MICROSPECTROPHOTOMETRIC DETERMINATION OF NUCLEIC ACIDS IN NORMAL AND IN VIRUS-INFECTED TISSUE CULTURE CELLS

by

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I hereby recommend that this dissertation prepared under my direction by Carl Arthur Bailey

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be accepted as fulfilling the dissertation requirement of the degree of DOCTOR OF PHILOSOPHY

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ABSTRACT

Experiments were conducted, using microspectrophotometry and radioautography, to determine the effect of four different viruses on the nucleic acid metabolism of the mouse cell line F120E90. The viruses utilized for this study were Herpes simplex virus, Western equine encephalomyelitis virus, Type I poliovirus, and Friend leukemia virus. For microspectrophotometry, nucleic acid metabolism was studied by methylene blue, which stains both DNA and RNA, and the Feulgen stain, which is specific for DNA. The stained cells, virus-inoculated and non-inoculated, were measured by the two-wavelength method on a Leitz microspectrophotometer. The methylene blue preparations were read at 600 mμ and 570 mμ; the Feulgen preparations were read at 550 mμ and 500 mμ. The relative amount of bound chromophore for each cell was computed by a CDC 6400 computer. For radioautography, tritiated thymidine and tritiated uridine were utilized for DNA and RNA, respectively. Grain counts were performed to delineate differences between virus-inoculated and non-inoculated cells.

Each of the viruses with the exception of poliovirus affected a measurable change in either DNA, RNA, or both. These changes represented the nucleic acid metabolic pattern of virus-cell interaction.

Herpes simplex virus increased both DNA and RNA during a 10 hour interval following inoculation. Western equine encephalomyelitis virus increased RNA from 6 to 12 hours post inoculation. Type I poliovirus did not affect nucleic acid metabolism. Friend leukemia virus increased both DNA and RNA during a 10 hour interval following inoculation. During a six day study, Friend leukemia virus also increased RNA; however, this increase reached a maximum at three days PI.

It is suggested that in situations where virus-cell interaction is not grossly apparent, microspectrophotometry utilizing methylene blue and Feulgen might be used to detect and quantitate the respective interaction.

CHAPTER 1

INTRODUCTION

The significance of intracellular nucleoproteins in structure and metabolism was discovered by Miescher (24) and Kossel (16) in the 1890's. Since that time and particularly during the last 15 years, the mechanisms by which deoxyribonucleic acid, DNA, and ribonucleic acid, RNA, control cellular metabolism, growth, and structure have been elucidated. The study of intracellular nucleoproteins has shown that DNA is found predominantly within the nucleus of the cell. There it is associated with the chromosomes, and its concentration remains remarkably constant from one cell to another during interphase. In contrast, RNA is associated with both the nucleus and cytoplasm and varies from cell to cell, depending upon the cell's metabolic state.

The binding of dyes by cells depends upon the chemical composition of the cellular components. Thus, DNA and RNA, as chemical constituents of all cells, have an affinity for specific dyes. In 1913, Van Herwerden (44) showed cytoplasmic basophilia to be attributable to cytoplasmic RNA. Feulgen and Rossenbeck (11), in 1924, developed a technique which would stain DNA but not RNA, thereby allowing the intracellular differentiation of the two nucleoproteins. In 1929, Stearn

and Stearn (40) showed the binding of basic dyes to nucleoproteins.

Although qualitative cytochemical analyses of intracellular nucleoproteins could be made, it was not until 1936 that Casperson (4) demonstrated that the microscope could be used quantitatively in conjunction with natural ultraviolet absorption spectra for the analysis of cellular constituents.

In microspectrophotometry, the amount of light absorbed by an intracellular chemical constituent is measured by a photometric device. The measured amount of absorbed light is the basis for the quantitative analysis of that particular constituent. Utilizing the basic work of Casperson (4), Pollister and Ris (31) measured the nucleic acid content of grasshopper testis by the Feulgen Method. This study contained the first estimates of the DNA content per nucleus. Ris (32) measured light absorption at wavelengths characteristic for the specific DNA-dye complex. Since 1948 a number of other specific stains have been employed in the analysis of intracellular chemical constituents. These early photometric studies were subject to a certain criticism due to the distributional error introduced by the uneven distribution of absorbing material within the measuring area. In 1952, Ornstein (27) and Patau (30) independently developed a two-wavelength method for measuring heterogeneously distributed absorbing material. Utilizing this technique, the previous distributional errors were kept within acceptable units.

The validity of the Feulgen reaction for quantitative determinations in individual cells was established by Leuchtenberger (19) in 1958. In 1964, Deitch (7) described a method using the basic dye methylene blue for the quantitative determination of DNA and RNA.

There are many applications of microspectrophotometry to the field of microbiology. One is the ability to measure the concentration of specific substances in individual cells as opposed to fractionation of total cell populations. Another is that nucleic acid quantities as small as 10^{-12}g can be measured accurately. Yet another application is the possibility of detecting nucleic acid changes within cells undergoing normal or abnormal alterations. Cellular changes occur normally by mitosis, mutation, and degeneration; or these changes may be induced by outside influences. Viruses, for example, affect cellular metabolism in various ways. Some viruses stimulate both cellular and viral nucleic acid synthesis. Some stimulate cellular nucleic acid syntheses which lead to cell "transformations" without the formation of competent viral nucleic acid. Others have no detectable effect on certain cells or their metabolism.

Utilizing Feulgen microspectrophotometry, Crouse, et al. in 1950 (5) showed that Herpes simplex virus increased the nuclear DNA level of infected cells. Bloch and Godman in 1957 (1) reported that in viral papilloma cells, the DNA level was increased markedly in comparison with normal skin cells. Boyer, Leuchtenberger, and Ginsberg

in 1957 (3) reported that the DNA level increased in adenovirus-infected cells but not in non-infected cells. Inui and Oota in 1965 (15) reported that the DNA level increased in spleen cells from animals infected with Friend leukemia virus.

Leuchtenberger, Boyer, and Strain (20) and Leuchtenberger and Leuchtenberger (21), using ultraviolet microspectrophotometry as well as cytochemical techniques, studied the effects of both DNA and RNA viruses on cell cultures. She concluded that in the virus-cell systems studied, a characteristic increase in nucleic acid of virus-infected cells occurred. This DNA or RNA increase depended upon whether the virus was a DNA or RNA virus. Leuchtenberger (22) concluded that microspectrophotometry was an accurate, sensitive, and quantitative method for determining changes in intracellular nucleic acid metabolism.

The purpose of this investigation was to study the quantitative changes in nucleic acid metabolism invoked in the deer mouse cell line F120E90 by four viruses using microspectrophotometric techniques. This was effected by the use of methylene blue and Feulgen staining of coverglass monolayer cultures previously inoculated with viruses. The intrinsic properties of the viruses selected included (a) cytolytic or non-cytolytic for F120E90 cells, (b) oncogenic or non-oncogenic for the BALB/c mouse, and (c) those properties with a distinct pattern of quantitative change in nucleic acid metabolism. As a

further check on the validity of the findings, standard radioautography techniques using tritiated thymidine and uridine were employed.

CHAPTER 2

MATERIALS AND METHODS

Cells

A fibroblastic-like cell line designated F120E90, derived from the skeletal muscle of a deer mouse, Peromyscus maniculatus, by Shaw and Barto (34) in 1960 and maintained in vitro or stored since then in liquid nitrogen by Ludovici (23) was used throughout this study. The cells were grown and subcultured twice weekly in 160 ml pyrex milk dilution bottles using a growth medium composed of Eagle's basal medium (EBM) containing Earle's balanced salt solution (BSS) supplemented with 10 per cent calf serum, 100 units penicillin and 100 μg of streptomycin per ml. The F120E90 cell line has retained its diploid chromosome constitution despite repeated subcultures over a five to eight year period.

Preparation of Coverglass Cultures

Stock cultures of cells were trypsinized with 0.25 per cent trypsin-Eagles solution, and 10^5 cells were seeded onto 9 x 22 mm Corning coverglasses previously inserted into 16 x 100 mm culture tubes. The coverglass tube cultures were grown to monolayers in growth medium in three days. Twenty-four hours prior to virus

inoculation the cultures were allowed to equilibrate in a maintenance medium containing EBM with 2 per cent Agamma calf serum, 100 units penicillin, and 100 μg of streptomycin per ml.

Viruses

1. Herpes simplex virus (HSV).

   A stock of O'Connel strain HSV was grown in F120E90, harvested, and frozen at -65C in a Revco freezer until used. The TCID50 of the virus on F120E90 cells was $10^{-5.5}$ per 0.1 ml.

