EFFECTS OF PHYTOHEMAGGLUTININ

ON RAPIDLY-GROWING MAMMALIAN CELLS

by

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INTRODUCTION

STATEMENT OF THE PROBLEM

The awareness that phytohemagglutinin (PHA*) had growth-inhibitory properties originated with an observation in our laboratory. The observation was made in conjunction with continuing studies on the catabolic effects of cortisol on lymphoid tissue. Gabourel and Aronow (1) had previously shown that cortisol would inhibit multiplication of Mouse Lymphoma (ML-388) cells growing *in vitro*. Because of its reported mitogenic effects on lymphocytes (2, 3), PPHA** was tested for its ability to reverse the growth-inhibitory effects of cortisol in this tissue culture system.

Mitogenic concentrations of PPHA (4, 5) were added to replicate cultures of ML-388 cells which had been treated with various concentrations of cortisol. No reversal was observed, but PPHA was found to be growth-inhibitory to control cultures which had not received cortisol. This growth inhibition was seen with concentrations of PPHA known to induce blastogenesis and mitogenesis in nongrowing cultures of peripheral lymphocytes.

Therefore, I have undertaken the investigation of the growth-inhibitory effects of PPHA on several rapidly-growing mammalian cell lines, both *in vivo* and *in vitro*, with particular emphasis on the effects on DNA, RNA and protein synthesis.

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*The abbreviation "PHA" will be used to designate any phytohemagglutinin preparation when a particular preparation is not indicated. Other appropriate abbreviations will be used when necessary to specify the source or degree of purity (See Methods and Materials).

**PPHA: purified protein phytohemagglutinin.

GENERAL INTRODUCTION

The term, "phytohemagglutinin", (PHA) means any substance isolated from plants, which is capable of agglutinating erythrocytes, but their agglutinating activity is often not limited to red blood cells alone (6-12). The subject of plant hemagglutinins, also called "lectins", was reviewed by Boyd (13). He reported that shortly after the discovery of plant agglutinins in 1888, Ehrlich and Landsteiner showed that these substances possessed blood group specificity (14, 15). This interest in plant agglutinins continues today for, during the ensuing 80 years, a number of remarkable effects have been observed in a wide variety of biological systems.

There have been several comprehensive reviews in the last two years concerning the biological effects of PHA (16-18). A partial list of these biological actions of PHA in addition to the agglutination of many cell types includes: a) blastogenesis and mitogenesis in both lymphoid and nonlymphoid tissues (16-19); b) possible immunosuppression activity (20-23); c) inhibition of growth both *in vivo* (24-29) and in some specialized systems *in vitro* (10, 29, 30); and d) the suggestion of therapeutic actions (10, 11, 31-37).

ISOLATION, PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF PHA

While hemagglutinins have been isolated from various plant sources (11-13, 24, 30, 38-42), the seeds of leguminasae have been the most common source (3-9, 13, 16-18, 43-52). Boyd (13) and others (16-18, 43-45, 49, 50) have reported that the plant hemagglutinin preparations described

and used by most early workers were purified to a greater or lesser extent, and this may account for some of the observed differences in biological activity.

Only a few investigators (4, 5, 25, 43-50) have attempted the characterization of PHA preparations isolated from different varieties of Phaseolus vulgaris and the results are summarized (Table 1). The first partially purified preparation of Rigas and Osgood (43) was a mucoprotein containing about 50 percent carbohydrate, with a high agglutination titer. This mucoprotein dissociated into an inactive polysaccharide and a protein hemagglutinin when exposed to an environment of low pH. The mucoprotein form and the protein form were powerful panagglutinins since they agglutinated all types of human and animal erythrocytes tested. The fact that both forms possessed mitogenicity indicated that the polysaccharide part was not essential for activity.

Börjeson et al. (45) noted that as the carbohydrate content of their preparation decreased there was an increase in mitogenic activity. The hemagglutinating property was still present but they claimed that it could be removed by adsorption with erythrocytes without a detectable loss of mitogenicity. This suggested that the two activities of agglutination and mitogenicity were not exerted by the same molecular entity.

