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# NUTRITIONAL STUDIES, IN VITRO, OF CHICK EMBRYO FIBROBLASTS INFECTED WITH ROUS SARCOMA VIRUS

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BERNARD A. BROWN, Jr.

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NUTRITIONAL STUDIES, IN VITRO, OF CHICK EMBRYO FIBROBLASTS INFECTED WITH ROUS SARCOMA VIRUS

APPROVED BY

H. Vernon Scott

Florence C. Kelly

John R. Schultz

E. C. Backus

A. T. Beyer

Philip E. Smith

DISSERTATION COMMITTEE

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# NUTRITIONAL STUDIES, *IN VITRO*, OF CHICK EMBRYO FIBROBLASTS INFECTED WITH ROUS SARCOMA VIRUS

## CHAPTER I

## INTRODUCTION AND HISTORY

Virus-host cell interaction is complex and difficult to study. Since viruses are so closely associated with the cell, an investigation of virus production involves an examination of this complex, and a study of the nutritional requirements of the virus-infected cell offers a good point at which to begin.

### The Rous Sarcoma Virus

Rous (1910) first investigated an avian neoplasm, a spindle-celled sarcoma of a barred Plymouth Rock chicken. He demonstrated that this neoplasm was transmissible by cell-free extracts of the tumor (Rous, 1911a; 1911b).

Keogh (1938) showed that inoculation of the Rous sarcoma virus onto the chorioallantoic membrane (CAM) of embryonated eggs resulted in the formation of ectodermal lesions, commonly called pocks. Rubin (1955) confirmed this and showed further that each tumor on the membrane is initiated by a single virus particle. This was established by demonstrating a linear correlation between the concentration of

virus in the inoculum and the number of tumors produced. Prince (1958a) and Vigier (1959) also confirmed the reliability of titration of Rous sarcoma virus on the CAM of embryonated eggs.

The first clear demonstration that chick embryo cells grown in culture were changed into the Rous sarcoma cells by infection with Rous sarcoma virus was given by Halberstaedter, Doljanski and Tenenbaum (1941). They exposed chick fibroblasts in culture to pieces of intensely irradiated Rous sarcoma cultures and observed cytological changes similar to those present in cultures of Rous sarcoma cells derived from a chicken sarcoma. Lo, Gey and Shapras (1955) succeeded in changing fibroblasts, in vitro, to rounded degenerating cells, many of which were hypertrophied. They also showed that the culture which contained changed cells produced virus for several months. Manaker and Groupe (1956) demonstrated discrete foci of changed cells upon treatment of monolayer cultures of chick embryo cells with Rous sarcoma virus. Temin and Rubin (1958) developed a method of assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. Foci of infection or infective centers which developed under agar overlay in these infected cultures, were composed of rounded cells growing in grape-like clusters.

## Nutritional Studies of Animal Cells in Culture

It has been shown that virus production by cultured cells of animal origin, varies with the composition of the medium. Before such studies can be undertaken, something must be known about the nutritional requirements of the animal cells in which the viruses are to be propagated.

A number of workers have studied the nutrition of animal cells. Eagle (1955c) first reported on the nutrition of animal cells in tissue culture and devised a medium which would support the growth of these cells. He determined that at least 12 amino acids were essential for the growth of HeLa cells. In Eagle's medium, which was deficient in purines and pyrimidines, fat soluble-vitamins and tissue extracts, and which was supplemented by five percent dialyzed serum, the cells required isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. These amino acids are also essential for man. In addition, arginine, cyst(e)ine, tyrosine, and histidine were also shown to be essential for multiplication of the HeLa cell. Alanine, aspartic acid, proline, hydroxyproline and serine proved to be non-essential, as determined by growth and multiplication of the cells. Failure of the cells to grow in a medium deficient in a single essential amino acid indicated that serum protein was not broken down sufficiently to provide either the essential amino acids or substances which could be substituted for them.

In two other studies, Eagle (1955b) and Eagle, et al. (1956) reported the nutritional needs of Earle's L cells and HeLa cells in culture. Glutamine was shown to be essential for both cell lines, and glutamic acid, ammonium ion and adenosinetriphosphate (ATP) failed to substitute for glutamine in permitting growth of the L cells. Ten to twenty times as much glutamic acid as glutamine was required for growth of the L cell. Proline, ornithine, alpha-ketoglutaric acid and asparagine failed to substitute for glutamine with either cell.

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cell. Proline, ornithine, alpha-ketoglutaric acid and asparagine failed to substitute for glutamine with either cell.

Eagle (1955a) also established the requirement of HeLa and L cells in culture for the water-soluble vitamins, choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin and thiamin. He was unable to demonstrate any requirement for an exogenous supply of fat-soluble vitamins, probably due to unexhausted cellular stores of the vitamin, its precursor or conjugates in which it is incorporated. If the cells were not grown too long in a vitamin-deficient medium, the cytopathogenic effect of the vitamin deficiency was reversible. Nicotinic acid had the same qualitative effect as nicotinamide, and pyridoxal, pyridoxine, pyridoxamine and pyridoxal phosphate were similarly interchangeable although not quantitatively equivalent. Biotin, tocopherol, vitamins A, D, K, inositol and lipoic acid were not essential. Vitamin precursors and conjugates diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin mononucleotide, flavin adenine dinucleotide, coenzyme A and cocarboxylase had not been evaluated at that time for their activity as substitutes as corresponding vitamins.

Pasieka, Morton and Morgan (1958b) studied amino acid changes in the medium during cultivation of L cells. Chromatographic technics were used to study the patterns of uptake and accumulation of amino acids in medium 150 (medium 199 prepared in Hanks balanced salt solution with an increase in bicarbonate ion concentration). They found that alanine accumulated in the medium and that if glutamine was omitted from the medium there was an increased uptake of the other amino acids

which resulted in the prevention of the accumulation of amino acids in the medium. However, omission of glutamine and glutamic acid from the medium resulted in cessation of amino acid changes in the medium. Adenine was taken up from the medium with a small increase in production of hypoxanthine and these changes were not altered by changes in the concentration of amino acids or content of the medium. There was no appreciable decrease in cell population or apparent degeneration of the culture area over a 30 day period when glutamine and glutamic acid were omitted from the medium. Pasieka, Morton and Morgan (1956) also studied amino acid metabolism of chick embryonic heart fibroblasts by chromatographic technics and showed that after seven days there was a decrease of alanine in the medium and an increase in glutamic acid. After 19 days cystine, lysine, histidine, and arginine had been almost completely removed from the medium, and there was a slight decrease in isoleucine and leucine. Some changes in alanine, methionine, and valine also occurred. After 21 and 25 days cystine and the basic amino acids lysine, histidine and arginine were almost completely removed from the medium. By the twenty-sixth day amino acid utilization had decreased and the cells began to die. Tryptophan was taken up while methionine concentration increased in the medium. There was also some utilization of phenylalanine, tryptophan, isoleucine and lysine. Glutamine, serine, glycine, threonine, alanine and methionine increased in the medium. There was a slight increase in aspartic acid, hydroxyproline and tyrosine. Variable results were obtained with glutamic acid.

Morgan and Morton (1957), while studying amino acid requirements of chick embryonic heart fibroblasts, found arginine, histidine, lysine, tryptophan, phenylalanine, tyrosine, cyst(e)ine, methionine, threonine, leucine and valine to be essential and to disappear from the medium. Conversely, glutamic acid, aspartic acid and alpha alanine had an inhibitory effect. The latter group of amino acids were found by these workers to accumulate in the medium. Chick embryonic heart fibroblasts were shown to possess an active transaminating system which caused accumulation of excessive and possible inhibitory amounts of certain amino acids in the medium. Glutamine was not required nor was it essential. Cystine was shown to be specific for survival of chick embryo fibroblasts. Pasieka, Morton and Morgan (1958a) confirmed the above data, and studied amino acid content of the medium by paper chromatography.

Mouse L cells in a medium of lactalbumin hydrolysate and yeast extract in HBSS supplemented with a reduced concentration of Eagle's basic vitamin and amino acid mixture, as studied by Munyon and Merchant (1959), showed glucose uptake and rapid glycolysis during early stages of growth; the lactic acid production rate paralleled the rate of removal of glucose from the medium during the first part of the logarithmic growth period. Lactic acid is taken up by the cells while glucose is present in relatively high concentration. During the last half of the logarithmic growth period, lactic acid was taken up rapidly and the rate of glucose uptake was low. Lactic acid accumulated in the medium as the growth became constant. Later, lactate was reduced to low concentration and glucose was exhausted from the medium after

192 hours. These workers also correlated pH, wet weight, protein and packed cell volume.

In contrast to the work of Pasieka, Morton and Morgan (1956) and Morgan and Morton (1957), Fischer (1955) as well as Ehrensvard, Fischer and Stjernholm (1949), found that glutamine was required by chick embryo tissue.

## Nutritional Studies of Virus-Infected Cells

The propagation of animal viruses in tissue culture has enabled the investigator to make more precise studies of the dynamics of virus multiplication in infected cells and has permitted observation on the growth cycles of numerous virus-cell systems (Scott, et al., 1953; Vigier and Golde, 1959; Temin and Rubin, 1959, Dulbecco and Vogt, 1954).

That virus production in animal cell cultures is dependent upon the composition of the culture medium and that it varies according to whether or not the cells are deprived of certain components prior to infection, was ably demonstrated by Hare and Morgan (1954) in the case of psittacosis virus propagated in chick embryo cells which had been maintained in a deficient medium consisting of inorganic salts and glucose. These cells lost their ability to support growth of psittacosis virus, but this capacity could be restored by a variety of nutrient materials such as embryo extract or the synthetic medium 199 of Morgan, Morton, and Parker (1950). Morgan (1956) demonstrated that chick embryo cells maintained in a balanced salt solution for 13 days before infection with psittacosis virus and thereafter for an

additional 15 days in balanced salt solution would produce virus if beef embryo extract were added on the 16th day. This emphasized the close relationship of the virus to the cells, since the non-infective stage persisted until beef embryo extract was added. Johnson and Morgan (1956) determined that a latent infection of chick embryo tissue with psittacosis virus could be activated in medium 199 (Morgan, Morton and Parker, 1950) from which purines, pyrimidines, ATP, adenylic acid, ribose and deoxyribose had been omitted and in which cystine, glutathione and ascorbic acid concentrations had been increased. Inorganic salts, glucose, amino acids and water-soluble vitamins were stimulatory to production of virus. Cysteine, glutathione and ascorbic acid, singly or in combination, were not capable of stimulating virus production. These experiments were performed with medium containing colchicine which suppresses cellular multiplication. By establishing a latent infection with psittacosis virus in mouse fibroblasts (L cells) maintained in a deficient medium (balanced salt solution), Bader and Morgan (1958) demonstrated that tyrosine, threonine, methionine, isoleucine, phenylalanine, tryptophan, leucine, valine and cyst(e)ine are essential components of a medium which supports propagation of virus. Lysine, arginine, histidine, hydroxyproline, glutamic acid, aspartic acid, serine, alanine and glycine were not essential nor was glutamine required for stimulation of viral growth. It was determined that a glucose-deficient but otherwise complete medium would not stimulate virus production. In a similar study, Bader and Morgan (1961) found that thiamine was the only specific vitamin requirement for production

of psittacosis virus in L cells. Lack of production of virus in thiamine-deficient cells is associated with failure of the cells to recover from the morphological changes associated with the nutritional depletion. Thiamine is an important factor in metabolism of the alpha-keto acids and is probably necessary for aerobic oxidation of carbohydrates. Kuwata and Shiba (1955) observed that chick embryo cells grown in Tyrode's solution and infected with ornithosis virus would produce virus in the presence of D-glucose, but the quantity of virus produced gradually declined. These cells would not produce virus in a glucose-deficient medium, and neither amino acids nor folic acid would substitute for glucose. These authors recall Eaton's work (1952) which indicated that reproduction of rickettsia occurs when the cell metabolism is rather low.

Ackermann (1951) demonstrated the effect of methionine and methionine antagonists on production of type A PR-8 influenza virus by minced chick chorioallantoic membrane (CAM) in Warburg flasks. He observed that graded concentrations of methoxinine (a methionine analog) produced, in vitro, a proportional inhibition of the propagation of influenza virus, as measured by the titration of the virus in eggs and by hemagglutination. This was competetive inhibition and not virucidal activity since the inhibition of viral production could be reversed by addition of methionine. DL-ethionine was also shown to be competetively antagonistic to methionine.

Zinsser and Schoenbach (1937) presented evidence that optimal growth of equine encephalomyelitis virus was obtained in actively metabolizing tissue cultures. Later, Daniels, Eaton and Perry (1952)

showed that maximum infectivity titer of influenza virus was produced in 48 hours by CAM in culture, regardless of whether glucose was added to the medium. They also showed that de-embryonated egg tissue infected with the virus produced only small increments of hemagglutinin and infective virus in the absence of glucose. This effect of glucose deprivation could be reversed by addition of glucose to the de-embryonated egg at 48 hours, and to infected tissue culture as late as five days after infection. Decreased respiration was observed in glucose-deprived infected tissue cultures. Burr, et al. (1954-1955) demonstrated that cell cultures of CAM infected with type A influenza virus, type B influenza virus and mumps virus, produced virus equally well in medium 199, Earle's balanced salt solution, and Hanks-Swims' ultrafiltrate. If the cells were first starved in HBSS there was no difference in the production of virus. Previous authors had demonstrated a requirement for methionine in the biosynthesis of virus by the use of methionine analogs. Since glucose was the only source of organic supplement in the medium, it was concluded by these authors that the endogenous nutrients were more important than the exogenous ones.

Eagle and Habel (1956) reported the nutritional requirements for propagation of the virus of poliomyelitis in HeLa cells. They observed that the omission of vitamins, 12 of 13 essential amino acids, and of protein from a complete medium had only a minor effect on the amount of virus formed by these cells. Only two components other than inorganic salts were essential for the optimal propagation of polio virus, namely glucose and glutamine. If the cells were maintained in a

medium containing only salts, glucose and glutamine, they were capable of producing virus. If these cells were deprived of glucose or glutamine their ability to produce virus was greatly diminished. Glutamine was more effective than glucose in restoring the virus-producing capacity of glucose-deficient cells. Employment of $C^{14}$-labelled glucose and glutamine showed that the cells produced virus protein from their own protein or amino acid pool. Since virus is produced by infected cells in a glucose-glutamine-salts medium, the amino acids of the viral protein must come from the host-cells own protein or from its amino acid pool.

Darnell and Eagle (1958) in a similar study, showed that if used in a higher concentration, fructose could substitute for glucose. Substitution of galactose, ribose, pyruvate or ribose-5-phosphate or combinations of these substances, even at high level concentrations, elicited only slight virus production. High concentrations of glutamic acid could substitute for glutamine, but ammonium chloride did not augment production of virus with glutamic acid. Hsuing and Melnick (1958) demonstrated a bicarbonate ion requirement for plaque formation by monkey kidney cells under agar infected with certain strains of poliovirus. However, virulent strains of poliovirus could produce plaques (cytopathogenic effect) under agar in the presence of either high or low concentrations of bicarbonate. This requirement for the bicarbonate ion by attenuated strains of poliovirus was shown to be specific since adjustments of initial pH of the media by NaOH or $CO_2$ did not influence plaque formation by these strains.

Alteration in HeLa cell metabolism resulting from infection with polio virus was shown by Salzman, et al. (1959). They observed inhibition of net synthesis of ribonucleic acid (RNA), deoxyribonucleic (DNA) and protein to the extent that there was no measureable increase in the concentration of these components in the first six hours after infection, even though the cells were maintained in complete medium sufficient for optimal growth rates of uninfected cells. After six hours there was a sharp loss of cellular material, including a large amount of RNA, into the medium. There was no measurable loss of DNA, although 20 to 30 percent of the protein was released into the medium by the ninth to the twelfth hour.

Melnick, et al. (1957) used poliovirus to infect Patas and rhesus monkey kidney cells which had been cultivated in Rappaport's synthetic medium, SM-2. The infected cells were maintained in an inorganic salts-glucose medium and virus production was determined. It was not possible to show a vitamin requirement for monkey kidney cells during the first three weeks of in vitro life. The rate and amount of virus produced during the first 24 hours in a salt solution was independent of exogenous sources of nitrogen and carbon; all the energy for synthesis of virus was derived from endogenous reserves of the cell. After 24 hours glucose was required to maintain the cells in a salt solution. Pyruvate only partially satisfied the requirement for glucose. Cyst(e)ine and glycine were found to affect the course of virus propagation. Cysteine could serve as a source of pyruvate. Glycine, which is required for cell growth, inhibited virus propagation in a salt-glucose medium. The authors concluded that no general theory can yet be

advanced to explain the propagation of animal viruses.

Chang (1959) showed the bicarbonate ion was required by infected cells in order to produce poliovirus and Coxsackie virus. Bicarbonate is known to be a precursor of nucleic acids in many systems and ribonucleic acid is related to protein synthesis. Virus multiplication in human cells could be suppressed by bicarbonate depletion. Chang was able to show a bicarbonate requirement by HeLa and human conjunctival cells infected with Coxsackie and poliovirus. He raised the question as to whether this effect of bicarbonate deficiency might be due to the virus inducing formation of protein necessary for virus precursor production, or whether the cells failed to form viral nucleic-acid.

Bather and Pepper (1961), made the observation that healthy Rous sarcoma cells incorporated uridine-$H^3$ into the nucleus more rapidly than uninfected chick fibroblasts, and there was no evident RNA synthesis occurring in the cytoplasm during the $H^3$ uptake. During the transfer of the $H^3$ to the cytoplasm, some cells showed cytoplasmic radioactivity which could not be removed by ribonuclease (RNA-ase). The proportion of cells showing RNA-ase resistant radioactivity rose to a maximum by 12 hours and fell at 48 hours. This, the authors comment, may represent $H^3$ incorporation into Rous virus. The preceding observations are in keeping with the findings of Prince (1958b) who observed that Rous sarcoma virus lost its infectivity upon entering an ectodermal cell of the chorion, and that infective virus appeared about 16 hours later. Thereafter, the number of infectious

particles found in the CAM increased rapidly until 48 hours when the increase became more gradual, and paralleled the increase in weight of the infected membrane.

Levy and Baron (1956) recorded several observations concerning the metabolic effects of poliomyelitis viruses on tissue culture. They noted that, within 30 minutes after infection, the glycolytic activity of poliovirus-infected monkey kidney cells increased markedly, in contrast to uninfected kidney cells. These authors also noted that glycine 2-$C^{14}$ uptake was depressed early in infected cells, but not so in uninfected cells.

Herpes simplex virus infection of HeLa cells was shown by Newton and Stoker (1958) to result in an increase in DNA content within six to nine hours after infection, although infective virus was not detectable until 12 hours later. By 72 hours after infection the cells contained twice as much DNA as non-infected cells and it was confined to the nucleus. There was no detectable change in RNA content of the cells.

Rich, Eidinoff and Stonehill used 5-fluorodeoxyuridine (FUDR) and 5-fluorodeoxycytidine (FCDR) to study pathways of thymine synthesis, and the effect of these compounds on the replication of Rous sarcoma virus by chick embryo fibroblasts. Both of these compounds, at a concentration of 0.3 uM to 4.0 uM, were capable of inhibiting the growth of chick embryo fibroblasts. This inhibition was reversible by thymidine in a 0.4 uM concentration. Low concentrations of 5-methyldeoxycytidine (0.4 uM) were capable of partially reversing this

inhibition, while high concentrations (4.0 uM) completely abolished the inhibitory effect. Thymine was completely inactive in regard to reversal of inhibition by either FUDR or FCDR. The authors were of the opinion that these studies support the hypothesis that the primary site of inhibition by FUDR and FCDR, in chick embryo cells, is de novo pathway for synthesis of the thymine moiety. Preincubation of the cells with FUDR prior to infection with Rous sarcoma virus had little effect on subsequent development of Rous cell foci.

Tyndall and Ludwig (1960) observed that poliomyelitis, Coxsackie B-3 and vaccinia viruses were produced by rabbit kidney, chick embryo vascular epithelium, monkey heart and HeLa cells in a medium composed of balanced salt solution, amino acids of medium 199, glucose and L-glutamine, to the same extent that the virus was produced by cells in medium 199. They confirmed the report of Darnell and Eagle (1958) that HeLa cells in balanced salt solution plus glucose and glutamine produce a maximum yield of poliovirus. Furthermore, Tyndall and Ludwig reported that maximum production of type II poliovirus in monkey heart cells requires the addition of the amino acid group. They also determined that amino acids are not required for adsorption of Coxsackie B-3 virus to monkey heart cells, that cystine is required for production of Coxsackie B-3 virus by monkey heart cells, and that thioglycollate, ascorbic acid or glutathione cannot replace cyst(e)ine in this instance.

Ackermann (1951) demonstrated the requirement of L-methionine in the production of influenza A virus by chick CAM cultures. Ackermann,

Rabson and Kurtz (1954) have likewise demonstrated the reversal of fluorophenylaline inhibition of type III poliovirus production by excess phenylalanine, and Dubes (1956) noted a cystine requirement in order to produce the cytopathogenic effect in poliovirus-infected monkey kidney cells.

The present study was undertaken to determine some of the nutritional requirements of chick embryo fibroblasts infected with Rous sarcoma virus. It is not intended to be a complete investigation of the nutritional and metabolism of such cells. Rous sarcoma virus is capable of initiating malignancies in vivo. It is hoped that a study of the nutritional requirements of cells infected with this tumor virus may contribute to an understanding of neoplastic disease in general.

## CHAPTER II

## MATERIALS AND METHODS

### Preparation of Virus Inoculum

Rous sarcoma virus, lot #CT842, was obtained from Dr. W.Ray Bryan of the National Cancer Institute at Bethesda, Maryland, in the form of partially purified virus preparation, (Bryan, Maloney and Calnan, 1954; Maloney, 1956). Each vial contained 0.15 ml. of a suspension of virus in citrate buffer. This stock preparation was used for the production of tumors in five to six week old white Leghorn chicks. Each chick was inoculated in both wingweb areas with 0.1 ml. of the virus preparation which had been diluted 1:100 in Hanks' balanced salt solution (HBSS), (Hanks and Wallace, 1949). The tumors were allowed to develop for 10 to 12 days and were then harvested aseptically, quick-frozen and stored in glass containers at -62 C.

The virus was harvested by making a 10 percent suspension of the frozen tumor in HBSS, which was subjected to high speed homogenization at 21,000 rpm in a Vir-Tis tissue homogenizer for 90 seconds. The HBSS contained 0.01 percent hyaluronidase (Wydase)[1]. The entire preparation was kept cold during homogenization by surrounding the container with an ice bath. The resulting suspension was allowed to incubate for four hours at 4 C and then centrifuged at 10,000

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[1]Wyeth Laboratories, Inc., Philadelphia, Pennsylvania.

x g for 30 minutes at 4 C. The supernatant fluid was removed and diluted at 1:100 in HBSS. Penicillin and Streptomycin were added in final concentration of 100 units and 100 mcg per ml, respectively. No antimycotic agents were used. The preparation was quick-frozen and stored at -62 C until required for titration or infection of tissue culture. The virus harvested in this manner served as the inoculum for infecting secondary cell cultures of chick embryo fibroblasts.

## Preparation of Cell Cultures

Primary cell cultures of chick embryo fibroblastic cells were prepared by the method of Dulbecco and Vogt (1954). White Leghorn embryos which had been incubated 9 to 11 days were removed from the shells aseptically, and the feet, wings and head were removed. Embryo carcasses were minced finely with scissors and washed three times with warm (37 C) HBSS. The minced tissue was suspended in a solution of warm, 0.25 percent trypsin and incubated 10 minutes at 37 C with occasional agitation. The trypsin solution was decanted and discarded. The minced tissue was resuspended in a fresh volume of warm trypsin, transferred to a fluted trypsinizing flask on a magnetic stirring apparatus (Mag-Mix) and stirred by a teflon-coated magnetic stirring bar. Care was taken to avoid foaming and cavitation in the liquid. The contents of the trypsinizing flask were kept warm during the stirring process by immersing the flask in a bath of warm water on the magnetic stirring apparatus. After the tissue had been stirred 10 minutes the flask was removed from the Mag-Mix and the larger particles were allowed to settle to the bottom of the flask. The supernatant fluid,

containing the cell suspension, was filtered through several layers of sterile gauze and received in a 250 ml centrifuge bottle contained in an ice bath. Another fresh portion of the warm trypsin was added to the minced tissue in the trypsinizing flask and the agitation was again carried out as described. This trypsinizing procedure was repeated three of four times until a sufficient quantity of cells were obtained. The centrifuge bottle containing the cells was removed from the ice bath and the cells were sedimented by centrifuging at 1000 rpm in a PR-2 International refrigerated Centrifuge in Head #284. This procedure was carried out at 10 C for a period of 20 minutes. The supernatant fluid was removed aseptically and discarded. The cells were resuspended in cold HBSS and were again sedimented by centrifuging at 1000 rpm for a period of 20 minutes. Finally, the cells were resuspended in 20 ml of cold lactalbumin medium (LYTS-10) in HBSS.

