8-1962

The Effect of Infection with Polyoma Virus on the Protein, RNA, and DNA of Mouse Embryo Cell Cultures

Lawrence E. McConnehey

LOMA LINDA UNIVERSITY

Graduate School

THE EFFECT OF INFECTION WITH POLYOMA VIRUS ON THE PROTEIN, RNA, AND DNA OF MOUSE EMBRYO CELL CULTURES

by

Lawrence E. McConnehey

A Dissertation in Partial Fulfillment

of the Requirements for the Degree

Master of Science in the Field of Microbiology

August 1962

I certify that I have read this thesis and recommend that it be accepted as fulfilling this part of the requirement for the degree of Master of Science in the field of Microbiology.

Robert L. Nutter Chairman

Robert L. Nutter, Associate Professor
Department of Microbiology

Jack D. Zwemer

Jack D. Zwemer, Associate Professor
Department of Microbiology

Donald M. Brown

Donald M. Brown, Research Associate
Department of Microbiology

Richard E. Beltz

Richard E. Beltz, Assistant Professor
Department of Biochemistry

Roger H. Helmendach

Roger H. Helmendach, Assistant Professor
Department of Physiology

ACKNOWLEDGMENTS

The investigation and writing of a thesis cannot be the words of a single author, but the combined efforts of many sympathetic friends. The author wishes to express deep appreciation to all who gave time and help in the preparation of this thesis.

Special thanks is expressed to Dr. Robert L. Nutter for his kind patience and encouraging assistance during the research and writing of the thesis. Sincere thanks to Dr. Ross H. Seasly for his instruction and permission to use the Coulter Counter in his laboratory. Thanks to Miss Barbara Close for typing and to Mrs. Jacqueline Meldrajs for helping with corrections and the assembly of the manuscript. Gratitude is deeply felt for my wife for her loving kindness and encouragment during the experiments and the writing of this thesis.

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INTRODUCTION

With increased study of many varied animal cell-virus interactions, an increased interest concerning the effect of virus infection on the nucleic acid and protein metabolism of the cell has been aroused. Since viruses are composed largely (some entirely) of nucleic acids and proteins, one might expect an increase in the total protein and nucleic acid of a host cell after infection with a virus, unless instead of viral nucleic acid and protein being synthesized from the media outside the cell, they are made from intracellular nucleic acid and protein.

Large increases in total nucleic acid and protein have been found in many bacteriophage infected bacteria. The bacteriophage T_2, which has Escherichia coli strain B as its host, is an example. In this case the viral DNA in the infected cell increased to an amount considerably greater than that for the host cell DNA (Nutter and Sinsheimer, 1959). The situation is very different in animal cells infected with animal viruses. Here, if the total nucleic acid per virus is multiplied by the average number of viruses produced per cell to get the viral nucleic acid in an infected cell, it is found that the value for the viral nucleic acid is much less (less than 1 per cent) than the value for the cellular nucleic acid. This is due to the very small size of the virus relative to its host. If the increase in nucleic acid in infected animal cells, therefore, were due only to

the build up of viral nucleic acid, the increase would be insignificant. Similar statements could be made for the protein. It has been found, however, that in certain instances, virus infected animal cells show very significant increases in proteins and nucleic acids (Green and Daesch, 1961).

The morphology and chemical structure of the viruses have been used to group the viruses for classification by some authors. The enteroviruses, which are RNA viruses and formed in the cytoplasm, are called Clathro (meaning lattice) R viruses. The polyoma, SV-40, papilloma, and wart viruses being DNA viruses and formed in the nucleus are referred to as Clathro D viruses. The herpes, varicella, and pseudorabies viruses, which are DNA viruses formed in the nucleus and which have an envelope around them, are called Clathro D Complex viruses (Howatson, 1962). The infective virus particle or virion includes a core which contains one kind of nucleic acid. This unit is encased in a protein shell or capsid. The capsid is built up of subunits called capsomeres (Lwoff et al., 1959).

Electron micrograph studies of the polyoma virus show it to be a sphericle particle having a mean diameter of 453 Å. The capsomere arrangement shows a 5:3:2 axial symmetry. This arrangement gives the polyoma virus an icosahedral symmetry with 42 capsomers (Wildy et al., 1960). Di Mayorca, et al.,

isolated from mouse embryo cells infected with polyoma virus an infectious nucleic acid which was resistant to the action of ribonuclease, but which was inactivated by deoxyribonuclease (Di Mayorca et al.,1959). Purified polyoma virus was shown to contain deoxyribonucleic acid and no other phosphorus containing compounds in appreciable amounts. The multiplication of the virus was shown to be inhibited by aminopterin, which is an inhibitor of DNA synthesis (Smith, Freeman, Vogt, and Dulbecco, 1960).

Electron micrographs of herpes virus showed that it is a sphericle particle with a mean diameter of 1050 Å. The particle is covered by a protein envelope which has an average diameter of 1800 Å. The 162 capsomeres are arranged, likewise, in a 5:3:2 axial symmetry. Thus the herpes virus capsid has an icosahedral symmetry similar to that of polyoma (Wildy et al., 1960). Purified herpes virus was shown to be a DNA virus and to contain DNA in the amount of 7.7×10^{-12} micrograms per virus particle (Ben-Porat and Kaplan, 1962).

Herpes virus and the polyoma virus have been compared as far as symmetrical architecture is concerned (Horne and Wildy, 1961).

Since the herpes virus and the polyoma virus are similar in structure, differing primarily in size, it is reasonable to assume than the fraction of total material which is DNA in the polyoma

virus will be the same as the fraction of the material of the herpes virus which is DNA. Two other assumptions are necessary before one can calculate the approximate amount of DNA per polyoma virus particle. One must assume that the virus particles in the two cases are spherical enough to allow one to use the formula for volume: $V = 4/3\ r^3$. Also, one must assume that the densities of the two types of virus are the same in order to assume that the masses are directly proportional to the volumes.

Using the three above-mentioned assumptions one may calculate the mass of DNA per polyoma virus particle as follows:

	Herpes virus	Polyoma virus
Diameter	1050 Å (given Wildy et al., 1960)	453 Å (given Wildy et al., 1960)
Volume	6.04×10^8 Å ^{3}	$.49 \times 10^8$ Å ^{3}
Mass of DNA	7.7×10^{-12} µgm DNA (given, Ben-Porat and Kaplan, 1962)	0.62×10^{-12} µgm DNA (calculated)

The average yields of polyoma virus particles were found to be 800-1000 PFU per cell based on the proportion of cells detected as virus producers (Winocour and Sachs, 1960). The product of the number of virus particles per cell (assume 1000) and the DNA per virus ($.62 \times 10^{-12}$ micrograms) is 6.2×10^{-10} micrograms of DNA per cell. We will show later that the mouse embryo cells used in this paper have an average of 17×10^{-6} micrograms of DNA per cell. It immediately becomes apparent that the DNA due to polyoma

virus in a polyoma infected cell represents only a fraction of the DNA of the cell (62/1,700,000 or 1/27,400 which is less than 0.004 per cent).

In accordance with the calculations shown above, one might not expect to find a significant increase in DNA in cells infected with polyoma virus compared with uninfected control cells. Should an increase in DNA be found in such a case, it most certainly would not be due to the DNA of the intracellular, newly synthesized virus.

CELLULAR CHANGES FOLLOWING VIRUS INFECTION.

Many workers have shown changes in the synthesis of viral components in viral infected cells and, more specifically, have shown changes in the nucleic acid and protein in infected cells, using uninfected cells or cells infected with heat killed virus as controls. The effect of viruses on the metabolism of their host cells has been studied from several approaches. The type of nucleic acid which the virus contains has been the basis for grouping the viruses as far as studies on the alteration of nucleic acid and protein in virus infected cells is concerned.

A brief review of the literature on cellular changes produced by the DNA viruses, on the one hand, and RNA viruses on the other, follows in the next several paragraphs. The DNA viruses will be discussed as the first group.

The Feulgen positive material in the cytoplasm of Hela cells

infected with vaccinia virus (a DNA virus) provided a means to demonstrate an increased utilization of thymidine-2-C^{14} and a concurrent alteration in the acid stability of the cellular DNA (Magee and Sagik, 1959).

An increase of DNA infected with herpes virus nearly double the normal amount has been shown in Hela cells. It was demonstrated that the increase of DNA was in the nuclear rather than in the cytoplasmic fraction (Newton and Stoker, 1958).

Kaplan and Ben-Porat showed that rabbit kidney cells infected with Pseudorabies virus gave a DNA to protein ratio 35 per cent higher than in non infected cells. During infection there was an increase in nuclei proliferation. The nuclei to cell ratio at 4 hours was 1.5. Although there is an increase in DNA per cell, there is not an increase per nucleus. They suggest that newly synthesized DNA is cellular and not viral DNA (Kaplan and Ben-Porat, 1959). In a later publication they showed that virus infection caused an increase of DNA in old cultures but not in young cultures. (Kaplan and Ben-Porat, 1960).

The DNA from Hela cells infected with adenoviruses was separated into saline soluble and water soluble DNA. The saline soluble DNA was shown to represent viral DNA and the water soluble represented cellular DNA. There was a marked increase of saline soluble DNA which derives its components from cell

precursors and medium. Synthesis of saline soluble DNA begins before newly formed infectious virus. RNA was also shown to increase (Ginsberg and Dixon, 1961). DNA and protein were shown to increase in KB cells infected with adenovirus. RNA increased more rapidly than DNA and protein but leveled off and in some experiments decreased after 36 hours (Green and Daesch, 1961).

Most of the work in the second group of viruses, the RNA viruses, has been done with poliovirus. In many cases cells infected with viruses have retained their cellular structure. In the case of poliovirus infected cells there are marked changes in the structure of the cells. The basophilia of the cytoplasm is noticed most. It is felt by some that chemical changes in the cell accompany these cellular changes. Incorporation of P^{32} into the medium of infected cells, showed that cytoplasmic RNA increased but that nuclear RNA and DNA decreased (Maassab, Loh, and Ackermann, 1957). It was later shown that RNA synthesis ceased in the nucleus at about four hours after infection and ceased in the cytoplasm at about six hours, but was enhanced at the four hour level. Protein continued at a constant rate until the seventh hour. Although there was no increase of nuclear RNA, there was extensive turnover of the RNA nuclear fraction (Ackermann, 1958).

On the other hand other workers noted no increase of RNA but rather a decrease of RNA in cells after infection (Rothstein and

Manson, 1959). Others showed that there was an increase in RNA of noninfected cells but no increase in infected cells. They showed a decrease in protein as well. There was an enhanced nucleotide pool early in infection, however (Salzman and Lockhart, 1959). These differences could be accounted for possibly through the fact that the first experiments were conducted with cells on glass surfaces, whereas the second experiments were conducted using cells in suspension.

Various workers have shown that even in uninfected cells there are differences in the rates of synthesis of DNA, RNA, and protein from one set of conditions to another. The main factors are: (1) the phase of growth of the cells, lag phase (where no cell division takes place), log phase, or stationary phase, and (2) the method of growth of the cells; on glass surfaces or in suspension. For cells grown on glass surfaces there is a lag phase of approximately 1 day, followed by a log phase of a few days after which the cells reach a stationary phase when a monolayer or near monolayer of cells exists on the glass. By decreasing the nutrients in a cell growth medium one may make a maintenance medium which has the effect, when used, of taking the cells from the log phase into a stationary phase before a complete monolayer (perhaps) is achieved. For cells grown in suspension the length of the lag phase depends upon the phase the seeding cells are in. Cells in the log

phase used for seeding a new culture will produce a very short lag phase, whereas cells in a stationary phase will produce a longer lag period similar to that for cells cultured on a surface. One advantage of a suspension culture is the fact that cells may be maintained in the log phase for extended periods of time by periodic dilutions of the cultures with fresh medium. Regardless of whether cells are grown on glass or in suspension, there is a rapid synthesis of RNA, DNA, and protein during the lag phase. During the log phase, the synthesis of these materials proceeds but at a reduced rate. In the stationary phase the amount of DNA appears to remain about constant whereas RNA, and in some cases protein, actually shows a decrease due to leakage from the cells (Salzman, 1959; Graham, 1959).

Goldé infected stationary chick embryo cells with Rous-sarcoma virus using uninfected cells as controls. RNA increased and reached a level 75 per cent higher than in the controls. The protein and the mean volume increased in parallel and at the end of the experiment reached values double those of the controls. DNA did not increase in the infected cells (Goldé, 1962).

It is obvious from this brief review that cellular changes in virus infected cells are many and varied. Among the changes observed have been increases in nucleic acids and protein which could hardly be due to newly synthesized intracellular viruses.

THE POLYOMA VIRUS - A TUMOR PRODUCING VIRUS

Viruses have been known to be a cause of tumors in animals for over fifty years. Not until about ten years ago, however, when the polyoma virus was discovered, has the oncogenesis been so apparent for a mammalian virus (Gross, 1951, 1953; Stewart, 1953). The polyoma virus has been shown to produce malignant tumors in many small laboratory mammals, including mice, rats and hamsters and is consequently of great interest to cancer research workers (Gross, 1957; Stewart, 1955; Dawe et al., 1959; Stewart et al., 1957, 1958; Eddy et al., 1958; Sachs et al., 1959). Biochemical studies of polyoma virus infection have been underway in the Virus Laboratory of the Department of Microbiology, Loma Linda University, since the fall of 1960.

A much more complete review of the literature on the polyoma virus has been included in the Introduction of the Master's thesis of Rheeta Stecker, who also worked on this project and will therefore not be repeated here (Stecker, 1961).

PURPOSE OF THE STUDY

The purpose of this study was to experimentally determine and analyze quantitative changes in the DNA, RNA, and protein of mouse embryo cells infected with polyoma virus, using uninfected mouse embryo cells as controls.