It had been argued whether these two properties were possessed by the same protein (8, 9, 43-45, 49, 50). Rigas and Johnson (4) undertook the task of preparing a protein PHA (PPHA) homogeneous by eleven different criteria, not counting the different pH's used with the varying types of electrophoresis. This material was found to possess both

TABLE 1

Comparison of the Physicochemical and Biological Properties of Phytohemagglutinins Isolated by Different Investigators from Different Varieties of *Phaseolus vulgaris*.

Property	Red Kidney Bean			Black Kidney Bean	Wax Bean
	A	B	C	D	E
Sedimentation Coefficient ($s_{20,w}$)	6.5S	-	6.5S	5.9S	5.37S
Diffusion Coefficient ($D_{20,w,}$) ($cm^2 sec^{-1} \times 10^{-7}$)	4.8	-	6.36	-	3.7
Molecular Weight	138000	-	89000	126000-130000	121000-132000
Isoelectric point, pH	6.5	-	-	4.9	5.5
Partial specific volume (ml/g)	-	-	-	0.683	0.728
Carbohydrate Content (%)	3.4	6	10.0	5.7	10.4
Optimum Mitogenic Dose (μg/ml culture medium)	5.0	0.2-1.0	2.0-3.0	-	-
Hemagglutination Property Tested	Yes	Yes	Yes	Yes	Yes
Toxic Properties Assayed	Yes	-	-	Yes	-

A - (4, 5, 44)
B - (45)
C - (46)
D - (24, 25, 48)
E - (47)

activities. Although a minor component was detected with reduced agglutinating ability when the material was adsorbed on erythrocyte stroma, the agglutinating activity and the mitogenic activity were both confirmed as properties of the major protein component. No conclusions were drawn concerning the relationship of the mitogenicity to the agglutinating ability of the major protein component.

In order to confirm the presence of hemagglutinin and mitogen properties on the same substance, Rigas et al. (16, 44) undertook structural studies of the PPHA molecule. They verified the possibility of a single substance possessing two different biologically active sites. Using a homogeneous preparation they demonstrated the complexity of the protein phytohemagglutinin molecule. Under the conditions of 8.0 M urea at neutral pH they were able to dissociate their PPHA into eight biologically active subunits with sedimentation coefficients of 1.5S ($s_{20,w}$) and separable by starch gel electrophoresis. One type of subunit was responsible for its mitogenic activity and another for its hemagglutinating activity. Thus the single protein (PPHA) contained both mitogenic and hemagglutinating activities and these activities were associated with different subunits of the protein (5).

A common problem encountered in the purification of PHA was the difficulty with which carbohydrate material could be dissociated from the protein. It therefore became important to establish that any carbohydrate associated with the purified protein was in fact covalently bound to the protein. Therefore Johnson (51) along with Takahashi and Leiner (52) isolated, purified and characterized the Phaseolus vulgaris glycopeptides of the PHA from the red kidney bean and the wax bean,

respectively. Johnson prepared a tryptic glycopeptide fraction with a molecular weight range of 3000-9000 from pure PPHA (44) which yielded eight glycopeptides. They were shown to be different in their amino acid composition. In contrast, Takahashi and Leiner (52) isolated a single glycopeptide after pronase digestion of PHA which had a molecular weight of about 4380. The carbohydrate was characterized as being a complex heteropolysaccharide. These differences may reflect either the bean source of the PHA (Table 1) or the method of digesting the PHA. The most homogeneous preparations of PHA from different laboratories have few similarities (Table 1). They are glycoproteins containing three to ten percent carbohydrate and have a molecular weight in the range of 89,000 to 138,000.

BIOLOGICAL PROPERTIES OF PHA

Agglutination. Rigas and Osgood (43) classified PHA as a panagglutinin after observing agglutination of the erythrocytes of the horse, pig, dog, cat, rabbit, chicken and frog. That this hemagglutination resulted from the action of a protein had been suggested even earlier (6, 10-15, 53) and was later confirmed (44-52). First, Hastings et al. (8) and later others (7, 9, 54) reported that leukocytes also formed clumps in the presence of PHA, and they speculated on the biological significance of this mixed agglutination. Later, Tunis (7) detected both the customary erythrocyte agglutinin and a second agglutinin reportedly devoid of hemagglutinating activity that was capable of agglutinating a variety of nucleated cells. Tumor cells grown in tissue culture

and transplantable mouse tumor cells in the ascites form, together with normal lymphocytes and human leukemic leukocytes, were also agglutinated.

