PHYTOHEMAGGLUTININ STIMULATION OF MOUSE SPLEEN LEUKOCYTES

THE EFFECT OF PHYTOHEMAGGLUTININ ON THE INFECTION OF MOUSE SPLEEN LEUKOCYTES WITH VESICULAR STOMATITIS VIRUS

By

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Mouse spleen leukocytes were stimulated with phytohemagglutinin and infected with Vesicular Stomatitis Virus. The virus titre from stimulated and unstimulated cells was determined and the number of infected cells in stimulated and unstimulated cultures was examined.

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ABBREVIATIONS

B particles: normal infectious bullet shaped particles of VSV.

BSA: Bovine Serum Albumin.

$CaSO_4$: calcium sulphate.

^{14}C: carbon fourteen.

CO_2: carbon dioxide.

^{o}C: degrees centigrade or Celsius.

ConA: Concanavalin A.

cpm: counts per minute.

cc: cubic centimeter (or millilitre).

Ci: Curie.

μCi: microCurie or 10^{-6} Curies.

DNA: deoxyribonucleic acid.

gm: gram.

mg: milligram or 10^{-3} grams.

μg: microgram or 10^{-6} grams.

HRLT PP_2: Heat resistant strain of VSV containing long truncated particles which has been plaque purified to produce a high proportion of B particles.

mmole: millimole or 10^{-3} moles.

mm: millimeter.

ml: milliliter.

MEM: Minimal essential medium.

Sp MEM: Joklik medified MEM.

MOI: multiplicity of infection.

NBCS: New born calf serum.

PBS: phosphate buffered saline.

PFU: plaque forming units.

pH: $\log_{10}$ concentration of hydrogen ions.

PHA: phytohemagglutinin.

PHA MR68: purified PHA.

KCl: potassium chloride.

KH_2PO_4: potassium phosphate monobasic.

RNA: ribonucleic acid.

rpm: revolutions per minute.

RPMI 1640: Roswell Park Memorial Institute 1640 medium.

NaCl: sodium chloride.

$NaHPO_4$: sodium phosphate.

^3H: tritium.

VSV: Vesicular Stomatitis Virus.

INTRODUCTION

Immune System

The immune system, which constitutes the major defense mechanism directed against invading organisms and foreign materials or tissues, is associated mainly with the lymphoid tissues. These include lymph nodes, spleen, thymus, gut associated lymphoid tissue or GALT (tonsils, Peyer's Patches, the Bursa of Fabricius in birds) and the lymphatic circulatory system, which carries lymph fluid (plasma) and lymphoid cells from one part of the system to another. The lymphatic circulatory system is apart from the blood circulatory system, but communicates with it via the thoracic duct, thus allowing lymphoid cells to recirculate from the blood to the lymph (Gowans, 1968).

The immune responses to antigens are generally of two types: (1) the cellular immune response, which is responsible for delayed hypersensitivity, tumour rejection and graft rejection, and (2) the humoral immune response, which is mediated by the production of circulating antibodies. In 1956, Glick *et al.* found that removal of the Bursa of Fabricius from neonatal chicks led to impaired humoral immune responses. Robert Good *et al.* (1962) later found that neonatal thymectomy of mice led to impaired cellular

immune responses and impaired humoral responses to some antigens, such as sheep red blood cells. This led to the discovery that two different types of lymphocytes are responsible for the two types of immune response: thymus derived cells (or T cells) are involved in the cellular immune response and humoral responses to some antigens, and bursal derived cells in birds or bursal equivalent cells in mammals, which lack a bursa, are responsible for the production of antibodies in the humoral immune response (Cooper et al., 1966).

Very little is known about the nature of the cellular immune response. The circulating cells which come in contact with the antigen are sensitized by an unknown mechanism, which may involve antibody-like receptors (Sell and Asofsky, 1968), although there are conflicting reports (Vitetta et al., 1973). The sensitized cells probably migrate via the lymph or blood from the site of antigenic contact to the site of sensitized cell production, such as the paracortical regions of the lymph nodes, where they undergo blast transformation and mitosis (Turk and Oort, 1967), thus producing larger numbers of specifically sensitized cells which are then disseminated via the lymph or blood back to the site of antigenic contact (Sell, 1972). Once in contact with the antigen-target cells, the sensitized cells secrete a number of substances which aid in the

destruction of the target cells: migration inhibitory factor (affecting macrophages), chemotactic factors (affecting macrophages, eosinophils and neutrophils), cytotoxic factors, skin reactive factor, mitogenic factor, interferon, immunoglobulin and transfer factor (David, 1971). The nature and purposes of these factors is not completely known.

The induction of the humoral immune response is better characterized and appears to involve interactions between at least two cell types and probably three. Mosier (1967) found that antibody formation in vitro requires the presence of two types of cells: plastic (or glass) adherent cells, which behave like macrophages, and non adherent cells, which have the morphology of small lymphocytes. The macrophages phagocytose the antigen (Frei et al., 1965) and may process it under the influence of a specific microenvironment since antigens phagocytosed in the liver are completely destroyed while those phagocytosed in the spleen are not (Franzl, 1972). The antigenic material is then transferred to the lymphocytes from the macrophages (Bona et al., 1972).

The role of the T cells in the humoral immune response is not known. Some antigens require the presence of T cells for both IgG and IgM responses while others can elicit either IgM or IgG and IgM responses in the absence

of T cells (Hunter and Munro, 1972). T cells may only be required where the antigenic dose is low (Tingle and Shuster, 1974) or where carrier-hapten molecules are used as antigen (Mitchison et al., 1970). Sell (1972) suggests that the T cell may present the T dependent antigen, which it received via the macrophage, to the B cell in a more reactive form. It is also possible that the sensitized T cell may secrete a substance, such as a mitogen (David, 1971), which reacts with the B-cell-antigen complex to stimulate the B-cell into blast transformation and mitosis, as seen in the germinal centres of the lymph nodes (Nossal et al., 1964).

Phytohemagglutinin Stimulation

In 1959, Hungerford et al. discovered that a glycoprotein extract of red kidney beans (Phaseolus vulgaris) called Phytohemagglutinin, which had previously been used to agglutinate red blood cells, caused peripheral blood lymphocytes to undergo blast transformation and mitosis in vitro. This opened up a new area of in vitro study of the immune system in general, and of lymphocytes in particular. Phytohemagglutinin (PHA) was found to induce blast transformation and mitosis in cells from different lymphoid tissues from a wide variety of animals (Naspitz and Richter, 1968). It was found to stimulate T cells specifically (Weksler et al., 1974; Piquet and Vassalli, 1973) although

conflicting results have been reported (Phillips and Weisrose, 1974). (Epstein et al., 1974, have reported that B cells can only respond to PHA in the presence of macrophages.) However, the evidence is so overwhelmingly in favour of the T cell specificity of PHA stimulation that it is being used as a diagnostic tool to test for cellular immunodeficiency states (Lawlor et al., 1973).

A vast amount of research has been directed at investigating the effect of PHA on various biochemical parameters in an attempt to determine the nature of the stimulatory event. Smith et al. (1971) reported an early rise followed by a decrease in the level of adenosine 3'5' monophosphate (cyclic AMP) in PHA stimulated human lymphocytes. This suggested that PHA may activate the adenylcyclase-cyclic AMP system of the lymphocytes in a manner similar to the activation of target cells by hormones. However, it was later reported (Smith et al., 1971a) that exogenous cyclic AMP inhibits the response of lymphocytes to PHA. DeRubertis et al. (1974) reported no change in the level of cyclic AMP following stimulation of mouse spleen lymphocytes with a variety of non specific mitogens, including PHA, and an inhibition of the mitogenic response following treatment with substances which increase the level of endogenous cyclic AMP. Thus, it appears that cyclic AMP is not directly involved in the response of lymphocytes to mitogens.

Other studies include the findings of Pernoud et al. (1973) that PHA stimulation is accompanied by an increase in the activity of βglucuronidase, which is involved in an alternate pathway of glucose catabolism. Kalwinsky and Lindquist (1973) found that rat lymphocyte acid phosphatase, which is involved in many catabolic reactions, acquires two new isoenzyme bands on acrylamide gel electrophoresis. Increases following stimulation with PHA have been observed in the pools of DNA precursor molecules (Munch-Petersen et al., 1973), the level of DNA polymerase (Tyrsted et al., 1973), the level of polyamines (Kay and Lindsay, 1973), the level and activity of RNA polymerases A and B (Cooke and Kay, 1973), ribosomal RNA synthesis (Kay, 1968), polysome content (Sörén and Biberfeld, 1973, Rabinowitz et al., 1973), and in DNA, RNA and protein synthesis (Sell et al., 1965), to name just a few examples. All of these findings suggest that PHA causes an increase in cell activity. However, the signal stimulating event is still unknown.

Stimulation of lymphocytes with PHA results in differentiation of the cells into apparently active immune cells. This has been supported by the findings of a number of workers that PHA stimulated cells produce, in vitro, substances similar to those produced following antigenic stimulation. Papageorgiou and Sorokin (1974) found that PHA stimulated human lymphocytes produce migration inhibitory

factor (MIF). Colony stimulating factor was found to be secreted by PHA stimulated mouse spleen cells (Parker and Metcalf, 1974), while Proliferation Inhibitory Factor (PIF) was found in the supernatant of human peripheral lymphocytes stimulated with PHA (Badger et al., 1973). A number of workers have observed that mitogen stimulated lymphocytes produce cytotoxic factors in vitro (Holm et al., 1973; Skejskal et al., 1973; Peter et al., 1973) and interferon (Wheelock, 1965; Friedman and Cooper, 1967; Wallen et al., 1973). All of these factors were shown to be produced by sensitized cells during cellular immune responses (David, 1971). For these reasons, it is thought that PHA induces changes in the cells similar to those produced by antigenic stimulation.

Another lymphocyte mitogen which has been studied widely in Concanavalin A. This is also a plant lectin, and appears to stimulate T cells specifically (Stobo and Paul, 1972). The nature of ConA stimulation is generally similar to that of PHA, although some reports suggest that the two are not identical (Perlmann and Nilsson, 1970). ConA has been found to agglutinate neoplastically transformed cells, while having no effect on normal cells unless they have been treated with trypsin (Inbar and Sacks, 1969; Inbar et al., 1969). It has also been found to inactivate enveloped viruses (Okada and Kim, 1972), prevent adsorption of enveloped viruses to host cells treated with ConA

and agglutinate cells infected with enveloped viruses (Becht et al., 1972). Because of the latter properties mentioned, ConA presented rather awkward problems with respect to the present study.

Viral Infection of Lymphocytes

Lymphocytes are generally poor host cells for viral infection. However, a number of viruses have been demonstrated to replicate in lymphocytes from a variety of species. These include Fowl Plague Virus (grown in chicken leukocytes); Rous Sarcoma Virus (chicken); Vaccinia Virus (rabbit); Newcastle Disease Virus (chicken); Hog Cholera Virus (pig); African Swine Fever Virus (pig); measles (human and monkey); Echovirus 9 (human) and Poliovirus I (human) (Gresser and Lang, 1966). After stimulation with a mitogen, however, lymphocytes are able to support the replication of some viruses to which they are normally refractory. Duc-Nguyen and Henle (1966) demonstrated that mumps virus would replicate in PHA stimulated human peripheral leukocytes. Vaccinia Virus was shown to grow to higher titres in PHA stimulated leukocytes than in unstimulated cells (Miller and Endus, 1968), and Nahmias et al. (1964) showed that while Herpes Simplex Virus will not grow in resting leukocytes, it will in PHA stimulated cells.

In 1967, Edelman and Wheelock found that human monocytes, which are macrophage precursors, are normally

susceptible to infection with Vesicular Stomatitis Virus (VSV), while human peripheral lymphocytes are not. However, upon stimulation with phytohemagglutinin, human lymphocytes become permissive to infection with VSV (Edelman and Wheelock, 1968). Other workers have also found that antigen or mitogen stimulated human peripheral, mouse lymph node or spleen, and guinea pig lymph node lymphocytes will support the replication of VSV while unstimulated cells will not (Rossier and Landry-Pigeon, 1972; Eustatia and Van der Veen, 1971; Kano et al., 1973; Nowakowski et al., 1973). Bloom and his co-workers used this property of stimulated lymphocytes to develop an assay system to enumerate cells stimulated either by antigen or mitogen.

The apparent ability of lymphocytes to undergo a change from VSV resistant to susceptible cells following stimulation suggested that a study of the infection of unstimulated and stimulated cells would provide some insight into the molecular events following stimulation. In this sense, VSV would act as a molecular probe to indicate the level of viral replication which is affected by the change in the lymphocytes following stimulation.

Vesicular Stomatitis Virus

Vesicular Stomatitis Virus (VSV) is an enveloped RNA virus and a member of the Rhabdovirus group. The morphology

and replication of VSV have recently been reviewed by Howatson (1970) and Galet (1973). Adsorption and penetration may occur by fusion of the viral envelope with the cell membrane, resulting in the release of the ribonucleoprotein core into the cytoplasm (Heine and Schnaitman, 1969), or the virions may be introduced into the cytoplasm by viropexis, a process similar to phagocytosis, with the uncoating occurring in the resulting vesicles (Howatson, 1970). The viral RNA, which is complementary to the messenger RNA, is transcribed by a virion associated RNA polymerase to form messenger RNA. Protein synthesis and replication of virion RNA occur in the cytoplasm and assembly of virions occurs at the cell membrane where the viral proteins associated with the envelope have been incorporated. The location of the membrane seems to be species dependent, since VSV assembles at the surface of mouse L cells (Howatson and Whitmore, 1962) and at intracellular membranes in chick embryo fibroblasts (Hackett et al., 1968). The completed virions are then extruded from the cell, enveloped in the cell membrane containing the viral proteins (Howatson, 1970).

Purpose of Study

The present study is concerned with an attempt to determine the initial effect of PHA on mouse spleen leukocytes by looking at its effect on the replication of

VSV in stimulated cells. This is a preliminary study whose purpose was to define more specifically the growth of VSV in mouse spleen leukocytes before and after stimulation with PHA with respect to the number of cells in the population which will support VSV replication. An attempt was made to determine whether PHA affects adsorption and penetration of VSV to mouse spleen leukocytes.

MATERIALS AND METHODS

Materials

Bacto phytohemagglutinin-P (PHA-P) was purchased from Difco Laboratories and reconstituted in phosphate buffered saline (PBS) or twice distilled water at a concentration of 0.8 mgm or 1.0 mgm per ml. Because the activity of PHA-P degrades after 2 or 3 weeks in solution, only small quantities were reconstituted at a time. Reconstituted PHA-P was sterilized by filtration through a nitrocellulose filter of pore size 0.20 μ.

Purified phytohemagglutinin MR68 (PHA MR68) was purchased from Wellcome Reagents Limited, and reconstituted in PBS at a concentration of 1.0 mgm per ml. This was used in experiments where the DNA synthetic response of mouse spleen leukocytes was examined.

Thymidine-methyl-^{3}H (^{3}H-TdR) was purchased from New England Nuclear. The specific activities of successive lots used were 15.6 Ci/mmole; 20.0 Ci/mmole; 12.4 Ci/mmole and 15.3 Ci/mmole. In all cases, the thymidine was diluted to 100 μCi per ml in PBS.

5-^{3}H-Uridine was purchased from Amersham Searle. The specific activity was 27 Ci/mmole and the final concentration of the stock solution after dilution in PBS was 100 μCi/ml.

Amino acid-^{14}C mixture was purchased from New England Nuclear. The final concentration of the stock solution was 100 μCi/ml.

^{3}H-alanine was purchased from New England Nuclear. The final concentration of the stock solution was 100 μCi/ml.

Sodium Heparin was obtained from Connaught Medical Research Laboratories at a concentration of 1,000 U. S. P. Heparin units per ml.

Eagle's minimal essential medium was obtained from Grand Island Biological Company in powder form and reconstituted such that a final solution was twice as concentrated as directed by the formula (2X MEM). This was sterilized by filtration through a nitrocellulose filter with an average pore size of 0.22 μ and was used for monolayer cultures after dilution in an equal volume of sterile water or agar.

Joklik-modified Eagle's minimal essential medium was also obtained from Grand Island Biological Company in powder form and was reconstituted according to directions. This was also sterilized by filtration and was used for suspension cultures (Sp-MEM).

RPMI 1640 medium was obtained from Grand Island Biological Company in the reconstituted sterilized form.

Phosphate buffered saline (PBS) was made by mixing 8.0 gm of NaCl, 0.20 gm of KCl, 1.15 gm of $NaHPO_4$ and 0.20 gm of KH_2PO_4 in 1.0 liter of glass distilled water. The pH was adjusted to 7.5.

Scintillation fluid was made by mixing (overnight) 4 gm of Omnifluor (New England Nuclear) with 1.0 liter of toluene.

Methods

1. Preparation of Leukocyte Cultures from Mouse Spleens.

Spleens from ICR Swiss mice or from Swiss Webster mice were used for all experiments. Mice were killed by CO_2 asphyxiation and cervical dislocation. Spleens were removed aseptically and placed in a sterile disposable tissue culture dish, 60 x 15 mm (Falcon Plastics) containing 1.5 ml Sp-MEM supplemented with 5% newborn calf serum, or 5% normal rabbit serum plus 1% non essential amino acids. The spleens were cut into smaller pieces aseptically, and the pieces and medium were transferred to a sterile ground glass Tenbrock tissue grinder (Wheaton Glass Co.). The cells were gently pressed from the spleen matrix and then transferred, suspended in medium, to a sterile disposable plastic test tube, 17 x 100 mm (Falcon Plastics). Cells were counted on a haemocytometer after 1/200 dilution in 3% acetic acid in a red blood cell counting pipette (Fisher Scientific Company).

2. Purification of Mouse Spleen Leukocytes by Glass Bead Columns.

Spleen leukocytes were purified on glass bead columns according to the method of Rabinowitz (1964), with some

modifications. Disposable syringes (Plastipak 50 cc) were used as columns. These were packed with three alternating layers of glass wool (Pyrex) and glass beads (3 mm diameter). The glass beads were previously washed for two days in dichromate-sulphuric acid cleaning solution, and then rinsed extensively in tap water and distilled water. They were then coated with silicone by soaking for five seconds in a solution of Siliclad (Clay-Adams, Inc.), followed by rinsing with distilled water. Columns were sterilized by autoclaving, 20 gauge sterile disposable needles (Becton-Dickinson) were fitted on the bottoms and number six sterile rubber stoppers were fitted on the tops.

Cultured spleen leukocytes were suspended in 20 to 30 ml of medium and introduced to the top of the column by pipette. Columns were incubated for 30 minutes at 37°C and then eluted with 20 ml of medium. The cells were counted in a haemocytometer after diluting them 1/10 in 3% acetic acid in a white blood cell counting pipette (Fisher Scientific Co.). The cells were then suspended at 1-2 x 10^6 cels/ml in medium and incubated at 37°C in 90% humidity and 5% CO_2.

3. Purification of Mouse Spleen Leukocytes by Velocity Sedimentation.

The apparatus, described by Miller and Phillips (1969) as STA-PUT, was used to separate the cells on the basis of

velocity sedimentation. The cells were suspended in 0.3% bovine serum albumin in PBS and loaded on top of a linear 1-2% bovine serum albumin gradient. Sedimentation was performed under unit gravity for 3 to 4 hours at 4°C, following which equal volume fractions of the linear part of the gradient were collected. The sedimentation value of each fraction was determined, according to the method described by Dr. R. A. Phillips (personal communications), by calculating the distance of each fraction band from the centre of the input cell band in the linear gradient. Fractions containing cells with sedimentation velocities between 2.5 and 3.5 mm/hr were pooled. These contained lymphocytes (S value 2.8 ± 0.2 mm/hr) and rosette forming cells (3.0 ± 0.3 mm/hr). Rosette forming cells are thought to be T lymphocytes (Silveira et al., 1972).

4. Culture of Human Peripheral Leukocytes.

Human peripheral leukocytes were cultured according to the method of Edelman and Wheelock (1968). Venous blood of a healthy adult (50 cc) was drawn into a sterile disposable syringe, put into a sterile glass bottle containing 1.0 ml of sodium heparin (Connaught Medical Laboratories) and incubated for one hour at 37°C to allow sedimentation of the red blood cells. The leukocyte-rich upper phase was aspirated and the cells were pelleted by centrifugation

at 1,100 rpm for 10 minutes. The cells were resuspended in PBS and pelleted again. The cells were then suspended in medium RPMI 1640 supplemented with 5% NBCS at a concentration of 10^6 cells/ml. Cell counts were done on a haemocytometer after dilution of an aliquot of cells 1/200 in 3% acetic acid.

5. Labelling of Cells with Radioisotopes.

Cultured or cultured and purified spleen leukocytes were incubated overnight at 37°C in 90% humidity and 5% CO_2. After addition of PHA to the appropriate cultures, 2.0 ml aliquots were labelled for 5 hours with ^{3}H-thymidine (1 µCi/ml) (Dr. P. Dent, personal communication), ^{3}H-uridine (1 µCi/ml), ^{14}C-amino acid mixture (0.5 µCi/ml) or ^{3}H-alanine (1 µCi/ml) in triplicate at various times. At the end of the labelling period, 5 ml of cold PBS were added to each aliquot, and 0.45 mg of non radioactive thymidine were added to the samples labelled with ^{3}H-thymidine. The samples were washed once with cold PBS on nitrocellulose filters having an average pore size of 0.22 µ, and then precipitated three times with cold 10% trichloroacetic acid. The filters were placed in scintillation vials, dried and 5.0 ml of scintillation fluid were added to each vial. Radioactivity was counted in a Beckman scintillation counter.

6. Preparation of Rabbit anti Vesicular Stomatitis Virus Serum.

Rabbit anti Vesicular Stomatitis Virus serum was used to inactivate exogenous virus in the virus growth curve experiments and the infectious centre assays.

Antiserum to Vesicular Stomatitis Virus (VSV) was raised in laboratory rabbits. VSV was grown in human KB cells and purified on sucrose gradients (C-Y Kang, 1971). The purified virus was suspended at 10^9 plaque forming units (PFU) per ml. The rabbits were given primary injections consisting of 0.5 ml of a 1:1 mixture of the virus suspension and Freund's adjuvant intraperitonealy, and 0.1 ml of the virus suspension intravenously. Fourteen days later secondary injections, consisting of 0.5 ml of the virus-Freund's mixture intraperitonealy and 0.1 ml of the virus suspension intramuscularly, were given. Similar secondary injections were given twice more at seven day intervals. A few days after the last secondary injection schedule, a few ml. of blood were removed from the ear artery and allowed to clot. The serum was tested for specific antibody titre (see below). One week after the last injection, the blood was collected by exsanguination via a carotid catheter, was allowed to clot and the serum was collected and tested for specific antibody titre and stored at $-70^{\circ}C$.

7. Assay of Antiserum.

Specific anti VSV antibody titre was tested by mixing different dilutions of serum with the same amount of virus for 30 minutes at 37°C. The mixtures were then assayed for plaque forming units as described below. Anti serum was inactivated by heating at 56°C for 30 minutes to remove complement.

Since large quantities of anti serum was required, two different sera was used in this study. Serum A, which was used in the virus growth curve experiments, and Serum B, which was used in the infectious centre assays. Both sera were prepared in the same manner (Fig. 1).