MATERIALS AND METHODS

CELLS

Primary cultures of mouse embryo cells were made using modifications of the techniques of Youngner and of Stewart and Eddy (Youngner, 1954; Stewart and Eddy, 1958). A pregnant Swiss-Webster mouse (14-18 days of gestation) was chosen and killed by expansion of the spinal column. The entire mouse was placed in a solution of Wescodyne and drained. The uterine sack was excised and placed in tris-buffered saline at a pH of 7.4. The embryos were extracted from the uterine sack, decapitated, placed in a trypsinizing flask with 50 milliliters of tris-buffered saline and minced with scissors in order to drain as much blood as possible from the embryos and also to speed separation of the embryo cells.

The flask was placed on a magnetic stirrer for three minutes after which the stirrer was stopped and the large debris allowed to settle. The supernatant fluid was poured off and discarded which removed most of the red cells. Fifty milliliters of tris-buffered saline containing .025 per cent trypsin was added to the trypsinizing flask and placed on the magnetic stirrer for three minutes. The flask was then removed and the large debris allowed to settle for about one-half minute. The supernatant fluid, containing single cells or very small clumps, was poured into a 50 milliliter centrifuge tube and centrifuged at 3,000 rpm for three minutes. Trypsinization of

the tissue remaining in the flask was repeated three more times. The supernatant fluid remaining after centrifugation was poured off the cell buttons and replaced with 20 milliliters of Eagle's medium containing 20 per cent calf serum.

The cells were pooled and diluted to 100 milliliters with more medium in order to make a preliminary cell count in a hemocytometer. After counting, the cells were diluted with medium to 7.5×10^5 to 1×10^6 cells per milliliter. Twenty-five milliliters of cells were seeded into 1000 milliliter prescription bottles with a flat side (Duraglas or Label-rite). The cells were incubated at 37°C in approximately 5-10 per cent CO_2.

After the cells had covered the glass (three to four days) the fluid medium was removed and the cell layer washed with 10 milliliters of tris-buffered saline two times. Twenty milliliters of .025 per cent trypsin in tris-buffered saline was added to each bottle for five to ten minutes. The bottles were rocked back and forth by hand several times during this period to aid in removing the cells from the glass. The cells were then poured into 50 milliliter centrifuge tubes and centrifuged five minutes at 3,000 rpm. The cells were drained, pooled, and diluted with medium to 7.5×10^5 to 1×10^6 cells per milliliter. Each 1000 milliliter flat-sided bottle was seeded with 25 milliliters of cells and incubated at 37°C at 5-10 per cent CO_2. In approximately two days these secondary cell monolayers were ready to use.

MEDIA AND SOLUTIONS

TRYPSIN

Trypsin was dissolved in .85 per cent saline to make a 5 per cent stock solution. The stock was filtered through Seitz and Millipore filter pads with a positive pressure of four pounds and dispensed into screw cap tubes in 15 milliliter amounts and stored frozen. At the time of use the trypsin was thawed and diluted to .025 per cent in tris-buffered saline.

TRIS-BUFFERED SALINE

Tris-buffered saline was made according to a formula used by Dr. Harry Rubin at the Virus Laboratory, University of California at Berkeley and given to Dr. Nutter by his technician.

NaCl	14.4	grams
KCl	0.684	gram
Na_2HPO_4	0.18	gram
Dextrose	1.8	grams
Sigma 7-9	5.4	grams
Penicillin	$2x10^5$	units
Streptomycin (dihydrous)	0.1	gram
Water to make	1.8	liters

Adjust the pH to 7.4 with approximately 3.2 milliliters of concentrated HCl.

Tris-buffered saline was sterilized by positive pressure filtration with Seitz and Millipore filters, dispensed into appropriate sterile bottles and stored at 4°C.

EAGLE'S BALANCED SALT SOLUTION

Eagle's balanced salt solution, less sodium carbonate, was made at 10x concentration as follows: (Eagle, 1959).

NaCl	6.8	grams
KCl	0.4	gram
$CaCl_2$	0.2	gram
$MgCl_2 . 6 H_2O$	0.2	gram
$NaH_2PO_4 . H_2O$	0.132	gram
Glucose	1.0	gram
Phenol red	0.02	gram
Water to make	100	milliliters

The Eagle's balanced salt solution was dispensed into containers appropriate for use and autoclaved at 252°C for 15 minutes.

EAGLE'S MINIMUM ESSENTIAL MEDIUM (MEM)

At the time of use 1000 milliliters of this medium which was used for growth and maintenance of mouse embryo cells, was made up. To a 1000 milliliter sterile bottle, 100 milliliters of Eagle's balanced salt solution (10X) with indicator was added followed by the addition of 10 milliliters of glutamine[1], 20 milliliters of MEM amino acids,[2]

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1. Microbiological Associates Cat # 17-605F Glutamine 200 mM 100X.

2. Microbiological Associates Cat # 13-606 MEM Essential Amino Acids 100X.

20 milliliters of vitamins,[3] 20 milliliters of penicillin-streptomycin stock solution,[4] and 1 milliliter of Fungizone stock solution.[5] The medium was diluted to 750 milliliters with sterile distilled water. Forty milliliters of 5 per cent $NaHCO_3$ was added (This is equivalent to 2 grams/liter) and the final volume raised to 1000 milliliters. (Eagle, 1959)

Calf serum was added to complete the medium. The concentration of calf serum used depended on whether cell multiplication or whether cell maintenance was desired. Ten to 20 per cent serum was used to grow the cells and 4 per cent serum was used to maintain them after growth.

Many bacteria are able to grow on media with bare essentials for maintenance of life. Tissue cultures on the other hand must have very carefully balanced salt bases with the addition of closely controlled amino acid, vitamin, one specific type of serum, and

3. Microbiological Associates Cat # 13-607F MEM Vitamin Mixture 100X.

4. Penicillin-G (Parke, Davis and Company) and Streptomycin Sulfate (Chas. Pfizer and Co., Inc.) stock solution. Five-hundred thousand units of Penicillin-G and 0.5 gm of Streptomycin Sulfate is dissolved in 100 ml total volume of sterile water and frozen until used.

5. Fungizone (Squibb Amphotericin B) stock solution. A 50 mg vial is dissolved in 20 ml total volume of sterile water. The stock should be frozen for prolonged storage.

mineral supplements. All of these factors tend to enhance the growth of bacteria and fungus and these agents are constantly a threat to the growth of tissue cultures. In all the media used in this work, penicillin-streptomycin and Fungizone were used. Very rarely did a bacterial contaminant appear while growing cells and no contaminant grew during the experiment. In early studies we were plagued with fungus contaminants but found that Fungizone not only prevented their growth, but on repeated occasions, tended to cure cultures of fairly heavy fungus growth.

CALF SERUM

The serum was procured from various slaughter houses in the area. Blood from newborn calves was allowed to flow freely from the incised jugular vein into wide mouth gallon jars. After the clots had formed, a period of one hour was allowed to pass without disturbing the jars. After this period the jars were transported to the laboratory and the clot allowed to retract for four to six hours at 4°C. The serum and free red cells were centrifuged to remove most of the cells. The serum was then centrifuged at 8,000 rpm in a continuous flow centrifuge to remove the remaining red cells. The recovered serum was filtered through Seitz filter pads and dispensed into appropriate containers. A sterility check was made on each dispensed sample of serum by inoculating 10 milliliters of thioglycollate medium with

1 milliliter samples of serum to be tested and incubating the tubes at 37°C for ten days. No growth in the tubes at the end of this ten day period indicated that the serum was sterile. This sterile serum was labeled and stored in a freezer until used.

VIRUS

The virus used in the experiments for this paper were received October, 1958 from Dr. C. Dawe (National Cancer Institute). Dawe isolated this tumor agent (called "parotid tumor agent" at that time) from a leukemic mouse which he received from Gross. The virus was maintained in P 388D_1 cells (mouse lymphoma cell line) for several months. The medium containing virus from these cultures was stored at 4°C until December, 1960. The agent was then passed once through milk adapted P 388D_1 cells and once through mouse embryo cells. The virus titers of the fluids from the cultures were estimated by hemagglutination of guinea pig red blood cells. The infectivity of the fluid containing virus was shown by tumor production in hamsters. Since 1958, the "parotid tumor agent" of Dawe was shown to be the same virus as the "polyoma virus" of Stewart and Eddy.

INFECTION OF CULTURES

The necessary prescription bottles containing secondary cell monolayers were chosen for the experiment. The bottles were paired: one bottle to receive the virus inoculum, and the other

bottle to receive an equal amount of tris-buffered saline and thus serve as the control. The monolayers were washed two times with 10 milliliters of tris-buffered saline. The monolayers marked "infected" received 3 milliliters of stock virus freshly thawed. This was calculated to be a dose sufficient to infect all the cells. The monolayers marked "control" received 3 milliliters of tris-buffered saline. The virus was allowed to adsorb for one and one-half hours. The monolayers were then washed with 10 milliliters of tris-buffered saline and 25 milliliters of nutrient medium containing 4 per cent calf serum was added. The bottles were then placed in a 37°C incubator maintained with a controlled CO_2 concentration between 5 and 10 per cent. At predetermined times the media were removed and held for virus assay. The monolayers were trypsinized for five minutes with 15 milliliters of tris-buffered saline containing .025 per cent trypsin, and then trypsin and cells were poured into a 50 milliliter centrifuge tube. The cells were centrifuged at 3,000 rpm for two minutes. The trypsin solution was poured off and the cell button washed with 10 milliliters of tris-buffered saline and centrifuged at 3,000 rpm, after which the supernatnat was poured off. Eleven and one-half millliliters of new tris-buffered saline was added. The cell button was broken up by the use of a pipette by drawing fluid into the pipette and expelling it rapidly into the tube. Ten milliliters of the cell mixture was placed into a polypropylene

centrifuge tube made especially for the Spinco Model L ultra centrifuge. One milliliter of the cell mixture was added to the 10 milliliters of cells already added to the polypropylene tube and the total of 11 milliliters was mixed thoroughly. One milliliter was extracted from the 11 milliliters and placed with 9 milliliters of filtered saline in a medicine vial. This sample was for the cell count which was made in the Coulter Counter. The remaining 10 milliliters left in the centrifuge tube was centrifuged at 3,000 rpm for two minutes and the fluid poured off. The cell button was then frozen rapidly in alcohol and dry ice and stored at -20°C.

CELL COUNT

A cell mixture was counted at various thresholds on the Coulter Counter (an electronic counter used to count blood cells in a clinical laboratory) and a plateau was found with the center at a threshold setting of 54. All counts thereafter were made at a threshold of 54 and a current setting of 4. The cells, diluted 1:10, were counted four times in the Coulter Counter and an average count calculated. Reference was made to a coincidence correction chart furnished by the Coulter Company. This is to correct for the probability of two or more cells passing through the counting orifice at the same time.

ASSAY FOR VIRUS

The growth medium from each bottle was recovered at the

time of cell trypsinization and an aliquot of the fluid was tested by hemagglutination with guinea pig red blood cells to determine the hemagglutination titer. The growth media and virus were diluted by two-fold serial dilutions (1:2, 1:4, 1:8, 1:16, etc.) in saline with the pH adjusted to 7.2-7.8. Guinea pig red cells were collected using Alsever's solution as an anticoagulent. The cells were washed two times in 0.85 per cent saline and a final 1 per cent solution of packed red cells in 0.85 per cent saline was made. To 0.5 milliliter of each dilution in a serology tube, 0.15 milliliter of red cells was added and the tubes stored undisturbed at 4°C and read after three hours. The tubes were read immediately after removal from the refrigerator since polyoma agglutination is destroyed at room temperature.

CHEMICAL ASSAYS

The chemical assay procedures used were modifications of two previously published techniques (Tyner et al., 1953 Green and Daesch, 1961).

Five milliliters of cold 0.2 molar perchloric acid were added to each tube containing cell buttons and immersed in an ice-water bath. The pellet was resuspended and mixed thoroughly and allowed to stand for 30 minutes for precipitation of the acid insoluble fraction. The precipitate was centrifuged at 15,000 rpm for 10 minutes in the Spinco Model L ultracentrifuge and the supernatant

poured off carefully so as not to disrupt the precipitate. Fifteen thousand rpm was found necessary to hold the precipitate pack so that it did not dislodge when the fluid layer was poured off. This was repeated two more times allowing 15 minutes before centrifuging each time. Three milliliters of 95 per cent ethanol containing 0.1 milliliter potassium acetate was added to each pellet, the pellet resuspended, and the suspension centrifuged at 15,000 rpm for 10 minutes. The supernatant was poured off with care as the precipitate was very powdery at this point. The preceeding step was repeated two times with ethanol-ether (3:1) at 50°C in a water bath, and once with ether with no heat added. These steps removed the lipid fractions. Where possible, lambda micropipettes were used for all the following procedures. Three hundred lambdas of 5 per cent trichloroacetic acid was added to each pellet and heated at 90°C in a water bath for 15 minutes. The tubes had to be capped during this procedure; otherwise, the small amount of fluid would have evaporated at 90°C. The tubes were centrifuged at 15,000 rpm for 10 minutes and great care used to pour off the supernatant fluid into a separately marked tube. This step was repeated two more times and the nucleic acid fraction from each individual pellet combined. From this 900 lambda nucleic acid fraction the RNA and the DNA analyses were performed.

The protein pellet, remaining after the preceeding steps, was

dissolved in 3 milliliters of 1 N NaOH at 37°C. After the protein was in solution, 3 milliliters of 1 N HCl was added to neutralize the NaOH which gave a total of 6 milliliters of protein solution. The protein, RNA, and DNA analyses were all carried out in serology tubes.