Earlier, Nungester with Van Halsema (10) and others (30) had investigated the possibility that biologically active substances such as phytoagglutinins from a variety of sources might selectively interact with the surface of mammalian tumor cells due to the complexity of their membranes. Steck and Wallach (12) showed that although the precise mechanism for agglutination is not known it appears to be an intercellular ligand function of the PHA molecule. It has been shown that PHA can affect the surface charge of lymphocytes as seen by alterations in the electrophoretic mobility of the cells (55, 56).

The nature of the relationship between erythroagglutinating, leukoagglutinating and lymphocyte mitogenic properties of PHA was obscure until attempts were made to separate these actions. An early approach to the problem involved the fractionation of a PHA preparation and then testing the various fractions for agglutinating and mitogenic activities. Early results using this approach were variable since preparations of variable purity were used (24, 25, 43, 45-50). More recently Rigas et al. (4, 44) demonstrated that homogeneous PPHA preparations under certain conditions could be dissociated into eight subunits with separate biological activities. Rivera and Mueller (62) also presented data for the separation of the hemagglutinating activity from the mitogenic activity of PHA.

Others (7, 9, 54, 57, 58, 60-62) used a second approach to demonstrate separation of the properties. Common to the second approach was the preabsorption of their preparations with red blood cells and/or

leukocytes before assaying for agglutination or mitogenicity. According to the reports hemagglutinating activity could consistently be removed without apparent alteration of either leukoagglutinating or mitogenic activities. When the leukoagglutinating activity was removed, however, the mitogenic activity was lost. These findings were viewed by many as evidence that leukoagglutination was involved in lymphocyte transformation in vitro. Yet there were reports that lymphocyte transformation could occur in the absence of leukoagglutination when other mitogens were employed (59). Tunis (60) found further that ethylenediaminetetraacetic acid would inhibit only the erythroagglutinin activity.

Borberg (63, 64) was the first to report inhibition of the PHA-agglutinating activity by N-acetyl-D-galactosamine and a number of simple sugars. Börjeson, Chessin and Landy (59) were recently successful in dissociating the leukoagglutinating activity and transforming properties of a purified PHA as well. They achieved this by coating human peripheral lymphocytes with Vi polysaccharide (a highly polymerized acidic polysaccharide of N-acetyl galactosaminuronic acid polymer) from E. coli. This material prevented the lymphocytes from being clumped by PHA yet these cells were capable of transformation to blast cells. The Vi polysaccharide was shown to act by altering the physical character of the cell surface in such a way that fixed antibody could no longer affect lattice formation and bring about agglutination. The authors claim that by itself Vi antigen did not produce any significant transformation. Their results appeared to rule out leukoagglutination as being causally related to lymphocyte transformation.

Mitogenesis. The idea that PHA might possess mitogenic activity originated with Li and Osgood (65) as they were attempting to develop methods for long-term culture of human leukemic leukocytes in vitro. According to Rigas (44), Li had speculated that the bean seed, a plant embryo, might contain growth-promoting factors analogous to those found in chick embryo extracts used for other mammalian cell cultures. Although they did not demonstrate the presence of a growth-stimulating factor (mitogen) in their red kidney bean extracts, Li and Osgood (65) did report a simple method employing PHA for rapid and efficient separation of leukocytes from whole blood.

Hungerford et al. (2) and later Nowell (3) observed an increased number of mitotic figures in their leukocyte preparations obtained by the PHA separation method of Li and Osgood (65). This demonstration that PHA was responsible for the mitogenic effect in mononuclear leukocytes, lymphocytes and macrophages sparked an interest in lymphocyte transformation.

The benefits derived from Nowell's original observation have been many and include a technic for chromosome preparations for karyotyping cells used in the study of genetic disorders (66, 67), a test system to study immune responses in vitro (68, 69), a cell culture technic for transformation of many different and otherwise non-proliferative tissues in vitro (70, 71), and a tool for the study of the mechanisms of cell differentiation and division (72, 73).

Mitogenic transformation by phytohemagglutinin is characterized by blastoid formation and the subsequent induction of mitosis. Studies comparing normal and PHA-treated tissue established (16-19, 74-81) the

criteria for demonstrating the morphological and biochemical events of transformation. They include increases in the following: cell size, percentage of chromatin as euchromatin, rate of radioactive precursor uptake into RNA, DNA and protein, rate of synthesis of RNA, protein and DNA, mitotic index, and cell number. Therefore, transformation of target cells is a process which is manifested by both biochemical and morphological alterations.