2. Western equine encephalomyelitis virus (WEE).

   A stock of WEE virus was prepared in F120E90 and kept at -65C until used. The TCID50 by tube titration on F120E90 was 10^{-6} per 0.1 ml.

3. Type I poliovirus.

   A stock of Type I poliovirus (Sabin strain) was prepared in HEp 2 cells and kept at -65C until used. The TCID50 of the virus was 10^{-6} per 0.1 ml on HEp 2.

4. Friend leukemia virus (FLV).

   Friend leukemia virus (13) has been maintained in the University of Arizona's BALB/c mouse colony since the virus was originally received from Dr. Howard A. Fieldsteel, Stanford Research Institute, Menlo Park,

California, in 1964. The virus suspension used in this study was prepared from infected BALB/c mice. The animals were bled with syringes previously coated with heparin. The blood was centrifuged at 2000 x g for ten minutes, after which the plasma was collected and filtered through a 0.22 μ Millipore membrane filter pad. The filtrate was used as the virus suspension throughout this study. The LD50 of the virus was $10^{-4.8}$ per 0.2 ml as assayed in mice.

Preparation of Specimen for Microspectrophotometry.

Coverglasses containing a complete monolayer of F120E90 cells were inoculated with 0.4 ml of an undiluted suspension of each virus; the virus was then allowed to adsorb for one hour at 37C. Following adsorption, the coverglasses were washed in BSS, and 1 ml of maintenance medium was added to each coverglass tube culture. The cultures were incubated at 37C. At periodic intervals, the coverglasses were removed from the tubes, washed in BSS, fixed in absolute methanol for fifteen minutes, air dried, and stored at -65C until used for the cytochemical determinations. Normal non-infected control coverglass tube cultures were prepared by the same procedure, except that maintenance medium was used in place of the virus suspension.

Staining Procedures.

The coverglasses were thawed at 37C and stained with either methylene blue or Feulgen. While methylene blue binds stoichiometrically to both DNA and RNA (7), the Feulgen stain is specific for DNA only (17).

1. Methylene blue.

   The coverglasses were stained for DNA or RNA in accordance with the procedure described by Deitch (7). For DNA preparations, the RNA was removed by 1N HCl hydrolysis at 60C for ten minutes. Fig. 1 shows the hydrochloric acid hydrolysis curve. For RNA preparations, the DNA was removed by enzymatic hydrolysis with deoxyribonuclease, 5 mg per ml, at 37C for three hours. Once stained, the coverglasses were mounted on slides in oil of ND=1.550, matching the average index of refraction of cellular material. The individual cells were then measured.

2. Feulgen.

   The coverglasses were stained for DNA in accordance with the procedure described by Deitch (7). Once stained, the coverglasses were mounted on slides in oil of ND=1.550, matching the average index of refraction of cellular material. The individual cells were then measured.

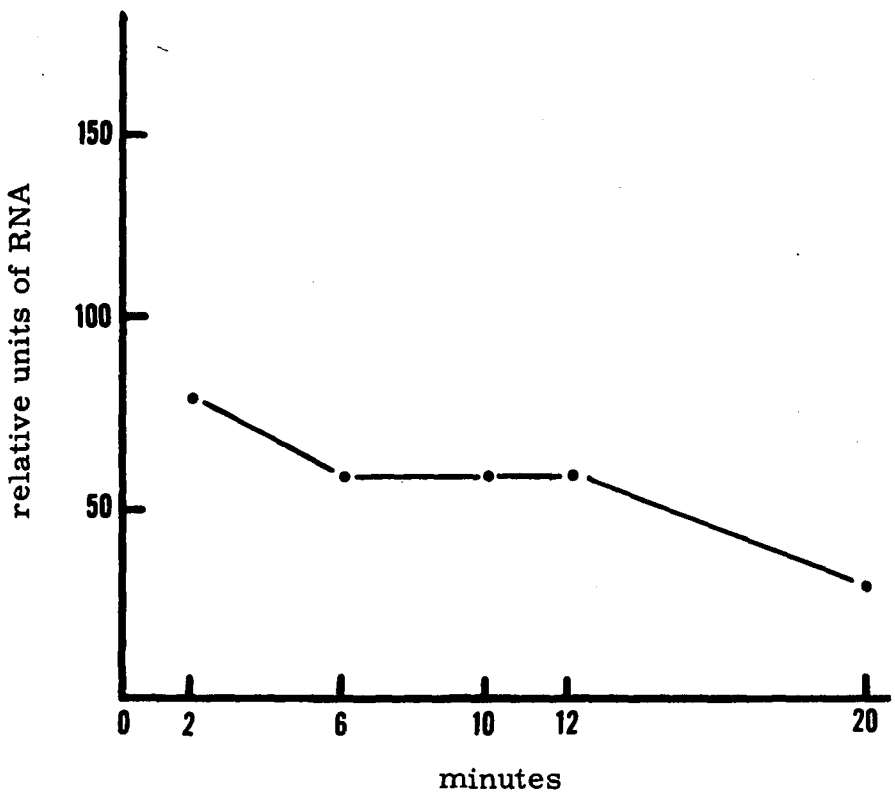

Fig. 1. The hydrochloric acid hydrolysis curve for F120E90 cells.

Microspectrophotometry.

1. Measurement of cells.

   The measurement of the amount of dye bound in individual cells was performed on a Leitz microspectrophotometer. The component parts consisted of a Leitz monochromator, Ortholux microscope, and photomultiplyer with an EMI type 6094A attaching phototube. Cell measurements were made by the two-wavelength method described by Ornstein (27) and Patau (30), at 600 mμ or 570 mμ for the methylene blue and at 550 mμ or 500 mμ for the Feulgen reaction. Fig. 2 and Fig. 3 show the absorption curves for these reactions, from which the suitable wavelengths were determined.

2. Analysis of data.

   The computations for the determination of the amount of chromophore bound in individual cells were carried out on the CDC 6400 computer at the University of Arizona.

Experimental Procedure for Radioautography.

Coverglass cultures were inoculated with 0.4 ml of the undiluted virus suspension, and the virus was allowed to adsorb for one hour at 37C. Following the adsorption period, the coverglasses were washed in BSS, and 1 ml of maintenance medium was added to each culture.

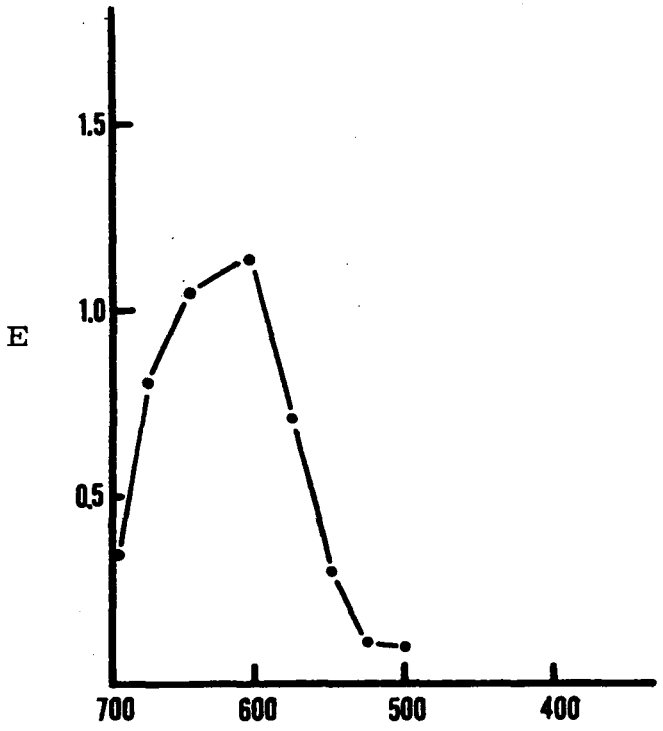

Fig. 2. The methylene blue absorption curve.