An aliquot of the cell suspension was diluted 1:10 in a cell-counting fluid consisting of 0.1 percent crystal violet in 0.1 M citric acid (Sanford, et al., 1951). This cell suspension in counting fluid was incubated at 37 C for a period of 10 to 15 minutes before the cells were enumerated in a hemocytometer by direct microscopic observation. the number of cells per square was multiplied by 100,000 to determine the number of cells per ml in the stock suspension. In enumerating the cells, care was taken to count only those cells having both cytoplasm and a nucleus. The counting of nucleated red cells was avoided. Approximately 12 to 15 million cells from the stock suspension of cells were used to initiate primary cultures in rectangular, 200 ml bottles.

Sufficient LYTS-10 medium was added to bring the final volume to 12 ml in each bottle. The bottles were sealed with rubber-lined screw caps and incubated in a horizontal position at 37 C. No attempt was made to adjust the gas phase in these bottles with carbon dioxide.

After a period of four to five days incubation the cells in the bottles had grown to confluent monolayers and were ready for trypsinization. The medium was removed by aspiration and 10 ml of a warm trypsin solution were added to each bottle. The bottles were resealed and incubated in a horizontal position at 37 C until the cells began to come loose from the glass. The cell suspension from these bottles was pooled in several 50 ml centrifuge tubes and sedimented by centrifugation at 1000 rpm for a period of 20 minutes at 10 C. The trypsin was decanted and the cells, which had been pooled in a single 50 ml centrifuge tube, were resuspended in cold HBSS and centrifuged as before in Head #823. The HBSS was removed and the packed cells were resuspended in 20 ml of Medium 199, Medium G or HBSS containing five percent calf serum and enumerated as before.

Secondary cultures of these cells were prepared by seeding stationary 16 x 100 mm tubes with one ml of the medium containing $5 \times 10^5$ cells. Five tubes of cells were prepared for each virus sample. The tubes were sealed with rubber stoppers and incubated at 37 C in a tissue culture rack in the horizontal position to allow attachment of the cells to the glass.

After an incubation period of 16 to 18 hours, the medium was removed from the cells, the cells were washed once with warm HBSS and

the cell cultures were ready for inoculation with the virus suspension.

## Infection of Secondary Cell Cultures

The medium was removed from each tube by aspiration and the cell culture was washed once with one ml of warm HBSS per tube. The HBSS was removed from each tube by aspiration, and 0.1 ml of the stock virus suspension in HBSS was inoculated into each tube. The virus was allowed to adsorb to the cells for 40 minutes at 37 C. The fluid containing the unadsorbed virus was removed from each tube, pooled and labeled post-adsorption virus (PAV). This suspension was centrifuged at 1500 rpm for 10 minutes at 10 G to remove cells and debris (Head #823); the supernatant fluid was removed, transferred to another tube, quick-frozen and stored at -62 C until assayed. Residual, unadsorbed virus was removed from each tube by washing two times with one ml portions of cold HBSS per tube. These washings were pooled, centrifuged and labeled post-adsorption wash number one and number two ($PAW_1$ and $PAW_2$). The washings were quick-frozen and stored at -62 C until assayed for viral content. One ml of medium was added to the cell cultures in each tube, and the tubes were reincubated at 37 C. The medium added to these tubes was either Medium 199 with five or 10 percent calf serum or the experimental medium.

## Titration of Virus Produced in Tissue Culture

Every four hours from zero through 32 hours, the supernatant fluid was removed from five tubes, pooled, centrifuged to remove cells, quick-frozen and stored at -62 C until assayed. In some cases, samples

were taken every three hours, from zero to 30 hours or longer. Supernatant fluids were removed from tubes of infected cells which contained either test medium or control medium.

The control cultures were drawn from the same population of cells as the experimental cultures, and were treated like the experimental cultures. All control cultures had a complete medium replaced on them. Negative controls were inoculated with virus diluent only.

In those cases in which the virus content of cells was to be determined, after the supernatant fluid had been removed from the tubes and the cells had been washed, one ml of warm trypsin was added to each tube and the cultures were reincubated at 37 C until the cells came loose from the glass. This usually required five to 10 minutes. The suspension of cells from each of the five tubes was pooled and centrifuged. The sedimented cells were washed two times in cold HBSS, then finally resuspended in one ml of cold HBSS, quick-frozen and stored at -62 C until assayed. Before the virus content of the cells was determined the cells were subjected to three alternate cycles of quick-freezing and thawing. This resulted in the disruption of almost 100 percent of cells.

Determination of the presence of virus in supernatant fluids or of cell-associated virus is a biological assay in the case of Rous sarcoma virus since no enzymatic or chemical method is known. There are three methods for assay of Rous virus; (a) wing-web inoculation of chicks (Bryan, 1946a, 1946b), (b) inoculation of the chorioallantoic membrane of embryonated eggs (Keogh, 1938; Rubin, 1955; Prince, 1958a) and (c)

infective center formation in tissue culture (Temin and Rubin, 1958; Vigier, 1959). The inoculation of the chorioallantoic membrane (CAM) was selected as the most sensitive and the easiest to use. White Leghorn embryonated eggs, from pullets rather than from hens, which had been incubated 11 days, were used in the assay of the virus (Prince, 1958a). Six embryonated eggs were used for each sample of virus preparation or supernatant taken at a given time interval, and 0.1 ml of the sample was inoculated onto the CAM of the embryonated eggs.

The CAM were prepared by conventional methods while the egg was illuminated by an egg candler. A small hole was punched into the air sac end of the egg, and a second small hole was punched through the shell, but not through the shell membrane, at the equator of the egg over a well vascularized area. Gentle suction was applied to the hole at the air sac end of the egg while a needle was used to penetrate the shell membrane, and a false air sac was thus created by the dropped CAM. After the false air sac formed, a third hole was punched through the shell over the area of the dropped membrane. This served as a pressure relief exit when the inoculum was injected through hole number two.

After the inoculum had been deposited on the CAM, the two holes at the equator of the egg were sealed with sterile, hot paraffin and the egg was then rotated gently to distribute the inoculum over the surface of the membrane. Inoculated eggs were incubated, with dropped CAM uppermost, in a horizontal position at 37 C for seven days, in a non-humidified incubator in which the air was circulated by a fan.

Then they were chilled at 4 C for at least four hours to prevent bleeding, opened and the CAM removed. This membrane was washed in two changes of 0.85 per cent saline and stored at 4 C in Petri dishes containing a small volume of saline, until examined.

Enumeration of the plaques or pocks on each membrane was done by observation through a Bausch and Lomb stereomicroscope at a magnification of 10 x. Counting of plaques was facilitated by spreading the wet membrane on the bottom half of a Petri dish and by making a cut from the center of the membrane to one edge in order to flatten the membrane. As the membranes were observed microscopically, each plaque was marked with a red, water-soluble marking pencil. Every mark was recorded on a hand-tally. The arithmetic mean of the number of plaques on the six membranes which had received the same inoculum was calculated and multiplied by 10 to derive the number of plaque-forming units (PFU) per ml of inoculum. This number was recorded as the $\log_{10}$. The logarithms of the arithmetic means of the number of PFU were plotted, against the time in hours after adsorption, on semi-log graph paper. In control experiments the curve represented a one-step growth cycle.

Some of the membranes inoculated with a particular dilution had no pocks on them. If the average number of pocks per membrane was five or more, as calculated from the six membranes, the zeroes of the non-reactors were excluded. If this average did not total five, the zeroes of the non-reactors were used in the calculation of the arithmetic mean (Vigier, 1959).

## Experimental Animals

White Leghorn chicks five to six weeks of age were inoculated with the partially purified Rous sarcoma virus to cause the formation of tumors. The chicks were all Honegger white Leghorns from the Wendt flock, a selected flock from one chicken farm.

The embryonated eggs which served as a source for chick embryo cell cultures as well as for assay of virus, were from Honegger White Leghorns of the same flock.

## Reagents and Media

The standard medium used in the production of virus by the chick fibroblastic cells was medium 199 (Morgan, Morton and Parker, 1950) prepared in HBSS, with a bicarbonate concentration of 350 mg per liter, and supplemented with five percent undialyzed or dialyzed calf serum. Commercial (Difco)[1] and freshly prepared medium 199 were used in these experiments. The amino acid solutions were adjusted to a neutral pH by the slow addition of 1 N NaOH. Final pH of the medium was pH 7.2 to 7.4.

The medium used to initiate secondary culture of chick embryo cells, Medium G, was a growth medium containing the amino acids, balanced salt solution, glutamine, glutathione, ascorbic acid, phenol red, sodium bicarbonate and water in the concentrations found in medium 199. In addition, this medium was also supplemented with five percent dialyzed or undialyzed calf serum.

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[1]Digested Ferments Company, Detroit, Michigan.

Hanks' balanced salt solution was prepared according to the method of Hanks and Wallace (1949) and Earle's balanced salt solution according to the method of Earle (1943).

Trypsin solution, 0.25 percent, was prepared by the addition of 2.5 gm of trypsin 1:250 (Difco) to 1000 ml Earle's balanced salt solution (EBSS) from which calcium,magnesium, and $NaHCO_3$ had been omitted. The pH of this mixture was adjusted by the addition of 1 N NaOH to pH 7.3 to pH 7.4. This usually required the addition of approximately 0.9 ml of 1 N NaOH. This mixture was stirred on a magnetic stirring apparatus at room temperature for a period of 30 to 60 minutes. It was then incubated in a 37 C water bath for an additional 30 minutes, filter-sterilized, bottled, and stored in frozen state until required for use.

Lactalbumin hydrolysate medium, dissolved in HBSS, was composed of lactalbumin hydrolysate,[1] 0.5 percent, yeast extract,[2] 0.1 percent, tryptose phosphate broth,[2] 10 percent, and sodium bicarbonate, 0.35 percent. This medium designated as LYTS-10, was supplemented with 10 percent whole calf serum.

Pooled calf serum, obtained from a local abattoir, was used dialyzed or undialyzed, according to the design of the experiment. Dialysis was carried out against two changes, at 12 hour intervals, of HBSS from which glucose had been omitted. Fifty ml aliquots of the serum were dialyzed against 100 volumes of the glucose-free HBSS. The HBSS was changed at the twelfth hour, and the second period of dialysis was continued for an additional 12 hours. All dialysis was performed at 4 C.

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[1]Obtained from Nutritional Biochemical Company.

[2]Obtained from Difco, Detroit, Michigan.

## CHAPTER III

## EXPERIMENTAL RESULTS

### Titration of Virus

A new method of titration of Rous Sarcoma virus was attempted. A tissue culture method of assay utilizing the formation of micro-tumors, or foci, in cell cultures of chick fibroblasts, was developed. Figure 1 represents a typical monolayer culture of uninfected chick embryo fibroblasts after seven days incubation in lactalbumin medium. When this type of cell is infected with Rous sarcoma virus nodules or micro-tumors are formed, as depicted at a magnification of 100x in Figure 2 and at a magnification of 450x in Figure 3. It can be seen in this figure that the micro-tumor is composed of individual cells.

During careful microscopic examinations over a period of time, it was observed that the infected cells appeared to aggregate or pull together to form micro-tumors or foci of infection. The migration of individual cells of the monolayer into aggregates of cells which continue to replicate to form micro-tumors can be seen in Figure 4 (photomicrograph, 100x). In area A can be seen what appears to be strands of the monolayer pulling together to form a micro-tumor. There are other areas in this same figure where this phenomenon can be observed. Area B is an example of a young micro-tumor while C is a more fully developed one. A higher magnification of area C is depicted in Figure 5 in which

Figure 1. Photomicrograph of a monolayer of normal chick fibroblasts, magnification, 100X.

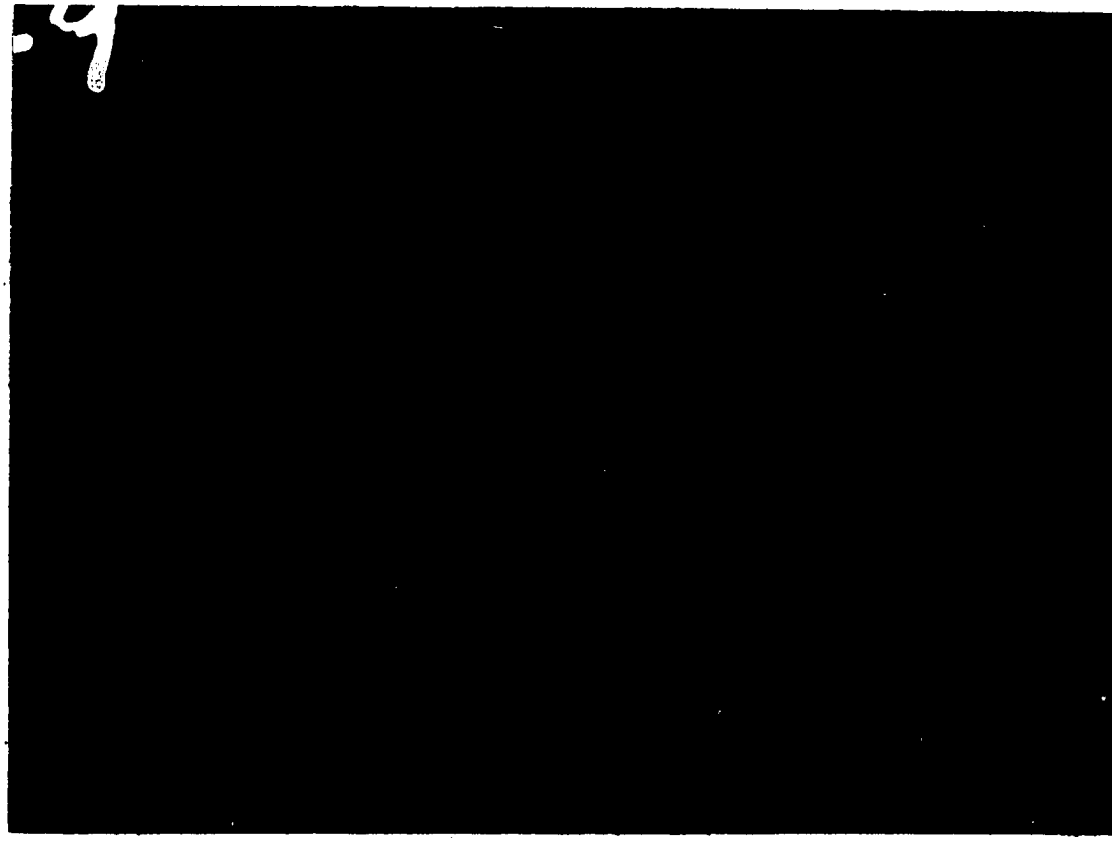

Figure 2. Photomicrograph of micro-tumor in chick fibroblasts, magnification, 100X.

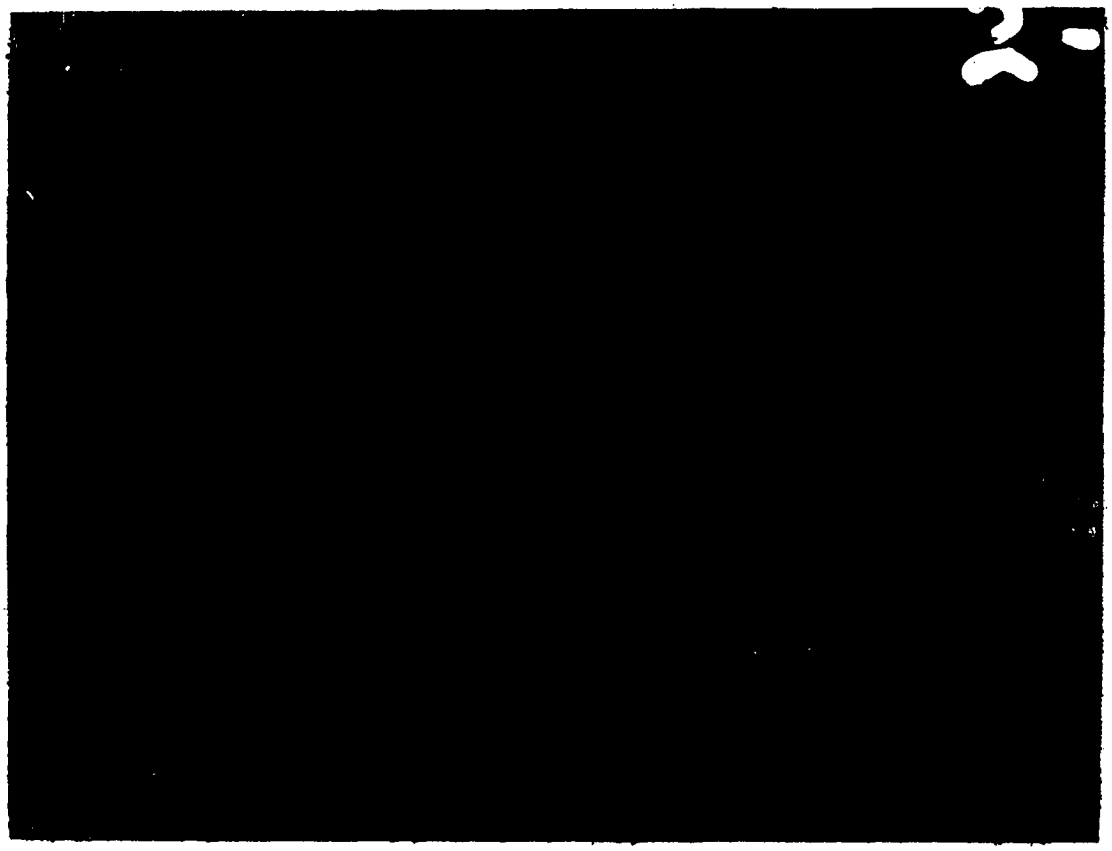

Figure 3. Photomicrograph of micro-tumor in chick fibroblasts, magnification, 450X.

Figure 4. Photomicrograph of cells in micro-tumor pulled away from the monolayer, 100X.

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A - strands of cells pulling away from the monolayer to form a micro-tumor.

B - a young micro-tumor

C - an older, more fully developed micro-tumor.

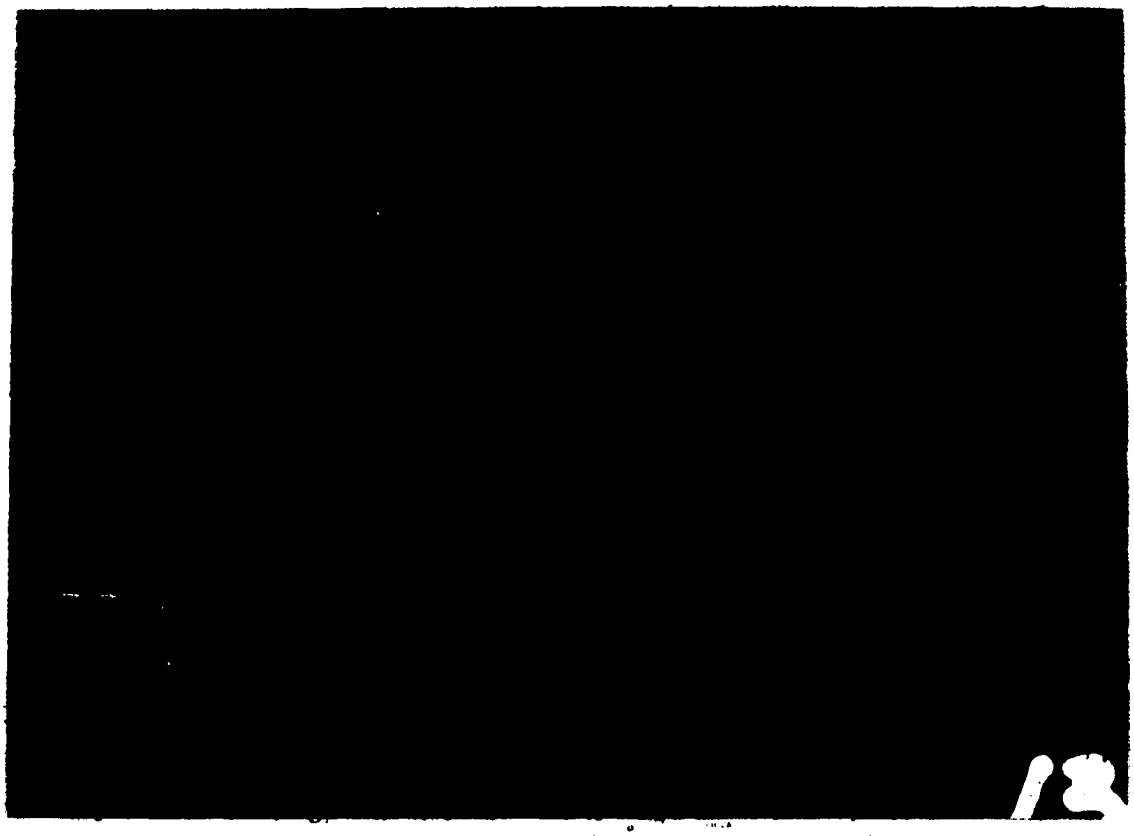

Figure 5. Photomicrograph of cells in micro-tumor pulled away from the monolayer, magnification, 450X.

the individual cells may be seen (450x).

Enumeration of the foci of infection offered the possibility of a fast and accurate method of assay of the virus. A comparison of this method with existing procedures for assay such as the CAM of embryonated eggs or inoculation of chicks, however, demonstrated that existing methods were more satisfactory for quantitative measurements. The insensitivity of the foci counting was due to infrequent and erratic tumor formation.

A further difficulty was encountered with this assay method. Rous sarcoma virus is produced slowly by the infected cell, but once produced, it is released readily within 20 hours into the supernatant fluid and thereafter a steady release of virus ensues. New virus may cause the formation of new foci of infection. Complete foci are formed within seven days and during this time additional new foci may have been initiated. That is, because of the length of time necessary for formation of the micro-tumors it is very difficult to distinguish between foci caused by virus of the inoculum and those due to subsequent multiplication of virus in the assay system. Because of these difficulties, previously established methods of viral assay were used in this study.

## Selection of Test Animal

Young chickens and embryonated eggs from several breeds were tested for their sensitivity to the Rous sarcoma virus. Among the breeds of chickens tested were New Hampshire Reds, Rhode Island Reds,

White Rock, Austria Whites, Indian River and the Honegger white Leghorn. The last two breeds mentioned were the most sensitive to the virus. The results reported herein are representative of comparative titration studies.

Embryonated eggs and three week old chicks of the Indian River strain were used in preliminary studies. Inoculation of virus into the wing-web area of three week old chicks resulted in tumor formation. The chorioallantoic membrane of the embryonated egg, however, was not satisfactory for use in these studies. When nine to 12 day old embryonated chick eggs of the Indian River strain were inoculated with 0.1 ml of half log dilutions of Lot #CT694 of Rous sarcoma virus, pock formation on the CAM was irregular and inconsistent. There was no correlation between dilutions, either as to the pock count on the individual membranes or as to the arithmetic mean of the pooled pock counts from the six to 10 membranes used for each dilution of the virus. Neither was there a linear dose-response curve expressed by the number of pocks formed on the membranes. The Indian River strain of chicken was therefore rejected for quantitative studies.

A white-shelled egg, obtained from the local hatchery, designated as Honegger white Leghorn was compared with the Indian River strain of egg for quantitation of virus. In a representative experiment virus was diluted in Earle's balanced salt solution containing five percent calf serum and the diluted virus was inoculated onto the CAM of 12 day old embryonated eggs of the Indian River and Honegger white Leghorn strains. Eight embryos were used for the inoculation of each dilution of the virus. The data for comparative titrations are

recorded in Table 1. Pock formation on the CAM of the Indian River strain of egg was not consistent, and membranes inoculated with a 1:10 dilution of the virus failed to respond. On the other hand, membranes of white Leghorn eggs responded to inoculation with graded concentrations of virus, and the number of pocks appearing on individual membranes of a group was quite consistent.

## Selection of a Basic Medium

A simple synthetic medium, such as that of Eagle (1955c), is required for nutritional studies. Unfortunately, in the present study replicate experiments proved that Eagle's basal medium was not satisfactory for growth of chick embryo fibroblasts. The growth was both slow and uneven. Lactalbumin hydrolysate in Earle's balanced salts solution (EBSS), which contained three to five percent calf serum, was capable of supporting growth of the chick embryo fibroblasts. Moreover, it was considerably less expensive than Eagle's medium, it did not deteriorate at 4 C and it underwent little, if any, pH change. For these reasons it was decided to use lactalbumin hydrolysate in EBSS containing five percent calf serum for the cultivation of the cells. In addition to this, Rappaport's SM-2 (Melnick, et al., 1957) and MB 752/1 (Waymouth, 1959) media were tested for their suitability. They did not support cell growth comparable to that in lactalbumin medium.

Since lactalbumin hydrolysate is not a synthetic medium and is not easily divided into its component parts, it would not serve as an appropriate medium for nutritional studies. Medium 199 (Morgan, Morton and Parker, 1950), supplemented with five percent calf serum

TABLE 1

COMPARATIVE SUSCEPTIBILITIES OF TWO STRAINS OF CHICK EMBRYOS TO ROUS SARCOMA VIRUS[a]

| Indian River | | | Honegger White Leghorn | | |
|---|---|---|---|---|---|
| Dilution of Virus | Pock Count | Mean | Dilution of Virus | Pock Count | Mean |
| 1:10,000 | 5,53,0,0,1, 0,0,0 | 19.6 | 1:10,000 | 31,26,30,10, 7,41,53 | 28 |
| 1:1,000 | 5,0,26,0,0, | 15 | 1:1,000 | 246,197,200, 150,223,167, 217,221 | 199 |
| 1:100 | 121,T[b],6,0, 0,0,0,0 | 63.5 | 1:100 | TNTC[c] | ....[d] |
| 1:10 | 0,0,0,0,0, 0,0,0 | 0 | 1:10 | TNTC and T | .... |

[a]Titration of virus in two strains of embryonated eggs to determine relative susceptibility of egg to infection.