The numerous materials with mitogenic properties capable of stimulating lymphocyte transformation have been classified into three groups: nonspecific stimulants, tissue antigens, and specific antigens (82-91). The nonspecific classification of some agents refers to their unknown mechanism of action and to distinguish them from the specific agents which require prior sensitization of the donor cells for effectiveness. It is assumed that there are specific sites for all of these mitogens on the target cells.

PHA is a prototype of the nonspecific stimulants. A partial list includes: streptolysin-S (82) and staphylococcal filtrate (83, 84) as well as phytomitogens such as pokeweed mitogen from Phytolacca americana (75) and other plant extracts (39). The tissue antigen group of blastogenic agents includes both mixed lymphocytes cultured from two unrelated (homologous) donors (75, 83) and leukocyte antiserum (8, 84, 88). The last group of mitogens includes such specific antigens as tuberculin-purified protein derivative (PPD) (89-91), streptolysin-O (82, 83) and tetanus toxoids (91).

TARGET CELLS FOR BIOLOGICAL ACTIONS

There is extensive and compelling evidence relating the "small" lymphocyte to a number of important functions, both immunological and non-immunological (92-97). The "small" lymphocyte was considered the primary target for the blastogenic action of PHA and other mitogens by Nowell (3). Similar observations were described by others (16-19, 92-97). Spontaneous transformation of lymphocytes to blastoid forms in culture has been suggested by Sabesin (98) and others (99) and refuted by both Hashem (100) and Caron (101).

The lymphocyte has played an important role in the study of blastogenesis and mitogenesis due to PHA. A variety of other normal tissues, both lymphoid and nonlymphoid from a number of species including man have been considered, as well. Together they comprise an extensive list of cell types which have been reported to be transformed by PHA. Some of these are the lymphocytes of man (92-101), rabbits (102), fish (103), rats (104), mice (105, 106), kangaroos (107), guinea pigs (108), monkeys (109), cows (110) and horses (111). Other lymphoid tissue of man and rodent stimulated by PHA includes the cells from lymph nodes (112), tonsils (113), spleen (114), appendix (115), thymus (116-118) and even the bursa of Fabricius of chickens (119). In addition, the stimulatory action of PHA has been investigated in such nonlymphoid tissue as epithelial cells (71) and certain tumor cell lines from long-term cultures (120). PHA has even been shown to be a mitotic stimulator of non-mammalian cells (121-123).

In addition to these PHA-induced transformations seen *in vitro*, there are reports of PHA-induced transformations *in vivo* for guinea pig skin (70, 124, 125) and mouse thymus, spleen and lymph node cells (126-129).

The bone marrow has also been proposed by some (34-36) as a target stimulated to erythropoiesis by PHA, both *in vivo* and *in vitro*, but the clinical and experimental data is vast, confusing and conflicting (37, 130-134).

There is considerable evidence that a number of tissues, both lymphoid and nonlymphoid, demonstrate a blastogenic response which is retarded, marginal, or fails entirely following mitogen exposure (135-137). Bone marrow and thymus cells are examples of lymphoid tissues in which such retarded or marginal responses occur following exposure to PHA(116, 118, 138-142).

One of the most useful methods for studying the mitogenic effects of PHA is the tissue culture system described by Nowell (3), Moorehead et al. (66) and others (143-147). The tissue is added to the complete growth medium in the form of a cell suspension. PHA is then added and the culture is incubated for periods up to one week. PHA induces lymphocyte transformation in this tissue culture system characterized by enlargement of small lymphocytes to larger blast cells. The changes resemble, both morphologically and biochemically, those seen in antigen-stimulated lymphocytes *in vivo*.