PROTEIN ANALYSIS

Six hundred lambdas of each of the 6 milliliter protein samples were pipetted with lambda pipettes into respectively marked serology tubes. Six hundred lambdas of 0.5 N NaCl was pipetted the same way for a blank to correspond to the 0.5 N NaCl formed in the samples by the 1 N NaOH and the 1 N HCl. Bovine albumin powder from Armour Pharmaceutical Company was weighed out and diluted with 0.5 N NaCl so that 600 lambdas represented 150 micrograms of protein. Normal Clinical Chemistry control serum from Hyland Laboratories was diluted with 0.5 N NaCl so that 600 lambdas represented 150 micrograms of protein. Since almost identical results were found when both of these standards were used, the Hyland standard was used exclusively because of its keeping qualities. Using the same technique as for the blank and unknown, 600, 300, 200, and 100 lambda samples of protein standard representing 150, 75, 50, and 25 micrograms of protein were pipetted into four marked tubes. The standards were brought to the 600 lambda volume with appropriate lambda

pipettes. To each sample (including blank and standard) 3 milliliters of reagent C were added with a volumetric pipette, mixed immediately and allowed to stand for 10 minutes at room temperature. To each of the above tubes, 0.3 milliliter of reagent D was rapidly added with a 300 lambda pipette and mixed in a second or two and then allowed to stand at room temperature for 30 minutes. With the wavelength of the Beckman DU Spectrophotometer set at 750 mu, the absorbancies of the sample were read and recorded. (Lowry et al., 1951).

REAGENTS

A. 2 per cent Na_2CO_3 in 0.1 N NaOH.

B. 0.5 per cent $CuSO_4.5H_2C$ in 1 per cent Na tartrate.

C. 50 milliliters of A and 1 milliliter of B.

D. Phenol reagent (Folin and Ciocalteu) 1 N in acid.

Titrate against NaOH to phenolphthalein end point.

Because of instability, reagents A, B, and C were made up fresh for each series of samples.

RNA ANALYSIS

One hundred lambdas of each nucleic acid extract was pipetted into 1.4 milliliters of 5 per cent trichloroacetic acid. The blank was made up of 1.5 milliliters of 5 per cent trichloroacetic acid. Twenty-five and fifty lambda RNA standards representing 10 and 20 micrograms of RNA per milliliter were pipetted into 1.475

and 1.450 milliliters of 5 per cent trichloroacetic acid respectively. One and one-half milliliters of orcinol reagent were added to each tube (including blank and standard). (To make the orcinol reagent, 100 milligrams of reagent grade ferric alum was dissolved in 100 milliliters of concentrated reagent grade hydrochloric acid. Just before the test was carried out 1 gram of orcinol was added per amount of reagent containing 100 milliliters of concentrated HCl.) All tubes were heated in a boiling water bath for 30 minutes and then cooled in a water bath at room temperature. With the wavelength of the Beckman DU Spectrophotometer set at 680 mu, the absorbancy of each sample was read and recorded. (Miller et al., 1951).

DNA ANALYSIS

Two hundred lambdas of each nucleic acid extract was pipetted into 800 lambdas of 5 per cent trichloroacetic acid. A blank was prepared from 1000 lambdas of 5 per cent trichloroacetic acid. Twenty-five and fifty lambda DNA standards, representing 15.63 and 31.25 micrograms of DNA per milliliter, were added to 975 and 950 lambda volumes of 5 per cent trichloroacetic acid respectively. Two milliliters of diphenylamine reagent were added to each tube (including blank and standards). (To make diphenylamine reagent, 1.5 grams of steam distilled diphenylamine was dissolved in 100 milliliters of redistilled glacial acetic acid and 1.5 milliliters

of concentrated H_2SO_4 were added. Just before use, 0.1 milliliter of aqueous acetaldehyde, 16 mg/milliliter, was added for each 20 milliliters of reagent required). All the tubes were heated at 30°C in a water bath for 16-20 hours. With the wavelength of the Beckman DU Spectrophotometer set at 600 mu, the absorbancy of each of the samples was read and recorded. (Burton, 1956).

RESULTS

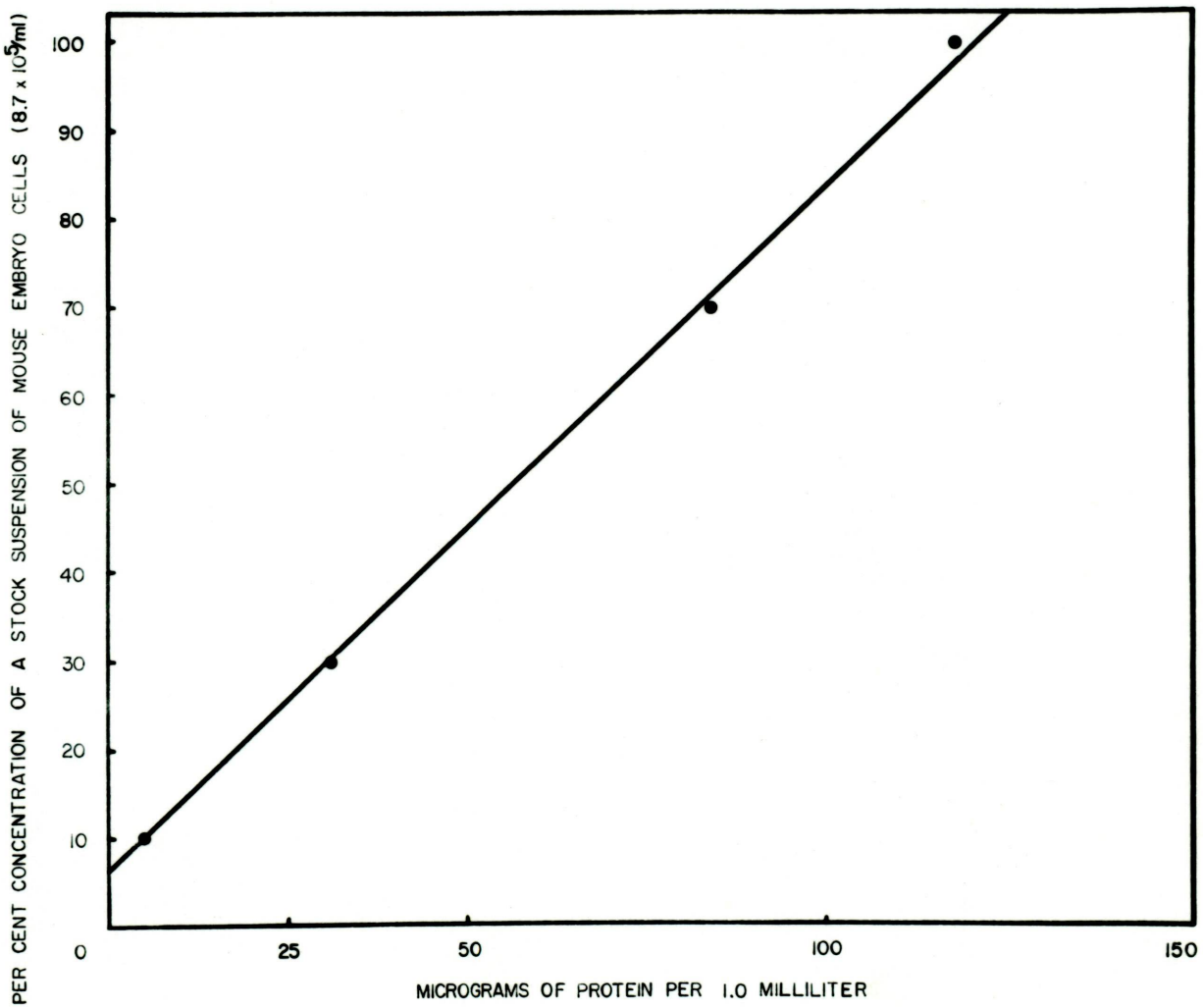
Four separate experiments to determine the change with time of total protein, RNA, and DNA in polyoma infected mouse embryo cells were performed using uninfected cells as controls. The methods employed for carrying out these experiments have been described in some detail in the previous chapter. The data collected and the results determined from the data will be included in the present chapter.

Secondary cells form a monolayer void of clumps of cells and tissue debris, therefore secondary mouse embryo cells were used. In primary cultures there is inevitably a certain concentration of red cells which tend to inhibit growth. These red cells are passed off in the process of making secondary cells. Secondary cells, by the process of elimination, represent a monolayer of cells more nearly uniform and, composed of cells already adapted to growth in vitro. The cells were allowed to grow to an almost confluent layer. By having an area of glass to grow further the cells more readily adsorb virus and are able to metabolize further.

The cells, being in the late log or early stationary phase, show little varying cellular metabolic activity. Therefore, they give evidence of small changes with time in chemical composities when not affected by some external factor.

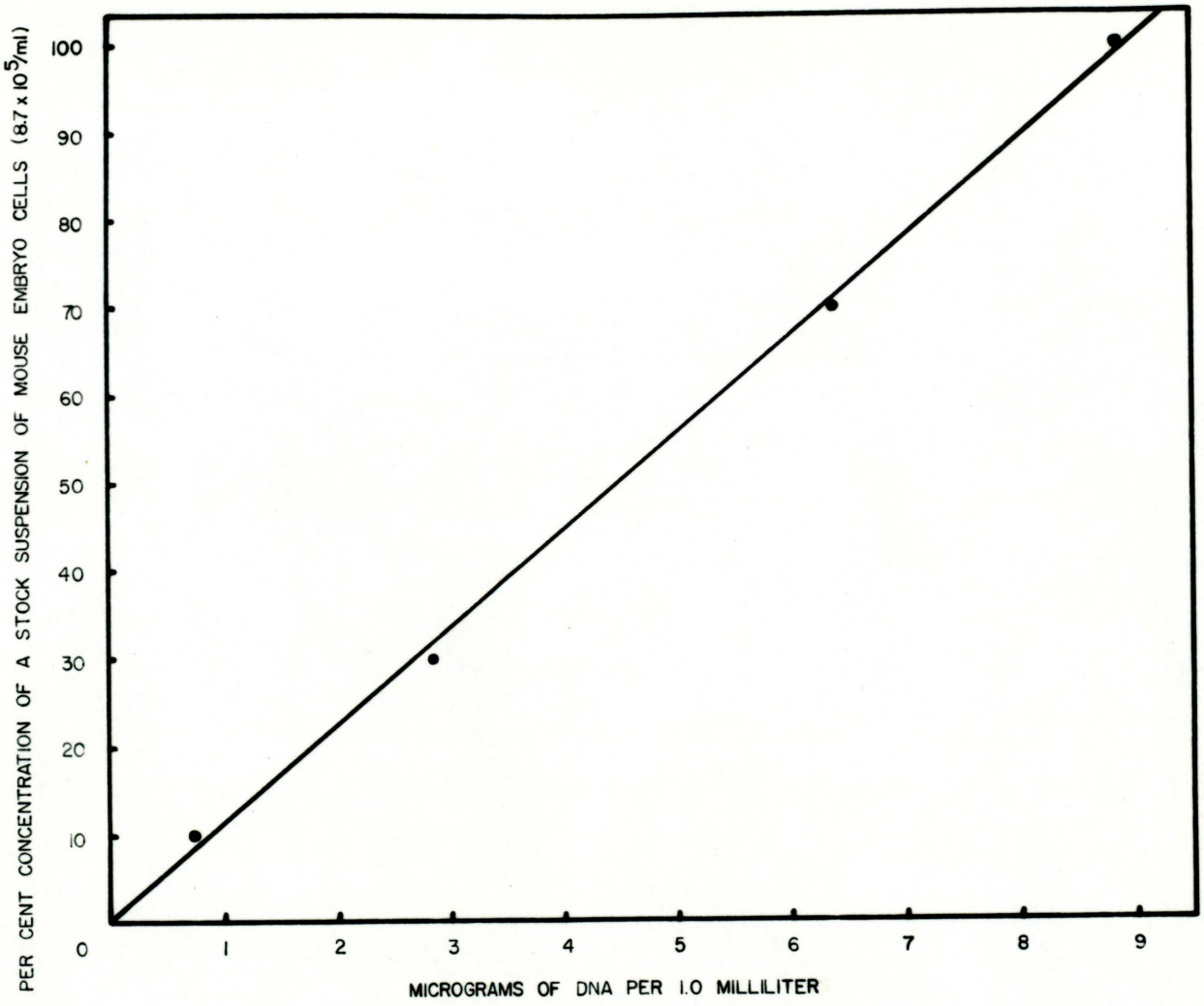
In order to evaluate the chemical analyses used in these experiments, protein and DNA determinations were performed on a particular sample of mouse embryo cells which had been counted and then diluted to various concentrations. The precision of the chemical analyses is indicated by the proximity of individual points to the straight lines in figures 1 and 2.

The Coulter Counter is an electronic counter used in many laboratories to count red cells, white cells, epithelial cells, etc., by counting the cells passing through a small orifice in one 0.5 milliliter volume. The change in resistance due to the cell in the orifice is picked up by the circuit and registered as one count. By varying the resistance necessary for a given cell to register, the smallness of the size of the particle can be limited so that only cells of a given size and larger will be counted. By making counts from a given sample of cells with a different threshold setting for each count, one finds, upon plotting the counts against threshold settings, a plateau in the curve (figure 3). This will show at the point where cells of a size most nearly alike are located. All debris and broken cells less than this size will not be counted. Some cells will pass through the orifice together. Therefore a count correction to correct for coincidence counts is made and added to the apparent value. The higher the count, the greater is the correction.



