

CULTURE AND CHARACTERIZATION OF CAT LYMPHOCYTES

BY

D. R. STOLTZ

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## ABSTRACT

This work presents an investigation of the behavior of cat lymphocytes in vitro. The responses of cat peripheral blood lymphocytes to phytohemagglutinin (PHA), pokeweed mitogen (PWM) and concanavalin A (Con A) were examined in detail in an attempt to find optimal culture conditions. Cat blood lymphocytes responded well to Con A and PWM but poorly or not at all to PHA. The observation that splenic lymphocytes responded well to PHA gave rise to several hypotheses to explain the difference between blood and splenic cells. It was concluded that a low number of PHA-responsive cells are present in cat peripheral blood.

In addition, the effects of ouabain, proteolytic enzymes, periodate and mercuric compounds upon cat lymphocytes were investigated.

## RESUME

Ce travail rapporte une étude du comportement des lymphocytes du chat in vitro. Les réponses des lymphocytes circulants du chat à la phytohémagglutinine (PHA), au pokeweed mitogene (PWM), à la concanavaline A sont examinées en détail afin de déterminer les conditions optimales de culture. Les lymphocytes circulants du chat présentent une bonne réponse à la Con A et au PWM mais sont peu ou pas du tout stimulés par la PHA. L'observation que les lymphocytes de la rate répondent bien à la PHA permet de proposer plusieurs hypothèses pour expliquer la différence entre les lymphocytes circulants et ceux de la rate. On conclut qu'un faible nombre de cellules répondant à la PHA est présent dans les lymphocytes sanguins de chat. En plus on a recherché les effets de l'ouabaine, des enzymes protéolytiques, du periodate et des composés mercuriques sur les lymphocytes de chat.

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## 1. INTRODUCTION

### 1.1 Scope and Purpose of the Investigation

When leukocytes of peripheral blood are cultured in vitro in the absence of any extraneous mitogen, few, if any, of the cells enlarge and undergo mitosis. Hungerford et al. (1959) found that phytohemagglutinin (PHA), used in the separation of leukocyte-rich plasma from erythrocytes, caused the appearance of more blastoid and dividing cells in his cultures than had usually been reported by previous investigators. Subsequently the small lymphocyte was identified as the cell undergoing blastogenesis in the presence of PHA.

The advent of the short-term peripheral leukocyte culture technique as developed by Hungerford et al. (1959) and Nowell (1960) and modified repeatedly thereafter, has provided a convenient tool for several disciplines. Geneticists were then able to prepare karyotypes without sacrificing the subject or resorting to skin biopsy and monolayer tissue culture. Immunologists could deal directly with several of their favourite cell types. And cell biologists in general had a new experimental animal that could be readily caged and cared for in any laboratory. 5

Although simple leukocyte culture systems have been described for many species, most investigators found that feline lymphocytes generally responded poorly if at all to mitogens under standard culture conditions (Gregson and Ishmael, 1971; Loughman et al., 1970). Aside from two reports that a complex medium supplemented with 30% pig serum and glutamine provided a suitable milieu for stimulation by pokeweed mitogen (PWM) or PHA (Bilz et al., 1967; Hare et al., 1966), there is little

literature regarding the culture and mitogen stimulation of feline lymphocytes.

The relative scarcity of published data on cat immunobiology is somewhat surprising in view of the fact that the cat is gaining wider acceptance as an experimental animal. Recently, the cat has proved valuable in toxicity studies, especially of methyl mercury, and for general teratogenicity testing. Furthermore, cats are susceptible to a defined viral leukemia, thus providing another animal model for cancer studies (Perryman et al., 1972).

The present investigation includes:

- 1) development of a reliable feline leukocyte culture system
- 2) characterization of various factors affecting the response of cat lymphocytes in culture
- 3) comparison of certain aspects of the feline immune response to those of other species.

## 1.2 Cellular Components of the Immune System

### 1.2.1 Lymphocytes

The ontogenesis of the immune system culminates in a dichotomous system responsible on one hand for cellular immunity and on the other for humoral immunity. Cellular immunity includes protection against certain virus, fungal and intracellular bacterial pathogens, immunological surveillance for mutant cells, transplantation immunity and delayed skin hypersensitivity reactions. Humoral immunity is manifested by the production of specific immunoglobulins or antibodies.

T lymphocytes responsible for cellular immunity and B lymphocytes responsible for the humoral system have a common progenitor in the lymphoid bone marrow stem cell (Ritzmann et al., 1973). Stem cells destined to become B cells migrate through the Bursa of Fabricius in birds or through the bursa-equivalent (unidentified) in mammals, emerge immunologically competent and settle preferentially in peripheral lymphoid organs (germinal centers and cortical areas of lymph nodes, medullary cords of the nodes, red pulp of the spleen, lamina propria of the gastrointestinal tract and bone marrow). T cells are derived from stem cells that migrate through or are influenced by the thymus (Goldstein, 1974) before occupying the paracortical areas of lymph nodes and the periarteriolar regions of the spleen (Ritzmann et al., 1973).

Differences between T and B lymphocyte populations have been demonstrated on the basis of 1) mitogen activation, 2) surface immunoglobulins, 3) theta antigen, 4) electrophoretic mobility, 5) ultrastructure, 6) rosette formation, etc. Certain differences have been exploited in the physical separation of these cell populations (Nordling et al., 1972).

#### 1.2.2 Macrophages

Another important component of the immune system is the macrophage. Although the precise role of the macrophage and of other adherent cells is not defined, it appears that these cells participate in antigen acceptance, antigen processing and presentation of antigen to

lymphocytes (Katz and Unanue, 1973). The physical form of the antigen appears to determine the need for macrophages in immune responses in vitro as well as in vivo. For example, polymerized bacterial flagellin is a macrophage independent antigen, in contrast to monomeric flagellin which requires macrophages (Feldman and Unanue, 1971).

### 1.3 Mitogens

Substances which stimulate the growth and division of lymphocytes may be classified into three groups - antigens, phytomitogens and similar compounds, and miscellaneous mitogens.

#### 1.3.1 Antigens

The in vitro activation of lymphocytes by specific antigens is found only in lymphocytes from donors who have been sensitized with these agents. Examples of commonly employed antigens include tuberculin (PPD), tetanus toxoid and streptolysin O (SLO). The magnitude of the response to antigens is generally much less than that elicited by phytomitogens. The response peaks later, five to eight days after initiation of the culture. There is an absolute requirement for the presence of adherent cells.

#### 1.3.2 Phytomitogens and Similar Compounds

The response to phytomitogens occurs in the absence of prior sensitization. In addition, the magnitude of the response is high, reaching a peak three to four days after addition of the mitogen. The commonly-used phytomitogens are the plant extracts PHA, concanavalin A (Con A) and PWM. A lipopolysaccharide (LPS) extract prepared from gram negative bacteria (usually E. coli or S. typhimurium) induces a lymphocyte culture response similar

in magnitude and time course to that caused by the phytomitogens. Although LPS stimulates cells from several species it does not affect human lymphocytes (Peavy et al., 1970).

### 1.3.3 Miscellaneous Mitogens

This group of mitogens is characterized by the diversity of its members. Although the miscellaneous mitogens do not require prior sensitization to elicit a response, they have little else in common. Members of this class include  $Hg^{++}$ ,  $Zn^{++}$ , proteolytic enzymes, sodium periodate and others. Both the magnitude and the time course of the response depend upon the particular mitogen.

### 1.3.4 Interaction of Mitogens and Cells

Current information indicates that each mitogen reacts with specific determinants on the lymphocyte surface. Furthermore it appears that different populations of lymphocytes possess different determinants, accounting for their different reactions with various mitogens. Thus, PHA apparently primarily stimulates T cells, while PWM stimulates both T and B cells. However, possession of determinants for the binding of a mitogen does not guarantee that such binding will produce blastogenesis. For example, cortical thymus cells bind both PHA and Con A but only Con A induces mitosis in these cells (Greaves et al., 1972).

Detailed information on the binding of Con A to cells was rapidly generated following the demonstration that cells transformed by tumor viruses or chemical carcinogens are agglutinated by Con A more readily than are

normal cells (Burger and Noonan, 1970). Although it was originally thought that the specificity of Con A was directed exclusively toward a single saccharide residue, there is now evidence of less specificity. The binding of Con A to cells can be conveniently reversed by competition with alpha-methyl-D-glucopyranoside and other carbohydrates (Lindahl-Kiessling et al., 1973).

Light and electron microscopic studies with mitogens tagged with fluorescein, ferritin,  $^{125}\text{I}$  or other markers indicated that soon after binding the marked mitogens were distributed to limited and distinct areas of the cell membrane (Stobo et al., 1972). This "capping" was followed by internalization of the mitogen (Pauli et al., 1973). The limited evidence available fails to establish conclusively a relation between cap formation and lymphocyte activation. In similar studies with radiolabelled Con A, Andersson et al.: (1972) found that the maximum number of molecules which could be bound per mouse thymus lymphocyte was in the order of  $10^7$  to  $10^8$  although only about 3 to 10% of the available receptors are bound when thymocytes are optimally stimulated by Con A.

The kinetics of mitogen binding to lymphocyte has not been studied in detail. It is known that after a 1 hour exposure of cells to PHA, the stimulation cannot be reversed by repeated washing of the cells (Lindahl-Kiessling, 1972). What is unclear is whether any or sufficient PHA can indeed be removed by mere washing. In other words one cannot conclude that a 1 hour exposure to PHA is all that is required to effect stimulation. On the

other hand, since it is possible to remove bound Con A with certain carbohydrates, the time sequence of stimulation can be more conveniently studied with Con A. Lindahl-Kiessling has demonstrated that complete inhibition can be obtained if the competing carbohydrate is added up to 10 hours, but even at 24 hours and later some inhibition is seen (Lindahl-Kiessling, 1972).

### 1.3.5 Selective Activation

The selectivity of mitogens for T and B lymphocytes has been investigated in detail in the mouse (Greaves and Janossy, 1972). Janossy and Greaves (1971) pioneered with observations that PHA stimulates DNA synthesis in T but not in B lymphocytes whereas PWM activates both T and B cells. Subsequently it was found that Con A stimulates only T lymphocytes (Janossy and Greaves, 1972) while LPS activates only B cells (Andersson et al., 1972).

The selective response of lymphocyte classes to certain mitogens was necessarily determined with purified cells. The assumption that the selective response would be maintained in mixed T and B cell cultures is not entirely supported, perhaps because activated lymphocytes release mitogenic and other factors which might facilitate the activation of otherwise inactive cells (Andersson et al., 1972a). On the other hand, mitogen adsorbed to cells or to other surfaces might possess similar properties to mitogen purposely insolubilized by binding to sepharose beads or by crosslinking to the bottom of plastic petri dishes to prevent internalization. Such insoluble mitogen is still

stimulatory but the selectivity of activation may be lost or altered. Thus insoluble PHA stimulates T and B cells (Greaves and Bauminger, 1972) whereas insoluble Con A stimulates only B lymphocytes (Andersson et al., 1972). Nevertheless, for the mouse, this selectivity is generally retained over a range of B and T cell mixtures (Janossy et al., 1973).

Autoradiographic ascertainment of the number of cells synthesizing DNA has shown that, even under conditions ideal for the selective induction of a response, not all the cells within the target class will respond. This seems to be particularly true for B lymphocytes (Janossy et al., 1973).

#### 1.4 Events in lymphocyte Activation

##### 1.4.1 Metabolic Activity

The earliest measurable effects of mitogens on lymphocytes are changes in properties of the cell membrane. Directly after PHA administration the normally high electrical resistance of the membrane is reduced at sites of cell contact (Hulser and Peters, 1971). Within the first three minutes Fisher and Mueller (1971) found a four-fold increase in turnover of phosphatidyl inositol, which was almost completely restricted to the plasma membrane. Stimulation of de novo synthesis of lecithin and neutral fats is also very rapid (Resch et al., 1973) and membrane glycoprotein turnover is doubled in 3 hours (Hayden et al., 1970). After five to thirty minutes increases in  $K^+$  (Quastel and Kaplan, 1970) and  $Ca^{++}$  (Whitney and Sutherland, 1973), glucose (Averdunk, 1972), uridine

(Peters and Hausen, 1971) and amino acid (Mendelsohn et al., 1971) transport are demonstrable followed by increased uptake of phosphates (Cross and Ord, 1971) and later folate (Das and Hoffbrand, 1970).

Activity of membrane enzymes is also increased, including adenyl cyclase within 10 minutes (Smith et al., 1971) and a Na<sup>+</sup> K<sup>+</sup> independent ATPase after 30 minutes (Novogrodsky, 1972). The activity of the former results in a cAMP level in the stimulated cell that is 2-3 times that of resting cells. However, cGMP levels are soon raised 20-50 fold; this may be a more important signal (Hadden et al., 1972). Changes in carbohydrate metabolism are detectable 15 minutes after PHA addition (Roos and Loos, 1970). Mitogen-induced protein synthesis is first measurable after a few hours and reaches a peak after 24-48 hours (Neiman and MacDonnell, 1970).

#### 1.4.2 RNA Synthesis

PHA increases the combined rate of synthesis of all forms of RNA about 4 times. It also increases the halflife of the labile heterogeneous nuclear RNA, changes the pattern of methylation of tRNA and increases the intracellular level of a ribonuclease inhibitor (Cooper, 1972).

PHA affects ribosome activity by causing more efficient utilization of pre-existing ribosomes, by stimulating both the synthesis of the 45S precursor of rRNA and the processing of this precursor into 32S and 18S subunits, and by reducing the rate of 18S RNA wastage,

\* which in resting cells amounts to the loss of half of the units before complete ribosome assembly (Cooper, 1972).

This general stimulation of RNA metabolism may be partly a result of gene activation. Acceleration of acetylation of histones and phosphorylation of nuclear proteins, and an increase in template activity of isolated nuclei have been reported, and the ratio of euchromatin to heterochromatin changes from 0.56 to 10.8 in the stimulated cells (Cooper, 1972).

### 1.4.3 DNA Synthesis

Mitogens cause asynchronous DNA synthesis detectable after about 24 hours and reaching a maximum several days later. Then the total DNA synthesis declines but the cells may be restimulated by fresh mitogen (Polgar and Kibrick, 1970).

At the peak of PHA-induced DNA synthesis 70-90% of the cells undergo blast transformation and synthesize DNA. However, less than 40% of these lymphocytes actually proceed through mitosis, while 35-90% of the newly synthesized DNA is excreted into the medium. This release of DNA is apparently selective since RNA is not lost similarly. The process may well be related to the reversion of blast cells to small lymphocytes (Rogers et al., 1972; Sarma and Zubroff, 1973).

### 1.4.4 Morphological Changes

Morphological aspects of lymphocyte transformation have been described in detail by Ling (1968) and Biberfeld (1971). Briefly, the small lymphocyte, about 10 microns in diameter, has a heterochromatic nucleus surrounded by a thin rim of cytoplasm containing few organelles. The transformed lymphocyte or blast cell, 15-30 microns in diameter, is characterized by a large lightly-staining nucleus with a prominent nucleolus. There is more cytoplasm rich in organelles.

### 1.4.5 Time Course of Response to Mitogens

After exposure to a phyto mitogen a large proportion of lymphocytes are transformed into blast cells. The blast cells start synthesizing DNA after about 24

hours. Earlier opinions that the blasts started DNA synthesis synchronously were not supported by Soren (1973) who found cells in their first S phase between 24 and 124 hours, with a majority of cells in their first S phase at 72 hours after initiation of the culture. Blasts may proceed through several cell cycles.

The response of a culture to a phyto mitogen as measured by radiolabelled thymidine incorporation peaks on day 3 to 4 for PHA, LPS and Con A and on day 3 to 5, or even later for PWM (Douglas, 1972). Culture conditions, the number of responding cells and other factors may affect the time course of the response.

The response of a culture to an antigen follows a slower course, reaching a peak on day 5 to 8. There is some question whether the number of activated lymphocytes measured represents only those cells derived by clonal proliferation of specific mitogen sensitive cells, or includes a population, possibly also clonally proliferating, recruited by the antigen activated cells early in the response. Experimental evidence supports the latter theory (Mills, 1970).

Mitogens in the miscellaneous group may elicit a response with a short [sodium periodate (Parker et al., 1973)] or a long [Hg<sup>++</sup> (Caron et al., 1970)] time course.

### 1.5 Immunogenetics

The nature of the results of this research, coupled with the fact that random-bred animals were employed throughout, necessitates a discussion of genetic control of immune responses.

The immune response consists of a process of multiplication and differentiation of immunocytes induced by antigen stimulation. This process may be genetically controlled at two different levels: antigen recognition and antibody synthesis. Genetic control of the initial discriminative phase, antigen recognition, is generally attributed to T lymphocytes although macrophages may play a role (Shevach and Rosenthal, 1973). The productive phase of antibody synthesis depends on genetic regulation of the life cycle of B lymphocytes.

The discovery of individual immune response (Ir) genes depended upon experiments where the immunological system was presented with unique challenges (Benacerraf et al., 1971). Three types of approaches have been utilized to characterize specific Ir genes:

1. The study of the antigenicity of synthetic polypeptides with restricted heterogeneity to present the immunological mechanism with molecules with limited structural diversity.
2. The study of the antigenicity of complex natural proteins injected in a dose range which is immunogenic only for some individuals or certain inbred strains in a species.
3. The study of immune responses to weak native antigens which differ only slightly from the corresponding host

proteins restricting the possible specific interactions with the host immune mechanism.

The contention that the detection of specific Ir genes depends upon the singularity of the challenge was supported by studies of the immunogenicity of a synthetic polypeptide poly-L-(Phe, Glu)poly-L-Pro-poly-L-Lys in two different mouse strains. Both strains responded well but the antibodies produced were of different specificities (Shearer, et al., 1971).

Although it might be concluded that strain-dependent variations in the antigen recognition phase of the immune response to complex antigens possessing many determinants might not be detected due to the animals' ability to respond to a portion of the total number of determinants presented, it is certainly possible to detect such strain-dependant variations in the phase of antibody synthesis elicited by complex antigens. For example, in two lines of mice separated by selective breeding for the character "agglutinin production to heterologous erythrocytes", an analysis in terms of rosette forming cells (RFC) showed that the doubling time of RFC was 9 hr in high responder and 16 hr in low responder mice. In this case, genetic control was shown to regulate the life cycle of RFC (Biozzi, et al., 1972).

Despite the complexity of the immunological system, the experimental approaches mentioned above have identified numerous distinct autosomal dominant Ir genes in guinea pigs and mice, the species most intensively investigated. It is indeed noteworthy that most of these genes have been shown to be very closely linked with the major loci controlling histocompatibility specificities (McDevitt et al., 1972).

Williams and Benacerraf (1972) have obtained evidence consistent with autosomal dominant non-H-2-linked, polygenic control of Con A and PHA stimulation of in vitro DNA synthetic response of mouse spleen cells. Williams et al. (1973a,b) found the rat T cell response to be similarly controlled. The only difference in response of two rat strains to PHA and Con A was in the Con A concentration required for maximal stimulation. Genetic control of mitogen-stimulated DNA synthesis in vitro could be manifested as strain differences at many levels including differences that affect the ability of the cells to function optionally under the culture conditions employed and differences in the proportion of an essential cell type in the organ studied.

The foregoing simplistic review of immunogenetics is deceptive. In reality, many facets of the genetics of the immune response are still unknown, for example:

1. the size of the specific Ir gene pool.
2. the identity of the cell type where specific Ir genes are expressed.
3. the significance of linkage between Ir genes and histocompatibility loci.
4. the possible mechanisms of action of Ir genes.

## 1.6 Factors affecting the response of lymphocytes

### 1.6.1 Endogenous Factors

#### 1.6.1.1 Age

The lymphoid system undergoes marked age-related changes during adulthood and senescence as well as during early development. Morphological changes with aging consist of thymic involution, a degree of lymphocyte depletion in peripheral lymphoid organs and the appearance of more reticuloendothelial cells in tissues. These changes are accompanied by decreased immunological capacity. Although the evidence is not complete, it appears that both humoral and cell-mediated responses may be affected (Kishimoto and Yamamura, 1971; Adler et al., 1971; Blazkovec et al., 1973; Mathies et al., 1973).

The decline with age of in vivo immunological function has been attributed not only to decreasing immunocompetence of individual cells but also to age-related factors in the cellular environment (Walford and Tunis, 1971). Thus in vitro assays of immune response may or may not detect age-related changes.

#### 1.6.1.2 Sex

Sex differences in terms of susceptibility to infections and immunoglobulin synthesis have been reported for the human and some experimental species (Washburn et al., 1970; Rifkind and Frey, 1972). In one study, the primary immune response patterns were compared in young adult male and female hamsters injected with sheep red blood cells (SRBC). The

females produced more plaque-forming cells per spleen than did the males. The effects of pre- and post-pubertal gonadectomy suggested that the female advantage in terms of specific immunoglobulin synthesis depends primarily on increased splenic weight, cellularity and hematopoietic activity due to a relative absence of male gonadal hormones (Blazkovec et al., 1973; Ravines, 1961).

Sexual dimorphism of in vitro lymphocyte responses to plant mitogens or specific antigens has not been reported.

#### 1.6.1.3 Disease State: Viruses and Leukemia

Naturally occurring viral infections can have a variety of effects upon the immune system, consisting of morphologic changes, lymphocytopenia, an altered distribution of lymphocytes in body compartments, a reduction in the cell-mediated immune response and resistance to stimulation by mitogens. These responses are due to the lymphocyte's capacity to serve both as a potential host for viral replication and as a mediator of the immune response to the virus (Wheelock et al., 1971).

The various leukemia viruses have different effects on the host's immune mechanism. For example, the Friend and Rauscher murine leukemia viruses markedly depress both cell-mediated and humoral immune responses whereas the Gross murine leukemia virus causes thymic atrophy and decreased cell-mediated immunity (Friedman and Ceglowski, 1971).

The responses of leukemic lymphocytes to plant mitogens have been investigated in detail in the hope of identifying the cell types responsible for lymphoid neoplasms. However, lymphocytes from patients with chronic lymphocytic leukemia generally show marked suppression of transformation with PHA, PWM and Con A compared with normal cells, suggesting that, irrespective of their origin and the presence of receptors, they may be metabolically unable to respond to mitotic stimulants. The small proportion of normal lymphocytes among the leukemic lymphocytes may account for whatever response can be elicited (Melief et al., 1973; Smith et al., 1972).

Feline leukemia (lymphosarcoma) is a virus-induced lympho-proliferative disease with a high spontaneous incidence in random bred cats and a latent period of 3-4 months (Jarrett et al., 1973). Immunological function during feline leukemogenesis has been studied. Histological correlates of immunological impairment, i.e. thymic atrophy, paracortical lymphoid depletion, runting disease, increased susceptibility to intercurrent infections, have been associated with the preleukemic state (Essex et al., 1973; Hoover et al., 1973). In addition, the assessment of immunocompetence during preleukemia, as measured by rejection of cutaneous allografts and antibody response to SRBC, showed that significant depression of cell-mediated immunity, but not of humoral antibody response, occurs in the preneoplastic

phase of experimental feline lymphosarcoma (Perryman et al., 1972).

In mice, SRBC have been shown to be thymic-dependent antigens (Munro and Hunter, 1970). The question then arises why the humoral antibody response was not depressed in the preleukemic cats similarly to the cell-mediated response. Either SRBC are not thymic-dependent antigens in the cat, or these cats had sufficient functional T-cells to satisfy the carrier recognition function, which thus allowed B-cells to make enough antibody (Perryman et al., 1972).

#### 1.6.2 Exogenous Factors

##### 1.6.2.1 Ouabain

The cardiac glycoside, ouabain, is a potent inhibitor of the biochemical, physiological and morphological parameters of human lymphocyte transformation. It acts as a specific inhibitor of Na<sup>+</sup>K<sup>+</sup> activated ATPase to prevent the K<sup>+</sup> influx essential for some step(s) in the transformation process (Quastel and Kaplan, 1968; Wright et al., 1973).

The lymphocytes of certain species, e.g. rats and mice, are insensitive to ouabain at concentrations several orders of magnitude greater than that required to completely inhibit transformation of human lymphocytes. Several explanations may be proposed:

1. K<sup>+</sup> influx may not be a (necessary) step in transformation in certain species.

2. the ATPase may be inaccessible, have lower affinity for ouabain or the complex may dissociate faster (Tobin et al., 1972).
3. transport enzymes may be more numerous, requiring more inhibitor to saturate the cell.

Curiously, cats do not maintain much of a gradient of either Na<sup>+</sup> or K<sup>+</sup> across their erythrocyte membranes, and, correlated with this, cat red cells have a minimal amount of ATPase activated by Na<sup>+</sup> and K<sup>+</sup> and show no inhibition by ouabain (Bonting et al., 1961; Sha'afi and Lieb, 1967).

#### 1.6.2.2 Enzymes

The enzymatic removal of lymphocyte cell surface components can modify the cells' response to mitogens. An increased response to mitogens following treatment of lymphocytes with the proteolytic enzymes trypsin and pronase was thought to be due to removal of peptides adjacent to or overlying the mitogen binding sites, resulting in an unmasking of cryptic binding sites, increased exposure of the cell to mitogen or exposure of critical sites. More recent evidence suggests that proteolysis might facilitate rearrangement, eg. clustering, of sites favourable to cell agglutination and stimulation (Burger, 1970; Sharon and Lis, 1972). Although several investigators have described enhanced responses to mitogens following enzyme treatments (Goodall et al., 1971) the opposite has also been reported (Adler et al., 1972;

Lindahl-Kiessling and Peterson, 1969). Since immunoglobulin patches on the cell surface can be removed by trypsin (Pernis et al., 1971) it seems likely that mitogen binding sites per se are also susceptible to prolonged or specific enzyme treatment.