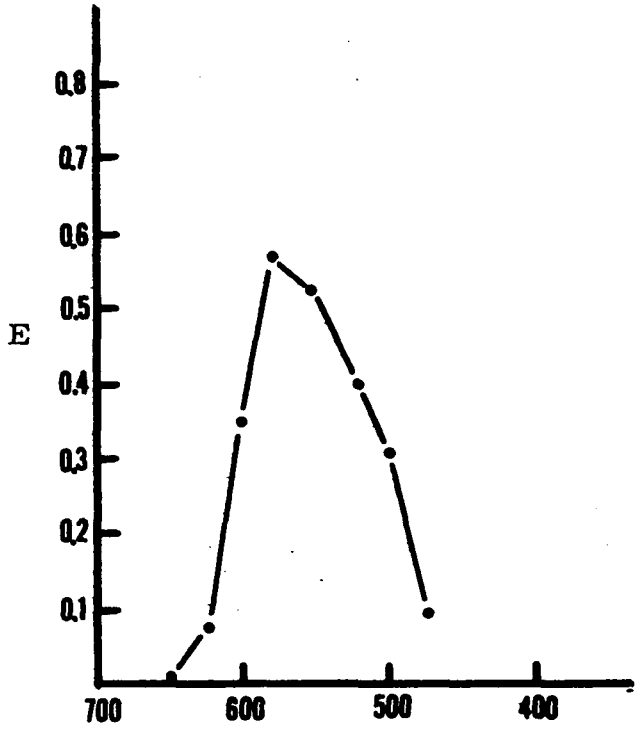

Fig. 3. The Feulgen absorption curve.

They were incubated at 37C; at periodic intervals the cells were pulsed with 0.1 μC of either tritiated thymidine or tritiated uridine for twenty minutes. Following pulsations, the coverglasses were washed several times in warm BSS, fixed in absolute methanol for fifteen minutes, allowed to air dry, mounted on slides in Permount, and stored at room temperature until processed as follows:

1. Eastman Kodak's nuclear tract emulsion, NTB3, was melted in a water bath at 43C, in the dark room. The slides were then coated with the emulsion and allowed to air dry for one hour.
2. The dry slides were placed into black, light-tight boxes, which were sealed with black friction tape.
3. The tritiated thymidine and uridine slides were exposed at 4C for four and three days, respectively.
4. Following exposure, the slides were developed in Eastman Kodak's D 19 developer for five minutes, placed in SB5a stop bath for fifteen seconds, fixed in Eastman Kodak's fixer for five minutes, and washed in running tap water for twenty minutes.
5. The cells were stained in 0.25 per cent toluidine blue for four minutes, destained in 70 per cent ethanol for five seconds, and air dried.

6. The uptake of isotope was measured by counting the number of grains per cell and expressed as the ratio of the mean grain count of virus-inoculated to non-inoculated cells.

Statistical Method of Analysis

Analysis of variance by a factorial design was chosen for the data derived from both the cytochemical and radioautography experiments. This type of analysis allows the simultaneous study of the effects of several factors and their interactions. It was expected from the type of experimental situation that interaction might be significant, so the selection of a factorial design was mandatory.

The data are presented in analysis of variance tables in Appendix A. The raw data, i.e., the amounts of chromophore bound in relative units or mean grain counts, were entered into the computation. For illustrative purposes the data are also presented in graphs throughout the text, where a relative scale was chosen for the ordinate, with the results obtained for the controls set to unity. The 95 per cent confidence limits, as based upon the error mean square of the analysis of variance, are illustrated as an example in Fig. 4 only, where they are converted into the relative units used for the graphic presentation. One can see that the experimental error for these experiments is on the order of magnitude of 1 to 4 per cent.

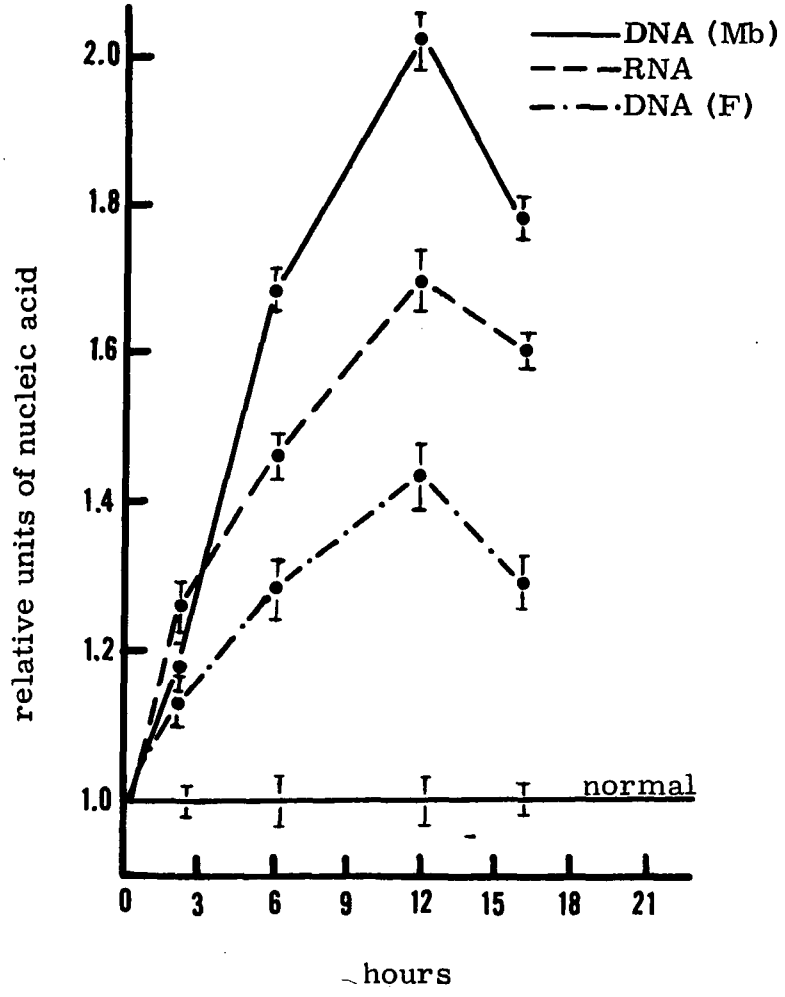

Fig. 4. The effect of Herpes simplex virus on nucleic acid metabolism as determined by cytochemical methods.

Chemicals and Isotopes

The basic fuchsin, C.I 42510, utilized for the Feulgen stain, was obtained from J. T. Baker Chemical Co. Methylene blue, No. B 341, was obtained from Matheson Co., Inc. Tritiated thymidine, 16.1 C/mMole, and tritiated uridine, 26.6 C/mMole, were purchased from New England Nuclear Corp., Boston, Mass. The nuclear tract emulsion, NTB3, was purchased from Eastman Kodak Co., Rochester, New York.

CHAPTER 3

RESULTS

The Effect of Herpes Simplex Virus on Nucleic Acid Metabolism

In each experiment a series of coverglass tube cultures was inoculated with a multiplicity of infection (MOI) of six; at periodic intervals, until cytopathogenicity (CPE) was observed, coverglasses were removed, fixed, and stained. Fig. 4 shows the effect of HSV on the nucleic acid metabolism utilizing the methylene blue and Feulgen stains.

The data from the methylene blue curves indicate that there is an initial increase in DNA at 2 hours post inoculation (PI). DNA increases to a maximum at 12 hours, followed by a decrease to 16 hours, when the first signs of CPE were observed. Table 1 shows the statistical analysis of variance for the DNA data. The RNA curve indicates a similar effect. Table 2 shows the statistical analysis of variance for the RNA data.

The data from the Feulgen curve simulates the methylene blue data for DNA synthesis. There is an initial increase, with the maximum effect at 12 hours, followed by a decrease to 16 hours. Table 3 shows the statistical analysis of variance for this DNA data.

Fig. 5 shows the effect of HSV on nucleic acid metabolism utilizing tritium radioautography. The tritiated thymidine curve for DNA metabolism indicates a sharp increase in the uptake of label from 2 hours PI to a maximum at 12 hours. At this time the amount of uptake decreases to a point which, at 16 hours, is still 88 per cent above normal uptake. Table 4 shows the statistical analysis of variance for this DNA data. The tritiated uridine curve for RNA metabolism shows an initial increase in uptake of label to 6 hours. From 6 to 12 hours the level of uptake remains somewhat constant; at 16 hours there is a slight decrease. Table 5 shows the statistical analysis of variance for this RNA data.

The Effect of Western Equine Encephalomyelitis Virus on Nucleic Acid Metabolism

In each experiment the cultures were inoculated with an MOI of twenty; coverglass preparations were removed, fixed, and stained periodically until CPE was observed. Fig. 6 shows the effect of WEE infection on nucleic acid metabolism, utilizing the methylene blue and Feulgen stains.