8. Growth of L-60 Cells.

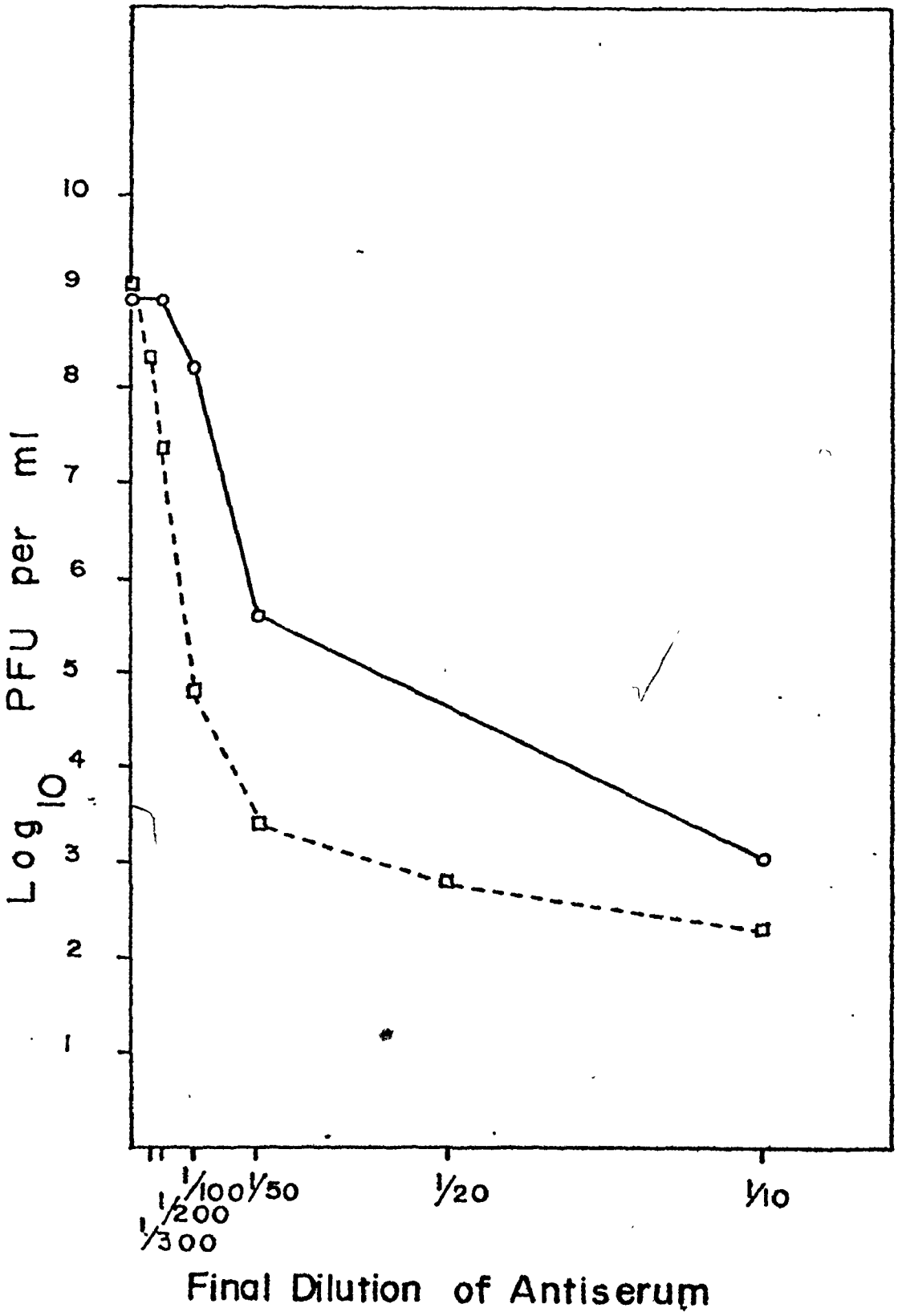
L-60 cells, a continuous passage line of mouse L-cells obtained from Dr. A. F. Howatson, Department of Medical Biophysics, University of Toronto, were maintained in suspension culture at 37°C by growing in erlenmeyer flasks fitted with a teflon coated magnetic stirrer and a tight fitting rubber or nalgene stopper. The growth medium used for suspension culture was Joklik-modified Eagle's minimal essential medium (Sp-MEM) supplemented with 5% newborn calf serum (NBCS).

Figure 1. Neutralization of VSV with Varying Concentrations of Rabbit anti VSV Serum.

Fixed volumes of stock virus were mixed with equal volumes of Rabbit anti VSV serum of different concentrations so that the final concentrations of anti serum was 1/10; 1/20; 1/50; 1/100; 1/200 or 1/300. (Control (C) samples consisted of virus diluted 1/2 with PBS.) The samples were incubated at 37°C for 30 minutes and then assayed for PFU according to the method outlined in "Methods".

0————0 Serum A

□------□ Serum B


Log10 PFU per ml
10
9
8
7
6
5
4
3
2
1
1/100
1/50
1/200
1/300
1/20
1/10
Final Dilution of Antiserum

L-60 cell monolayers for virus plaque assay and for infectious centre assay were made by seeding sterile disposable tissue culture dishes, 60 x 15 mm with 2-3 x 10^6 cells and incubating overnight at 37°C in 90% humidity and 5% CO_2. The medium was removed by aspiration just prior to plating.

9. Infection of Mouse Spleen Leukocytes by VSV.

Cultured, purified leukocytes were pelleted by centrifugation at 1,000 rpm for 10 minutes and resuspended at approximately 1 x 10^7 cells per ml in Joklik-modified Eagle's minimal essential medium (Sp-MEM) supplemented with 2% newborn calf serum (NBCS). Virus was added and the infected cells were incubated at 37°C in 90% humidity and 5% CO_2 for varying lengths of time.

For virus growth curves, antiserum (A) at approximately 1/50 dilution was added after an adsorption period of 1 hour and incubated for 30 minutes at 37°C in 90% humidity and 5% CO_2. The cells were then washed three times by centrifugation and resuspended in fresh medium at 10^6 cells per ml. Samples of the culture fluid were taken at various times and assayed for PFU.

10. Growth of VSV in Mouse L-60 Cells.

Mouse L-60 cells were pelleted by centrifugation at 1,000 rpm for 10 minutes and resuspended at 10^7 cells/ml

in Sp-MEM supplemented with 2% NBCS. VSV was added at a multiplicity of infection of 1.0 and the cells were incubated at 37°C for 30 minutes in a suspension culture. After adsorption, the cells were pelleted, resuspended in fresh medium at 10^6 cells per ml and incubated at 37°C in suspension culture. For virus growth curves, samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6 and 7 hours after the final resuspension and assayed for PFU as described in the following section.

11. Assay of VSV.

VSV was assayed by the plaque forming technique and the titre was expressed in plaque forming units (PFU) per ml. After appropriate serial dilutions of each virus sample, 0.1 ml aliquots were pipetted onto monolayers of mouse L-60 cells in sterile disposable tissue culture dishes, 60 x 15 mm. These were incubated for 30 minutes at 37°C in 90% humidity and 5% CO_2, after which 5.0 ml of Eagle's MEM supplemented with 5% NBCS and containing 0.9% Noble agar (Difco Laboratories) were added to each dish. The dishes were incubated overnight at 37°C in 90% humidity and 5% CO_2 and then fixed with 1 to 2 ml of Carnoy's fixative. The agar overlay was washed off and plaques, which appeared as clear spots in the cell sheet, were counted.

12. Infectious Centre Assay.

Cultured purified mouse splenic leukocytes were incubated overnight with or without PHA as directed by the experimental conditions. The cells were pelleted by centrifugation at 1,000 rpm for 10 minutes and resuspended in Sp-MEM supplemented with 2% NBCS. They were then infected with VSV at a multiplicity of infection (MOI) of 50 and incubated for one hour at 37°C in 90% humidity and 5% CO_2. After adsorption, residual virus was inactivated by treatment with a 1/10 dilution of rabbit anti VSV serum (serum B) for 30 minutes at 37°C. In some experiments, the infected cells were washed partly free of virus by pelleting and resuspension prior to treatment with antibody. After treatment with anti serum the cells were washed once by centrifugation at 1,500 rpm for 5 minutes and resuspended in a small volume of fresh medium. The cell number was determined by haemocytometer count after dilution of an aliquot of cells 1/20 in 3% acetic acid in a white blood cell counting pipette. The cells were suspended at appropriate concentrations, usually 10^7 cells per ml, 10^6 cells per ml, and 10^5 cells per ml, in fresh medium and 0.2 ml aliquots were placed in small sterile polypropylene test tubes 12 x 75 mm (Falcon Plastics). To each aliquot 0.8 ml of a 1:1 mixture of melted agar of the appropriate concentration (kept at 45°C) and 2 x MEM containing 10%

NBCS (prewarmed to 37°C) was added. Two samples, each 0.4 ml, of the resulting mixture were introduced to L-60 cell monolayers in sterile plastic tissue culture dishes, 60 x 15 mm. The resulting overlay was an average of 0.17 mm in thickness, although the thickness was often uneven because the medium gelled before it was completely spread over the entire area of the plate. After the overlay containing the infected cells had gelled, a second nutrient overlay of 4.5 ml of the agar-MEM mixture was added gently to each plate. Plates were incubated overnight at 37°C in 90% humidity and 5% CO_2, fixed with 1-2 ml of Carnoy's fixative for 10-15 minutes and the agar was washed off in warm running water. Infectious centres were indicated by clear plaques in the L-cell sheet.

13. Growth of Virus Stock.

A heat resistant stock of Vesicular Stomatitis Virus of the Indiana serotype, designated HR-LT PP_2 was used for all experiments. This stock has been plaque purified five times before preparation of the stock in order to ensure that the proportion of normal B particles to defective LT particles would be high. The plaque purification and growth of this stock has been described previously (Galet 1973).

RESULTS

I. Mitogenic Response of Mouse Spleen Leukocytes.

The mitogenic response of mouse spleen leukocytes to PHA, ConA and PHA MR68 were examined by measuring the rate of incorporation of radioactively labelled precursors of DNA, RNA and protein into acid insoluble material.

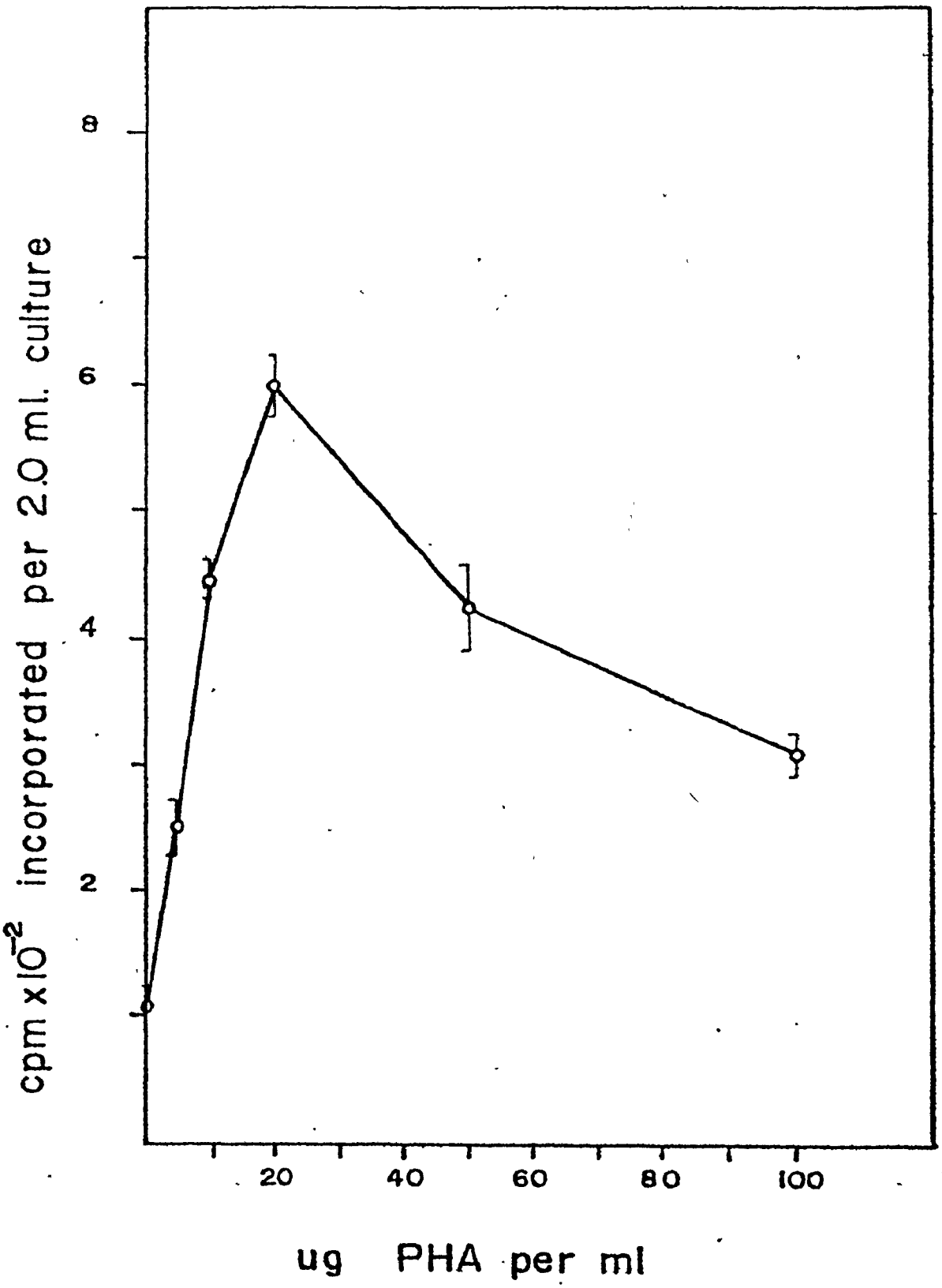
A. Response to PHA.

i) Optimum concentration of PHA for purified leukocyte cultures.

To determine the optimum concentration of PHA-P required to stimulate purified mouse spleen leukocytes cultures, the following experiment was performed: A purified leukocyte culture was incubated for 48 hours with varying concentrations of PHA-P, following which 2.0 ml samples were labelled with 1 μCi/ml of ^{3}H-thymidine for 5 hours. The samples were then precipitated with 10% trichloroacetic acid and counted in a scintillation counter to determine the amount of radioactivity incorporated into acid insoluble material. Fig. 2 indicates that the optimum concentration of PHA-P required to stimulate DNA synthesis in mouse spleen leukocytes appears to be 20 μg per ml of culture.

Figure 2. Dose Response of Cultured Purified Leukocytes to Varying Concentrations of PHA-P.

Cultured, purified mouse spleen leukocytes were suspended in medium at 1 x 10^6 cells per ml. Ten ml aliquots of culture were treated with the following concentrations of PHA-P for 48 hours: 0, 5, 10, 20, 50 and 100 µg per ml of culture. The aliquots were then divided into five 2.0 ml cultures and each culture was labelled for five hours with 1 µCi per ml of ^{3}H-thymidine (12.4 Ci per mmole). Radioactive label incorporated was determined as outlined in "Methods". Each point represents the average of five determinations.


cpm x10⁻² incorporated per 2.0 ml. culture
8
6
4
2
20
40
60
80
100
ug PHA per ml

Several workers using PHA-P to stimulate mouse lymphoid cells employed concentrations ranging between 10 µg/ml and 100 µg/ml (Watson et al., 1973; Lindahl-Magnusson et al., 1972; Gershon et al., 1974), although concentrations as low as 5 ng/ml (Rodney and Good, 1969) and as high as 400 µg/ml (Bryan and Hybertson, 1972) have been used. The amount of PHA-P used to stimulate unpurified mouse leukocytes was the same as that used by Dr. P. Dent (personal communications), which was 40 µg/ml.

ii) Mitogenic response.

The stimulatory effect of PHA-P on mouse leukocyte DNA synthesis was examined in a number of experiments. At various times after addition of PHA-P, aliquots of the stimulated and unstimulated cultures were labelled for five hours with ^{3}H-thymidine. These aliquots were then precipitated with trichloroacetic acid on nitrocellulose filters and the radioactivity incorporated into acid insoluble material was determined. After a number of experiments in which the only apparent variable was the spleen from which the cell cultures were obtained, it became evident that the mitogenic response to PHA-P as determined by incorporation of radioactive precursors was not a predictable observation. Figures 3 and 4 illustrate two divergent results using "unpurified" spleen cell preparations. The culture

Figure 3. Incorporation of ^{3}H-Thymidine into Mouse Spleen Leukocytes After Stimulation with PHA-P.

Cultured mouse spleen leukocytes were incubated overnight and aliquoted into 2.0 ml cultures. To half of the cultures was added 40 µg per ml of PHA-P. At 24, 48 and 72 hours after addition of PHA-P, triplicate cultures of stimulated and unstimulated cells were labelled for five hours with 1 µCi per ml of ^{3}H-thymidine. At the end of the labelling period, the amount of radioactivity incorporated into DNA was determined by precipitation of the cells with 10% trichloroacetic acid on nitrocellulose filters, followed by counting in a scintillation counter, as described in "Methods". Each point represents the average of three determinations.

0————0 without PHA-P

□-------□ with PHA-P

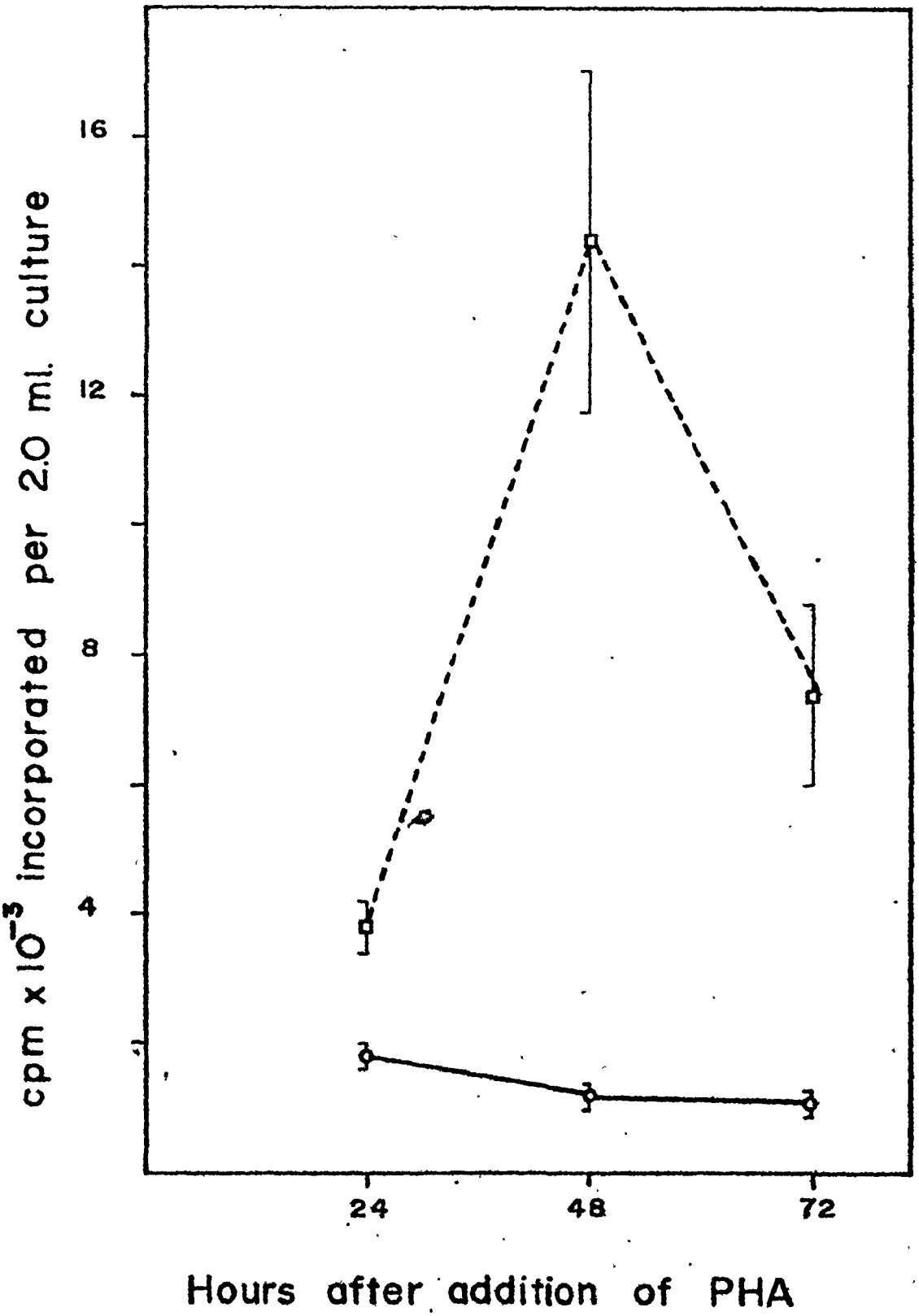
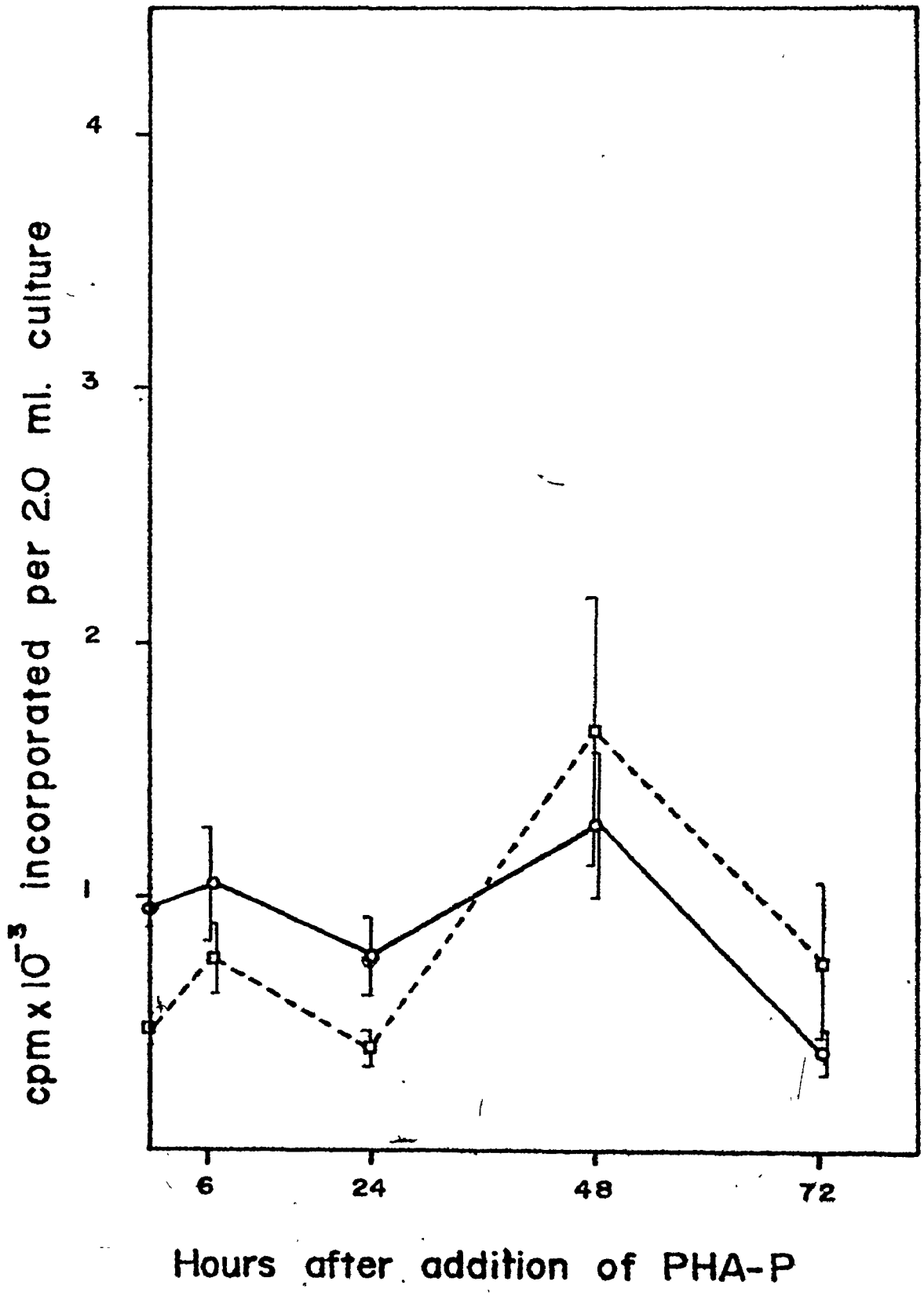

16
12
8
4
cpm x 10⁻³ incorporated per 2.0 ml. culture
24
48
72
Hours after addition of PHA

Figure 4. Incorporation of ^{3}H-Thymidine into Mouse Spleen Leukocytes After Stimulation with PHA-P.

Cultured mouse spleen leukocytes were incubated overnight and aliquoted into 2.0 ml cultures containing 1-2 x 10^{6} cells per ml. To half of the cultures was added 40 µg/ml of PHA-P. At 0, 6, 24, 48 and 72 hours after addition of PHA-P triplicate cultures of stimulated and unstimulated cells were labelled with 1 µCi/ml of ^{3}H-thymidine for 5 hours. At the end of the labelling period, the amount of radioactivity incorporated into DNA was determined by precipitation of the cultures with 10% trichloroacetic acid on nitrocellulose filters followed by counting in a scintillation counter, as described in "Methods". Each point represents the average of three determinations.