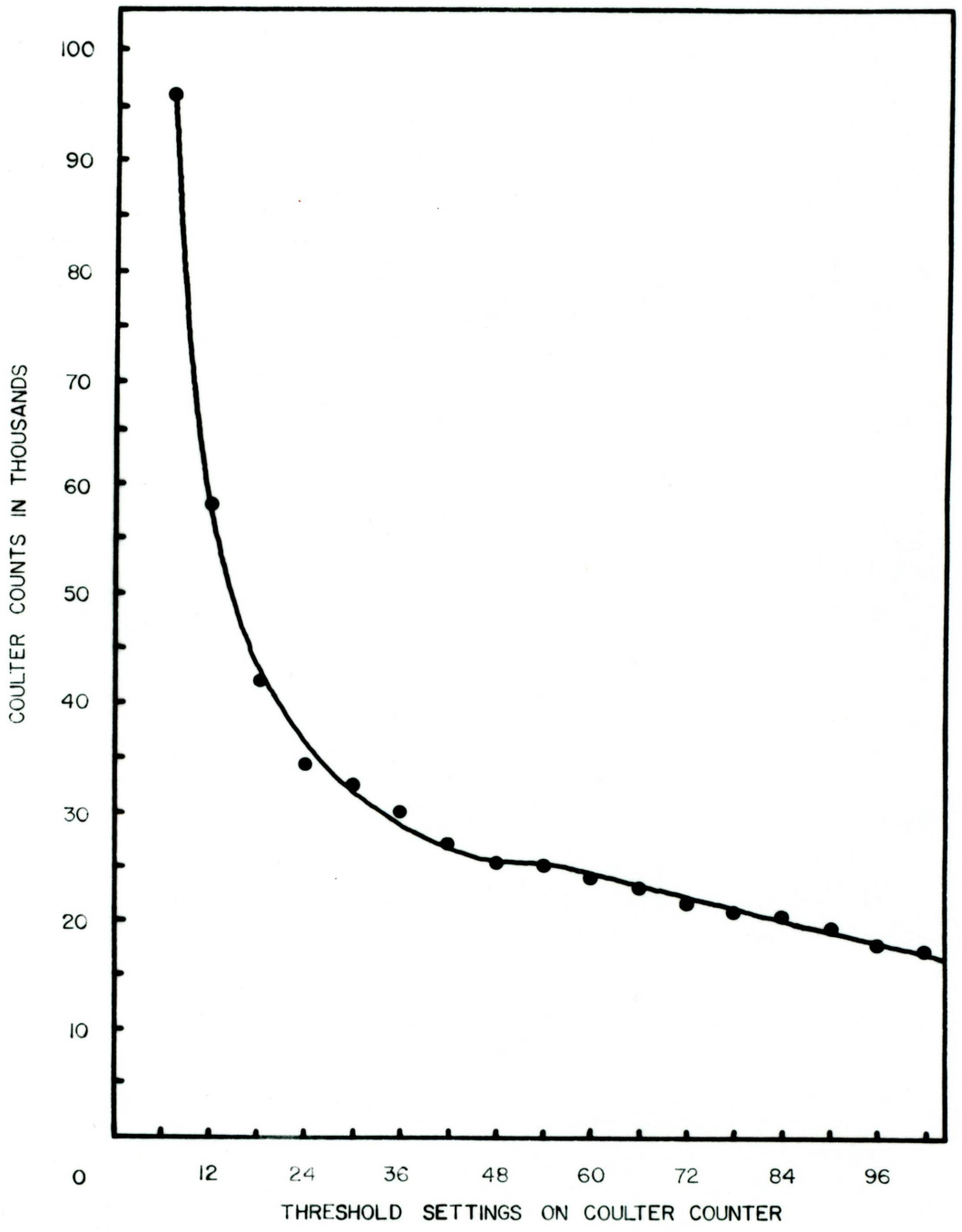
EVALUATION OF THE PROTEIN ASSAY

FIGURE 1



EVALUATION OF THE DNA ASSAY

FIGURE 2



PLATEAU CURVE FOR DETERMINATION OF OPTIMAL THRESHOLD SETTING

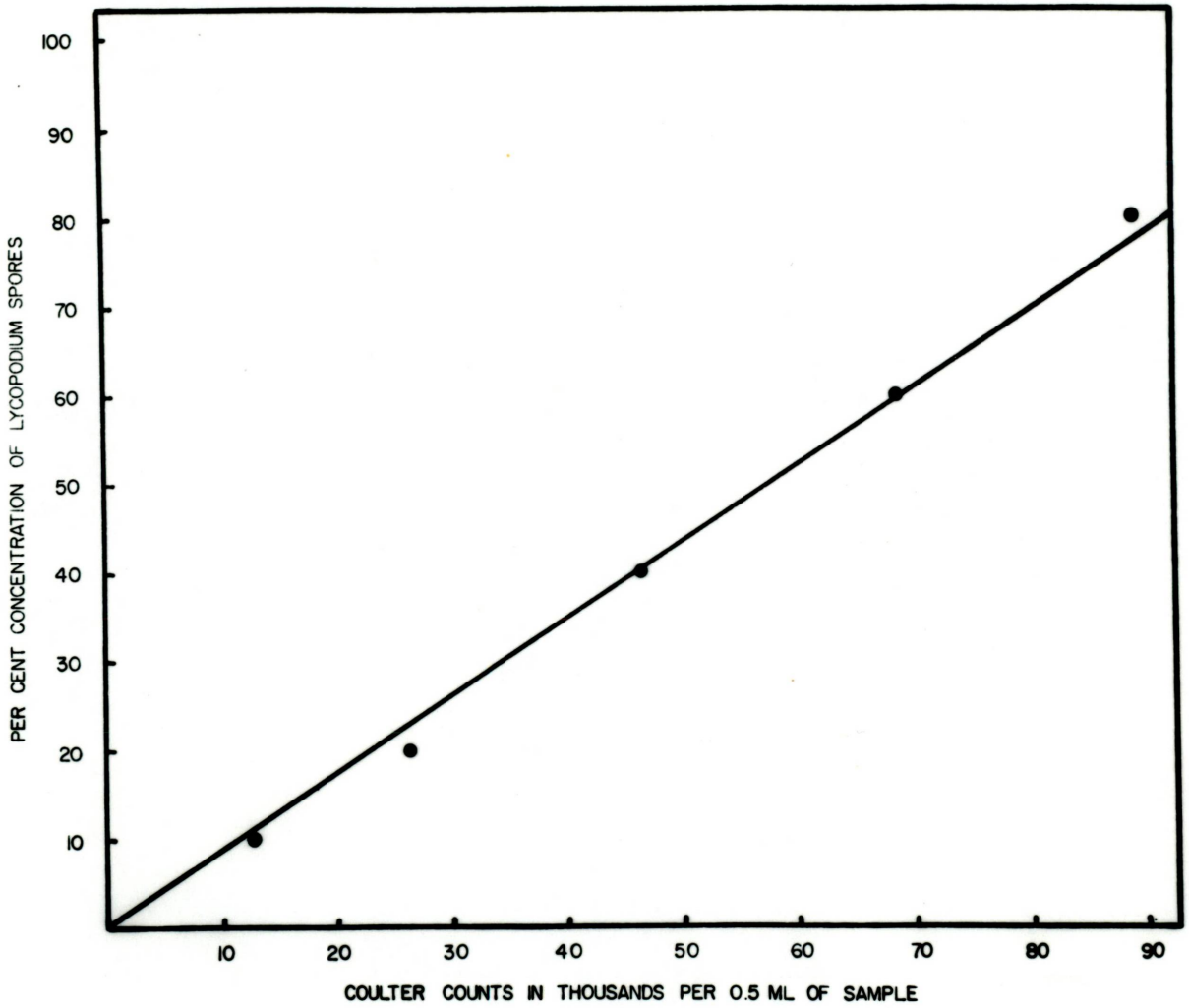
FIGURE 3

To assure that high to low Coulter counts were consistent, an experiment to evaluate the Coulter counter was performed. A sample of Lycopodium spores was diluted to a non-specific concentration to form a stock. Lycopodium spores tend to float and are not easily made wet. In order to place them in a homogeneous suspension it was necessary to wet the spores with a solution of detergent. Tamol which is a dispersing agent was used to hold the spores in suspension and prevent their settling out. Specific dilutions were made representing per cent concentrations of the Lycopodium spore stock suspension. These several samples were read in the Coulter Counter and plotted, after coincidence correction, in figure 4.

So as to show further the variation in the counts of the Coulter Counter, a second sample was prepared similar to that just described. Counts were made, at the time of various sampling periods, over the interval of a week. The counts were averaged as follows:

No.	Counts	Variation from mean	No.	Counts	Variation from mean
1	18,115	532	7	18,782	135
2	18,115	532	8	18,025	622
3	18,125	522	9	19,435	788
4	19,149	502	10	18,779	185
5	18,622	25	11	18,747	100
6	19,042	395	12	18,832	132
				223,768/12	4,470/12
				= 18,647	= 373

373/18,647 = .02 = 2%



EVALUATION OF THE COULTER COUNTER

FIGURE 4

In the preceding calculations, the mean value for the 12 counts was first found (18,647), then the deviation of each value from the mean was determined. The mean deviation from the mean (373), calculated in a similar manner, was found to represent two per cent of the mean count.

In order to indicate how the results in tables I to III were derived, a single calculation is included as an example. The sample chosen is the first in experiment 1. Three counts were made in the Coulter Counter, were averaged, and corrected.

13,413
13,164
13,413
39,990/3 = 13,330 corrected to 13,830

Sample of Coincidence Correction Chart

130	134	For counts in terms of hundreds
31	136	(130 represents 13,000.)
32	137	
33	138	
34	139	
35	140	
36	141	
37	142	
38	143	
39	144	

As was shown in the methods chapter, one milliliter of cells was diluted to ten milliliters for counting. This represents only one tenth of the cells in the tube on which the chemical assays were performed. Thus the count 13,830 is multiplied by two, to get cells per milliliter, by ten to correct for the dilution made for

TABLE I

PROTEIN IN MICROGRAMS PER ONE MILLION CELLS

Time after infection	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Control	Infected	Control	Infected	Control	Infected	Control	Infected
0 hrs.	329	381	360	500	163	119	94	114
1.5 hrs.	462	332	486	427	105	99	107	142
16 hrs.	316	249	309	362	131	136	182	129
20 hrs.	251	286	256	311	135	117	120	128
24 hrs.	300	276	242	277	124	134	146	186
28 hrs.	309	301	285	312	97	113	106	113
32 hrs.	267	356	315	269	112	84	111	109
40 hrs.	313	369	356	239				
44 hrs.					152	97	128	149
48 hrs.	212	179	185	267				
66 hrs.	141	181	237	288				
72 hrs.					113	94	100	102
120 hrs.	194	201	227	195	72	50	77	45
168 hrs.	196	108	155	160	61	39	46	41

TABLE II

RNA IN MICROGRAMS PER ONE MILLION CELLS

Time after infection	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Control	Infected	Control	Infected	Control	Infected	Control	Infected
0 hrs.	15.0	18.4	19.8	23.3	20.0	13.3	11.5	12.7
1.5 hrs.	27.3	20.6	29.0	19.6	12.3	10.9	13.9	12.0
16 hrs.	23.6	24.3	21.9	24.5	10.0	15.2	17.6	15.5
20 hrs.	18.0	20.8	18.3	20.8	12.9	14.7	13.8	16.0
24 hrs.	23.2	18.0	17.7	20.9	16.9	16.4	15.8	21.7
28 hrs.	19.9	20.4	16.5	23.0	9.2	12.8	12.4	10.8
32 hrs.	22.1	26.2	25.4	18.8	10.5	13.7	11.4	13.1
40 hrs.	23.0	26.8	26.1	14.0				
44 hrs.					10.1	6.2	15.9	16.8
48 hrs.	14.8	10.7	11.4	16.7				
66 hrs.	8.1	10.1	16.1	19.5				
72 hrs.					11.8	11.7	10.8	11.0
120 hrs.	11.6	12.8	16.4	11.2	6.0	4.1	6.2	2.7
168 hrs.	14.5	2.3	4.2	2.9	4.3	1.1	1.7	2.2

TABLE III

DNA IN MICROGRAMS PER ONE MILLION CELLS

Time after infection	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Control	Infected	Control	Infected	Control	Infected	Control	Infected
0 hrs.	12.0	14.3	12.8	20.3	11.0	17.7	14.2	16.4
1.5 hrs.	15.4	16.6	17.6	14.1	16.3	14.8	18.2	11.2
16 hrs.	12.6	17.1	13.5	15.6	16.9	18.5	23.4	16.2
20 hrs.	10.4	11.7	16.4	14.7		19.0	17.0	16.8
24 hrs.	16.9	13.6	17.2	20.4	45.2	36.1	28.1	32.5
28 hrs.	13.7	12.7	18.5	17.2	14.1	18.5	22.4	12.0
32 hrs.	14.0	16.1	18.1	14.1	17.7	21.0	23.0	16.9
40 hrs.	18.1	17.2	22.9	11.1				
44 hrs.					23.4	14.5	22.4	24.9
48 hrs.	12.2	18.7	14.8	17.3				
66 hrs.	10.3	11.6	14.6	23.2				
72 hrs.					18.5	23.3	24.0	26.7
120 hrs.	16.8	14.6	14.9	25.8	10.4	18.0	18.3	8.2
168 hrs.	15.5	4.8	12.2	7.6	5.4	4.4	4.9	6.3

counting, and once more by ten, the reciprocal of the fraction of cells used for counting from the tube for chemical analyses.

13,830 × 2 × 10 × 10 = 2,766,000 (total number of cells in this sample used for chemical analyses.)