[b]T denotes presence of one or more tumors on the chorioallantoic membrane.

[c]TNTC - too numerous to count.

d.... - unable to determine mean of pock count.

supported growth of the chick cells very well, and this was chosen as the basic medium for the nutritional studies. Medium 199, as well as lactalbumin medium, was prepared in HBSS. The latter medium was also supplemented with 0.1 percent yeast extract, 10 percent tryptose phosphate broth, and 10 percent calf serum. These supplements increased the rate of cells growth and thus a large population of cells was available in a relatively short period of time.

The lactalbumin medium was used to initiate primary cultures of chick embryo fibroblasts. Medium 199 was used to initiate secondary cultures of chick embryo fibroblasts and was the basic medium for the nutritional studies.

The composition of medium 199, as seen in Table 2, is somewhat complex although of a synthetic nature. This medium is supplemented with calf serum which is the only unknown component.

The composition of Hanks' and Earle's balanced salt solutions is given in Table 3.

Medium 199 was selected for these studies and certain components were omitted from this medium in order to determine the nutritional requirements of the infected cells.

## Growth Curves of Rous Sarcoma Virus

Before the nutritional studies could be undertaken it was necessary to know at what time after infection the cells started to produce virus and, once virus production had started, how long a period of time was required to complete a one-step growth curve.

In the first experiment, secondary cultures of cells were

TABLE 2

COMPOSITION OF MEDIUM 199[a]

| Ingredients per Liter | | | Ingredients per Liter | | |
|---|---|---|---|---|---|
| arginine[b] | 70 | mg | pantothenate | 0.01 | mg |
| histidine | 20 | mg | biotin | 0.01 | mg |
| lysine | 70 | mg | folic acid | 0.01 | mg |
| tyrosine | 40 | mg | choline | 0.50 | mg |
| tryptophan[c] | 20 | mg | inositiol | 0.05 | mg |
| phenylalanine | 50 | mg | p-aminobenzoic acid | 0.05 | mg |
| cystine | 20 | mg | vitamin A | 0.10 | mg |
| methionine | 30 | mg | calciferol | 0.10 | mg |
| serine | 50 | mg | menadione | 0.01 | mg |
| threonine | 60 | mg | a-tocopherol phosphate | 0.01 | mg |
| leucine | 120 | mg | ascorbic acid | 0.05 | mg |
| isoleucine | 40 | mg | glutathione | 0.05 | mg |
| valine | 50 | mg | cholesterol | 0.20 | mg |
| glutamic acid | 150 | mg | glutamine | 100 | mg |
| aspartic acid | 60 | mg | adenosinetriphosphate | 1 | mg |
| alanine | 50 | mg | adenylic acid | 0.20 | mg |
| proline | 40 | mg | ribose | 0.50 | mg |
| hydroxyproline | 10 | mg | deoxyribose | 0.50 | mg |
| glycine | 50 | mg | tween 80 | 5 | mg |
| cysteine | 0.1 | mg | sodium acetate | 50 | mg |
| adenine | 10 | mg | ferric nitrate | 0.10 | mg |
| guanine | 0.3 | mg | sodium chloride | 8 | gm |
| xanthine | 0.3 | mg | potassium chloride | 0.4 | gm |
| hypoxanthine | 0.3 | mg | calcium chloride | 0.14 | gm |
| thymine | 0.3 | mg | magnesium sulfate | 0.20 | gm |
| uracil | 0.3 | mg | disodium phosphate | 0.06 | gm |
| thiamine | 0.01 | mg | monopotassium phosphate | 0.06 | gm |
| riboflavin | 0.01 | mg | sodium bicarbonate | 0.35 | gm |
| pyridoxine | 0.025 | mg | glucose | 1 | gm |
| pyridoxal | 0.025 | mg | phenol red | 0.02 | gm |
| niacin | 0.025 | mg | triple distilled water | 1000. | ml |
| niacinamide | 0.025 | mg | | | |

[a]Medium 199 is prepared according to the formula given by Morgan, Morton, and Parker (1950), except containing Hanks' balanced salt solution, rather than Earle's salt solution as recommended by Burr, et al. (1954), Salk, Youngner and Ward, (1954), and Morgan, Campbell and Morton, (1955).

[b]L-isomers of arginine, histidine, lysine, tyrosine, cystine, proline, hydroxyproline, cysteine, and glutamine.

[c]DL-isomers of tryptophan, methionine, serine, threonine, leucine, isoleucine, valine, glutamic acid, aspartic acid, and alanine.

TABLE 3

COMPOSITION OF BALANCED SALT SOLUTIONS

| | Hanks'[a] | Earle's[b] |
|---|---|---|
| Component | Grams per Liter | Grams per Liter |
| sodium chloride | 8.00 | 6.80 |
| potassium chloride | 0.40 | 0.40 |
| calcium chloride | 0.14 | 0.20 |
| magnesium sulfate | 0.10 | 0.20 |
| magnesium chloride | 0.10 | ..... |
| disodium phosphate | 0.06 | ..... |
| monosodium phosphate | ..... | 0.125 |
| monopotassium phosphate | 0.06 | ..... |
| glucose | 1.00 | 1.00 |
| phenol red | 0.02 | ..... [c] |
| sodium bicarbonate | 0.35 | 2.20 |
| triple distilled water | 1000.00 ml | 1000.00 |

[a]From the formula of Hanks and Wallace (1949).

[b]From the formula of Earle (1943).

[c]Phenol red is added to Earle's salt solution later.

initiated in tubes with medium 199 containing 10 percent calf serum and 10 percent tryptose phosphate broth. These cells were infected with $4.7 \times 10^6$ plaque forming units (PFU) of the Rous virus. After the 40 minute adsorption period, the unadsorbed virus was removed and the cells were washed twice in cold HBSS. The washings were pooled and stored at - 62 C until assayed. One ml of medium 199 containing 10 percent calf serum and 10 percent tryptose phosphate broth was added to each tube of infected cell cultures and the tubes were incubated at 37 C.

Samples of supernatant fluid were removed from the infected cell cultures at two hour intervals from zero through 96 hours. These samples were frozen and stored at - 62 C until assayed for viral content.

After the medium was removed for assay, one ml of warm 0.25 percent trypsin in EBSS was placed on the cells in each tube and the tube was incubated at 37 C for five minutes in order to remove the cells from the glass. Cells from the five tubes were pooled, washed twice in cold HBSS and resuspended either in 0.2 the original volume or in the original volume of HBSS. These suspensions were stored at - 62 C until assayed for virus. Before the cell preparations were inoculated onto the CAM of embryonated eggs, the cell-associated virus was liberated from the cells by three alternate cycles of quick-freezing in alcohol and solid $CO_2$ and rapid thawing in a 37 C water bath. The quantity of free virus (FV) and cell-associated virus (CAV) in the inoculum was determined by inoculation of these preparations onto the CAM of embryonated eggs.

Pock counts from the membranes inoculated with the supernatant fluids containing the FV, and with the freeze-thaw preparations containing the CAV, are recorded in Table 4. The arithmetic mean of the pock counts derived from eggs inoculated with FV were multiplied by 10 to determine the mean number of pocks per ml of inoculum. Since the pooled infected cells were resuspended in one ml instead of the original five ml volume, the mean of these pock counts is divided by five, and this result multiplied by 10 to determine the average or mean number of PFU per ml of inoculum. An example will help to clarify this explanation. If the cells from five tubes were resuspended in one ml of HBSS, after washing, then frozen and thawed, and inoculated onto the CAM of embryonated eggs, the pock counts might be distributed in the following manner; 7, 9, 10, 23, 22, 48. The mean of this group of figures is 19.85, and since the cells were resuspended in one ml, this figure is divided by five to give 3.97, which is multiplied by 10 to yield a final figure of 39.7 PFU of virus in one ml of CAV inoculum. The logarithm of this number is 1.597.

Examination of the curves in Figure 6, which were plotted through the forty-eighth hour, reveals that the quantity of FV declined over a period of time from zero to the sixteenth hour. After the sixteenth hour, an increase in concentration of FV occurred, which continued to the thirty-fourth hour, and then virus concentration leveled off until the forty-second hour. At this time the concentration of FV again decreased until the forty-fourth hour, at which time the concentration again became constant.

TABLE 4

GROWTH CURVE OF ROUS SARCOMA VIRUS IN CHICK EMBRYO FIBROBLASTS

| Free Virus[a] | | | | Cell-Associated Virus[b] | | | |
|---|---|---|---|---|---|---|---|
| Hour | Pock Count | $10(\bar{x})$[c] | Logarithm[d] | Hour | Pock Count | $10(\bar{x})$ | Logarithm |
| 0 | tube broken[e] | ..... | ..... | 0 | 56,90,56,142 | 172 | 2.236 |
| 2 | 17,91,116,28,65 | 634 | 2.80 | 2 | 185,88,153,36,126 | 235.2 | 2.37 |
| 4 | 43,117,92,44,31,125 | 753.3 | 2.87 | 4 | 15,5,15 | 23.3 | 1.368 |
| 6 | 59,97,83,88,144 | 942 | 2.974 | 6 | 10,24,11,38,52 | 54 | 1.73 |
| 8 | 0,0,0,0,0,0 | 0 | ..... | 8 | 7,9,10,23,22,48 | 38.7 | 1.597 |
| 10 | 39,24,107,111,71,18 | 940 | 2.973 | 10 | 1,1,16,12,4,20 | 21.32 | 1.328 |
| 12 | 121,13,81,41,23 | 558 | 2.746 | 12 | 48,5,19,4,25 | 40.4 | 1.606 |
| 14 | 107,31,69,38,20,44 | 498 | 2.697 | 14 | 39,12,20,79,31,42 | 74.3 | 1.87 |
| 16 | 89,9,12,43,53 | 412 | 2.61 | 16 | 36,30,22,37,92 | 86.8 | 1.938 |
| 18 | 192,69,48,160,162,15 | 1076 | 3.031 | 18 | 27,94,113,17,0 | 126.5 | 2.104 |
| 20 | 574,432,390,183,127,60 | 2943 | 3.469 | 20 | 458,392,503,250,432,67 | 700.6 | 2.845 |
| 22 | 382,465,600,325,220,283 | 3791 | 3.579 | 22 | 127,357,294,77,340,447 | 547.3 | 2.738 |
| 24 | 179,264,256,248,181,392 | 2533 | 3.404 | 24 | 133,147,241,295,300,418 | 418 | 2.625 |
| 26 | 271,485,581,680 | 5092.5 | 3.707 | 26 | 404,7,306 | 478 | 2.679 |
| 28 | 404,434,154 | 3306.6 | 3.486 | 28 | TNTC[f] | ..... | ..... |
| 30 | 73,199 | 1360 | 3.133 | 30 | tube broken | ..... | ..... |
| 32 | 76,321,212,28,17,173 | 13,783[g] | 4.139 | 32 | 339,297,197,28,472 | 5260[g] | 3.721 |
| 34 | 253,91,266,312 | 23,050 | 4.368 | 34 | 104,114,205,106,64,112 | 2350 | 3.373 |
| 36 | 156,214,106,324,352 | 23,040 | 4,368 | 36 | 155,145,3,302,50,232 | 2956.6 | 3.471 |
| 38 | 342,16,288,480 | 28,150 | 4.99 | 38 | 246,165,196,355,336 | 5152 | 3.712 |
| 40 | tube broken | ..... | ..... | 40 | 132,124,120,119,195,273 | 3210 | 3.507 |
| 42 | 120,419,499,110,184 | 29,150 | 4.469 | 42 | 38,293,356,351 | 519 | 2.715 |
| 44 | 204,252,164,162,172 | 1908 | 3.28 | 44 | 178,269,166,369,175 | 462.8 | 2.665 |
| 46 | 262,158,228,332 | 2450 | 3.389 | 46 | 134,264,197,283 | 434 | 2.637 |
| 48 | 138,280,192,112 | 1805 | 3.257 | 48 | 214,206 | 220 | 2.34 |

[a]Virus free in the medium, not attached to the cell.

[b]Virus associated with the cell, and not free in the medium.

[c]Denotes 10 times the mean pock count.

[d]Logarithm to the base 10, Of $10(\bar{x})$.

[e]Tube broken in deepfreeze, unavailable for assay.

[f]Too numerous to count.

[g]See text, referring to Table 4, for these calculations.

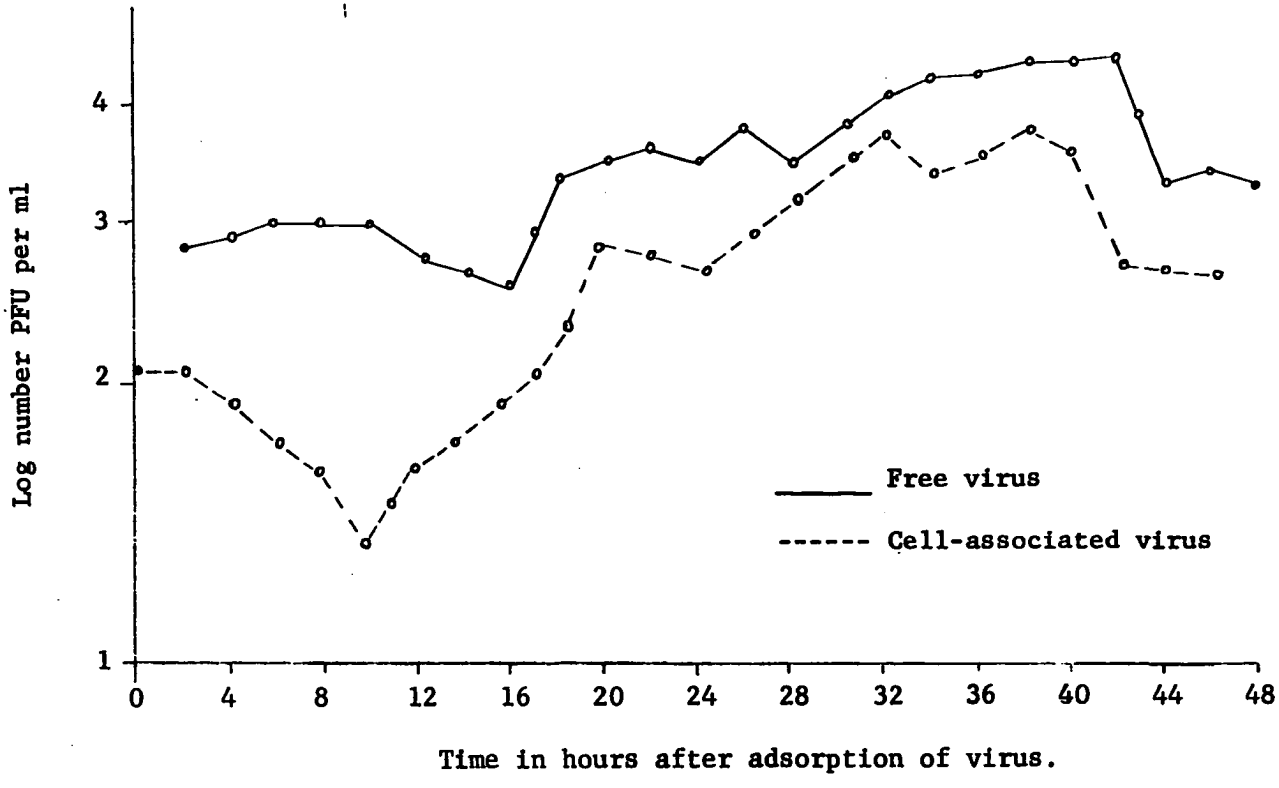


Fig. 6. Growth curve of Rous sarcoma virus in chick embryo fibroblasts, in vitro.

The curve of the CAV paralleled that of the FV at about one log lower concentration.

An eclipse period (represented by the dip in the two curves) occurred at the sixteenth hour in the case of the FV and at the tenth hour in the case of the CAV. The eclipse period was followed by a period of increased virus production. The final titer of the virus was well above that observed at the zero hour.

Starting at the thirty-second hour, concentrations of FV and CAV became so high that the inocula had to be diluted 1:10. The mean of these counts is multiplied by 100 to determine the mean number of PFU per ml of inoculum. This high level of virus continued until the forty-second hour, at which time it had decreased sufficiently so that the inocula did not have to be diluted 1:10. The preceding paragraph is an explanation of foot note g in Table 4.

A second experiment, similar in design and procedure to the previous one, was carried out over a period of 30 hours, and samples for viral titration were obtained every two hours. The cultures were initiated in the same medium, and treated just as the cultures in the first experiment with the exception that the virus input was $4.7 \times 10^5$ PFU, about one-tenth that of the previous experiment. Assay for the FV and the CAV was performed in the same manner as in the preceeding experiment. Pock count distribution on the membranes inoculated with samples obtained from the cultures are recorded in Table 5 and in Figure 7.

Examination of the growth curve in Figure 7 will reveal that in the case of the FV an eclipse period occurred at about the sixteenth hour, followed by a period of increased virus production, the level of

TABLE 5

GROWTH CURVE OF ROUS SARCOMA VIRUS IN CHICK EMBRYO FIBROBLASTS

| Free Virus[a] | | | | Cell-Associated Virus[b] | | | |
|---|---|---|---|---|---|---|---|
| Hour | Pock Count | $10(\bar{x})$[c] | Logarithm[d] | Hour | Pock Count | $10(\bar{x})$ | Logarithm |
| 0 | 19,3,5,3,0,0 | 75 | 1.875 | 0 | 7,4,1,4,7,5 | 9.23 | 0.97 |
| 2 | 3,10,1,31,5,11 | 38.3 | 1.583 | 2 | 4,2,0,2,1 | 3.6 | 0.556 |
| 4 | 4,7,2,2 | 37.5 | 1,574 | 4 | 1,6,4,5,0,5 | 10.3 | 1.001 |
| 6 | 5,10,14,1 | 75 | 1.875 | 6 | 6,5,3,2 | 8.0 | 0.903 |
| 8 | 2,11,0,6,15 | 85 | 1.93 | 8 | 9,10,4,11,8 | 16.8 | 1.226 |
| 10 | 8,14,7,7 | 90 | 1.955 | 10 | 7,9,8,1,1 | 10.4 | 1.001 |
| 12 | 19,8,45,5,2 | 158 | 2.198 | 12 | 0,0,1,0 | 0.5 | 0.069 |
| 14 | 7,5,4,8,3,7 | 73.3 | 1,865 | 14 | 3,7,10,6,5 | 12.4 | 1.093 |
| 16 | 3,2,2,1,0 | 20 | 1.301 | 16 | 12,0,2,2,4 | 10 | 1.000 |
| 18 | 3,5,2,2,5,4 | 35 | 1.544 | 18 | 1,1,2,36 | 20 | 1.301 |
| 20 | 4,1,286,184,12 | 974 | 2.989 | 20 | 31,23,28,17 | 49.5 | 1.694 |
| 22 | 9,159,19,21,22,151 | 635 | 2.803 | 22 | 20,22,22,15 | 39.5 | 1.597 |
| 24 | tube broken[e] | ... | ..... | 24 | 30,37,24,25,19 | 54 | 1.733 |
| 26 | 41,58,59,77 | 587.5 | 2.769 | 26 | 126,109,154, 73,67,100 | 209.6 | 2.311 |
| 28 | 27,16,111,13,353 | 1040 | 3.017 | 28 | 73,72,5,44 101 | 118 | 2.072 |
| 30 | 132 | 1320 | 3.121 | 30 | 56 | 112 | 2.05 |

[a]Virus free in the medium.

[b]Virus associated with the cell, not free in the medium. See text for method of calculation of the mean pock count.

[c]Denotes 10 times the mean pock count.

[d]Logarithm to the base 10, of $10(\bar{x})$.

[e]Tube broken in deepfreeze. Unavailable for assay.

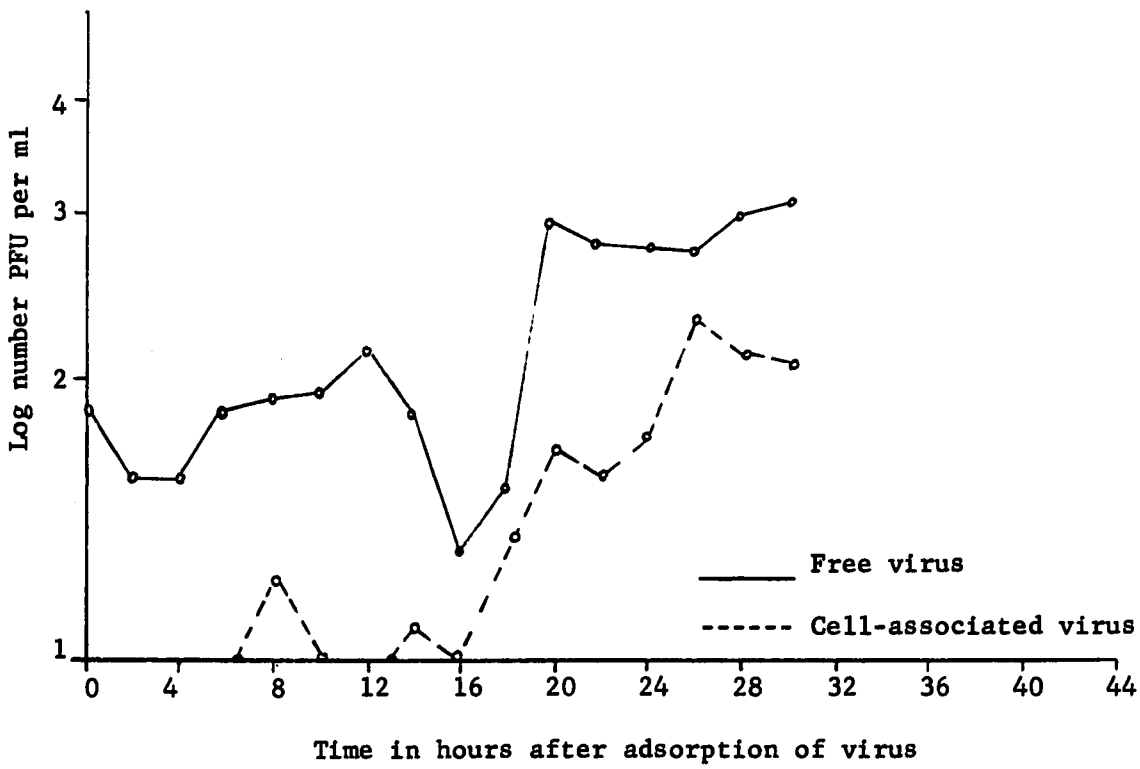


Fig. 7. Growth curve of Rous sarcoma virus in chick embryo fibroblasts, *in vitro*.

which almost equaled the peak titer at the twentieth hour. There was a slight decrease after this, followed by a small increase to the peak titer which occurred at the thirtieth hour.

The curve of the CAV followed closely the curve of the FV, although at about one log difference. It can be seen in the figure that the level of virus in the CAV never exceeded that of the FV.

Effect of Serum on the Proliferation of Rous Sarcoma Virus

Using the information gained in the above experiments, other studies were planned to determine some of the nutritional requirements of the infected cells. The next group of experiments were designed to determine if the cell-virus system required serum for virus-production.

Two experiments are reported here in which serum was omitted from the medium. In the first study secondary cell cultures were initiated in medium 199 containing 10 percent calf serum. After an 18 hour incubation period, the cells were washed with cold HBSS and infected with $2.3 \times 10^3$ PFU of virus which was allowed to adsorb for 40 minutes. The unadsorbed virus was removed, pooled and frozen until it could be assayed. The infected cells were washed two times in cold HBSS. The washings were pooled and stored at - 62 C. One ml of experimental medium consisting of HBSS and glutamine without serum was placed on the cells in each tube. Medium 199 containing 10 percent calf serum was added to the infected cells in each control tube. Samples for virus titration were removed every four hours from the experimental and control cultures. The effect of serum on the proliferation of virus, as reflected by the pock counts, can be seen in Table 6. The results of the analyses of

these data are shown in Figure 8.

Examination of Figure 8 reveals two curves for cultures in control medium. The smallest amount of virus that can be recorded in Figure 8 is 10 PFU and it was not possible to plot the production of virus in experimental media as recorded in Table 6. In the case of virus isolation during the experiment, six PFU per ml of inoculum were recovered at the zero hour, and no virus was recovered during the remainder of the period represented by the growth curve. In the case of the control cultures which contained undialyzed serum (US), virus was released from the cells through the twelfth hour, after which time an eclipse period occured. The lowest level of virus production was reached at about the sixteenth hour. Following this there was an increase in the amount of virus with peak production at the thirty-second hour.