0————0 without PHA

□-------□ with PHA


cpm x 10⁻³ incorporated per 2.0 ml. culture
4
3
2
1
6
24
48
72
Hours after addition of PHA-P

illustrated in Fig. 3 showed a PHA-P dependent increase in the incorporation of ^{3}H-thymidine, with the maximal response occurring at 48 hours after the addition of PHA. The unstimulated culture exhibited no change in the rate of DNA synthesis, which was at all times lower than that of the stimulated culture. In contrast to this result, the culture presented in Fig. 4 showed no stimulation of ^{3}H-thymidine incorporation at any time points in the presence or absence of PHA-P.

When glass bead column purified spleen leukocytes were examined for stimulation of DNA synthesis, no apparent mitogenic response was observed, as can be seen in Figures 5A and 6. Figure 5A indicates that both stimulated and unstimulated cultures exhibited no increase in DNA synthesis and that the level of incorporation of ^{3}H-thymidine was similar for both cultures at each time point. Figure 6 indicates that of six spleens tested, six PHA stimulated cultures exhibited a slight stimulation of DNA synthesis at 24 hours after addition of PHA, while 2 unstimulated cultures appeared to have undergone a similar slight increase in DNA synthesis. The difference between the stimulated and unstimulated cultures at 24 hours after addition of PHA ranges from 0 to 860 cpm per 2.0 ml culture, which suggests that the slight apparent stimulation observed may be insignificant as a result of the scale used to plot the results. The incorporation of ^{3}H-thymidine after stimulation

Figure 5. Incorporation of ^{3}H-Thymidine, ^{3}H-Uridine and ^{3}H-Alanine into Purified Mouse Spleen Leukocytes After Stimulation with PHA-P.

Cultured, purified mouse spleen leukocytes were incubated with and without 20 µg per ml of PHA-P. At 0, 5, 24, 48 and 72 hours after addition of PHA, triplicate cultures were labelled with 1 µCi per ml of ^{3}H-thymidine (A), ^{3}H-uridine (B) or ^{3}H-alanine (C). Radioactivity incorporated into acid insoluble material was determined by precipitation with 10% trichloroacetic acid as described in "Methods". Each point represents the average of three determinations.

0————0 without PHA

□-------□ with PHA

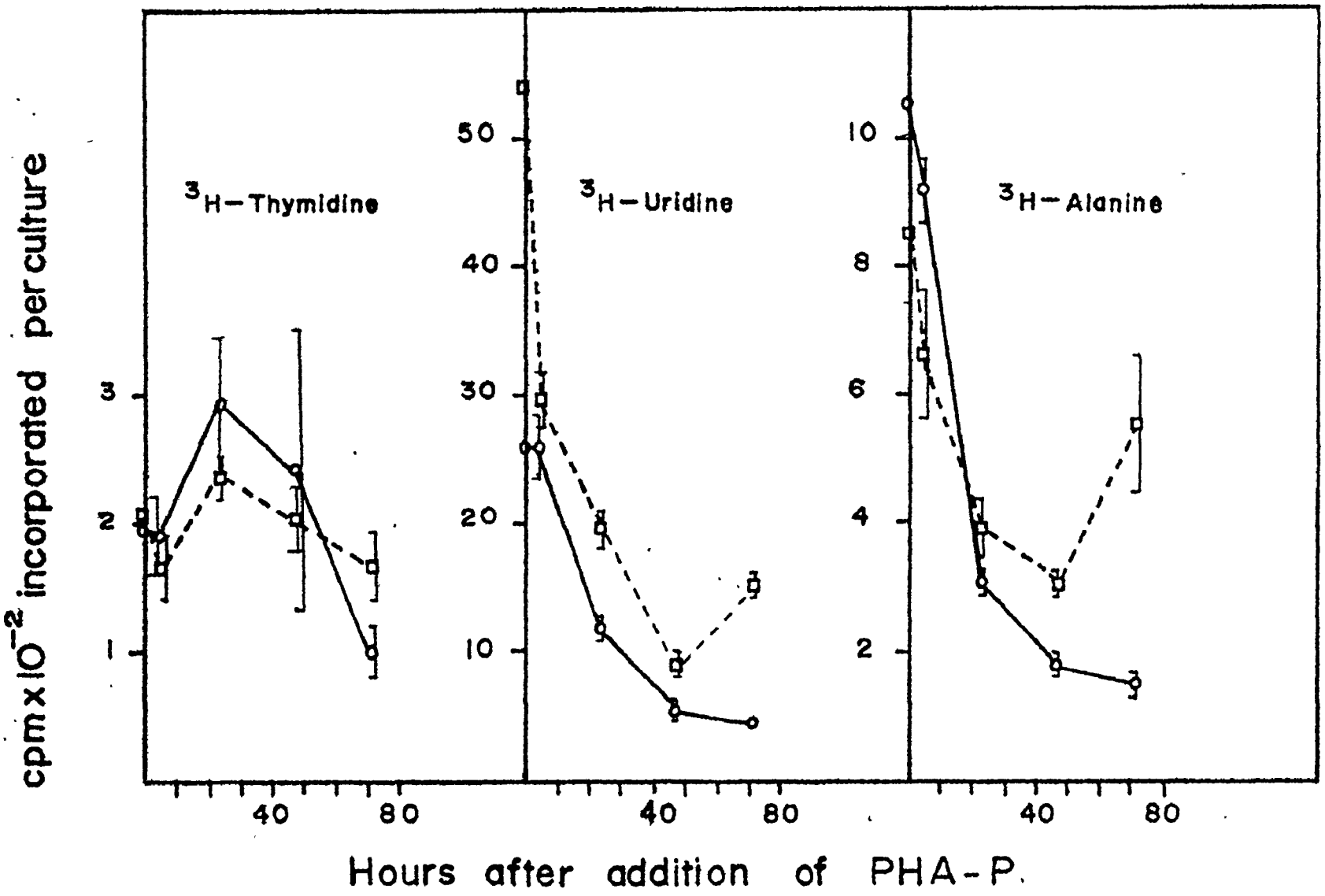
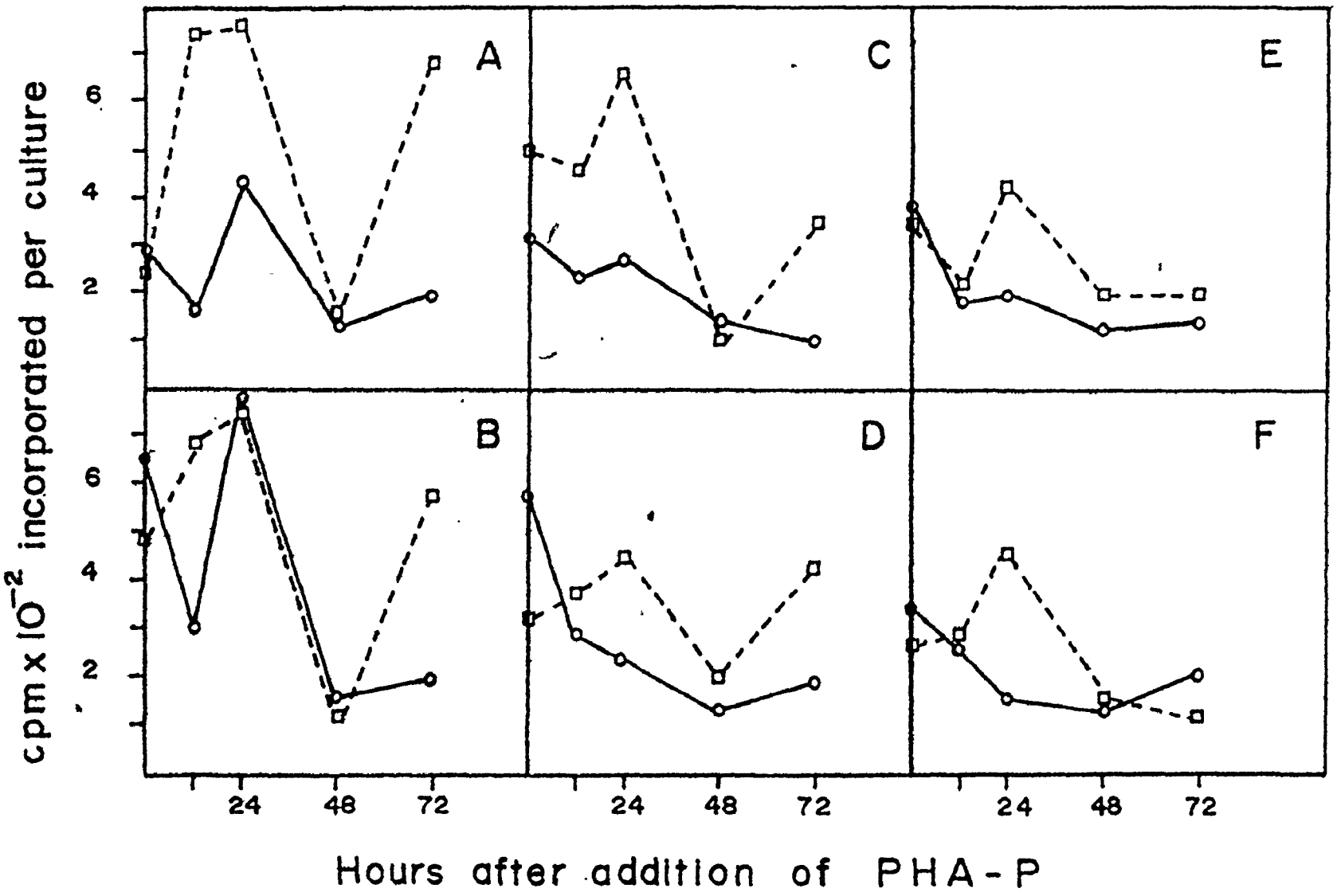

cpm x 10⁻² incorporated per culture
³H—Thymidine
³H—Uridine
³H—Alanine
Hours after addition of PHA-P.

Figure 6. Incorporation of ^{3}H-Thymidine into Purified Mouse Spleen Leukocytes After Stimulation with PHA-P.

Cultured, purified mouse spleen leukocytes were incubated overnight and aliquoted into 2.0 ml cultures containing 1 x 10^{6} cells per ml. To half of the cultures was added 20 µg/ml of PHA-P. At 12, 24, 48 and 72 hours after addition of PHA-P, triplicate cultures of stimulated and unstimulated cells were labelled with 0.75 µCi/ml of ^{3}H-thymidine for 5 hours. At the end of the labelling period, radioactivity was determined by precipitation of the cells with 10% trichloroacetic acid followed by counting in a scintillation counter, as described in "Methods". Figures A through F represent the results of six different spleens cultured at the same time. Experiments represented by graphs A and B, C and D, and E and F were performed on spleens from litter mates. Each point represents the average of three determinations with a few exceptions.

0————0 without PHA-P.

□------□ with PHA-P.


A
B
C
D
E
F
2
4
6
24
48
72
cpm x 10^{-2} incorporated per culture
Hours after addition of PHA-P

of "unpurified" cells with the mitogens Concanavalin A and PHA MR68 was at least ten times higher (see below), and other workers have observed levels of incorporation under similar conditions using PHA-P, which are two orders of magnitude higher than those observed here (Eustatia and Van der Veen, 1971).

The mitogenic response of the cells in terms of ^{3}H-uridine incorporation was somewhat less ambiguous. Figures 7A and 8 indicate that an observable RNA synthetic response can be elicited from unpurified leukocytes following stimulation with PHA-P, with the maximum incorporation occuring at 50 and 24 hours after addition of PHA-P, respectively. On the other hand, purified leukocytes exhibit no apparent RNA response in that there was no difference in the level of incorporated ^{3}H-uridine between the stimulated and unstimulated cultures (Fig. 5B). The same apparently holds for the response of protein synthesis following stimulation with PHA-P. While unpurified cells respond with an increased level of incorporation of ^{14}C-amino acids between 12 and 50 hours after addition of PHA-P (Fig. 7B), purified cells exhibit the same level of incorporation of ^{3}H-alanine with or without PHA-P at all time points following addition of the mitogen (Fig. 5C).

Where a stimulatory effect was observed, the maximal response, in terms of incorporation of radioactive

Figure 7. Incorporation of ^{3}H-Uridine and ^{14}C Amino Acids into Mouse Spleen Leukocyte Cultures Following Stimulation with PHA-P.

Mouse spleen leukocytes were incubated for 24 hours after culturing. PHA-P was added to a final concentration of 40 µg per ml and triplicate 2.0 ml cultures were labelled for five hours with 1 µCi per ml of ^{3}H-uridine and 0.5 µCi per ml of ^{14}C amino acids at 0, 12, 24, 50 and 74 hours after addition of PHA. Radioactivity incorporated into acid insoluble material was determined as described previously.

O———O without PHA

□------□ with PHA.

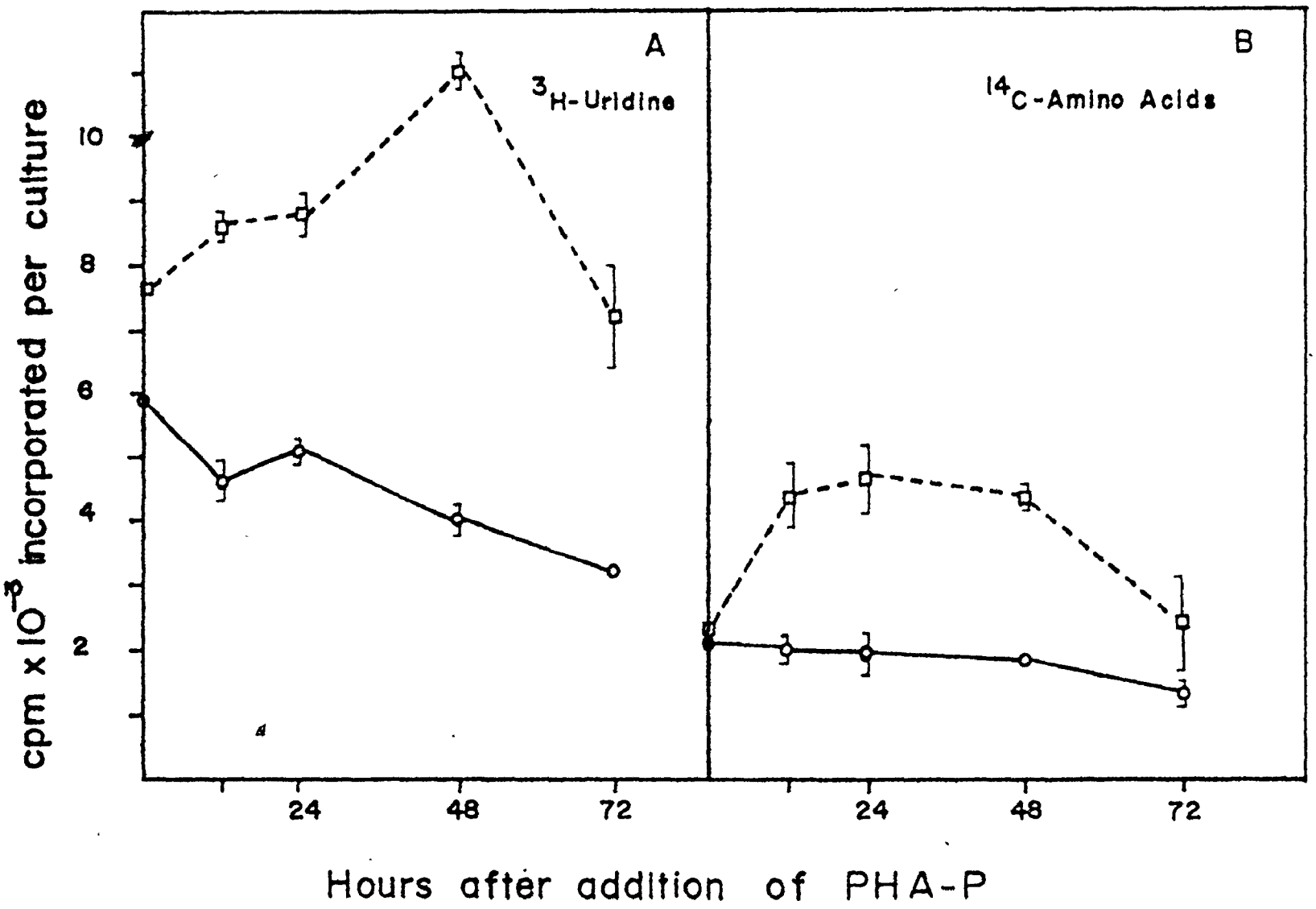
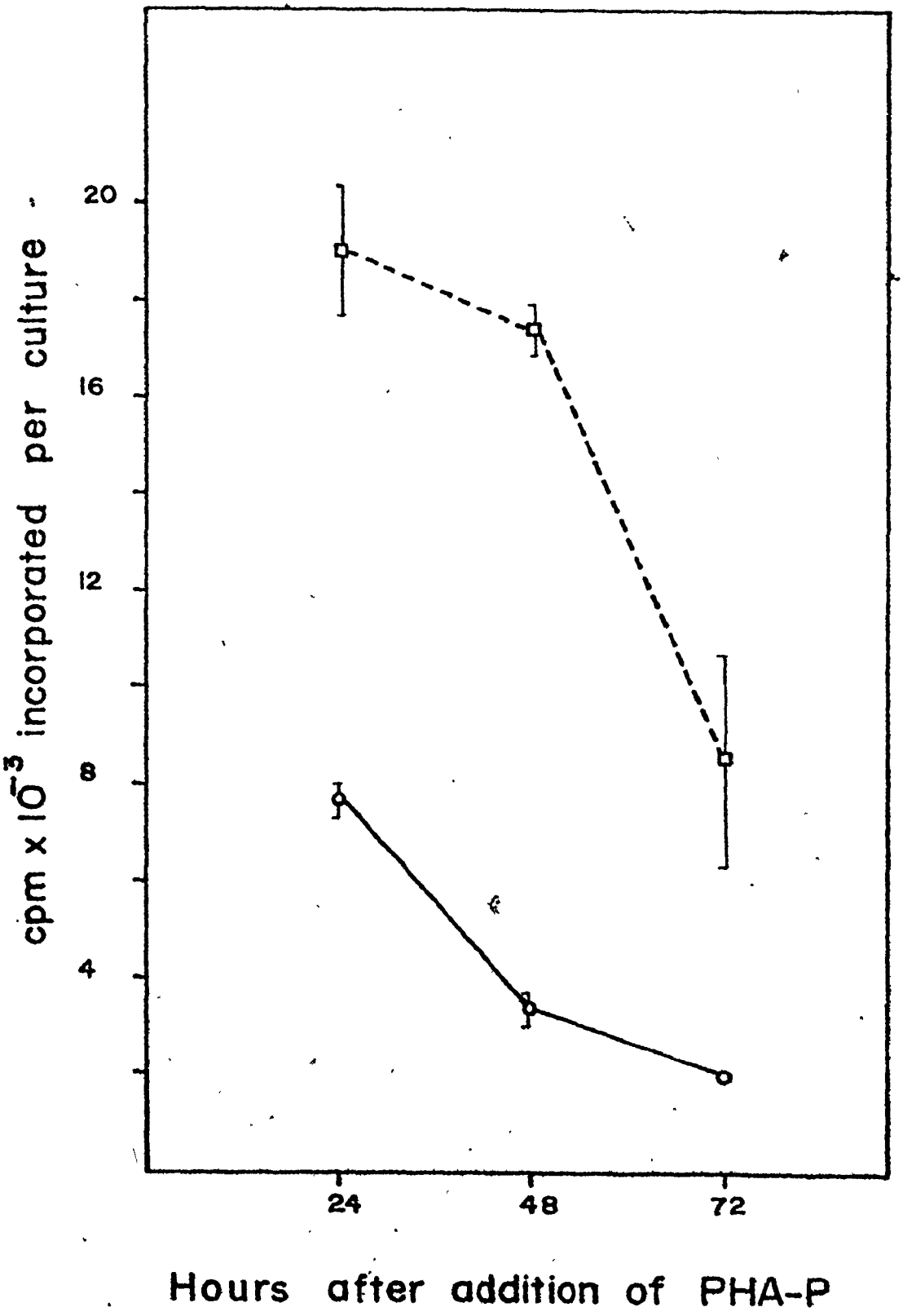

A
^{3}H-Uridine
B
^{14}C-Amino Acids
cpm x 10^{-3} incorporated per culture
10
8
6
4
2
24
48
72
24
48
72
Hours after addition of PHA-P

Figure 8. Incorporation of ^{3}H-Uridine into Mouse Spleen Leukocytes Following Stimulation with PHA-P.

Twenty four hours after culturing, the mouse spleen leukocytes were aliquoted into 2 ml cultures containing 2 x 10^{6} cells per ml and incubated with or without 40 µg/ml PHA-P. At 24, 48 and 72 hours after addition of PHA-P, triplicate cultures were labelled for 5 hours with 1 µCi/ml ^{3}H-uridine. Radioactivity incorporated was determined by precipitation of each culture on a nitrocellulose filter with 10% trichloroacetic acid, followed by counting in a scintillation counter, as described in "Methods".

□------□ with PHA

0————0 without PHA


20
16
12
8
4
cpm x 10^{-3} incorporated per culture
24
48
72
Hours after addition of PHA-P

precursors into DNA, RNA or protein, occurred between 12 and 48 hours after addition of PHA-P.

An attempt to resolve the apparently ambiguous effect of PHA-P on unpurified mouse spleen leukocytes was made by measuring the incorporation of ^{3}H-thymidine in two different strains of mice, after stimulation for 48 hours with two different lots of PHA-P. The results are presented in Table I. There was no difference between the two lots of PHA-P and no difference between the ICR Swiss and the C3H spleens. All three spleens tested exhibited higher incorporation of ^{3}H-thymidine following stimulation with PHA-P.

B. Response to Concanavalin A and PHA MR68.

The response of mouse spleen cells to ConA was considerably less ambiguous than the PHA-P response, although only unpurified cells were examined. The effect of ConA on the incorporation of ^{3}H-thymidine is illustrated in Figure 9. A very strong response was elicited by either 5 µg/ml or 25 µg/ml ConA, with the maximal response occurring at 24 hours or 48 hours respectively after addition of the mitogen. The incorporation of ^{3}H-uridine (Figure 10A) indicated that a definite RNA synthetic response occured between 48 and 72 hours after addition of ConA.

Results similar to those observed for ConA were obtained when PHA MR68 was used as mitogen. Figure 11

Table I

Effect of Different Lots of PHA-P on incorporation of ^{3}H-thymidine into Mouse Spleen Leukocyte Cultures obtained from either C_3H mice or ICR Swiss mice.

Expt. No.	CPM incorporated per 2.0 ml culture					
	ICR Swiss			C_3H		
	PHA-1	PHA-2	Control	PHA-1	PHA-2	Control
1	1,203	1,514	644	923	745	389
2	3,155	2,402	373			

Spleens were cultured, aliquoted into 2.0 ml cultures and incubated for 48 hours with or without 40 µg/ml PHA-P. Duplicate or triplicate cultures were labelled for 5 hours with 1 µCi/ml ^{3}H-thymidine and radioactivity incorporated was determined by precipitation of cultures on nitrocellulose filters with 10% trichloroacetic acid as described in "Methods". Experiment no. 1 was performed in triplicate and experiment no. 2 was performed in duplicate.

Figure 9. Incorporation of ^{3}H-Thymidine into Mouse Spleen Leukocytes After Stimulation with Concanavalin A.

Cultured mouse spleen leukocytes were incubated overnight at 37°C in 90% humidity and 5% CO_2. ConA was added in varying concentrations and triplicate 2.0 ml samples were labelled with 1 µCi per ml ^{3}H-TdR for 5 hours at 0, 12, 24, 48 and 72 hours after addition of ConA. Radioactivity incorporated into acid insoluble material was determined as described in "Methods" by precipitation with 10% trichloroacetic acid.

0———0 without ConA.

□———□ with 5 µg ConA per ml.

△-------△ with 25 µg ConA per ml.

●-------● with 0.5 µg ConA per ml.

▲———▲ with 100 µg ConA per ml.