Many conflicting reports, using the tissue culture system, have appeared in the literature concerning target cells for the mitogenic effects of PHA (135-142). Inconsistencies between results of various laboratories were explained on three bases: a) possibility of multiple mitogens in crude PHA preparations used (137); b) heterogeneity of cell types present in cultures (135); and c) reflection of the nature of the particular mitogen used (137). Results obtained with this system are

are now known to be affected by a number of parameters which most investigators failed to consider. To explain (at least in part) the confusion which now exists, a partial list of those factors known to affect the culture system itself is given below:

a) cell number, duration and surface area of the culture (148);

b) components present in the serum or plasma added to the medium (149-152);

c) concentration and relative mitogenic potency of the PHA used (153-155);

d) incubation conditions and cell survival (156, 157);

e) contamination of cell cultures by mycoplasma (158-160) or viruses (161-163);

f) action of chemical (164-171) and humoral (172-176) agents used in conjunction with PHA;

g) action of anti-PHA antibodies (177-179).

A partial list of those factors related to pretreatment or prior conditioning of the tissues before being placed in the culture system includes:

a) effect of prior treatment of donor with PHA *in vivo* (180);

b) age and specie of the donor (181, 182);

c) relative immunocompetence of the tissue (183-185);

d) abnormal condition of donor at time of sampling (186-190);

e) responsiveness of tumor cells (191-196).

ON THE MECHANISM OF THE MITOGENIC ACTION OF PHA

Although the factors which determine cell responsiveness to the mitogenic effects of PHA (or other mitogens) are not understood, much

data is available concerning the morphological and biochemical events which take place in the cells which are transformed.

Morphological Events of Transformation. Direct examination of the morphology for gross changes was undertaken, using both light and phase microscopy (72, 73, 80, 81, 97). For detailed ultrastructure evaluation electron microscopy was used (74, 76, 198-200). Less direct approaches were used to correlate the related biochemical and immunological events, including cytochemical technics (138, 201-204), histochemical methods (205-207) and autoradiography (208-210).

According to Daniels, Ritzmann and Levin (97) and others (92-96), during stimulation cells undergoing transformation exhibit intermediate blastoid forms characterized by increases in both nuclear and cytoplasmic volume until finally a large cell results, capable of undergoing mitosis. In small lymphocytes stimulated to blastoid formation, the cell diameter may be three times that of the non-stimulated cell. One of the earliest changes occurs in the nucleus and is the conversion from a pachychromatic (heterochromatic) state to a leptochromatic (euchromatic) state. The cytoplasmic alterations follow those of the nucleus and the entire process can require a period up to 72 hours. The details of blastoid morphology have been described (92-97).

Biochemical Events of Transformation. The biochemical events of PHA-induced transformation in lymphocytes have received extensive study through the direct application of the sophisticated methods of biochemical analysis currently available. The site of action of PHA has received

some consideration in addition to the nature and sequence of biochemical events which occur during blastogenesis and mitogenesis. Investigators have attached various labels to PHA molecules such as fluorescein isothiocyanate (211, 212), ^{125}iodine (213), and mercury (214), with inconclusive results with respect to the cellular localization of PHA when the labelled molecule is used to stimulate target cells. Stanley, Frenster and Rigas (215) have added tritiated-phytohemagglutinin (highly purified) to human peripheral leukocytes. They prepared ultrathin sections for electron microscopic autoradiography and quantitated the observed grains. By 24 hours after PHA localization the grains within cells were found in both PHA-stimulated lymphocytes and in non-PHA-stimulated granulocytes. Most of the grain counts which occurred in the granulocytes were found in the cytoplasm, while the grains within lymphocytes were present over both cytoplasm and nucleus. Within the lymphocyte nucleus the grains were localized almost exclusively over condensed masses of repressed heterochromatin, as compared to the extended microfibrils of active euchromatin.

In addition, there is circumstantial evidence that PHA can, and probably does, enter stimulated cells at very early times after its addition to the cultures, and that it soon begins to exert its effect. The uptake of foreign proteins into mammalian cells in culture has been demonstrated (216). Adsorption has been shown to occur within a few minutes and is independent of time and temperature, while the subsequent rate of uptake is a function of both time and temperature.