Such experiments, yielding either enhanced or diminished mitogen responses after removal of cell surface components, suggest means by which the rate of resynthesis or at least reappearance of these cell components might be determined. For example, Lindahl-Kiessling and Peterson (1969) found the reappearance of PHA response to be complete in a number of hours following trypsinization of lymphocytes. Similarly, molecules which maintain the normal surfaces of contact-inhibited monolayer cells are known to turn over rapidly (Baker and Humphreys, 1972).

Paradoxically, it has even been possible to effect lymphocyte stimulation by enzyme treatment in the absence of mitogens. Several authors have described stimulation by mere treatment with neuraminidase (Kirchner, 1969), which removes sialic acid, and proteases (Goodall et al., 1971; Mazzei et al., 1966a and b). A theory of lymphocyte transformation based on proteolytic cleavage of a trigger peptide has been proposed (Kast, 1974).

Recent evidence suggests that in the mouse trypsin is a B cell specific mitogen and that pronase affects both B and T lymphocytes (Kaplan and Bona, 1974; Vischer, 1974). The evidence is derived from

experiments in which cells were exposed to proteases for 3 to 5 days in serum-free medium. Lymphocytes treated with proteases for only 30 minutes and then cultured in medium with serum gave less clear-cut results.

Endogenous proteolytic enzymes have also been implicated in the complex cell interactions and release of various soluble mediators during delayed hypersensitivity reactions. Havemann *et al* (1973) presented evidence that in the presence of circulating antibody and antigen, protease release occurs from adherent cells, leading to local activation of sensitized lymphocytes and migration inhibition of macrophages.

#### 1.6.2.3 Periodate

Lymphocytes can be stimulated to transform by treatment with sodium periodate ( $\text{NaIO}_4$ ) at concentrations ranging from  $10^{-4}$  to  $5 \times 10^{-3}$  M (Novogrodsky and Katchalski, 1971). The mode of action is presumably by oxidation of carbohydrates in surface glycoproteins or glycolipids. The response to periodate is reported to depend to some extent upon the presence of macrophages (Biniaminov *et al.*, 1974). Only a brief exposure to periodate (approx. 10 seconds) is required to stimulate lymphocytes. In fact exposures longer than one hour are toxic, but only to those cells that initially respond to the treatment. After the killing of periodate-sensitive cells, the remaining cells retain responsiveness to

PHA and PWM. Thus the periodate-responsive subpopulation is distinct from or overlaps minimally with the PHA and PWM responsive populations (Parker et al., 1973).

#### 1.6.2.4 Mercury

The toxicity of mercury is currently of considerable interest because of the widespread environmental contamination with this metal. The symptoms of chronic mercury poisoning in man are the result of progressive neurological damage. Mercury poisoning in the cat is a workable model for the human disease since the symptomatology and pathogenesis are similar in the two species (Charbonneau et al., 1974). Furthermore, analogous to human exposure conditions, cats will readily consume a diet containing fish naturally contaminated with mercury.

Few immunological effects of mercury poisoning have been reported. In general hematologic parameters in mercury-poisoned animals are within normal ranges. However, as with many chemicals, it is possible to develop contact hypersensitivity to mercury compounds. During lymphocyte culture studies on a patient with allergic contact dermatitis from mercury, Schopf et al. (1967) discovered that  $\text{HgCl}_2$  at  $7.4 \times 10^{-5}\text{M}$  caused blast transformation of normal human peripheral lymphocytes from individuals who showed no evidence of allergy to mercury as well as causing transformation of sensitized lymphocytes. This finding placed mercury in a group of

miscellaneous nonspecific mitogens alongside  $Zn^{++}$  and  $NaIO_4$ .

Three mercuric salts, the chloride, acetate and nitrate, proved to be mitogenic in human lymphocyte cultures. Of the two complex organic mercurials tested, merbromin (Mercurochrome) was effective whereas thimerosal (Merthiolate) was toxic at the concentrations tested (Caron et al., 1970; Yachnin, 1972). Since the mercury in merbromin is covalently bound and does not ionize in solution, there is some question whether its mode of action is similar to that of the simple mercuric salts.

The toxicity of mercuric ions appears to be due to their affinity for sulfhydryl (SH-) groups of biologically active molecules, and can be neutralized with SH-containing chemicals such as cysteine and 2,3-dimercaptopropanol (Ramel, 1969). It is not known whether these chemicals will also abolish the mitogenic activity of mercury.

5  
It is difficult to accept mercury as a "nonspecific" mitogen for two reasons. To begin with, since mercury is ubiquitous in the environment, all individuals are exposed to some extent, although sensitization may not be detectable. Secondly, the DNA synthetic response to mercury peaks on the sixth day of culture as does the response to antigens (Caron et al., 1970). In contrast, the response to plant mitogens peaks two to three days earlier.

## 2. MATERIALS AND METHODS

### 2.1 Techniques for Lymphocyte Culture

#### 2.1.1 Peripheral Blood Lymphocytes

Random bred adult cats (Felis catus) served as blood donors. Feline lymphocytes were separated from blood drawn from different anatomical sites depending upon the blood volume required to yield the number of cells desired (Table 2.1.1.). If a large blood volume was desired the cat was killed by exsanguination under nembutal or CO<sub>2</sub> anesthesia.

When human lymphocytes were required for comparative purposes, blood was obtained from the peripheral anticubital vein and processed in the same manner as cat blood.

Table 2.1.1.

#### COLLECTION OF BLOOD FROM CATS

<u>Blood Volume (ml)</u>	<u>Anesthetic</u>	<u>Venipuncture Site</u>
1-5	none	cephalic or saphenous ve
5-20	nembutal	cardiac puncture
20-200	nembutal or CO <sub>2</sub>	posterior vena cava

#### 2.1.2 Defibrination

The blood was collected into a syringe without anticoagulant, quickly transferred to an Erlenmeyer flask containing glass beads (approx. 5 mm diameter) and defibrinated by rotating either by hand or on a New Brunswick rotary shaker for 20 minutes. Clotting of the blood during the defibrination procedure was a hazard to be avoided since lymphocytes tended to adhere to or be trapped

within the clot. To minimize clotting it was necessary to use a flask of sufficient volume with enough glass beads, rotated with vigor to quickly remove the fibrin. For example 5 ml of blood would be defibrinated in a 20 ml flask with 5 beads whereas 50 ml of blood required a 250 ml flask and 30 beads. Furthermore when successive syringes full of blood were taken from the same animal (eg. for complete exsanguination), it was found necessary to put each in a separate defibrinating flask otherwise the risk of clotting was greatly enhanced.

#### 2.1.3 Lymphocyte Separation

Lymphocytes were separated from whole blood by the method of Coulson and Chalmers (1964). Defibrinated blood was mixed with a 3% weight/volume solution of gelatin in physiological saline (three volumes of blood to one volume of gelatin solution). The mixture was allowed to stand in a graduated cylinder for 1/2 hour at 37°C. At this time the clear supernatant contained primarily lymphocytes. The lymphocytes were washed twice with PBS.

#### 2.1.4 Lymphocytes from Other Sources

Lymphocytes were prepared from mesenteric lymph nodes, spleen and thymus by removing the tissue aseptically, mincing in PBS and washing to eliminate red cells.

#### 2.1.5 Cell Counting

0.1 ml of cells in PBS was added to 1 ml of neutral red solution (.08%). A drop of the mixture was examined in a hemocytometer under the microscope. Viable

cells (stained) were readily distinguished from dead cells (unstained), as were lymphocytes from neutrophils.

#### 2.1.6 Culture Conditions and Medium

Cells were grown in liquid tissue culture medium HB597 (Connaught Medical Research Laboratories, Toronto) containing fetal calf serum (#614, Grand Island Biological Company, New York) which had been heat inactivated at 56°C for 30 minutes. For most experiments the medium was supplemented with 10% serum. For uniformity and sterility disposable plastic culture vessels were used. 0.2 ml cultures were set up in each of the wells of the 96 well microplate (#3040, Falcon Plastics, Oxnard, California) and covered with the solid lid (#3041, Falcon Plastics). Cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37.5°C. The usual culture duration was 72 hours.

#### 2.1.7 Mitogens

The mitogens were reconstituted according to the directions of the supplier (Table 2.1.2) and stored frozen in small aliquots. Unless otherwise stated, the final concentrations of mitogens in culture were PHA  $5 \times 10^{-3}$  ml/ml, PWM  $10^{-2}$  ml/ml and Con A 20ug/ml.

Table 2, 1.2Source of Mitogens

<u>Mitogen</u>	<u>Catalogue No.</u>	<u>Supplier</u>
Phytohemagglutinin M	0528-57	Difco Labs, Detroit, Michigan
Pokeweed Mitogen	536	Grand Island Biological Co., New York
Concanavalin A	234567	Calbiochem, La Jolla, California

2.1.8 Prevention of Contamination

To reduce the risk of contamination of cultures by micro-organisms, normal precautions were taken. All manipulations were carried out in a laminar flow hood (Conn. Environmental R & D Corp., New Britain, Conn.). Liquids were sterilized by autoclaving or by filtration (type HA, pore size .45u, Millipore Ltd., Montreal). In addition the culture medium contained 200 ug/ml streptomycin, 10 ug/ml neomycin and 40 units/ml polymyxin.

2.1 Methods with Radioactive Tracers2.2.1 Measurement of Incorporation of  $^3\text{H}$ -TdR into DNA by Scintillation Counting

The in vitro response of lymphocytes to mitogens was assessed by means of tritiated thymidine ( $^3\text{H}$ -TdR) incorporation into newly-formed DNA followed by liquid scintillation counting. Cells were incubated with 1 uCi/ml thymidine  $\alpha$ -methyl- $^3\text{H}$  (20 Ci/m mole, NET-027X, New England Nuclear, Boston, Massachusetts) for 3 to 5 hours.

Following the isotope incorporation, cultures were harvested onto glass fiber filters (grade 934AH, Reeve Angle, Clifton, New Jersey) by a semi-automatic multiple sample precipitator (Otto Hiller, Madison, Wisconsin). The use of this machine is described in detail by Hartzman et al (1972). Each filter was placed in a scintillation vial (Minivials, Picker Nuclear, Montreal, Quebec) to which was added 3 ml of scintillation fluid (Aquasol, NEF-934 or Econofluor, NEF-941, New England Nuclear). The vials were counted in a liquid scintillation spectrophotometer (LS-233, Beckman Instruments Inc., Fullerton, California) equipped with plexiglass holders (Picker Nuclear) to accommodate the minivials.

#### 2.2.2 Expression of Data

There are several modes of expression for LSC data. Raw CPM can be converted to DPM and/or the square root transformation can be used since the Poisson distribution describes the random decay of radioactive atoms. Data from replicate samples are reduced to a mean and an indication of variability. Different treatment groups with their respective controls can be compared by 1) presenting both treatment and control data, 2) subtracting control CPM from treated group CPM, or 3) taking the treated group CPM as a ratio of the control CPM. Since there appears to be no consensus as to which method of data expression is most valid (Schechter and McFarland, 1972) and since certain manipulations have been shown to lead to incorrect conclusions, (Daniels et al., 1970) it seems preferable to present both control and treatment data.

Data in CPM were converted to their square roots to equalize the variance (Bach et al., 1970).

Analyses of variance were conducted on several sets of square root transformed data. The error mean square was used to compute the standard error of a difference between means. Means were compared by the least significant difference (lsd) method which is basically a Student's t test using a pooled error variance (Steel and Torrie, 1960).

## 2.3 Insoluble PHA

### 2.3.1 Coupling of PHA to agarose beads

PHA was coupled to agarose beads using methods similar to those of Porath et al. (1967) and Greaves and Bauminger (1972). One gram of cyanogen bromide activated Sepharose 4B (#9787 Pharmacia, Uppsala) was swollen and washed for 15 minutes on a glass filter with 250 ml  $10^{-3}M$  HCl. 82 mg of PHA in 20 ml 0.1M NaHCO<sub>3</sub> buffer, pH 8.5, containing 0.5M NaCl was mixed with the gel for 2 hours at room temperature. Unbound PHA was washed away with the coupling buffer and remaining active groups were allowed to react with 20 ml 1M ethanolamine at pH 8 for 2 hours. After removal of the ethanolamine, the gel was washed twice alternately with acetate buffer (0.1M, pH 4, containing 1M NaCl) and borate buffer (0.1M, pH 8, containing 1M NaCl). Finally the gel was washed with sterile medium HB 597, resuspended in 20 ml HB 597 and stored at 4°C.

Control beads were prepared similarly without PHA. The final products proved to be free of microbial contamination.

### 2.3.2 Attachment of lymphocytes to beads

10<sup>6</sup> lymphocytes in 1 ml HB 597 containing 10% fetal calf serum were mixed with 0.5 ml of Sepharose beads and incubated for 90 minutes. The mixture was then layered onto a 5 ml column of HB 597 containing 10% serum in a test tube. In 5 to 10 minutes the beads settled to the bottom leaving most of the unbound cells near the top. The beads were examined by light microscopy for the presence of attached cells.

### 2.3.3 Response of lymphocytes to insoluble PHA

Lymphocytes were cultured with several concentrations of Sepharose beads and Sepharose-PHA to assess the mitogenicity of insoluble PHA. The actual quantity of PHA (free and bound) in these preparations was unknown.

### 2.4 Ficoll-Hypaque (F-H) separation

Gelatin-sedimented lymphocytes were further purified on Ficoll-Hypaque gradients. Ficoll, a nonionic synthetic polymer of sucrose, MW=400,000, was obtained from Sigma Chemical Co., St. Louis, Mo. Nine percent aqueous Ficoll was mixed with 34% Hypaque (50% sodium diatrizoate, Winthrop Labs, Aurora, Ont.) in a ratio of 4 volumes Ficoll to 1 volume Hypaque. The specific gravity, as measured with a hydrometer (#S41885G, and F Sargent Welch, Montreal, range 1.060-1.130 and 1.000-1.070), was about 1.075. Mixtures with specific gravities ranging from 1.055 to 1.095 in 0.005 increments were prepared by varying the F-H ratio or by adding water. The solutions were sterilized by filtration, stored at 4°C and used within a month of preparation.

The gradient was prepared by gently layering 1.5 ml F-H, specific gravity 1.055, on top of an equal volume of F-H of specific gravity 1.095 in a 16x125mm tube. About  $10^7$  gelatin-sedimented lymphocytes in 0.6ml medium without serum were carefully layered on top. The gradient was centrifuged for 40 minutes at 400g. This procedure was adopted after experimenting briefly with a number of gradients of continuous and discontinuous densities. It was modified from that described by Thurman et al. (1973).

### 3. RESULTS

#### 3.1 Response of peripheral blood lymphocytes to mitogens

The response (measured by  $^3\text{H}$ -TdR incorporation) to a range of concentrations of mitogens, cells and serum was measured in order to determine optimal culture conditions. Within the framework of these experiments, optimal culture conditions were defined as those conditions consistent with maximal  $^3\text{H}$ -TdR incorporation by 3 day old cultures. Since the dose-response relationships investigated here yielded more or less bell-shaped graphs, the optimal conditions as defined above were also those conditions at which minor technical inaccuracies were least likely to influence the response.

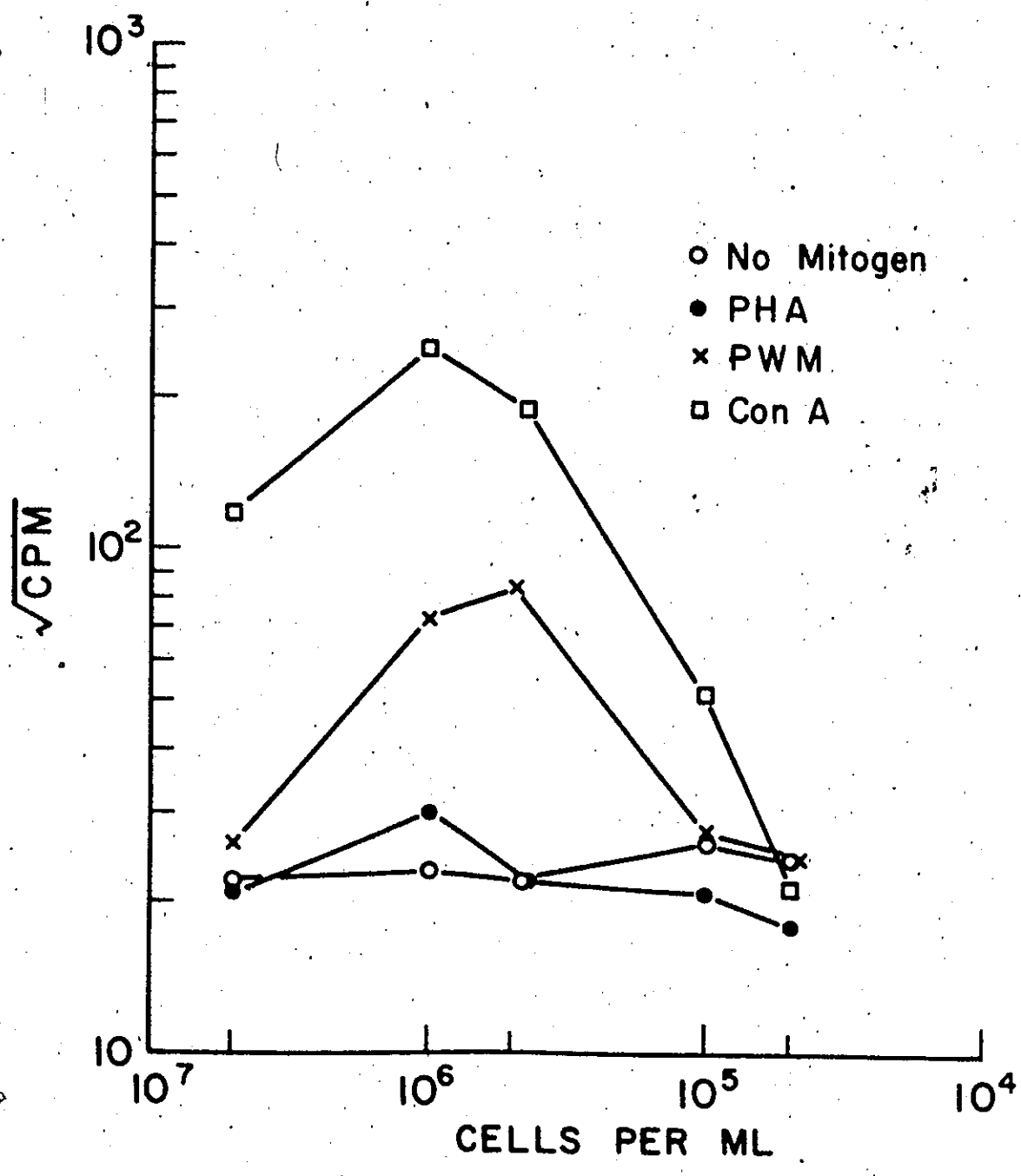
##### 3.1.1 Cell Dose Response

If the lymphocyte concentration is varied while the mitogen and serum concentrations are held constant it is found that a minimal concentration of cells ( $10^4$ - $10^5$ /ml) is required in order to initiate a response. It has been suggested that at low cell concentrations the time course of the response is delayed so that the peak occurs later (Melief et al., 1973). For the cat as well as for most species a cell concentration of about  $10^6$  lymphocytes/ml gives an optimal response to mitogens at three days (Fig. 3.1.1). Higher cell concentrations limit the response presumably because of rapid nutrient depletion, exhaustion of the buffering capacity and pollution of the medium with cell wastes.

##### 3.1.2 Mitogen Dose Response

In the absence of mitogen, lymphocytes incorporate very little  $^3\text{H}$ -thymidine. As the mitogen concentration

Fig: 3.1.1. Cell dose response. The optimal response of blood lymphocytes occurred near  $10^6$  cells/ml.

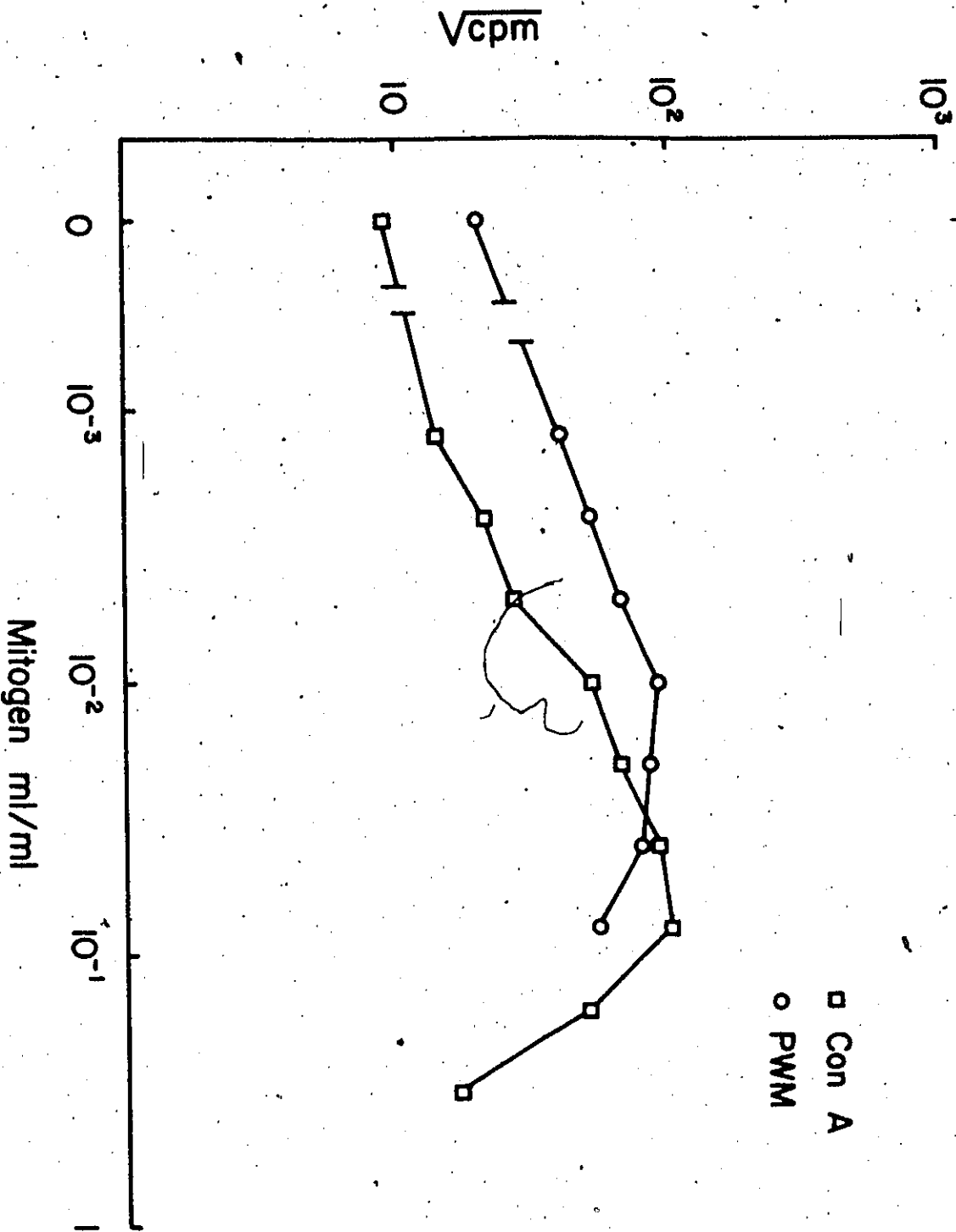


increases more and more cells respond. As discussed previously (Interaction of Mitogens and Cells), either critical binding sites must be saturated or the total membrane mitogen binding must reach a certain level before the cell is stimulated to transform and synthesize DNA. At high concentrations of mitogen the response is abolished as a result of cytotoxicity (Milthorpe and Forsdyke, 1970) or suppression without cell death (Andersson et al., 1972).

The available commercial preparations of PHA, PWM and Con A differ in purity and activity partly because the compounds have not been fully characterized (Uchida, 1972). Thus comparison of quantitative data between laboratories using different mitogen preparations and culture systems is fraught with difficulty. Nevertheless, the optimal Con A and PWM concentrations found for feline lymphocytes under the present culture conditions (Fig. 3.1.2) are comparable to those reported for other species and other culture situations. On the other hand, feline peripheral blood lymphocytes consistently responded either poorly or not at all to PHA under a number of experimental conditions.

The poor response of cat peripheral blood lymphocytes to PHA is an unusual finding. However it is interesting to note that McIntyre and Segel (1966) reported that PHA did not stimulate human thymus cells, and several years thereafter confessed that their results were due to the failure to consider the dose response curve to PHA. The importance of constructing a mitogen dose response curve when dealing with refractory cells was further emphasized by Ron et al. (1973) who found that lymphocytes

Fig. 3.1.2. Mitogen dose response. Cat blood lymphocytes were cultured in the presence of different concentrations of Con A or PWM. The Con A stock solution contained 1 mg/ml. PWM was reconstituted according to the manufacturer's instructions.