The data from the methylene blue curves indicate that there is no effect on DNA metabolism. Table 6 shows the statistical analysis of variance for this DNA data. RNA, after an initial increase to 2 hours PI, remains somewhat constant to 6 hours. From 6 to 12 hours, the RNA increases to maximum, followed by a decrease at 16 hours,

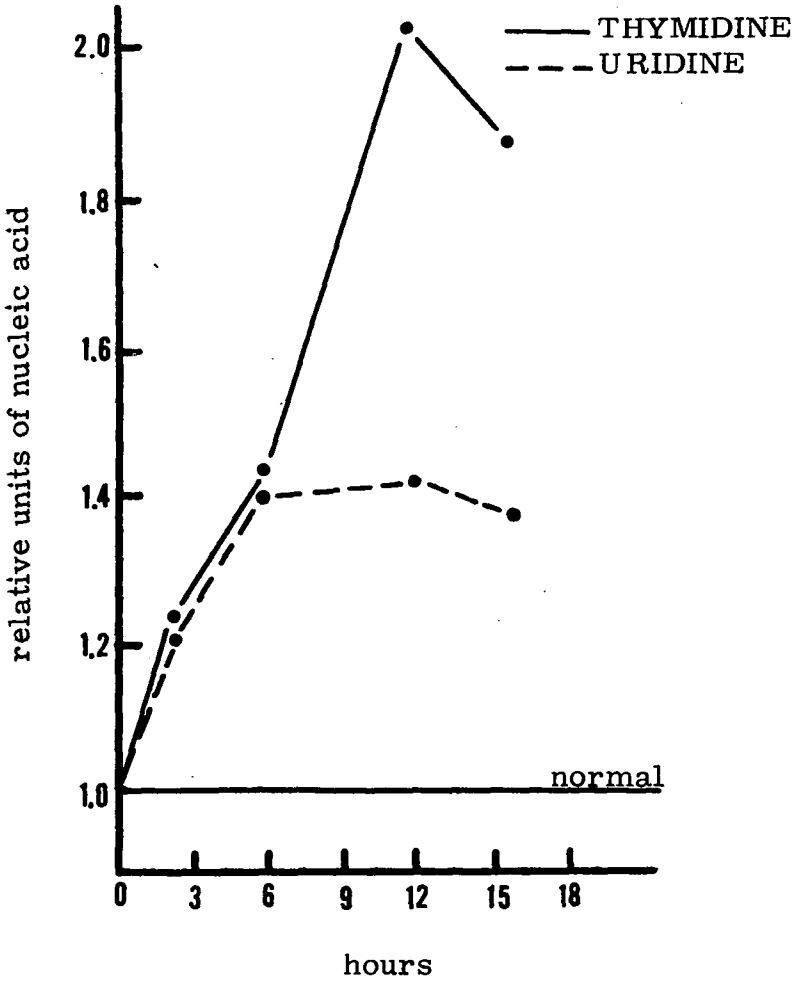

Fig. 5. The effect of Herpes simplex virus on nucleic acid metabolism as determined by tritium radioautography.

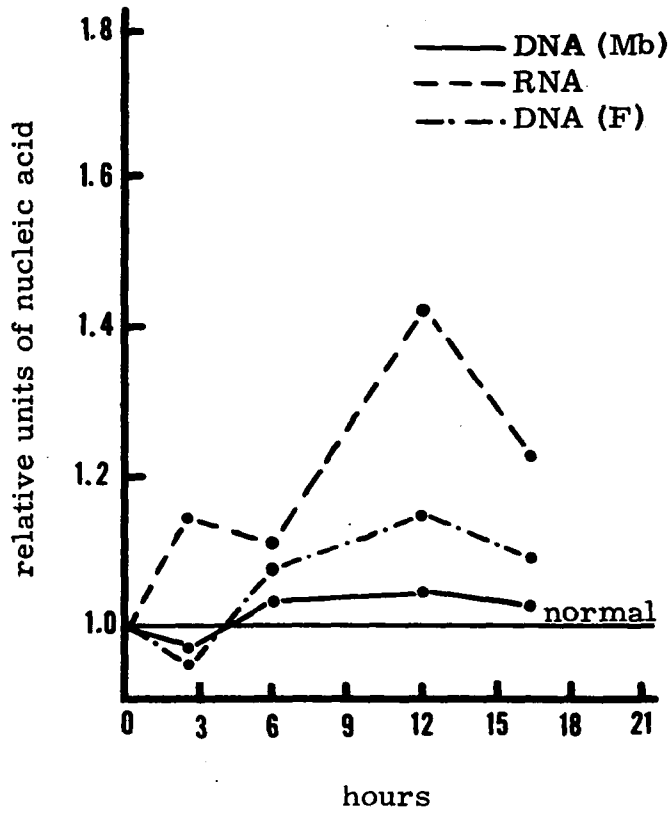

Fig. 6. The effect of Western equine encephalomyelitis virus on nucleic acid metabolism as determined by cytochemical methods.

when the first signs of CPE were observed. Table 7 shows the statistical analysis for this RNA data.

The data from the Feulgen curve indicate that there is no effect on DNA metabolism; however, the statistical data indicate an effect on DNA metabolism. This effect is found in the time factor and not in the virus-cell interaction factor. Table 8 shows the statistical analysis of variance for this DNA data.

Fig. 7 shows the effect of WEE on nucleic acid metabolism using tritium radioautography. There is no difference in the uptake of tritiated thymidine between virus-inoculated and normal cells, indicating that WEE has no effect on DNA metabolism. Table 9 shows the statistical analysis of variance for this DNA data. The tritiated uridine curve for RNA metabolism shows an initial increase at 2 hours PI. From 2 to 6 hours there is a slight decrease in the uptake of label, followed by an increase, reaching a maximum at 12 hours. From 12 to 16 hours there is a decrease in the uptake of label. Table 10 shows the statistical analysis of variance for this RNA data.

The Effect of Type I Poliovirus on Nucleic Acid Metabolism

In each experiment the cells were inoculated with an MOI of twenty; coverglass preparations were removed, fixed, and stained at periodic intervals up to 16 hours PI. At no time was there any evidence of CPE, even in coverglass cultures held for 72 hours or longer.

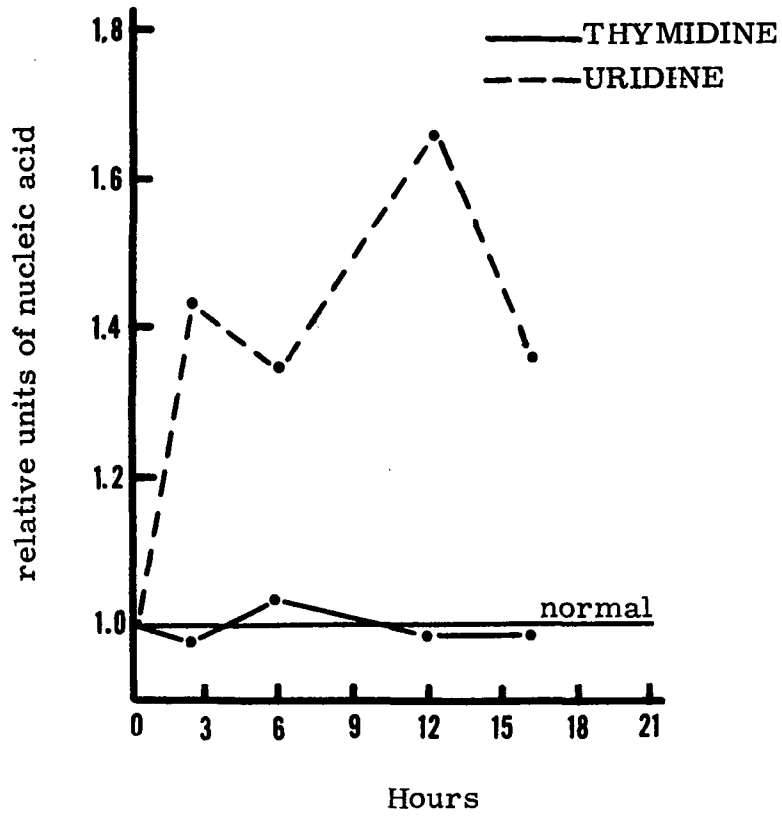

Fig. 7. The effect of Western equine encephalomyelitis virus on nucleic acid metabolism as determined by tritium radioautography.

Fig. 8 shows the effect of Type I poliovirus on nucleic acid metabolism utilizing the methylene blue and Feulgen stains.

