretreatment and by continuous treatment of DDT (Table 3). Protein synthesis specified by virus in infected cells was much reduced (Figure 5) by DDT. It would be reasonable for one to ask the question whether DNA synthesis would also be suppressed in CEC receiving a DDT pretreatment. Incorporation of radioactive thymidine into DNA is a measure of DNA synthesis in the cells.

The incorporation of ^{3}H-thymidine into DNA was inhibited by about 27% (Figure 6) in the cells pretreated with DDT 3 μg/ml for six hours before measurement compared to the cells that received no treatment. In another experiment, DNA synthesis was inhibited by only 10-15% in cells treated with DDT for 24-72 hours. This percent inhibition of DNA synthesis was not sufficient to account for all of the inhibition observed in virus production, in virus-specified protein synthesis and in cell transformation. Activation (step 3) of virus-specified RNA and protein, according to the proposed life cycle (53), required cell division. Inhibition of DNA synthesis would certainly block the activation process and contributes partially to the inhibition in virus synthesis, virus-specified protein and cell transformation.
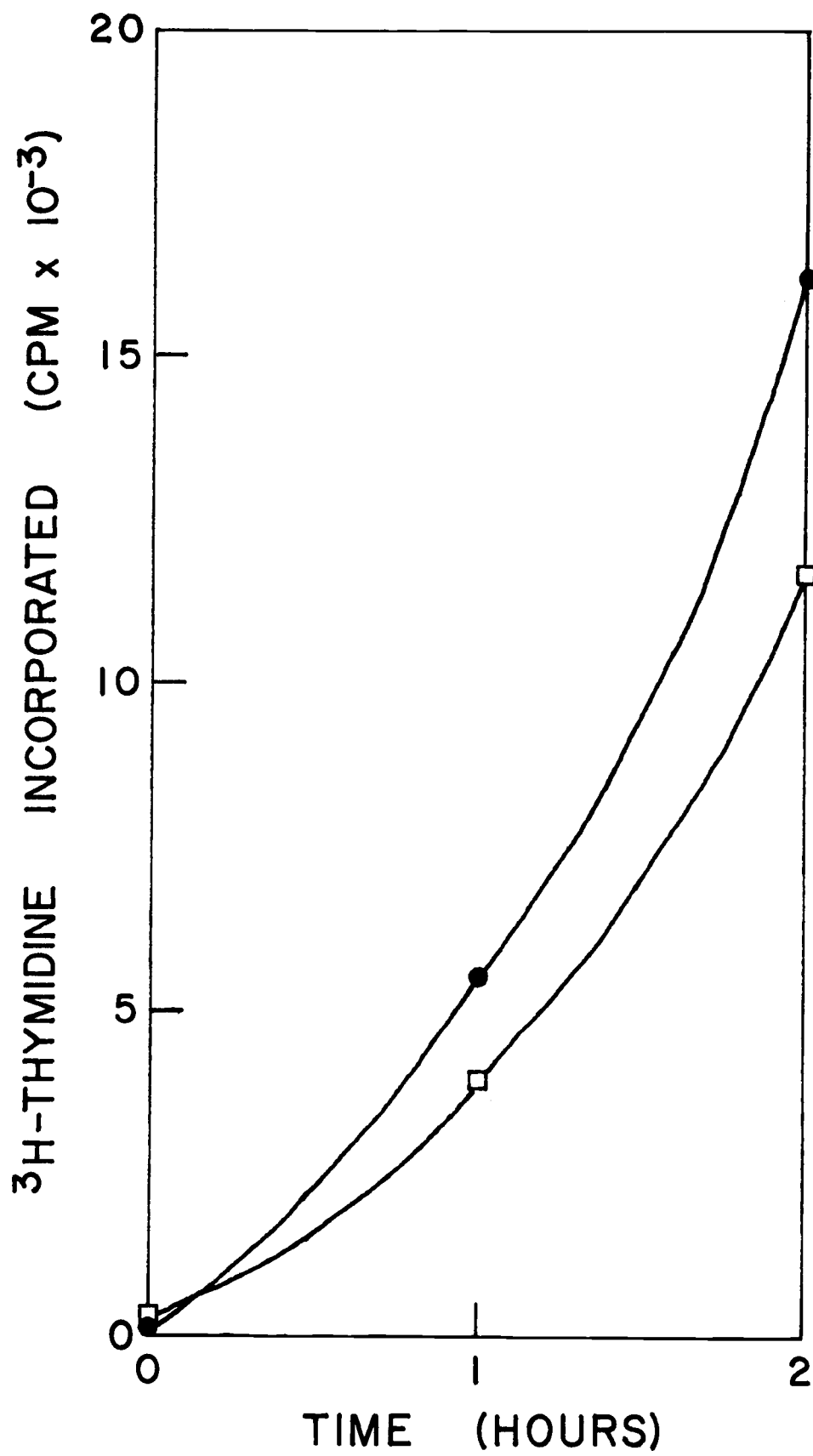
DISCUSSION