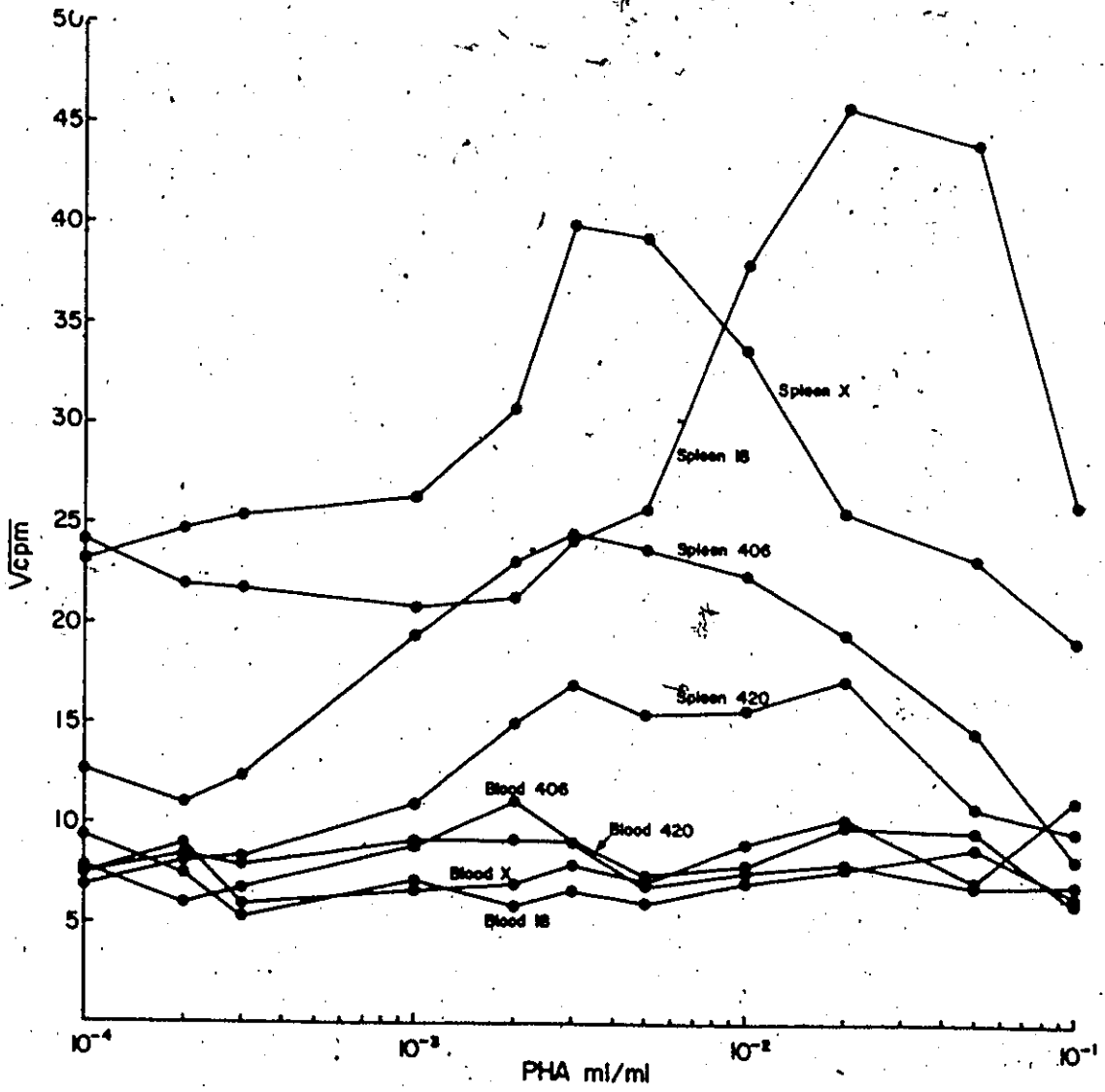
from hamsters required 10 to 50 times more PHA for optimal stimulation than cells from other rodents. In their hands the dose responses to Con A and PWM were similar in hamsters, guinea pigs, rats and mice. In addition, a genetically controlled intra-specific difference in optimal Con A concentration has been reported between Lewis and Brown Norway rats (Williams et al., 1973).

Several PHA dose response curves with cat lymphocytes failed to elicit a large PHA response from peripheral blood cells although splenic lymphocytes from the same individuals gave good responses at PHA concentrations effective for other species (Fig. 3.1.3). Some variation between cats was noted in the PHA concentration for optimal stimulation of splenic lymphocytes.

### 3.1.3 Serum Dose Response

Protein-free chemically defined synthetic media are of great use in analyzing the effects of certain substances on cells, especially in cases where the substances may first interact with serum proteins in the media. Although many studies have been carried out, a limited number of kinds of cells have been grown in protein-free media (Katsuta and Takaoka, 1973). Defined tissue culture media is usually supplemented with serum as a source of lipids, vitamins, hormones, trace metals, peptides, etc., not otherwise provided for (Waymouth, 1972; Temin et al., 1972). The routine supplement, fetal calf serum, is high in  $K^+$  (16 mM) which may be a contributing

Fig. 3.1.3. PHA dose response. The response of blood and splenic lymphocytes to a range of concentrations of PHA was assayed in 4 cats (#406, 420, 18 and X). Blood lymphocytes responded poorly to concentrations of PHA which stimulated spleen cells. Splenic lymphocytes from different cats exhibit maximal responses at different PHA concentrations.



factor in its effectiveness in media (Scott and Fisher, 1970). Media intended for short term cultivation can be less complete than those for long term studies since the absence of certain elements may not produce significant metabolic or growth deficiencies for several weeks.

The origin of serum is an important consideration. Claims have been made for the excellence of autologous, homologous and heterologous sera. Since Bilz *et al.* (1961) indicated that pig serum supported mitogen-induced transformation of feline lymphocytes, various concentrations of pig, cat (homologous), horse and fetal calf sera were tested. Fetal calf serum proved superior in terms of cell response, convenience and expense.

For lymphocyte culture as well as for most other tissue culture applications serum constitutes 5 to 20% of the medium. The optimal response to mitogens is reported to occur at a wide range of serum concentrations. The results for feline lymphocytes are not exceptional (Figs. 3.1.4 and 5; 3.3.3-5). The stimulation of lymphocytes in "serum-free" media observed with feline lymphocytes and with those of other species is intriguing (Forsdyke, 1973; Novogrodsky and Katchalski, 1971). Such observations have been questioned on two counts: (1) the cells might not have been adequately washed free of serum components, and (2) the cell response was assessed after several days in culture during which time serum components or substitute molecules could be secreted by live cells or released from degenerating cells (Forsdyke, 1973).

Fig. 3.1.4. Serum dose response. Cat blood lymphocytes were cultured in different concentrations of fetal calf serum. Note the response to PWM in serum-free medium.

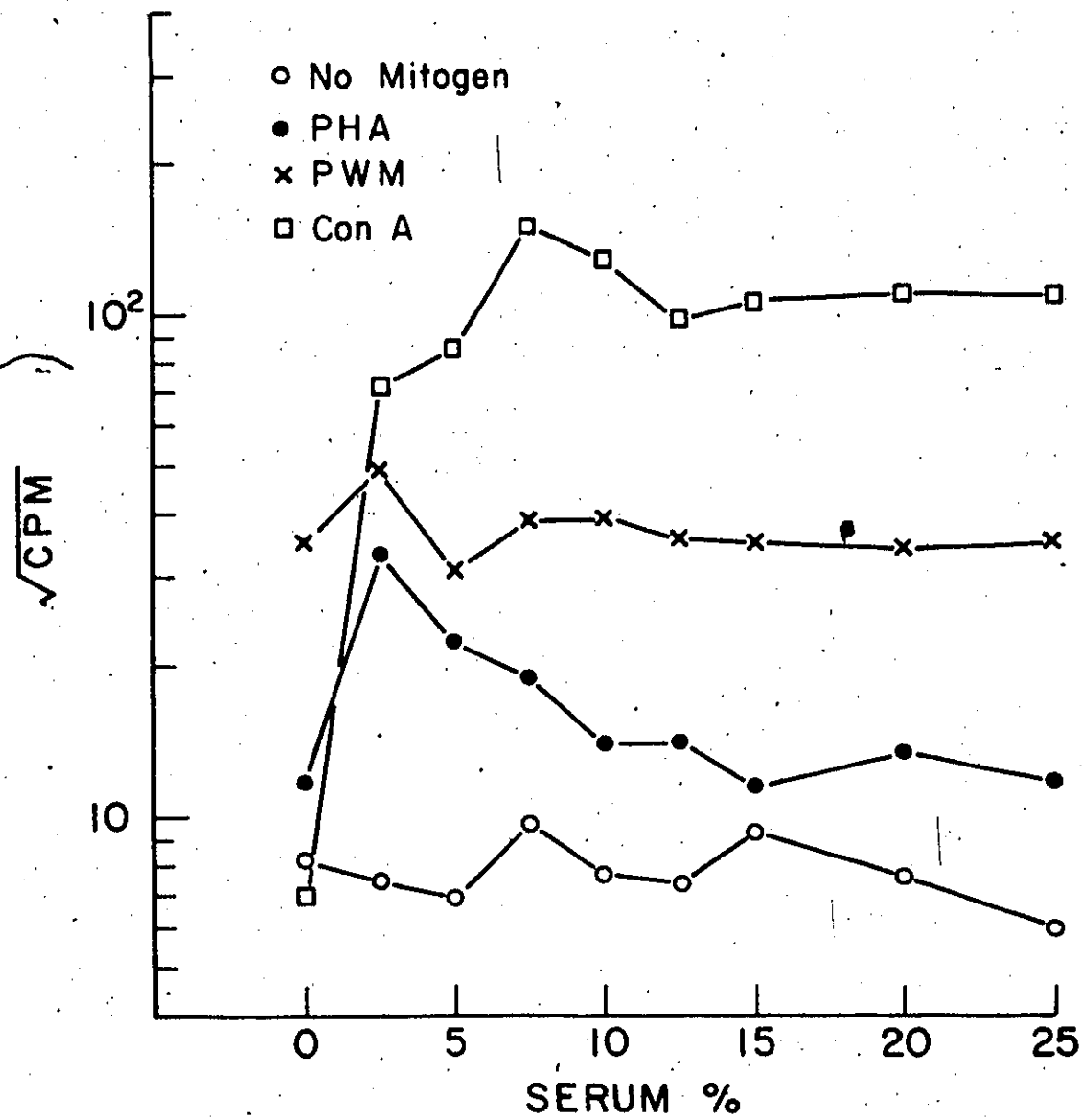
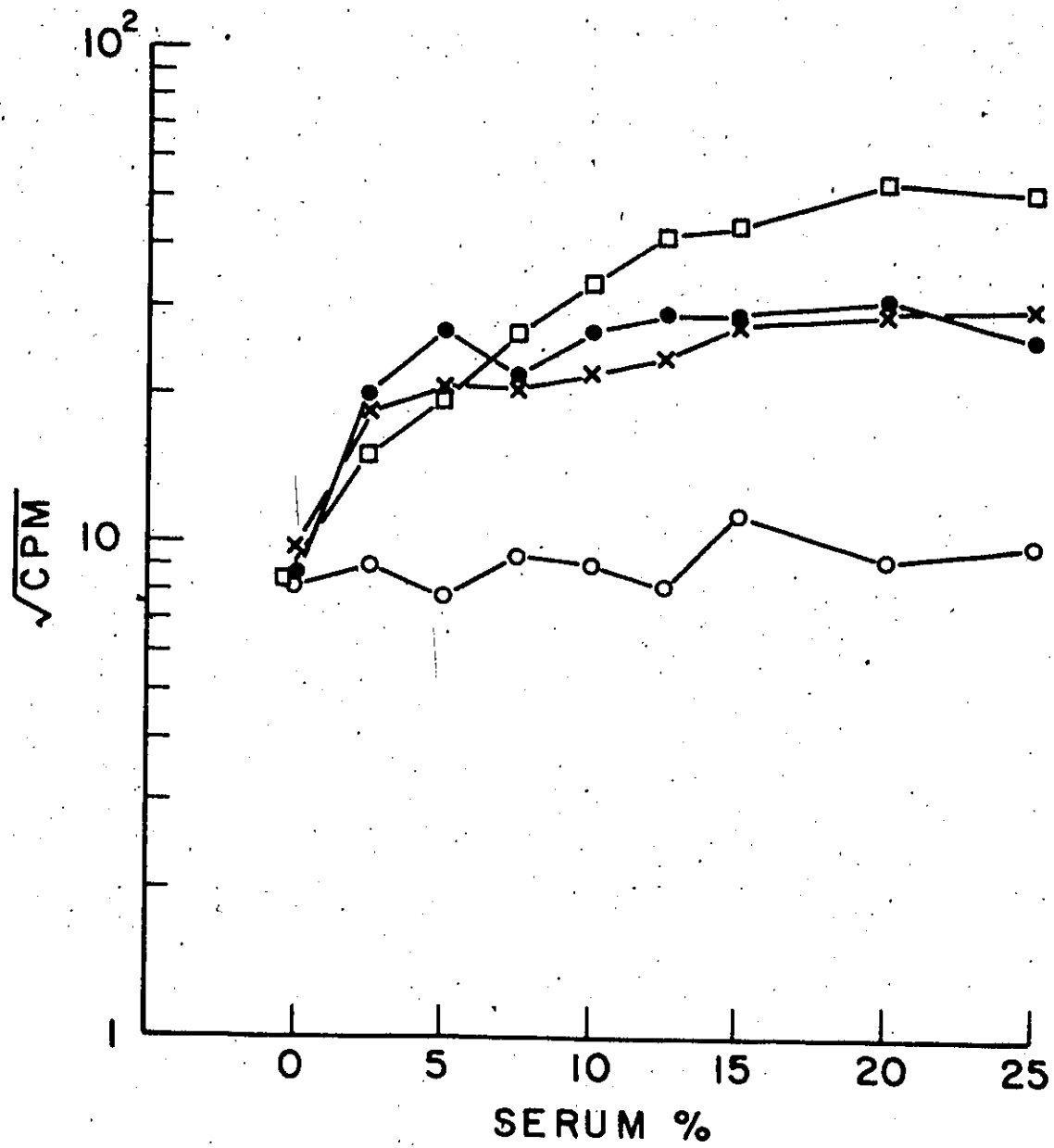


Fig. 3.1.5. Serum dose response. Splenic lymphocytes from the same cat as in Fig. 3.1.2. were cultured in different concentrations of fetal calf serum. In this case there was no response in serum-free medium.



Two substances inhibiting mitogenesis have been identified in serum. First, serum complement has a detrimental effect on cultured cells which can be avoided simply by routinely heating serum at 56°C for 30 minutes (Milthorpe and Forsdyke, 1970). Second, carbohydrate-containing -glycoproteins bind mitogens thus reducing the amount of mitogen available to stimulate cells (Chase, 1972). The latter circumstance suggests the existence of an optimal mitogen/serum ratio which may be derived from a range of absolute concentrations of mitogen and serum. The work of Forsdyke (1973) supports this theory.

#### 3.1.4 Time Course

The time course of response of lymphocytes to mitogens, as measured by <sup>3</sup>H-TdR incorporation, varies with the mitogen used. For instance, cultures treated with PHA or Con A usually show maximum response on day 3 or 4 while PWM is often reported to induce a peak response a day or so later. On the other hand, the response to antigens peaks on day 5 to seven. Why cultures treated with different mitogens might require varying periods of time before showing maximum <sup>3</sup>H-TdR incorporation is not definitely known. Although inherent qualities of different responding cell populations may be a factor, consideration must be given to the actual number of responding cells and the culture conditions. Melief et al. (1973) presented data indicating that diluted lymphocytes responded to PHA with a delayed time course. In another study however, a delayed response to PWM, peaking on day 7, was merely ascribed to

culture methodology, the mitogen preparation or possibly related to the blood donor (Wu et al., 1973).

The presence of cells other than lymphocytes in the culture milieu is also reported to affect the time course. Gajl-Peczalska et al. (1969) compared the time course of the response of purified and non-purified cell cultures to PWM. They found that the removal of macrophages and neutrophils shifted the peak response from day 3 to day six. The magnitudes of the day 3 impure and day 6 pure culture responses were similar.

Regardless of the identity of the major contributing factors to the course of the response with time, it is apparent that a time course study is indicated especially when lymphocytes fail to respond under routine conditions.

Cat blood lymphocytes responded best to Con A and PWM on days 3 and 4 of culture. The PHA response did not improve with longer culture times (Fig. 3.1.6).

### 3.2 Organ Differences

Although the bulk of the present work concerned cat peripheral blood lymphocytes, cells from other lymphoid organs were used occasionally. The responses of cells from blood, spleen, lymph node and thymus of three cats are shown in Figure 3.2.1. In spite of considerable inter-individual variability, several trends emerged. Splenic lymphocytes were generally responsive to PHA (see also Fig. 3.1.3). Cells from mesenteric lymph nodes responded inconsistently to all mitogens. Thymic lymphocytes usually responded poorly to all mitogens. The serum

Fig. 3.1.6 Time course of response to mitogens. Cat blood lymphocytes responded maximally to mitogens on days 3 and 4 of culture. No mitogen o; PHA ●; PWM x; Con A □.

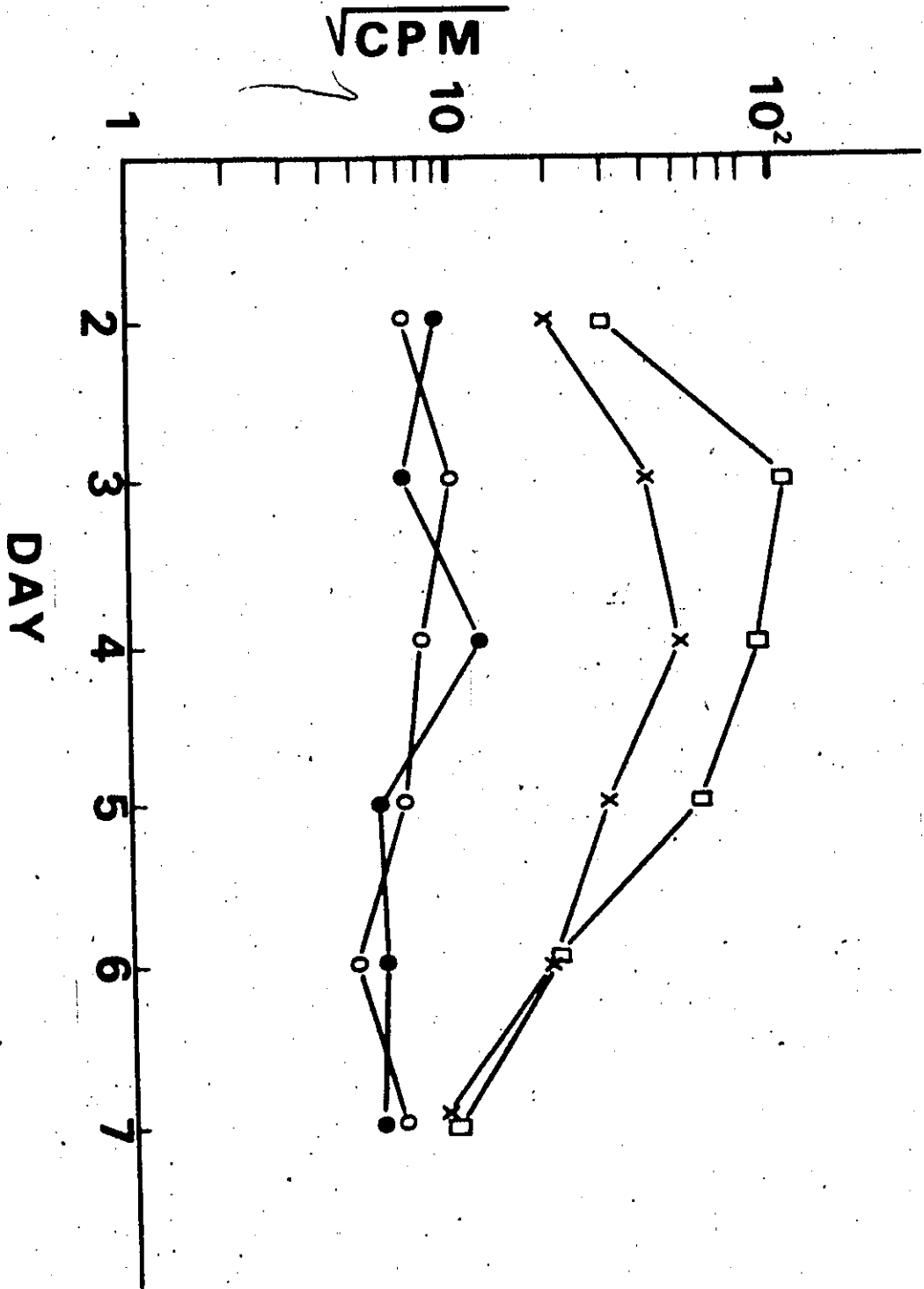
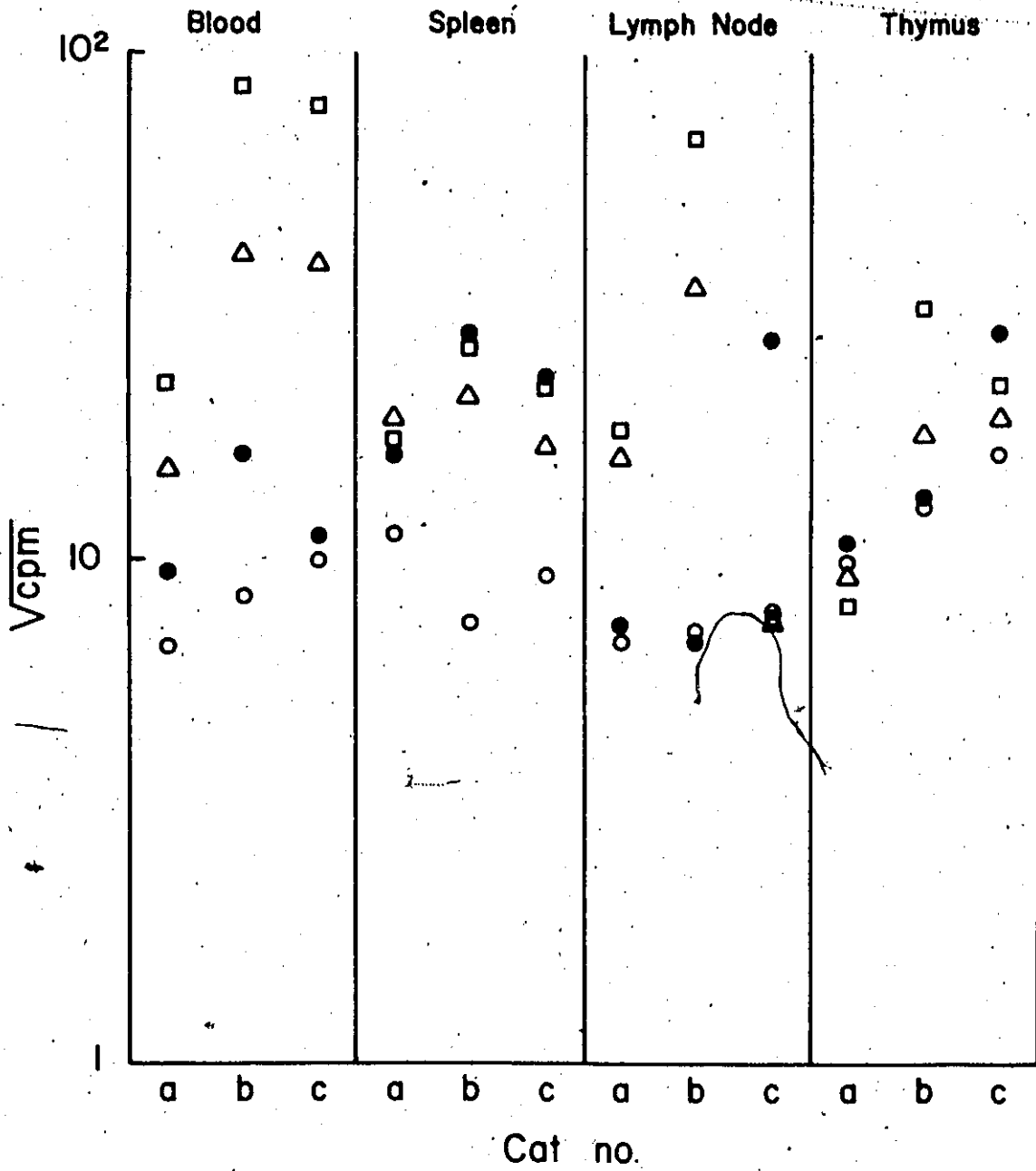


Fig. 3.2.1. Organ differences. Lymphocytes from cat blood, spleen, lymph node and thymus were cultured in 10% fetal calf serum at a density of  $10^6$  cells/ml.

- No Mitogen
- PHA
- △ PWM
- Con A



dependence of the response of lymphocytes from different organs is shown in Figure 3.2.2a, b, c.

The most striking and consistent observation from experiments with central lymphoid organs is the superior response to PHA of splenic cells compared to peripheral blood lymphocytes. A similar pattern of response to PHA has been reported for the rabbit (Mansfield and Wallace, 1973; Sell and Sheppard, 1973), but the converse appears to hold for mice (Fowler *et al.*, 1971).