The other experiment represented in Figure 8 is one in which secondary cultures of cells were initiated in HBSS containing five percent dialyzed calf serum. The cultures in this experiment were examined for virus over a period of 48 hours. After infection and washing, the cells were recultured in medium 199 which contained no serum. No virus was produced by the cells cultured in this experimental medium, as recorded in Table 6. After viral adsorption control cultures that were treated in the same manner as the experimental cultures were recultured in medium 199 which contained five percent dialyzed calf serum. The virus inoculum for the experimental cultures contained 4,383 PFU, and the titered virus input for the control cultures was 6,430 PFU.

Data relative to virus production as reflected by pock counts of these two experiments are recorded in Table 6. Pock counts for a

TABLE 6

EFFECT OF SERUM ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| HBSS[b] | | | | Medium 199[c] | | | |
|---|---|---|---|---|---|---|---|
| Hour | Pock Count | $10(\bar{x})$[d] | Logarithm[e] | Hour | Pock Count | $10(\bar{x})$ | Logarithm |
| 0 | 0,1,2,0,0 | 6 | 0.778 | 0 | 0,0,0,0,0,0 | ..... | ..... |
| 4 | 0,0,0,0,0,0 | ..... | ..... | 4 | 0,0,0,0,0,0 | ..... | ..... |
| 8 | 0,0,0,0,0,0 | ..... | ..... | 8 | 0,0,0,0,0,2 | 3.33 | 0.523 |
| 12 | 0,0,0,0,0 | ..... | ..... | 12 | 0,0,0,0,0,0 | ..... | ..... |
| 16 | 0,0,0,0,0,0 | ..... | ..... | 16 | 0,0,0,0,0 | ..... | ..... |
| 20 | 0,0,0,0,0,0 | ..... | ..... | 20 | 0,0,0,0,0,0 | ..... | ..... |
| 24 | 0,0,0,0,0,0 | ..... | ..... | 24 | 0,0,0,0,0,0 | ..... | ..... |
| 28 | 0,0,0,0,0,0 | ..... | ..... | 28 | 0,0,0,0,0,0 | ..... | ..... |
| 32 | 0,0,0,0,0,0 | ..... | ..... | 32 | 5,0,0,0,0,0 | 8.33 | 0.926 |
| | | | | 36 | 0,0,0,0,0,0 | ..... | ..... |
| Positive Control[f] | | | | 40 | 0,0,6,0,0,0 | 10 | 1.000 |
| 0 | 1,1,1,1,0,0 | 8.33 | 0.920 | 44 | 0,0,1,0,0,0 | 1,66 | 0.220 |
| 4 | 5,6,1,0,1 | 26 | 1.415 | 48 | 0,0,0,0,2,0 | 3.33 | 0.523 |
| 8 | 17,0,1,0,1 | 63.3 | 1.801 | | | | |
| 12 | 2,0,22,1,38,4 | 134 | 2.127 | Positive Control | | | |
| 16 | 3,0,0,4,3,4 | 23 | 1.361 | 0 | 9,5,7,0,4,7 | 64 | 1.806 |
| 20 | 7,24,3,26,7 | 134 | 2.127 | 12 | 3,11,1,30,23 | 98 | 1.991 |
| 24 | 41,13,9 | 210 | 2.322 | 24 | 7,3,4,6,4 | 48 | 1.681 |
| 28 | 22,1,36,10,12 | 202.5 | 2.306 | 36 | 0,3,1,4,1 | 18 | 1.25 |
| 32 | 106,42,1,77,12 | 476 | 2.678 | 48 | 121,84,67,67, 59,20 | 696.6 | 2.842 |
| Negative Control[g] | | | | | | | |
| 32 | 0,0,0,0,0,0 | ..... | ..... | | | | |

[a]Serum omitted from the experimental media.

[b]Medium consisting of Hanks' balanced salt solution and glutamine constituted the experimental medium.

[c]Experimental medium consisted of medium 199 without serum.

[d]Denotes 10 times the mean pock count.

[e]Logarithm to the base 10, of $10(\bar{x})$.

[f]Positive control medium consisted of medium 199 supplemented with calf serum.

[g]Negative control contained uninfected cells maintained in medium 199 supplemented with calf serum.

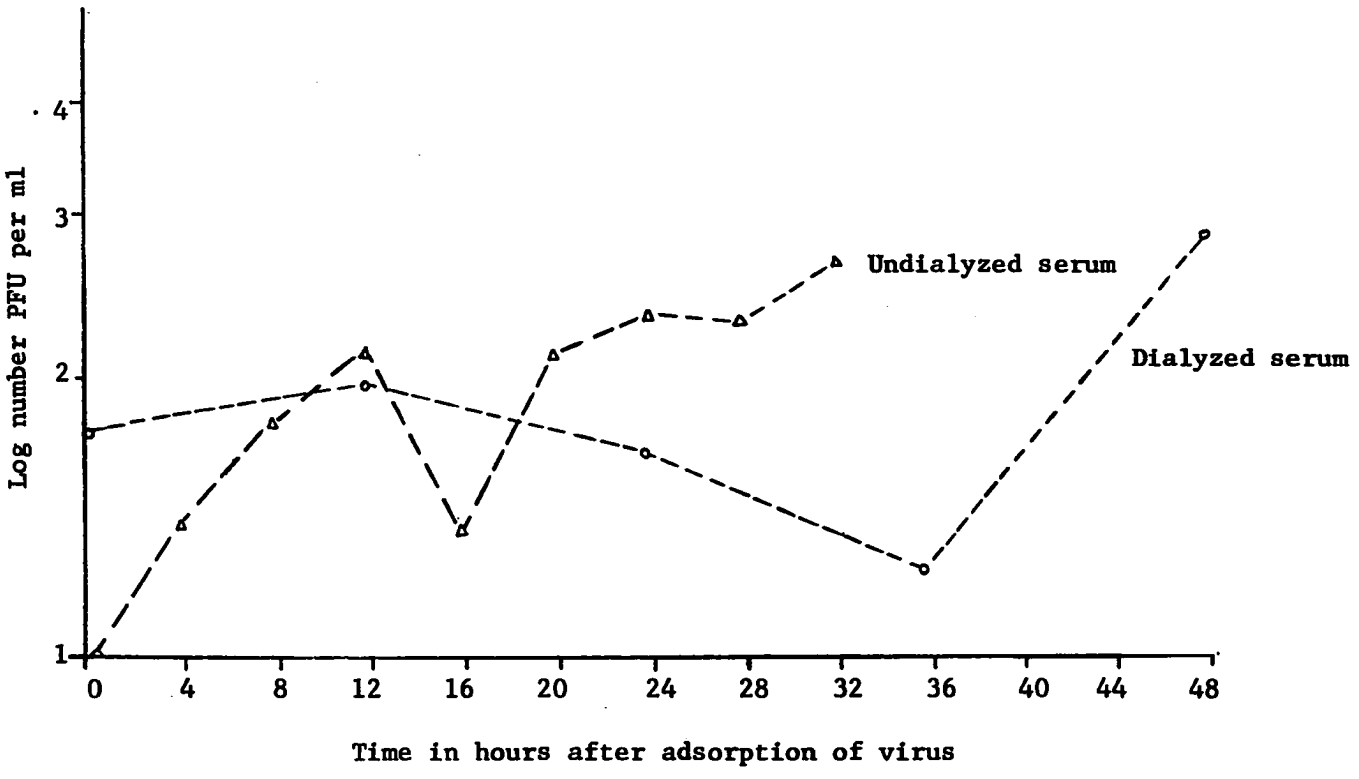


Fig. 8. Effect of serum on the proliferation of Rous sarcoma virus

negative control which was sampled at the thirty-second hour are also included in the table. A negative control is one in which cells are not infected with virus.

As in the previous experiment, little or no virus was detected in cultures bathed in serum-free medium. In this case the recovery of virus in PFU per ml amounted to 3.3 at the eighth hour, 8.33 at the thirty-second hour, 10 at the fortieth hour and 3.33 at the forty-eighth hour. Since in only one sample was it possible to find 10 PFU per ml, the results of the experimental curves were not graphed, except for the control cultures in dialyzed serum (DS). It can be seen that the eclipse period occurred later than usual, as exemplified in the control culture. Samples from the control cultures were taken only every 12 hours. This was done to economize on time and the number of embryos required. The controls show the rate of virus production by these cells, as well as the amount of virus.

## Effect Of Vitamins On the Proliferation Of Rous Sarcoma Virus

Since the data in the foregoing experiments indicated that serum was required for the production of virus, the effect of the omission of vitamins from the medium was the next course of interest. Many cell lines have an excellent endogenous supply of many of the vitamins and it is difficult to exhaust these reserves. Three experiments were included in this study. Secondary cultures for the first experiment were initiated in medium 199 containing 10 percent undialyzed calf serum. After incubation and washing they were infected with 2,120 PFU of virus. The

experimental medium which was placed on the cells after infection consisted of medium 199 from which all vitamins had been omitted and which was supplemented by the addition of 10 percent calf serum. Samples for titration of virus were taken every three hours from zero through 30 hours.

The data concerning the pock counts are found in Table 7. The plots for the free and the cell-associated virus can be seen in Figure 9. The two curves are quite similar, and the one representing the cell-associated virus indicates a higher concentration of virus up to the

TABLE 7

EFFECT OF ALL VITAMINS ON PROLIFERATION OF ROUS SARCOMA VIRUS

| Free Virus | | | Cell-Associated Virus | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[a] | Logarithm[b] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 23.3 | 1.368 | 0 | 36.66 | 1.564 |
| 3 | 22.5 | 1.355 | 3 | 43.3 | 1.637 |
| 6 | 35 | 1.544 | 6 | 64 | 1.806 |
| 9 | 38 | 1.58 | 9 | 38 | 1.58 |
| 12 | 83.3 | 1.921 | 12 | 136 | 2.143 |
| 15 | 55 | 1.74 | 15 | 36 | 1.556 |
| 18 | 20 | 1.301 | 18 | 50 | 1.699 |
| 21 | 47.5 | 1.677 | 21 | 28.33 | 1.454 |
| 24 | 125 | 2.097 | 24 | 25 | 1.398 |
| 27 | 214 | 2.33 | 27 | 63.3 | 1.803 |
| 30 | 1123 | 3.05 | 30 | 182 | 2.26 |
| | Negative Control | | | | |
| 30 | 0 | ..... | | | |

[a]Denotes 10 times the mean pock count.

[b]Logarithm, to the base 10, of $10(\bar{x})$.

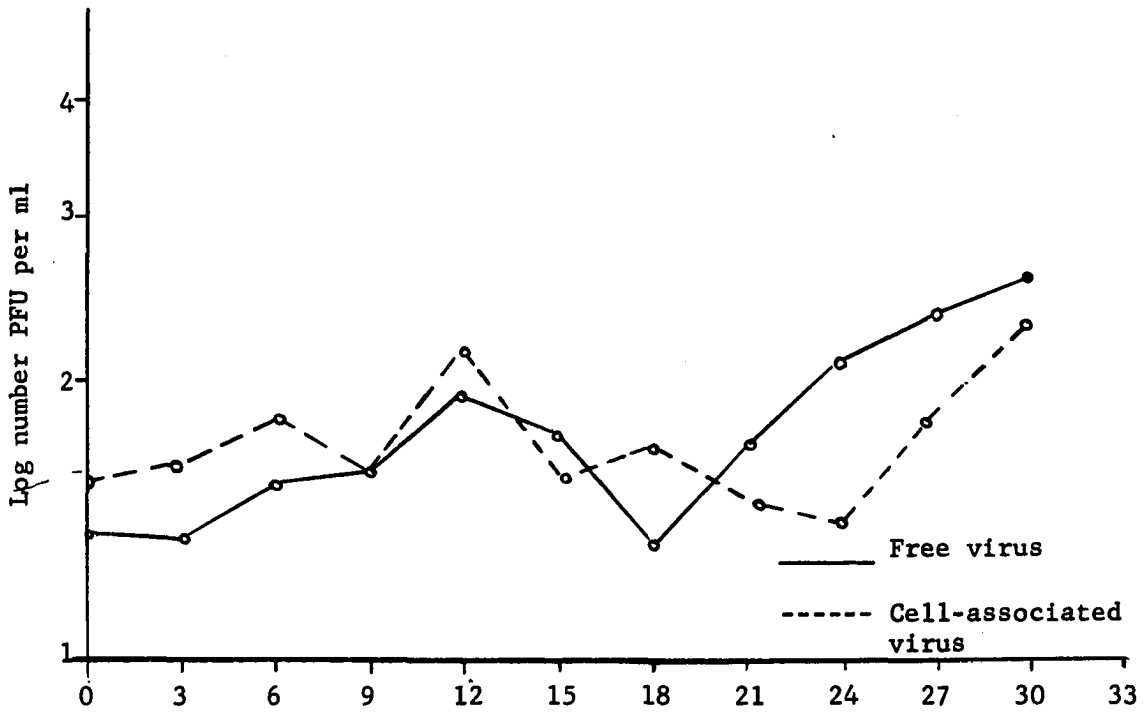


Fig. 9. Effect of the vitamins in medium 199 on the proliferation of Rous sarcoma virus.

eclipse period. After the eclipse period the concentration of the cell-associated virus followed that of the free virus, but at about 0.1 that of the FV. The cells were able to produce virus in the absence of any exogenous source of vitamins, aside from what might be found in the undialyzed serum.

It was realized that not all of the virus used for infection was adsorbed to the cells. Vigier and Golde (1959) also noted this in their studies. In this experiment and others to follow, virus adsorption as well as virus input was measured. "Adsorption" now refers to the difference between the input and the amount recovered after attachment of the virus to the cells. The post-adsorption virus was recorded as PAV, and the post-adsorption washes as $PAW_1$ and $PAW_2$. The term "adsorption" also included the difference between virus input and virus recovery as affected by thermal inactivation and non-specific adsorption to glass.

The next experiment involved the omission of fat-soluble vitamins (A, D, K, and E) and accessory components, choline, cholesterol, Tween 80 and ethyl alcohol; the last served as a solvent for some of the vitamins.

Secondary cultures were initiated in medium 199 containing 10 percent calf serum, and after infection and washing, the experimental medium, medium 199, from which the fat-soluble vitamins had been omitted, was placed on the cells. Control cultures received medium 199 containing 10 percent calf serum. The virus input was 6900 PFU, and the amount of virus recovered in the form of PAV and PAW was 2400 PFU. The virus adsorbed was 4500 PFU, the difference between the virus

input and the recovery. Pock count data for this experiment are recorded in Table 8. The individual pock counts were omitted to save space and are represented by the mean pock count from one ml of inoculum ($10(\bar{x})$. This procedure will be used in the remainder of the tables. The data are plotted in Figure 10.

TABLE 8

EFFECT OF FAT-SOLUBLE VITAMINS ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 13.3 | 1.124 | 0 | 8.33 | 0.920 |
| 4 | 0.0 | ..... | 4 | 26 | 1.415 |
| 8 | 28 | 1.447 | 8 | 63 | 1.801 |
| 12 | 20 | 1.301 | 12 | 134 | 2.127 |
| 16 | 78 | 1.892 | 16 | 23 | 1.36 |
| 20 | 295 | 2.469 | 20 | 134 | 2.127 |
| 24 | 370 | 2.568 | 24 | 210 | 2.322 |
| 28 | 354 | 2.549 | 28 | 202.5 | 2.306 |
| 32 | 375 | 2.574 | 32 | 476 | 2.678 |

[a]Vitamins A, D, K, and E, as well as cholesterol, choline, Tween 80, and ethyl alcohol omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm, to the base 10, of $10(\bar{x})$.

Examination of the curve for the control reveals that, as in the growth curves, virus was released by the cells up to the sixteenth hour, at which time an eclipse occurred, followed by an increase in production of virus through the thirty-second hour. Examination of the curve for the experimental medium from which the fat-soluble vitamins and accessory components were omitted, indicated that the

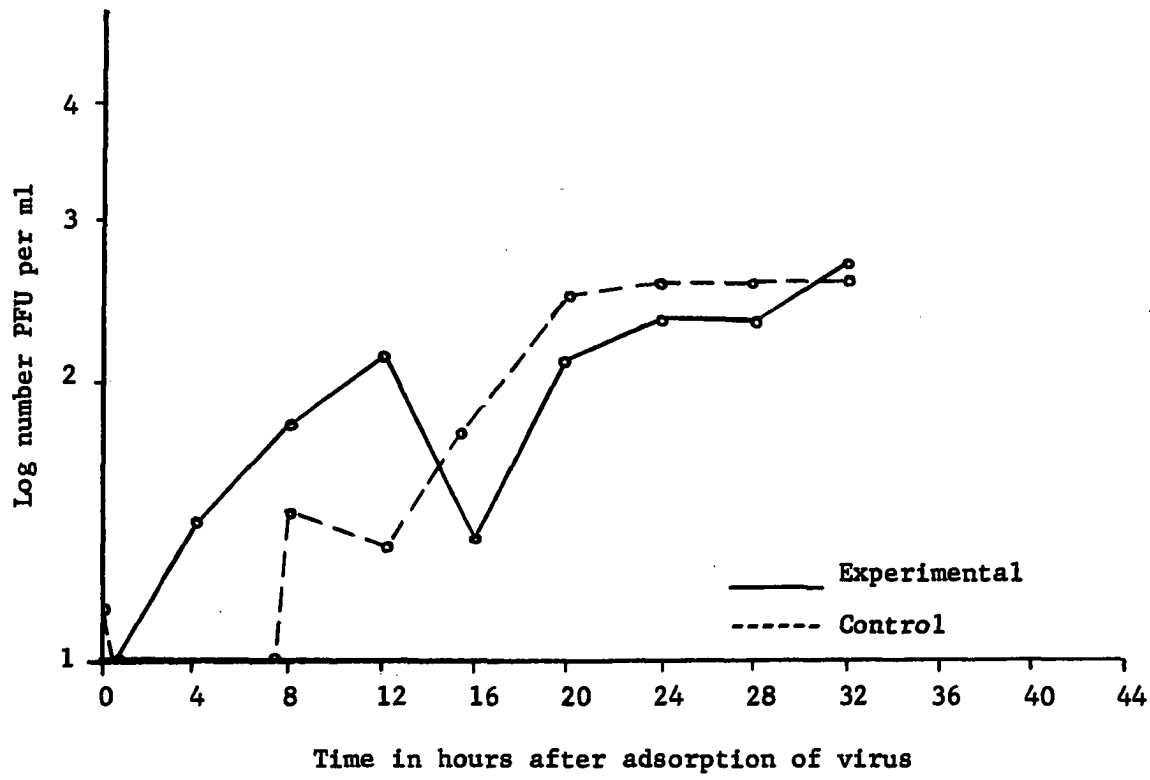


Fig. 10. Effect of fat-soluble vitamins on the proliferation of Rous sarcoma virus.

eclipse period and viral production occurred earlier and at a higher concentration than in the control medium. The final concentration of virus at 32 hours was approximately the same in both media.

Since the medium in which the secondary cultures of the previous experiment were initiated contained vitamins, and the serum in the medium was not dialyzed, another experiment was done to determine whether these factors had any effect on the production of virus in a vitamin-free medium.

Secondary cultures of cells were initiated in a balanced salt solution (HBSS) which contained five percent dialyzed calf serum, and the cells were incubated 18 hours in this vitamin-free medium. After washing and infection of these cells, a medium devoid of vitamins, with the exception of ascorbic acid, was placed on the cells. Medium G, supplemented with five percent calf serum, was used for this purpose. Control cultures received medium 199 containing five percent undialyzed calf serum. The components of medium G are recorded in Table 9.

Virus input for this experiment was $12 \times 10^3$ PFU, and the virus recovered in the form of PAV and PAW was $7.66 \times 10^3$ PFU. Virus adsorbed in this experiment was $4.33 \times 10^3$ PFU.

Examination of the experimental and control curves in Figure 11, as plotted from the pock count data recorded in Table 10, shows that infected, vitamin-starved cells, grown in a vitamin-deficient medium did not require an exogenous source of vitamins. An eclipse period was observed in both curves, and was followed by an increased virus production which progressed through the thirty-second hour.

TABLE 9

COMPOSITION OF MEDIUM G[a]

| Ingredients per Liter[b] | |
|---|---|
| L-arginine hydrochloride | 70.00 mg |
| L-histidine hydrochloride | 20.00 mg |
| L-lysine hydrochloride | 70.00 mg |
| DL-tryptophan | 20.00 mg |
| DL-phenylalanine | 50.00 mg |
| DL-threonine | 60.00 mg |
| L-leucine | 60.00 mg |
| L-isoleucine | 20.00 mg |
| DL-valine | 50.00 mg |
| DL-serine | 50.00 mg |
| L-glutamic acid | 75.00 mg |
| DL-aspartic acid | 60.00 mg |
| DL-alpha alanine | 50.00 mg |
| L-hydroxyproline | 10.00 mg |
| L-proline | 40.00 mg |
| glycine | 50.00 mg |
| L-methionine | 30.00 mg |
| L-tyrosine | 40.00 mg |
| L-cystine | 20.00 mg |
| L-cysteine hydrochloride | 0.01 mg |
| L-glutamine | 100.00 mg |
| glutathione | 0.05 mg |
| ascorbic acid | 0.05 mg |
| sodium chloride | 8 g |
| potassium chloride | 0.40 g |
| calcium chloride | 0.14 g |
| magnesium sulfate | 0.20 g |
| disodium phosphate | 0.06 g |
| monopotassium phosphate | 0.06 g |
| sodium bicarbonate | 0.35 g |
| D-glucose | 1 g |
| phenol red | 0.02 g |
| triple distilled water | 1000 ml |
| calf serum | 50 |

[a]Medium devised in this laboratory for cultivation of chick embryo cells.

[b]Concentration of ingredients per liter is the same as in medium 199. pH of amino acid solution is adjusted to neutrality with sodium hydroxide.

TABLE 10

EFFECT OF VITAMINS ON THE PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Medium G | | | Medium 199 | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(x)$ | Logarithm |
| 0 | 53.3 | 1.726 | 0 | 30 | 1.477 |
| 4 | 106 | 2.025 | 4 | 91.6 | 1.961 |
| 8 | 48.3 | 1.684 | 8 | 48.3 | 1.684 |
| 12 | 28.3 | 1.452 | 12 | 73.3 | 1.865 |
| 16 | 25 | 1.398 | 16 | 36 | 1.556 |
| 20 | 16.6 | 1.222 | 20 | 5.0 | 0.699 |
| 24 | 25 | 1.398 | 24 | 6.66 | 0.823 |
| 28 | 11.6 | 1.164 | 28 | 20 | 1.301 |
| 32 | 57.5 | 1.759 | 32 | 33.3 | 1.522 |

[a]Cells cultured in vitamin-free medium for 18 hours before infection.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

Effect of Glutamine on the Proliferation of Rous Sarcoma Virus.

Eagle and Habel (1956) reported that glutamine was required for the production of poliovirus in HeLa cells. Lewis and Scott (1961) concluded that glutamine was not required by HeLa cells for the Production of herpes simplex virus. No studies have been reported of the requirement for glutamine by chick fibroblasts for the production of Rous sarcoma virus.

Several experiments were done to investigate the glutamine requirements of virus-infected chick embryo fibroblasts and representative experiments are recorded here. In the first experiment secondary cultures of chick embryo fibroblasts were initiated in medium 199 containing 10 percent calf serum, and 10 percent tryptose phosphate broth. After incubation, infection and washing of the cells, medium 199 from

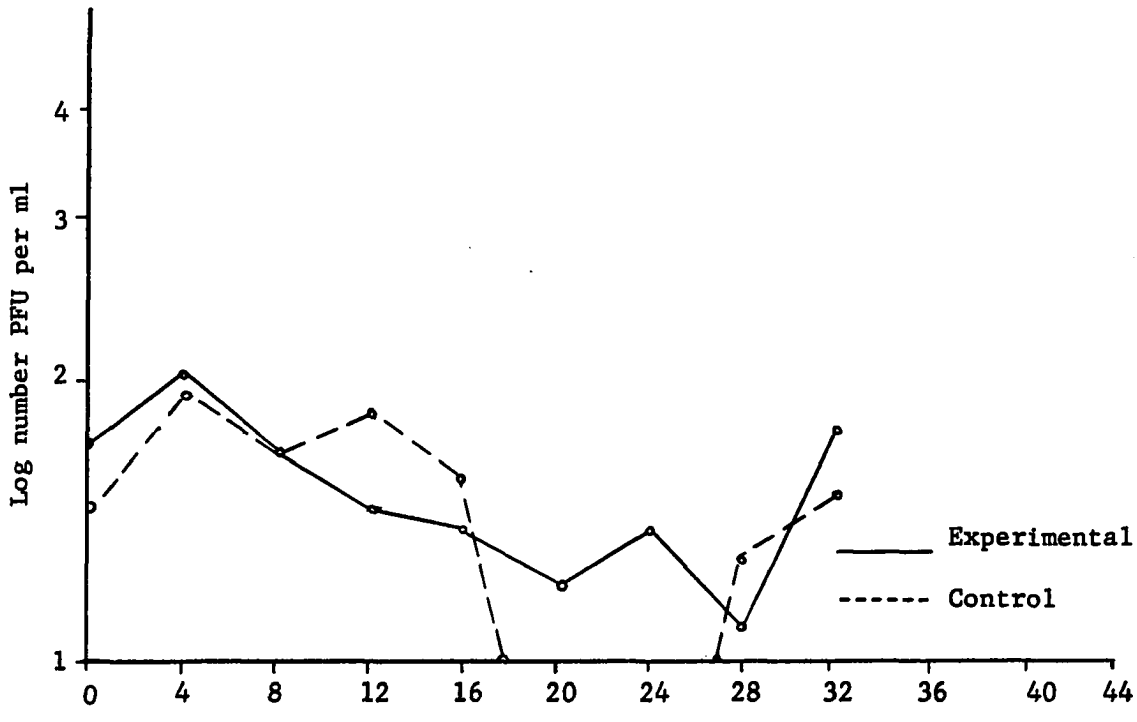


Fig.11. Effect of vitamins on the proliferation of Rous sarcoma virus.

which glutamine had been omitted and supplemented with 10 percent calf serum and 10 percent tryptose phosphate broth, was added to the cells. Control cultures received medium 199 supplemented with 10 percent calf serum and 10 percent tryptose phosphate broth. Virus input was 1875 PFU. The control cultures in this experiment, drawn from the same population of cells and treated the same, were infected with 5150 PFU of virus. The curves for this experiment have been plotted in Figure 12 from the pock count data as recorded in Table 11.