The results of this research showed that infectivity of MC29 virus

in CEC was inhibited by 3 μg DDT/ml culture medium. CEC <u>in vitro</u>

was able to withstand the toxicity of DDT at concentrations below

10 μg/ml without losing their fibroblastic spindle appearance. The cor-

responding ethanol concentration (0.3%) added to the culture medium

had insignificant effect on virus infectivity resulting in virus synthesis

(Table 1). Thus the effect observed on the infectivity of MC29 virus

in CEC was due solely to the DDT present in the culture medium.

The inhibition of virus infection by DDT was reflected by virus

release, cell transformation and protein synthesis. Virus release

takes place between 12-24 hours after infection by MC29. Kinetics

studied of reverse transcriptase activity from 24-72 hours indicated

that virus released in DDT treated CEC was reduced by about 80%

in 72 hours compared to the untreated and infected CEC. Our labora-

tory has shown that the reverse transcriptase was produced in the

infected cells just prior to virus release. A variant of Rous sarcoma

virus, RSVα(0), was able to transform chicken cells, but no infectious

progeny virus was produced. RSVα(0) does not exhibit the reverse

transcriptase activity. Thus, this enzyme is essential for the produc-

tion of infectious progeny viruses.

According to the hypothetical life cycle of RNA tumor viruses

proposed by Temin (53), all events from step 1 to step 5 inclusive

are possible sites acted upon by DDT. The evidence that virus adsorption to the CEC was not affected arose from the observation that pretreated CEC for a short period of time before infection gave nearly as much inhibition of virus synthesis as continuous treatment after infection (Table 3). Experiments that compared adsorption rate of virus in treated and untreated cells supported these results. Both conditions showed 60-70% of MC29 virus adsorbed during a six-hour period (Figure 4), suggesting that the cell receptor sites were not influenced by DDT. Studies undertaken by Toyoshima and associate (57) on the effect of polycations on infectivity of virus showed that virus infection was enhanced by polycations, but was inhibited by polyanions. The assumption was that viruses possess negatively charged surfaces and adsorb poorly to the negatively charged cell surfaces. The addition of the polycations neutralizes this repulsive force. DDT is highly chlorinated and one would predict that the chlorine atoms withdraw electrons from the two electron-rich phenyl groups, inducing weak negative charges on the outside of the molecule. Induced charge is comparatively weaker than ionic charge. The induced negative charges on DDT molecules are unable to inhibit virus adsorption.

The observed inhibition of virus synthesis appeared to be irreversible, or slowly reversible. The percent inhibition of virus release was only slightly decreased from 84% to 72% during the time

interval of 72-96 hours. Reversibility had only been observed when

DDT was removed from cells after chronically treated with DDT for

seven days or more at concentrations exceeding the toxic level. The

cells reverted back to the normal spindle appearance from the vacuo-

lated appearance within 72 hours.

Cell transformation is a consequence of virus infection (53) and

can be determined by the number of focus forming units in the infected

cultures. It gives measure of the infectious state of the CEC. The

number of susceptible cells was greatly suppressed in CEC receiving

a six hour pretreatment and followed by continuous DDT treatment

after infection and was relatively less suppressed in CEC receiving

only a six hour pretreatment. This was in coordination with the per-

cent of inhibition on virus production. The amount of virus synthesized

was a direct result of the number of susceptible cells present.

There was evidence to suppose that an early function essential

for virus infection was blocked by DDT. This evidence stemmed from

the fact that short-time pretreatment of CEC cultures with DDT

resulted in nearly as much inhibition of virus production as continuous

treatment with DDT and that DDT had no effect on chronically infected

cells. Two possible modes of action could be derived. Firstly, DDT

might act on a cellular function. CEC pretreated with DDT at various

times (Table 3), even as low as two hours, blocked virus synthesis.

This information suggested that a cell-specified function was inhibited

by DDT. Secondly, DDT might act on a virus-specified expression.
DDT was made available during the pretreatment period and dissolved
in the lipid layer of the cell membrane. Afterwards it may be slowly
released into the cells and blocked virus expression in the early
infection period.