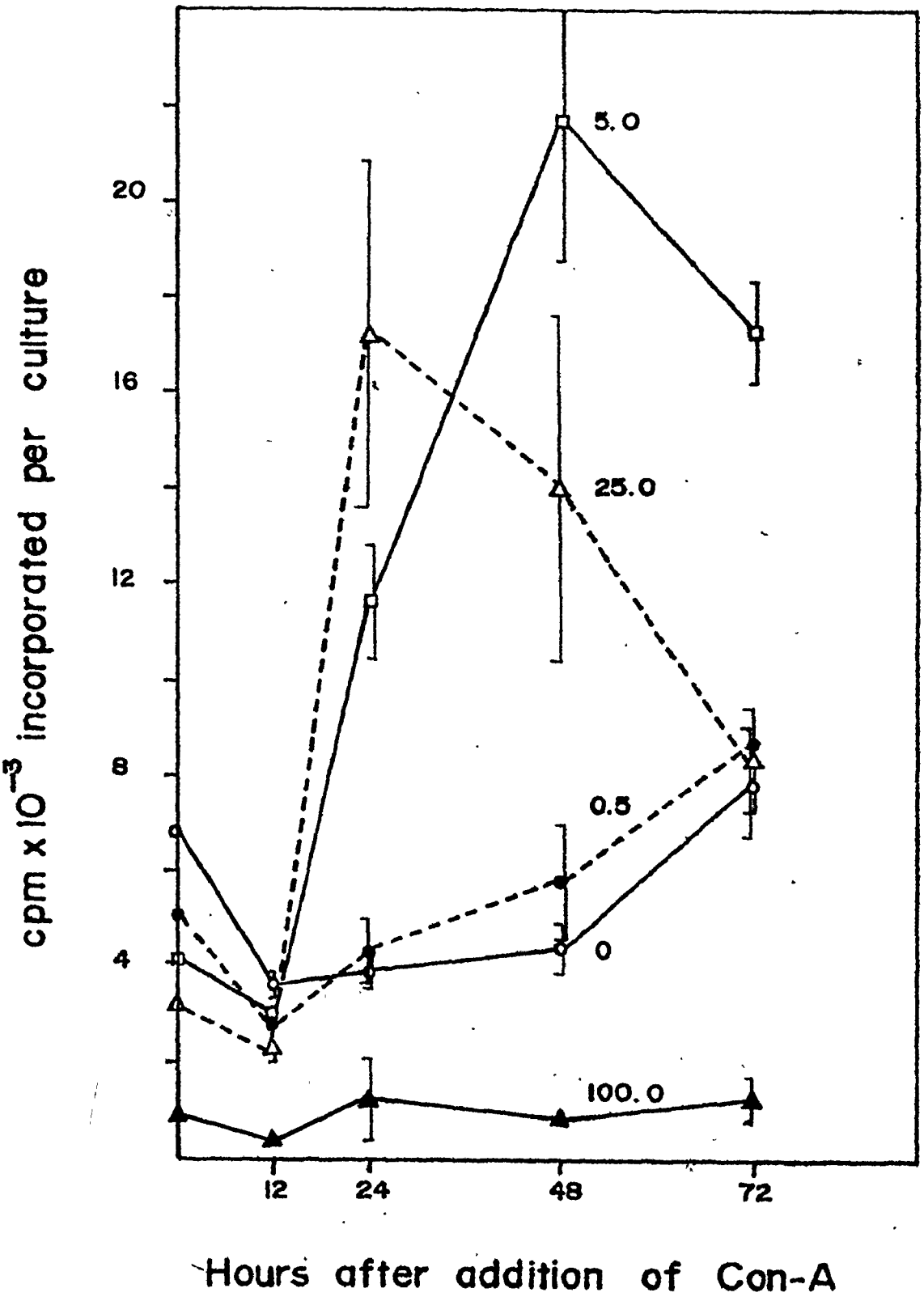

cpm x 10⁻³ incorporated per culture
20
16
12
8
4
5.0
25.0
0.5
0
100.0
12
24
48
72
Hours after addition of Con-A

Figure 10. Incorporation of ^{3}H-Uridine into Mouse Spleen Leukocytes After Stimulation with Concanavalin A or PHA MR68.

Mouse spleen leukocytes were cultured and incubated overnight. Concanavalin A (A) or PHA MR68 (B) were added to varying concentrations and at 0, 12, 24, 48 and 72 hours after addition of the mitogens, triplicate 2.0 ml samples were labelled with 1 µCi per ml of ^{3}H-uridine for 5 hours. At the end of the labelling period, radioactivity incorporated into acid insoluble material was determined by precipitation of the cells with 10% trichloroacetic acid, followed by counting in a scintillation counter, as described in "Methods".

(A)

0————0 without ConA.

□————□ with 5 µg/ml ConA.

Δ------Δ with 25 µg/ml ConA.

(B)

0————0 without PHA MR68.

□————□ with 2.5 µg/ml PHA MR68.

Δ-------Δ with 5.0 µg/ml PHA MR68.

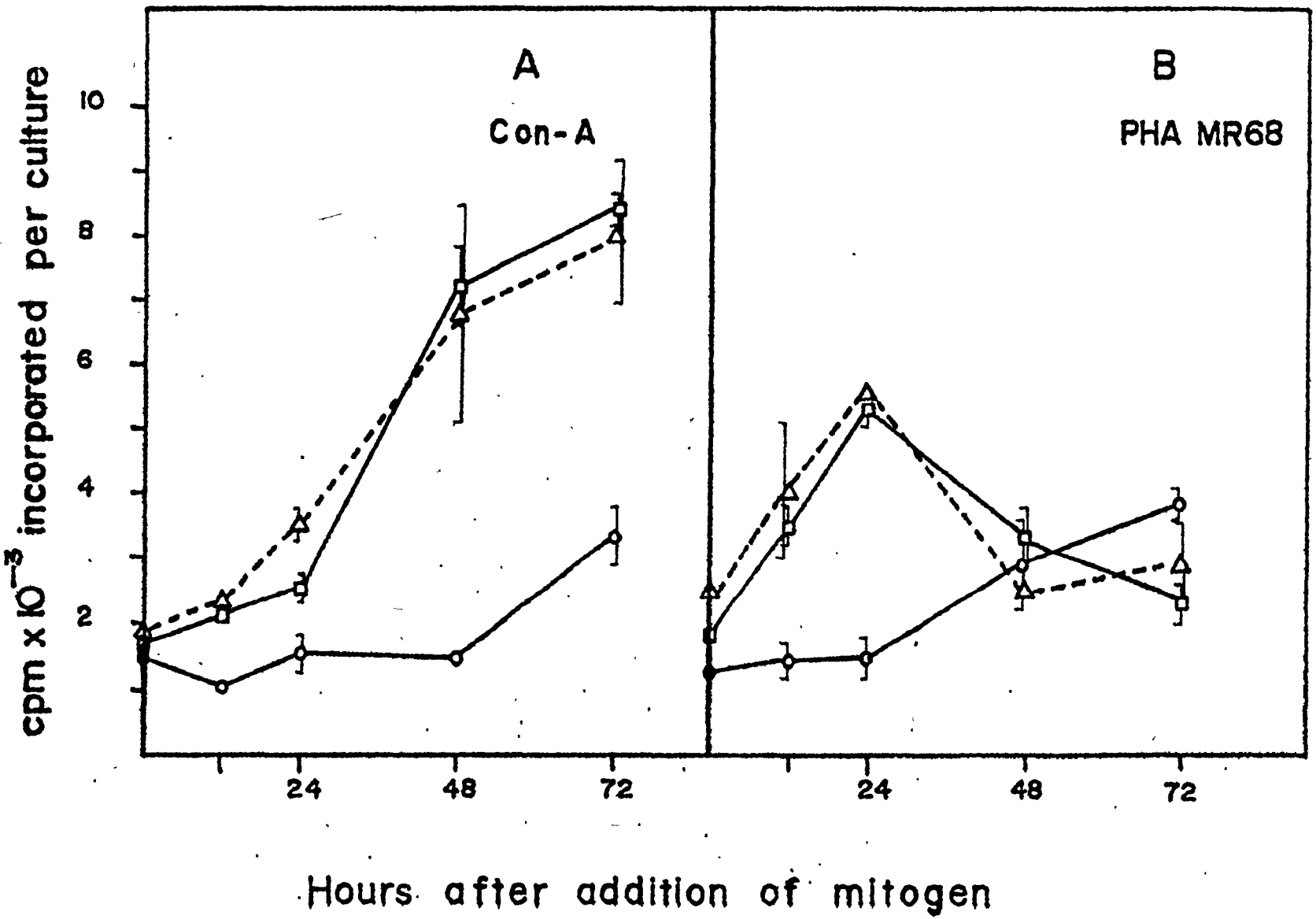

A
Con-A
B
PHA MR68
cpm x 10⁻³ incorporated per culture
2
4
6
8
10
24
48
72
24
48
72
Hours after addition of mitogen

Figure 11. Incorporation of ^{3}H-Thymidine into Mouse Spleen Leukocytes After Stimulation with PHA MR68.

Cultured mouse spleen leukocytes were incubated overnight and PHA MR68 was added in varying concentrations to aliquots of the cells. At 0, 12, 24, 48 and 72 hours after addition of the mitogen, triplicate 2.0 ml samples were labelled with 1 µCi per ml of ^{3}H-thymidine for five hours. At the end of the labelling period, radioactivity incorporated into acid insoluble material was determined by precipitation of the cells with 10% trichloroacetic acid followed by counting in a scintillation counter as described in "Methods".

0———0 without PHA MR68.

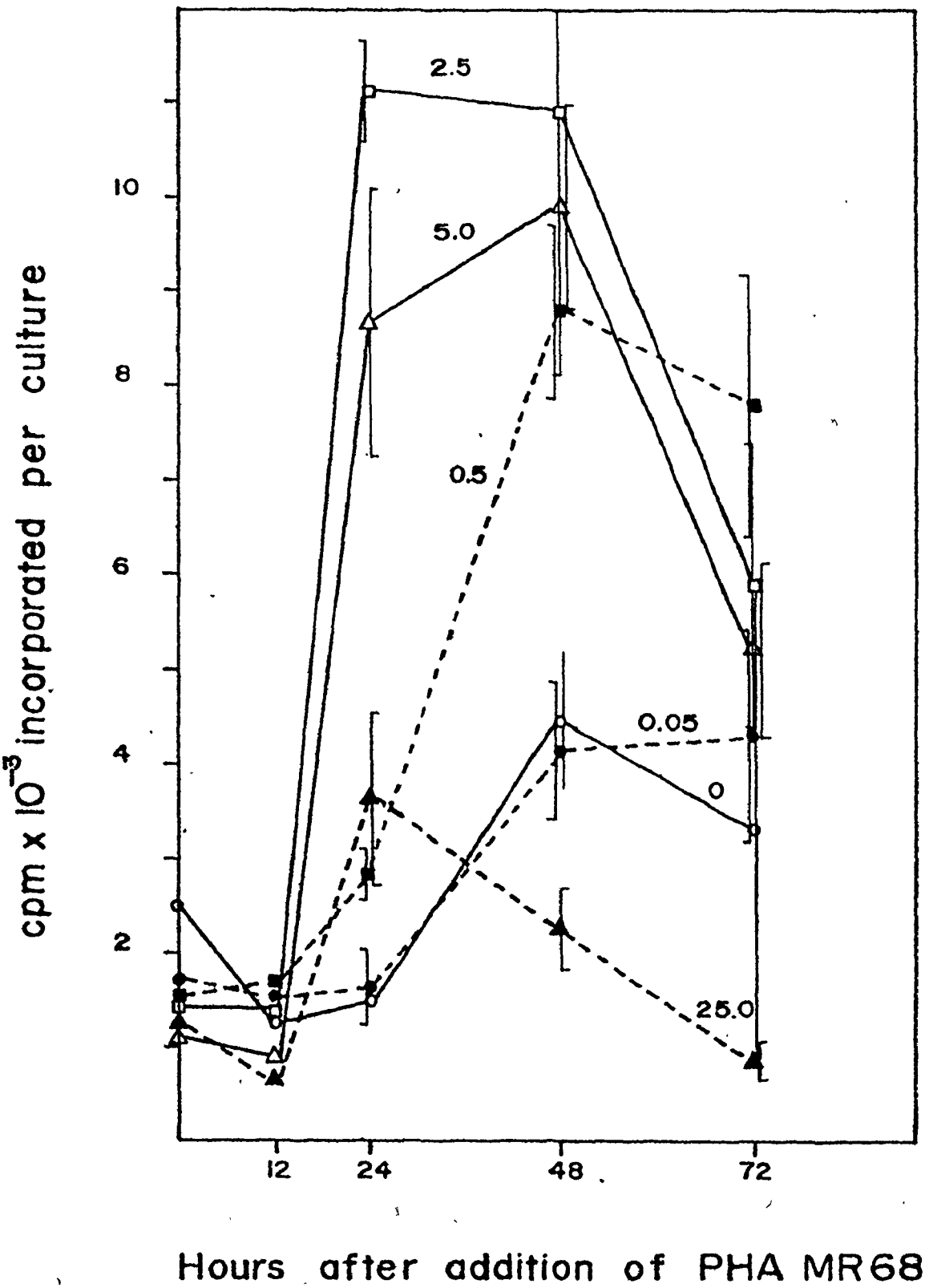
●-------● with .05 µg per ml PHA MR68.

■------■ with 0.5 µg per ml PHA MR68.

□———□ with 2.5 µg per ml PHA MR68.

△———△ with 5.0 µg per ml PHA MR68.

▲------▲ with 25.0 µg per ml PHA MR68.


2.5
5.0
0.5
0.05
0
25.0
10
8
6
4
2
12
24
48
72
cpm x 10^{-3} incorporated per culture
Hours after addition of PHA MR68

indicates that the maximal DNA synthetic response occurred at approximately 24 and 48 hours after stimulation with concentrations of 0.5, 2.5 or 5.0 µg/ml respectively. Figure 10B shows that the maximal RNA response of "unpurified" cells to PHA MR68 occured at 24 hours after addition of mitogen.

While the effect of PHA-P on DNA , RNA and protein synthesis in mouse spleen leukocytes is unclear from this study, the effects of ConA and PHA MR68 resemble those observed by other workers. Similar experiments have been performed by various other workers on lymphocytes from different lymphoid tissues and different species, including mouse spleen (Williams and Benacerraf, 1972). All of these experiments have indicated that the maximal response in terms of the rate of DNA, RNA and protein synthesis occurs at approximately 24 to 48 hours after addition of the mitogen to the cells.

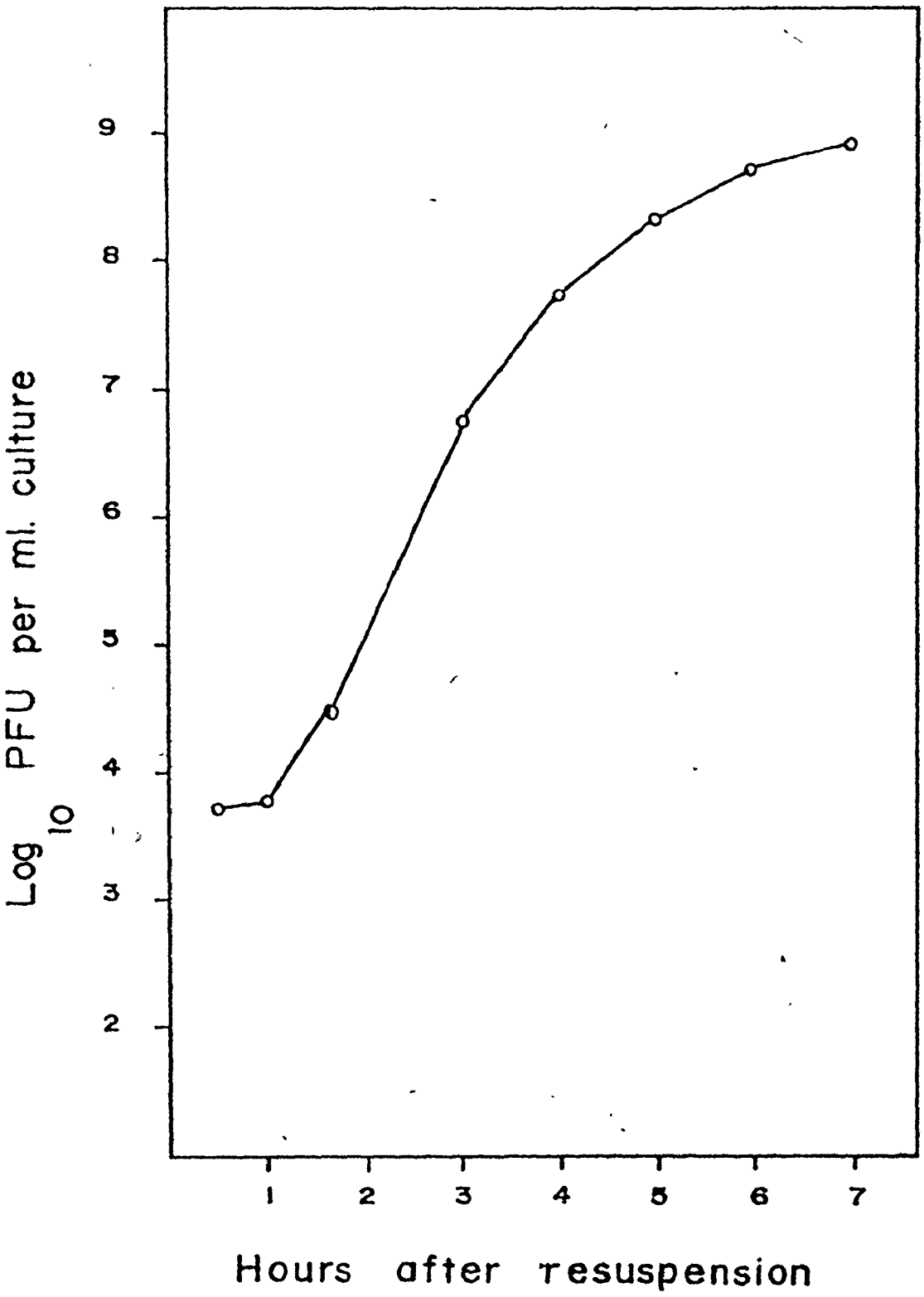
II. Growth of VSV in Cultured Leukocytes.

A. Growth of VSV in L-60 Cells.

A typical single step growth experiment was performed in normally susceptible cell line in order to provide a standard with which to compare later experiments. Mouse L-60 cells were chosen because VSV replicates well in them and because they are of the same species origin as the spleen leukocytes used in this study. Figure 12 indicates that

Figure 12. Growth of VSV in L-60 Cells.

L-60 cells were infected at MOI = 1.0 for 0.5 hours at 37°C and pelleted and resuspended at 10^6 cells per ml in fresh medium. Samples were assayed for PFU at 0.5, 1, 2, 3, 4, 5, 6, and 7 hours after resuspension as described in "Methods".


Log10 PFU per ml. culture
9
8
7
6
5
4
3
2
1
2
3
4
5
6
7
Hours after resuspension

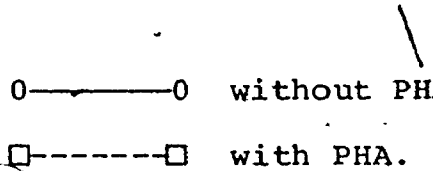
when the cells are infected with an average of one plaque forming unit (PFU) per cell, the virus yield reaches a plateau between 6 and 7 hours after resuspension. Because virus is continually released from the cell by the budding process, the kinetics of virus appearance in the supernatant of a population of infected cells approximates the kinetics of virus production by a single cell.

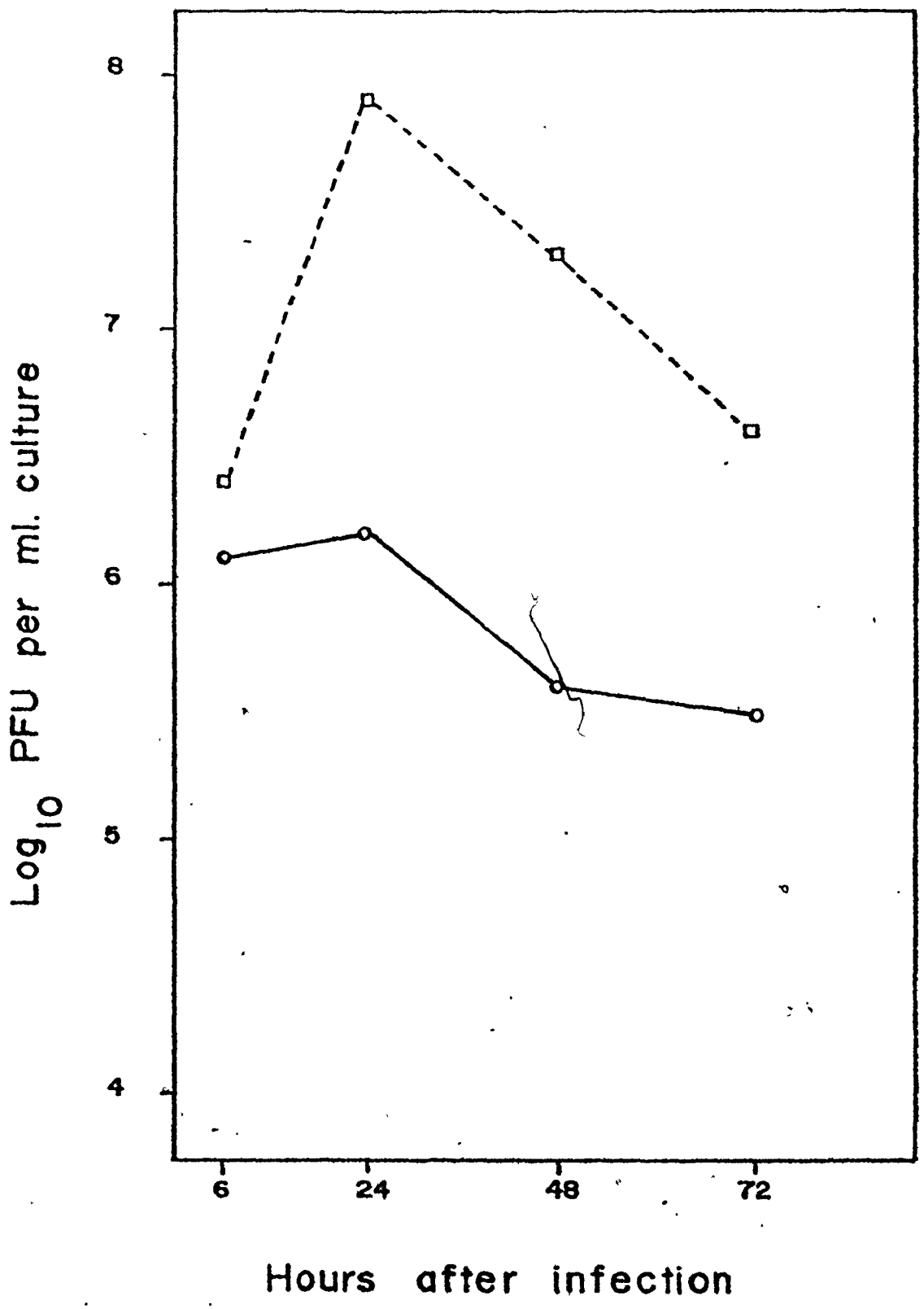
B. Growth of VSV in Human Peripheral Leukocytes.

Edelman and Wheelock (1967; 1968), grew VSV in peripheral human leukocytes, and therefore, their experiment was reproduced to provide a leukocyte standard with which to compare later experiments. PHA-P stimulated and unstimulated cultured human leukocytes were infected with VSV at a multiplicity of 5 for one hour, pelleted and resuspended in fresh medium. Samples were assayed for PFU up to three days after infection. Figure 13 indicates that the maximum virus yield ($\log_{10} = 7.9$) from the stimulated cells was reached by 24 hours after infection, after which the virus titre declined, probably as a result of thermal inactivation, with kinetics similar to those of the virus samples taken from the unstimulated culture. No apparent viral growth occured in the unstimulated cells, although this could be due to an initially high background exogenous virus titre which masked any low level of virus production.

Figure 13. Growth of VSV in Human Peripheral Leukocytes After Stimulation with PHA-P.

Cultured human peripheral leukocytes were incubated with or without 50 µg per ml PHA-P, for 24 hours, pelleted and resuspended in RPMI 1640 supplemented with 5% NBCS at 1 x 10^7 cells per ml. The cells were infected with VSV at MOI = 5, washed once after an adsorption period of one hour and resuspended at 1 x 10^6 cells per ml. Samples were taken at 6, 24, 48 and 72 hours after infection and assayed for plaque forming units as described in "Methods".




8
7
6
5
4
Log10 PFU per ml. culture
6
24
48
72
Hours after infection

It should be noted that while virus grows to higher titres in the PHA-P stimulated cells, no apparent mitogenic response in terms of ^{3}H-thymidine incorporation was observed in cells taken from the same donor. This is illustrated in Table II.

C. Growth of VSV in Mouse Spleen Leukocytes.

i) ConA and PHA MR68 stimulated cells.