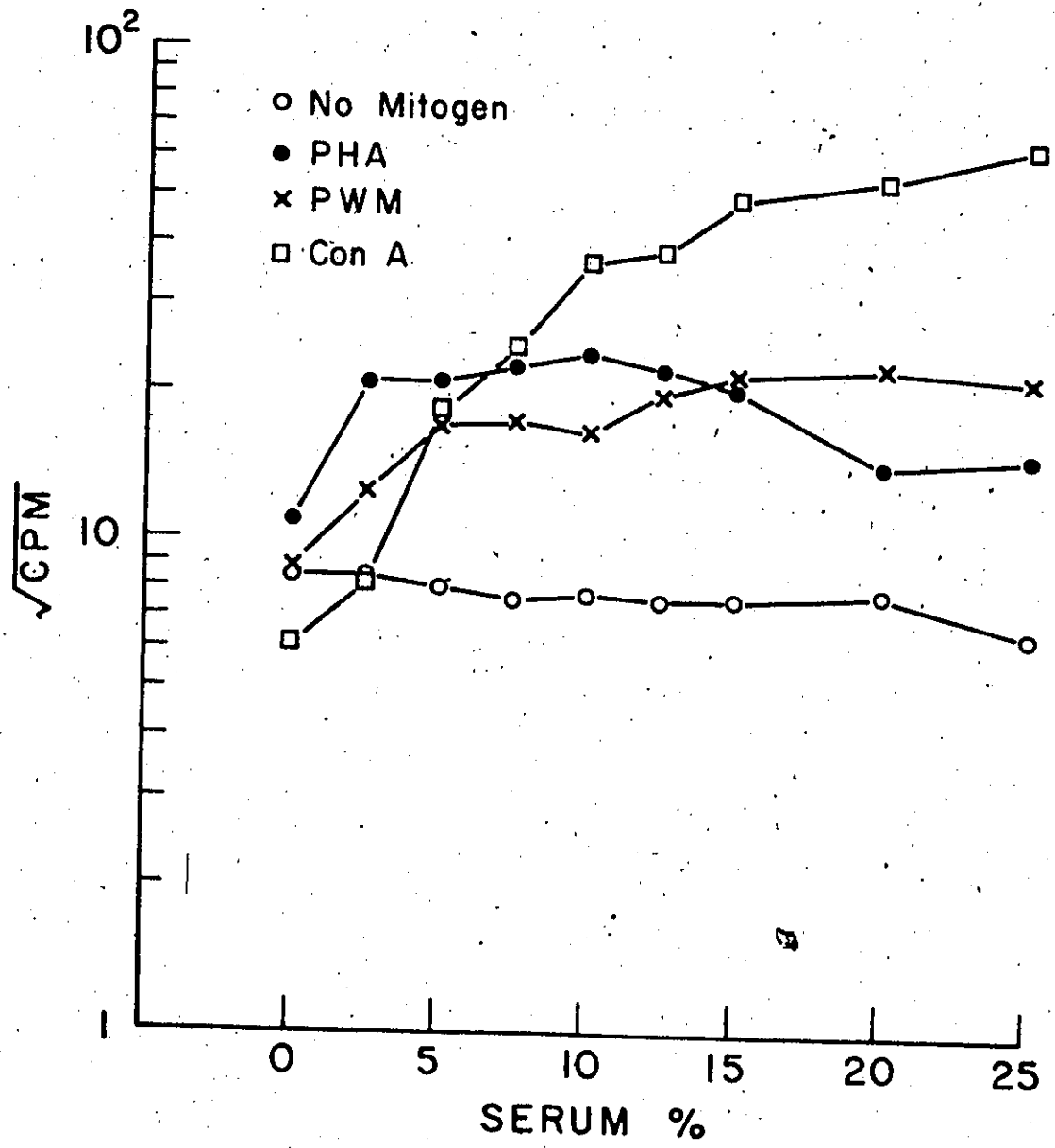
### 3.3 Inter- and Intra-individual Variation

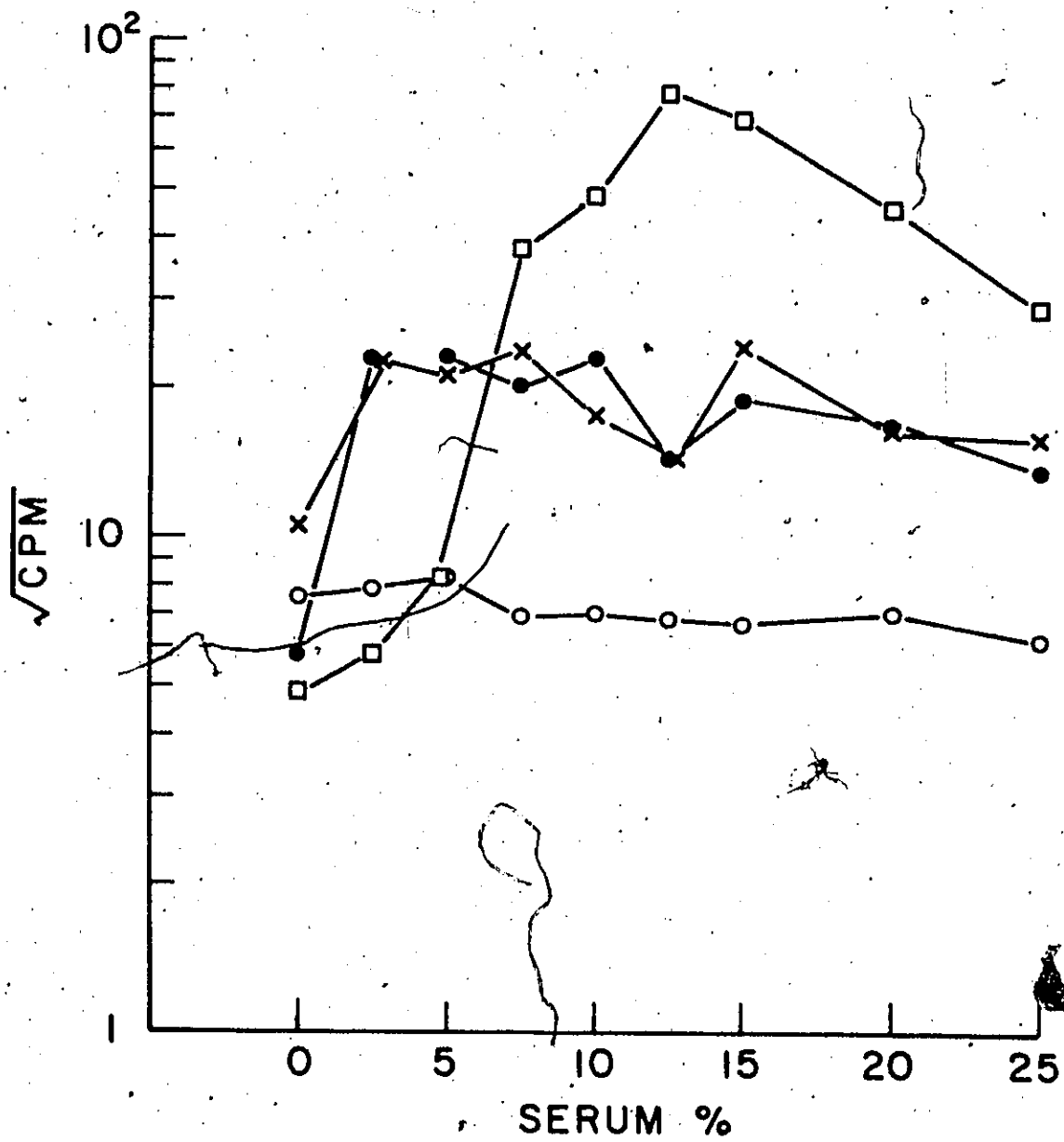
Variation in mitogen-induced thymidine uptake by lymphocytes was examined at three levels: 1) among different individuals, 2) among replicate cultures established at the same time from one individual and 3) among cultures initiated from the same individual at different times. The responses of 24 cats assayed in a single experiment (Figure 3.3.1) allow several generalizations. The magnitude of the response to phyto mitogens was, in descending order, Con A > PWM > PHA. All cats responded to Con A and PWM. The response to PHA varied from moderate to nil. Thus, although the responses to Con A and PWM showed considerable quantitative variation, the responses to PHA varied qualitatively as well. The data from Figure 3.3.1, summarized in Figure 3.3.2, reveal considerable inter-individual variation in mitogen-induced lymphocyte thymidine uptake.

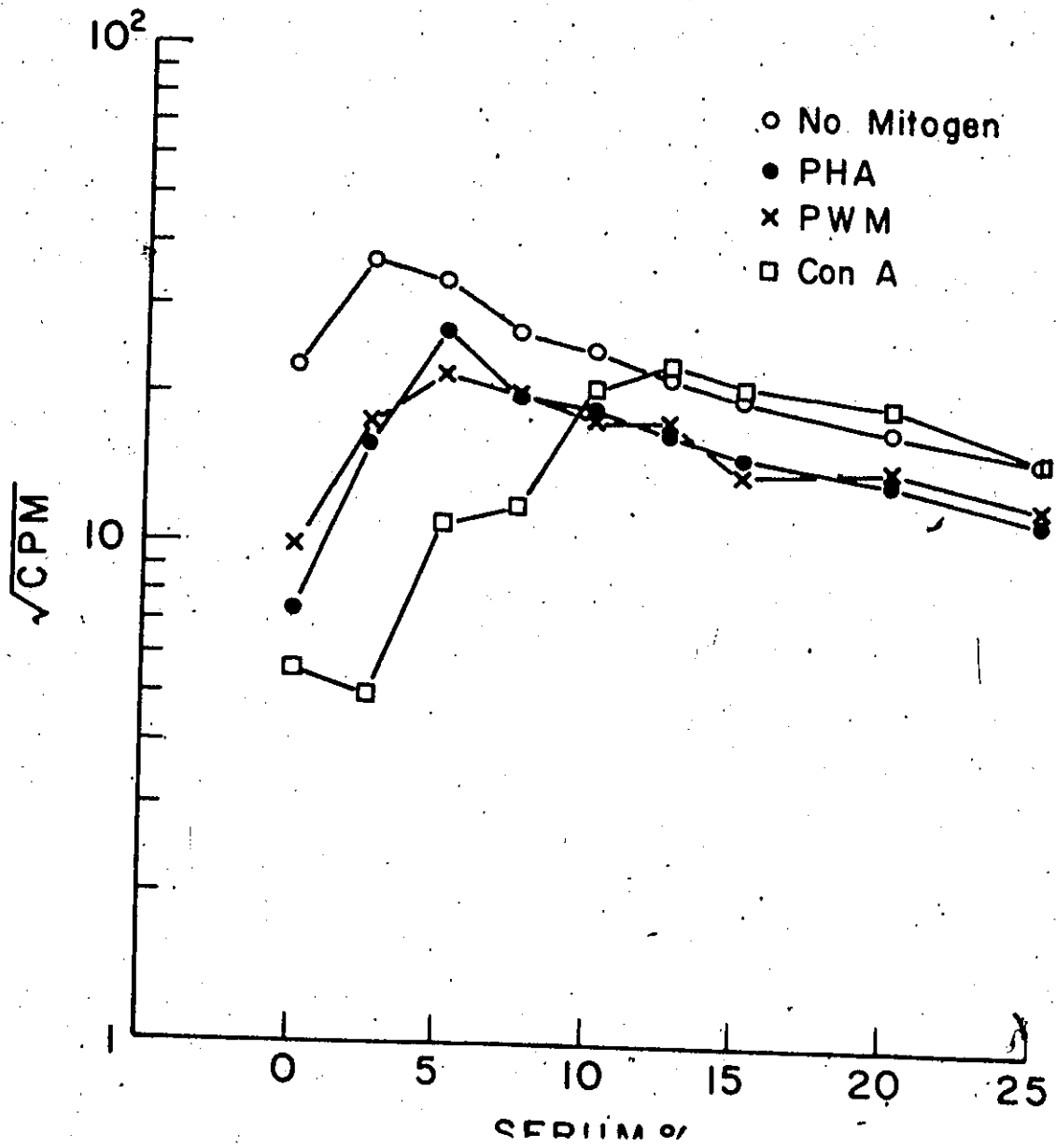
The variation among replicate cultures established at the same time from one individual was usually negligible.

Others have reported major variation in repeated experiments using cells from the same donor (Hagen and Froland, 1973; Hinz and Chickosky, 1972). To determine the extent of variation in mitogen responses of cats three individuals were

Fig. 3.2.2a,b,c. Organ differences in serum requirements. Cat lymphocytes from spleen (a), lymph node (b) and thymus (c) were grown in medium with different concentrations of calf serum. Splenic and lymph node lymphocytes were maximally stimulated by PHA and PWM at 5% or greater serum concentrations. The response to Con A required more serum and the shape of the dose response curve differed between the organs. The response of thymic cells was masked by a high background.



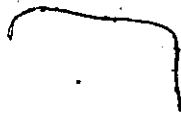




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Fig. 3.3.1. Inter-individual variation. The raw data are shown from an experiment in which the phyto-mitogen responses of 24 cats were determined. Although considerable variation is evident, cat lymphocytes consistently responded poorly to PHA, better to PWM and best to Con A. No mitogen o; PHA ●; PWM x; Con A □. The response to PHA of cats #4, 7, 14-18 and 20 is significant at the 5% level.

3



CPM

$10^2$   $10^3$   $10^4$   $10^5$

2 4 6 8 10 12 14 16 18 20 22 24

CAT NO.

MEDIUM  
CONTROLS

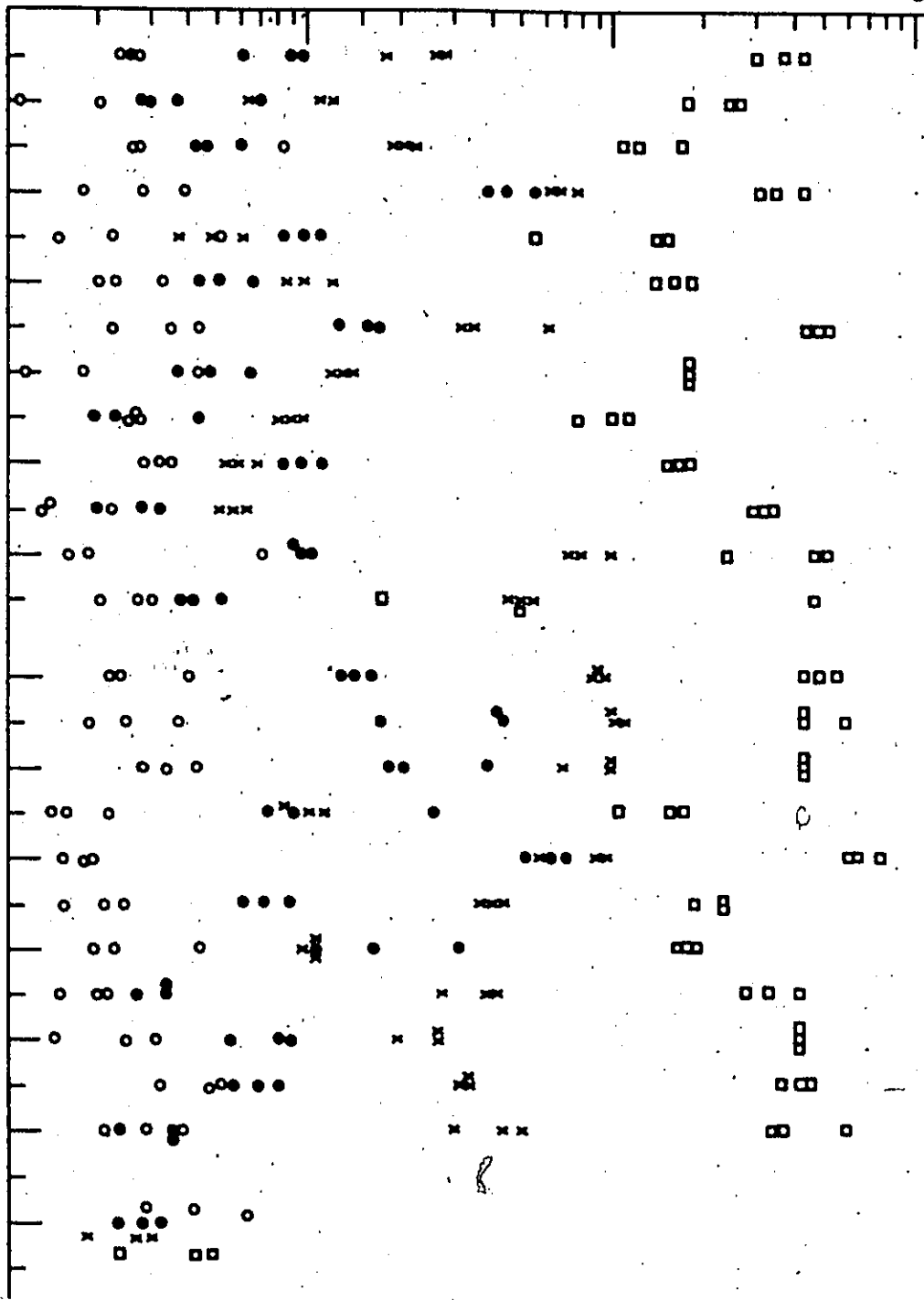
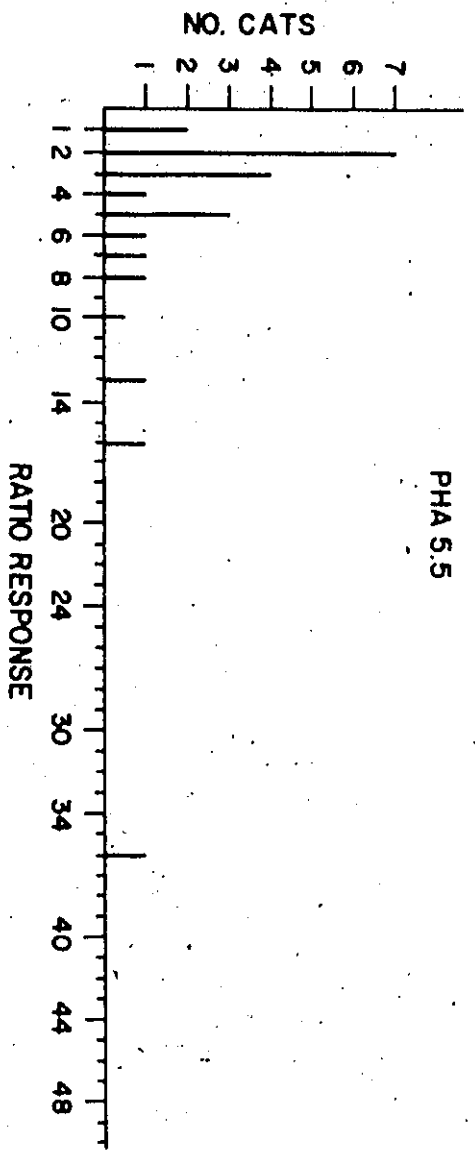
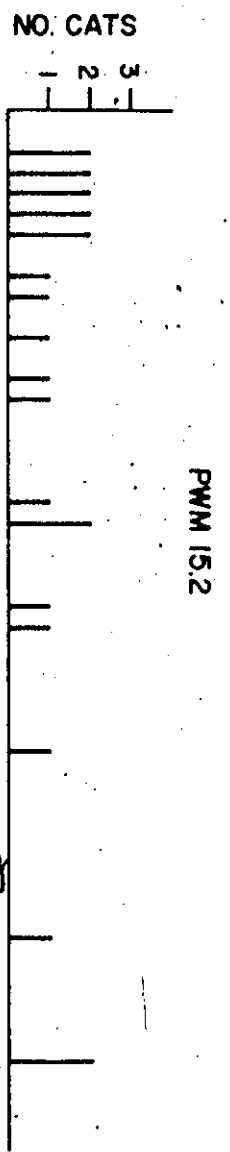
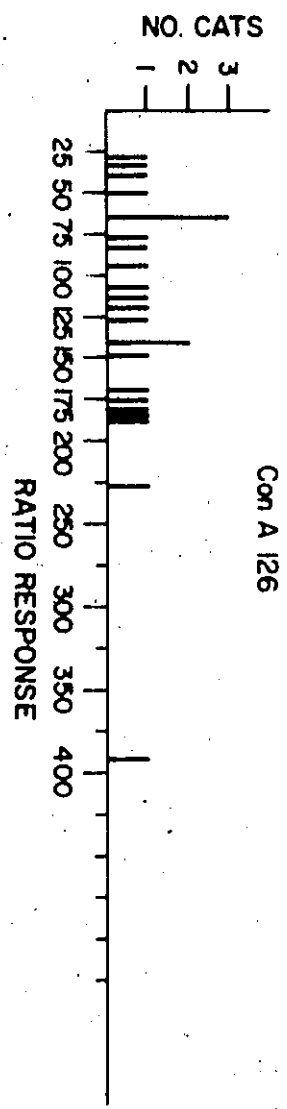


Fig. 3.2.2. Inter-individual variation. The data in Fig. 3.3.1. are expressed by the ratio method. The mean of the response to each mitogen are shown.



tested 6 times at three serum concentrations (Figures 3.3.3-5). The time intervals between bleedings are given in Table 3.3.1. The greatest variation occurred in serum-free medium, suggesting that some plasma components indigenous to the donor were not completely removed from the lymphocytes during the preparatory washing procedures. The same explanation was offered by Fowler et al. (1971) to explain the marked variation of the influence of serum concentration on PHA stimulation between individual mice of an inbred strain.

Table 3.3.1

Time Intervals Between Bleedings

<u>Bleeding interval</u>	<u>Cat Number</u>		
	<u>6</u>	<u>18</u>	<u>406</u>
A-B	14 days	14d	11d
B-C	24d	24d	25d
C-D	6 months	6m	6m
D-E	39d	39d	39d
E-F	27d	59d	42d

3.4 Effects of Miscellaneous Treatments

The characterization of feline lymphocytes was extended to include a comparison among several species of the effects of a variety of agents. In general the results obtained here parallel those published for other species.

3.4.1 Quabain

Mitogen induced lymphocyte transformation was completely inhibited by ouabain at  $3.5 \times 10^{-9}M$  and unaffected by concentrations lower than  $3.5 \times 10^{-9}M$ . The

Fig. 3.3.3. Intra-individual variation. Cat #406 was bled on 6 occasions. Each time the response to mitogens was assayed in 3 concentrations of fetal calf serum. No mitogen o; PHA ●; PWM x; Con A □. None of the responses to PHA are significant at the 5% level.

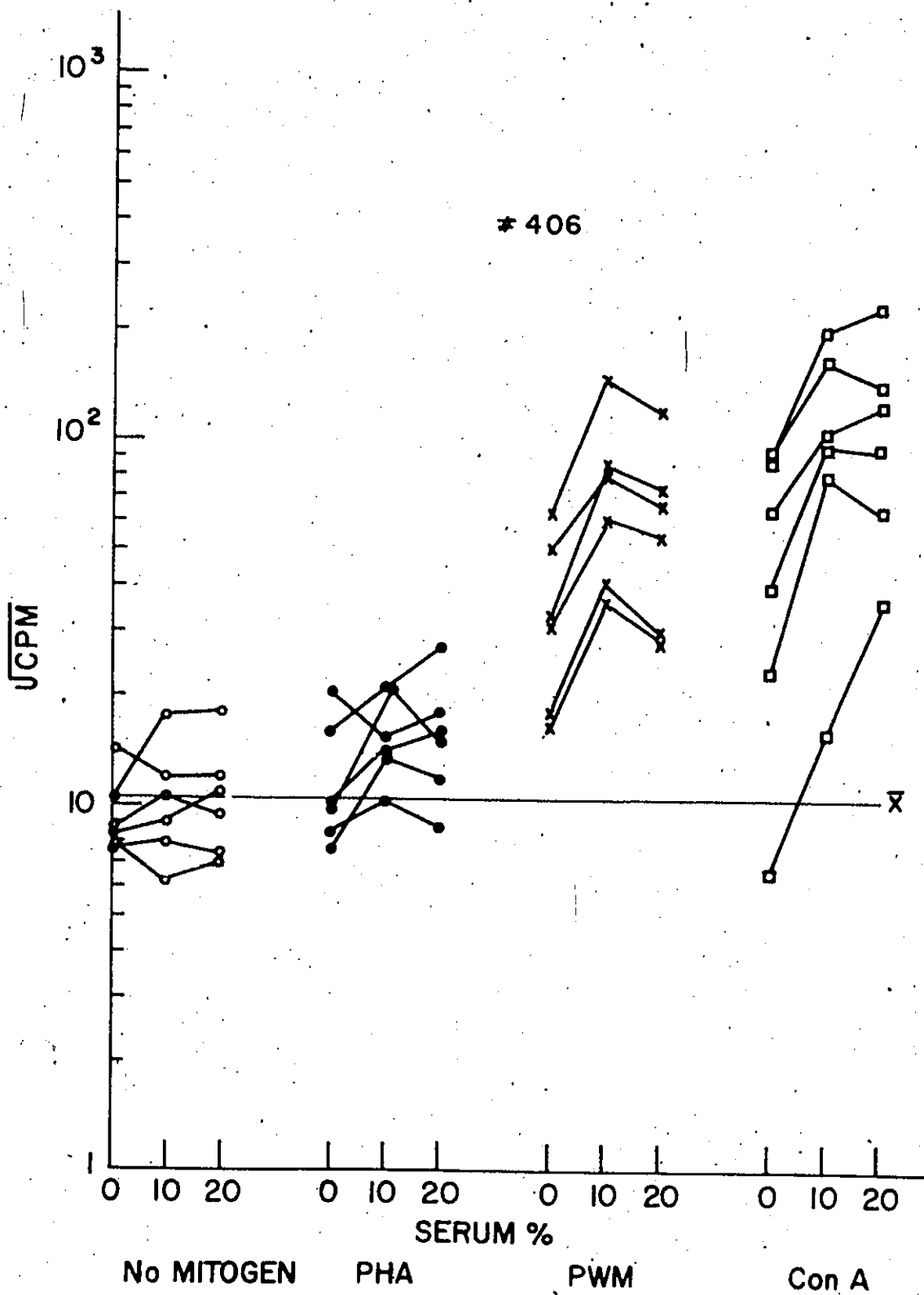


Fig. 3.3.4. Intra-individual variation. Cat #18 was bled on 6 occasions. Each time the response to mitogens was assayed in 3 concentrations of fetal calf serum. No mitogen o; PHA ●; PWM x; Con A □.