That similar mechanisms control PHA uptake into leukocytes is suggested by the results of several investigators (217-221). Stefani

and Schrek (217) examined the cytotoxic effect of nitrogen mustard (H_2N) on PHA-treated lymphocytes. They found that 56 percent of their control cells survived seven days in culture. The addition of PHA to the H_2N-treated cultures permitted 40 percent of the treated cells to survive seven days at a dose of H_2N which killed all cells when PHA was omitted. This protective effect of PHA was seen if it was added from four days to thirty minutes before H_2N. If PHA was added up to 24 hours after H_2N, the survival rate was greatly reduced. Elves, Gough and Israels (218) reported that prednisolone added to lymphocyte cultures before or simultaneously with PHA produced significant inhibition of lymphocyte transformation. Lesser inhibition was reported if prednisolone was added at only five minutes after PHA; after ten or more minutes following PHA addition, prednisolone caused no inhibition. Thus, the time dependence of the protective effect of PHA against the cytolytic effect of steroids is suggestive of a rapid mechanism for this effect.

Naspitz and Richter (219) have reported that the incubation of leukocytes with PHA for only five minutes followed by an incubation for three days in PHA-free medium resulted in transformation. However, for maximum blastogenesis an incubation time of six hours with PHA was required. They further showed that cell-bound PHA was not metabolized by the cells during incubations up to three days. Sonicated preparations of PHA-treated cells were tested for their ability to induce blastogenic activity in previously untreated target cells. The sonicated preparations from cells incubated with PHA for as little as six hours displayed maximum stimulation. Further incubation of the cells for three days before testing for activity did not significantly decrease

the mitogenic activity of the preparations. Preparations from cells not treated with PHA or from PHA-treated cells subsequently subjected to heat inactivation did not display blastogenic activity. Finally, Kay (220) has reported an increase in the rate of protein synthesis as early as two to three hours after PHA was added to lymphocyte cultures. The earliest detected increase in the incorporation of uridine into RNA was found one hour after PHA (221). Larger stimulations in incorporation rate were seen after incubations of three to six hours.

The PHA-stimulated lymphocyte has provided a convenient test system in which to investigate the role of nucleohistones in the regulation of DNA-dependent RNA synthesis. Several authors (111, 222-226) have suggested that acetylation and phosphorylation of histones appear to be early events in the process of derepression. These processes were shown to reach a maximum rate before there is a significant increase of RNA synthesis in lymphocytes undergoing PHA-induced mitogenic stimulation. Acetylation and phosphorylation did not necessarily require new histone synthesis in order to occur.

Until now only a few results have been reported on the phospholipid synthesis of lymphoid cells. Among the first were those of Rose and Frenster (227) who isolated two forms of chromatin from calf thymus lymphocytes which they demonstrated to be free of nuclear membranes. One form of chromatin was repressed (heterochromatin) and the other active (euchromatin) in RNA synthesis. The lipids of these chromatin fractions were isolated and identified and their ability to incorporate ^{14}C-glycerol was measured. They concluded that active chromatin was much richer in phospholipids than repressed chromatin, and that the lipids of active chromatin had a much more active metabolism.

Three different laboratories have reported on the lipid and phospholipid metabolism of PHA-stimulated lymphocytes (228-230). As early as 30 minutes after PHA addition to lymphocyte cultures, Fisher and Mueller (228) observed accelerated incorporation of $^{32}PO_4^{\equiv}$ into phosphotidyl inositol; by 12 hours after PHA addition they detected a stimulated incorporation into phosphotidyl choline. Conversion of newly synthesized phosphotidyl inositol into phosphotidic acid was observed within six to seven hours after PHA addition. Fisher and Mueller proposed that the activated phospholipid metabolism facilitated protein secretion such as gamma globulin.

Kay (229) noted that the rapid increase in the rate of incorporation of labelled lipid precursors into PHA-activated lymphocytes was not mediated through the effects of PHA on RNA and protein synthesis. His evidence was that ^{14}C-choline incorporation was not affected either by the addition of a concentration of actinomycin D sufficient to inhibit the incorporation of ^{3}H-uridine into RNA by 85-90 percent or by the addition of a concentration of cyclohexamide sufficient to inhibit protein synthesis by 95 percent. It remains to be established whether this rapid increase in turnover or metabolism of lymphocyte lipid after PHA addition is causally related to lymphocyte transformation or whether it is prerequisite to some subsequent event.

Huber et al. (230) failed to detect any difference in the distribution of ^{14}C-acetate in phospholipid fractions separated by thin layer chromatography from both normal and PHA-treated lymphocytes. But they did confirm earlier reports (231) that mitochondria and microsomes play a leading part in the synthesis of phospholipids, at least in lymphocytes.