The growth of VSV in ConA and PHA MR68 stimulated mouse spleen leukocytes was examined to determine whether a good mitogenic response in terms of ^{3}H-thymidine incorporation could be used to predict good virus replication. Since ConA is capable of inactivating enveloped virus (Okada and Kim, 1972), α methyl mannoside was added to the cells 0.5 hours before infection to inhibit the ConA. The results are presented in Figure 14. While the stimulated cultures appear to contain more virus particles than the unstimulated cultures, no apparent growth occured. The lag of approximately 15 hours before heat inactivation kinetics of virus became apparent in the stimulated cultures may be due to virus production in these cells. However, a more sensitive assay is required to give more positive results.

These results suggest that a good mitogenic response in terms of incorporation of radioactive precursors of DNA is not necessary to predict good virus production. The

Table II

Dose Response of Human Peripheral Leukocytes to PHA-P: Incorporation of ^{3}H-thymidine

Concentration of PHA (µg/ml)	CPM incorporated per 2.0 ml (average of three determinations)
0	599
0.1	418
1.0	564
10.0	531
80.0	229
160.0	162

Human peripheral leukocytes were cultured as described in "Methods", aliquoted into 2.0 ml cultures and incubated for 24 hours with or without varying concentrations of PHA-P. Triplicate cultures were labelled for five hours with 1 µCi/ml of ^{3}H-thymidine and radioactivity incorporated was determined by precipitation of each culture on nitrocellulose filters with 10% trichloroacetic acid as described in "Methods".

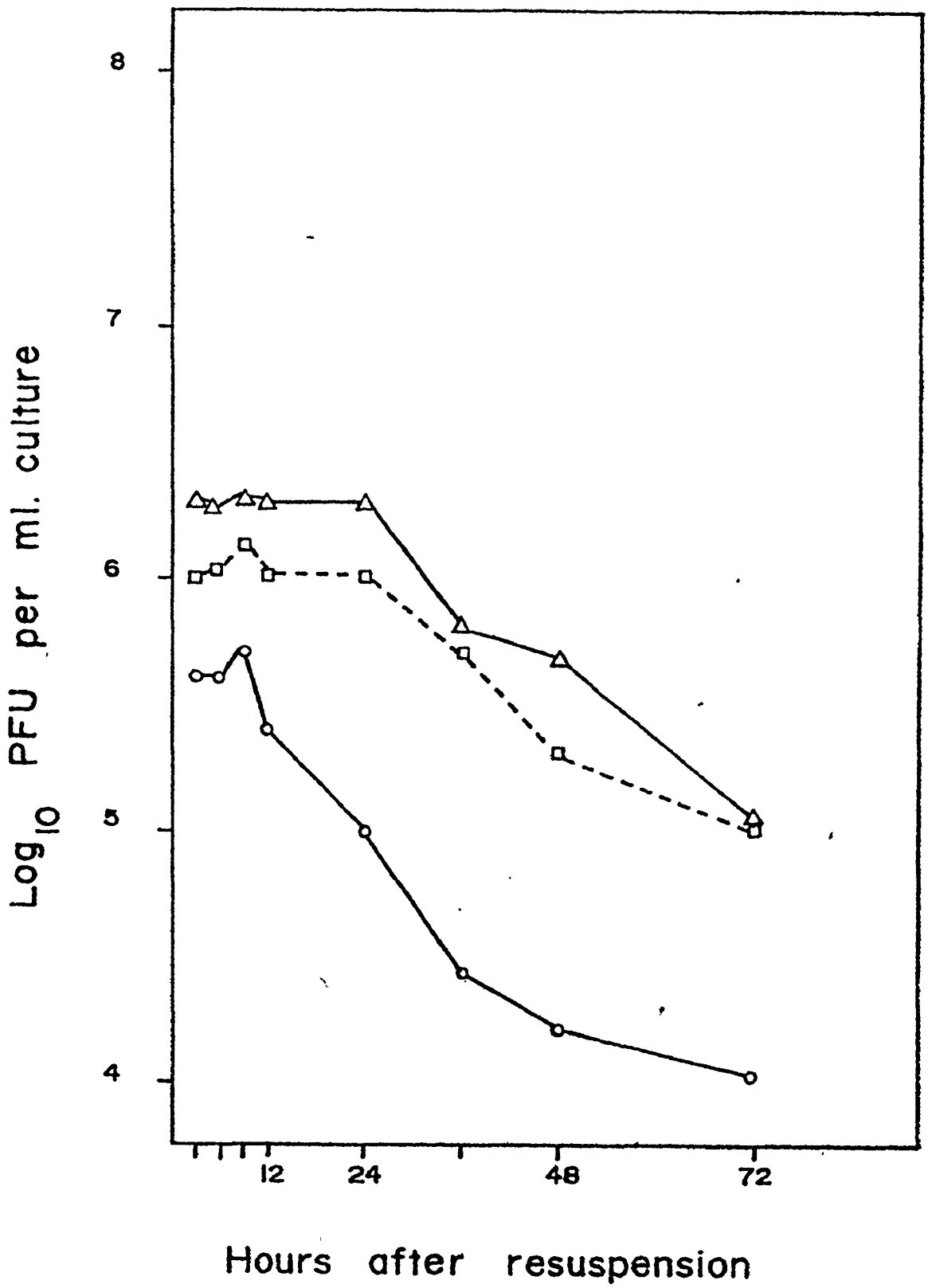
Figure 14. Growth of VSV in Mouse Spleen Leukocytes After Stimulation with ConA or PHA MR68.

Cultured mouse spleen leukocytes were incubated overnight at 37°C in 90% humidity and 5% CO_2 and then stimulated with 10 µg/ml ConA, 5 µg/ml PHA MR68 or nothing for 24 hours. The cells were pelleted, resuspended in fresh medium at 1 x 10^7 cells per ml and infected at MOI = 5 for 1.5 hours. The cells were then washed once by centrifugation and resuspension, resuspended at 10^6 cells per ml and samples were assayed for PFU as described in "Methods" at 3, 6, 9, 12, 24, 36, 48 and 72 hours after resuspension. One hour before infection, the ConA stimulated culture was treated with 19.4 µg per ml α methyl mannoside.

O———O without mitogen.

□-------□ with ConA.

Δ———Δ with PHA MR68.


8
7
6
5
4
Log10 PFU per ml. culture
12
24
48
72
Hours after resuspension

human leukocytes, which did not incorporate significant amounts of precursor after stimulation with PHA-P, produced higher titres of VSV than the unstimulated cells. The mouse leukocytes on the other hand, produced proportionately less virus than the human cells, even though they responded well to the mitogens ConA and PHA MR68 when incorporation of ^{3}H-thymidine was measured.

ii) PHA-P stimulated cells.

Since it appears that a good mitogenic response was not necessary to predict good virus production, the latter was examined in PHA-P stimulated mouse spleen leukocytes. These were infected in the same manner as has been described for human leukocytes and ConA or PHA MR68 stimulated mouse leukocytes, with the exception that PHA was added at the same time as the virus. Figure 15 illustrates the results. No apparent virus growth occured, although the PHA-P stimulated culture contained 0.4 logs more virus at 48 hours after infection.

These results suggest that if any virus is being produced by the cells it is not enough to be detected above the background exogenous virus.

iii) Infection at low MOI.

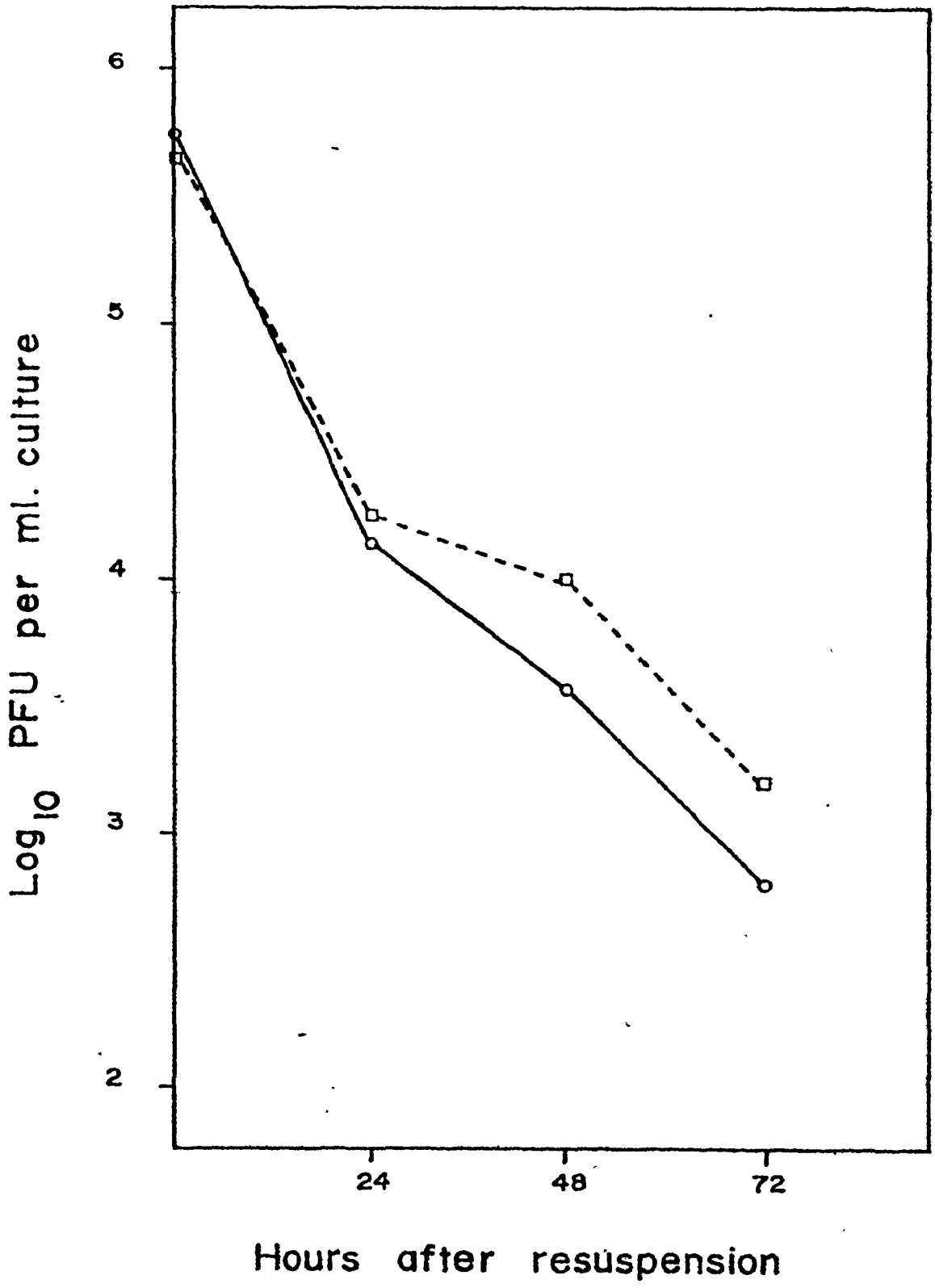
Eustatia and Van der Veen (1971) demonstrated production of VSV in mouse lymph node lymphocytes after

Figure 15. Growth of VSV in Mouse Spleen Leukocytes After Stimulation with PHA-P.

Cultured mouse spleen leukocytes were treated with or without 50 µg per ml PHA-P and infected at MOI = 5 for one hour. The cells were pelleted and resuspended in fresh medium with or without PHA at 10^6 cells per ml and samples were assayed for PFU at 0, 24, 48 and 72 hours after resuspension as described in "Methods".

O————O without PHA.

□------□ with PHA (50 µg/ml).


6
5
4
3
2
Log10 PFU per ml. culture
24
48
72
Hours after resuspension

infection at low multiplicities. The most dramatic increase in VSV titre was observed when the cells were infected at a multiplicity of approximately 1×10^{-5} PFU per cell. To determine whether VSV does indeed replicate in mouse spleen leukocytes, similar experiments were performed. Figures 16, 17 and 18 illustrate the results of three experiments. All three indicate that VSV does grow in mouse spleen leukocytes and that the maximum titre reached is about 1×10^{6} PFU per ml. All three experiments also indicate that the unstimulated cultures produce, by approximately 72 hours after infection, the same amount of virus as the PHA stimulated culture (in Figure 18, the unstimulated culture had produced about 1.6 logs less virus by 72 hours, but the curve had not reached a plateau). However, Figures 17 and 18 indicate that the unstimulated cultures lag behind the PHA stimulated cultures by about 24 hours in virus production. No lag is seen in the unstimulated culture, illustrated in Figure 16, infected at the higher MOI (0.003); however, no plaques were observed in the unstimulated culture infected at the lower multiplicity (0.0003) until 72 hours after infection, when 1×10^{6} PFU per ml were assayed. This suggests that a similar lag occured as that seen in Figures 16 and 17.

iv) Infection at high MOI followed by antiserum treatment.

Having determined that VSV grows in mouse spleen

Figure 16. Growth of VSV in Mouse Spleen Leukocytes Infected at Low Multiplicity of Infection.

Cultured mouse spleen leukocytes were suspended at 2 x 10^6 cells per ml in RPMI 1640 medium supplemented with 10% NBCS and incubated overnight. PHA was added to the appropriate cultures to a final concentration of 40 µg/ml and one hour later the cultures were infected with .00003 PFU per cell, .0003 PFU per cell or .003 PFU per cell. At 4, 12, 24, 48 and 72 hours after infection, samples were assayed for PFU as described in "Methods". No plaques were detected in any cultures infected with .00003 PFU per cell and none were detected in the unstimulated culture infected at .0003 PFU per cell until 72 hours after infection, when 1 x 10^6 PFU per ml were detected.

O———O without PHA, infected at MOI = .003

Δ———Δ with PHA, infected at MOI = .0003.

□------□ with PHA, infected at MOI = .003.

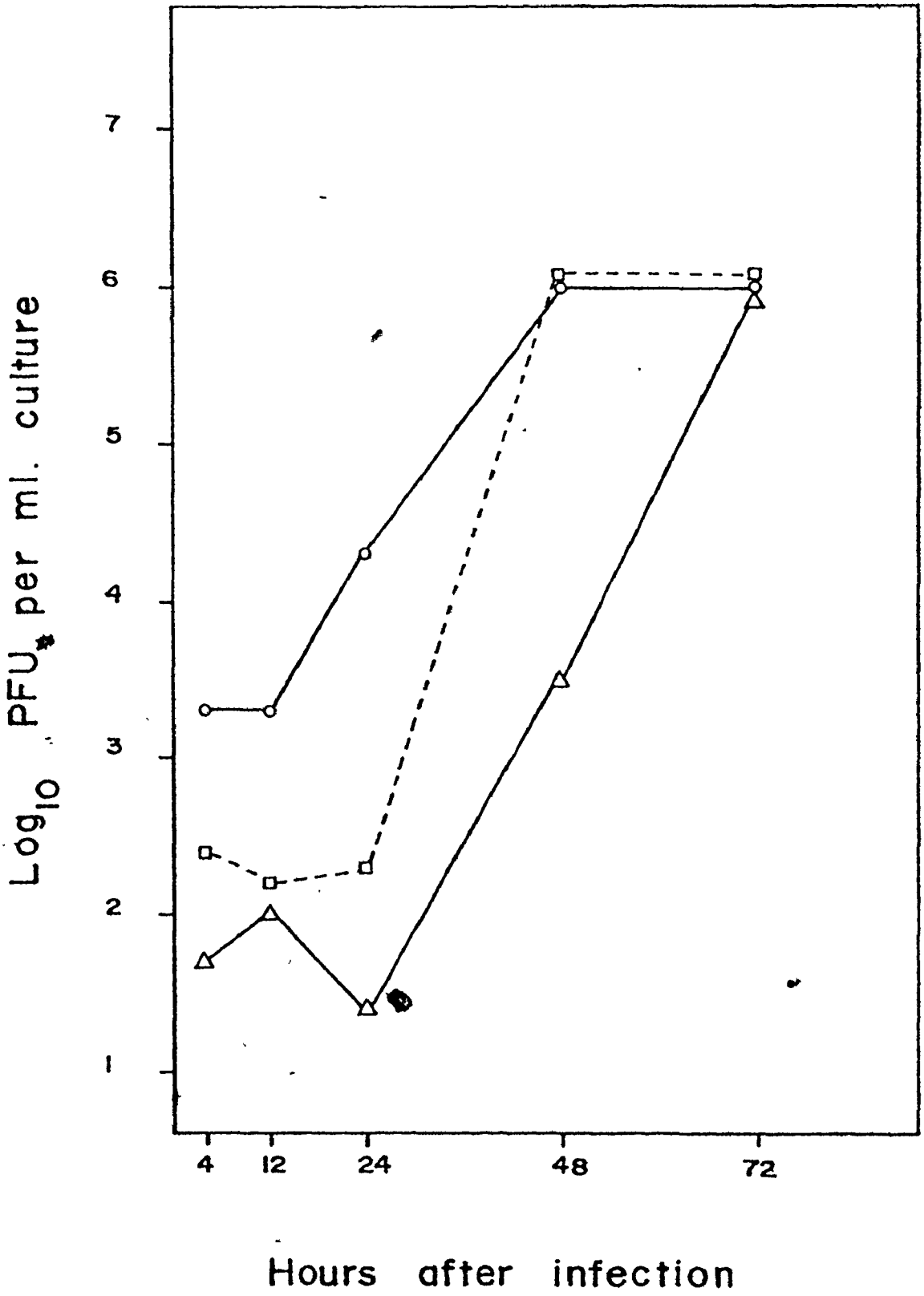

7
6
5
4
3
2
1
Log10 PFU* per ml. culture
4
12
24
48
72
Hours after infection

Figure 17. Growth of VSV in Mouse Spleen Leukocytes Infected At Low Multiplicity.

Cultured mouse spleen leukocytes were incubated with or without 20 µg per ml of PHA-P overnight at 37°C. The cells were pelleted and resuspended at about 2×10^7 cells per ml and infected at MOI = .007 for 1/2 hour at 37°C. The cells were then diluted to 1×10^6 cells per ml and samples were assayed for PFU as described in "Methods" at 0, 12, 24, 48 and 72 hours after dilution.

0————0 without PHA

□------□ with PHA.

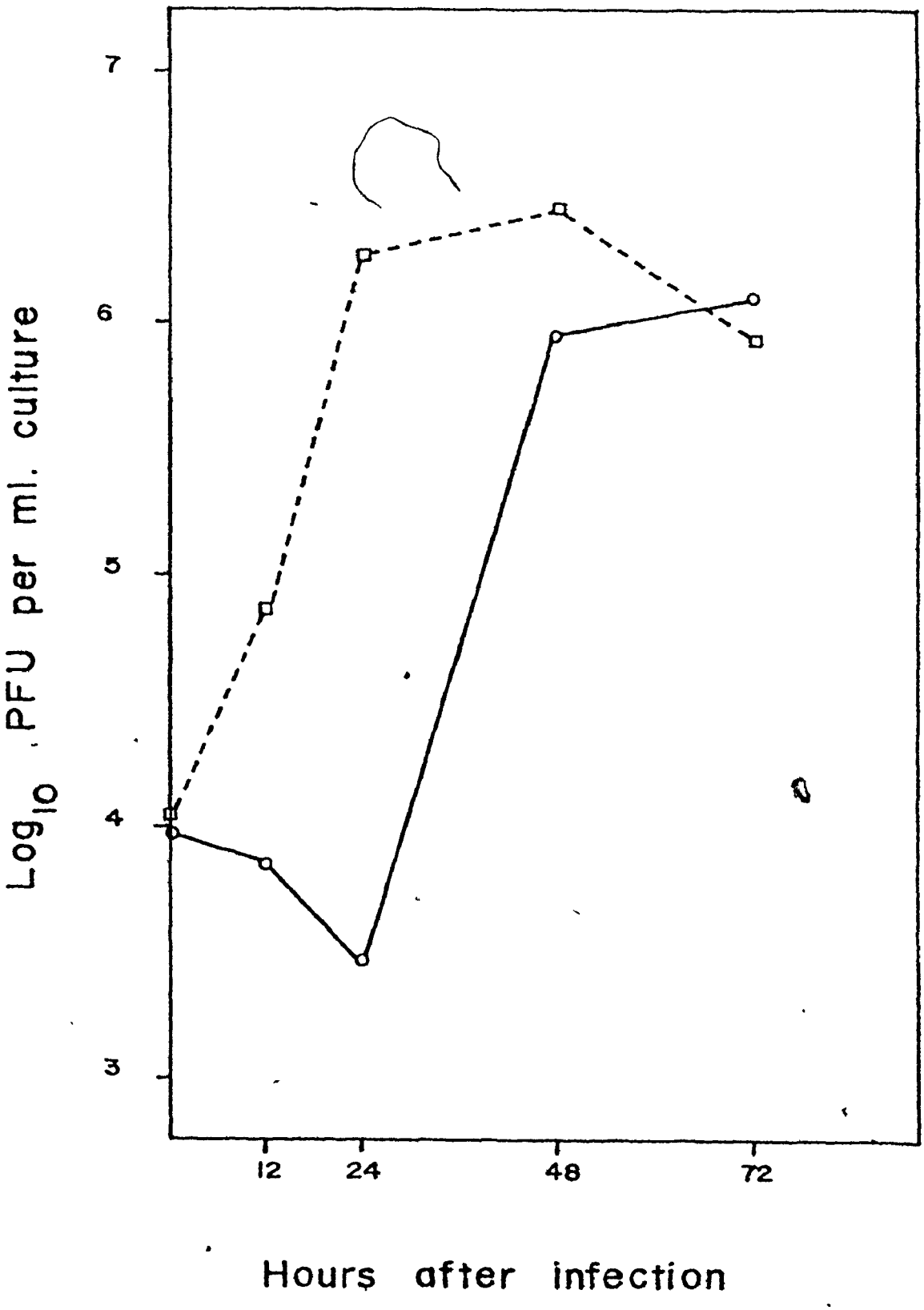
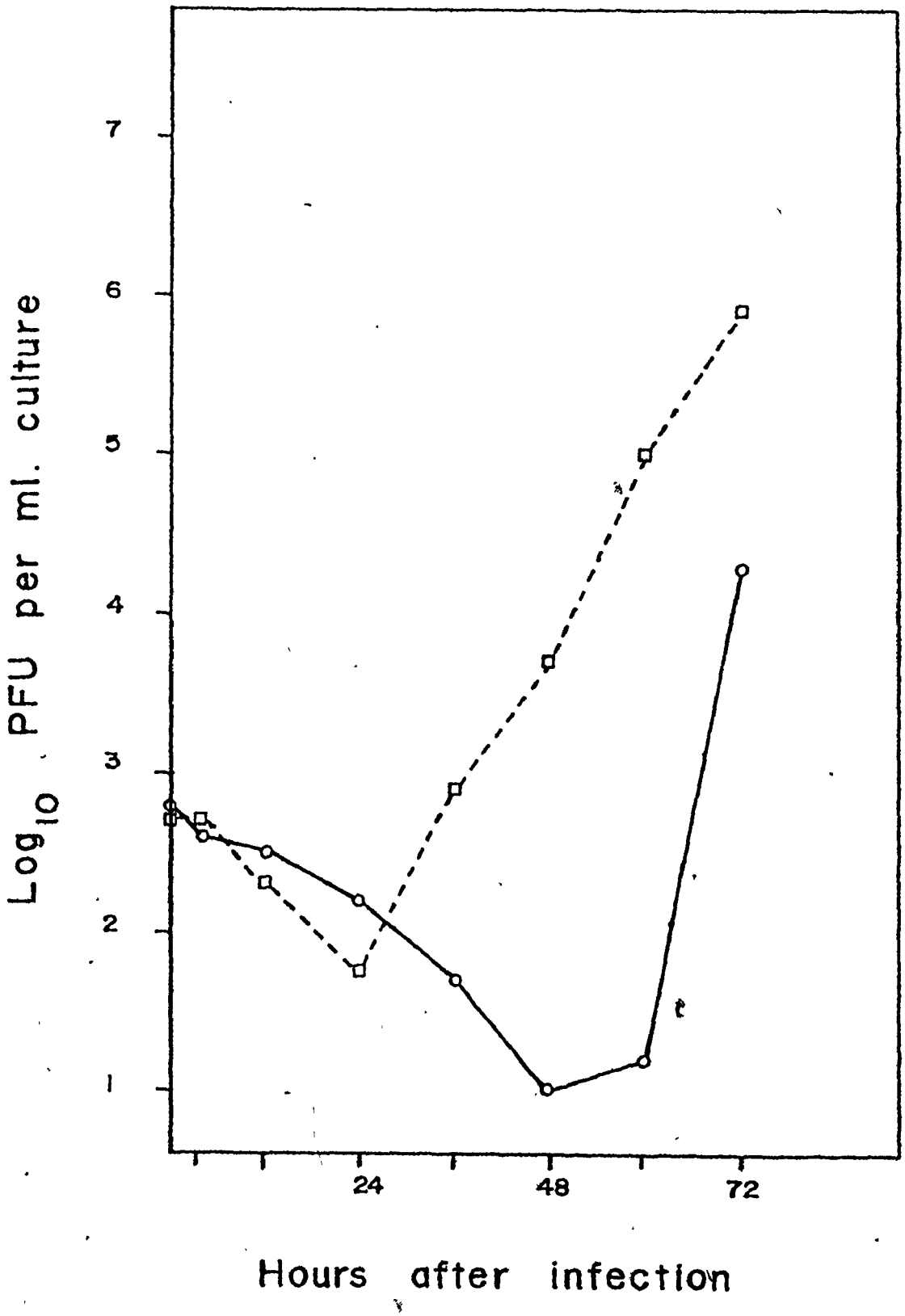

7
6
5
4
3
Log10 PFU per ml. culture
12
24
48
72
Hours after infection

Figure 18. Growth of VSV in Cultured Mouse Spleen Leukocytes Infected At Low MOI.