Examination of the curve representing virus production in the experimental medium which contained tryptose phosphate broth, reveals that relatively little virus was produced or released early in the growth curve. At the twenty-fourth hour an increase in virus production occurred which continued through the thirtieth hour.

It can be seen in Figure 12 that, except for the early part of the curve, the control and experimental curves of the medium containing tryptose phosphate broth closely approximate each other. This indicates lack of a requirement for glutamine for the production of the Rous sarcoma virus over a period of 32 hours.

The secondary cultures in the second experiment to investigate the requirement for glutamine were initiated in medium G containing five percent dialyzed calf serum. The cells were infected with approximately $8.5 \times 10^4$ PFU of virus, of which $7.3 \times 10^4$ PFU were recovered in for form of PAV and PAW. The other $1.2 \times 10^4$ PFU remained to be adsorbed. The experimental cultures received medium 199 from which glutamine had been omitted, and which was supplemented with five percent dialyzed calf serum. Tryptose phosphate broth was not used as a supplement in either

TABLE 11

EFFECT OF GLUTAMINE ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Medium with TP Broth[b] | | | Medium without TP Broth[c] | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[d] | Logarithm[e] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 10 | 1.00 | 0 | 51.66 | 1.713 |
| 3 | 11.6 | 1.064 | 4 | 184 | 2.264 |
| 6 | 10 | 1.00 | 8 | 98 | 1.991 |
| 9 | 0 | ..... | 12 | 33.3 | 1.523 |
| 12 | 0 | ..... | 16 | 21.66 | 1.336 |
| 15 | 6 | 0.778 | 20 | 38.33 | 1.583 |
| 18 | 18 | 1.255 | 24 | 20 | 1.301 |
| 21 | 4 | 0.602 | 28 | 150 | 2.176 |
| 24 | 5 | 0.699 | 32 | 84 | 1.924 |
| 27 | 116 | 2.064 | | | |
| 30 | 148 | 2.170 | | | |
| | Control Medium | | | Control Medium | |
| 0 | 86.6 | 1.937 | 0 | 76 | 1.88 |
| 4 | 78 | 1.892 | | | |
| 8 | 26.6 | 1.424 | | | |
| 12 | 0 | ..... | 12 | 60 | 1.778 |
| 16 | 0 | ..... | | | |
| 20 | 0 | ..... | 20 | 82 | 1.914 |
| 24 | 67.5 | 1.829 | | | |
| 28 | 150 | 2.176 | | | |
| 32 | 105 | 2.021 | 32 | 281.66 | 2.45 |
| | Negative Control | | | | |
| 30 | 0 | ..... | | | |

[a]Glutamine omitted from the experimental medium.

[b]Experimental medium, devoid of glutamine, contained 10 percent tryptose phosphate broth.

[c]Experimental medium, devoid of glutamine, contained no tryptose phosphate broth.

[d]Denotes 10 times the mean pock count.

[e]Logarithm to the base 10, of $10(\bar{x})$.

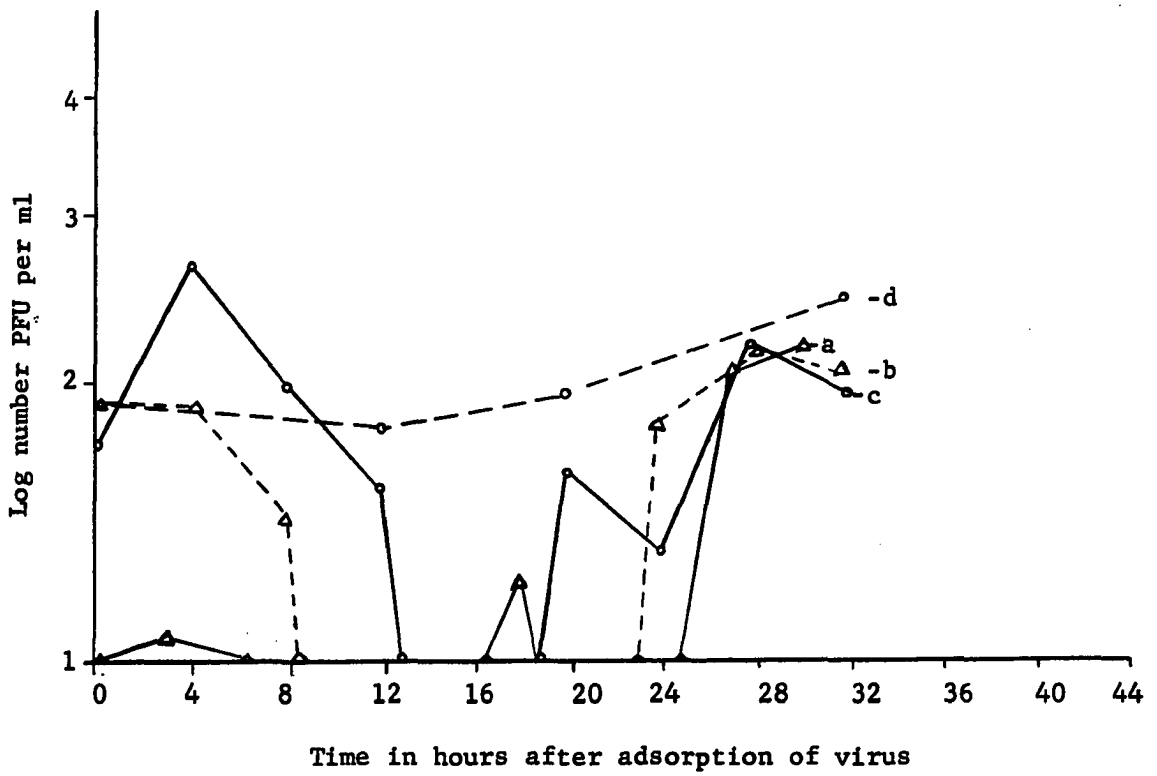


Fig.12. Effect of glutamine on the proliferation of Rous sarcoma virus.

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[a]Cultures initiated in medium with tryptose phosphate broth.

[b]Controls to cultures of a.

[c]Cultures not initiated in medium with tryptose phosphate broth.

[d]Controls to cultures of c.

the experimental or the control medium. The control cultures received medium 199 containing five percent dialyzed calf serum. The curves of virus production in the experimental and control media are plotted in Figure 12 and the pock count data are recorded in Table 11.

Examination of the control curve in Figure 12 reveals that there was a decrease in the amount of virus released through the twelfth hour, followed by an increase in production of virus which continued through the remainder of the observation time.

It can be seen by examination of the curve for the production of virus in the experimental medium that an eclipse phase occurred about the sixteenth hour. This was followed by an increase in production of virus which at the twenty-eighth hour approached the concentration of virus produced by the control culture, and then decreased slightly. It can be noted that virus was produced in a glutamine-deficient medium. It was not produced as rapidly nor in as high a concentration as in a complete medium. Near the end of the growth period the amount of virus produced in the glutamine-deficient medium nearly equaled that in the complete medium.

The secondary cultures of the third experiment to determine the glutamine requirements for virus production were initiated in medium 199 containing five percent calf serum. After incubation, the washed cells were infected with $2.9 \times 10^4$ PFU of virus, of which $4 \times 10^3$ PFU were recovered as PAV and PAW. According to calculation $2.5 \times 10^4$ PFU of virus were adsorbed. The cells were washed and overlaid with an experimental medium composed of medium 199 from which glutamine had been omitted . This was supplemented with five percent calf serum. Control

cultures were overlaid with medium 199 which contained five percent calf serum.

The effect of glutamine on the proliferation of Rous sarcoma virus can be seen in Figure 13, as drawn from the pock count data recorded in Table 12.

Examination of the control curve from which samples were taken for virus titration at 0, 12, 20 and 32 hours reveals a decrease in the amount of virus released through the twelfth hour, followed by a slight increase in virus produced through the twenty-fourth hour. After this there was an abrupt increase in virus production through the thirty-second hour.

An inspection of the experimental curve reveals that virus was released from the cells through the eighth hour, after which no virus could be detected until the thirty-second hour, when a small amount of virus was produced. Production of virus continued through the forty-eighth hour.

In the last representative experiment of this series concerning the role of glutamine in the production of Rous sarcoma virus, the secondary cells were initiated in a balanced salt solution (HBSS) containing five percent dialyzed calf serum. The cells were infected with approximately $3.1 \times 10^4$ PFU of virus, of which $2.4 \times 10^4$ PFU were recovered as PAV and PAW. This allowed $7 \times 10^3$ PFU to be adsorbed. The experimental cultures were overlaid with medium 199 from which glutamine had been omitted and which was supplemented with five percent dialyzed calf serum. The control cultures received medium 199 containing five percent

TABLE 12

EFFECT OF GLUTAMINE ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Initiation in Medium 199[b] | | | Initiation in HBSS[c] | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[d] | Logarithm[e] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 13.3 | 1.125 | 0 | 62 | 1.792 |
| 4 | 32.5 | 1.512 | 4 | 128 | 2.112 |
| 8 | 0 | ..... | 8 | 56.6 | 1.753 |
| 12 | 1.66 | 0.456 | 12 | 36 | 1.556 |
| 16 | 0 | ..... | 16 | 16.6 | 1.220 |
| 20 | 0 | ..... | 20 | 20 | 1.301 |
| 24 | 0 | ..... | 24 | 6.66 | 0.823 |
| 28 | 0 | ..... | 28 | 0 | ..... |
| 32 | 13.33 | 1.124 | 32 | 0 | ..... |
| 48 | 35 | 1.544 | 36 | 0 | ..... |
| | | | 40 | 0 | ..... |
| | | | 44 | 0 | ..... |
| | | | 48 | 18.3 | 1.262 |
| | Control Medium | | | Control Medium | |
| 0 | 70 | 1.845 | 0 | 64 | 1.806 |
| 12 | 46.6 | 1.668 | 12 | 98 | 1.991 |
| 24 | 51.66 | 1.714 | 24 | 48 | 1.681 |
| 32 | 244 | 2.387 | 36 | 18 | 1.25 |
| | | | 48 | 696.6 | 2.842 |

[a]Glutamine omitted from the experimental medium.

[b]Secondary cultures initiated in medium 199 supplemented with serum.

[c]Secondary cultures initiated in HBSS supplemented with serum.

[d]Denotes 10 times the mean pock count.

[e]Logarithm to the base 10, of $10(\bar{x})$.

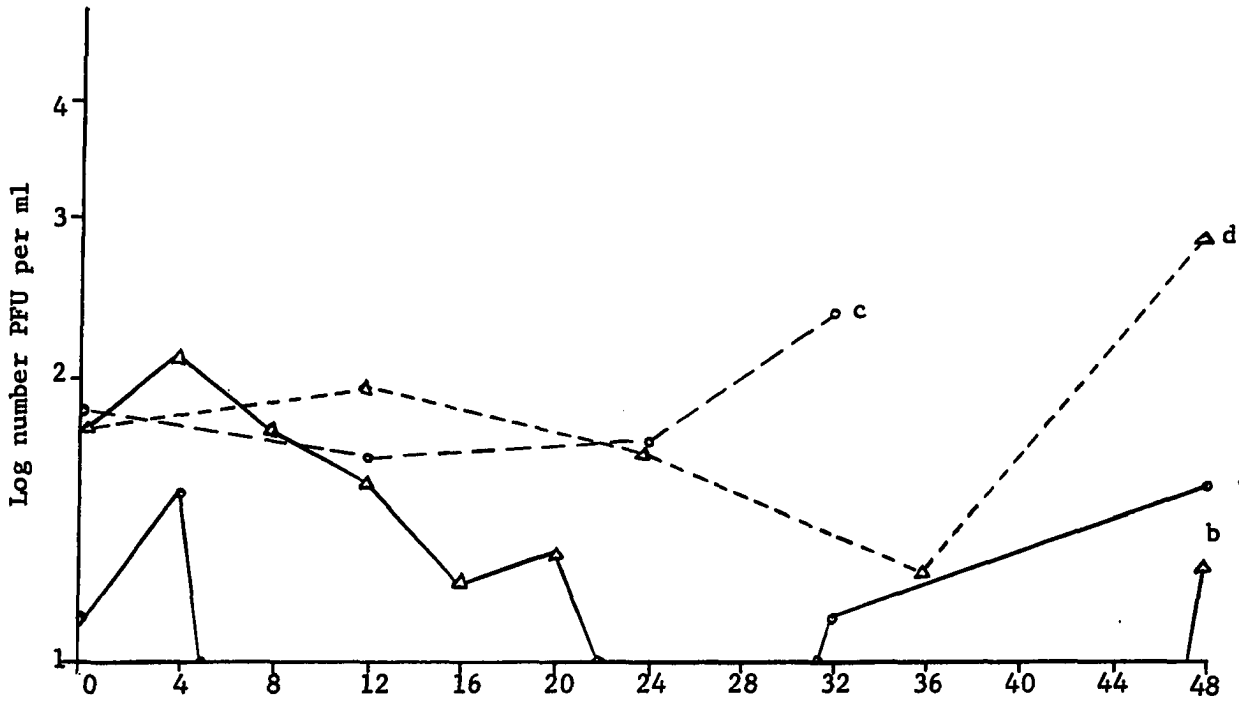


Fig.13. Effect of glutamine on the proliferation of Rous sarcoma virus.

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[a]Cultures initiated in medium 199.

[b]Control to cultures of a.

[c]Cultures initiated in medium HBSS

[d]Control to cultures of c.

dialyzed calf serum. The pock count data, representing synthesized virus, are recorded in Table 12; the experimental and control curves are plotted in Figure 13. This experiment was carried out over a period of 48 hours to determine if the effect of the omission of glutamine from the medium might manifest or fail to manifest itself at a later period in the growth curve.

A careful scrutiny of the control curve in Figure 13 reveals a long period during which there was a decrease in virus released. At the thirty-sixth hour viral proliferation was evident and it continued through the forty-eighth hour. The amount of virus released at the latter time was approximately 38 times that released at the thirty-sixth hour.

Decreased virus release as shown in the experimental curve began at the fourth hour and continued through the sixteenth hour. There was a small increase in virus produced between the sixteenth and the twentieth hour. This in turn was followed by a decrease in virus concentration which dropped to zero by the twenty-eighth hour, and did not rise again until the forty-eighth hour. At this time there was a small increase of virus in the inoculum.

## Effect of Amino Acids on the Proliferation of Rous Sarcoma Virus

One of the major components of medium 199 is the 20 amino acids. Some of these are required for nitrogen balance in humans, and many are known to be required for cell culture maintenance. The status of the amino acid requirements for virus-infected chick embryo fibroblasts, *in vitro*, is as yet unreported.

A representative experiment in the investigation of amino acid requirements is one in which all of the amino acids were omitted from the experimental medium. The secondary cultures were initiated in a balanced salt solution (HBSS) which contained five percent dialyzed calf serum. The washed cells were infected with $7.9 \times 10^4$ PFU of virus of which $5 \times 10^4$ PFU were recovered as PAV and PAW. Thus, $2.9 \times 10^4$ PFU remained to be adsorbed. The experimental medium added to the cells was medium 199 from which all the amino acids, except the amide, glutamine, and glutathione, a tripeptide, had been omitted. This medium was supplemented with five percent dialyzed calf serum. The control cultures received medium 199 containing five percent dialyzed calf serum.

The curves representing virus production in experimental and control media have been plotted in Figure 14, from the pock count data recorded in Table 13. Examination of the control curve in Figure 14 reveals a steady decrease in virus release which was followed by an increase in virus production starting at the twentieth hour. An inspection of the experimental curve reveals that there was an initial decrease in virus. The experimental curve follows closely the control curve. From the twelfth to the twenty-fourth hour there was a decrease to almost zero of the virus released. By the thirty-second hour there was a slight increase in concentration of virus but this did not approach the amount of virus in the control. About 10 times as much virus was produced by the control cultures at the thirty-second hour as compared to the experimental cultures.

One can infer from the results of this experiment that one or several amino acids were required for production of the Rous

TABLE 13

EFFECT OF AMINO ACIDS ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 814 | 2.910 | 0 | 1574 | 3.197 |
| 4 | 613 | 2.787 | | | |
| 8 | 858 | 2.933 | | | |
| 12 | 546.6 | 2.747 | 12 | 276 | 2.44 |
| 16 | 52 | 1.716 | | | |
| 20 | 16.6 | 1.22 | 20 | 128.3 | 2.108 |
| 24 | 21.6 | 1.334 | | | |
| 28 | 5 | 0.699 | | | |
| 32 | 18.3 | 1.262 | 32 | 220 | 2.332 |

[a]All amino acids omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

sarcoma virus by chick embryo fibroblasts, in vitro. Thus, the amino acids in the medium were grouped for investigation according to the requirements of chick embryonic heart fibroblasts (Morgan and Morton, 1957). In the experiment involving a group of non-essential amino acids, the secondary cultures were initiated in medium G containing five percent calf serum. The washed cells were infected with $5.6 \times 10^4$ PFU. After adsorption $2.5 \times 10^4$ were recovered as PAV and PAW, which left $3.1 \times 10^4$ PFU for adsorption. The experimental medium placed on the cells consisted of medium 199 from which serine, glutamic acid, aspartic acid, hydroxyproline, proline and glycine had been omitted, and which was supplemented with five percent calf serum. The infected control cultures received medium 199 containing

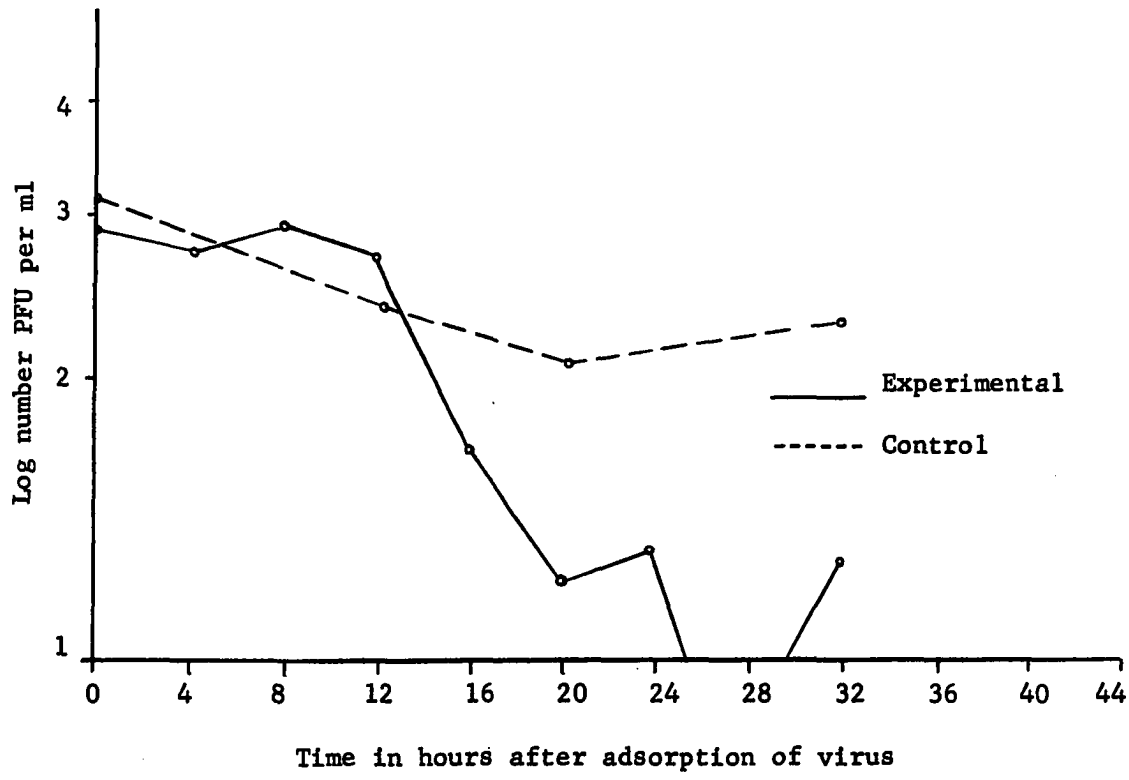


Fig.14. Effect of amino acids on the proliferation of Rous sarcoma virus.

five percent calf serum. The curve for virus production in experimental and control media are plotted in Figure 15 which is a graphic representation of the pock count data recorded in Table 14.

An examination of the control curve in Figure 15 reveals a decrease in virus released until the 20th hour, at which time an increase in the concentration of virus was noted. This increase continued through the thirty-second hour. The experimental curve follows the control curve closely through the twelfth hour, after which time the virus concentration in the experimental medium dropped to a low level by the sixteenth hour. By the twentieth hour there was a marked increase in virus production which allowed the experimental curve to approximate the control curve through the thirty-second hour. The curve which represents virus production by cells in experimental medium is, with the

TABLE 14

EFFECT OF AMINO ACIDS ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 246.6 | 2.392 | 0 | 222 | 2.346 |
| 4 | 105 | 2.021 | | | |
| 8 | 126 | 2.100 | | | |
| 12 | 91.6 | 1.962 | 12 | 112.5 | 2.051 |
| 16 | 41.66 | 1.619 | | | |
| 20 | 45 | 1.653 | 20 | 106.6 | 2.028 |
| 24 | 141.6 | 2.151 | | | |
| 28 | 391.66 | 2.592 | | | |
| 32 | 683.3 | 2.834 | 32 | 893 | 2.951 |

[a]Serine, glutamic acid, aspartic acid, alanine, hydroxyproline, proline, and glycine omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

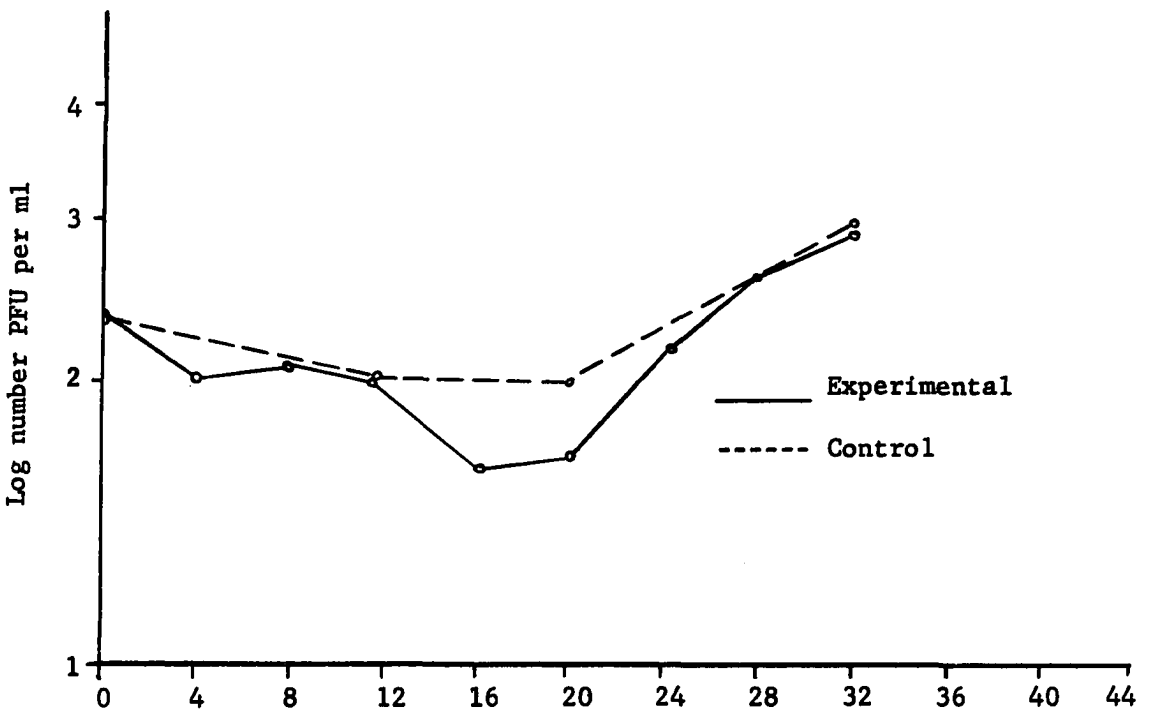


Fig.15. Effect of amino acids on the proliferation of Rous sarcoma virus.

exception of the four hour delay following the eclipse period, almost identical to that curve which represents virus production by cells in control medium.