The data from the methylene blue curves indicate that Type I poliovirus has no effect on DNA metabolism. Table 11 shows the statistical analysis of variance for this DNA data. The RNA curve indicates that there is no effect on RNA metabolism; however, the statistical data indicate that there is an effect on RNA metabolism. This effect is found in the time factor and not in the virus-cell interaction factor. Table 12 shows the statistical analysis of variance for this RNA data.

The data from the Feulgen curve indicate that there is no effect on DNA metabolism; however, the statistical data indicate an effect on DNA metabolism. This effect is found only in the time factor. Table 13 shows the statistical analysis of variance for this DNA data.

Fig. 9 shows by tritium radioautography that there is no difference between virus-inoculated and non-inoculated cells in the uptake of tritiated thymidine. Table 14 shows the statistical analysis of variance for this DNA data. The uridine curve indicates that there is no effect on RNA metabolism. Table 15 shows the statistical analysis of variance for this RNA data.

The Effect of Friend Leukemia Virus on Nucleic Acid Metabolism

In each experiment the cells were inoculated with an MOI of one; coverglass preparations were removed, fixed, and stained at

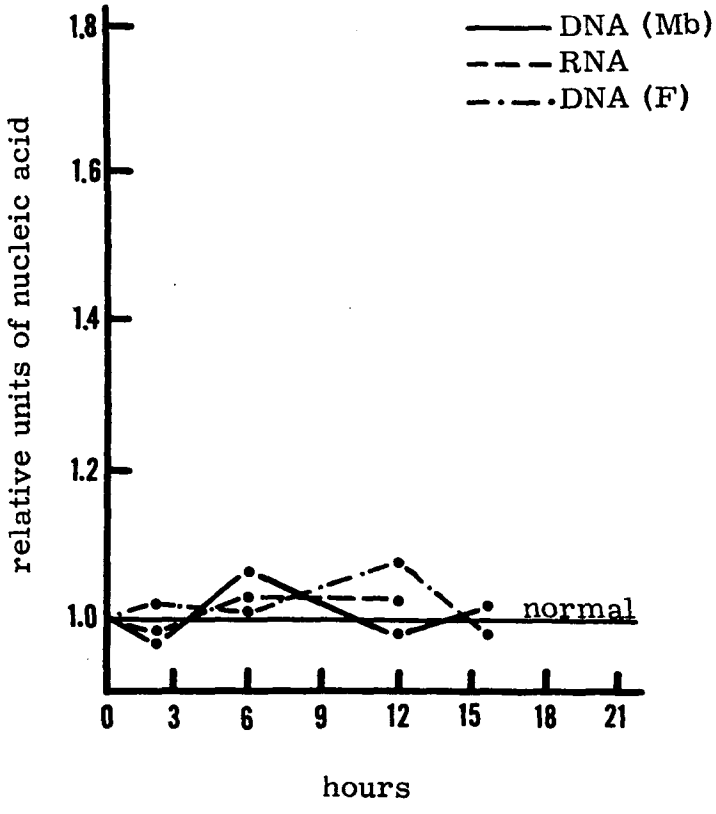

Fig. 8. The effect of Type I poliovirus on nucleic acid metabolism as determined by cytochemical methods.

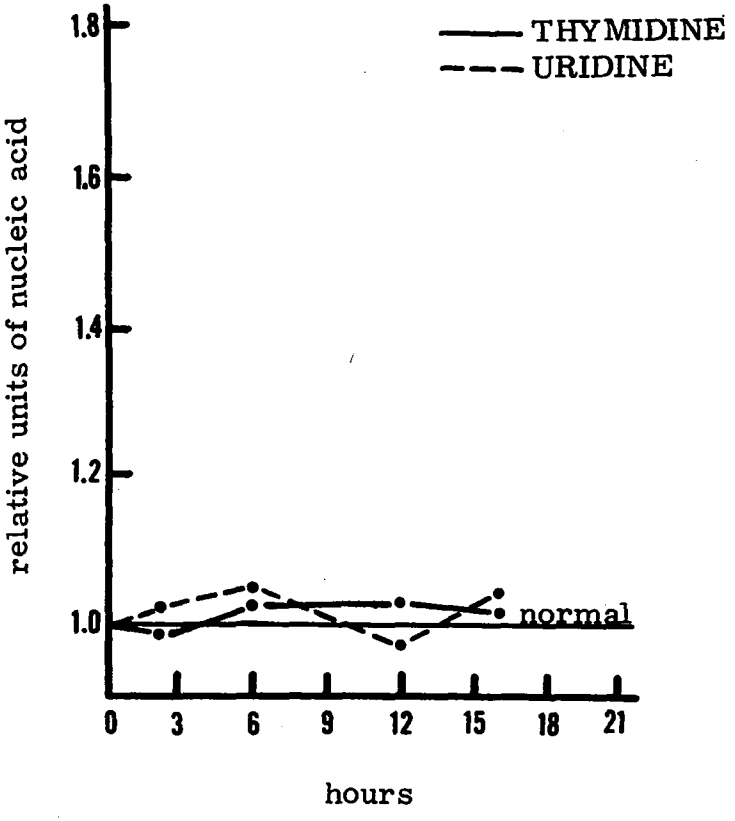

Fig. 9. The effect of Type I poliovirus on nucleic acid metabolism as determined by tritium radioautography.

periodic intervals up to 24 hours, PI. There was no evidence of CPE in any preparation, even in cultures held for nine days or longer. Furthermore, the pooled cells and supernatant of inoculated cultures, injected intraperitoneally into BALB/c mice, were negative. Fig. 10 shows the effect of FLV on nucleic acid metabolism utilizing the methylene blue and Feulgen stains.

The data from the methylene blue curve indicate an initial increase in DNA at 2 hours PI, followed by a decrease to 6 hours; when an increase in DNA starts, reaching a maximum at 12 hours. By 24 hours, the DNA level has decreased to normal. Table 16 shows the statistical analysis of variance for this DNA data. The RNA curve indicates an initial increase in RNA at 2 hours PI. The RNA remains constant from 2 to 6 hours, at which time there is an increase which reaches a maximum at 12 hours. Table 17 shows the statistical analysis of variance for this RNA data.

The data from the Feulgen curve simulates the methylene blue curve, with an increase in DNA reaching a maximum at 12 hours. By 24 hours there is no difference between virus-inoculated and non-inoculated cells. Table 18 shows the statistical analysis of variance for this DNA data.

Fig. 11 shows the effect of FLV on nucleic acid metabolism using tritium radioautography. There is no difference in the uptake of tritiated thymidine up to 6 hours PI. At that time there starts an

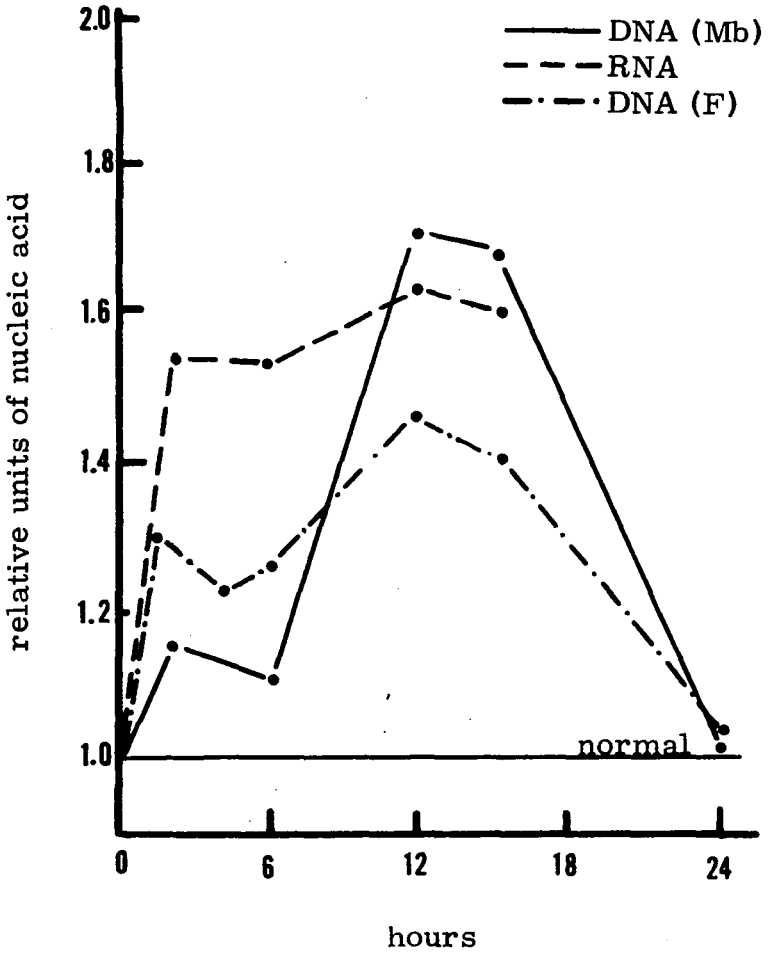

Fig. 10. The effect of Friend leukemia virus on nucleic acid metabolism as determined by cytochemical methods.