Cultured mouse spleen leukocytes were suspended at 1-2 x 10^6 cells per ml. PHA was added to a final concentration of 40 µg per ml to half of the cells and the cells were infected with VSV at MOI = .0003. Samples were assayed for PFU at 0, 3, 12, 24, 48, 60 and 72 hours after infection.

0————0 without PHA.

□------□ with PHA.


7
6
5
4
3
2
1
Log10 PFU per ml. culture
24
48
72
Hours after infection
t

leukocytes after infection at low multiplicity, it seemed desirable to examine the single step growth kinetics by infecting at a higher multiplicity to determine whether the kinetics of VSV production in mouse spleen leukocytes are similar to those in human leukocytes (same cell type) and to those in mouse L-60 cells (same species). In order to reduce the background exogenous virus, the infected cells were treated with rabbit anti VSV serum (serum A, see "Methods") at a 50 fold dilution after the adsorption period. Figure 19 indicates that both stimulated and unstimulated mouse spleen leukocytes infected at MOI = 10 reached the plateau of virus titre by 3 to 6 hours after resuspension. However, unlike the previous low MOI experiments, the unstimulated culture reached a virus titre plateau approximately 1.5 logs lower than that of the stimulated culture and did not increase above this level.

v) Infection of purified mouse spleen leukocytes.

Edelman and Wheelock (1967) showed that human monocytes were capable of supporting virus production without any prior stimulation. Eustatia et al. (1972) demonstrated the same for mouse macrophages. This suggested that the virus growth observed in unstimulated cultures may have been due to an infection of monocytes and macrophages. Therefore, an attempt to eliminate virus production in unstimulated cells was made by removing the normally

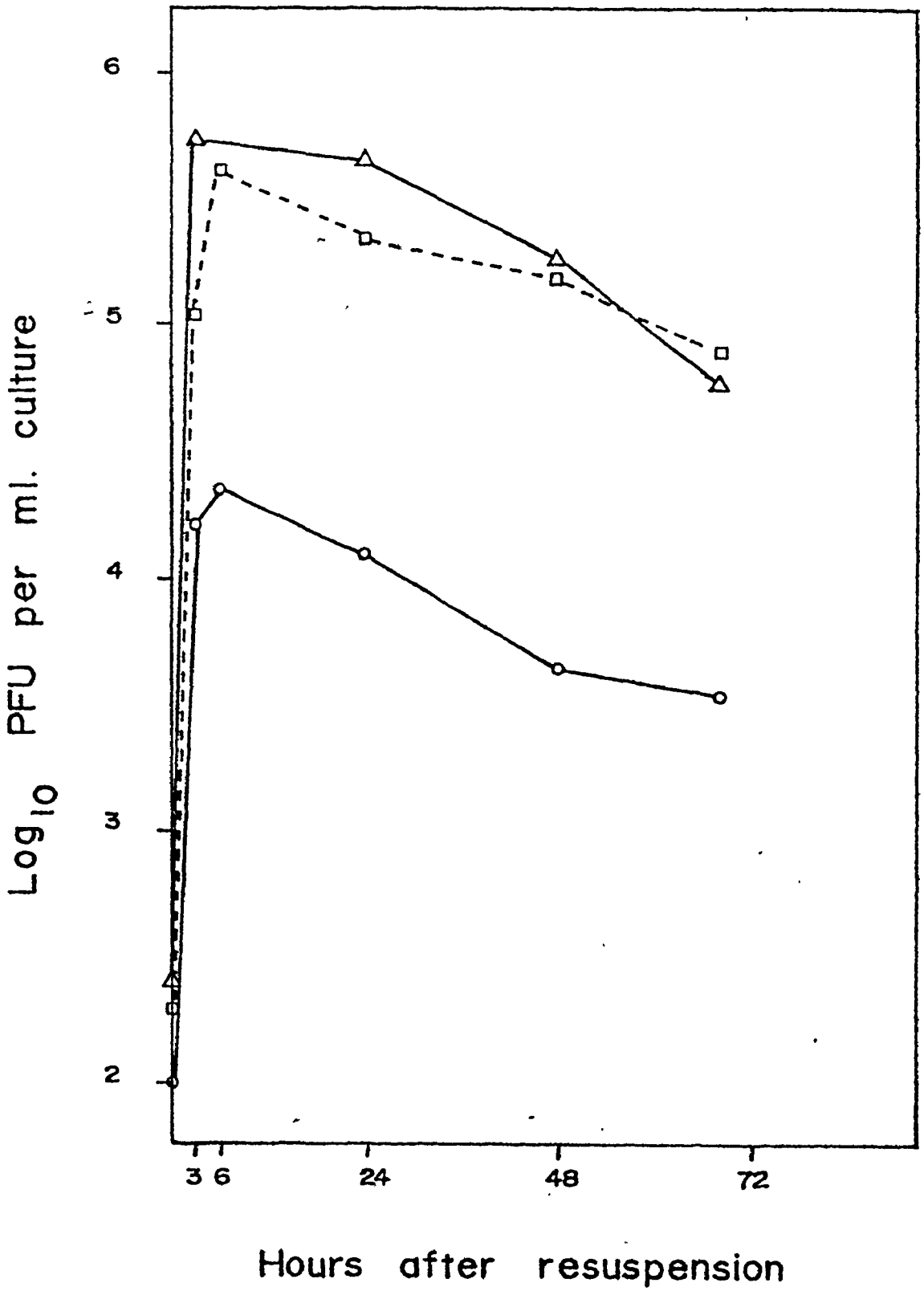
Figure 19. Growth of VSV in Mouse Spleen Leukocytes Infected at High MOI and Treated with Anti Serum. -

Cultured leukocytes were incubated with 20 µg/ml PHA for 24 or 48 hours, or without PHA overnight. The cells were pelleted by centrifugation at 2,000 rpm for 5 minutes and resuspended at 1 x 10^7 cells per ml in Sp-MEM supplemented with 2% NBCS. The cells were then infected with VSV at MOI = 10 for 2 hours, washed twice with fresh medium and treated with Rabbit anti VSV serum (final dilution about 1/50) for 1/2 hour. After washing three times by centrifugation and resuspension in fresh medium the cells were resuspended at 1 x 10^6 cells per ml and samples were assayed for PFU as described in "Methods" at 0, 3, 6, 24, 48 and 72 hours after resuspension.

O———O without PHA

□------□ PHA treatment 24 hours before infection.

△———△ PHA treatment 48 hours before infection.


6
5
4
3
2
Log10 PFU per ml. culture
3 6
24
48
72
Hours after resuspension

susceptible cells - monocytes and macrophages.

Two different methods of purification were used. The first method was originally described by Miller and Phillips (1969) and employed the velocity sedimentation properties of cells on bovine serum albumin gradients. Lymphocytes sediment at a rate of 3.0 ± 0.3 mm per hour, while colony forming cells (i.e. blast cells) sediment at a rate of 3.9 ± 0.2 mm per hour (Dr. R. Phillips, personal communication). The second method utilized the glass adherent properties of monocytes and macrophages. Originally described by Rabinowitz in 1964, the separation of glass adherent and non adherent cells was achieved by incubating the cells on glass bead columns and eluting the non adherent cells with medium. Infection with VSV of PHA stimulated and non stimulated mouse spleen leukocytes purified by the two techniques resulted in similar growth curves (Figures 20 and 21). The virus titre reached a plateau between 3 and 6 hours after resuspension in both stimulated and unstimulated cultures purified by either technique, and the stimulated cultures produced 1.5 logs (glass bead column purified, Figure 21) to 2.0 logs (STA PUT purified, Figure 20) more virus than the unstimulated cultures. These results are not significantly different from those of the unpurified cells (Figure 19), which suggests that the macrophages and monocytes are not significantly involved in virus production in cultures infected at high multiplicity.

Figure 20. Growth of VSV in Purified Cultured Mouse Spleen Lymphocytes Infected at High MOI.

Cultured mouse spleen leukocytes were purified on a STA-PUT BSA gradient as described in "Methods". The cells were incubated with or without 20 µg per ml of PHA for 48 hours, pelleted, resuspended at 1×10^7 cells per ml and infected with VSV at MOI = 10 for 2 hours. After two washes with fresh medium, the cells were treated with Rabbit anti VSV serum (final dilution 1/50) for 1/2 hour, washed twice with fresh medium and resuspended at 10^6 cells per ml. Samples were assayed for PFU at 0, 1/2, 1, 2, 3, 4, 5, 6, and 7 hours after resuspension as described in "Methods".

0————0 without PHA.

□------□ with PHA.

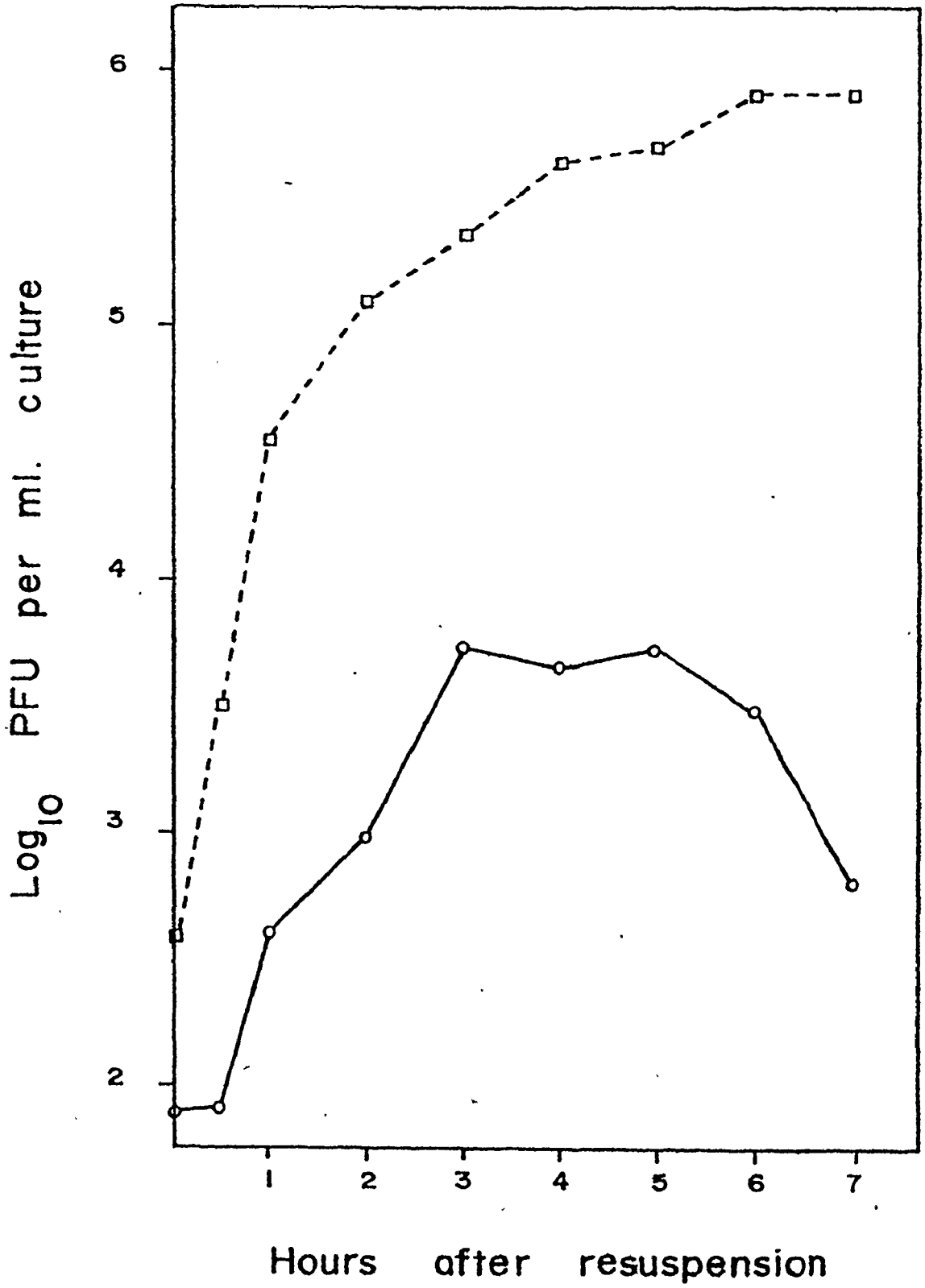
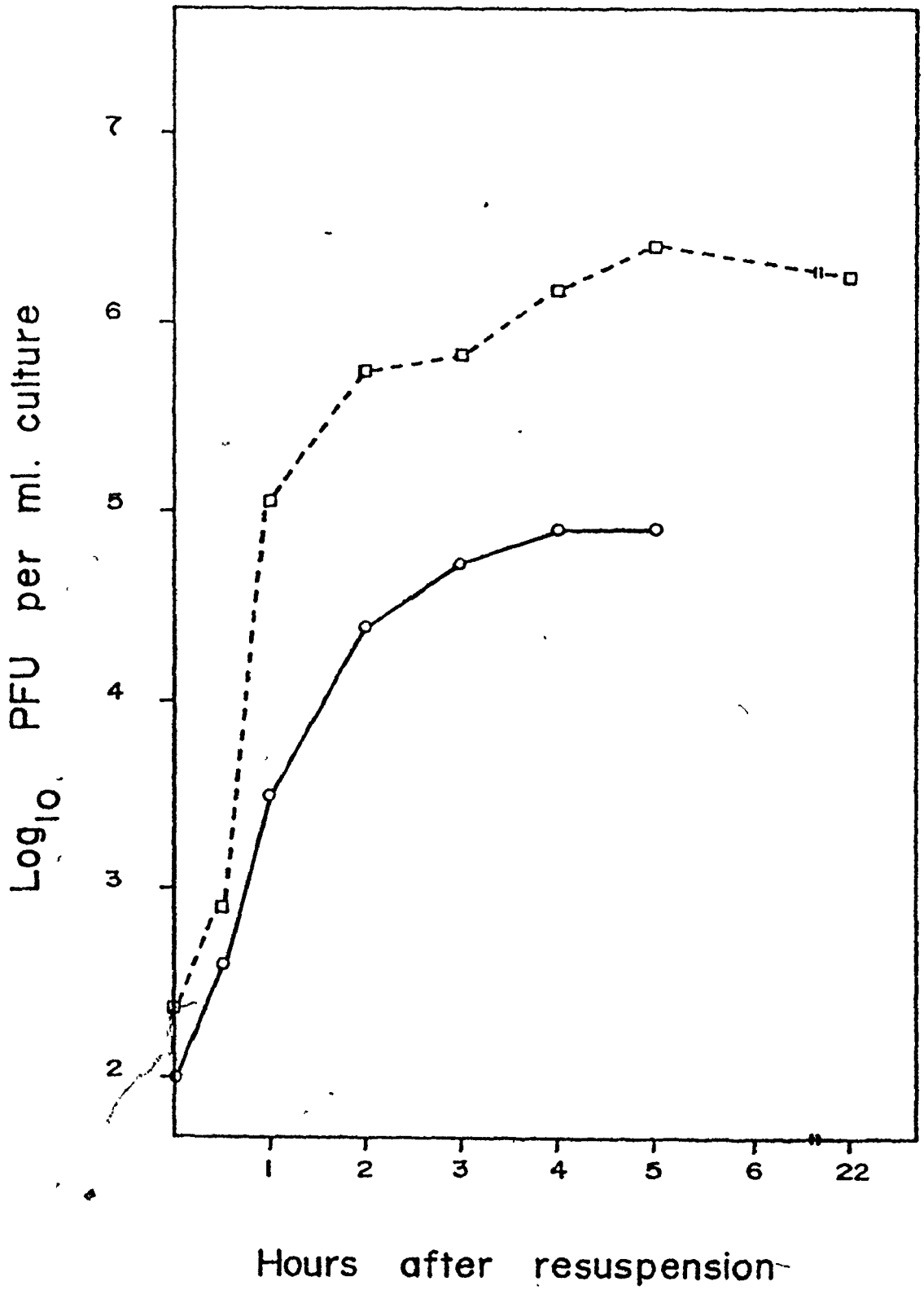

6
5
4
3
2
Log10 PFU per ml. culture
1
2
3
4
5
6
7
Hours after resuspension

Figure 21. Growth of VSV in Mouse Splenic Leukocytes Infected At High MOI.

Cultured, glass bead column purified mouse spleen leukocytes were incubated at 37°C in 90% humidity and 5% CO_2 for two days before infection. The cells were pelleted and resuspended in Sp MEM + 2% NBCS at 10^7 cells per ml. They were then infected at MOI = 10 for one hour, following which Rabbit anti VSV serum (Serum A) was added, to a final concentration of about 1/40, for 1/2 hour. The cells were washed three times by centrifugation and resuspension in fresh medium and resuspended after the final wash in medium to a concentration of 10^6 cells per ml. Samples were taken at the indicated times and assayed for plaque forming units.

□------□ 20 µg PHA/ml added just after purification.

0————0 without PHA.


7
6
5
4
3
2
Log10 PFU per ml. culture
1
2
3
4
5
6
22
Hours after resuspension

Because there was no difference in virus yield between gradient purified cells and glass bead column purified cells, the latter technique was used in all subsequent experiments because it was more expedient.

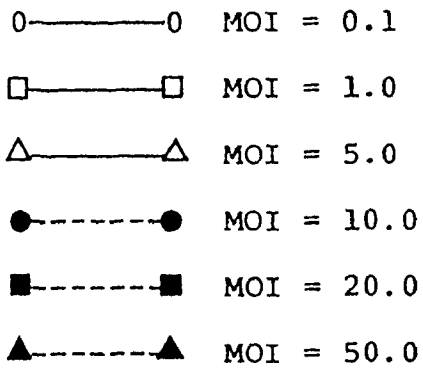
The virus growth curve experiments all indicate that there is a population of lymphocytes which is normally susceptible to infection with VSV without any prior PHA stimulation. This will be discussed later.

Attempts to determine whether the yield of virus could be increased by increasing the multiplicity of infection were unsuccessful (Figure 22). At multiplicities above 1.0, the maximum yield from stimulated cultures was approximately 1×10^6 PFU per ml. However, as might be expected, the rate of virus production increased with increasing multiplicities of infection.

Varying the adsorption period from 0.5 hours to 1.5 hours also had no effect on the virus yield (Figure 23) from unstimulated cells. A survey of all the virus growth experiments indicates that the virus yield from PHA stimulated cells ranged from 3.2×10^5 to 2.5×10^6 PFU per ml with an average of 6.5×10^5 PFU per ml, while the unstimulated cells produced between 5.0×10^3 to 3.0×10^4 PFU per ml at an average of 2.0×10^4 PFU per ml

Figure 22. Optimum MOI of Mouse Spleen Leukocytes Infected with VSV.

Cultured purified mouse spleen leukocytes were stimulated for 48 hours in the presence of 20 µg/ml of PHA-P, following which they were infected with VSV at one of the following multiplicities of infection: 0.1, 1.0, 5.0, 10.0, 20.0, or 50.0. After one hour of adsorption, the cells were treated with rabbit anti VSV serum (Serum A) for 0.5 hours, washed three times by centrifugation and resuspension, and resuspended at 10^6 cells per ml. Samples were assayed for PFU at 0, 1, 3, 5 and 7 hours after resuspension.



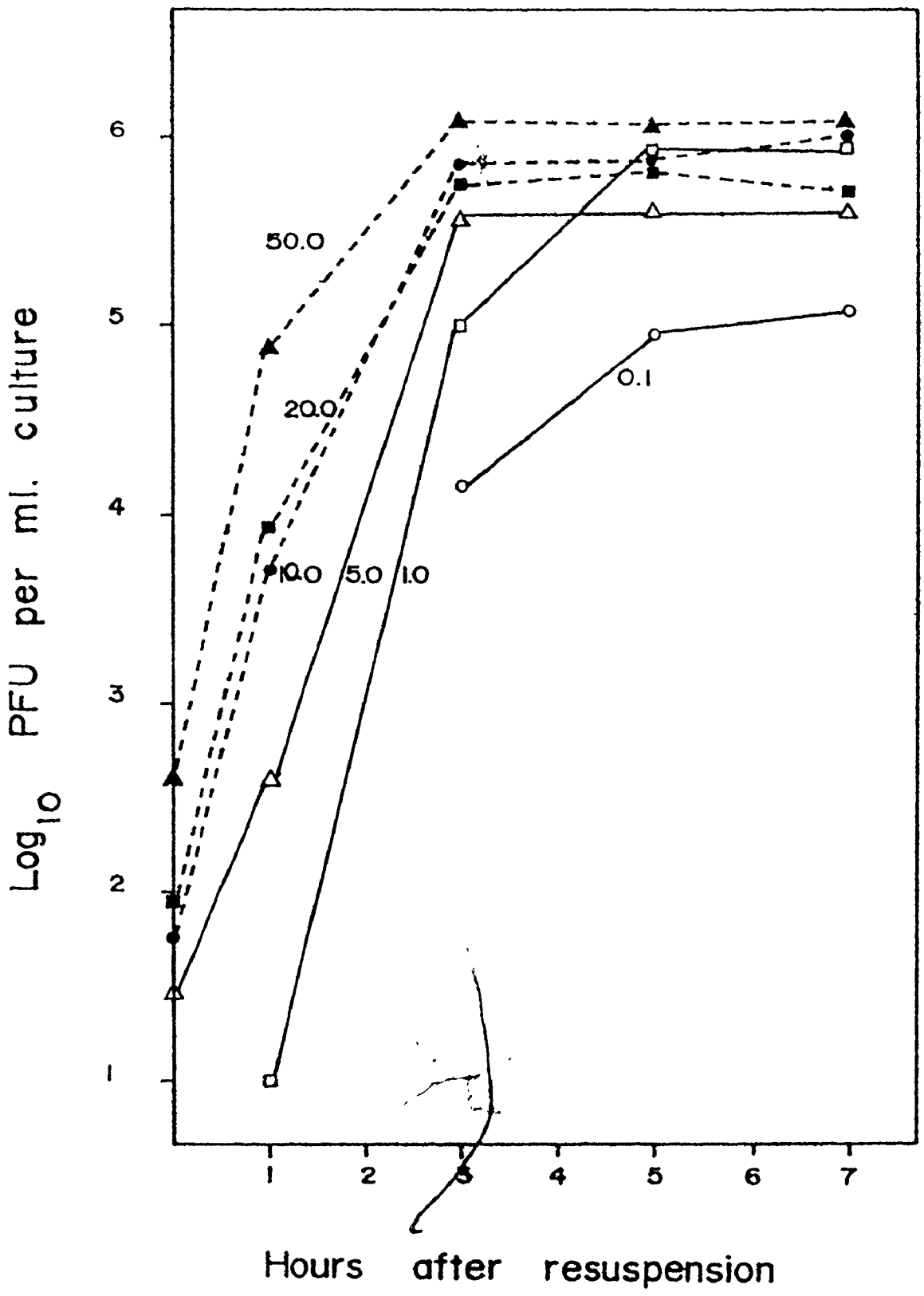
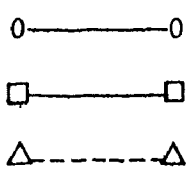

Log10 PFU per ml. culture
6
5
4
3
2
1
50.0
20.0
10.0
5.0
1.0
0.1
1
2
3
4
5
6
7
Hours after resuspension

Figure 23. Optimum Adsorption Period for VSV Infecting Mouse Spleen Leukocytes.