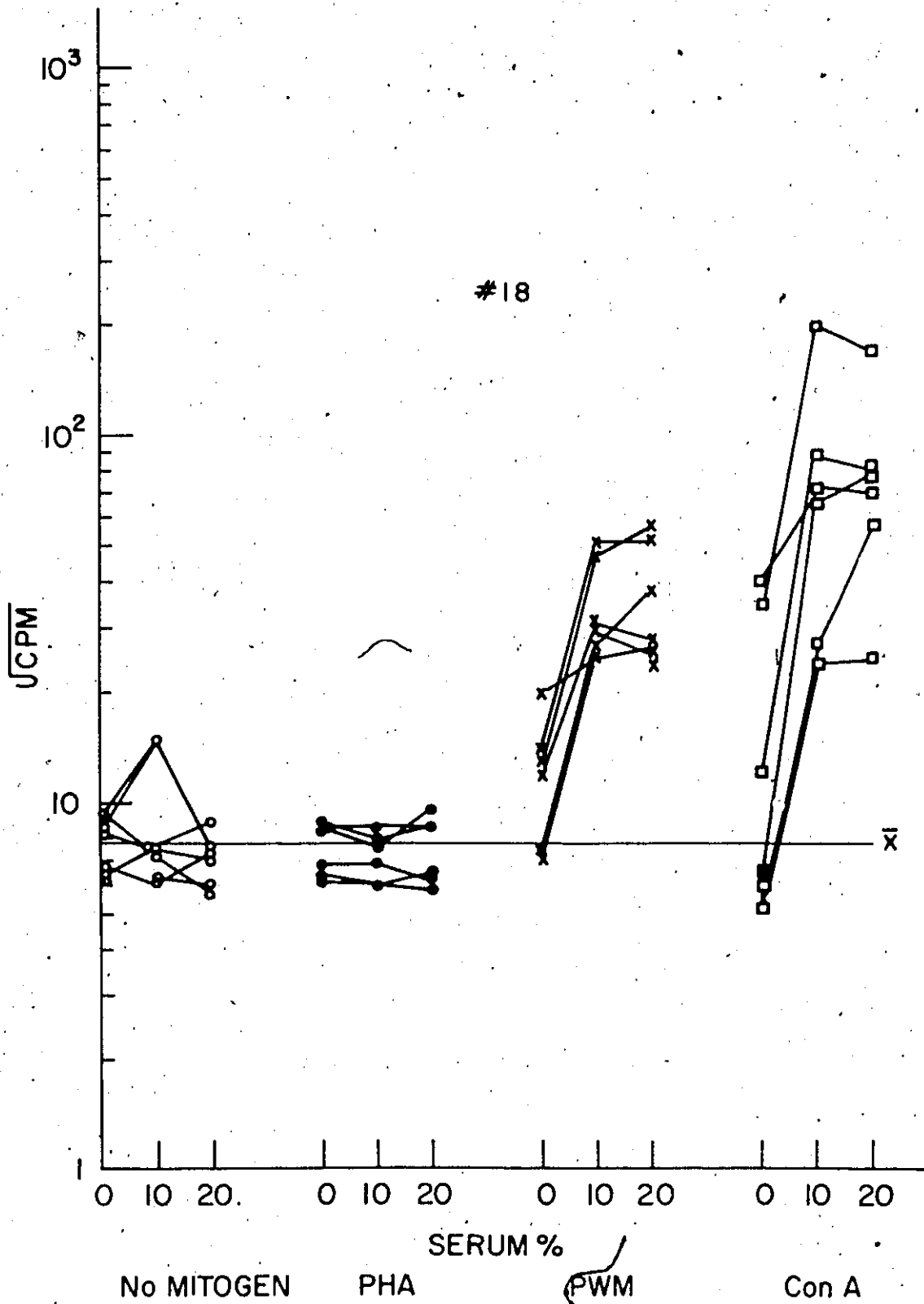
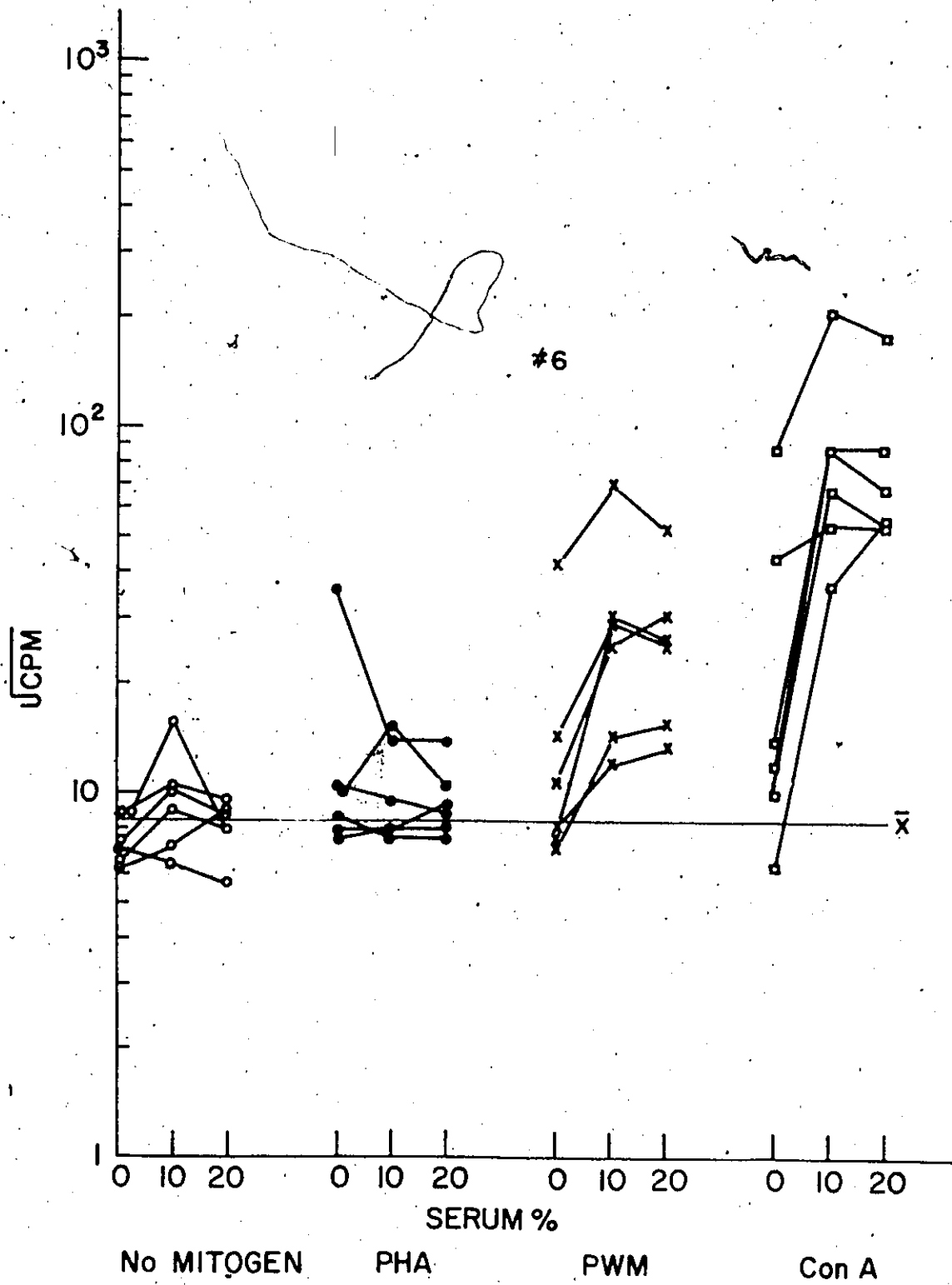


Fig. 3.3.5. Intra-individual variation. Cat #6 was bled on 6 occasions. Each time the response to mitogens was assayed in 3 concentrations of fetal calf serum. No mitogen o; PHA ●; PWM x; Con A □.



responses evoked by Con A, PHA and PWM in spleen and blood lymphocytes were inhibited similarly (Fig. 3.4.1 and 2). The inhibitory action of ouabain was observed at concentrations slightly lower than those employed by Quastel and Kaplan (1968) to inhibit transformation of human peripheral blood lymphocytes.

Most cat tissues have relatively low  $\text{Na}^+ \text{K}^+$  activated ATPase levels. However the ciliary body has a comparatively high enzyme activity which is inhibited by ouabain at the same concentration as human red blood cell ATPase (Bonting et al., 1961). Although cat lymphocyte ATPase activity has not been measured, this enzyme activity is essential for mitogen stimulation of cat lymphocytes.

#### 3.4.2 Enzymes

The treatment of lymphocytes with enzymes has been reported to activate lymphocytes in the absence of mitogen (Mazzei et al., 1966a and b) and to modify the response of lymphocytes to mitogens (Goodall, et al., 1971; Adler et al., 1972). Experiments with cat lymphocytes were done in an attempt to effect stimulation in the absence of mitogen and to expose mitogen binding sites. Feline peripheral blood and spleen lymphocytes treated with the proteolytic enzymes trypsin and pronase were not activated in the absence of phytomitogens. However pretreatment with enzymes did affect the response to mitogens. Although in most cases the mitogen response was diminished by enzyme pretreatment, the response of the spleen cells of one cat was consistently enhanced by pronase. The mitogen responses after enzyme treatment appeared to follow no

Fig. 3.4.1. Inhibition of feline peripheral blood lymphocytes by ouabain. Ouabain was added at the initiation of 3 day cultures. Total inhibition was achieved with  $3.5 \times 10^{-8}$  M ouabain.

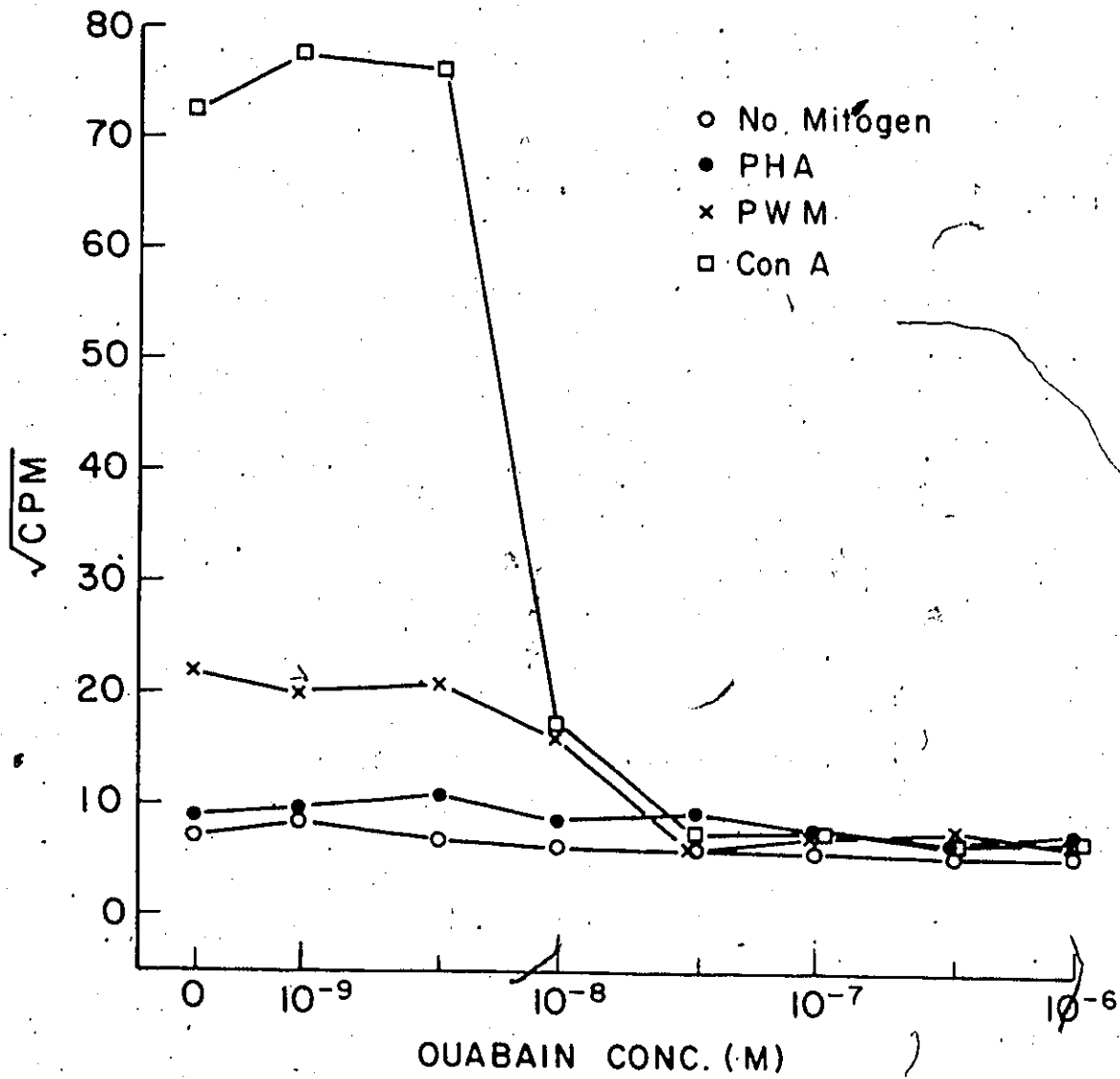
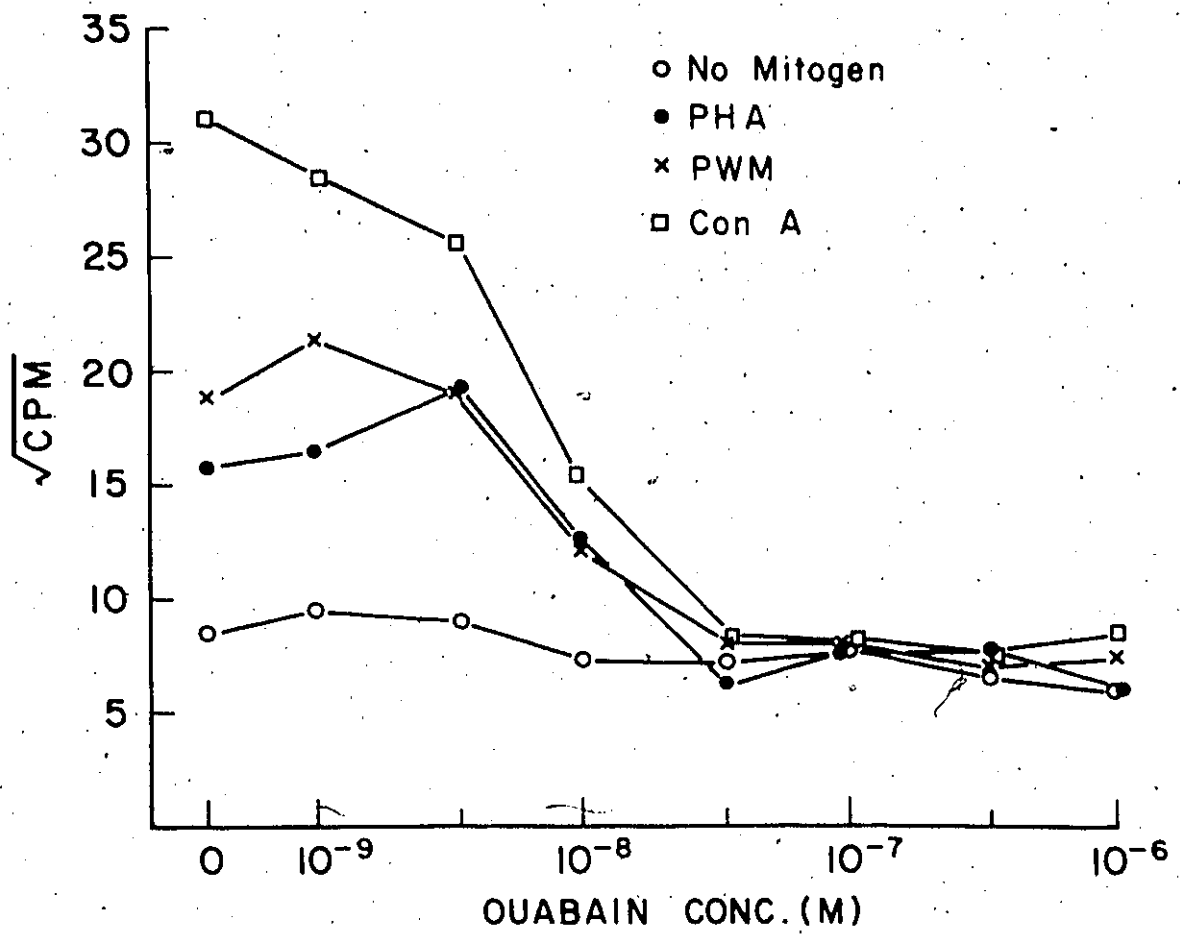


Fig. 3.4.2. Inhibition of feline splenic lymphocytes by ouabain. Ouabain was added at the initiation of 3 day cultures. Total inhibition was achieved with  $3.5 \times 10^{-8}$  M ouabain.



recognizable pattern, except that, as reported by Goodall et al., (1971), pronase was the more effective enzyme (Figs. 3.4.3-6).

Attempts to effect stimulation by incubation of lymphocytes with trypsin alone in serum-free medium for 3 to 5 days were unsuccessful (Table 3.4.1). The phytomitogen response in the serum-free medium used in this study was never ideal.

Lymphocytes from most cats show little or no response to PHA. Cells that were refractory to PHA stimulation did not consistently gain responsiveness after proteolytic enzyme treatment. Nevertheless the results support the concept that removal of cell surface components can unmask mitogen binding sites and/or allow rearrangement of sites to facilitate mitogen-induced activation.

### 3.4.3 Periodate

Although periodate is reported to be mitogenic for human lymphocytes, the response of cat cells to periodate was not striking. In some cases a ten minute periodate treatment induced slight stimulation at concentrations of  $5 \times 10^{-4}$  to  $10^{-3}M$ . Periodate followed by phytomitogen treatment yielded results illustrated in Figure 3.4.7. Periodate at  $10^{-2}M$  abolished the mitogen responses but seemed to augment these responses at lower concentrations. The results show that periodate is a poor mitogen for cat lymphocytes possibly because the oxidizable sugar is absent or inaccessible or that its oxidation does not result in stimulation.

Fig. 3.4.3. Effects of enzyme pretreatment on mitogen response. Following the 30 minute enzyme treatment (0.1% trypsin; 0.05% pronase), feline peripheral blood lymphocytes were washed and then cultured in medium with serum for 3 days. Note the apparent depression of the PHA response and enhancement of Con A and PWM responses.

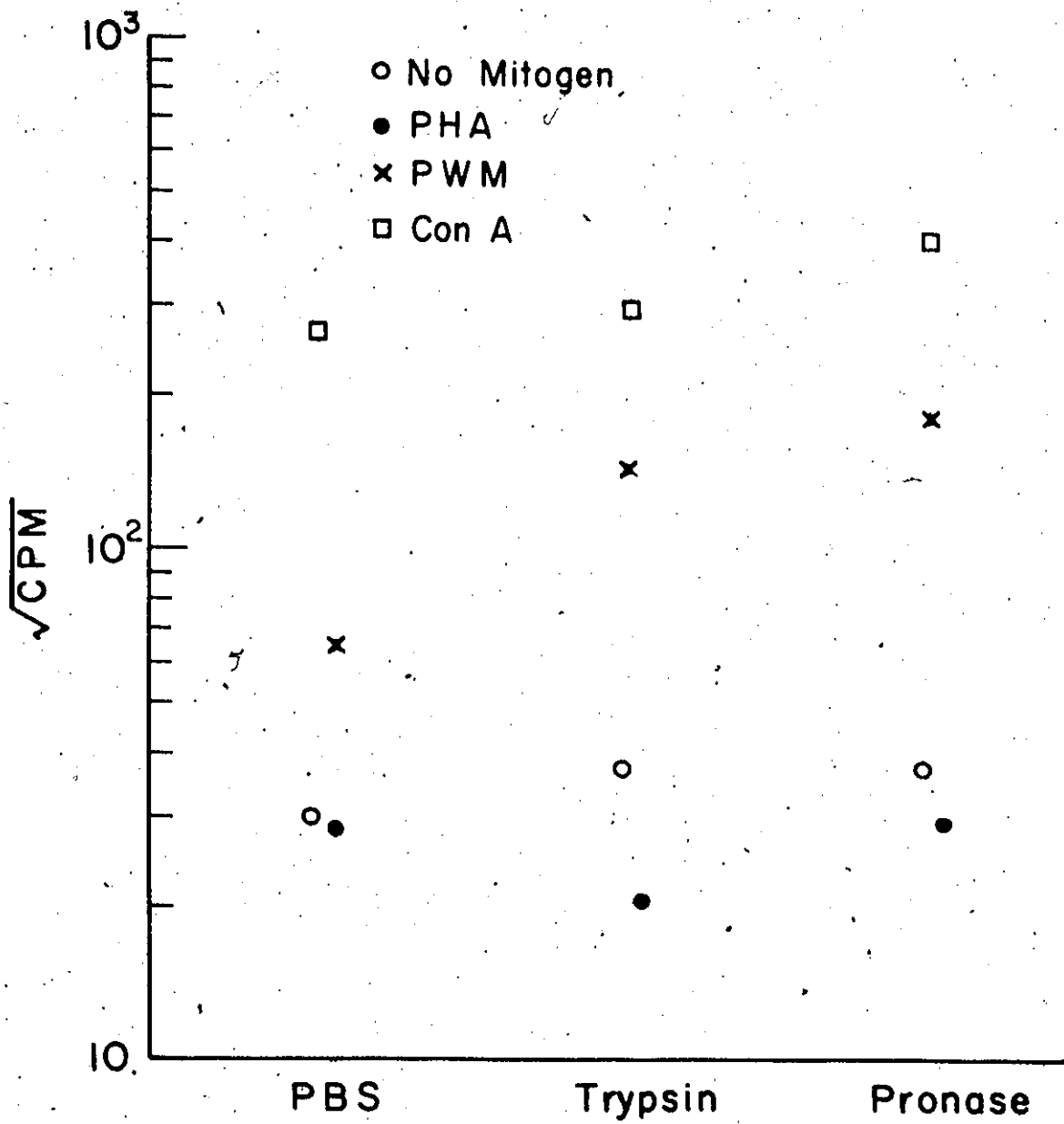


Fig. 3.4.4. Effects of enzyme pretreatment on mitogen response. When the data from Fig. 3.4.3. are expressed by the ratio method, the apparent depression of the PHA response and enhancement of the Con A response disappear.