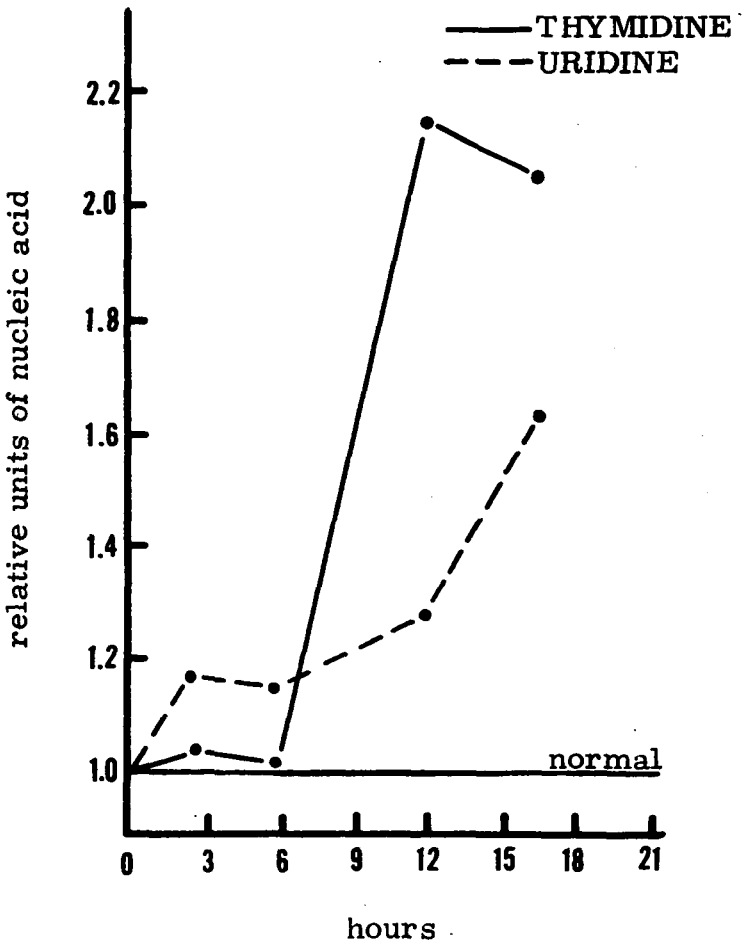

Fig. 11. The effect of Friend leukemia virus on nucleic acid metabolism as determined by tritium radioautography.

increase reaching a maximum at 12 hours and followed by a decrease at 16 hours. Table 19 shows the statistical analysis of variance for this DNA data. The data from the tritiated uridine curve shows that there is an initial increase in uptake of label at 2 hours. From 2 to 6 hours the uptake remains somewhat constant, followed by an increase in uptake which reaches a maximum at 16 hours. Table 20 shows the statistical analysis of variance for this RNA data.

Since FLV takes a minimum of 4 days PI to produce a histopathological effect in BALB/c mice, coverglass preparations were inoculated with FLV and allowed to progress for 6 days. No CPE was observed in any preparation during the six-day period, and the cells as well as the supernatant failed to produce leukemia when injected intraperitoneally into BALB/c mice. Fig. 12 shows the effect of FLV on nucleic acid metabolism during the six-day interval, utilizing the methylene blue stain.

The data indicate that from 1 to 6 days PI, there is no effect on DNA metabolism. Table 21 shows the statistical analysis of variance for this DNA data. In contrast, the RNA curve shows an increase at 2 days, reaching a maximum at 3 days. There is a decrease in RNA from 3 to 5 days, at which time there is no difference between the virus-inoculated and non-inoculated cells. Table 22 shows the statistical analysis of variance for this RNA data.

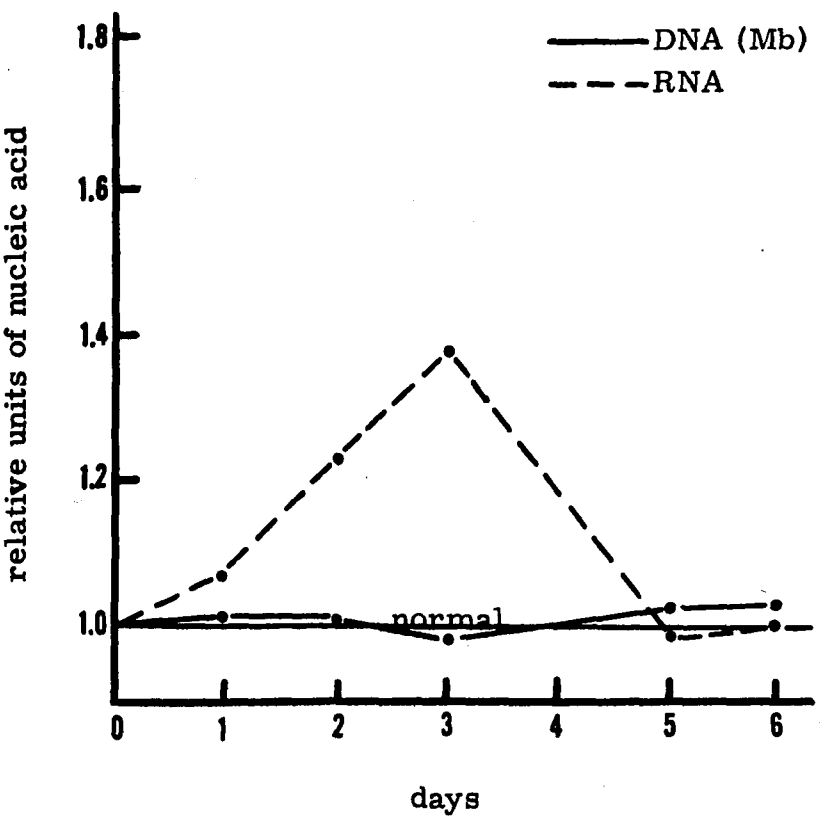

Fig. 12. The effect of Friend leukemia virus on nucleic acid metabolism over a six day interval as determined by cytochemical methods.

CHAPTER 4

DISCUSSION

The F120E90 cells were used for this study because of their rapid growth characteristics, virus susceptibility, and diploid nature, i.e., DNA constancy. Of diploid fibroblasts, Levine et al. (18) said, "When normal diploid fibroblasts growing on a glass surface come into contact, an adhesion forms and cell movement in that direction stops. As the resulting 'monolayer' of diploid cells becomes confluent, the rate of DNA and RNA and protein synthesis is progressively depressed." From his statement it can be seen that changes in nucleic acid invoked by viral effect can be more easily detected within the individual cells of the diploid population than would be possible within the individual cells of a polyploid population, which are characterized by varying amounts of DNA and an excessive growth potential.

The cytochemical data provides evidence that for HSV-inoculated cells, the greatest effect on DNA metabolism is from 2 to 12 hours PI, which probably corresponds to that interval for maximum viral DNA synthesis. These results are in agreement with Darlington and Moss (6), Roane and Roizman (33), and Feldman, Sheppard, and Bornstein (10), all of whom used HSV in different cell systems and

determined the effect on nucleic acid metabolism by methods other than cytochemical. The effect at 2 hours is probably due to the initial stimulatory response of the cell following adsorption and penetration of the viral genome. The RNA increases at 2 hours and reaches a maximum at 12 hours PI. Since RNA is necessary for protein synthesis of capsid, it can be assumed that the RNA increase is needed to complete the viral cycle. Tamm and Bablanian (43) have shown that RNA synthesis is necessary for the replication of vaccinia virus, adenovirus, and herpes simplex virus.

The results of the radioautography confirm and corroborate the cytochemical data. Both DNA and RNA increase in the early stages of infection, as shown by the increase in uptake of tritiated thymidine and tritiated uridine, respectively. There is a decrease in the uptake of thymidine between 12 and 16 hours PI. This is probably due to the cellular shutdown mechanism, in that there is sufficient pooled thymidine to complete the viral requirements for viral DNA synthesis. These results for HSV are consistent with the fact that HSV is a DNA cytolytic type virus for the F120E90 cell line.