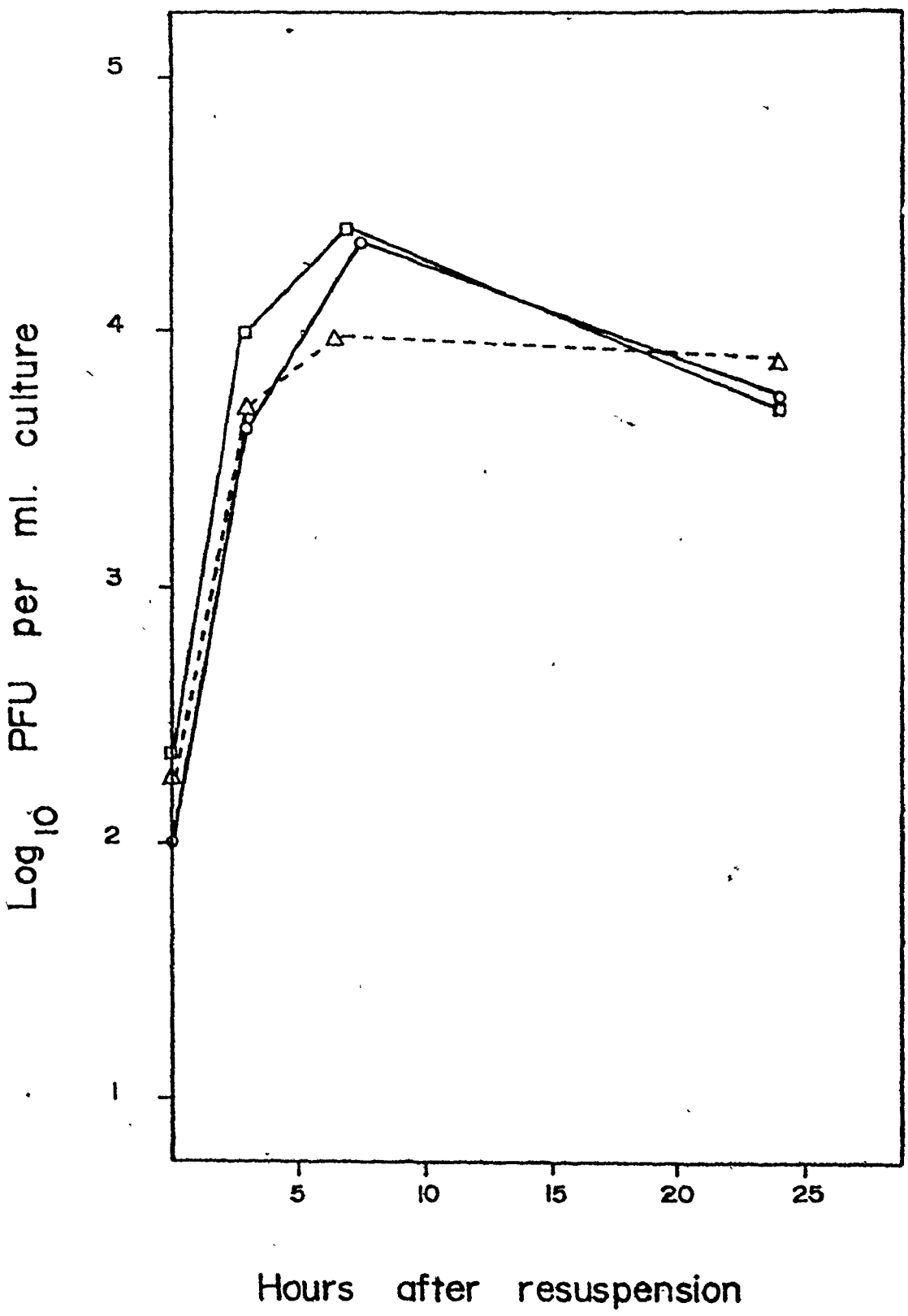
Cultured mouse spleen leukocytes were infected with VSV at MOI = 10, and incubated at 37°C for 0.5, 1.0 or 1.5 hours, following which the cells were washed once by centrifugation and resuspension and treated with Rabbit anti VSV serum at a final concentration of about 1/40 for 1/2 hour at 37°C. The cells were then washed three times and resuspended at 10^6 cells per ml. 1.0 ml samples were taken at the indicated times and assayed for plaque forming units.



0———0 0.5 hours adsorption.

□———□ 1.0 hour adsorption.

△------△ 1.5 hour adsorption.


5
4
3
2
1
Log10 PFU per ml. culture
5
10
15
20
25
Hours after resuspension

D. Stimulation Post Infection.

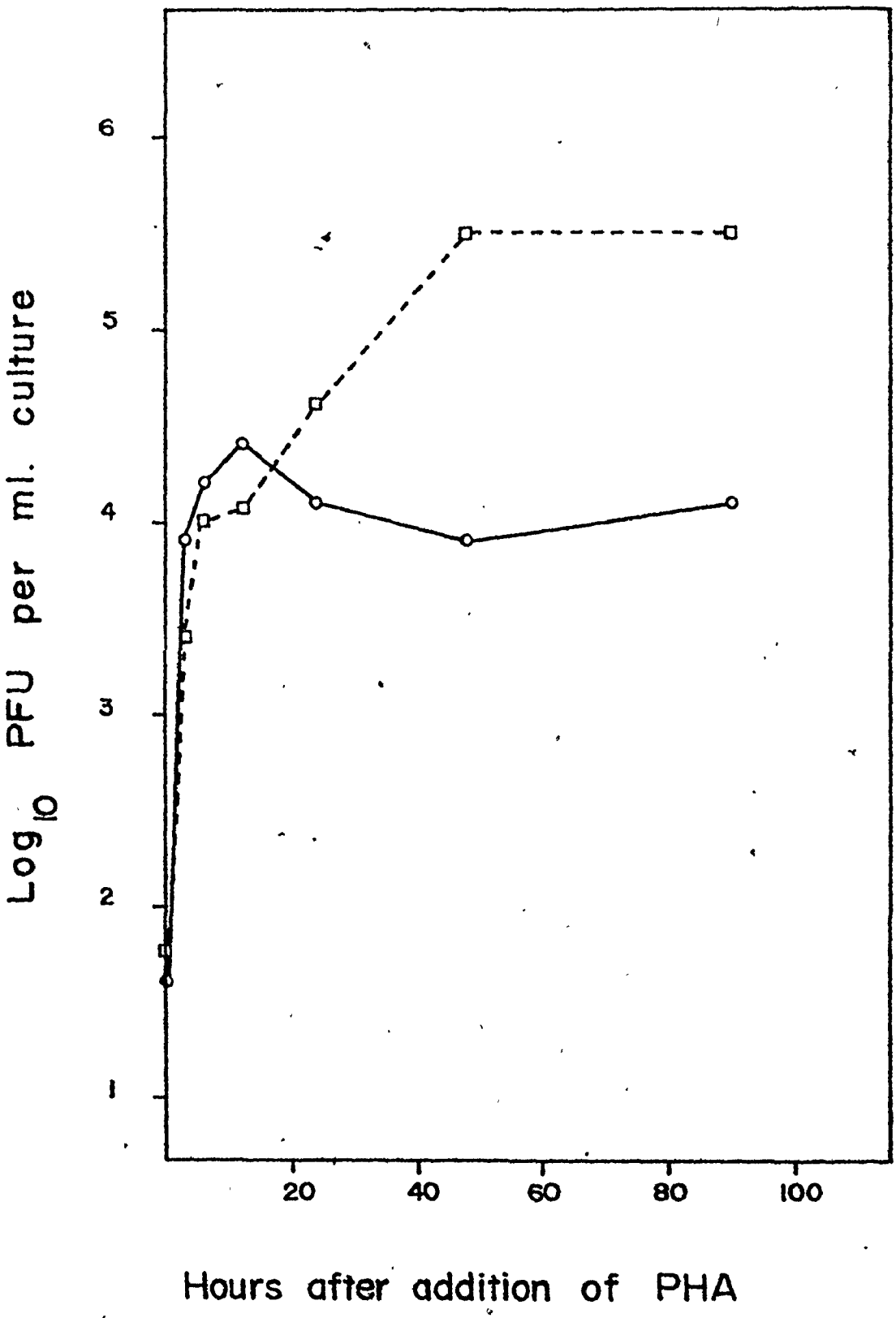
A crucial point which needs elucidation in this system is whether stimulation of leukocytes with PHA affects infection with VSV at the level of adsorption, or at some other intracellular stage in virus replication. For this reason it seemed desirable to infect the cells first and stimulate them later. Figure 24 illustrates the results. By 12 hours after resuspension the PHA stimulated and unstimulated cultures had produced about the same amount of virus. Between 12 and 48 hours after resuspension, the PHA treated culture produced more virus, reaching a plateau of about 3.2×10^5 PFU/ml. The unstimulated culture, on the other hand, did not produce any more virus after 12 hours, and the virus titre levelled off at 1×10^4 PFU per ml. This suggests that the stimulated cells went through a second round of virus production while the unstimulated cells did not. The interpretation of this experiment is complicated by the fact that unstimulated cells are also capable of limited VSV production. It can not be determined whether the virus production seen after PHA stimulation is the result of cells which had been preinfected at the time of mitogen addition or of those cells which were infected post stimulation by virus produced by unstimulated cells. This leaves the original question of whether PHA stimulation facilitates adsorption and penetration or some later intracellular step in viral production unanswered. An

Figure 24. Growth of VSV in Mouse Spleen Leukocytes: Stimulation After Infection.

Cultured leukocytes were infected at MOI = 10 with VSV for 2 hours, washed twice by centrifugation and resuspension and treated with Rabbit anti VSV serum (final dilution 1/40) for 1/2 hour at 37°C. The cells were washed three times and resuspended in fresh medium. PHA-P was added to the experimental culture at a concentration of 20 µg/ml culture. 1.0 ml samples were taken at the indicated times and assayed for virus plaque forming units as described in "Methods".

O———O without PHA.

□-------□ with PHA.


Log10 PFU per ml. culture
6
5
4
3
2
1
20
40
60
80
100
Hours after addition of PHA

attempt to further elucidate the point was made by infecting the cells with VSV for 24 hours, removing exogenous virus with antiserum and then stimulating with PHA. Figure 25 indicates that stimulated and unstimulated cells had produced the same amount of virus by 48 hours after stimulation. It is interesting to note that the amount of virus produced by both stimulated and unstimulated cells (3.5×10^5 PFU per ml) is similar to the yields of cells infected at low multiplicity, where it was also observed that both stimulated and unstimulated cells produced the same amount of virus by 72 hours after infection. This suggests that unstimulated cells infected at high multiplicity are inhibited from producing the maximum yield of virus, and that this inhibition can be reversed by treating the cells with antiserum and/or washing the cells. It is possible that the antiserum treatment may constitute a stimulation of the cells. However, this is difficult to visualize when similar treatment in previous experiments one hour after infection did not result in increased production of virus.

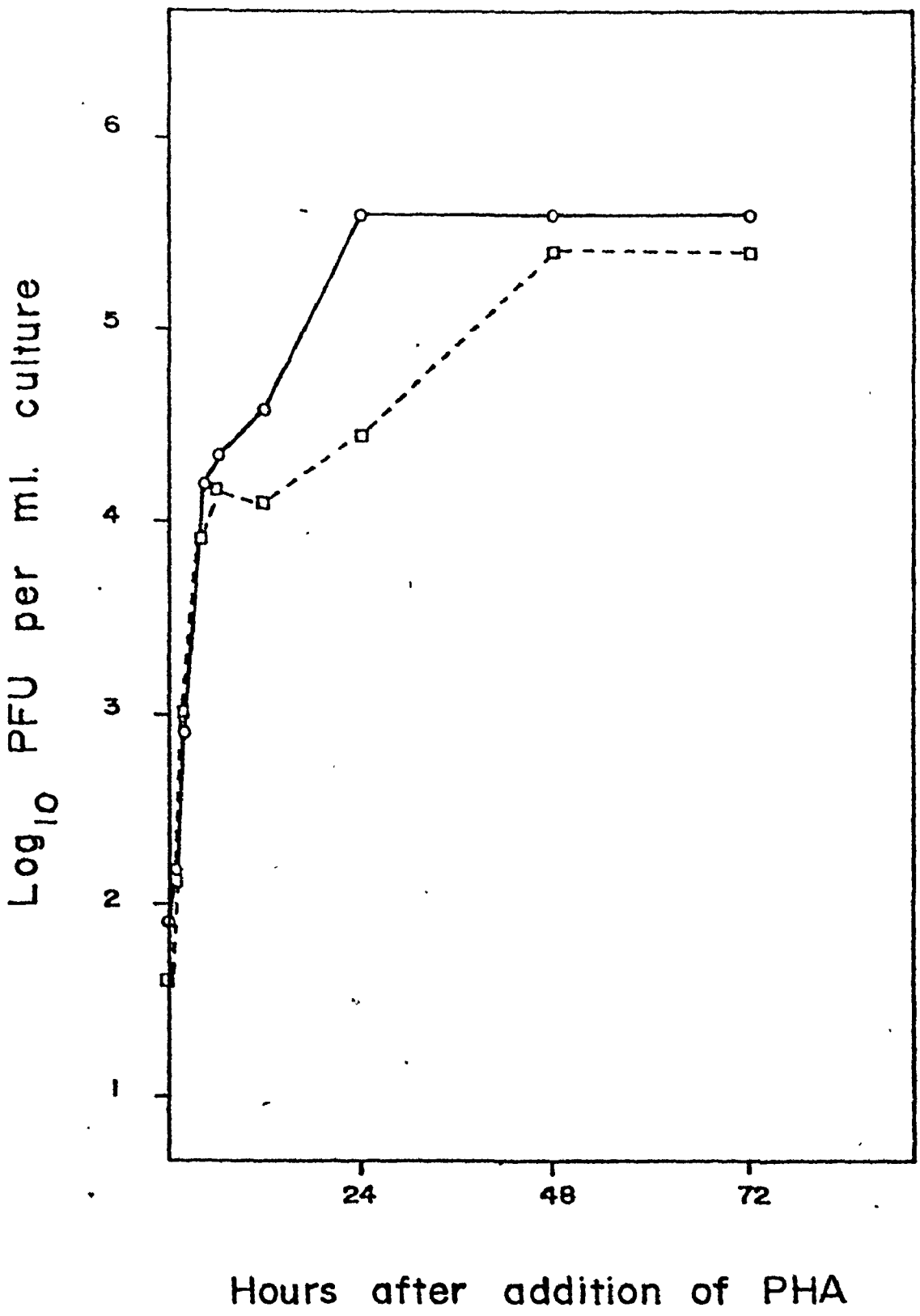
This experiment still leaves the original question regarding the effect of PHA unanswered, and in order to further investigate this problem, a variation of the infectious centre assay was used.

Figure 25. Stimulation Post Infection.

Cultured mouse spleen leukocytes at a concentration of 1 x 10^7 cells per ml were infected at a multiplicity of 10 for 2 hours and then diluted to 1 x 10^6 cells per ml. The cells were incubated for 24 hours at 37°C in 90% humidity and 5% CO_2, washed twice in medium and treated with Rabbit anti VSV serum (final dilution approx. 1/50) for 1/2 hour at 37°C. The cells were washed twice with fresh medium, resuspended at 1 x 10^6 cells per ml and incubated for three days with or without 20 µg/ml PHA. Samples were assayed for PFU per ml at 0, 2, 4, 6, 12, 24, 48 and 72 hours after addition of PHA.

O———O without PHA.

□------□ with PHA.


Log10 PFU per ml. culture
6
5
4
3
2
1
24
48
72
Hours after addition of PHA

III. Infectious Centre Assays.

The infectious centre assay was employed to enumerate the number of infected cells in the population which were producing virus. The rationale behind the technique was to suspend the infected cells, after treatment with anti VSV serum to neutralize any exogenous virus, close to a monolayer of susceptible cells (mouse L-60 cells) in medium containing agar. The virus produced by the infected cells would diffuse only as far as the susceptible cells in the immediate vicinity of the infected cells and form clear plaques in these regions.

A. Validity of Infectious Centre Assay.

A number of experiments were performed to ensure that the infectious centre assay was indeed measuring infected cells. An important consideration to be made was whether or not any of the plaques formed were made by exogenous virus which survived the procedure. Therefore, a sample of virus was carried through the infectious centre assay procedure, with the exception that no cells were present, to determine the ability of free virus to survive the treatment and yield an infectious centre. The results are presented in Table III. Assaying the virus suspended in agar reduced the titre by 1 to 2 logs. However, treatment with anti serum virtually eliminated all of the infectious virus. Only one experiment resulted in

detectable plaques following anti serum treatment, both with and without suspension of virus in agar, and this experiment indicated that treatment with anti serum reduced the virus titre by about seven logs. This is in good agreement with the neutralization curve of Serum B (Figure 1, "Methods") which indicates that a 10 fold dilution of anti serum reduces the virus titre by seven logs.

These experiments indicated that anti serum treatment combined with suspension of the virus in agar reduced the free virus titre to a very small fraction of the original. However, the infectious centre assay was performed in such a manner that the exogenous virus and the anti serum were removed before the cells were added to the susceptible monolayers. Any infectious virus escaping the wash would no longer be vulnerable to neutralization since the anti serum would have been diluted beyond its effective concentration. In order to determine the proportion of plaques formed by this exogenous virus in an infectious centre assay, reconstruction experiments were performed. Anti serum-treated virus was mixed with uninfected cells just prior to the washing of the cells. The cells were then assayed for "infectious centres" with and without suspension in agar. Parallel experiments in which infected cells were assayed were also performed. The results are presented in Table IV. Four different samples

Table III

The Effect of Plating Virus in Medium containing Agar on the Apparent VSV Titre Before and After Treatment with Rabbit Anti VSV Serum.

Experiment Number	VSV titre (PFU/ml)			
	with agar		without agar	
	with antiserum	without antiserum	with antiserum	without antiserum
1	1	2×10^7	80	1.1×10^9
2	ndp	2.9×10^8	ndp	2.4×10^9
3	nd	9.6×10^7	nd	1.3×10^9

The virus stock was serially diluted in medium containing 2% NBCS and 0.1 ml aliquots were either pipetted directly onto L-60 cell monolayers and incubated at 37° for 1/2 hour, or mixed with 0.4 ml of MEM containing 2% NBCS and 0.9% Noble agar and then pipetted onto L-60 cell monolayers. After either incubation of plates or gelling of agar-medium, a 4.5 - 5.0 ml overlay of agar-medium was added, gelled and the plates were incubated overnight at 37°C in 90% humidity and 5% CO_2. Plaques were counted the next day.

The virus was treated with anti VSV serum by incubating with rabbit anti VSV serum (Serum B, see "Methods") at a final

Table III (cont'd)

dilution of 1/10 for 1/2 hour at 37°C, followed by serial dilution and treatment as described above.

ndp = no detectable plaques

nd = not done.

of uninfected cells, including one PHA stimulated sample, assayed with or without agar resulted in a relatively uniform low level of background plaques per 10^6 cells, while the parallel samples of infected cells yielded larger and widely varying numbers of plaques per 10^6 cells. Suspension of the samples in agar, in all cases, resulted in a decrease in the number of plaques formed. Thus, in the samples containing uninfected cells, the plaques formed per 10^6 cells plated in agar were reduced to between 0 and 3. This indicates that a very low residual background of plaques formed in the infectious centre assay can be attributed to exogenous background virus.

As yet another check to ensure that the plaques were produced principally by the infected cells, the latter were assayed before and after disruption by freeze thawing. Table IV, Expt. 4 indicates that at the time of assaying, the cells had not produced any significant amount of virus. Without agar, the frozen thawed culture contained 5 plaque forming units per 10^6 cells while the whole cells produced 239 plaques per 10^6 cells. Suspension of the samples in agar again resulted in approximately a 10 fold decrease in the number of plaques formed. As seen in Table V, the freeze thawing procedure employed did not affect the titre of exogenous virus to a large degree; nor was the virus eclipsed by adsorption to cell membranes exposed by the disruption of the cells, as can be seen in Table V. This

Table IV

Assay of Exogenous Virus, With and Without Agar in the Medium, in the Infectious Centre Assay.

Expt. No.	Sample type	Plaques formed per 10^6 cells with agar	without agar
1	infected, unstimulated cells	5	116
	virus + Ab + uninfected cells	ndp	64
2	infected, unstimulated cells	50	460
	virus + Ab + uninfected cells	ndp	12
3	infected, PHA stimulated cells	560	1,280
	virus + Ab + PHA stimulated cells	1.7	43
	infected, unstimulated cells	25	215
	virus + Ab + unstimulated cells	3	51
4	infected, unstimulated cells	82	239
	frozen/thawed infected, unstimulated cells	ndp	5

Unstimulated or PHA stimulated cultured purified mouse spleen leukocytes were infected at MOI = 50 for 1 hour, treated with anti VSV serum for 1/2 hour, and washed by centrifugation and resuspension in fresh medium. The cells were counted and suspended at the appropriate concentrations and 0.1 ml

Table IV (cont'd)

aliquots were either plated directly on L-60 cell monolayers and incubated for 1/2 hour, or mixed with 0.4 ml medium containing agar and plated. After incubation or gelling of agar, a 5.0 ml overlay of medium containing agar was added to each plate. The same volume of virus stock which was used to infect the parallel sample of infected cells was mixed with anti VSV serum at a final dilution of 1/10 for 1/2 hour at 37^{o} and then mixed with uninfected cells. The cells were washed immediately and treated in the same manner as the infected cells. For experiment number 4, infected cells were frozen in dry ice and ethanol, and thawed at 37^{o} twice, diluted to the same degree as the infected live cells and plated in the same manner.

ndp = no detectable plaques.

indicates that the reduction in plaques formed per 10^6 cells following disruption of the cells by freeze thawing (Table IV, Expt. 4) is due to destruction of the infectious centres and not to any artifact of the procedure.

B. Efficiency of Plating.

A number of experiments were performed in an attempt to increase the efficiency of plating of the infectious centre assay. The first of these involved assaying the infected cells in medium containing varying concentrations of agar. The results are summarized in Table VI. The optimum concentration of agar appeared to be 0.72%, which yielded 1,060 plaques per 10^6 cells, although the difference in plaques formed per 10^6 cells was quite small. The decrease in plaques observed at the lower concentrations of agar was probably due to mixing of the cell-containing agar with the second overlay as a result of unstable agar gels. All infectious centre assays performed subsequent to this experiment employed 0.72 % agar in the overlay medium.

Several attempts to increase the efficiency of plating in the infectious centre assay were made by centrifuging the plates after addition of the infected cells and prior to solidification of the agar-medium. The rationale behind this was to pellet the infected cells closer to the L-60 cell monolayer in order to reduce the distance for the virus to diffuse. The results are presented

Table V

The Effect of Freeze-Thawing on Virus Infectivity and the Effect of Mixing Virus with Frozen-Thawed Cells on Infectivity.

Sample Assayed	PFU/ml
dilute virus	7.9×10^3
dilute virus + PHA stimulated frozen-thawed cells (A)	9.3×10^3
A frozen-thawed once	8.8×10^3
A frozen-thawed twice	7.5×10^3
dilute virus + unstimulated frozen-thawed cells (B)	10.6×10^3
B frozen-thawed once	8.9×10^3
B frozen-thawed twice	6.6×10^3
virus stock	7.4×10^8
virus stock frozen-thawed twice	4.5×10^8

Virus stock was diluted to approximately the titre expected if all plaques formed per 10^6 cells in the infected cell sample from Experiment 4, Table V were formed by exogenous virus. PHA stimulated cells or unstimulated cells were disrupted by freezing in dry ice and ethanol and thawing at 37°C twice. Dilute virus was mixed with disrupted PHA stimulated cells (mixture A) and with disrupted unstimulated cells (mixture B).

Table V (cont'd)

Both mixtures were frozen in dry ice and ethanol and thawed at 37°C twice and each mixture was assayed for virus before and after each freeze-thaw according to the method described in "Methods".

Table VI

Efficiency of Plating VSV Infected Mouse Spleen Leukocytes in Medium Containing Varying Concentrations of Agar.