The next experiment in this group was one in which the amino acids required by chick embryonic heart fibroblasts were studied as a group. The secondary cultures were initiated in medium G containing five percent calf serum. The washed cells were infected with $4.5 \times 10^4$ PFU, of which $5.6 \times 10^3$ were recovered as PAV and PAW, thus leaving $3.94 \times 10^4$ PFU for adsorption. After infection of the cells, medium 199, from which arginine, histidine, lysine, tryptophan and phenylalanine had been omitted, was added to the experimental cultures. This was supplemented with five percent calf serum. Medium 199 containing five percent calf serum was placed on the control cells. The curves representing virus production by the cells are plotted in Figure 16, from pock count data recorded in Table 15.

From the control curve in Figure 16 it can be determined that there was a decrease in virus released through the twelfth hour, followed by an increase in concentration of virus, which continued through the thirty-second hour.

Examination of the experimental curve reveals that there was a sharp decrease in virus between zero and fourth hours followed by an increase in release of virus between the fourth and eighth hours. The concentration of virus in the supernatant fluid then decreased through the twentieth hour, increased again at the twenty-eighth hour and decreased by the thirty-second hour.

In the last of the experiments to determine the effect of

amino acids, the secondary cultures were initiated in medium G containing five percent dialyzed calf serum. After washing, the cells were infected with $1.76 \times 10^4$ PFU. There were $3 \times 10^3$ PFU recovered in PAV and PAW which left $1.46 \times 10^4$ for adsorption. Experimental

TABLE 15

EFFECT OF AMINO ACIDS ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 608.3 | 2.783 | 0 | 1050 | 3.116 |
| 4 | 0 | ..... | | | |
| 8 | 40 | 1.602 | | | |
| 12 | 22 | 1.342 | 12 | 256.6 | 2.409 |
| 16 | 16 | 1.176 | | | |
| 20 | 12 | 1.079 | 20 | 452 | 2.655 |
| 24 | 3.33 | 0.482 | | | |
| 28 | 43.3 | 1.636 | | | |
| 32 | 8.33 | 0.92 | 32 | 1348 | 3.130 |

[a]Arginine, histidine, lysine, tryptophan, and phenylalanine omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

cultures received medium 199 from which threonine, leucine, isoleucine and valine had been omitted. This was supplemented with five percent dialyzed calf serum. The control cultures were covered with medium 199 containing five percent dialyzed calf serum. The representative experimental and control curves were plotted in Figure 17, and the pock count data were recorded in Table 16.

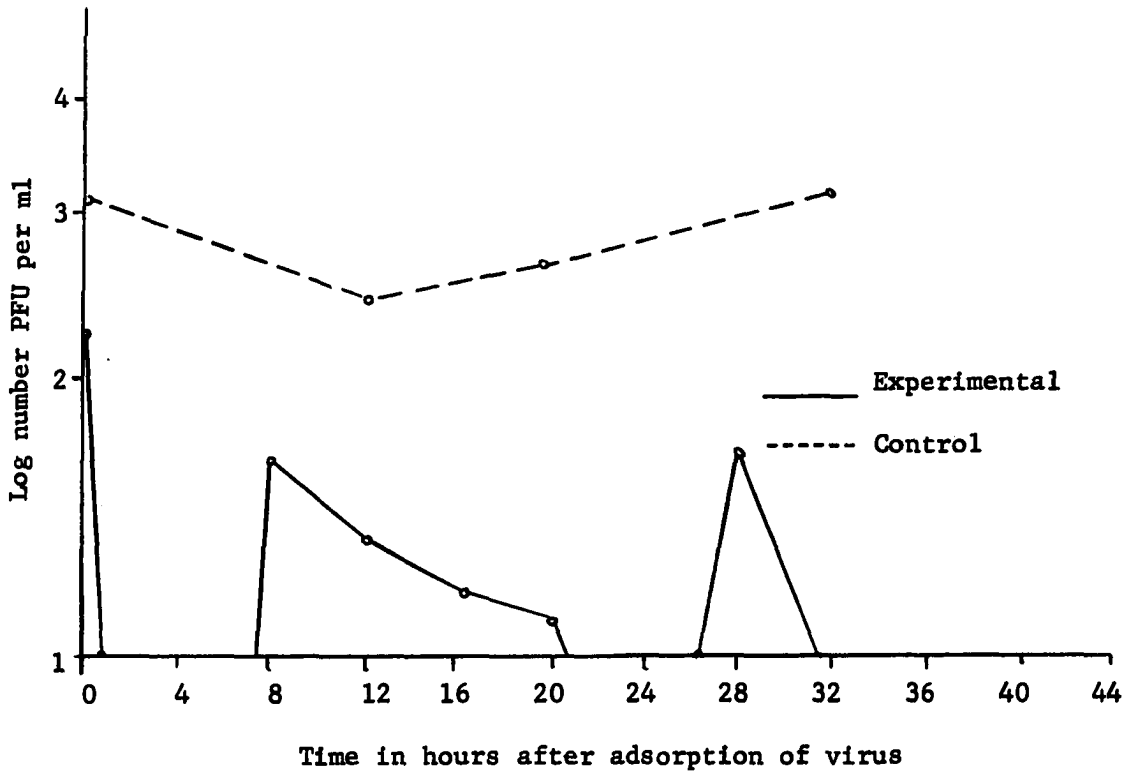


Fig.16. Effect of amino acids on the proliferation of Rous sarcoma virus.

TABLE 16

EFFECT OF AMINO ACIDS ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 141.6 | 2.151 | 0 | 133.3 | 2.125 |
| 4 | 115 | 2.061 | 4 | | |
| 8 | 95 | 1.977 | | | |
| 12 | 45 | 1.653 | 12 | 63.3 | 1.801 |
| 16 | 38.33 | 1.583 | | | |
| 20 | 58 | 1.764 | 20 | 318.3 | 2.503 |
| 24 | 171 | 2.233 | | | |
| 28 | 170 | 2.230 | | | |
| 32 | 318.3 | 2.503 | 32 | 705. | 2.848 |
| | | | 48 | 2930 | 3.466 |

[a]Threonine, leucine, isoleucine, and valine omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

An inspection of the control curve in Figure 17 reveals a decrease in virus released through the twelfth hour, followed by an increase in production of virus which became evident at the twentieth hour and continued through the thirty-second hour. The forty-eighth hour virus production indicated an increase.

A study of the experimental curve in Figure 17 discloses a steady decrease in virus released through the sixteenth hour, which was followed by a slow increase in virus production through the thirty-second hour. The final titer of the virus produced by the cells in the experimental medium did not quite attain the same level as that of the control medium. However, there is little difference between these curves,

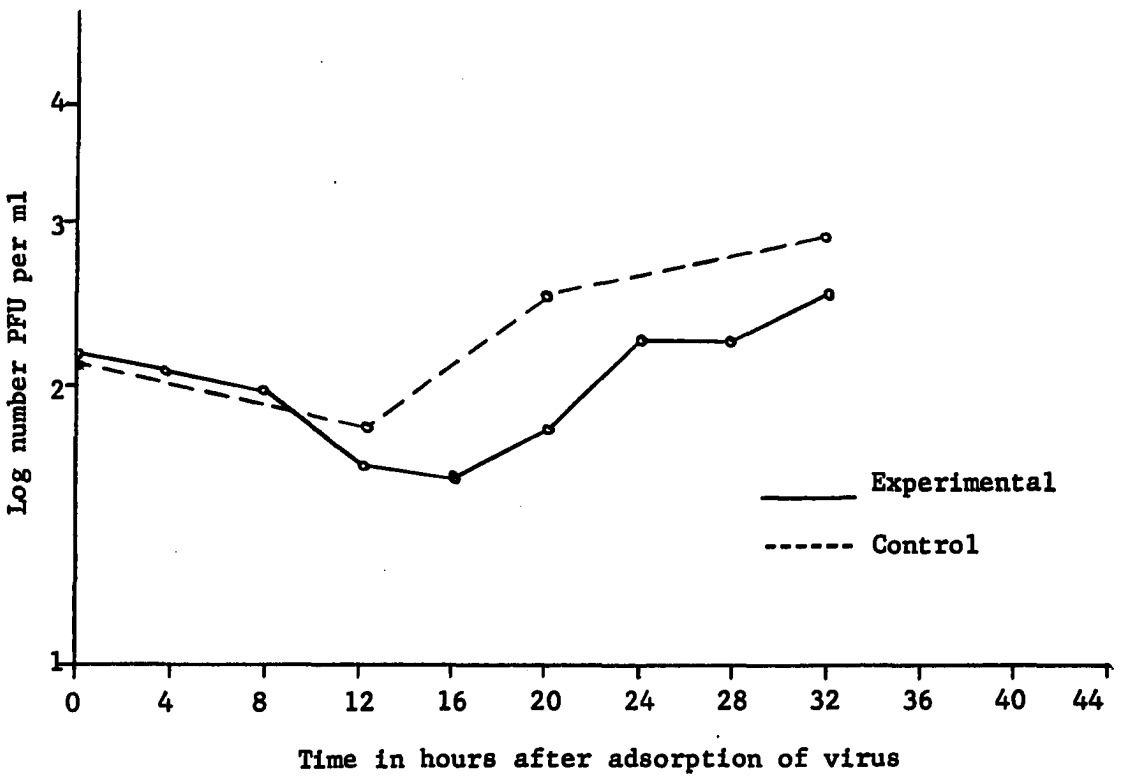


Fig.17. Effect of amino acids on the proliferation of Rous sarcoma virus.

and the experimental curve roughly parallels the control curve, at a lower level.

## Effect of Glucose on Rous Sarcoma Virus Proliferation

Actively growing cells require energy in the form of an oxidizable carbon source, and in medium 199 glucose fills this requirement. Two representative experiments are described to show the effect of glucose on the production of virus by chick embryo fibroblasts.

In the first experiment the secondary cultures were initiated in medium G containing five percent dialyzed calf serum and the cells were infected with $1.76 \times 10^4$ of virus, of which $3 \times 10^3$ PFU were recovered in the PAV and the PAW. This left $1.46 \times 10^4$ PFU to be adsorbed. The experimental medium which was placed on the cells consisted of medium 199 from which glucose had been omitted. Five percent dialyzed calf serum was added to this medium. The control medium consisted of medium 199 supplemented with five percent dialyzed calf serum. The experimental and control curves which have been plotted in Figure 18 are a graphic representation of the pock count data recorded in Table 17.

An examination of the control curve in Figure 18 reveals that there was an initial decrease in the amount of virus released through the twelfth hour, followed by an increase in virus production through the thirty-second hour.

Similarily an examination of the experimental curve reveals an initial decrease in release of virus. This is followed by an increase

of virus through the twentieth hour, at which time a plateau occurred in the virus concentration curve. This was followed by a decrease of virus through the next four hours to the twenty-fourth hour. Starting at this time, there was an initial increase in virus through the twenty-eighth hour, followed by a slight decrease through the thirty-second hour. This curve has two major areas in which a decrease of virus occurred. Each decrease was followed in turn by an increased production of virus which made this a bimodal curve.

TABLE 17

EFFECT OF GLUCOSE ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 150 | 2.176 | 0 | 133.3 | 2.125 |
| 4 | 103.3 | 2.014 | | | |
| 8 | 106 | 2.025 | | | |
| 12 | 75 | 1.875 | 12 | 63.3 | 1.801 |
| 16 | 141.6 | 2.151 | | | |
| 20 | 151.6 | 2.180 | 20 | 318.3 | 2.503 |
| 24 | 88.3 | 1.946 | | | |
| 28 | 536 | 2.730 | | | |
| 32 | 336.6 | 2.526 | 32 | 705 | 2.848 |

[a]Glucose omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

Another experiment in this group also involved medium G which contained five percent calf serum, for the initiation of the secondary cultures. The cells were infected with $1.15 \times 10^4$ PFU, of which $1 \times 10^3$ PFU were recovered in the PAV and the PAW. A PFU count of

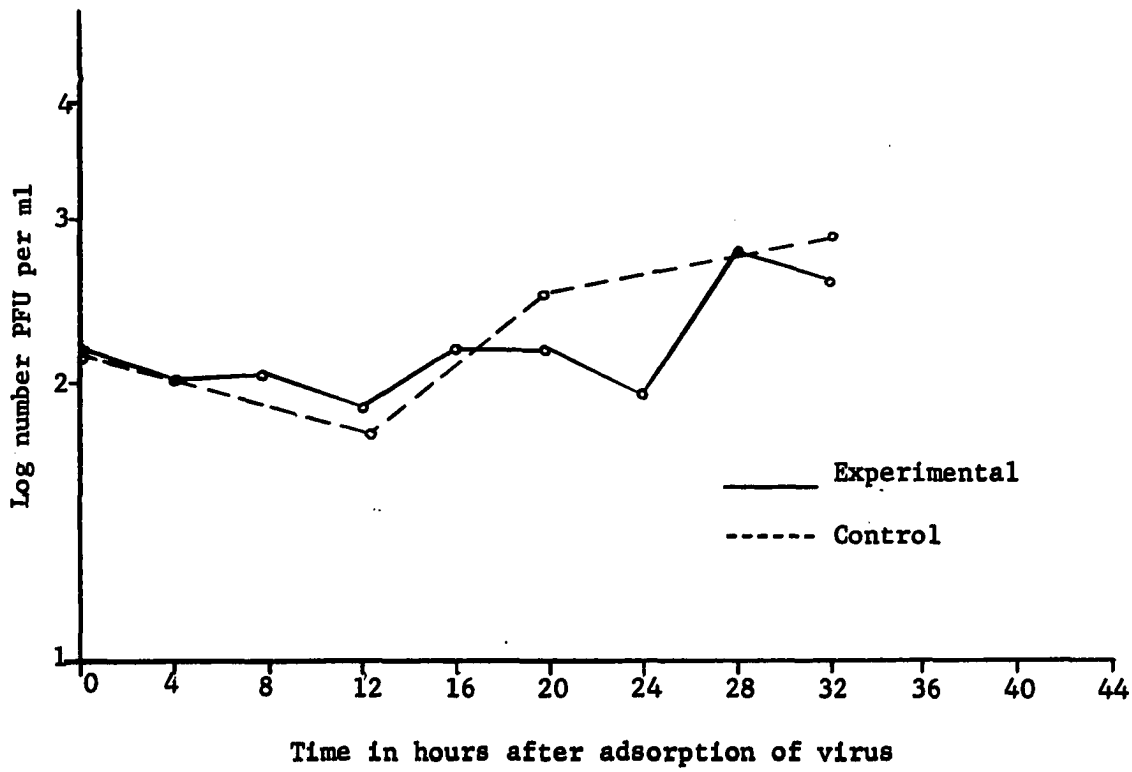


Fig.18. Effect of glucose on the proliferation of Rous sarcoma virus.

$1.05 \times 10^4$ was available for adsorption. The experimental medium consisted of medium 199 from which glucose had been omitted. A supplement of five percent calf serum was added to the medium. The control medium consisted of medium 199 supplemented with five percent calf serum. The experimental and control curves were plotted in Figure 19, from the pock count data recorded in Table 18.

TABLE 18

EFFECT OF GLUCOSE ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 165 | 2.217 | 0 | 50 | 1.699 |
| 4 | 152 | 2.182 | | | |
| 8 | 140 | 2.146 | | | |
| 12 | 26.6 | 1.424 | 12 | 18.3 | 1.262 |
| 16 | 200 | 2.301 | | | |
| 20 | 323 | 2.509 | 20 | 116 | 2.064 |
| 24 | 106 | 2.025 | | | |
| 28 | 21.6 | 1.334 | | | |
| 32 | 291.6 | 2.464 | 32 | 1250 | 3.097 |

[a]Glucose omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

An investigation of the control curve in Figure 19 discloses an initial rapid decrease of virus during the first 12 hours which was followed by an increase in virus through the thirty-second hour.

An examination of the experimental curve of Figure 19 reveals a curve very similar to the experimental curve of Figure 18. There was a slight decrease of virus through the eighth hour followed by a

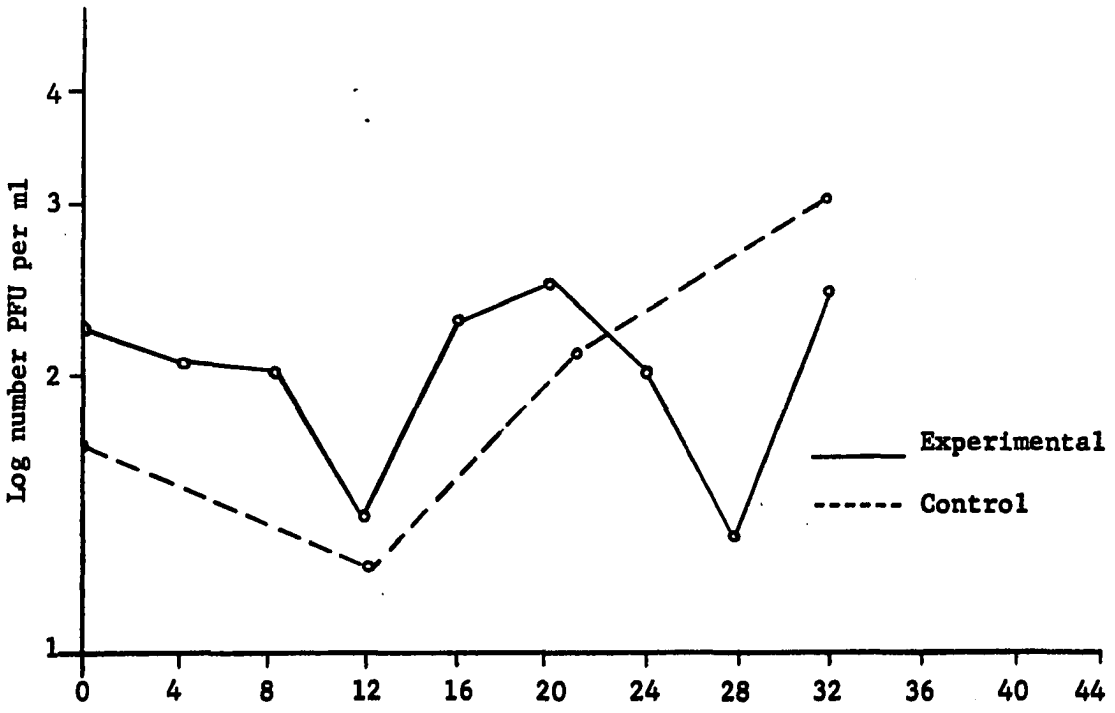


Fig.19. Effect of glucose on proliferation of Rous Sarcoma virus.

precipitous drop in virus concentration through the twelfth hour. After this eclipse there was an increase of virus production through the twentieth hour, at which time the level of virus concentration exceeded that produced in the control medium. After the twentieth hour there was a decrease in the amount of virus produced which continued through the twenty-eighth hour. This decrease was followed by another increase in virus production through the thirty-second hour. Like the experimental curve of Figure 18, this is a bimodal curve, with two areas in which there was a marked decrease in virus, followed by a remarkable increase in virus.

From the data presented in the two sets of curves (Figures 18 and 19), it appeared that some energy source was required, but that it need not be glucose. Two compounds, pyruvic acid and glycerol which occur in the Embden-Meyerhoff-Parnas pathway of glucose fermentation were investigated as substrates which might be substituted for glucose.

In the first experiment, the secondary cultures were initiated in medium G containing five percent calf serum. The cells were infected with $1.15 \times 10^4$ PFU, of which $1 \times 10^3$ PFU were recovered in the PAV and PAW. There were $1.05 \times 10^4$ remaining for adsorption. The experimental medium consisted of medium 199 in which 0.011 M pyruvate was substituted for 0.0055 M glucose. This was supplemented with five percent calf serum. The control cultures received medium 199 supplemented with five percent calf serum. The experimental and control curves plotted in Figure 20, and the pock count data are recorded in Table 19.

TABLE 19

EFFECT OF PYRUVIC ACID ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 183 | 2.262 | 0 | 50 | 1.699 |
| 4 | 120 | 2.079 | | | |
| 8 | 150 | 2.176 | | | |
| 12 | 190 | 2.278 | 12 | 18.3 | 1.262 |
| 16 | 290 | 2.462 | | | |
| 20 | 274 | 2.437 | 20 | 116 | 2.064 |
| 24 | 80 | 1.903 | | | |
| 28 | 280 | 2.446 | | | |
| 32 | 848.3 | 2.926 | 32 | 1250 | 3.097 |

[a]Pyruvic acid, 0.011 M, substituted for 0.0055 M glucose in the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

An inspection of the control curve in Figure 20 will disclose an initial decrease in viral concentration through the twelfth hour. This was followed by an increase in viral production through the thirty-second hour. One can discern from the experimental curve in Figure 20 that a large amount of virus was released through the twentieth hour, followed by a slight decrease in virus through the twenty-fourth hour. An increase in virus concentration became apparent through the thirty-second hour. This curve of pyruvate utilization approached the control curve closely and in the last one-third of the two curves, and the final concentrations of virus were quite similar.

In the next experiment involving a substitution of glycerol for glucose, the secondary cultures were initiated in medium G

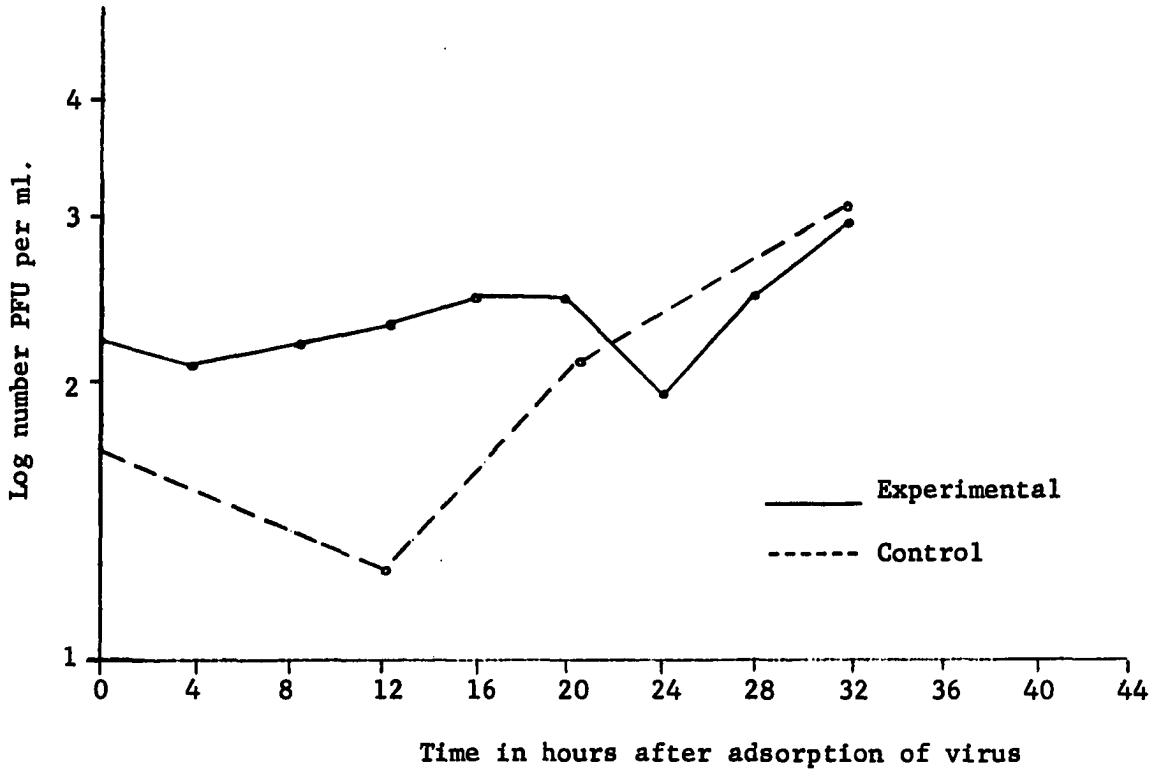


Fig. 20. Effect of pyruvic acid on the proliferation of Rous sarcoma

containing five percent calf serum. The cells were infected with $4.25 \times 10^4$ PFU of virus, of which $3.7 \times 10^4$ PFU were recovered in the PAV and PAW, and $5.5 \times 10^3$ PFU remained to be adsorbed. The experimental medium consisted of medium 199 in which 0.011 M glycerol was substituted for 0.0055 M glucose. Five percent calf serum was added to this medium. The control medium consisted of medium 199 supplemented with five percent calf serum. The experimental and control curves were plotted in Figure 21 from the pock count data as recorded in Table 20.