Particular attention has been focused on RNA synthesis by a number of investigators (178, 179, 233-242) and their results have led to the following conclusions:

a) Characterization of the rapidly synthesized RNA obtained from PHA-treated lymphocyte cultures was achieved by sucrose density gradient centrifugation. These studies revealed progressive changes in the synthesis patterns of different classes of RNA. The newly synthesized polydisperse RNA was relatively unstable (233-235);

b) The rapidly synthesized RNA which followed PHA stimulation of lymphocytes was mostly nonribosomal as determined from its sedimentation profile, its relative instability during "pulse chase" experiments (232*) in the presence of actinomycin D, and by its nucleotide composition. In contrast, lymphocytes stimulated to transform by a specific antigen such as streptolysin O produced a 45S material consistent with the accepted precursor which subsequently was converted to 18S and 28S ribosomal RNA (233, 235);

c) The sequence of events for ribosomal RNA production in PHA-activated cells was essentially similar to that reported for HeLa cells but represented only a small portion of the new, rapidly synthesized RNA (235);

d) Base composition determinations by ^{32}P incorporation indicated that the newly synthesized material was neither ribosome-like nor

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*Technic whereby the biological system is exposed to labelled nucleoside precursor for a brief period of time before incorporation is abruptly terminated by the addition of actinomycin D. The fate of the incorporated radioactivity is then examined with time in the absence of further incorporation.

DNA-like, since it was high in uridylic acid and low in adenylic acid content (236);

e) The active methylation observed after pulse labelling with methyl-^{3}H-methionine was strongly suggestive that the new RNA was non-messenger in nature, and probably consisted mainly of transfer RNA, known to be rich in methylated bases (237, 238);

f) The exponential acceleration of RNA synthesis in PHA-stimulated lymphocytes preceded the stimulation of protein synthesis and the initiation of DNA synthesis and the induction of mitosis. This PHA-induced stimulation of RNA synthesis was dose-dependent (239-242).

The synthesis of a variety of specific proteins appears to be stimulated by the addition of PHA to lymphocyte cultures such as gamma globulin, glycolytic enzymes, lysozymal enzymes and even interferon. Results have been presented (243-246) which show that the magnitude of stimulation of gamma globulin synthesis was insufficient to account for the total increase in cell protein synthesis seen after treatment with PHA.

Parenti et al. (246) also observed that the glycolytic activity of lymphocytes is considerably stimulated by PHA. They suggested that the active uptake of amino acids may also be stimulated because of the well-known relationship between energy metabolism and active uptake. The observed stimulatory effect of PHA on carbohydrate catabolism is well documented in the literature (78, 247-251). MacHaffie and Wang (249) have reported a significant enhancement of the pentose phosphate shunt for glucose utilization. They interpret these findings to be a reflection of an increased TPNH demand by the lymphocytes. Pulgar, Foster and

Cooperband (250) have noted that the increased cellular activities following PHA activation are dependent on glycolysis rather than on respiration. This observation resembles in many respects what has been reported for other rapidly-dividing cells (251).

Several laboratories (205, 207, 252, 253) have submitted histochemical evidence suggestive of a high activity by the dehydrogenase enzymes of the glycolytic and Krebs cycle enzymes as well as those of the pentose phosphate shunt. These dehydrogenases have received much attention (254-256). Other enzymes studied include specific esterases (207), phosphatases (205, 207), G-6-PD (204), lysozyme (257, 258) and uridine kinase (259, 260). PHA was also reported to be one of the nonviral inducers of interferon production (261).

As noted earlier by Bender and Prescott (262) and others (72, 79, 177, 263), PHA addition induced a very great increase in the rate of DNA synthesis and mitosis in transformed cells. This activity was generally measured by observing ^{3}H-thymidine incorporation by autoradiography (208-210) or by quantitative measurement (44, 101, 265). The first wave of mitosis following PHA addition was started by 42 to 48 hours and was preceded by DNA duplication. Studies of the growth phases of transformed lymphocytes after PHA indicated that a minimum of 24 hours was required for the initial G-1 phase and a minimum of 12 hours was required for the S or DNA synthetic phase. Peak activity of DNA synthesis occurred at about 72 hours and the G-2 phase occupied a minimum of two hours, but no more than six hours before mitosis (79, 177, 262).