Concentration of agar (w/vol.)	Plaques formed per 10^6 cells
0.90%	860
0.81%	1,000
0.72%	1,060
0.63%	910
0.54%	630
0.45%	690

Cultured, purified mouse spleen leukocytes were infected with VSV at MOI = 50 for 1 hour, treated with a 10 fold dilution of rabbit anti VSV serum for 0.5 hours, pelleted and resuspended in fresh medium at 10^7 cells per ml and 10^6 cells per ml. Aliquots of 0.1 ml were mixed with 0.4 ml of medium containing varying concentrations of agar and plated on L-60 cell monolayers in disposable tissue culture dishes, 60 x 15 mm. After the medium had gelled, an additional 4.5 ml of medium containing the same concentration of agar was added to each plate and plates were incubated at 37°C in 90% humidity and 5% CO_2 overnight. The plates were fixed with Carnoy's fixative and plaques were enumerated.

Table VII

Efficiency of Plating VSV Infected Mouse Spleen Leukocytes After Centrifuging the Plates.

Expt No	Sample description	Plaques formed per 10^6 cells	
		Before cent.	After cent.
1	unstimulated cells	20	20
2	PHA stimulated cells	1,410	1,010
	unstimulated cells	210	190
3	unstimulated cells	507	690

Cultured purified mouse spleen leukocyyes were incubated overnight with and without PHA-P. The cells were resuspended at 10^7 cells per ml, infected with VSV at MOI = 50 for 1 hour, treated with a 10 fold dilution of rabbit anti VSV serum for 1/2 hour, pelleted and resuspended at the appropriate concentrations. Aliquots of 0.1 ml were mixed with 0.1 ml of medium containing agar and introduced to L-60 cell monolayers in disposable tissue culture dishes 60 x 15 mm. The plates were centrifuged for 10 minutes at 1,000 rpm at 37° and 4.5 ml of medium containing agar was overlaid. The plates were incubated at 37°C in 90% humidity and 5% CO_2 overnight, fixed with Carnoy's fixative and examined for plaques.

in Table VII. Centrifuging the plates after addition of the cells in agar-medium did not affect the efficiency of plating. There was no difference in plaques formed per 10^6 cells in four different samples assayed before and after centrifuging.

All of these preliminary experiments indicated that the infectious centre assay is a valid assay in that it measures infected cells rather than free virus particles. While it is possible that the efficiency of plating can be improved, the proportion of infected cells in stimulated and unstimulated cultures can still be estimated using the present techniques.

C. Infectious Centre Assays of Stimulated and Unstimulated Cells.

The infectious centre assay was employed to enumerate the number of cells producing infectious virus particles in the PHA stimulated and unstimulated leukocyte populations. A number of experiments were performed on leukocyte cultures from normal spleens after incubation overnight with or without PHA-P. The cells were infected, treated with anti serum and plated in agar, as described in "Methods". Table VIII summarizes the results of several experiments. As can be seen, there is a wide variation in the number of infectious centres per 10^6 cells in the unstimulated cultures, ranging from 25 plaques per 10^6 cells for one spleen to 760 plaques per 10^6 cells for

another, a 30 fold difference. However, the variation in infectious centres per 10^6 cells is not as great in PHA stimulated cultures, ranging from 560 to 2,900 plaques formed per 10^6 cells, a 5 fold difference. In both cases however, the number of infectious centres per 10^6 cells is very low, indicating that only a small proportion of cells in either a PHA stimulated or an unstimulated culture is capable of supporting VSV replication.

Two similar experiments were performed on "abnormal spleen" cultures. One spleen came from an animal which had been immunized against rabbit erythrocytes after receiving three intravenous injections at seven day intervals of 2×10^7 rabbit red blood cells. Another spleen came from an animal which had received an intravenous injection of 5×10^3 spleen focus forming units of Friend Leukemia Virus three weeks prior to culturing to allow development of splenomegaly. Both spleens were cultured, purified and incubated with or without PHA-P for 24 hours before infection with VSV. The results are presented in Table IX. The preimmunized spleen cells do not exhibit abnormal behaviour with respect to the "normal spleen" cells in the infectious centre assay. The plaques formed per 10^6 cells for both PHA stimulated and unstimulated cells fall within the ranges seen in Table VIII, with the PHA stimulated cells producing 2,140 plaques per 10^6 cells and the unstimulated cells producing 250 plaques per 10^6 cells.

Table VIII

Infectious Centre Assays of PHA Stimulated and Unstimulated Mouse Spleen Leukocytes Infected with VSV.

No. plaques formed per 10^6 cells		Stimulation Ratio*
PHA stim.	Unstimulated	
1,990	760	2.6
560	25	22.4
810	50	16.2
1,410	210	6.7
2,900	360	8.0

Mouse spleen leukocytes were cultured and purified according to the procedure outlined in "Methods". PHA stimulated cultures received 20 µg/ml PHA-P 24 hours before infection. The cells were pelleted, resuspended at 10^7 cells per ml, infected with VSV at MOI = 50 for 1 hour and treated with a 10 fold dilution of rabbit anti VSV serum for 1/2 hour. The cells were then washed by centrifugation, resuspended at appropriate concentrations and distributed on L-60 cell monolayers in medium containing agar as described in "Methods". Plaques were observed the following day.

* $\frac{\text{No. plaques formed per } 10^6 \text{ PHA stimulated cells}}{\text{No. plaques formed per } 10^6 \text{ unstimulated cells}}$

However, the leukemic spleen reacted somewhat differently from the "normal spleens" in the infectious centre assay; the PHA stimulated and unstimulated cells both produced considerably more plaques per 10^6 cells, 4,400 and 2,300 respectively, than the "normal spleens". The reason for this is unclear, although it can be suggested that the erythroblasts formed by the Friend Virus infection are capable of producing VSV when infected with that virus.

A third experiment was performed to test Bloom's hypothesis that the virus plaque assay can be used to enumerate antigen stimulated cells. Spleen cells from an animal preimmunized with rabbit red blood cells were incubated in vitro for two days with or without the antigen (rabbit red blood cells), and then infected and assayed for infectious centres. As indicated by Table IX, the antigen stimulated cultures also contain higher numbers of infectious centres, although these are quite low when compared with PHA stimulated cultures. Whether these cells are B cells or T cells remains to be tested.

A general observation was made with respect to the infectious centre assay, namely that as the number of plaques formed per 10^6 unstimulated cells increased, the ratio of plaques formed per 10^6 PHA stimulated cells to plaques formed per 10^6 unstimulated cells (Stimulation Ratio) decreased. The stimulation ratio was computed in this manner

Table IX

Infectious Centre Assays of Stimulated and Unstimulated Mouse Spleen Cells from "Abnormal" Spleens Infected with VSV.

Spleen type	No. plaques formed per 10^6 cells			Stimulation ratio*
	with PHA	with RRBC	without PHA or RRBC	
immunized against RRBC	2,140		250	8.6
immunized against RRBC		76	14	5.4
FV induced splenomegaly	4,400		2,300	2.0

Mouse spleen leukocytes were cultured, purified and incubated with or without PHA-P overnight, or with or without 1×10^7 RRBC per ml for 2 days. The cells were pelleted, resuspended at 10^7 cells per ml, infected with VSV at MOI = 50 for 1 hour, treated with a 10 fold dilution of rabbit anti VSV serum for 0.5 hours and washed by centrifugation. The cells were resuspended at the appropriate concentrations and introduced to L-cell monolayers in medium containing agar as described in "Methods". Plaques were observed the following day.

* $\frac{\text{plaques formed per } 10^6 \text{ stimulated cells}}{\text{plaques formed per } 10^6 \text{ unstimulated cells}}$

in an attempt to eliminate the broad variation in the number of plaques formed per 10^6 unstimulated cells, by giving each set of data a common denominator. Figure 26 illustrates the observed relationship. When the stimulation ratio is plotted against the $\log_{10}$ plaques formed per 10^6 unstimulated cells, a straight line inverse relationship can be observed. The possible implications of this relationship will be discussed later.

D. Stimulation Post Infection.

In the preceding section an attempt was made to determine whether PHA stimulation of leukocytes facilitates adsorption and penetration of VSV or whether some later intracellular step in VSV production is affected. However, the experiment described previously, in which cells stimulated after infection produced higher titres of VSV than unstimulated cells, did not differentiate between infected cells which were subsequently stimulated, and stimulated cells which were then infected by exogenous virus produced by the normally susceptible cells. The experiment described here was designed in an attempt to overcome this problem. VSV infected leukocytes were suspended in a small volume of medium containing PHA and agar over a monolayer of L-cells and an overlay of medium containing PHA and agar was placed over this. The agar in

Figure 26. Stimulation Ratio Versus the Log_{10} (Number of Plaques Formed per 10^6 Unstimulated-Cells.)

The data from Tables VII and VIII were treated to obtain the log_{10} (number of plaques formed per 10^6 unstimulated cells) and plotted as shown.

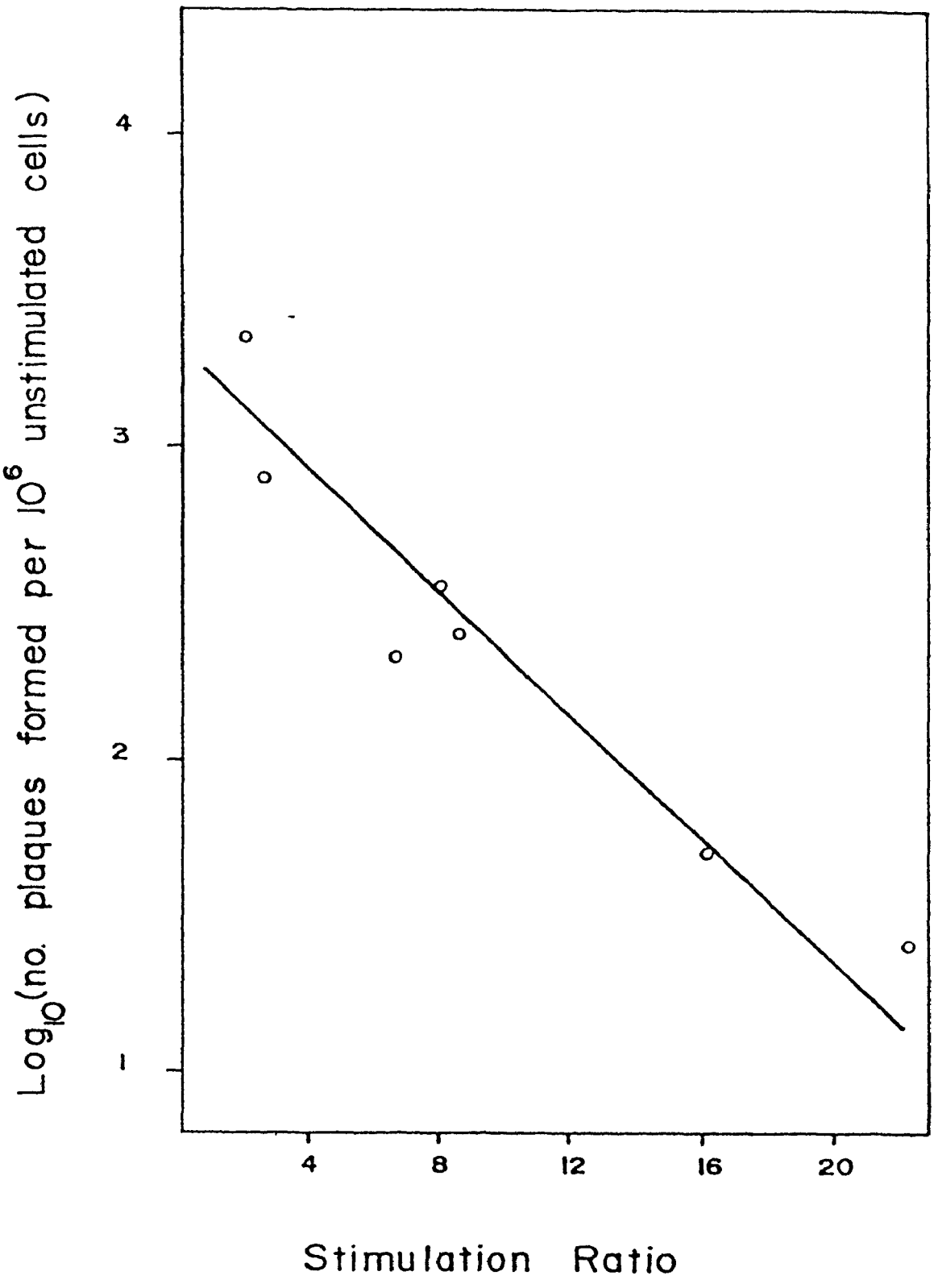

Log10(no. plaques formed per 10^6 unstimulated cells)
4
3
2
1
4
8
12
16
20
Stimulation Ratio

Table X

Stimulation Post Infection

Expt. No.	Mode of PHA treatment	No. of plaques formed per 10^6 cells with PHA	without PHA
1	20 µg/ml	240	220
2	40 µg/ml	5,000	5,000
	80 µg/ml	5,000	
	120 µg/ml	5,000	
3	20 µg/ml	26	15
	40 µg/ml	26	
	80 µg/ml	25	
4	20 µg/ml, 1 hour treatment in liquid medium before suspension in medium containing agar	330	350
5	20 µg/ml, 2 hour treatment in liquid medium before suspension in medium containing agar	10	11
6	20 µg/ml, 2 hour treatment in liquid medium before suspension in medium containing agar	35	42

Table X (cont'd)

Cultured, purified mouse spleen leukocytes were infected with VSV at a multiplicity of 50 for one hour at 37°, treated with rabbit anti VSV serum for 1/2 hour at 37° (final dilution of serum = 1/10), washed by centrifugation and resuspended in fresh medium. The cells were suspended over L-cell monolayers in medium containing agar. In experiments 4, 5 and 6, the PHA treated cells were treated with PHA in liquid medium for the specified times after treatment with anti serum and before suspension in medium containing agar.

the medium would prevent any secondary infection of leukocytes and, it was hoped, at the same time allow stimulation of the cells with the PHA in the medium. The results of these experiments are presented in Table X. In all of the experiments performed there was no difference in plaques formed per 10^6 cells between cells assayed with and without PHA in the agar-medium. Even when infected cells were treated with PHA in liquid medium for up to 2 hours before assaying, there was no difference between the stimulated and the unstimulated cells. Varying the concentration of PHA from 20 µg/ml to 120 µg/ml also produced no difference.

These results indicate one of two things: a) the agar prevented the PHA from stimulating the cells, or b) stimulation of leukocytes with PHA facilitates adsorption and penetration of VSV rather than some later intracellular step in VSV replication.

DISCUSSION

Mitogenic Response

The poor mitogenic response observed in mouse spleen cells following stimulation with PHA-P could be attributed to a number of possible reasons. Work with human peripheral leukocytes has indicated that different individuals require different concentrations of mitogens to induce maximal incorporation of radioactive precursors of DNA (Asquith et al., 1973; Richter and Naspitz, 1967; Fitzgerald, 1971). These workers have also noted a wide variation in the magnitude of the maximal response among individuals. These variations in both optimal dose and maximal response may be caused by genetic variation, or some factor related to the immunological state of the individual, or a combination of the two. A non inbred strain of mice whose immunological state was not controlled may well exhibit wide variation in mitogenic responses. Further investigation of this problem should probably be performed on spleens from inbred mice raised under rigidly controlled germ free conditions.

Williams and Benacerraf (1972) and Williams et al. (1973) observed that different strains of mice or rats respond differently to PHA and ConA in both maximal response and optimal dose. In surveying the literature, the author

has never seen a report in which Swiss mice were used to study the mitogenic response to PHA or other mitogens. It is possible that spleen cells from Swiss mice do not respond well to PHA-P in terms of incorporation of radioactive precursors of DNA. This would account for the low levels of incorporation observed in this study.

A more probable reason for the poor response observed is the possibility that both batches of PHA-P used in the study were of inferior quality since human, C_3H mice and Swiss mice lymphocytes all failed to respond significantly to the PHA-P.

Another explanation for the poor mitogenic response may be the age of the mice used. There is some evidence to suggest that the mitogenic response decreases with increasing age in rats (Nielsen, 1974; Folck and Waksman, 1974). The same may be true in mice. The mice used in this study were generally between 6 and 12 months old, although some mice may have been older.

The complete lack of any observable mitogenic response in purified leukocytes may have been a result of the purification procedure. There is some evidence suggesting that lymphocytes require the presence of macrophages in order to respond to PHA-P in terms of incorporation of radioactive DNA precursors (Heilman and Gambrill, 1974), and that removal of the macrophages abolishes this response.

However, the lack of a mitogenic response does not preclude the ability of the cells to undergo blast transformation. Some evidence indicates that the various responses to PHA-P stimulation are not necessarily associated with the DNA synthetic response (Badger et al., 1973).

Indeed, this appears to be the case with virus production by stimulated leukocytes. While the human leukocytes showed no mitogenic response to PHA-P in terms of ^{3}H-thymidine uptake, they supported replication of VSV to higher titres than the unstimulated cells. On the other hand, mouse spleen leukocytes stimulated by ConA or purified PHA incorporated proportionately high amounts of ^{3}H-thymidine, but produced only slightly more virus than the unstimulated cells. This suggests that the mitogenic response and the change allowing increased virus production following PHA stimulation may be two independent responses. Therefore, since PHA-P elicits a variable and poor mitogenic response in unpurified cells, and none at all in purified cells, its effect on virus production was examined to determine if the general observation noted above held true for mouse spleen leukocytes.

Virus Production by Mouse Spleen Leukocytes

A number of interesting observations were made when mouse spleen leukocytes were infected with VSV. The first

of these concerned the growth of VSV in unstimulated cells. Eustatia and Van der Veen (1971) observed no virus production in mouse lymph node cells infected at low multiplicity. However, in the present study, unstimulated cells produced consistently high titres of VSV after infection at low MOI. It is possible that this discrepancy may be due to the different sources of the cells. Spleen lymphocytes may be inherently different from lymph node lymphocytes with respect to virus production. However, this hypothesis remains to be tested. These results indicate that there is a population of cells in the mouse spleen which is normally susceptible to infection with VSV without any prior stimulation. It is also possible that some part of the culturing procedure is providing a stimulation which is not apparent to the experimenter.

The second observation presents a phenomenon which might be the subject of an interesting study. Stimulated cells infected at high or low multiplicities produce consistent titres of VSV - around 10^6 PFU per ml. However, unstimulated cells produce about 10^6 PFU per ml when infected at low multiplicities, but only 10^4 PFU per ml when infected at high multiplicities. This suggests that infection of unstimulated cells at high multiplicities inhibits viral viral replication. Interference of replication by defective T particles of VSV can be ruled out since Galet (1973) has shown that at least four high multiplicity passages of the virus used in this study, $HRPP_2$, are required before any

interference activity can be observed in virus grown in mouse L-cells. Therefore, the inhibitory effect must be a cellular phenomenon. It is possible that cells infected at high multiplicity produce a substance (interferon?) which inhibits viral replication in a population of cells which produce VSV with slower kinetics. Eustatia et al. (1972) have shown that mouse macrophages infected at high MOI reach the plateau of virus production by 24 hours after infection. However, the present study indicates that mouse lymphocytes produce the maximum yield of virus within six hours after infection. This indicates that there may be two populations of cells in mouse spleens which produce VSV with fast and slow kinetics. If the hypothesis is correct, then the lymphocytes (fast kinetics) are somehow preventing viral replication in the macrophages (slow kinetics) when the cells are infected at high MOI. This suggestion is partially supported by the observation that purified cells infected at high MOI produce the same amount of virus as unpurified cells, indicating that glass adherent cells (macrophages) are not significantly involved in virus production in cells infected at high MOI. A number of experiments remain to be performed to test this hypothesis; for example, purified cells should be infected at low MOI to see if the removal of macrophages lowers the virus titres produced by unstimulated cells; the supernatant of purified cells infected at high or low MOI should be assayed for interferon and for any ability to inhibit virus replication

Infectious Centre Assays

The amount of virus produced by both stimulated and unstimulated cells was quite low when compared with virus yields from infected L-60 cells. The yield per cell appeared to be 1 PFU per stimulated cell and .01 PFU unstimulated cell after infection at a multiplicity of 10. Therefore, infectious centre assays were performed to determine the actual size of the virus susceptible population. The most significant fact to emerge from this part of the study is that a very small population of cells produce infectious particles. It is possible that the infectious centre assay can be improved to increase the efficiency of plating. In fact, the results obtained from experiments where infected cells were assayed without suspension in agar suggest that this may be a more efficient method of assaying infectious centres. Other workers have found that rat lymphocytes will stick to mouse embryo fibroblast monolayers after stimulation with mitogens (Hollander and Ginsberg, 1972). The increase in infectious centres assayed without agar ranged from 2.3 times to 80 times those assayed with agar, with an average 21 fold increase. However, even if a comparable increase in plating efficiency had been observed, the resulting populations of virus producing cells would have ranged from 0.01 percent to 2.2 percent of unstimulated cells and from 1.2 percent to 6.0 percent of stimulated cells. Cells plated in agar

indicated populations of 0.0005 percent to 0.1 percent of unstimulated cells and 0.05 percent to 0.3 percent of stimulated cells which were producing virus. These are very small populations of virus susceptible cells. It is possible that the small virus susceptible population observed in PHA stimulated cells may be due to the apparent lack of stimulation observed in these cells and that a larger population may be elicited by another mitogen such as ConA or PHA MR68. This has yet to be investigated. Because of the interaction of ConA with enveloped viruses and the ensuing problems of infecting ConA stimulated cells, PHA MR68 would probably be a better choice of mitogen.

Even though the populations of virus susceptible cells in both stimulated and unstimulated cultures were very small, a stimulatory effect was nevertheless observed. This ranged from a 2.6 fold to a 22.4 fold increase in the number of virus susceptible cells following stimulation with PHA. The wide variation in stimulation ratios cannot be explained at this time. However, two interesting observations were made which might provide a suitable answer. The first is that there is a much wider variation in plaques formed per 10^6 cells in unstimulated cultures than in stimulated cultures (the former ranges from 5 to 1,060, while the latter ranges from 560 to 2,900). The second observation concerned the stimulation ratio. As the number of plaques formed

per 10^6 unstimulated cells increased, the stimulation ratio decreased. This suggests that the number of plaques formed per 10^6 stimulated cells is independent of the number of plaques formed per 10^6 unstimulated cells, i.e., that stimulation with PHA affects a different population of cells from those that are normally virus susceptible. It also suggests that the PHA responsive population is less variable in size from one animal to the next than the normally virus susceptible population. These observations pose some rather interesting problems which might prove worthy of further investigation. The most obvious question concerns the nature of the normally virus susceptible population of cells and the reasons for the wide variation among different animals. Investigation of this problem would require rigid control of antigenic stimulation of the animal and assay of the antigen sensitive cell population. Another line of investigation into the nature of the PHA stimulated population would determine whether PHA stimulation resulting in virus susceptible cells is indeed unrelated to stimulation of DNA synthesis. Eustatia and Van der Veen showed that mouse lymph node cells require stimulation of DNA synthesis before virus production can be observed. The results presented in this work contradict these findings, although there may be some fundamental difference between spleen cells and lymph node cells which would account for this apparent contradiction. Although the stimulation of virus susceptible

cells may be independent of stimulation of DNA synthesis, it is possible that the level of stimulation of Swiss mouse spleen cells by PHA-P is at the lower end of the spectrum and that another mitogen, which elicits a good DNA response, may also stimulate a larger proportion of cells to become virus susceptible. If this is the case, then the dependence or independence of virus production on DNA synthesis must be determined by manipulation of the cells with certain drugs which inhibit DNA synthesis and mitosis.

Stimulation Post Infection

The central question concerning which stage of virus production is affected by PHA stimulation remains unanswered. Nowakowski et al (1973a) have shown that a human lymphoblastoid cell line, Raji, which is refractory to VSV replication, transcribes the VSV messenger RNA but does not replicate the virion RNA. They have suggested that a similar intracellular block may prevent replication of VSV in unstimulated lymphocytes. However, they have reported no VSV production in mouse lymphocytes (Nowakowski et al., 1973), which is contradictory to the findings of this study. Until the adsorption and penetration of VSV into unstimulated cells has been demonstrated satisfactorily, this question will remain unanswered. The stimulation post infection experiments are rather ambiguous because stimulation of cells in agar could not be demonstrated. If this latter problem could be resolved, the experiment should provide some interesting information.

SUMMARY

No clear conclusions can be reached regarding the effect of PHA stimulation on virus production in mouse spleen leukocytes. However, some interesting facts have been established. Virus replicates normally in unstimulated cultures (assuming the culture conditions do not provide an unknown stimulus). Infection at high multiplicity appears to depress the normal replication in unstimulated, unpurified cultures. Only a very small population of cells are engaged in virus production and some evidence suggests that the PHA stimulated population of infected cells is different from the normally susceptible population. Several different areas of future investigation into different aspects of this work have been suggested.

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