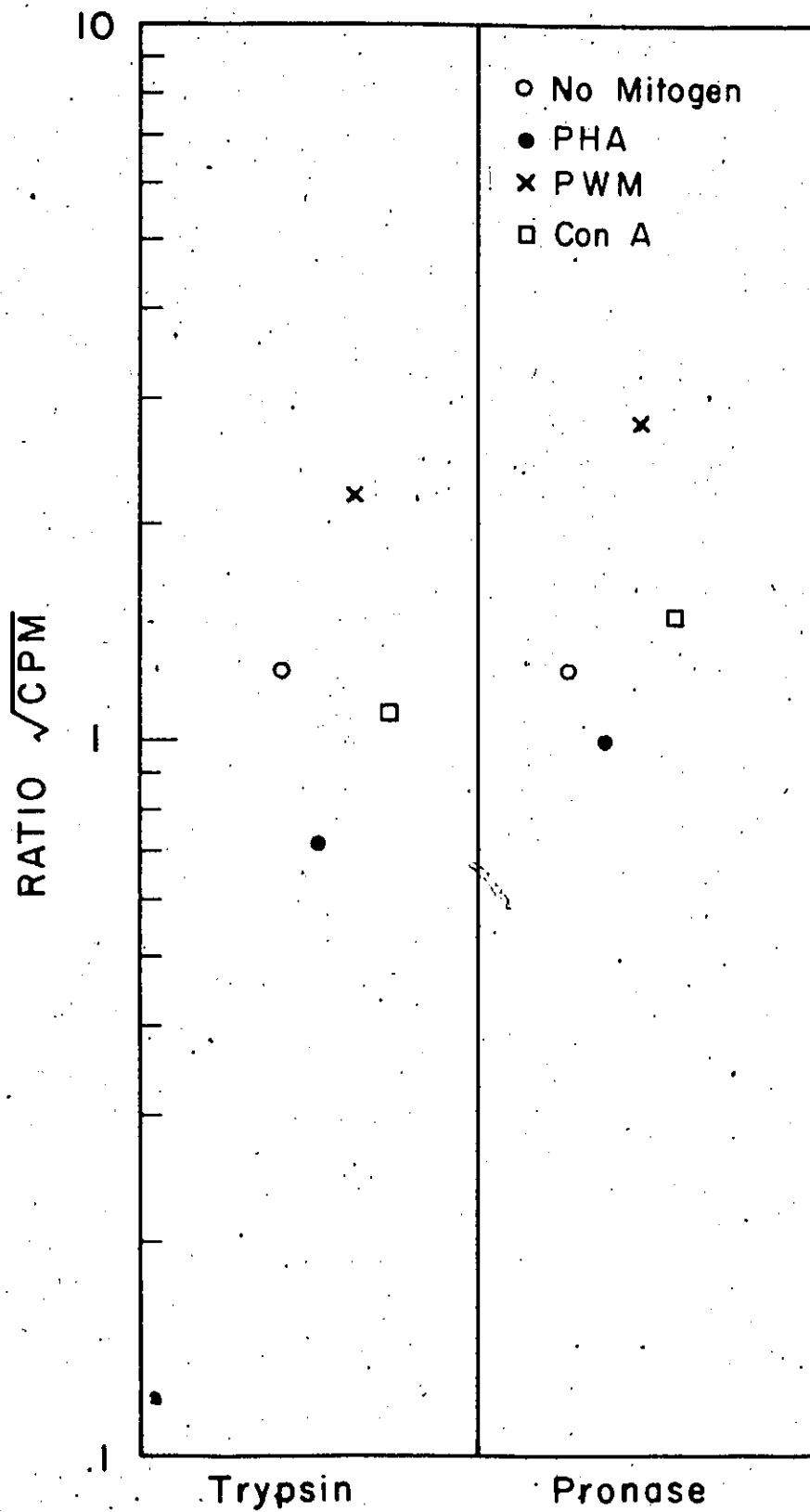


Fig. 3.4.5. Mitogen response following enzyme treatment. Blood lymphocytes from 2 cats (#12, #236) were treated for 30 minutes with saline (S), 0.1% trypsin (T) or 0.05% pronase (P) prior to culture in medium plus serum. The mitogen response was consistently depressed except in the case of cat #236 where the PHA response was enhanced by enzyme pre-treatment.

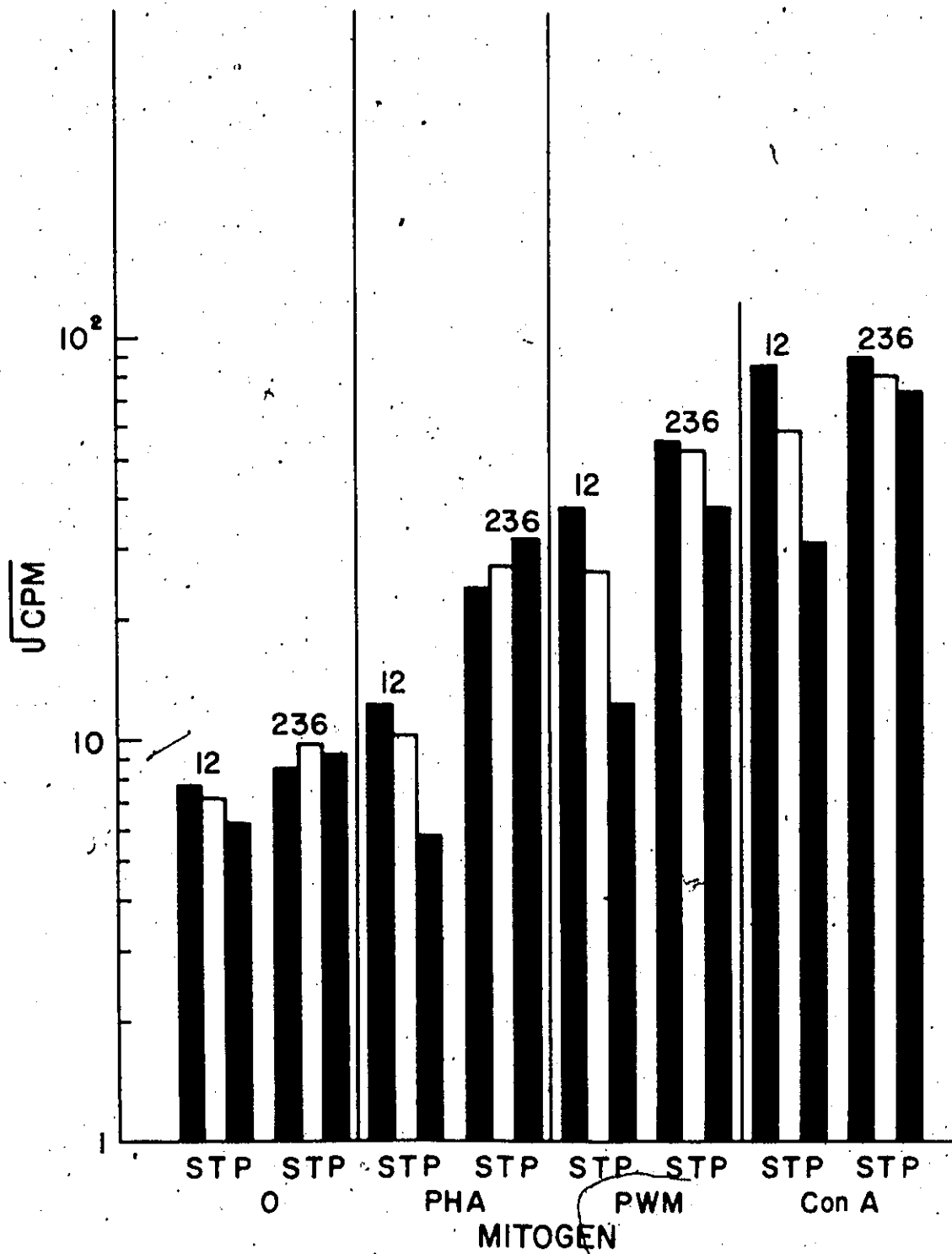


Fig. 3.4.6. Mitogen response following enzyme treatment. Splenic lymphocytes from 2 cats (#12, #236) were treated for 30 minutes with saline (S), 0.1% trypsin (T) or 0.05% pronase (P) prior to culture in medium plus serum. Although the responses of cat #12 were usually depressed, the responses of cat #236 were enhanced by pronase.

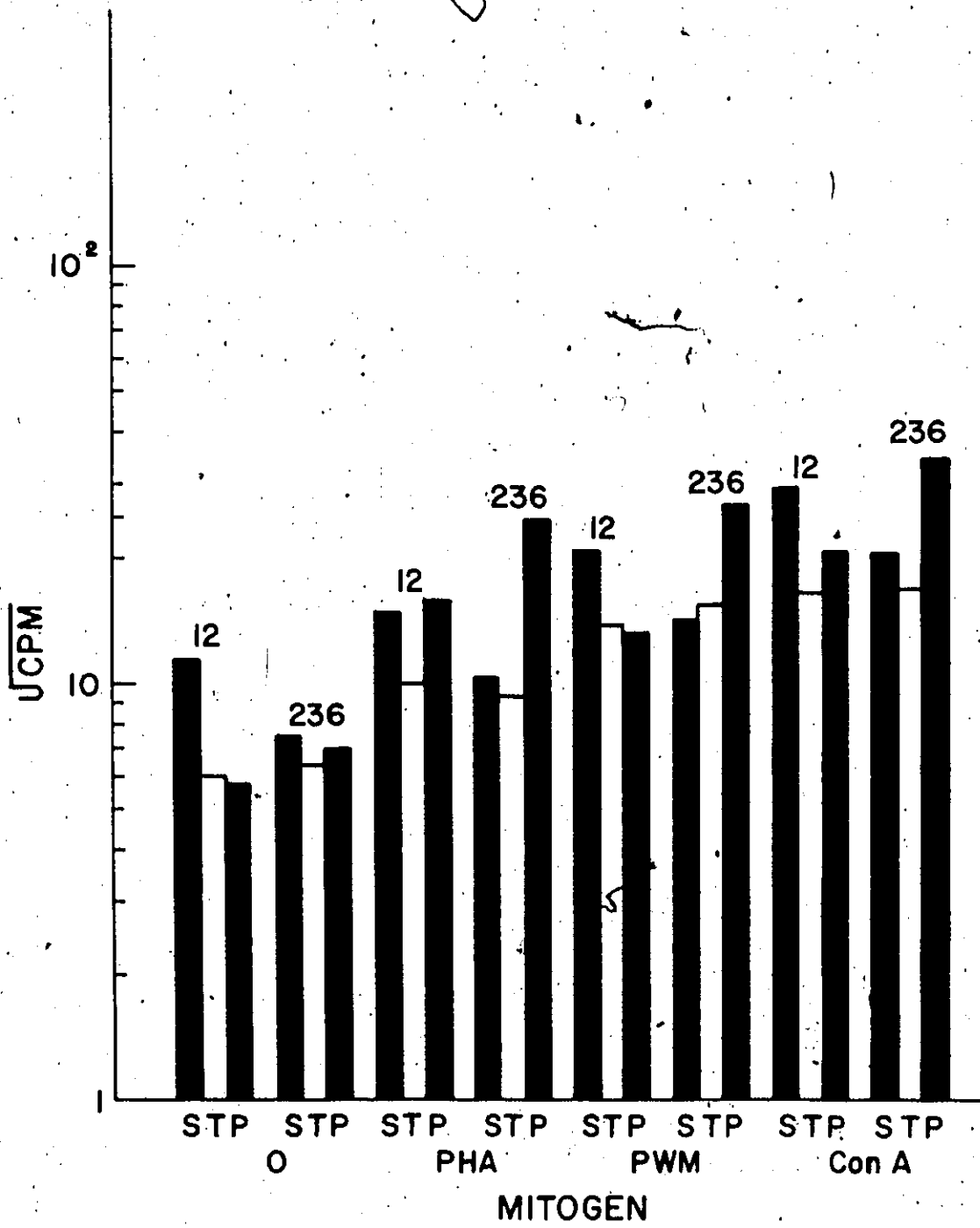


Table 3.4.1. Lymphocyte response to trypsin and mitogens in serum-free medium. Cat lymphocytes were cultured in serum-free medium with trypsin or mitogen for 3 or 5 days. In only one case (blood, 5 days incubation, 0.3 ug/ml trypsin) was there any indication of stimulation by trypsin. Lymphocytes responded normally to PWM but not to PHA or Con A in serum-free medium.

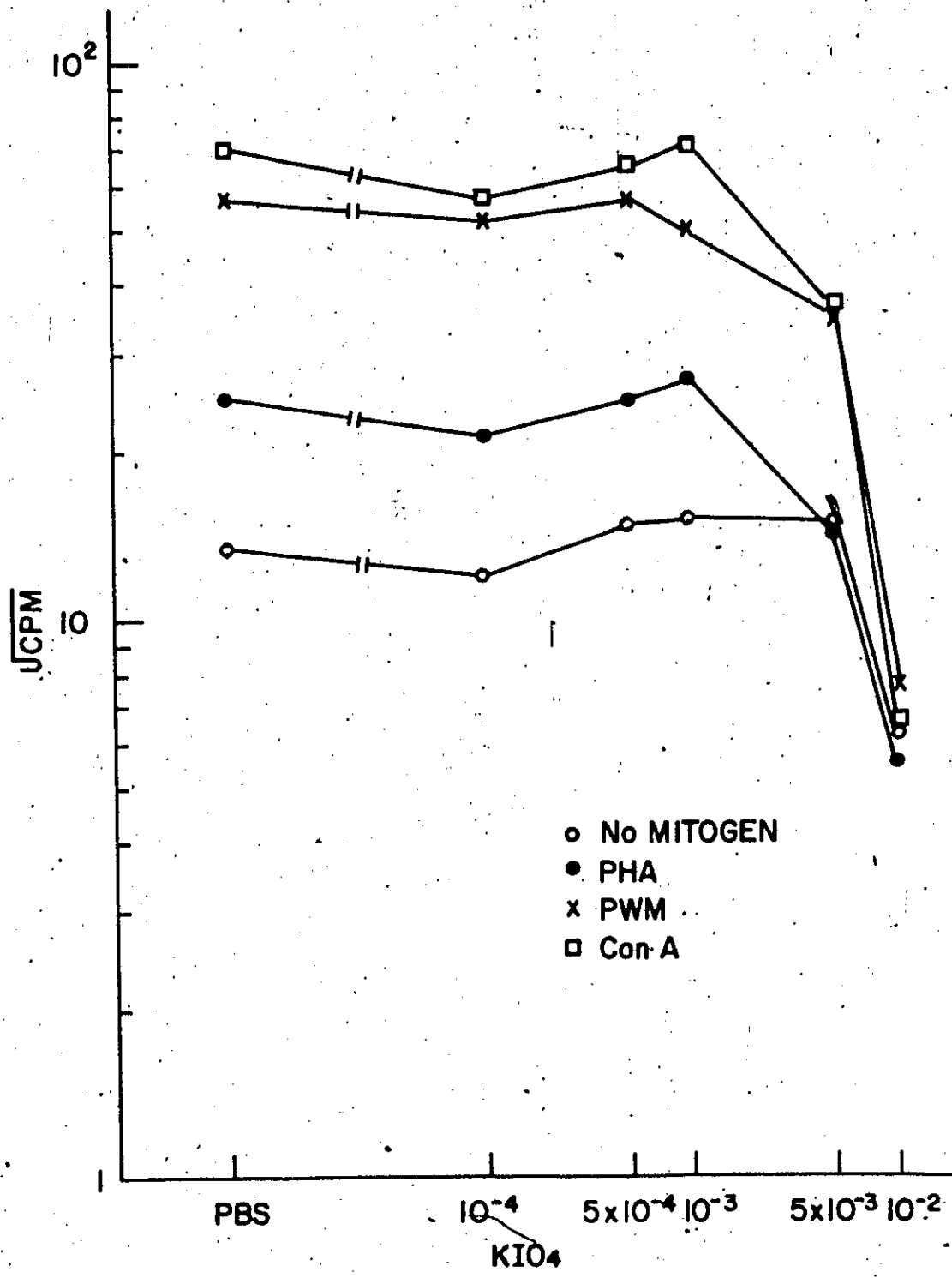
Table 3.4.1.

Lymphocyte response to trypsin and mitogens in serum-free medium.<sup>1</sup>

<u>Origin of Lymphocytes</u>	<u>Days of Incubation</u>	<u>Treatment</u>	<u>CPM</u>
Blood	3	0	10.4
		.1	9.6
		.3 µg/ml	12.3
		.6 trypsin	12.7
		.9	10.0
		PHA	11.0
	PWM	28.4	
	Con A	10.2	
	5	0	8.6
		.1	8.4
		.3 µg/ml	11.3
		.6 trypsin	8.3
		.9	8.8
		PHA	8.3
	PWM	14.6	
Con A	7.5		
Spleen	3	0	10.5
		.1	14.9
		.3 µg/ml	10.6
		.6 trypsin	11.2
		.9	9.6
		PHA	16.1
	PWM	24.0	
	Con A	9.1	
	5	0	9.1
		.1	9.9
		.3 µg/ml	9.8
		.6 trypsin	9.0
.9		11.2	
PHA		9.8	
PWM	17.6		
Con A	8.7		

1. Cat lymphocytes were cultured in serum-free medium with trypsin or mitogens for 3 or 5 days.

Fig. 3.4.7. Effect of periodate on splenic lymphocyte stimulation. Responses to mitogens were slightly augmented at low and abolished at high  $KIO_4$  concentrations. Periodate alone was weakly mitogenic. Identical results were obtained with blood and splenic lymphocytes from another cat.



#### 3.4.4 Mercury

The response of human and cat peripheral blood lymphocytes to mercury was compared. Mercuric chloride consistently stimulated lymphocytes from both species at concentrations ranging from  $4 \times 10^{-6}M$  to  $10^{-5}$  (Fig. 3.4.8 and 9). Concentrations of  $2 \times 10^{-5}M$  or higher were toxic. The effective mercury concentrations in this study are slightly lower than those published by others for other species (Schopf et al., 1967; Caron et al., 1970). Two explanations may be advanced. First, culture medium and serum Hg-sequestering agents (eg. cysteine and various proteins) could reduce the amount of mercury available to interact with lymphocytes. Secondly, it has been demonstrated recently that if dilute mercury solutions are filter-sterilized a significant proportion of the mercury may remain bound to the filter. In the present study a  $10^{-3}M$  stock solution was filter-sterilized then diluted, minimizing the proportion of mercury lost.

The response to mercury is reported to peak on the 6th day of culture (Caron et al., 1970). In the present study cultures were harvested on days 3, 5 and 7. Peak responses were found to be divided evenly between days 3 and 5, indicating a somewhat shorter time course than reported by others. The finding of an abbreviated time course tends to discount the hypothesis that mercury might be acting as an antigen. Further studies on the mechanism of Hg-induced lymphocyte transformation are required.

Attempts to stimulate human lymphocytes with methyl mercuric chloride were unsuccessful (Fig. 3.4.10a

Fig. 3.4.8. Response of cat Blood lymphocytes to mercuric chloride. Cat lymphocytes responded well to  $\text{HgCl}_2$ ; the response reached a peak at  $10^{-5} \text{M}$   $\text{HgCl}_2$  on day 5.

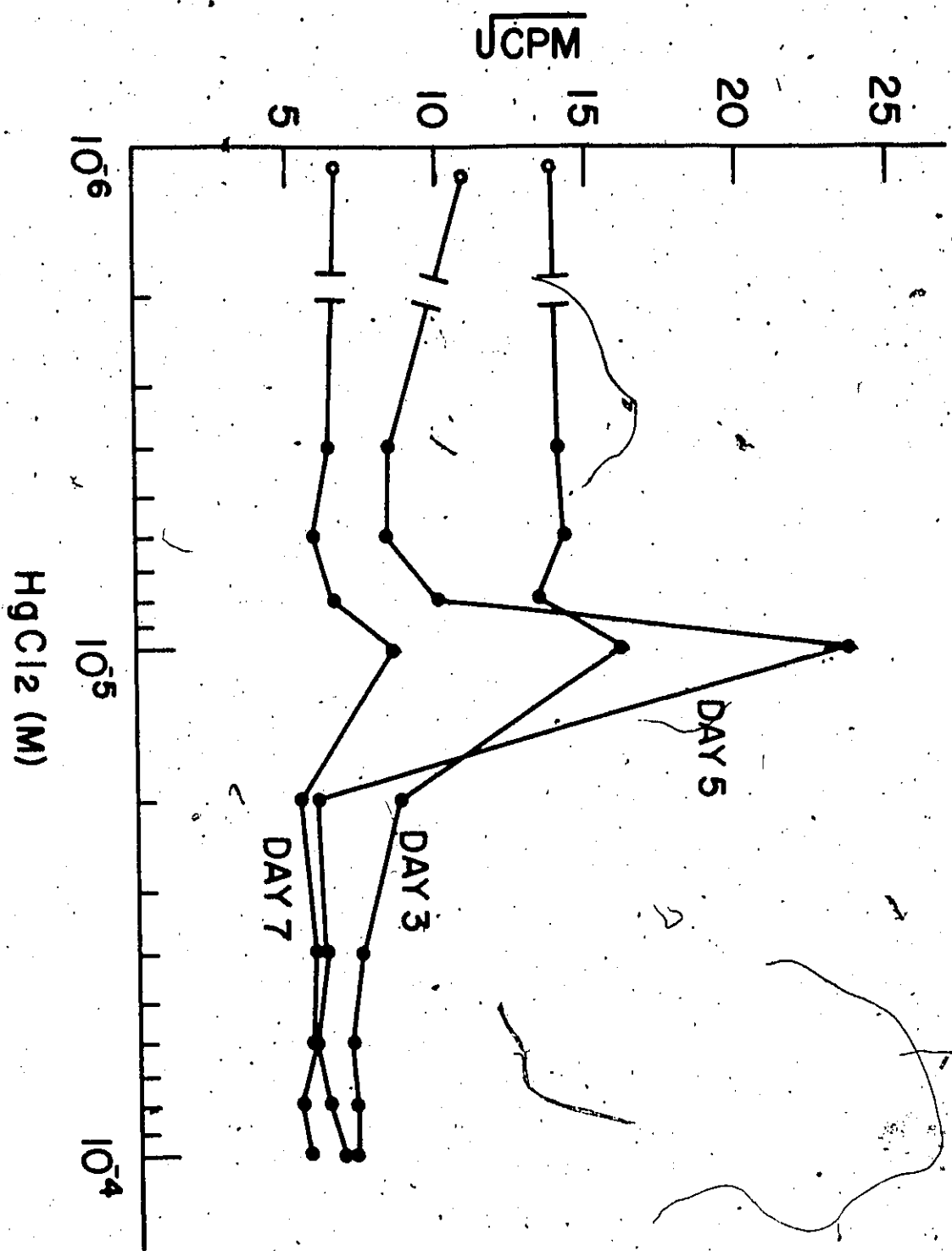


Fig. 3.4.9. Response of human blood lymphocytes to mercuric chloride. The results with human blood resemble those with cat lymphocytes (Fig. 3.4.8.) except that the high response extended over a longer time with human cells.