TABLE 20

EFFECT OF GLYCEROL ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 376.6 | 2.576 | 0 | 395 | 2.596 |
| 4 | 161.6 | 2.208 | | | |
| 8 | 200 | 2.301 | | | |
| 12 | 92 | 1.964 | 12 | 60 | 1.778 |
| 16 | 10 | 1.000 | | | |
| 20 | 92 | 1.964 | 20 | 430 | 2.633 |
| 24 | 201.6 | 2.304 | | | |
| 28 | 135 | 2.131 | | | |
| 32 | 281.6 | 2.449 | 32 | 250 | 2.398 |

[a]Glycerol, 0.011 M, substituted for 0.0055 M glucose in the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

An investigation of the control curve reveals an initial decrease in virus concentration through the twelfth hour, followed by

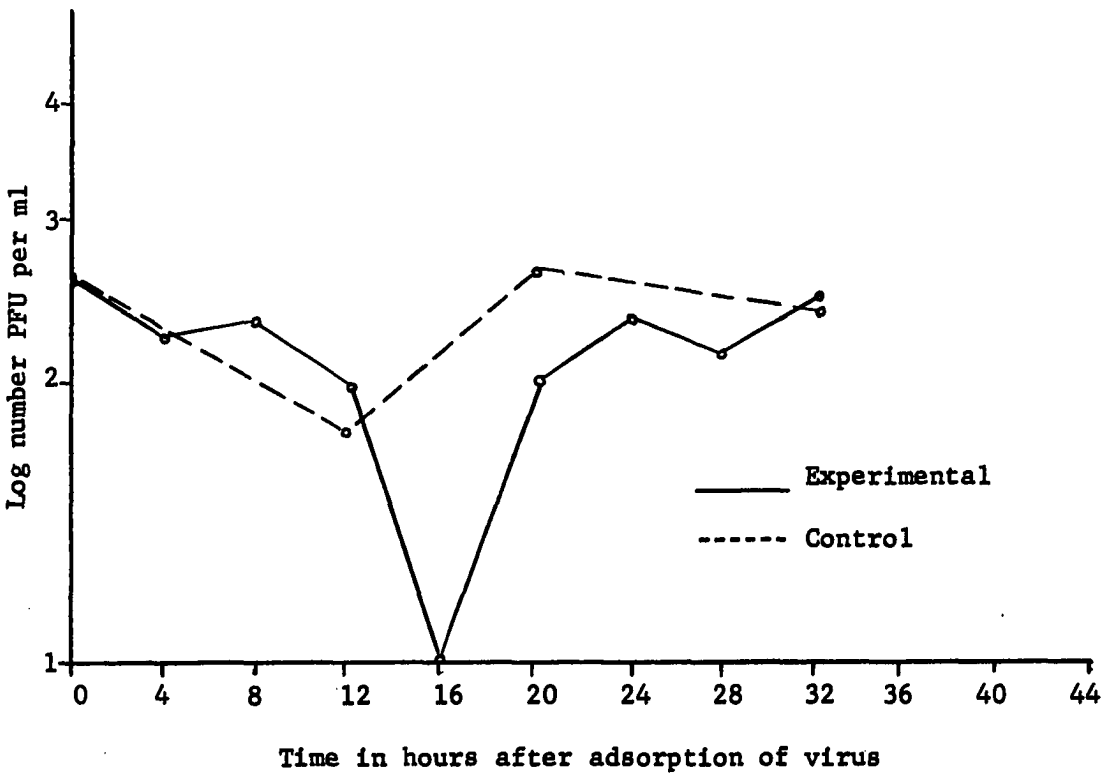


Fig.21 Effect of glycerol on the proliferation of Rous sarcoma virus.

an increased production of virus through the twentieth hour. A slight decrease in virus production occurred between the twentieth and the thirty-second hours.

The experimental curve follows the control curve quite closely through the twelfth hour, at which time the concentration of virus decreased markedly, through the sixteenth hour, which indicated an eclipse period. This was followed by a rapid increase in virus production through the twenty-fourth hour. From this time to the twenty-eighth hour a slight decrease in virus production occurred, followed by an increase in virus production through the thirty-second hour. At this point the concentration of virus in the control and experimental media was approximately the same.

In the next experiment the pentoses, ribose and deoxyribose, were omitted from medium 199. The secondary cultures were initiated in medium 199 containing five percent calf serum. The cells were infected with $1.94 \times 10^4$ PFU of virus. of which $8.4 \times 10^3$ PFU were recovered in the PAV and PAW, which left $1.1 \times 10^4$ PFU to be adsorbed.

The experimental medium consisted of medium 199, devoid of ribose and deoxyribose, supplemented with five percent calf serum. The control medium consisted of medium 199 to which five percent calf serum was added. The control was sampled for virus content every four hours from zero to 32 hours. The representative pock count data are recorded in Table 21 and the experimental and control curves depicting these data are plotted in Figure 22.

It is discernable by examination of the control and experimental curves that although virus production in the control medium

was slightly less than in the experimental medium, the two curves are quite similar after the twelfth hour. The final concentration of virus at 32 hours was approximately the same in both cases.

An evaluation of the experiments described, and a realization of the type of media in which chick embryo fibroblasts will grow prompted a comparison of two media, both of which have been used in this investigation.

TABLE 21

EFFECT OF PENTOSES ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Experimental Medium | | | Control Medium | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 38 | 1.579 | 0 | 38.3 | 1.580 |
| 4 | 66 | 1.82 | 4 | 10 | 1.000 |
| 8 | 62 | 1.783 | 8 | 28.3 | 1.452 |
| 12 | 58.3 | 1.766 | 12 | 18.3 | 1.262 |
| 16 | 200 | 2.301 | 16 | 53.3 | 1.726 |
| 20 | 72 | 1.857 | 20 | 18 | 1.261 |
| 24 | 223.3 | 2.349 | 24 | 120 | 2.079 |
| 28 | 258.3 | 2.412 | 28 | 63.3 | 1.801 |
| 32 | 563.3 | 2.75 | 32 | 652.5 | 2.815 |

[a]Ribose and deoxyribose omitted from the experimental medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

In the first experiment, the secondary cultures were initiated in medium G containing five percent calf serum. The cells were infected with $3.4 \times 10^4$ PFU of virus, of which $2.04 \times 10^4$ were recovered in the PAV and PAW. This left $1.36 \times 10^4$ PFU for adsorption. The

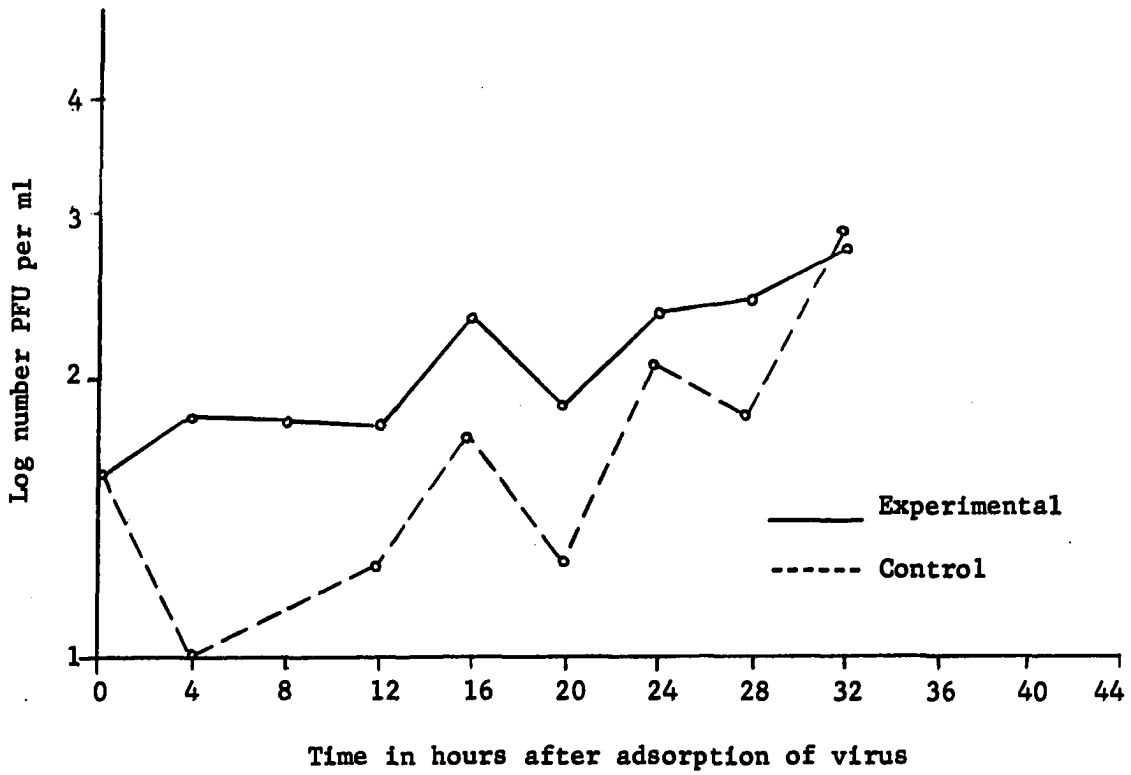


Fig.22. Effect of pentoses on the proliferation of Rous sarcoma virus.

experimental medium consisted of medium G containing five percent calf serum. The control medium consisted of medium 199 supplemented with five percent calf serum. From the pock count data, as recorded in Table 22, the experimental and control curves were plotted as shown in Figure 23.

TABLE 22

PROLIFERATION OF ROUS SARCOMA VIRUS IN MEDIUM G AND MEDIUM 199[a]

| Medium G | | | Medium 199 | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 256 | 2.408 | 0 | 0 | ..... |
| 4 | 253.3 | 2.403 | | | |
| 8 | 254 | 2.404 | | | |
| 12 | 160 | 2.204 | 12 | 102 | 2.085 |
| 16 | 280 | 2.447 | | | |
| 20 | 168.3 | 2.226 | 20 | 876.6 | 2.94 |
| 24 | 592 | 2.774 | | | |
| 28 | 313.3 | 2.496 | | | |
| 32 | 1615 | 3.208 | 32 | 875 | 2.942 |

[a]A study of the comparative abilities of medium G and medium 199 to support proliferation of virus by cells cultured in these media.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

An examination of the control curve in Figure 23 reveals that no virus was recovered at zero hours, probably due to insusceptibility of embryonated eggs used for assay. The zero hour figure was referred back to the zero hour figure of the experimental curve. At the twelfth hour the slope of the control curve which increased sharply through the twentieth hour remained the same through the thirty-second

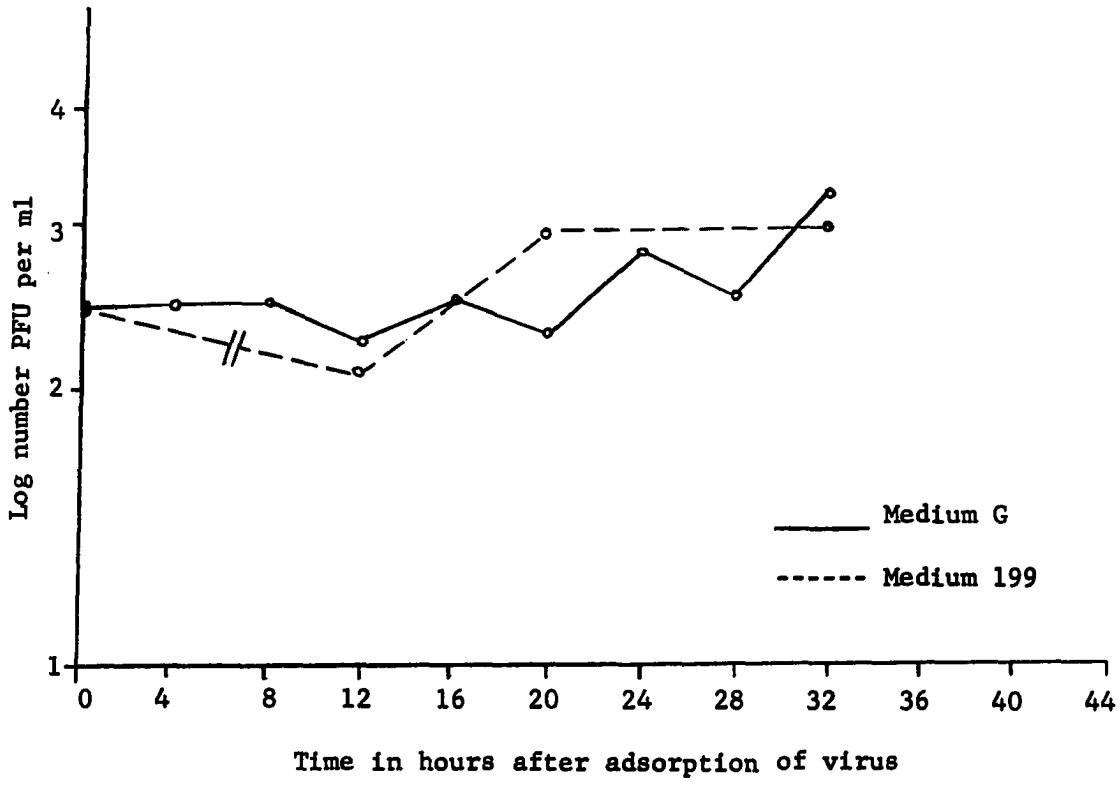


Fig.23. Proliferation of Rous sarcoma virus in medium G and medium 199.

hour.

Initially, there was a slight decrease in virus concentration as reflected in the experimental curve through the twentieth hour, followed by a slight peak at the twenty-fourth hour. Another slight decrease occurred at the twenty-eighth hour, followed by an increase in virus through the thirty-second hour. The level of virus in the experimental medium is approximately twice that in the control medium at the thirty-second hour.

Another experiment was one in which a balanced salt solution was compared with medium 199 in its ability to support virus propagation. The balanced salt solution was supplemented with glutamine and serum, and medium 199 was supplemented with serum.

The secondary cultures were initiated in medium G containing five percent calf serum. The cells were infected with $7.76 \times 10^4$ PFU of virus, of which $3.47 \times 10^4$ PFU were recovered in the PAV and the PAW. A PFU count of $4.29 \times 10^4$ for adsorption was made. The experimental medium consisted of Hanks' complete balanced salt solution containing 350 mg sodium bicarbonate per liter of medium which was supplemented with 100 mg of glutamine per liter and five percent calf serum. The control medium consisted of medium 199 supplemented with five percent calf serum. The experimental and control curves were plotted in Figure 24, from the pock count data recorded in Table 23.

An investigation of the control curve in Figure 24 discloses a rapid decrease in virus released through the twelfth hour, typifying the eclipse period, which is followed by rapid virus

production through the thirty-second hour.

TABLE 23

PROLIFERATION OF ROUS SARCOMA VIRUS IN HBSS AND MEDIUM 199[a]

| HBSS | | | Medium 199[a] | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 255 | 2.406 | 0 | 300 | 2.477 |
| 4 | 346.6 | 2.539 | | | |
| 8 | 295 | 2.469 | | | |
| 12 | 144 | 2.158 | 12 | 155 | 2.190 |
| 16 | 146 | 2.164 | | | |
| 20 | 40 | 1.602 | 20 | 555 | 2.744 |
| 24 | 48.3 | 1.684 | | | |
| 28 | 31.6 | 1.505 | | | |
| 32 | 80 | 1.903 | 32 | 1832 | 3.263 |

[a]A study of the comparative abilities of Hanks' balanced salt solution and medium 199 to support proliferation of virus by cells cultured in these media.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

The curve representative of virus release and propagation in the experimental medium reveals a steady decrease of virus released through the twenty-eighth hour, after which there was a slight increase in virus concentration, through the thirty-second hour. This slight increase resulted in a final concentration of virus of about 1/20 of that in the control medium at the thirty-second hour.

A similar experiment was done in which a comparison was made of the ability of a balanced salt solution and medium 199 to support the proliferation of Rous sarcoma virus. The pock count data are recorded in Table 24 and are plotted in Figure 25.

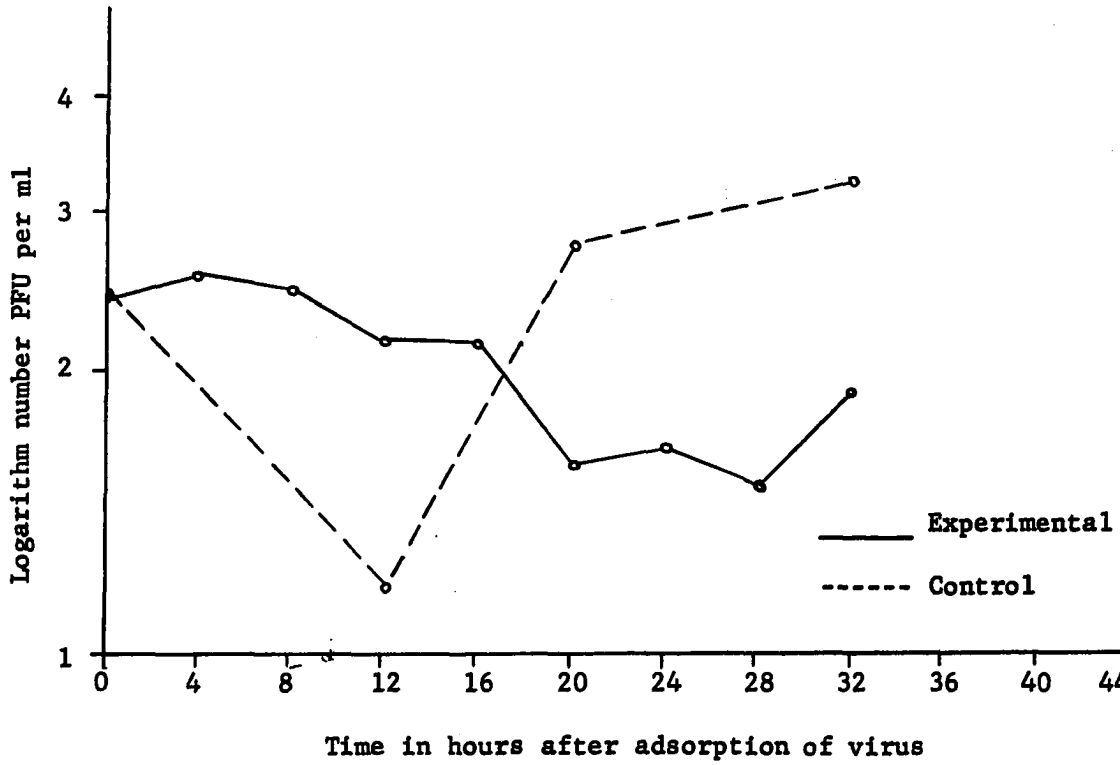


Fig.24. Proliferation of Rous sarcoma virus in HBSS and medium 199.

TABLE 24

PROLIFERATION OF ROUS SARCOMA VIRUS IN HBSS AND MEDIUM 199[a]

| HBSS | | | Medium 199 | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 1185 | 3.073 | 0 | 1574 | 3.197 |
| 4 | 1250 | 3.097 | | | |
| 8 | 596.6 | 2.775 | | | |
| 12 | 308.3 | 2.489 | 12 | 276 | 2.44 |
| 16 | 154 | 2.187 | | | |
| 20 | 41.6 | 1.619 | 20 | 128.3 | 2.108 |
| 24 | 40 | 1.602 | | | |
| 28 | 16.6 | 1.22 | | | |
| 32 | 90 | 1.954 | 32 | 220 | 2.332 |

[a]A study of the comparative abilities of Hanks' balanced salt solution and medium 199 to support proliferation of virus by cells cultured in these media.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

The secondary cultures were initiated in HBSS containing five percent dialyzed calf serum. After washing the cells were infected with $7.9 \times 10^4$ PFU, of which $4.97 \times 10^4$ were recovered in the PAV and PAW, thus leaving $2.92 \times 10^4$ PFU of virus for adsorption. The experimental medium placed on these cultures was HBSS containing five percent dialyzed calf serum. The control cultures were covered with medium 199 supplemented with five percent dialyzed calf serum.

Examination of the control curve reveals that the virus was released slowly until the twentieth hour, after which virus production increased through the thirty-second hour. Examination of the experimental curve reveals a steady decrease in the release of virus

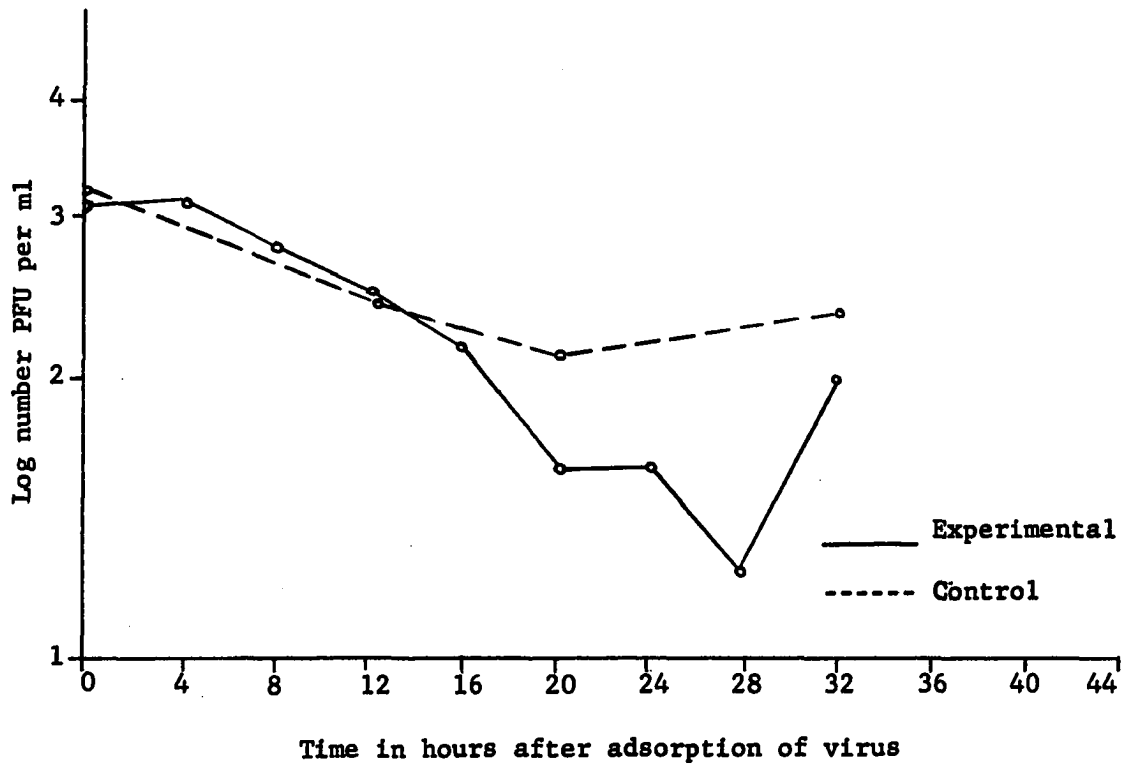


Fig.25. Proliferation of Rous sarcoma virus in HBSS and medium 199.

from the cells through the twenty-eighth hour. This was followed by a very slight increase in virus production through the thirty-second hour.

Dialyzed serum was used in some experiments and undialyzed serum in others. It was of interest to know the effect of dialyzed serum on the proliferation of the virus.

Secondary cultures were initiated in medium G containing five percent dialyzed calf serum. After washing the cells were infected with $8.5 \times 10^4$ PFU of virus of which $7.25 \times 10^4$ PFU were recovered in the PAV and PAW. Thus, the amount of virus adsorbed is $1.25 \times 10^4$ PFU. After infection the experimental cultures received medium 199 containing five percent dialyzed calf serum. The control cultures received medium 199 with five percent undialyzed calf serum. The virus produced in experimental and control media are represented by the curves in Figure 26 and the pock count data are recorded in Table 25.

Examination of the curves in Figure 26 reveals no essential difference in virus production in media containing dialyzed or undialyzed serum. There was an initial decrease in the amount of virus released through the sixteenth hour, followed by an increase in virus concentration through the thirty-second hour.

TABLE 25

EFFECT OF DIALYZED SERUM ON PROLIFERATION OF ROUS SARCOMA VIRUS[a]

| Dialyzed Serum | | | Undialyzed Serum | | |
|---|---|---|---|---|---|
| Hour | $10(\bar{x})$[b] | Logarithm[c] | Hour | $10(\bar{x})$ | Logarithm |
| 0 | 256 | 2.408 | 0 | 76 | 1.88 |
| 4 | 365 | 2.562 | | | |
| 8 | 91.6 | 1.962 | | | |
| 12 | 61.6 | 1.79 | 12 | 60 | 1.778 |
| 16 | 90 | 1.954 | | | |
| 20 | 82 | 1.913 | 20 | 82 | 1.913 |
| 24 | 115 | 2.061 | | | |
| 28 | 102.5 | 2.006 | | | |
| 32 | 345 | 2.537 | 32 | 281.6 | 2.45 |

[a]Medium 199, containing either dialyzed serum or undialyzed serum, was the basic medium.

[b]Denotes 10 times the mean pock count.

[c]Logarithm to the base 10, of $10(\bar{x})$.