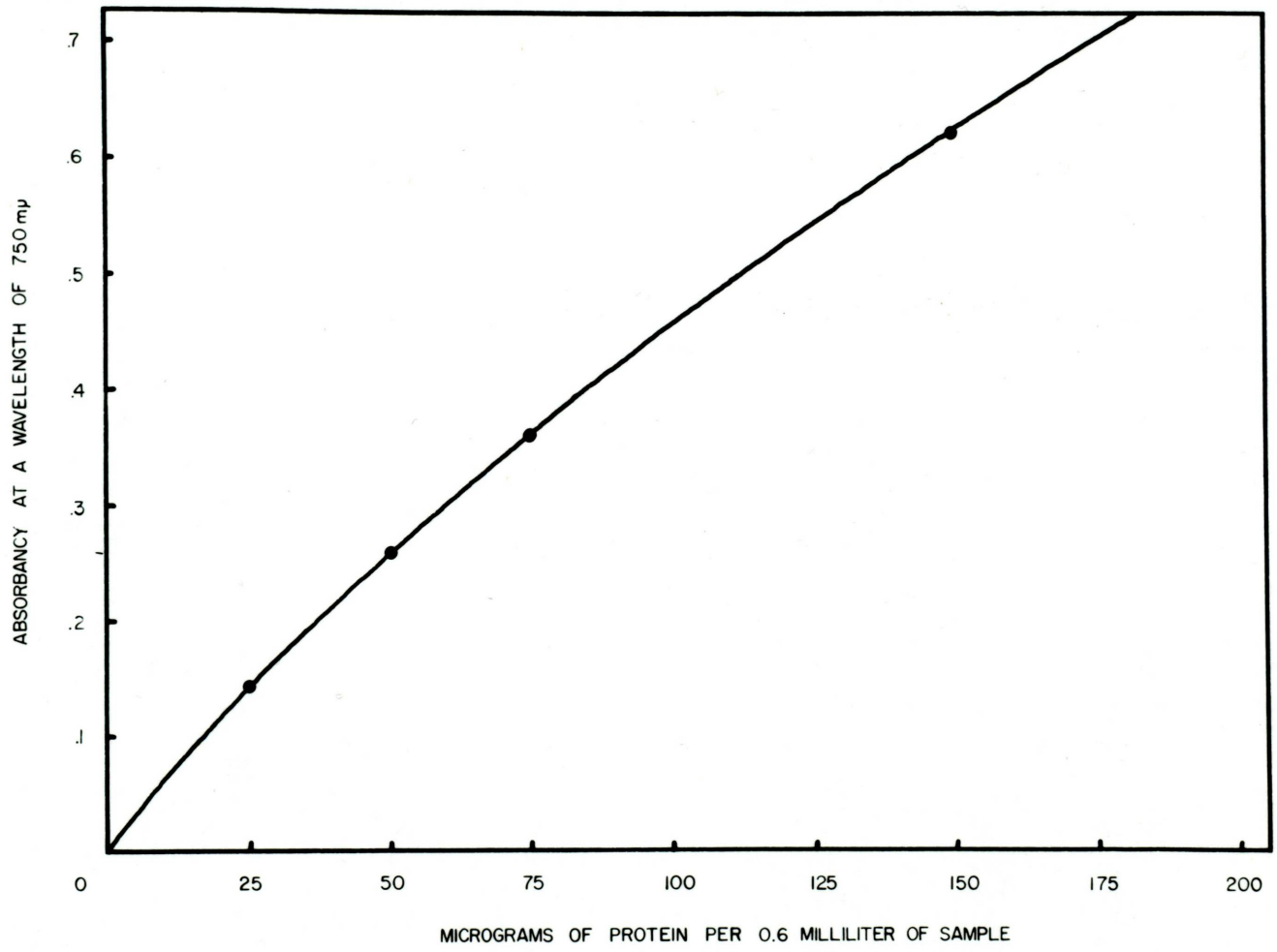
Each sample for final protein analysis was six milliliters in volume. For protein determination a volume of 600 lambdas was used which represents one-tenth the protein of all the cells in the sample.

The absorbancy at 750 mμ, from the colorimetric test for this particular sample, was found to be 0.418. Using the standard protein curve (figure 5), one finds this absorbancy to represent 91 micrograms of protein per six-tenths milliliters.

If 91 micrograms per 0.6 milliliters is multiplied by ten (the reciprocal of the fraction of total sample used) and divided by 2.766, the micrograms of protein per one million cells may be determined.

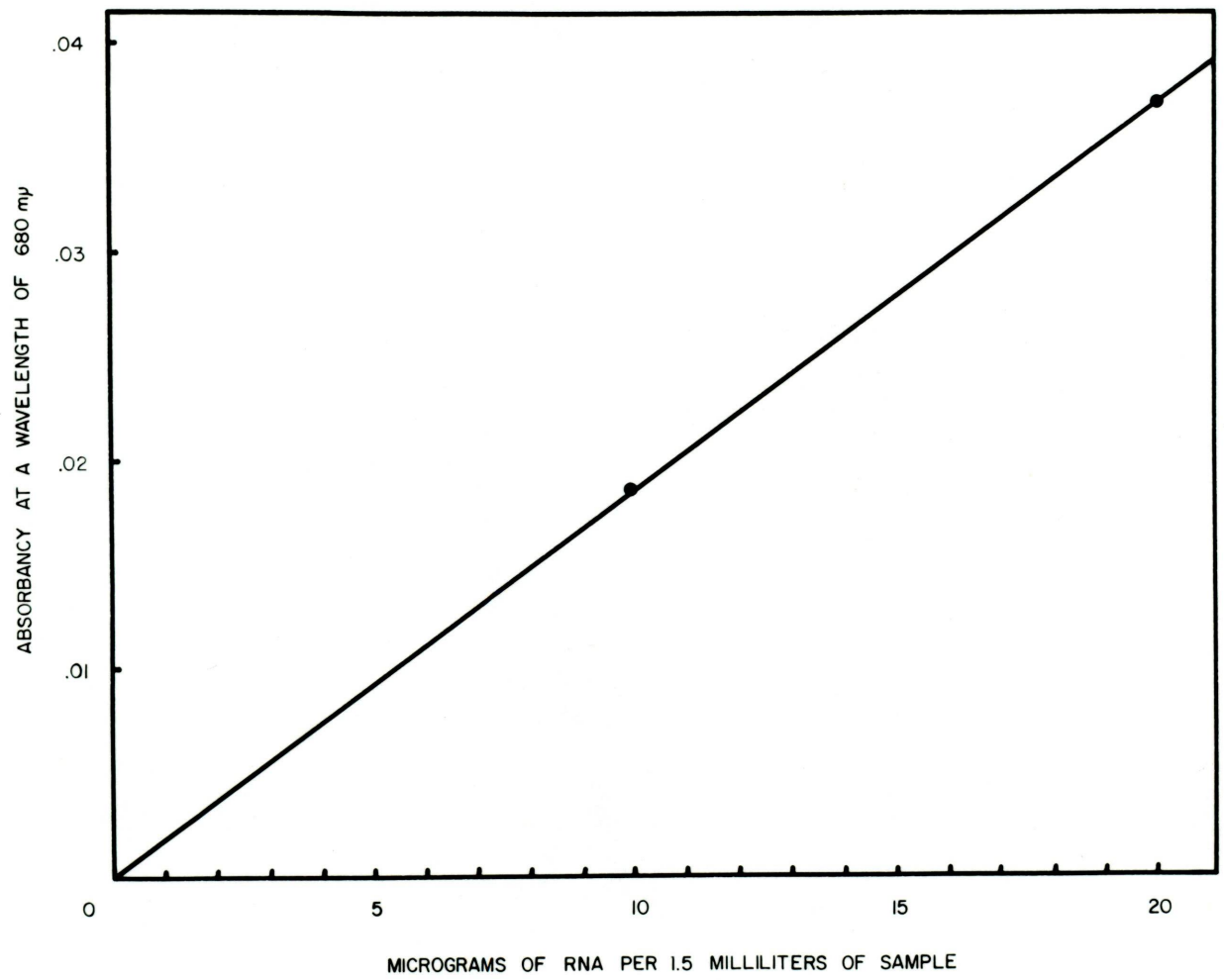
$$\frac{91 \times 10}{2.766} = 329 \text{ μg protein}/10^6 \text{ cells}$$

One hundred lambda samples were used for RNA analyses and the total volume of RNA per cell sample was 900 lambdas. If the absorbancy reading of 0.088 is checked using the RNA standard curve (figure 6) it will be found to represent 4.6 micrograms of RNA. This is one-ninth the total RNA in the cell sample and if multiplied



STANDARD CURVE FOR PROTEIN DETERMINATION

FIGURE 5



STANDARD CURVE FOR RNA DETERMINATION

FIGURE 6

by nine and divided by 2.766 will yield the amount of RNA per million cells.

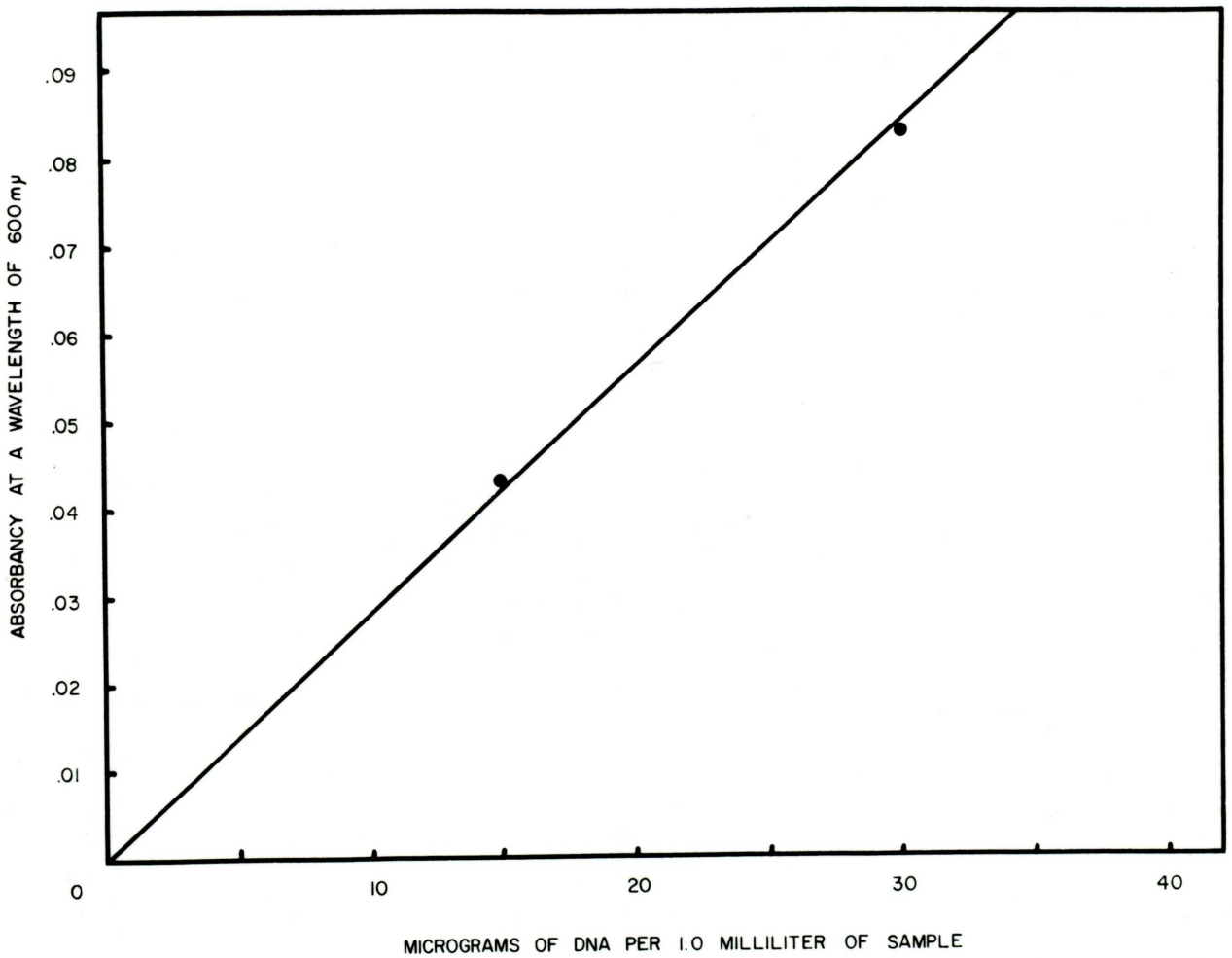
$$\frac{4.6 \times 9}{2.766} = 14.97 \text{ ug RNA}/10^6 \text{ cells}$$

The DNA is calculated similarly except that 200 lambda aliquots out of 900 lambda samples were used. An absorbancy reading of 0.020 represents 7.4 micrograms of DNA in the sample. (figure 7),

$$\frac{7.4 \times \frac{9}{2}}{2.766} = 12.02 \text{ µg DNA}/10^6 \text{ cells.}$$

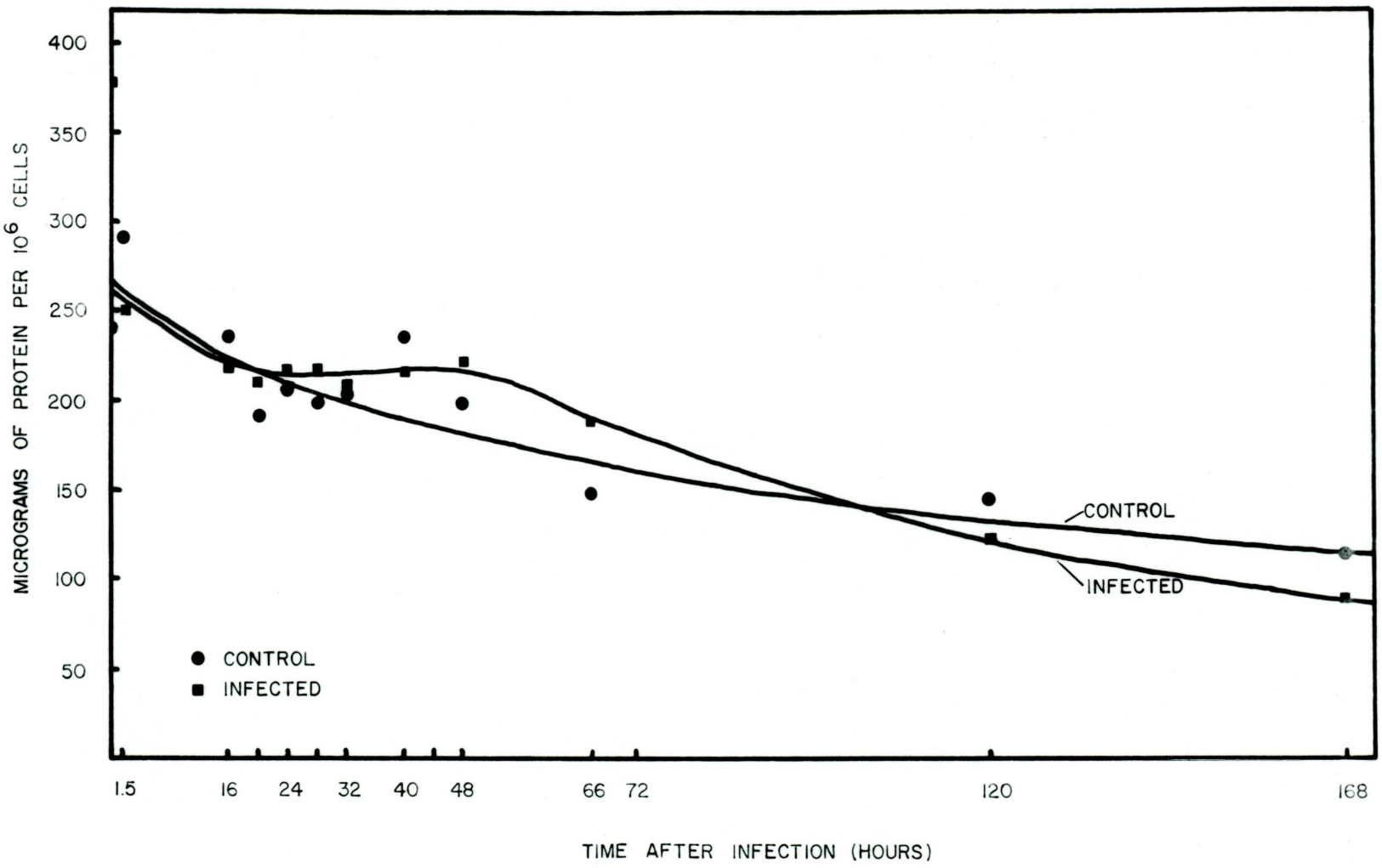
In each of the four experiments the amount of protein and RNA in cells infected with polyoma virus changed very little with time and paralleled the control cultures closely. For this reason the protein values for corresponding samples in the four experiments were averaged. The RNA values were averaged in the same manner as were the protein values. This procedure tended to level points which were separated due to variation in counts. This information has been plotted in figures 8 and 9. In experiments 1 and 4 small differences from the control were found in the amount of DNA in virus infected cells. Corresponding DNA values for these two experiments were, therefore, averaged and plotted together in figure 10.