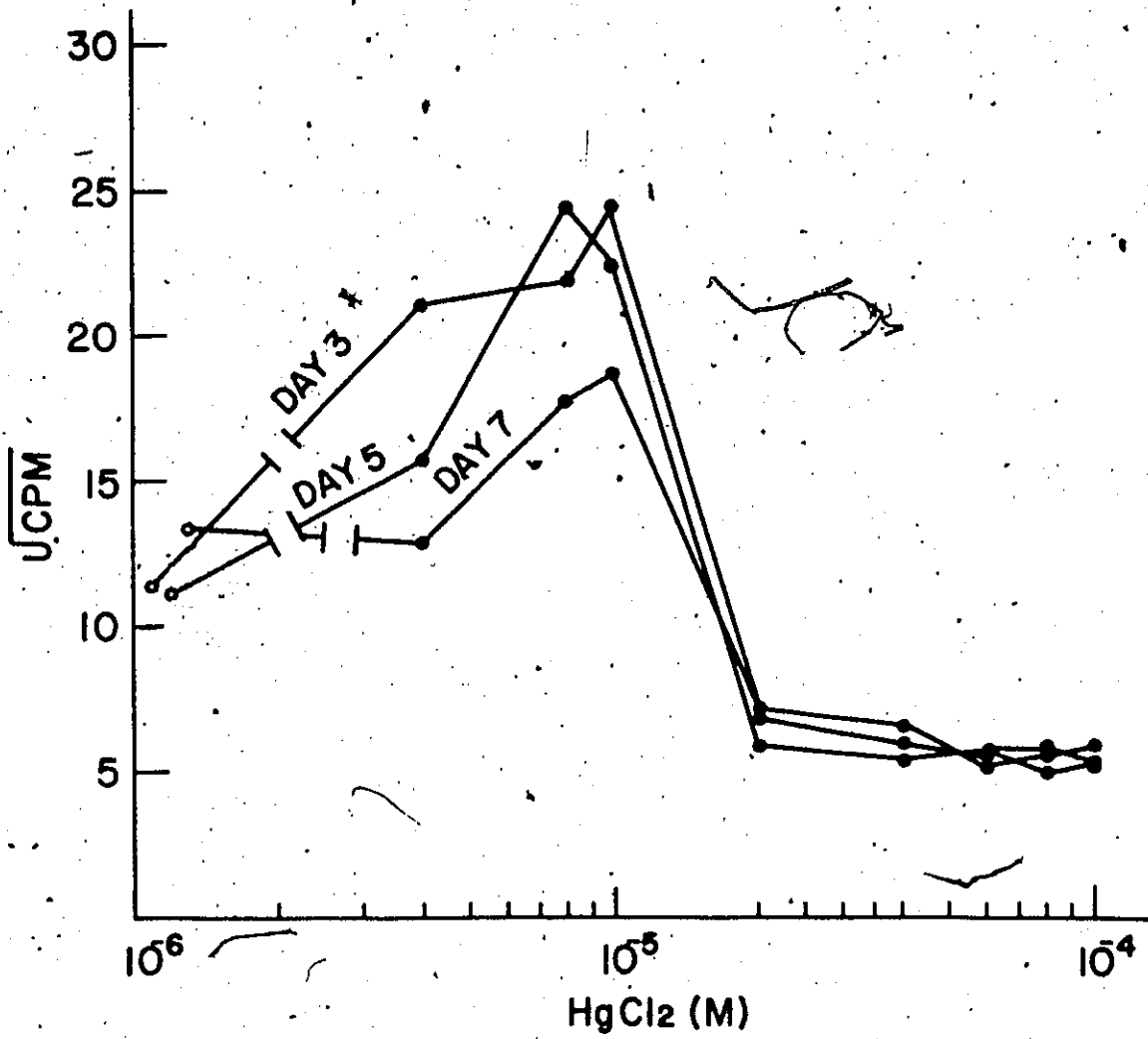
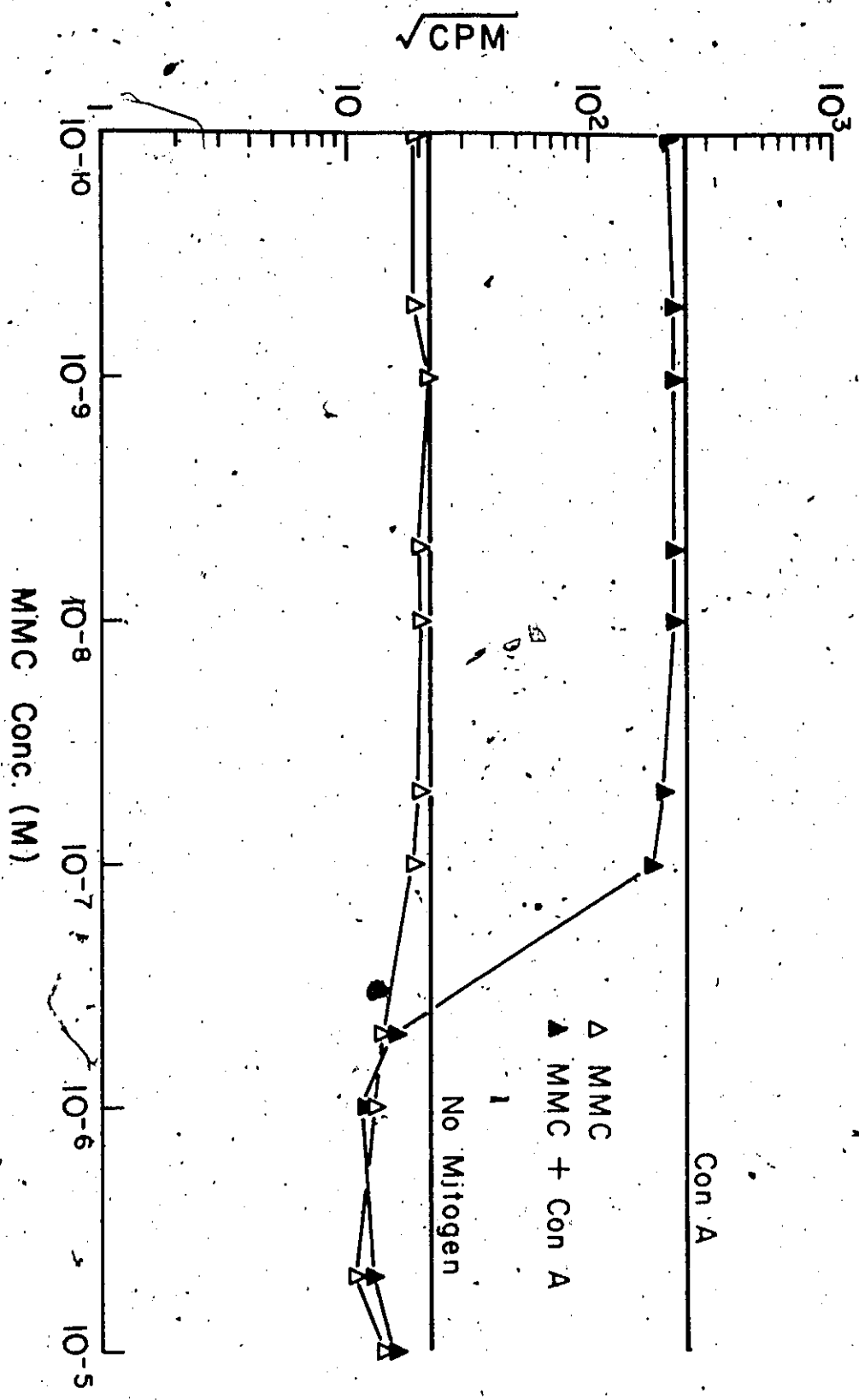
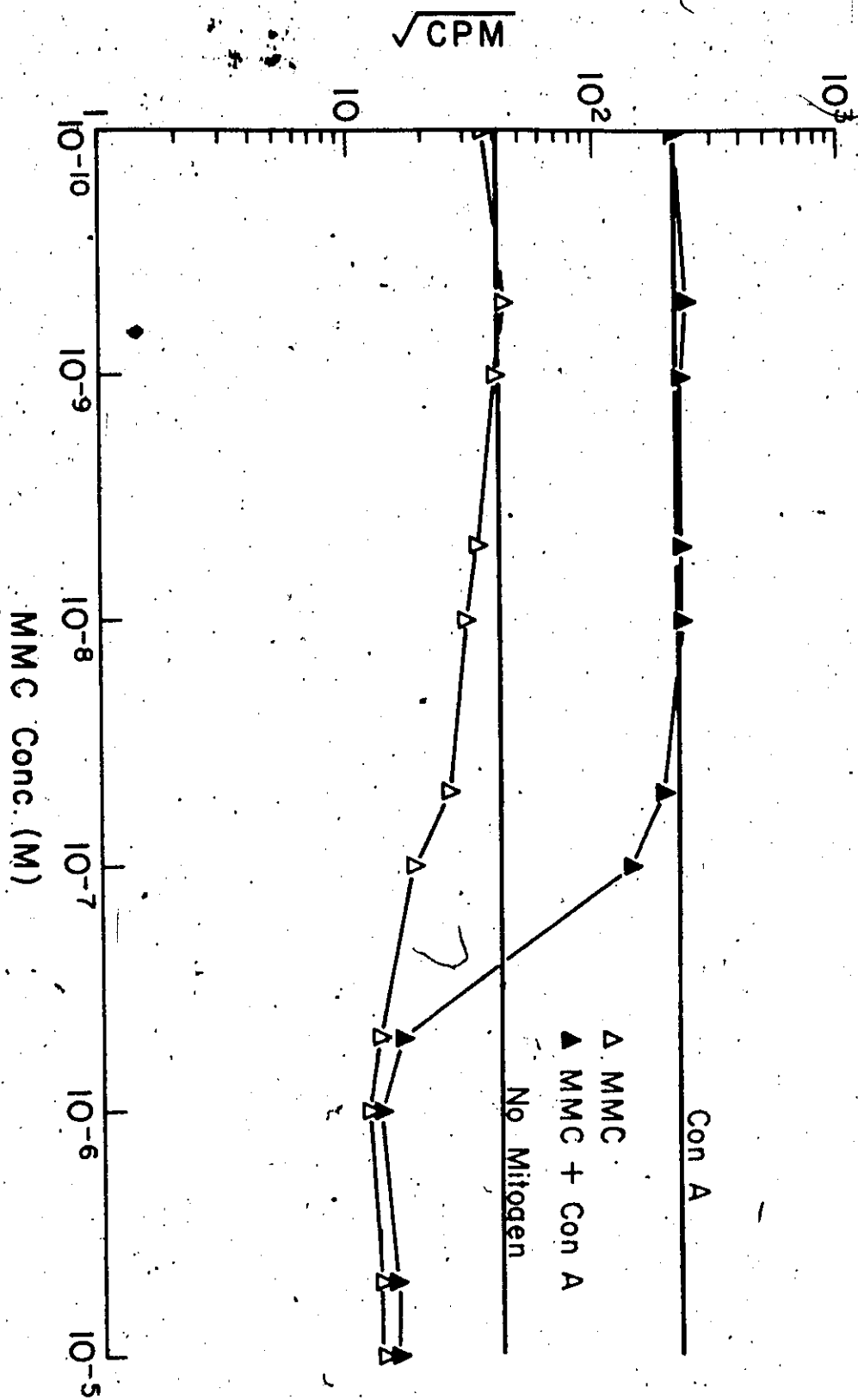


Fig. 3.4.10a and b. Response of human lymphocytes to methyl mercuric chloride. MMC failed to stimulate cells at concentrations well below those inhibiting the Con A response at either day 3 (a) and day 5 (b) of culture.





and b). Methyl mercury toxicity was evident at  $2 \times 10^{-7}M$ , a concentration 100 times lower than that at which the inorganic mercuric chloride was toxic. Organic mercurials have been reported to be more cytotoxic in tissue culture than inorganic compounds (Umeda et al., 1969). 3.5

### Insoluble PHA

#### 3.5.1 Attachment of cells to Sepharose-PHA

The binding of mitogens to cells has been demonstrated repeatedly with radio- or fluorescein-labelled mitogens. Such studies have led to the conclusion that both responsive and nonresponsive cells bind mitogens equally, except in the case of PWM which does not bind detectably to either B or T cells. Similar experiments can be performed with mitogens coupled to Sepharose beads (Greaves and Bauminger, 1972; Loor, 1974).

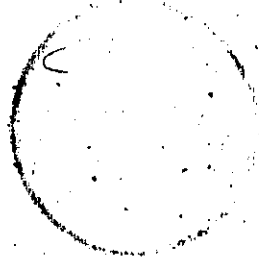
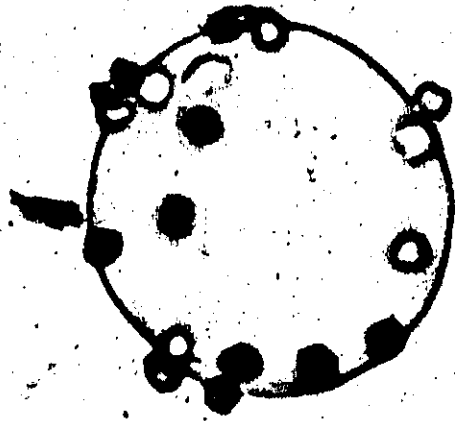
In four experiments, using human blood lymphocytes and cat blood and splenic lymphocytes, cells attached in greater numbers to Sepharose-PHA than to agarose beads alone (Fig. 3.5.1-5). One can readily conclude that PHA-binding cells were present in all samples tested.

Although cat and human peripheral blood lymphocytes appeared to bind equally well to Sepharose-PHA the conclusion that equal number of PHA-binding cells were present is not entirely justified in the absence of information on the ability of the system to detect quantitative differences in reactive cell numbers.

#### 3.5.2 Response of lymphocytes to insoluble PHA

Insoluble PHA has been reported to stimulate both B and T lymphocytes of those species for which soluble PHA

Fig. 3.5.1. Photomicrograph of cat blood lymphocytes  
attached to Sepharose-PHA (top). The lower  
photograph shows a control bead with  
adjacent lymphocytes.



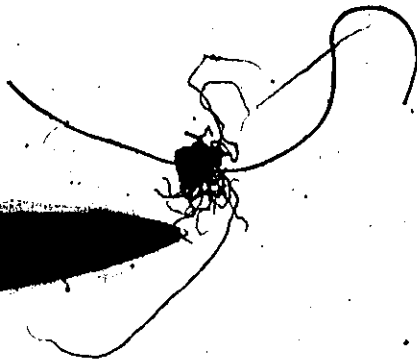


Fig. 3.5.2. Attachment of human blood lymphocytes to Sepharose-PHA. Lymphocytes in medium containing 10% fetal calf serum were incubated with Sepharose beads or with beads coupled to PHA for 90 minutes prior to examination by microscopy. Beads coupled to PHA bound more lymphocytes than did beads without attached PHA.

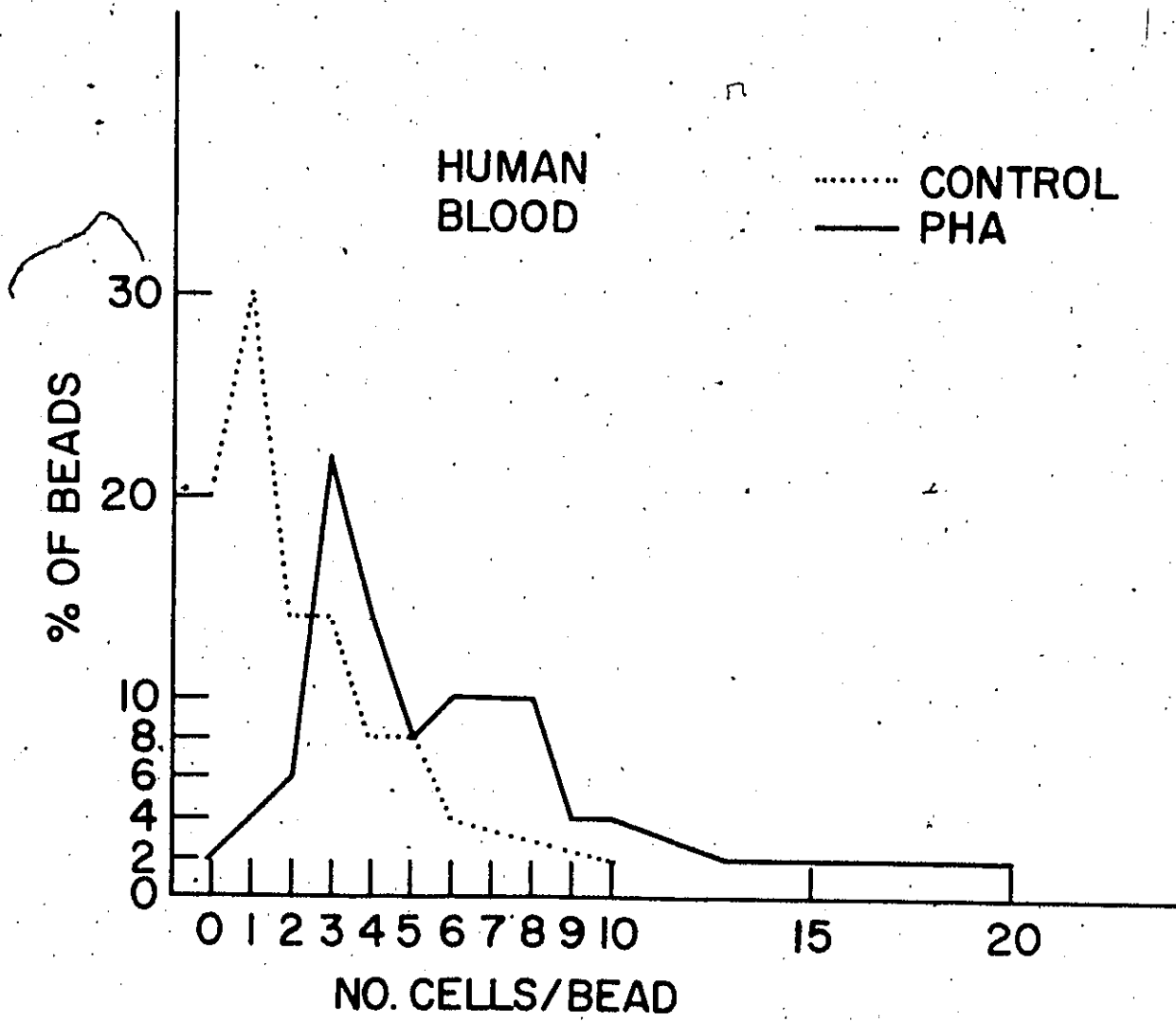


Fig. 3.5.3. Attachment of cat blood lymphocytes to  
Sephrose-PHA. See Fig. 3.5.2.

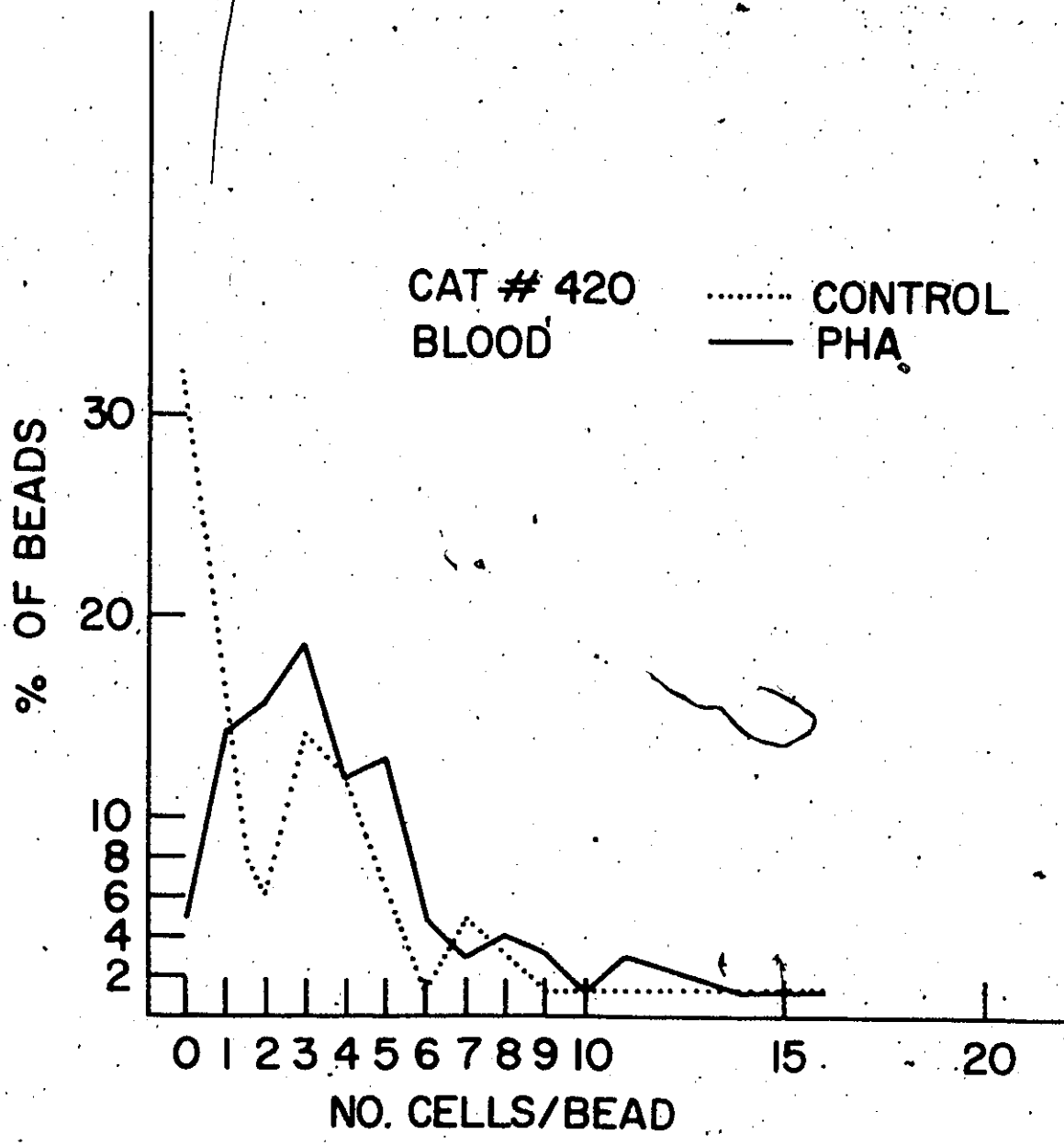


Fig. 3.5.4. Attachment of cat splenic lymphocytes to  
Sephrose-PHA. See Fig. 3.5.2.

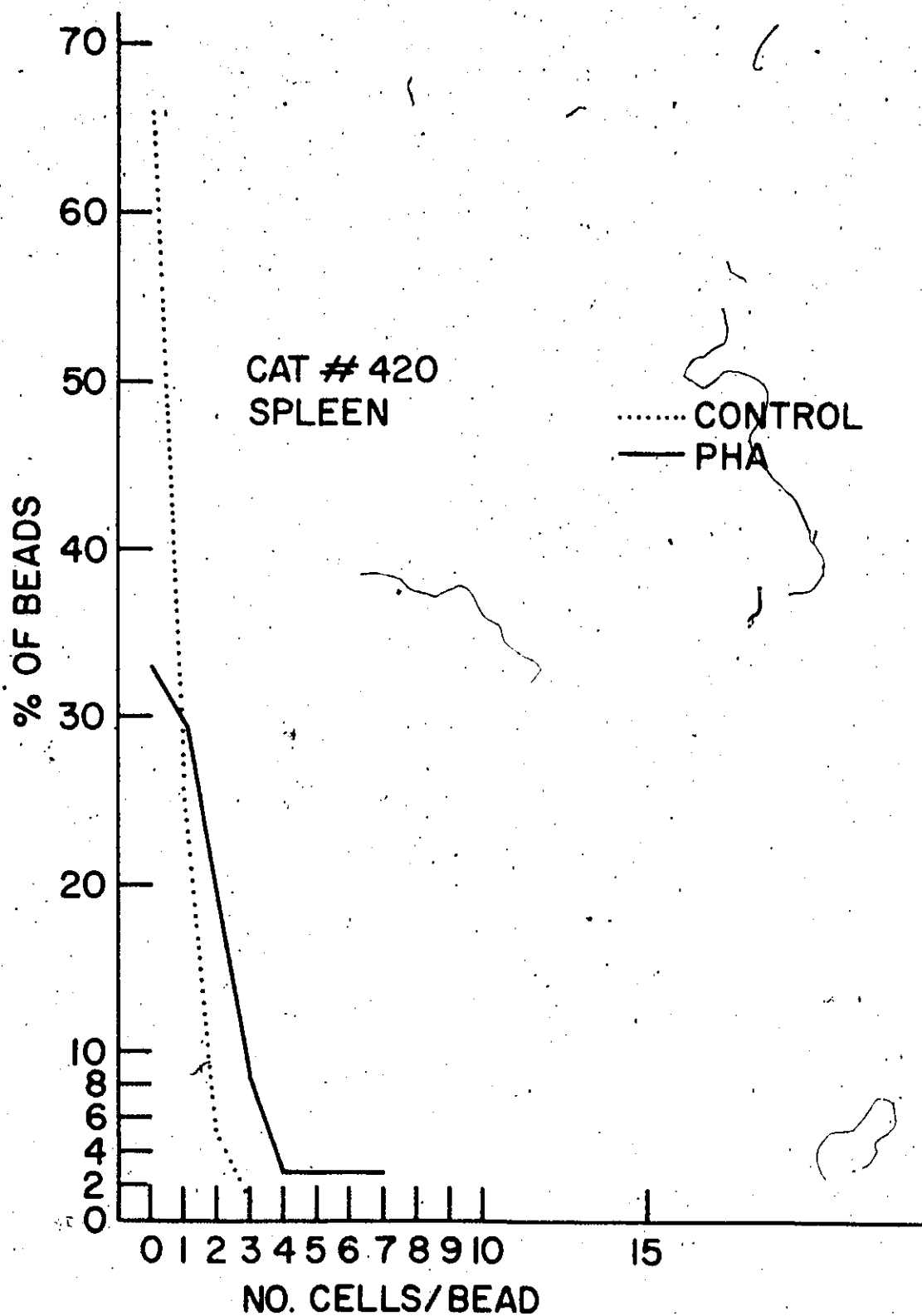
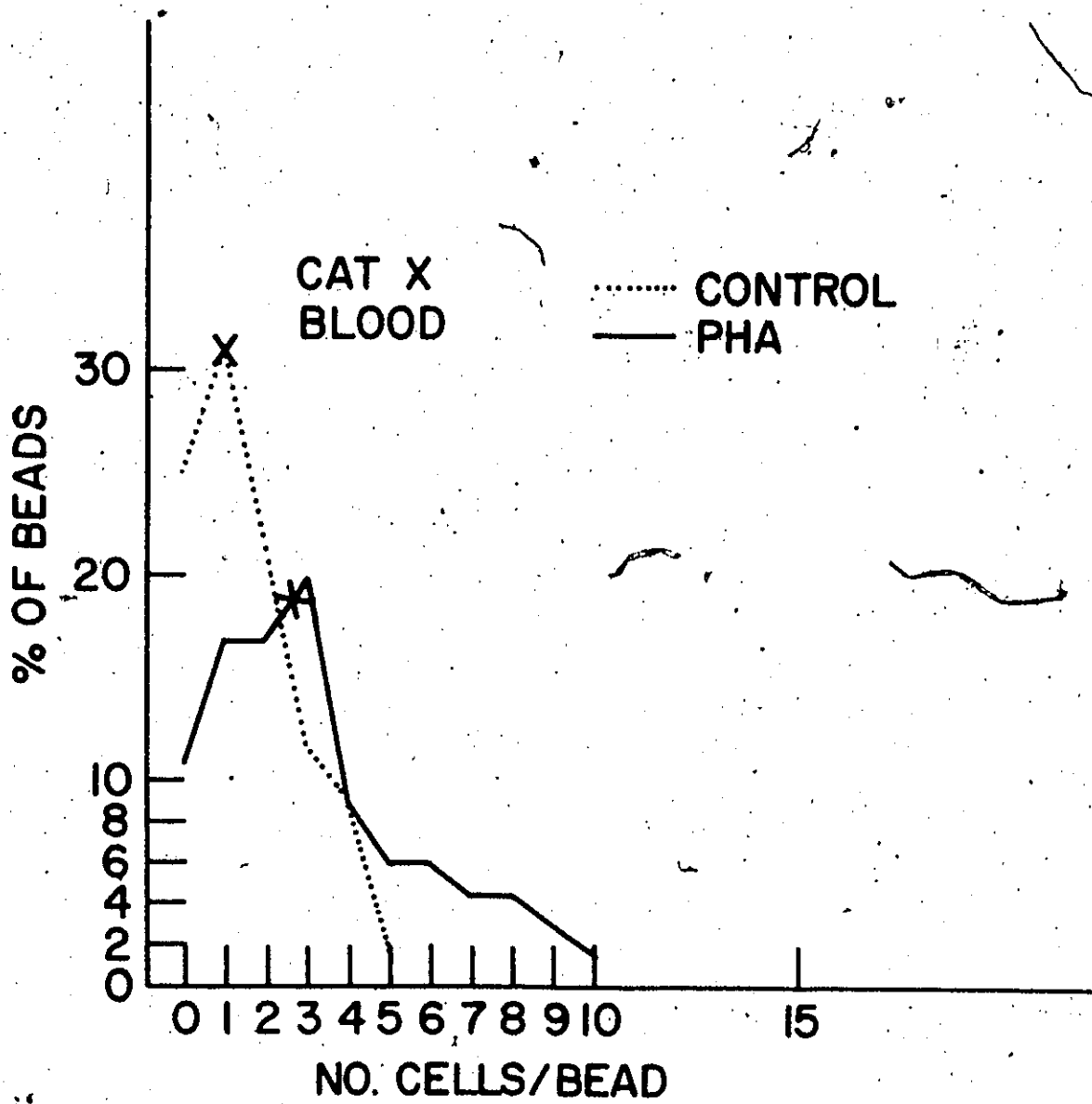


Fig. 3.5.5. Attachment of cat blood lymphocytes to  
Sephrose-PHA. See Fig. 3.5.2.



is T cell specific (Greaves and Bauminger, 1972; Loor, 1974). Attempts to stimulate blood and splenic lymphocytes from two cats as well as human blood cells with Sepharose-PHA failed. Although a wide range of concentrations of beads was tested, the possibility remains that a suitable concentration was not employed. The human blood and splenic lymphocytes from both cats were responsive to soluble PHA while the cat peripheral blood lymphocytes were not.