Possible Modes of Action

Cellular Function

DDT has a very high lipid solubility and is believed to penetrate
the cell membrane by dissolving in the lipid bi-layers of the membrane.
As soon as DDT reaches the cytoplasm of the cell, it distributes itself
in the membranes of some cellular organelles having similar chemical
composition as the cellular membranes. Such organelles have been
found to be the mitochondria and endoplasmic recticulum. Evidences
have been reported that DDT induces several microsomal drug meta-
bolizing enzymes in the cell (18, 19), but also inhibits reactions of
some enzyme systems (28, 56). The loss of regulatory control of
enzymes may play a role in the virus infection.

Temin (51) and Bader (4), based on experiments with inhibitors
of DNA, concluded that DNA synthesis is essential for the replication
of Rous sarcoma virus. It was found that Rous sarcoma virus infec-
tion was reduced by 50-80% when the cells were not infected during the

DNA synthesizing period (25). Experimentally, DDT was found to inhibit about 27% of DNA synthesis in the DDT pretreated cells (six hours). This percent of inhibition was not sufficient to account entirely for the inhibition of virus synthesis, cell transformation and viral-specified reverse transcriptase encountered. But in view of the requirement of cell division in the activation of viral-specified RNA and protein, this amount of inhibition in DNA synthesis may contribute partially to the inhibitions of virus synthesis, cell transformation and viral specified reverse transcriptase in the infected cells.

Viral-specified Expression

The virus cannot possibly express its genome unless it has penetrated into the inside of the host cells. The slow mobilization of DDT in the cell membrane may prevent the virus from penetrating the cell. When a virus infects a host cell, it has to recognize the receptor sites on the surface of the cell. Thus, adsorption is the rate limiting step. DDT was found to have no effect on the adsorption of MC29 in CEC (Figure 4). Penetration may happen very fast after the virus adsorbs.

After the virus penetrates into the cell nucleus, the viral genome is believed to form a proviral DNA intermediate and to integrate into the cell genome (53). This viral DNA intermediate has not been isolated and characterized. DNA inhibitor studies (4, 51) indicate

that the provirus is a DNA intermediate. Separate studies by Temin

(52) found that virus formation was resistant to cycloheximide treat-

ment, a specific and reversible inhibitor of protein synthesis, during

the first 12 hours after infection. Early protein synthesis was not

required. DNA synthesis continued in the absence of protein synthesis

and infectious virions were produced. The enzyme for provirus syn-

thesis must exist in the cell or in the virions before the virus enters

into the cell. Discoveries of the RNA-dependent DNA polymerase

confirmed that the enzyme is present in the virions. Other polymerase

activity dependent on DNA had also been isolated (41, 47). DNA made

in vitro by the two polymerase activities in the RNA tumor viruses

was much smaller than the template RNA. Endonuclease and also a

ligase were later found in the RNA tumor virions (36). The activities

of these two enzymes may play a role in the insertion of the proviral

DNA into the cell genome.

The virus-specified reverse transcriptase in MC29 infected cells

sediments at a characteristic sedimentation coefficient of 8.2S. Its

activity in DDT-treated and MC29 infected cells was reduced by about

50% (Figure 5). But Temin (52) had shown that formation of the virus

did not depend on the synthesis of protein. Thus, the provirus is

formed and inserted into the cell chromosome by the pre-existing

reverse transcriptase brought in with the virus and does not depend

on the synthesis of reverse transcriptase in the infected cells.

However, the number of infected cells depended on the formation of the proviral DNA was found to be reduced after DDT treatment (Table 4). DDT might affect the site of insertion of provirus into the cell chromosome. Studies on the endonuclease and the ligase were not made.

Direct addition of DDT to the reaction mixture of reverse transcriptase enzyme did not inhibit the activity of the enzyme. The observed reduction of enzyme activity in DDT-treated and infected cells might be due to the reduction of infected cells and led to the reduction of the enzyme synthesized.

Reduction in the synthesis of virus-specified reverse transcriptase in the infected cells after DDT treatment suggested that the site of provirus insertion into cell chromosomes was blocked. Determination of other virus-specified protein, such as the group-specific (gs) antiegens in the infected cells, may reveal the extent of provirus inserted into the cell chromosome. In view of the reduction of reverse transcriptase synthesis in the infected cells, production of the gs antigens might be reduced.

There exists a close interaction between virus and cells after the cells are infected by an RNA tumor virus. The information obtained from the results of the experiments does not distinguish which function, cellular or viral, is involved in the reaction mechanism

undertaken by DDT. Both cellular function and virus-specified expression may play an important role in the inhibition of virus infectivity under the effect of DDT.

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