The methylene blue data shows that WEE virus has no effect on DNA metabolism. There is no difference in the DNA curves between the virus-inoculated and non-inoculated cells. This would be expected since WEE is an RNA virus which has not been implicated as an RNA-DNA mediated-type virus. In contrast, the Feulgen data seem

to indicate an effect on DNA metabolism. Statistical analysis of this data, Table 8, provides evidence that this effect must be attributed to the time factor and is caused by a time-dependent trend in the binding of chromophore. It is not attributable to virus-cell interaction. The RNA increases from 6 to 12 hours PI. This increase probably corresponds to viral RNA synthesis as well as RNA-dependent protein synthesis, since WEE is a cytolytic virus for F120E90 cells. These findings are in agreement with Sreevalsan and Lockart (38), Sreevalsan and Allen (39), and Dulbecco and Vogt (8) who have shown that viral RNA of WEE is synthesized between 5 and 12 hours PI. These authors used density gradient centrifugation and plaque assay, respectively, as the assay method for viral RNA. Friedman, Levy, and Carter (12) have shown that viral RNA of Semliki Forest Virus, a Group A arbovirus which remarkedly resembles WEE in growth characteristics, is synthesized in infected cells between 3 and 9 hours PI.

The results of the radioautography confirm and corroborate the cytochemical data. There is no effect on DNA metabolism, and RNA increases in the early stages of infection between 6 and 12 hours PI.

Type I poliovirus was used in this study as a negative control. It is purported that poliovirus will replicate only in primate tissue cultures. Soloviev et al. (37) has shown that the mechanism of

resistance to some primate as well as cells of other species is due to the inability of the virus to adsorb; this property is genetically controlled. Therefore, poliovirus should not be cytolytic or have any effect on nucleic acid metabolism of mouse tissue cultures. The cytochemical DNA data and the radioautography indicate that Type I poliovirus has no effect on either DNA or RNA. The effects shown by the methylene blue RNA and Feulgen data were found to be associated with the time factor. They are not attributable to the virus-cell interaction. No cytopathogenicity of poliovirus was observed in the F120-E90 cells.

Moore and Friend (25) reported soon after the discovery of Friend leukemia virus that this virus would not replicate in tissue cultures. Cells and supernatant from inoculated cultures were not infective in susceptible animals, and thus it followed that FLV neither replicated nor altered the inoculated cells. In this study, the results of the cytochemical determinations and the radioautography for nucleic acid changes indicate that in FLV inoculated cells both DNA and RNA metabolism were affected. Virus-inoculated cells showed an increase in DNA beginning at 6 hours PI and reaching a maximum at 12 hours. The DNA decreased at 24 hours to a normal value and remained normal throughout a six-day study interval. These findings are in agreement with Inui and Oota (15) who, in 1965, using the Feulgen stain, reported that spleen cells from FLV-infected BALB/c mice

showed an increase in DNA. Dulbecco, Hartwell, and Vogt (9) in 1965 and Sheinin and Quinn (35) in 1965 reported that polyoma virus, an oncogenic virus, increased cellular DNA during the early phase of infection prior to viral DNA synthesis in infected tissue culture cells. Henry et al. (14) in 1966 reported that SV40 stimulated cellular DNA synthesis at 14 to 24 hours PI in infected tissue culture cells. Sundelin (41) in 1967 reported the Rous Sarcoma Virus, an RNA virus, stimulated cellular DNA synthesis, as shown by Feulgen staining, in chick embryo cells. Bolognesi et al. (2) in 1968 reported that strain MC29 Avian Leukosis Virus, an RNA virus, increased DNA in chick embryo cells as shown by tritiated thymidine uptake. Osato et al. (28) in 1966 reported that FLV transformed mouse embryo cells at 60 days PI. The early effect on DNA metabolism found in this study may correspond to the alterations involved in the transformations of cells which do not manifest themselves for a period of 60 days.

The RNA metabolism was also affected by FLV. The early increase in RNA from 2 to 16 hours PI probably corresponds to a stimulatory effect following adsorption and penetration of the viral genome. This initial effect was followed by a latent phase in which no changes were detected for 20 hours, at which time the RNA began to increase, reaching a maximum at 3 days PI. This secondary increase probably corresponds to a period of viral RNA and RNA-dependent protein synthesis. Silber (36) in 1967 reported that in FLV-infected mice,

RNA, as measured by ^{14}C-uridine uptake, was maximum at 4 days PI. Moore (26) and Osato, Mirand, and Grace (29) have reported that mouse embryo tissue culture cells were susceptible to FLV, but CPE was not observed. These authors found that cell sonicates and supernatants of FLV inoculated cells were leukemogenic in susceptible animals. It would seem that these results lend credence to the findings concerning RNA metabolism described in this study; however, cell sonicates and supernatants from FLV inoculated cultures in the present study were not leukemogenic in susceptible animals. One possible explanation for this discrepancy could be that even though viral RNA was synthesized, the mechanism of encapsidation was defective, thereby preventing the formation of mature viral particles necessary for leukemogenic activity in vivo. Takahashi, Van Hoosier, and Trentin (42) in 1966 reported that type 12 adenovirus, a cytolytic and oncogenic virus, has an incomplete growth cycle in hamster cells in which viral DNA is synthesized; however, mature viral particles are not produced. Rous sarcoma virus has been shown to be defective in that the virus can infect chick embryo cells and synthesize viral RNA, but mature viral particles are not produced due to a defective mechanism in encapsidation.

The results of the radioautography confirm and corroborate the cytochemical data. Both DNA and RNA increased in FLV-inoculated cells as compared with normal cells.

Overall, the data indicate that the methylene blue stain, the Feulgen stain and radioautography are sensitive, quantitative methods that can be used to detect viral activity in the form of nucleic acid changes within infected tissue culture cells. The validity of the Feulgen reaction and radioautography have been well established; however, prior to this communication, the methylene blue stain as a dual reactor of DNA and RNA had been utilized only in the determination of DNA-RNA ratios in non-inoculated HeLa cell nuclei. The data suggest that methylene blue can also be used, with quantitative results, to determine the effect of virus on tissue culture cells.

There were distinct patterns of nucleic acid changes that could be attributed to cytolytic viruses, providing these viruses adsorbed to and penetrated the cellular membrane. The predominant type of nucleic acid synthesized in infected cells was dependent upon the intrinsic nature of the infecting viral genome. These results are in agreement with Leuchtenberger (22). In contrast, Friend leukemia virus, a non-cytolytic virus, invoked both DNA and RNA synthesis in tissue cells. The oncogenic nature of this virus may account for the DNA changes observed, whereas the RNA changes may be invoked by the RNA genome of FLV.

CHAPTER 5

SUMMARY

It was established that methylene blue microspectrophotometry is a sensitive and accurate method that can be used to determine the effect of viruses on the nucleic acid metabolism of tissue culture cells. The Feulgen stain, long since established as a valid method for DNA quantitation as well as radioautography corroborated the methylene blue results. The effects of the various viruses, themselves, were found to agree with the current hypotheses concerning virus-cell interaction.

APPENDIX A

ANALYSIS OF VARIANCE TABLES

TABLE 1. Summary of Analysis of Variance for the Effect of HSV on DNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	4.550	689		
Treatments	2.279	7	0.3256	97.7771 **
virus x normal	1.745	1	1.7450	528.7878 **
time	0.286	3	0.0953	28.8787 **
virus x time	0.248	3	0.0826	25.0304 **
Error	2.271	682	0.0033	

** $P < 0.01$

TABLE 2. Summary of Analysis of Variance for the Effect of HSV on RNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	3.925	483		
Treatments	1.567	7	0.2240	45.2500 **
virus x normal	0.615	1	0.6150	125.5102 **
time	0.555	3	0.1850	37.7551 **
virus x time	0.397	3	0.1323	26.7272 **
Error	2.358	476	0.0049	

** $P < 0.01$

TABLE 3. Summary of Analysis of Variance for the Effect of HSV on DNA Metabolism as Determined by Feulgen stain.

Source	SS	df	ms	F
Total	0.216	308		
Treatments	0.068	7	0.0097	19.7759 **
virus x normal	0.037	1	0.0370	75.5120 **
time	0.011	3	0.0036	7.3469 **
virus x time	0.020	3	0.0066	13.4693 **
Error	0.148	301	0.0005	

** $P < 0.01$

TABLE 4. Summary of Analysis of Variance for the Effect of HSV on DNA Metabolism as Determined by Tritiated Thymidine.