The DNA curves for experiments 2 and 3 were plotted separately in figures 11 and 12 since, in these two experiments, the amount of DNA in the infected cells increased to values significantly higher



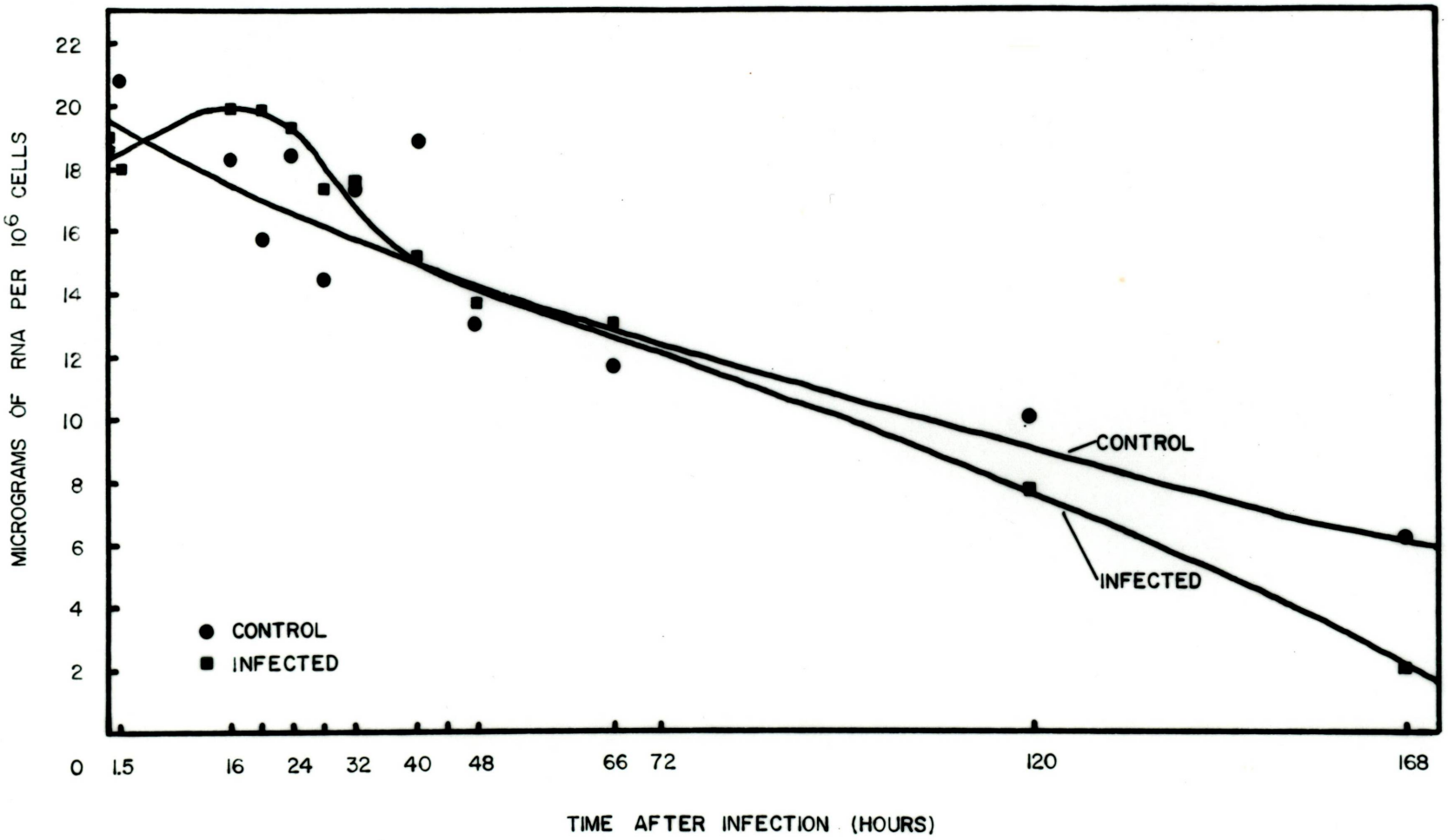
STANDARD CURVE FOR DNA DETERMINATION

FIGURE 7



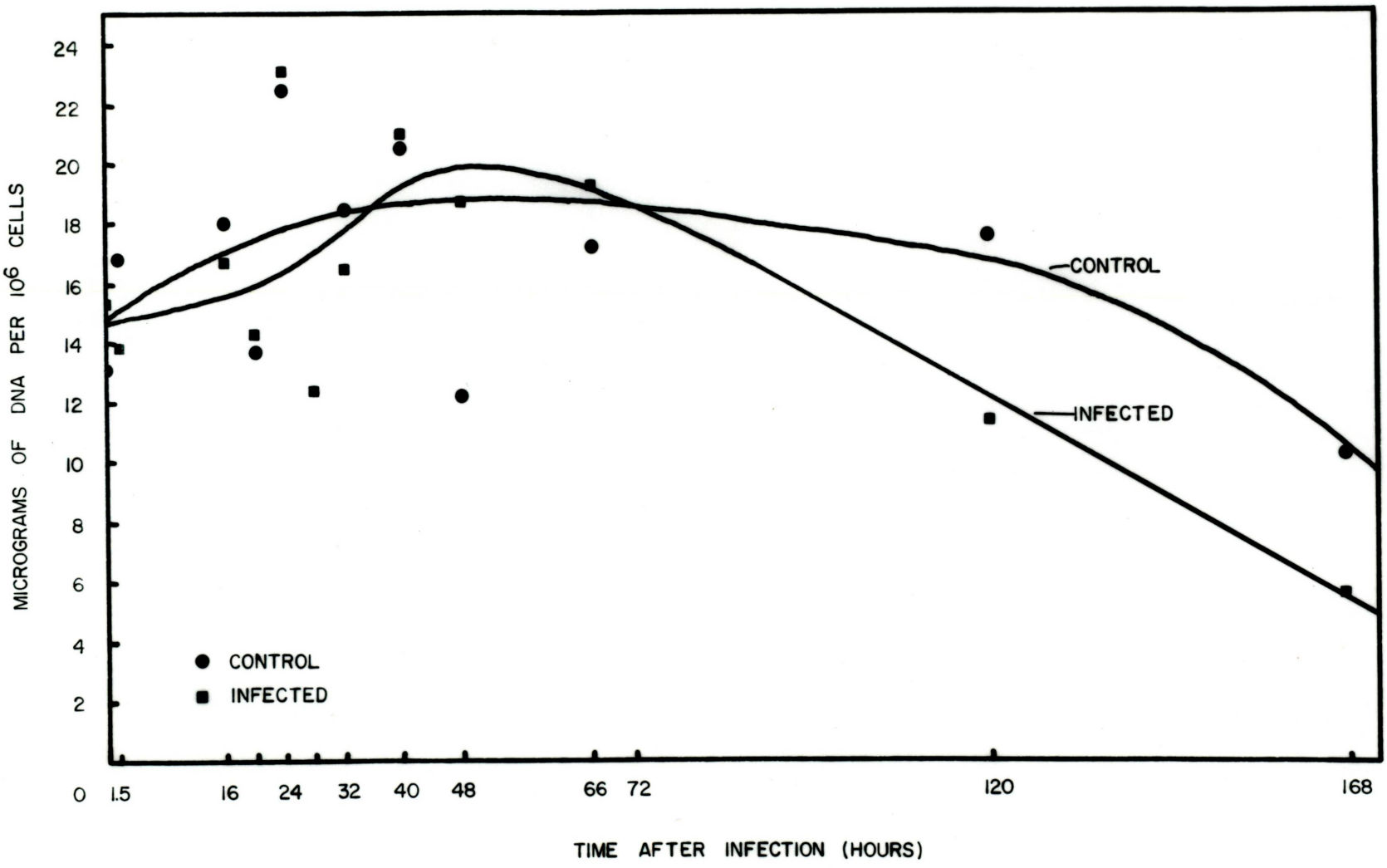
CHANGE OF PROTEIN IN MOUSE EMBRYO CELLS WITH TIME AFTER INFECTION WITH POLYOMA VIRUS; UNINFECTED CELLS USED AS CONTROL (EXPERIMENT 1 TO 4)

FIGURE 8



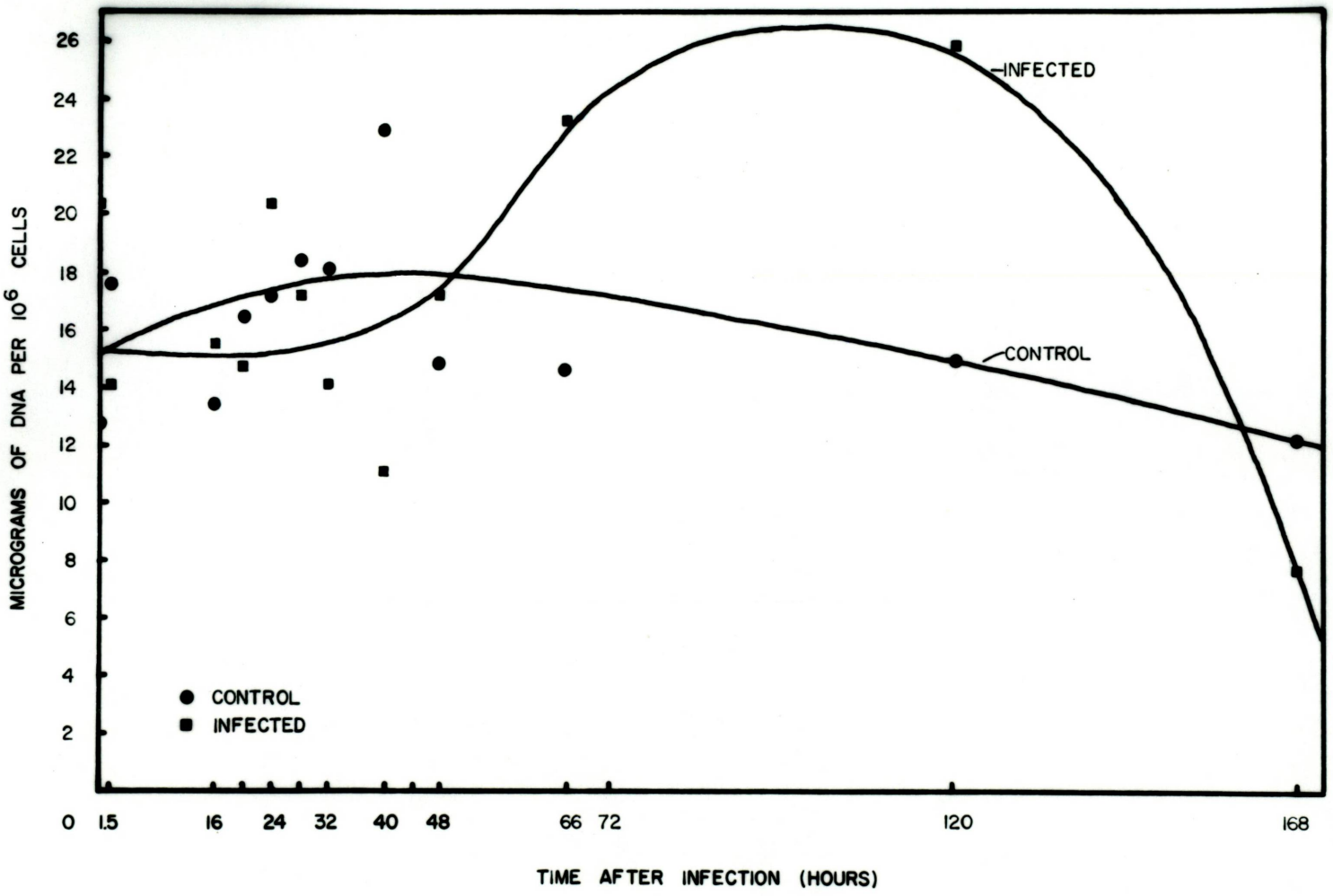
CHANGE OF RNA IN MOUSE EMBRYO CELLS WITH TIME AFTER INFECTION WITH POLYOMA VIRUS; UNINFECTED CELLS USED AS CONTROL (EXPERIMENT I TO 4)