### 3.6 Cell separation

Very recent reports have emphasized the importance of lymphocyte separation methods. Particularly relevant to the present work is the demonstration that one population of cells might inhibit the mitogen response of another group of lymphocytes. In addition it has been suggested that routine separation methods such as gelatin or dextran sedimentation might purify one lymphocyte class at the expense of others.

#### 3.6.1 Suppressor cells

Tardieu et al. (1974) reported that rat thoracic duct (TD) lymphocytes did not respond to PHA in medium supplemented with 10% agamma (gamma globulin free) fetal calf serum. However, in agamma calf serum or donor calf serum these cells responded well to PHA. Incubation of TD lymphocytes with bovine serum albumin (BSA) for 30 minutes abolished their ability to respond to PHA. These experiments suggest the existence in unfractionated TD lymphocytes of a cell which in the presence of BSA or fetal calf serum inhibits the response of T lymphocytes to PHA. These suppressor cells were isolated by density gradient

centrifugation and identified as relatively dense small lymphocytes.

To ascertain whether a similar situation might be responsible for the low response of feline peripheral blood cultures to PHA, cells were cultured in different concentrations of fetal and donor calf serum. The responses to PWM and Con A were clearly higher in medium supplemented with fetal calf serum. The response to PHA was similar in fetal and donor calf sera (Fig. 3.6.1), suggesting that the suppressor cell, if indeed present, did not require activation by a component of fetal calf serum.

A related phenomenon in an unrelated species, the nurse shark, was reported by Sigel *et al.* (1973). In this case unpurified shark blood lymphocytes responded to Con A but not to PHA. Fractionation of leukocyte populations on a Ficoll-Isopaque gradient yielded 3 lymphocyte-containing bands. One layer responded to both Con A and PHA but the response to PHA could be inhibited by cells from either of the two other layers. These results can be explained by invoking a suppressor cell not requiring activation.

### 3.6.2 Enrichment in B cells by separation methods

Gelatin and dextran sedimentation techniques have been employed routinely by many investigators to prepare lymphocytes from whole blood for diverse purposes, including the estimation of the proportions of T and B cells. Recently Singh *et al.* (1974) reported that these separation methods result in a selective separation of B lymphocytes and a considerable destruction of T cells. Table 3.6.1 shows the data of Singh *et al.* (1974) on the

Fig. 3.6.1. Response to mitogens in fetal and donor calf sera. Feline blood lymphocytes responded similarly or better in fetal calf serum (FCS) than in donor calf serum (DCS).

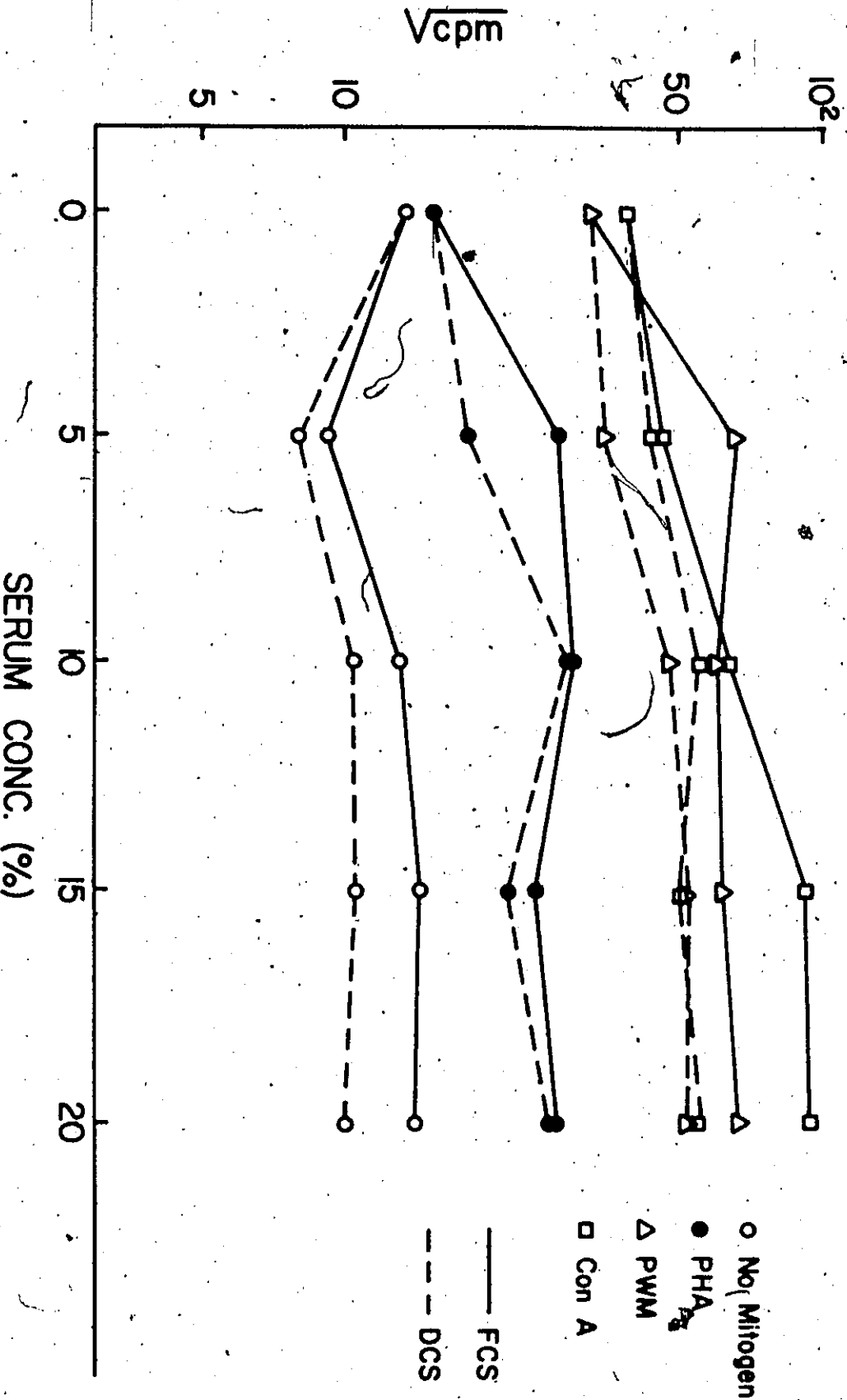


TABLE 3.6.1.

Enrichment in B cells by separation methods.<sup>1</sup>

% B lymphocytes

before separation                      after separation

mouse blood	25-30	60-72
spleen	49-53	67-69
rabbit blood	32	62-74

1. Data from Singh, et al. (1974).

percentage of B cells in unseparated cell suspensions and after separation in gelatin, dextran or Ficoll-Triosil.

In the present work, cat blood lymphocytes, separated from defibrinated blood by gelatin sedimentation, responded poorly to PHA whereas spleen cells, prepared directly from spleens, responded well to PHA. Since PHA might indeed be a T cell mitogen for cat lymphocytes and since T cells might be selectively lost in the routine separation method, it was necessary to compare the PHA response of cells purified by several methods as shown in Figure 3.6.2. The results (Fig. 3.6.3) do not support the hypothesis that PHA responsive cells were destroyed by the defibrination and gelatin sedimentation procedures.

### 3.6.3 Ficoll-Hypaque separation

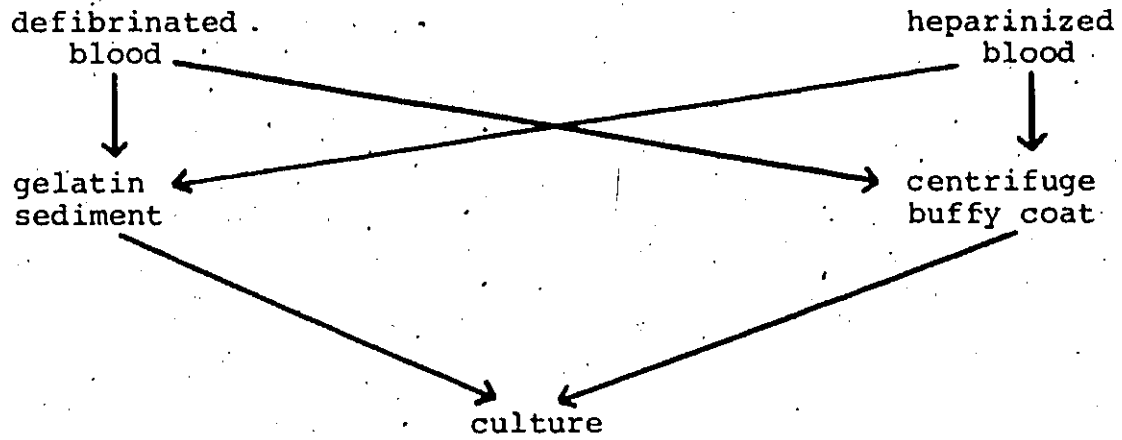
Cat lymphocytes were subjected to centrifugation on a discontinuous F-H gradient after routine defibrination and gelatin sedimentation. Lymphocytes were located in three areas (Fig. 3.6.4). Lymphocytes in these bands were recovered and cultured at a density of  $10^6$ /ml with PHA, PWM and Con A (Table 3.6.2). Five concentrations of PHA were used since it has been reported that purified cells may be responsive to mitogen over a narrower dose range than the parent cell mixture (Andersson *et al.*, 1972a).

Concentration "b" ( $5 \times 10^{-3}$  ml/ml) is the PHA dose used for most of their previous experiments. PHA concentrations a-e correspond to  $10^{-2}$ ,  $5 \times 10^{-3}$ ,  $3.3 \times 10^{-3}$ ,  $2.5 \times 10^{-3}$  and  $1.8 \times 10^{-3}$  ml/ml respectively.

The data in Table 3.6.2 and 3 indicate that cultures of lymphocytes from fraction B incorporate more

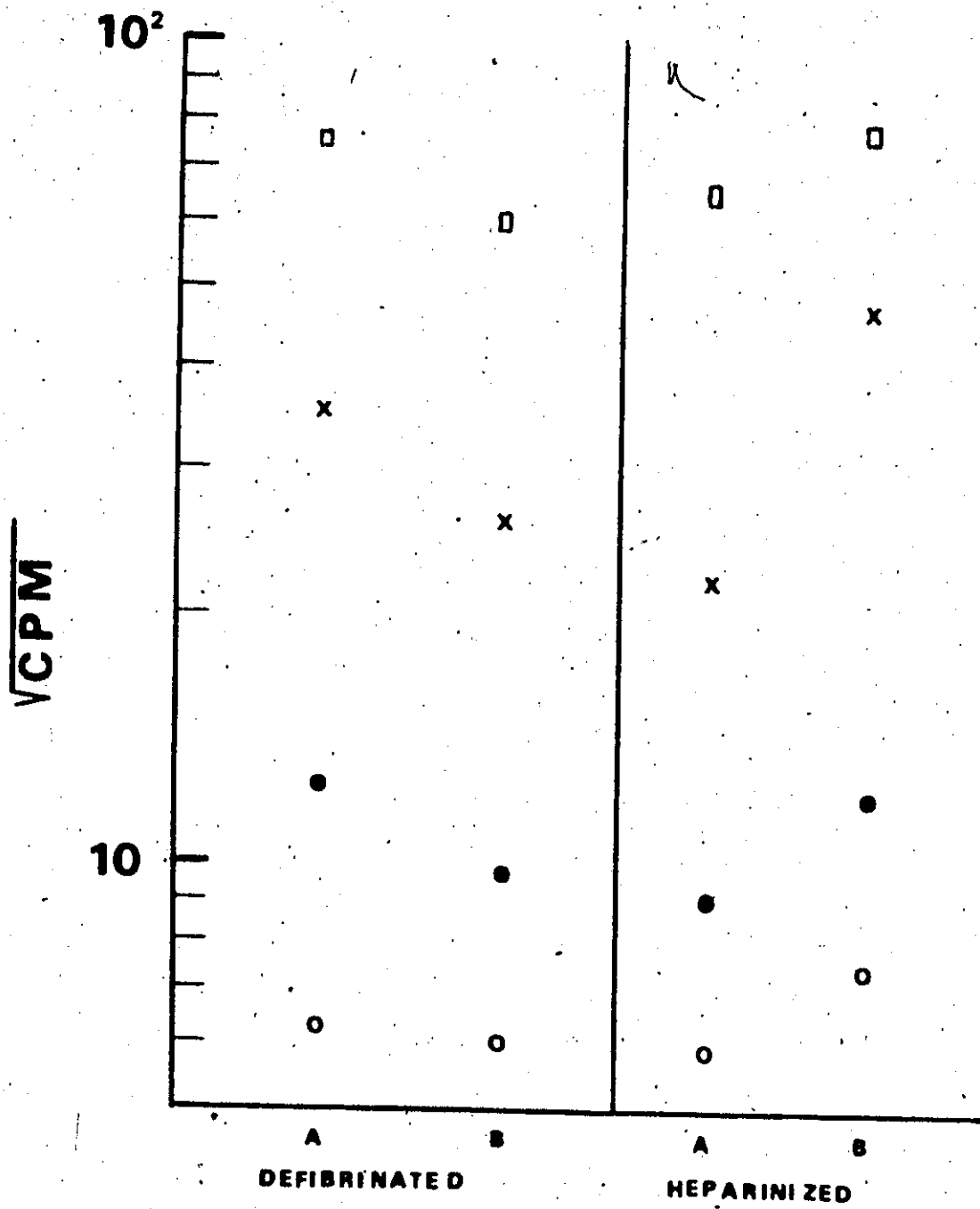
Fig. 3.6.2

Lymphocyte separation techniques.<sup>1</sup>



1. Cat peripheral blood lymphocytes were purified by several methods prior to culture. The mitogen responses are shown in Fig. 3.6.3.

Fig. 3.6.3. Effect of separation method on mitogen response. Gelatin sedimented cells from defibrinated blood responded as well as or better than cells prepared from cat blood by other methods.  
(A=gelatin sedimented; B=buffy coat; O=no mitogen; ●=PHA; x=PWM; □=Con A).



$^3\text{H-TdR}$  than cultures of cells from other fractions or cells prior to F-H separation. In the same experiments, lymphocytes from different fractions were mixed at a 9:1 ratio and cultured at a density of  $10^6$  cells/ml. Only those cultures containing mostly cells from fraction B responded to PHA.

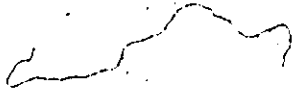



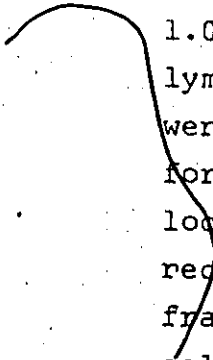


Fig. 3.6.4. Ficoll-Hypaque separation of cat peripheral lymphocytes. To prepare the column (a) 1.5 ml of Ficoll-Hypaque (F-H), specific gravity 1.055, was layered on top of an equal volume of F-H of specific gravity 1.095. About  $10^7$  gelatin-sedimented lymphocytes in 0.6 ml medium without serum were layered on top. After centrifugation for 40 minutes at 400g the cells were localized in three areas (b). Cells were recovered from these areas and designated fractions B, C and D. Gelatin-sedimented cells prior to F-H purification were designated Fraction A.



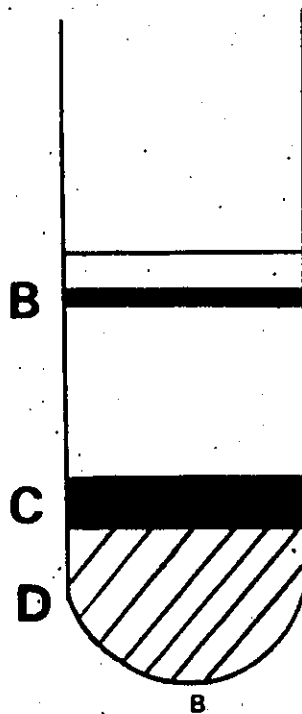
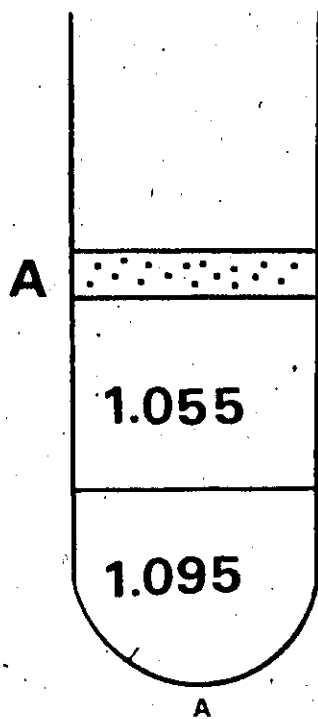


TABLE 3.6.2.

Mitogen response of Ficoll-Hypaque separated cells.<sup>1</sup>

Fraction	O	PHA					PWM	Con A
		a	b	c	d	e		
A	6.6	7.9	8.2	8.7	9.2	7.7	22.7	38.5
B	12.7	20.6	23.4	27.2	28.0	27.3	81.3	78.1
C	7.8	6.3	8.6	10.2	9.5	11.5	28.3	15.2
D	6.4	7.7	8.0	8.0	7.6	8.4	20.1	30.2
<u>9:1</u>								
C:B	6.4		8.9			12.9		
C:D	8.1		6.6			8.0		
D:B	6.9		11.0			12.9		
D:C	7.0		7.8			13.0		
B:D	11.6		27.8			29.7		

1. Cat peripheral lymphocytes were separated on a Ficoll-Hypaque column and the fractions were designated as shown in Fig. 3.6.4. Cells prior to F-H separation were designated fraction A. In all cases lymphocytes were cultured at a density of  $10^6$  per ml. The responses to no mitogen, 5 concentrations of PHA (a>e), PWM and Con A are expressed in square root CPM. The lower portion of the table shows the response to PHA of cultures containing cells from different fractions combined at a ratio of 9:1. Cells from fraction B show a dramatic response to PHA and improved responses to PWM and Con A.

TABLE 3.6.3.

Mitogen response of Ficoll-Hypaque separated cells.<sup>1</sup>

Fraction	O	a	PHA			d	e	PWM	Con A
			b	c					
A	5.0	9.2	8.3	9.4	11.3	10.9	43.2	57.8	
B	11.3	25.0	23.8	31.2	36.4	33.7	115.9	167.6	
C	5.9	4.9	5.6	5.5	5.6	5.9	7.4	5.3	
D	5.1	6.0	5.5	4.9	5.5	4.5	4.9	6.9	

9:1

C:B	5.8	5.9	7.3
C:D	5.7	5.1	5.2
D:B	5.4	6.2	5.5
D:C	5.2	5.0	5.4
B:D	7.4	17.4	23.8
B:C	9.2	25.5	24.8

1. Cat peripheral lymphocytes were separated on a Ficoll-Hypaque column and the fractions were designated as shown in Fig. 3.6.4. Cells prior to F-H separation were designated fraction A. The responses to no mitogen, 5 concentrations of PHA (a>e), PWM and Con A are expressed in square root CPM. The lower portion of the table shows the response of PHA of cultures containing cells from different fractions combined at a ratio of 9:1. Cells from fraction B show a dramatic response to PHA which is not suppressed by cells from fraction D or G.

#### 4. Discussion

##### 4.1 General discussion

The initial objective of the present investigation was readily attained. The development of a reliable cat lymphocyte culture system was accomplished simply by using the mitogen Con A in place of PHA. The stimulation of cat lymphocytes by PWM as advocated by Bilz et al. (1967) was invariably inferior to stimulation by Con A. Incidentally, the mitogenic properties of Con A were not recognized until 1968 (Leon and Powell, 1968).

The observation that cat peripheral lymphocytes responded poorly to PHA was intriguing since lymphocytes of most species respond well to this mitogen. Suspicions that culture conditions might not be optimal were not confirmed since varying the culture parameters, cell, serum and mitogen concentration failed to improve the response to PHA. Nevertheless, other experiments concerning diverse aspects of lymphocyte behavior, mercury stimulation, ouabain inhibition of mitogen stimulation, etc, demonstrated that cat lymphocytes reacted under these conditions similarly to cells of other species.

Experiments showing that peripheral lymphocytes of some cats do respond while similar cells from other individuals do not led to an hypothesis of inter-individual differences in PHA responsiveness, possibly genetic in origin. This reasoning was rejected when it became evident that the response to PHA of spleen cells was considerable even from cats whose blood cells were unresponsive. However, a suitable explanation for the poor PHA responsiveness of peripheral lymphocytes is lacking.

A number of hypotheses can be advanced to explain the slight or negligible response of cat peripheral lymphocytes to PHA:

- 1) anatomical compartmentalization of PHA-responsive cells.
- 2) cryptic PHA receptors on circulating cells.
- 3) destruction of PHA-reactive cells during separation.
- 4) presence in blood of PHA suppressor cells.

1) Anatomical compartmentalization of PHA-responsive cells. The data gathered in the present investigation appear to be entirely consistent with the contention that cat PHA-reactive cells are present in low numbers in the peripheral blood and are more concentrated in the spleen and/or other organs. A similar situation may exist in the rabbit where spleen cells respond much better to PHA than do peripheral blood cells (Mansfield and Wallace, 1973; Sell and Sheppard, 1973). On the other hand compartmentalization of PHA-responsive cells may be less pronounced in some other species such as the mouse (Fowler et al., 1971).

This hypothesis would be invalidated in the event of substantiation of any of the following hypotheses.

- 2) Cryptic PHA receptors on circulating cells. Two lines of evidence tended to discount this hypotheses. First, blood and spleen lymphocytes attached equally well to PHA coupled to agarose

beads, although the ability of this technique to distinguish quantitative differences was not established. In addition, the inability to consistently improve the PHA response of peripheral lymphocytes by pretreatment with proteolytic enzymes provided no evidence that the difference between spleen and blood cells is based on cryptic PHA receptors.

- 3) Destruction of PHA-reactive cells during separation. Experiments to compare the mitogen response of gelatin sedimented and buffy coat lymphocytes from both defibrinated and heparinized blood did not support this hypothesis.
- 4) Presence in blood of PHA suppressor cells. The concept of a cell population present in blood but not spleen that suppresses the response to PHA could account for the differential response of blood and spleen cells to PHA. The data in Tables 3.6.2 and 3 do not support this hypothesis. In these particular experiments cat peripheral lymphocytes were separated into three fractions on Ficoll-Hypaque columns. Cultures of cells from only one fraction responded to PHA. Cells from the two PHA-unresponsive fractions did not suppress the response of the responsive cells.

Hypothesis number one is a reasonable explanation for the poor PHA responsiveness of cat peripheral lymphocytes. It is supported by the data in Tables 3.6.2 and 3 which appear to show an enrichment of PHA reactive cells in fraction B. The alternate

hypotheses, numbers two to four, receive little support from this study.

#### 4.2 Conclusions

1. A reliable culture method for cat lymphocytes was developed.
2. The stimulation of cat lymphocytes was inhibited by  $3.5 \times 10^{-6}$ M ouabain.
3. Attempts to modify the response of lymphocytes to mitogens by proteolytic enzyme treatment gave variable results. Trypsin was not mitogenic to cat cells cultured in serum-free HB 597.
4. Periodate was a poor mitogen for cat lymphocytes.
5. Cat and human lymphocytes were stimulated by mercuric chloride; cells did not respond to methyl mercuric chloride.
6. Cat peripheral lymphocytes responded well to Con A and PWM, but poorly to PHA.
7. Splenic lymphocytes, even from cats whose blood cells were unresponsive, responded well to PHA.
8. The poor response of peripheral blood cells to PHA was explained on the basis of a low concentration of circulating PHA - responsive cells.

#### 4.3 Future directions suggested

Research in several areas seems indicated. First, breeding programs with PHA responders and non-responders to develop lines would provide enough animals for further investigation of various aspects of the PHA response. In addition, attempts should be

made to distinguish cat T and B lymphocytes on the basis of cell surface characteristics.

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