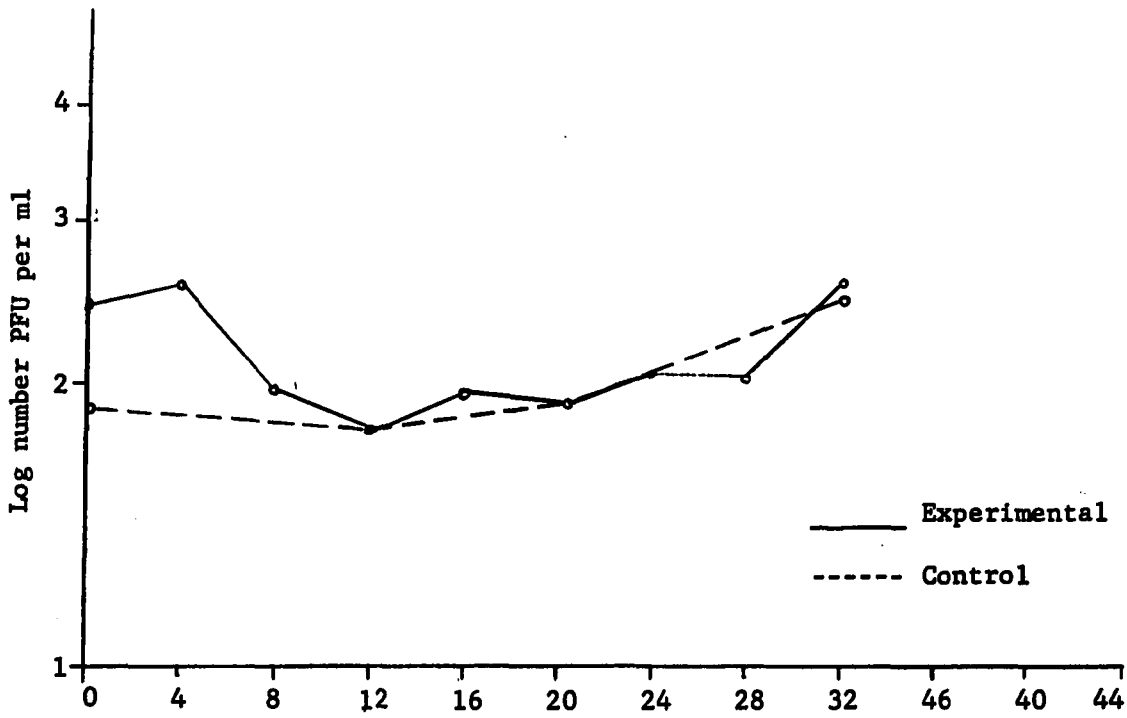


Fig. 26. Effect of dialyzed serum on the proliferation of Rous sarcoma virus.

## CHAPTER IV

## DISCUSSION

A suitable experimental animal and a source of cells susceptible to infection with the virus are essential for any nutritional study. Prince (1958a) investigated the susceptibility to infection with Rous sarcoma virus of embryos of nine strains of chickens and one strain of ducks. He demonstrated that embryos from only three of these breeds evidenced insusceptibility at a rate of less than 20 percent. The insusceptible embryos were designated as "non-reactors". One breed was shown to be non-reactive to infection at a rate of only 4.5 percent. A variety of strains of chickens have been used by other workers in the study of Rous sarcoma virus. In order to be sure that a suitable strain of chicken and the embryonated egg of this chicken were available for use in this research, a careful study was made to determine the relative susceptibilities to the Rous sarcoma virus of chicks and embryos from various strains of chickens. An evaluation of comparative viral titrations revealed that chickens and embryonated eggs of the Honegger white Leghorn breed were well suited to this investigation. The chickens and embryos of several other breeds, including the Indian River breed, were demonstrated to be less satisfactory for this study. These data corroborate the work of Rubin (1955), Prince (1958a) and Vigier (1959) who demonstrated that the chorioallantoic

membrane of embryonated eggs were suitable for titration of this virus.

Although the chorioallantoic membrane of the embryonated egg serves quite well for the titration of the Rous virus, the infected embryos must be incubated seven days before results can be obtained. In addition, the embryos are expensive and subject to biological variation. On the other hand, tissue culture methods of virus titration are relatively inexpensive. Many tubes of monolayers can be obtained from one chick embryo. The cell cultures system utilizes a more homogeneous population of cells. In some cases, titration of virus in tissue culture can be done more quickly.

Manaker and Groupe (1956) reported a method of titration of Rous sarcoma virus. They described the effect of the virus on the cell culture as "altered descrete foci". Because of the ease with which titrations are carried out in tissue culture, an attempt was made in this study to devise a method of titration of Rous sarcoma virus in tissue culture. The attempt was successful to the point that nodules or micro-tumors were formed by infected chick embryo fibroblasts in cultures but formation of micro-tumors was irregular and inconsistent. Later Temin and Rubin (1958) developed a method of titration of this virus in tissue culture, and designated the responsive areas as "foci of infection" or "infective centers." This method of titration was not useable in this study because of the lack of correlation of nodule formation between replicate cultures due to failure of consistent nodule or micro-tumor formation. No pictures were included in the report of Manaker and Groupe and it is assumed that their altered discrete foci and the foci of infection of Temin and Rubin are quite similar. The

nodules or micro-tumors reported in this study were more fully developed than the foci of Temin and Rubin and were composed of many cells which become rounded and piled on one another. Presumably all these effects on the cell cultures are caused by infection of the cell with the virus.

Temin and Rubin (1958) prepared their tissue culture for virus assay by plating 3.5 to 5 x $10^5$ cells. After overnight incubation of the cultures, Rous virus was allowed to infect the cells. These authors determined that the maximum number of cells which could be infected by this method varied between 0.5 to 10 percent. This means that when the amount of virus in the inoculum exceeds the number of cells which are physiologically competent to be infected that some virus is not adsorbed to these cells and consequently is not titrated. This phenomenon would preclude the use of this method of virus titration is quantitative studies. For this reason, inoculation of the virus onto the chorioallantoic membrane of the egg was selected for titration of the virus. It was also determined that embryonated eggs from pullets, eight to 15 months of age, were more sensitive to the virus than older hens.

The medium for the cultivation of infected and uninfected cells in a nutritional study should be a snythetic medium which can be prepared as needed. It should be defined to the extent that any of the several components could be omitted from the medium as desired. Eagle (1955c) devised such a medium for use in culturing cells of animal origin and it was thought that this basal medium would be suitable for the <u>in vitro</u> propagation of chick fibroblasts. However, Eagle's medium failed

to support adequate growth of chick fibroblasts. Another medium, an enzymatic hydrolysate of lactalbumin, which was used by many workers for the cultivation of many types of animal cells, was shown to support adequate proliferation of cells. Because this medium, supplemented with calf serum and tryptose phosphate broth, allowed the proliferation of large numbers of chick fibroblasts in a short period of time, it was selected for the initiation of primary cultures. However, this is not a chemically defined medium and it could not be used for the nutritional studies. Morgan, Morton and Parker (1950) devised a simply synthetic medium, medium 199, for the propagation of animal cells in tissue culture. An investigation of this chemically defined medium revealed that it would adequately support proliferation of chick fibroblasts. The expense of the medium precluded its use in initiation of primary cultures, but it was used to initiate secondary cell cultures and served as the basal medium for the nutritional studies.

Production of Rous sarcoma virus in chick embryo fibroblasts, in vitro, was studied over a period of 48 hours to determine when the cells started to produce virus and the duration of a one-step growth cycle. The studies were done to facilitate selection of a period of time when increased virus production could be observed. If certain components of the medium were required for the production of virus, this requirement could be determined by comparison of virus production in experimental and control growth media. It was observed that loosely adsorbed virus was dissociated from infected cells through the sixteenth hour and this could be detected as free virus in the supernatant

medium. At the sixteenth hour, an eclipse period occurred, when little or no free virus could be detected. Afterward, an increase in virus production occurred and continued through the thirty-second to the thirty-sixth hour. The increase was followed by decreased virus production through the forty-eighth hour. Thus, it was determined that a one-step growth curve occurred between the zero and thirty-second or thirty-sixth hour.

The virus associated with the cell, or contained within the cell, was also investigated to determine if the newly produced virus would accumulate in the cell and not be released into the medium as free virus. Such was not the case. The newly-formed virus was released into the medium soon after it had been formed. It was also determined that the concentration of cell-associated virus did not precede that of the free virus, nor did it exceed the concentration of the free virus at any time. This early release of the virus made it possible to do nutritional studies by the titration of free virus rather than to include procedures for the release of cell-associated virus for titration studies.

Serum has been used in tissue culture studies a long time, and those who have investigated its role in tissue culture systems still do not know exactly why it is required by most cells. Baron and Low (1958) were able to demonstrate that non-fat skim milk would serve as a substitute for the serum in a medium which would allow proliferation of LAC and HeLa cells. The role of serum in these media might be that of a protective colloid, since dialyzed serum usually serves as well

as undialyzed serum. However, accessory growth factors which cannot be removed by dialysis may be bound to the serum protein. It has also been shown by a number of workers that serum facilitates attachment of the cells to the substrate, but there are also strains of cells which do not require serum for any purpose. It was determined that serum is a necessary component of the chick fibroblast-Rous sarcoma virus system. Production of virus does not proceed in the absence of serum. Additional types of proteins or colloids were not examined for their capacity to replace serum in this system. In addition to the skim milk mentioned above, gelatin or starch might serve as protective colloids in the place of serum, if that is the role of serum in this system. On the other hand, an investigation of this factor could show that the cell requires a protein, specifically, or some other nutritive substance in the serum. All of the serum used in these studies were obtained from a large pool of serum collected at one time.

Vitamins, in their coenzyme form, are known to mediate a number of enzymatic reactions and it could reasonably be expected that they might function in the production of the virus. Thiamine, in the form of cocarboxylase, participates in all oxidative decarboxylations which lead to the formation of $CO_2$. Thiamine also has been shown to be required for production of psittacosis virus in L cells (Bader and Morgan, 1961). Riboflavin mononucleotide and the more complex flavin adenine dinucleotides are coenzymes and, with their apoenzymes, form a number of flavoprotein enzymes which can function in the electron transport system. Riboflavin itself (with pyridoxine) is involved

in the conversion of tryptophan to nicotinic acid. Folic acid is required either directly or indirectly for the synthesis of serine, or thymine, of purine bases and, hence, of nucleic acids. These are but a few of the functions of several vitamins, but it can be seen that they are extremely important. Since they play such an important role in cellular metabolism, they were investigated with respect to a requirement for virus production.

Examination of the results of several experiments involving vitamin requirements revealed that with the exception of ascorbic acid, any or all of the vitamins in medium 199 could be omitted from the medium without impairing the production of the virus. Even if the cells were starved for a period of 18 hours before infection, no vitamin requirement could be demonstrated within the 32 hour period of the growth curve. Neither could any requirement be demonstrated for choline, cholesterol nor Tween 80. The production of virus in a vitamin-free medium occurred at the same rate and at the same level as in the control medium which contained vitamins. Conceivably, if primary cultures of cells were initiated in a vitamin-free medium, by the time secondary cultures were initiated in a vitamin-free medium, a requirement for one or several vitamins could be shown.

In one part of the investigation concerned with vitamin requirements of Rous sarcoma virus-infected cells, dialyzed calf serum was used to supplement the medium, since it was thought that dialysis could probably remove many or most of the vitamins from the serum (Eagle, 1955c). No difference could be detected between dialyzed and undialyzed serum in their effect on the proliferation of Rous sarcoma

virus.

Glutamine is an important compound in nutrition and metabolism. It serves as a storage compound for nitrogen and by transamination reactions within the cell, glutamic acid, aspartic acid, asparagine and proline are derived from glutamine (Eagle, 1959; Levintow, 1957a). Levintow (1957b) described multiple functions of glutamine. Independent of glutamic acid it is incorporated as such into protein. Almost half of the pyrimidine carbons and approximately one-tenth that amount of the purine carbons are derived either directly or indirectly from the carbon skeleton of glutamine. Some of the nitrogen of adenine, guanine, cytosine, uracil and thymine is contributed directly and specifically from glutamine amide nitrogen.

Examination of the data in the study involving requirements for glutamine reveals that production of virus by cell cultures in a glutamine-deficient medium lags behind that production of virus in the control medium, but begins to increase, although at a lower level, at about the same time.

The chick embryo fibroblasts either have a store of glutamine, or precursors of purines and pyrimidines, since these substances are not required in the medium, as exemplified in the experiments in which secondary cultures were initiated in a purine and pyrimidine-free medium. The experimental medium also did not contain purines or pyrimidines. Some virus was produced in a glutamine-free medium, and it is conceivable that carbon and nitrogen for purines and pyrimidines came from another but limited source. No virus could be detected until a sufficient supply of purines and pyrimidines had been synthesized to provide the ribonucleic

acid of the virus. Salzman, Eagle and Sebring (1958) demonstrated the utilization of glutamine, glutamic acid and ammonia for the biosynthesis of nucleic acids in mammalian cell culture. Fairman (1959) showed inhibition of cell division by 5-fluorouracil, but infected cells were not prevented from producing Rous sarcoma virus if they were exposed to this compound. This analog inhibited DNA synthesis or utilization, but not that of RNA which is found in Rous sarcoma virus.

Three amino acids, (alanine, serine, and glycine) have been reported to be derived from glucose and four amino acids (glutamic acid, aspartic acid, asparagine, and proline) from glutamine (Eagle, 1959; Levintow, 1957a; and Levintow, 1957b). Since an exogenous source of some of the amino acids is not required, it was of interest to know what amino acids, if any, might be required by the cell for virus production. Morgan and Morton (1957b) determined that chick embryonic heart fibroblasts required arginine, histidine, lysine, tryptophan, phenylalanine, tyrosine, cyst(e)ine, methionine, threonine, leucine and valine for survival and growth since these amino acids disappeared from the medium. Conversely, glutamic acid, aspartic acid and alanine accumulated in the medium. These authors observed that chick embryonic heart possesses an active transaminating system which causes the accumulation of excessive and possibly inhibitory amounts of certain amino acids. In addition, proline and hydroxyproline were shown to be inhibitory, while glycine, serine, and glutamine were not required.

Certain amino acids, the building blocks of protein, have been found to be essential for proliferation of animal cells in tissue

culture by Morgan and Morton (1957b), Eagle, et al. (1956), Eagle, (1955b), Pasieka, Morton and Morgan (1956; 1958a;1958b), and others. Amino acids also have been shown to be required by some infected cells for the production of virus (Ackermann, 1951; Bader and Morgan, 1958: Dubes, 1956; Heggie and Morgan, 1956; Morgan, 1954). Formation of proteins as precursors of enzymes needed for production of virus may be inhibited by omission of a specific amino acid from the medium in which the cells are grown. In a few instances, an exogenous source of amino acids is not required. Eagle and Habel (1956) observed that amino acids were not necessary for the propagation of poliovirus in HeLa cells. In the present study the amino acids of medium 199 were grouped according to the requirements of chick embryo heart fibroblasts (Morgan and Morton, 1957b), and the experiments were carried out in the manner of those done by Tyndall and Ludwig (1960), who were studying amino acids essential for the production of poliovirus, Coxsackie B-3, and vaccinia viruses by cells in continuous culture. An investigation into the amino acid requirements of the Rous sarcoma virus-infected chick fibroblasts revealed that as a group, the amino acids, serine, aspartic acid, glutamic acid, alanine, proline, hydroxyproline, and glycine were not needed for the production of the virus. Neither were these amino acids required for the proliferation of the chick fibroblast (Morgan and Morton, 1957b). However, the group of amino acids comprised of arginine, histidine, lysine, tryptophan, and phenylalanine was shown to be necessary for the proliferation of the virus. Similarly, all of these amino acids were essential for the proliferation of the chick embryonic heart fibroblast (Morgan and Morton 1957b).

Threonine, leucine, isoleucine, and valine were also investigated as a group and it was disclosed that although virus production lagged behind the virus production by the control cultures, a large quantity of virus was produced by the cells in the experimental medium. Morgan and Morton (1957b) showed that of the amino acids in this group, only isoleucine was not essential for proliferation of the chick embryonic heart tissue. The first experiment in this study which indicated a need for some amino acid was one in which all the amino acids of medium 199, with the exception of glutamine and glutathione, were omitted from the medium. The cells in the experimental medium failed to produce virus, which indicated that some amino acid or amino acids were involved in the production of the virus. It was apparent from this experiment that glucose, glutamine, glutathione and serum in a balanced salt solution would not support viral proliferation. The amino acids methionine, tyrosine, cystine and cysteine were not investigated for their effect on the proliferation of the Rous sarcoma virus.

It is known that some of the amino acids (serine, alanine and glycine) are derived primarily from glucose, and other amino acids (glutamic acid, aspartic acid, asparagine, and proline) are derived primarily from glutamine. Fischer, et al. (1952) demonstrated that radioactivity of C-14 labeled-glucose appeared in aspartic acid, glutamic acid, alanine, serine, and proline of tissue proteins, after 72 hours. This explained why the group of amino acids composed of serine, alanine, aspartic acid, glutamic acid, proline, hydroxyproline and glycine were not essential for the proliferation of Rous sarcoma

virus.

Glucose is found in most tissue culture media and serves as an oxidizable carbon source. It was of interest to know if glucose specifically was required for this role in the production of Rous sarcoma virus by chick embryo fibroblasts. When glucose was omitted from the experimental medium, it was observed that the cells were capable of producing virus. It was also noted that there were two points in the curve representing virus production at which a decrease in the production of virus occurred. Kuwata and Shiba (1955), studying production of ornithosis virus in chick fibroblasts, showed that all the glucose in the medium was depleted by the cells within 24 hours, but that virus production did not begin until the thirty-sixth to the forty-eighth hour. However, no virus was produced by cells in a glucose-free medium. They also determined that neither folic acid nor amino acids could satisfy the glucose requirement. Weber, et al. (1960), who compared the metabolism of slices of Rous sarcoma and chorioallantoic membrane, discovered an increased glucose uptake in Rous sarcoma over that of the control tissue. In both types of tissue glucose was fermented to pyruvic acid which was converted to lactic acid. The lactic acid content of Rous sarcoma was greater than that of the CAM. A highly increased level of lactic dehydrogenase was observed in the tumor tissue which was correlated with the markedly increased lactate production in this tissue. Wilson, Jackson and Brues (1941-1942) also found glucose was converted to lactate by chick embryo tissue. They observed that the lactate could be derived from glucose through pyruvate or from certain amino acids. In a glucose-

free medium, the tissues produced 10 times as much ammonia nitrogen and urea as compared with those supplied with glucose. These authors also noted that pyruvic acid is rapidly metabolized by these tissues. Harris and Kutsky (1953) demonstrated that D-mannose and D-fructose were also utilized by chick heart fibroblasts and that D-maltose and glycogen were hydrolyzed to glucose by extracellular enzymes.

These observations concerning the relationship between glucose and lactic acid could explain the production of virus in a glucose-free medium if the cells were not starved of glucose. The first decrease in virus production corresponds to the time at which the eclipse period occurred. The second decrease in the concentration of the virus occurred at about the time the cells would have depleted the supply of glucose in the medium or of any form of glucose stored in the cell. The increase in the concentration of virus in the medium after the twenty-fourth hour could be explained by utilization of lactic acid by the cell for the production of virus. An alternate explanation is one which would allow production of virus through 20 hours by cells which utilized stored lactic acid. The proliferation of virus would be followed by a decrease in virus production during the period of time in which deamination of amino acids might be taking place, followed by conversion of these amino acids to glucose or to lactic acid through pyruvate. Sequence of events would explain the second increase in virus production and is in accordance with the findings of Wilson, Jackson and Brues (1941-1942).

It was also shown in the study reported here that if pyruvic acid were substituted for glucose at twice the molar concentration of glucose in the medium, virus production would occur at a level comparable

to that in the control medium. This observation also is in accordance with the findings of Wilson, Jackson and Brues (1941-1942) who noted rapid utilization of pyruvate by chick embryo tissue. Similarly, glycerol, at twice the molar concentration of glucose, would satisfy requirements for the production of virus. This finding is not unduly surprising, since both pyruvic acid and glycerol are intermediates in the Embden-Myerhoff-Parnas pathway of glucose fermentation. It was also shown that if the pentoses, ribose and deoxyribose, were omitted from the medium which contained glucose, there was no appreciable effect on the proliferation of the Rous sarcoma virus. The level of virus concentration at 32 hours was approximately the same in both the experimental and control media.

Thus far the amino acids, glucose, glutamine and the vitamins of medium 199 have been studied for their effect on the proliferation of Rous sarcoma virus. Eagle's medium is not as complex as medium 199, in that it does not contain purines, pyrimidines, fat-soluble vitamins, ATP, adenylic acid, pentoses, Tween 80, sodium acetate, ferric nitrate nor the non-essential amino acids, all of which are present in medium 199. It became of interest to know if these components could be omitted from medium 199 without interfering with the production of Rous sarcoma virus.

After an evaluation of these studies and a comparison of the components of Eagle's medium and medium 199, a new medium, known as G which stands for growth, was devised. The concentrations of the components of medium G are the same as those of the same components in medium 199. Evaluation of the results of two experiments reported

in this study revealed that medium G supported viral propagation by chick fibroblasts at least as well as did medium 199. The further use of this medium in nutritional studies could conceivably facilitate the demonstration of requirements for production of Rous sarcoma virus by chick fibroblasts. This is a medium in which many of the nonessential components of medium 199 have been omitted and is, therefore, a less complex medium. After medium G had been developed and used in these experiments, the work of Johnson and Morgan (1956) was discovered and their medium 635 is remarkably similar to medium G. In addition to the components omitted from medium 199 in the preparation of medium 635, Medium G also lacks all the vitamins except ascorbic acid, sodium acetate, ferric nitrate and Tween 80.

Further studies compared the abilities of Hanks' balanced salt solution and medium 199 to support the proliferation of Rous sarcoma virus by chick embryo fibroblasts. Even though the balanced salt solution was supplemented with serum and glutamine, it failed to support virus production by infected cells. This is in contrast to the production of virus in medium 199 supplemented with serum.

Upon examination of these and other data presented here, it is apparent that for the production of Rous sarcoma virus, chick embryo fibroblasts must be supplied with serum, amino acids and the inorganic ions of the balanced salt solution. Some of the amino acids can be omitted from the medium without impairing virus production but omission of all amino acids from the experimental medium resulted in production of no virus. Glucose and glutamine were found to stimulate proliferation of the virus but if they were omitted from the medium, virus

production was only delayed or slightly curtailed.

The unknown component of all media described is the serum and its role is not well understood. Eagle (1955c) reported that exahustive dialysis of serum rendered it unsatisfactory for supporting proliferation of L cells and HeLa cells, but that less exhaustively dialyzed serum was quite satisfactory for this purpose. When serum which was dialyzed 24 hours was compared with undialyzed serum in the present study, it was found that this period of dialysis had no effect on proliferation of the cells or production of the virus.

## CHAPTER V

## SUMMARY

The Honegger white Leghorn chicken and its embryonated egg were shown to be suitable for quantitative studies of Rous sarcoma virus.

Several media were investigated for their ability to support proliferation of chick embryo fibroblasts and for their suitability as basal media in nutritional studies. Lactalbumin hydrolysate medium supplemented with serum, yeast extract and tryptose phosphate broth was selected for the initiation of primary cultures of chick fibroblasts. Medium 199 supplemented with serum was shown to be suitable for the initiation of secondary cultures of chick fibroblasts and for a basal medium in nutritional studies.

An in vitro cell culture method was devised which adequately demonstrated viral activity by the formation of foci of infection which, when allowed to develop, formed micro-tumors. This method was shown to be less sensitive as an assay method than that of the chick embryo chorioallantoic membrane technic which was used for viral assay in this investigation.

As a baseline for comparison of viral production in experimental media, the production of virus by chick fibroblasts was determined in complete medium 199 during the period from zero to 48 hours after

viral adsorption. This was found to be a one-step growth cycle which ended between the thirty-second and the thirty-sixth hours. An eclipse period was observed at or about the sixteenth hour and thereafter virus production increased through the thirty-second hour. Therefore, the time selected for the observation of cellular production of virus in experimental medium was the period from zero to thirty-two hours.

Nutritional components were omitted in a stepwise fashion from medium 199 to determine the requirements for cellular proliferation and cellular production of virus. Omission of the vitamins from the experimental medium had no effect on virus production, and the use of a vitamin-free medium for the cultivation of secondary cultures of cells prior to infection also had no effect on the proliferation of virus.

Infected cells cultivated in a glucose-free medium continued to produce virus over a 32 hour period; pyruvic acid and glycerol had no appreciable effect on the production of the virus.

When glutamine was omitted from the medium, production of virus lagged behind virus production by cells in a control medium, but the cells in the experimental medium did produce virus. Presumably the cells were able to draw carbon and nitrogen from another source for synthesis of purines and pyrimidines which were to be incorporated into nucleic acid of the virus.

Serine, aspartic acid, glutamic acid, alanine, proline and hydroxyproline were not essential and arginine, histidine, lysine, tryptophan and phenylalanine, were shown to be required by the cells for the production of virus. Threonine, leucine, isoleucine, and

valine, when omitted from the medium had a slight effect on the proliferation of virus. The cells were capable of producing virus but at a rate slightly less than that of cells in the control medium.

A medium, known as medium G, devised in this laboratory for the in vitro growth of chick embryo fibroblasts, was as capable of supporting proliferation of virus by infected cells as was medium 199. This medium was used to initiate secondary cultures of cells in a number of the experiments. In addition Hanks' balanced salt solution supplemented with glutamine and serum would not support proliferation of Rous sarcoma virus by chick fibroblasts.

Serum was shown to be a necessary component of the medium in which infected cells would produce virus. If the serum were omitted from the medium, the cells failed to produce the virus. It was not possible to distinguish between dialyzed and undialyzed serum in their effect on the proliferation of Rous sarcoma virus.

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