FIGURE 9



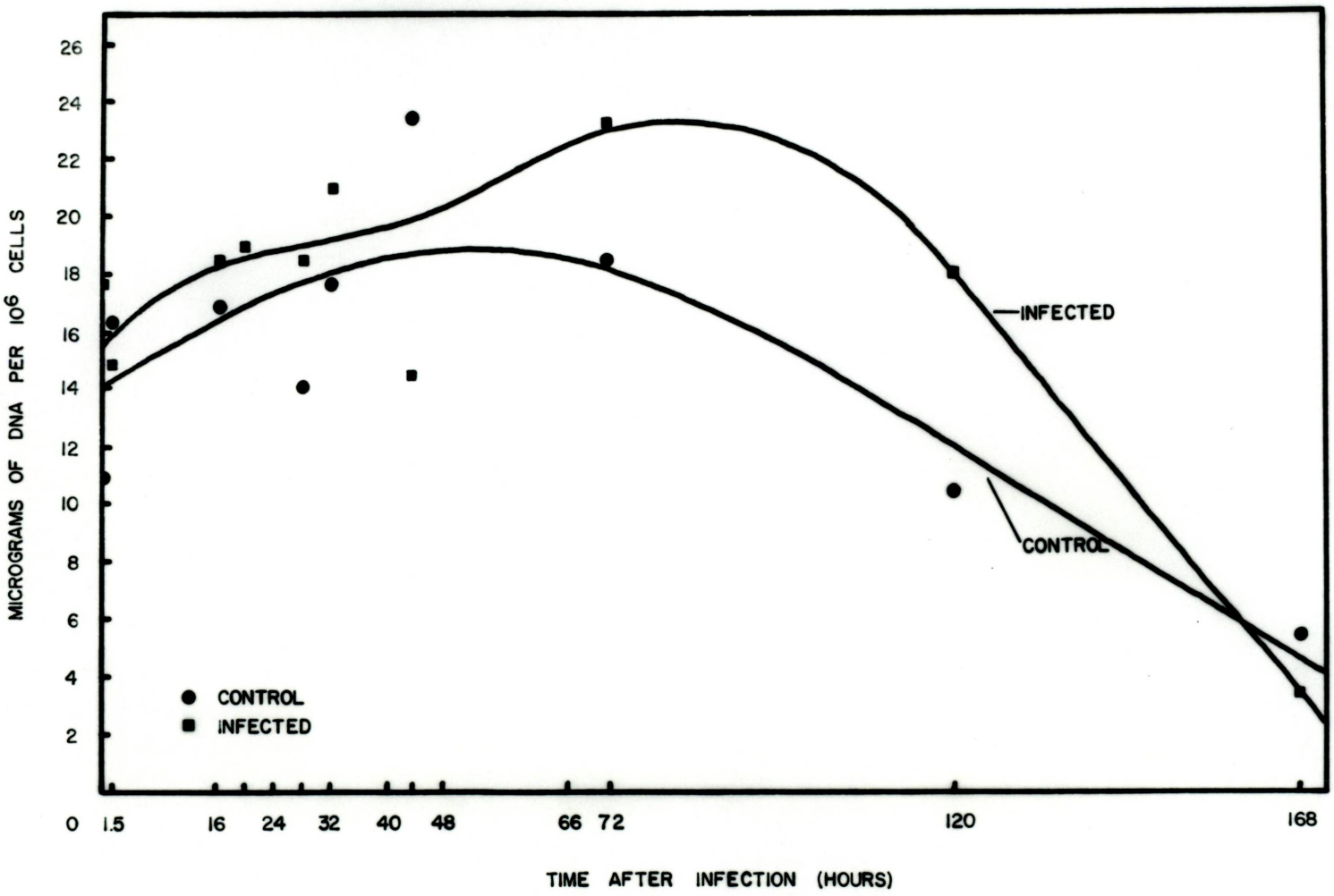
CHANGE OF DNA IN MOUSE EMBRYO CELLS WITH TIME AFTER INFECTION WITH POLYOMA VIRUS; UNINFECTED CELLS USED AS CONTROL (EXPERIMENT I AND 4)

FIGURE 10



CHANGE OF DNA IN MOUSE EMBRYO CELLS WITH TIME AFTER INFECTION WITH POLYOMA VIRUS; UNINFECTED CELLS USED AS CONTROL (EXPERIMENT 2)

FIGURE 11

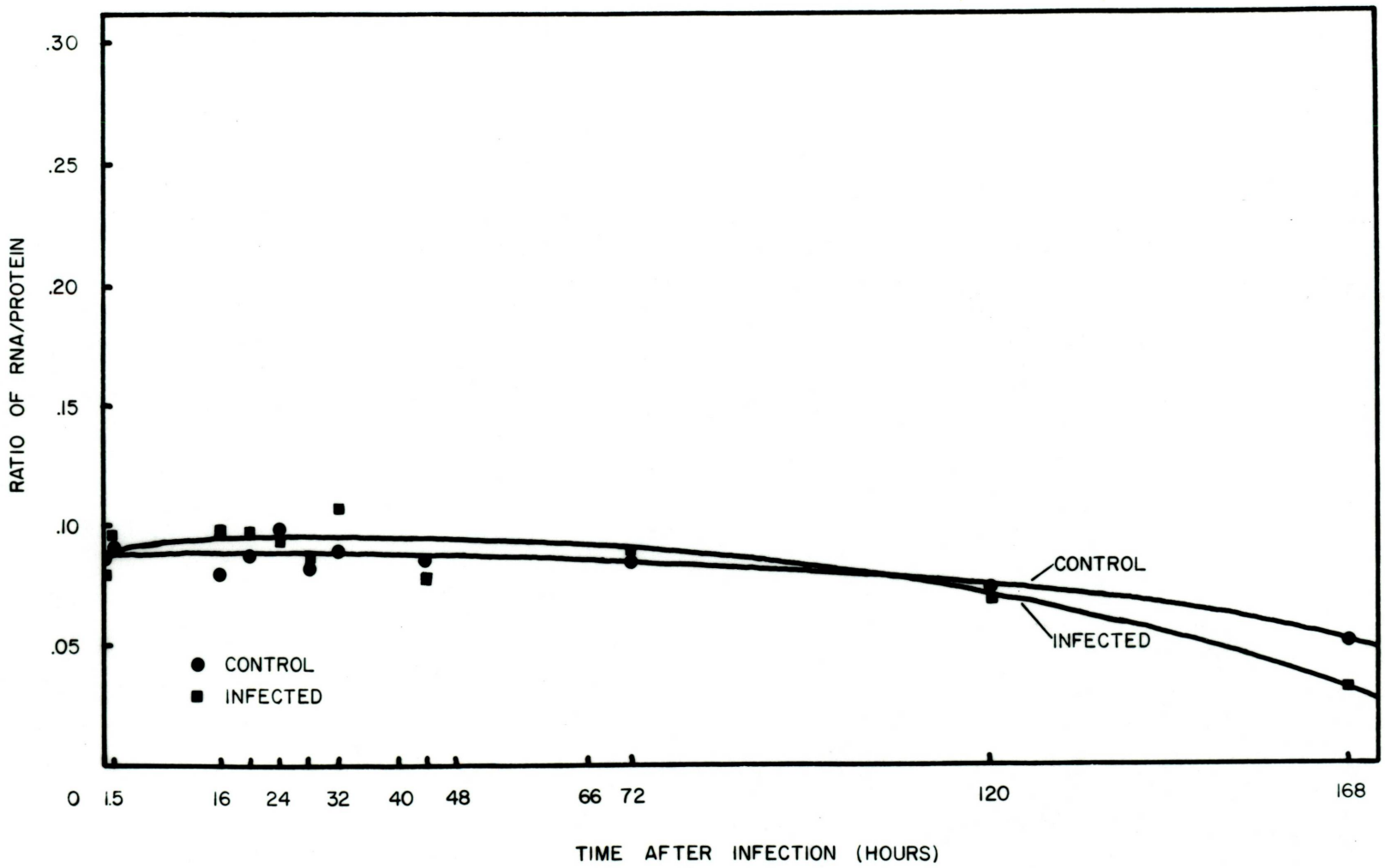


CHANGE OF DNA IN MOUSE EMBRYO CELLS WITH TIME AFTER INFECTION WITH POLYOMA VIRUS; UNINFECTED CELLS USED AS CONTROL (EXPERIMENT 3)

FIGURE 12

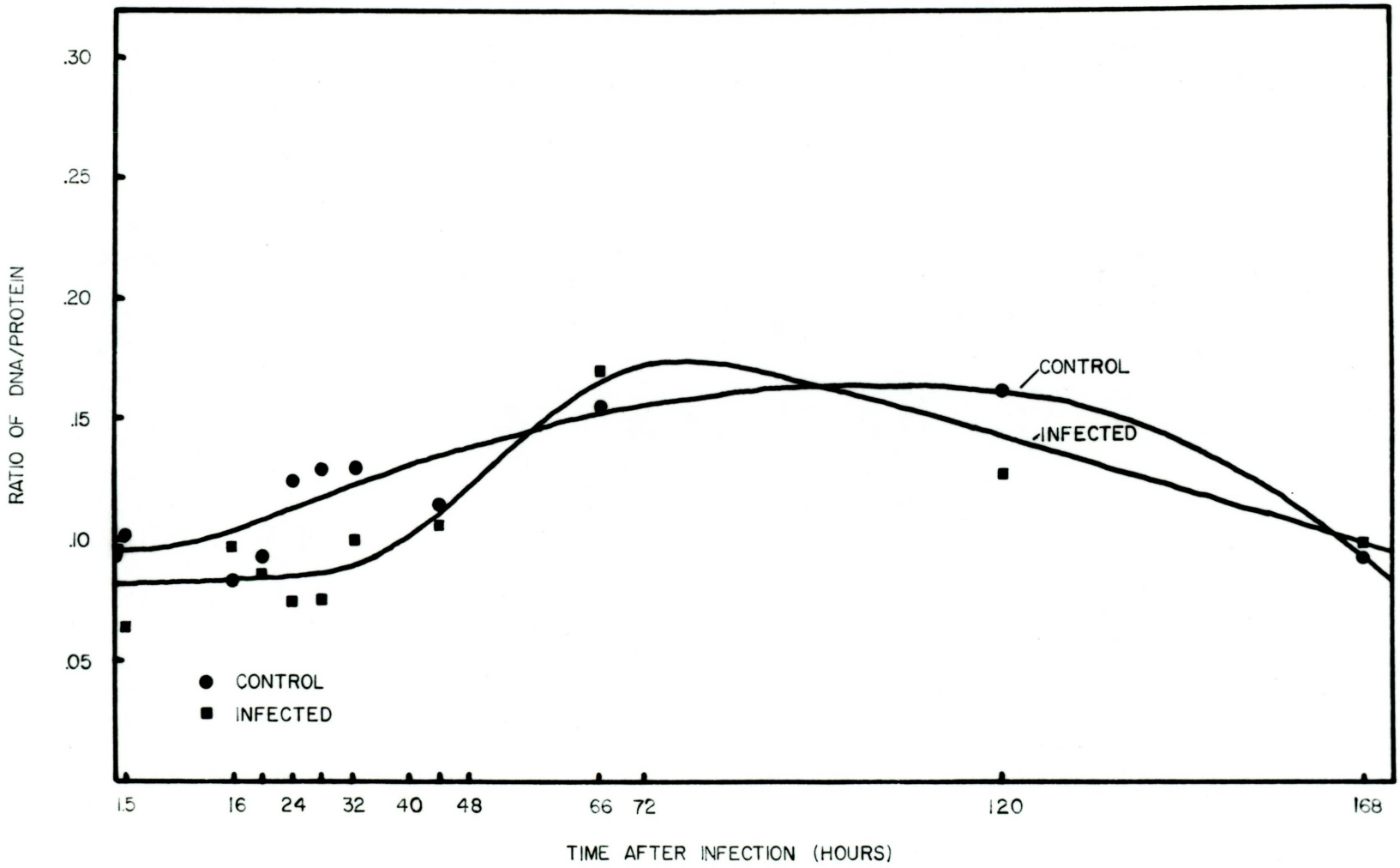
than in the control cultures. The curves may be compared with virus concentration figures indicated by hemagglutination techniques which appear in Table IV.

In the process of performing the four experiments it was noticed that there was more variation in the cell counts than in the chemical assays. This became very obvious when it was found that in many instances the amounts of protein, RNA, and DNA varied from sample to sample but increased and decreased together. When the protein increased, there was also an increase of RNA and DNA; when the protein decreased, the RNA and DNA decreased. Since this variation was probably due to inaccuracies in cell counts due to a certain amount of clumping of the cells or settling out, it was decided to plot graphs which would minimize this effect. In order to accomplish this, the ratios of RNA to protein, and DNA to protein were calculated and plotted. These appear as figures 13, 14, 15 and 16 and correspond to the data plotted originally in figures 9, 10, 11 and 12.