Source	SS	df	ms	F
Total	4540.1	51		
Treatments	2375.1	7	339.30	6.8963 **
virus x normal	1348.8	1	1348.80	27.4146 **
time	517.3	3	172.43	3.5046 **
virus x time	509.0	3	169.66	3.4483 **
Error	2165.0	44	49.20	

** $P < 0.01$

TABLE 5. Summary of Analysis of Variance for the Effect of HSV on RNA Metabolism as Determined by Tritiated Uridine.

Source	SS	df	ms	F
Total	10.70	40		
Treatments	5.83	7	0.8328	5.6653 **
virus x normal	2.09	1	2.0900	14.1694 **
time	2.02	3	0.6733	4.5647 **
virus x time	1.72	3	0.5733	3.9010 **
Error	4.87	33	0.1475	

** $P < 0.01$

TABLE 6. Summary of Analysis of Variance for the Effect of WEE on DNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	1.700	272		
Treatments	0.064	5	0.0128	2.0850 ns
virus x normal	0.008	1	0.0080	1.3114 ns
time	0.031	2	0.0155	2.5285 ns
virus x time	0.025	2	0.0125	2.0226 ns
Error	1.636	267	0.0061	

TABLE 7. Summary of Analysis of Variance for the Effect of WEE on RNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	1.911	483		
Treatments	0.533	7	0.0761	26.3322 **
virus x normal	0.154	1	0.1540	53.1034 **
time	0.254	3	0.0846	29.1724 **
virus x time	0.125	3	0.0416	14.3448 **
Error	1.378	476	0.0029	

** $P < 0.01$

TABLE 8. Summary of Analysis of Variance for the Effect of WEE on DNA Metabolism as Determined by Feulgen stain.

Source	SS	df	ms	F
Total	0.252	239		
Treatments	0.030	7	0.0042	4.3750 **
virus x normal	0.002	1	0.0020	2.0833 ns
time	0.025	3	0.0083	8.6458 **
virus x time	0.003	3	0.0001	0.1041 ns
Error	0.222	232	0.0009	

** $P < 0.01$

TABLE 9. Summary of Analysis of Variance for the Effect of WEE on DNA Metabolism as Determined by Tritiated Thymidine.

Source	SS	df	ms	F
Total	115.20	44		
Treatments	9.70	7	1.3857	0.4860 ns
virus x normal	0.24	1	0.2400	0.0841 ns
time	8.37	3	2.7900	0.9786 ns
virus x time	1.09	3	0.3633	0.1274 ns
Error	105.50	37	2.8510	

TABLE 10. Summary of Analysis of Variance for the Effect on WEE on RNA Metabolism as Determined by Tritiated Uridine.

Source	SS	df	ms	F
Total	17.55	40		
Treatments	11.29	7	1.6128	8.5018 **
virus x normal	3.88	1	3.8800	20.4533 **
time	4.70	3	1.5666	8.3857 **
virus x time	2.71	3	0.9033	4.7793 **
Error	6.26	33	0.1897	

** $P < 0.01$

TABLE 11. Summary of Analysis of Variance for the Effect of Poliovirus on DNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	1.381	388		
Treatments	0.058	7	0.0083	2.3714 ns
virus x normal	0.001	1	0.0010	0.2857 ns
time	0.034	3	0.0113	2.9736 ns
virus x time	0.023	3	0.0076	2.1714 ns
Error	1.323	381	0.0038	

TABLE 12. Summary of Analysis of Variance for the Effect of Poliovirus on RNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	30.014	234		
Treatments	3.751	5	0.7502	6.5462 **
virus x normal	0.217	1	0.2170	1.8935 ns
time	3.018	2	1.5090	13.1675 **
virus x time	0.516	2	0.2580	2.2513 ns
Error	26.263	229	0.1146	

** $P < 0.01$

TABLE 13. Summary of Analysis of Variance for the Effect of Poliovirus on DNA Metabolism as Determined by Feulgen stain.

Source	SS	df	ms	F
Total	0.169	223		
Treatments	0.018	5	0.0036	4.5568 **
virus x normal	0.001	1	0.0010	1.2500 ns
time	0.014	2	0.0070	8.7500 **
virus x time	0.003	2	0.0015	1.8750 ns
Error	0.171	216	0.0008	

** $P < 0.01$

TABLE 14. Summary of Analysis of Variance for the Effect of Poliovirus on DNA Metabolism as Determined by Tritiated Thymidine.

Source	SS	df	ms	F
Total	3151.9	53		
Treatments	164.4	7	23.485	0.3615 ns
virus x normal	109.0	1	109.000	1.6784 ns
time	12.8	3	4.266	0.0655 ns
virus x time	42.6	3	14.200	0.2186 ns
Error	2987.5	46	64.940	

TABLE 15. Summary of Analysis of Variance for the Effect of Poliovirus on RNA Metabolism as Determined by Tritiated Uridine.

Source	SS	df	ms	F
Total	2.91	40		
Treatments	1.16	7	0.1657	3.1264 ns
virus x normal	0.14	1	0.1400	2.6415 ns
time	0.50	3	0.1666	3.1433 ns
virus x time	0.52	3	0.1733	3.2698 ns
Error	1.75	33	0.0530	

TABLE 16. Summary of Analysis of Variance for the Effect of FLV on DNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	1.470	368		
Treatments	0.500	7	0.0710	27.3076 **
virus x normal	0.320	1	0.3200	123.0769 **
time	0.120	3	0.0400	15.3847 **
virus x time	0.060	3	0.0200	7.6923 **
Error	0.970	361	0.0026	

** $P < 0.01$

TABLE 17. Summary of Analysis of Variance for the Effect of FLV on RNA Metabolism as Determined by Methylene blue.

Source	SS	df	ms	F
Total	5.303	397		
Treatments	1.722	7	0.2460	26.7971 **
virus x normal	0.896	1	0.8960	97.3913 **
time	0.704	3	0.2346	25.5000 **
virus x time	0.122	3	0.0406	4.4130 **
Error	3.581	390	0.0092	

** $P < 0.01$

TABLE 18. Summary of Analysis of Variance for the Effect of FLV on DNA Metabolism as Determined by Feulgen stain.

Source	SS	df	ms	F
Total	0.135	277		
Treatments	0.033	7	0.0047	12.7292 **
virus x normal	0.015	1	0.0150	40.5405 **
time	0.012	3	0.0040	10.8108 **
virus x time	0.006	3	0.0020	5.4054 **
Error	0.102	270	0.0004	

** $P < 0.01$

TABLE 19. Summary of Analysis of Variance for the Effect of FLV on DNA Metabolism as Determined by Tritiated Thymidine.

Source	SS	df	ms	F
Total	5897.5	54		
Treatments	3132.0	7	447.428	7.6041 **
virus x normal	843.5	1	843.500	14.3354 **
time	881.5	3	293.833	4.9937 **
virus x time	1407.0	3	469.000	7.9707 **
Error	2665.5	47	58.840	

** $P < 0.01$

TABLE 20. Summary of Analysis of Variance for the Effect of FLV on RNA Metabolism as Determined by Tritiated Uridine.

Source	SS	df	ms	F
Total	10.44	40		
Treatments	8.31	7	1.871	29.0077 **
virus x normal	2.38	1	2.380	36.8992 **
time	4.10	3	1.366	21.1782 **
virus x time	1.83	3	0.610	9.4573 **
Error	2.13	33	0.0645	

** $P < 0.01$

TABLE 21. Summary of Analysis of Variance for the Effect of FLV on DNA Metabolism During a Six Day Interval as Determined by Methylene blue.

Source	SS	df	ms	F
Total	1.550	94		
Treatments	0.280	9	0.0311	1.8791 ns
virus x normal	0.030	1	0.0300	2.0314 ns
time	0.070	4	0.0175	1.1745 ns
virus x time	0.180	4	0.0250	1.6778 ns
Error	1.270	85	0.0149	

TABLE 22. Summary of Analysis of Variance for the Effect of FLV on RNA Metabolism During a Six Day Interval as Determined by Methylene blue.

Source	SS	df	ms	F
Total	0.166	92		
Treatments	0.074	7	0.0105	9.7870 **
virus x normal	0.020	1	0.0200	18.1818 **
time	0.035	3	0.0116	10.5454 **
virus x time	0.019	3	0.0063	5.7272 **
Error	0.092	85	0.0011	

** $P < 0.01$

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