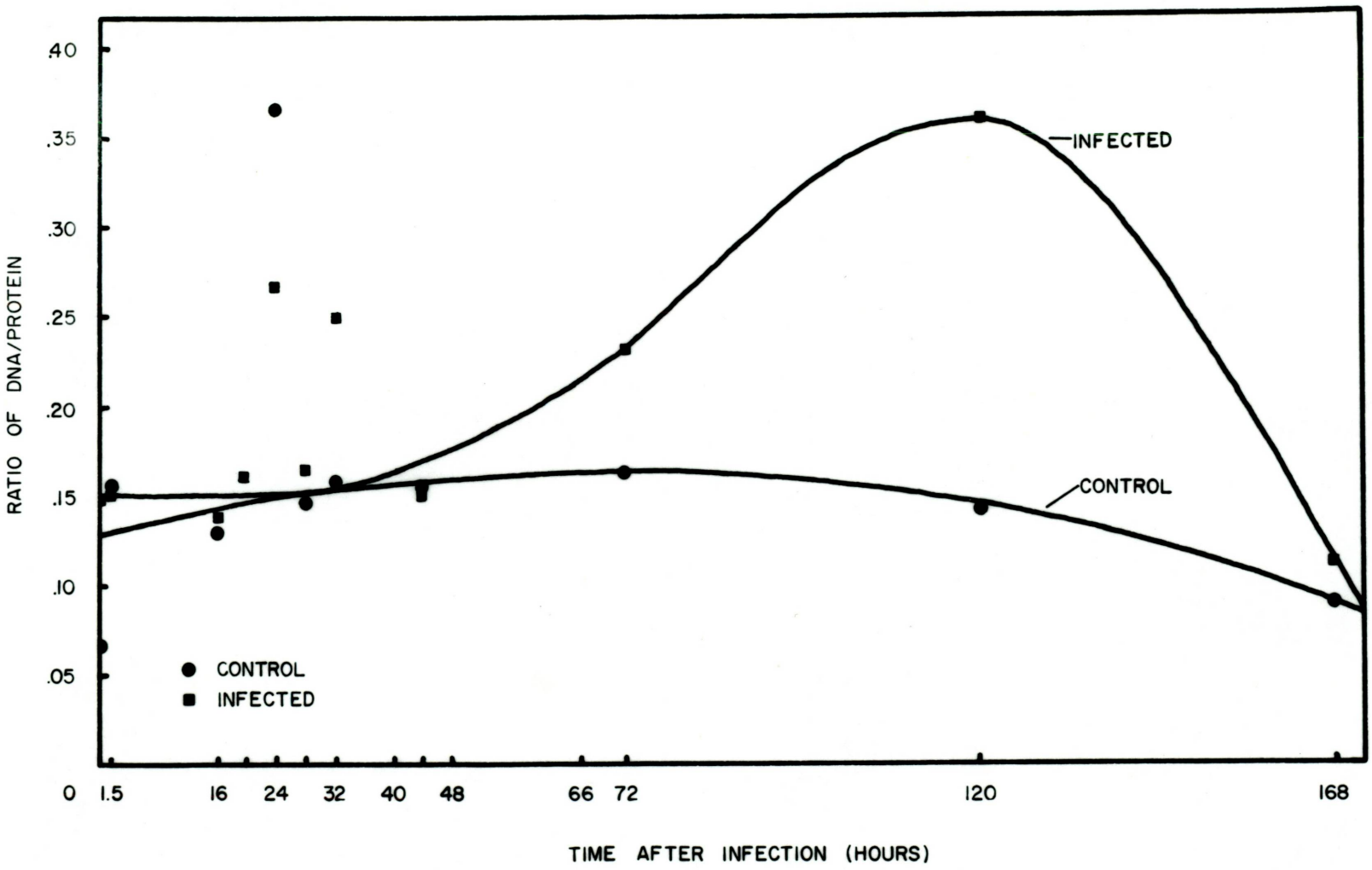
CHANGE OF RNA/PROTEIN RATIO WITH TIME (EXPERIMENT 1 TO 4)

FIGURE 13



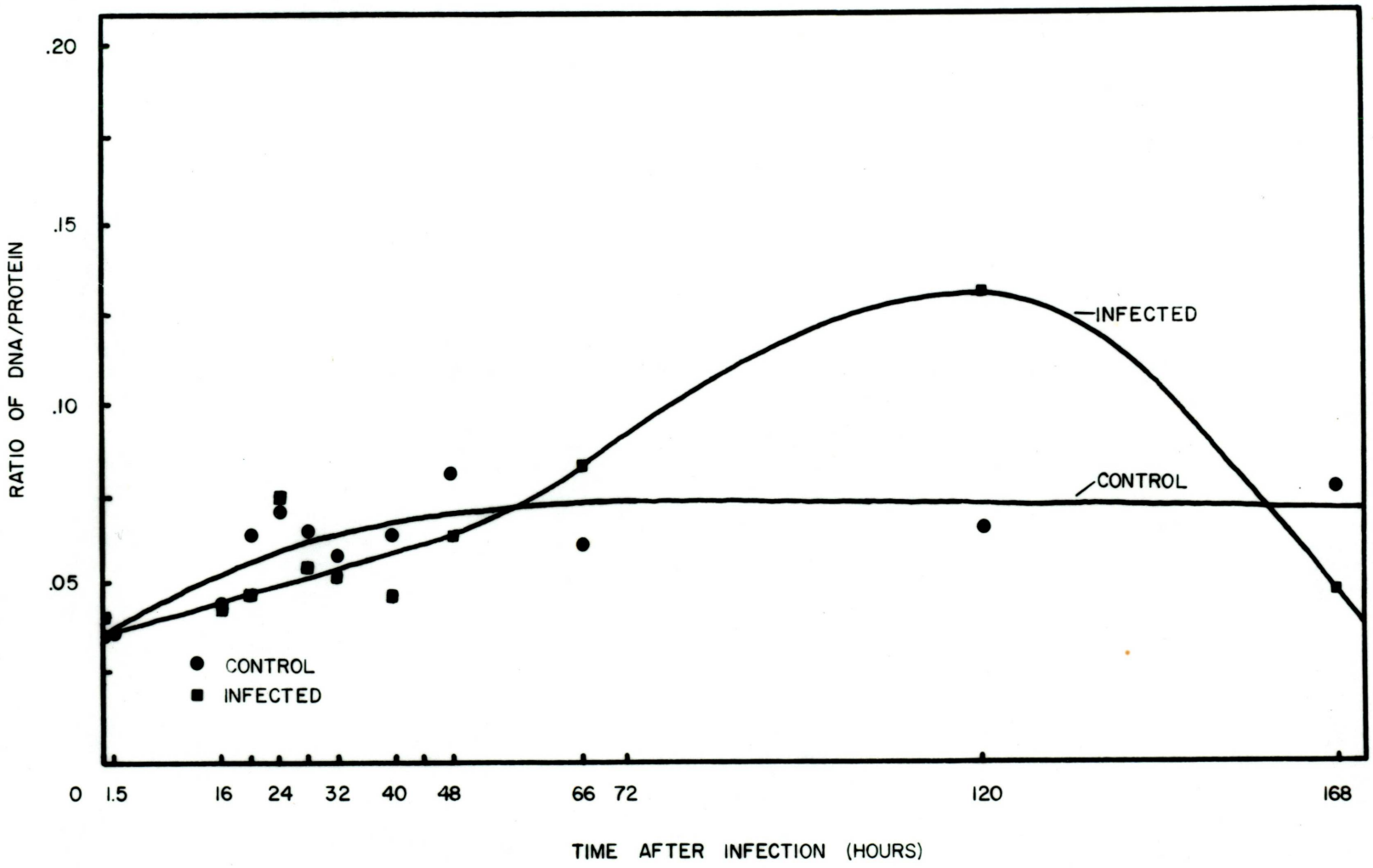
CHANGE OF DNA/PROTEIN RATIO WITH TIME (EXPERIMENT 1 AND 4)

FIGURE 14



CHANGE OF DNA/PROTEIN RATIO WITH TIME (EXPERIMENT 3)

FIGURE 15



CHANGE OF DNA/PROTEIN RATIO WITH TIME (EXPERIMENT 2)

FIGURE 16

DISCUSSION

The scatter of the points on the curves where various amounts of protein, RNA, and DNA per million cells are plotted against time, one might have desired to have had less than that found. There is probably little variation in the chemical analyses from inherent errors in the assay procedures when the close proximity to the standard is noted in the standardization curves. Also, if the curves to evaluate the Coulter counts through a range of dilutions and the variation of counts of a given dilution through a period of a week are examined, one might be tempted to make a similar statement for the Coulter counts. These statements, however, do not take into consideration the state of the mouse embryo cells and the conditions under which these cells were counted.

The experiments to evaluate the chemical assays and the counts were made on a single group of pooled mouse embryo cells. On the other hand, when experiments 1 to 4 were counted and the chemical assays performed, each control and each infected sample represented the cells of a single culture bottle. These cultures were each grown, infected, and removed from the glass separately. Even though great care was used to do the same manipulations on each culture, there are certain possible differences that can be noted. The reactions of the different bottles to the many washing procedures may vary considerably. When the cells are seeded

into the bottles there is often a tendency for cells to swirl and aggregate in the center of the bottle. This will cause the cells in these areas to progress rapidly to the stationary phase and tend to be fragile and fall from the glass during the washing and adsorption period. After the period for infection has passed, the cells are trypsinized, washed, and diluted for counting. During this procedure there is a great tendency for cell clumping. The cell fluids are forced from a pipette to separate the cells from one another, however, there is a tendency for the cells to collect and clump. The longer the period of time between dilutions and counting, the greater the tendency for this clumping to take place. Because of these factors, in some instances there were very low cell counts from the bottles manipulated in the same manner as other bottles. This will cause an apparent increase in the amount of chemical constituents per one million cells. These counts appear as high points on the graph. It is noted that the protein and RNA increase with the DNA in these instances. The points at 40 hours in experiment 2 represent an extreme example of this.

It has been already mentioned that the Coulter counter can be set to count cells of a given size or larger as determined by the plateau curve. Any cells present which are too small to count will still contribute to the chemical constituents. It is safe to assume that the cells too small to count will differ from one sample

to the next. This becomes especially evident when one considers that the growth conditions of each bottle may be somewhat different in spite of all attempts to make them similar.

In an attempt to minimize the variation of the points, a mean was determined for protein and RNA for each time a sample was counted. The determinations for protein and RNA in cells infected with virus did not seem to deviate appreciably from the assay of control cells which had not been infected. Since two of the experiments for DNA determination did not show any appreciable change in infected from control values these were also averaged to minimize the variation of the points.

In experiments 2 and 3 the DNA determination from infected cells did seem to show deviation from the control cells. For this reason these experiments were not averaged and therefore tend to show more variation from point to point.

During cell metabolism in the stationary phase there is a tendency for RNA and protein to decrease in cells whether infected or not. This is probably due to leakage from old cells or to the apparent increase in numbers of cells due to the counting methods used in most experiments to determine cell protein, RNA, and DNA. The cell may break up and each part counted as a single cell. Thus the increase in number of cells would decrease the apparent amount of constituent in each cell. The latter possibility

seems to be a less likely explanation for the general downward trend of these curves.

When the DNA to protein and RNA to protein ratios are determined, it is possible to minimize further the variation of the points. The RNA showed little deviation of infected cells from control cells and when plotted as a ratio against the protein an almost straight line for infected cell RNA and control cell RNA is seen.

The DNA in cells infected with polyoma virus did deviate from the DNA in control cells and when DNA to protein ratios were determined this deviation was not factored out. The deviation of DNA in infected cells tended to increase with time over that in control cultures. Indeed, the DNA in infected cells tended to almost double that of control cells. This is in agreement with the work by Newton and Stoker (1958) who showed that the DNA increase in HeLa cells infected with herpes virus almost doubled the amount in the uninfected cells.

It is not possible from these experiments to determine whether the DNA indicated in these experiments is cellular or viral in nature. We have already shown that most of the DNA increase is not due to whole intracellular virus but to DNA which was formed by the cell under viral stimulation.

The increase in hemagglutination titers tend to compare

favorably with the increase of DNA in infected cells. In experiment 2 the hemagglutination reached a titer of ± 512 and in the experiment the DNA reached the highest level of all the experiments. In experiment 3 the titer reached ± 128 and the DNA was lower than in experiment 2. The hemagglutination value of 128 read at 120 hours in experiment 1 does not seem to fit into the pattern of a high DNA value where there is a significant increase in hemagglutination titer. However, since the hemagglutination value at 168 hours in the same experiment is only 2, the former point might well be an artifact. There seems to be no other satisfactory explanation for it.

The fact that no significant rises in either DNA or hemagglutination titers appear in experiments 1 and 4 is somewhat disconcerting. It is likely that in these instances the active virus titer used for inoculation was too low to yield adequate multiplicity of infection.

It is probable that improvements could be made in further experiments of the type performed in this series. Cells grown in suspension would likely yield less variation. A chemical such as Versene to decrease cell clumping might be used to good advantage. Thirdly, an assay procedure for virus based on infectivity might be better than the hemagglutination assay as a measure of live virus concentration.

SUMMARY

Four separate experiments were performed in which mouse embryo cell monolayers were infected with polyoma viruses, with uninfected monolayers used as controls. Protein, RNA, and DNA determinations, in terms of micrograms per million cells, were made on samples withdrawn at various times after infection up to one week.

Curves plotted for protein and RNA individually against time were found to be similar in virus infected and control cultures. In two of the experiments the amount of DNA in virus infected cells rose to values considerably higher than those in controls.

Hemagglutination assays for polyoma virus performed on these samples indicated good correlation between increased DNA synthesis and increased concentrations of viruses.

The significance of these findings and their correlation with work reported by others is discussed.

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LOMA LINDA UNIVERSITY

Graduate School

THE EFFECT OF INFECTION WITH POLYOMA VIRUS ON THE PROTEIN, RNA, AND DNA OF MOUSE EMBRYO CELL CULTURES

by

Lawrence E. McConnehey

An Abstract of a Dissertation

in Partial Fulfillment of the Requirements

for the Degree Master of Science

in the Field of Microbiology

August 1962

ABSTRACT

In four separate experiments mouse embryo cell monolayers infected with polyoma virus and uninfected control mouse embryo cells were maintained in Eagle's Minimum Essential Medium for periods of time ranging from 0 to 168 hours. The cell layers were removed from the glass by trypsinization. Samples of the cells were counted in a Coulter Counter and the remainder of the cells frozen after centrifugation, in a button. Later, the buttons were thawed and protein, RNA, and DNA analyses were performed on each sample. The micrograms per million cells of protein, RNA, and DNA were determined for each sample and plotted against time.

The amounts of protein and RNA in infected cells showed no apparent change from the corresponding biochemicals in the uninfected control cells. However, in two experiments DNA in infected cells did show an increase with time when compared with DNA values in uninfected cells. In one experiment the DNA in infected cells almost doubled the DNA in uninfected cells.

Hemagglutination assays, for polyoma virus concentration were done on many of the cell samples. The hemagglutination titers were found to be elevated in the two experiments which yielded significant DNA increases with time in virus infected cells.

The amount of viral DNA in polyoma virus infected cells prior to their disintegration, could not account for the additional amount of DNA found in the cells. The DNA probably results, however, from stimulation by the infection with the virus.