Thus, a summary of the sequence of changes from early blastogenesis to mitogenesis after PHA stimulation includes:

a) First, changes in the deoxyribonucleoprotein (72, 79) with an increase in the acetylation of histones (222, 224, 225) plus the phosphorylation of histones as well as lipoproteins (223, 226) and phospholipids (227-230);

b) Then, RNA synthesis is accelerated as reflected in an exponential increase in the incorporation of uridine into RNA (232-242);

c) Somewhat later the cells in culture produce such proteins as gamma globulin (243-246) and a variety of cellular enzymes active in glycolysis (252-256) and other metabolic processes (204, 205, 207, 257-260);

d) Finally, there is initiation of DNA synthesis and the eventual induction of mitotic activity (72, 79, 177, 262, 263).

ON THE MECHANISM OF TARGET CELL SELECTION

A controversy exists in the literature as to why PHA stimulates transformation in some cells and not others. Some investigators have postulated a nonimmunological mechanism and others have suggested an immunological basis for PHA action in an attempt at clarification of the controversy.

Evidence Favoring a Nonimmunological Mechanism of Selection.

The recent studies on the cellular localization of PHA (215) suggest that absorption of PHA to the cell surface is not enough to explain the transformation of cells. On the basis of the available evidence Rigas (44) has suggested that the addition of PHA causes derepression of genetic information resulting first in RNA, then protein synthesis.

In turn, DNA synthesis and eventual cell division occur as a result of the synthesis of other necessary cell constituents. Induction of cell division in dormant cells has been associated with PHA localization within repressed heterochromatin (264). It has been suggested that purified PHA is polyanionic in character at physiological pH (4) and might be expected to localize within electrostatically-neutral heterochromatin, rather than within euchromatin already containing an excess of polyanions (264).

Additional supportive evidence of a nonimmunological action of PHA is suggested by Daniels, Ritzmann and Levin (97). They have summarized the differences between the nonspecific PHA-type stimulation and specific antigenic stimulation with respect to the number of mitoses seen and the manner of their formation. The evidence includes the observations that a) prior sensitization is required for specific antigen-induced transformation; b) only 5 to 47 percent of a lymphocyte population undergoes antigenic blastogenesis compared with greater than 80 percent for PHA; and c) blastogenesis is slower by nearly 48 hours in antigenic stimulation than in PHA cultures.

Some additional evidence suggesting a nonimmunological action of PHA includes a) the demonstration that PHA can increase the output of specific antibody in vitro from lymphocytes previously immunized to specific antigens (265); b) the repeated demonstration of PHA stimulation of nonlymphoid tissues (120, 124, 125) and even nonmammalian cells (121-123), and c) the lack of a summation of effects when lymphocytes sensitized to a variety of specific agents are incubated with a number of these specific antigens (266, 267).

PHA and other mitogens have been shown to increase the lysosomal enzyme activity of stimulated lymphocytes, as seen by histochemical technic. These studies suggest PHA-induced labilization of lysosomal membranes (257). Moreover, substances considered to stabilize lysosome membranes such as chloroquine and prednisolone also inhibit PHA stimulation (268).

The nonspecific attachment of PHA to lymphocyte cell surfaces has been observed during leukoagglutination (269, 270). Other effects related to cell surface changes have also been reported (55, 56), suggesting the hypothesis that cell clumping was required for stimulation. This appears unlikely since blastogenesis can occur without leukoagglutination (59).

Evidence Favoring an Immunological Mechanism of Selection.

The evidence favoring an immunological basis of PHA action reflects the selective stimulation of the lymphocyte after the addition of PHA but does not rule out the possibility that both specific antigens and phytomitogens may evoke a derepression phenomenon.

Some of the evidence offered in support of the immunological mechanism for target cell selection considers the morphological similarity of transformed lymphocytes as a consequence of incubation with PHA or specific antigens *in vitro* (271), the synthesis of gamma globulin by lymphocytes *in vitro* regardless of the mitogen (272-275), and the transformation of the small lymphocyte by PHA since it is the small lymphocyte which possesses all the attributes required for immunologic competence (92-97, 276, 277). There is also the correlation of impaired response to PHA and reduced immunocompetence seen with respect to thymus cells (139),

or leukemias (196, 278, 279) and related conditions (196, 280). Finally, it has been suggested (281) that PHA may be a heterogenetic antigen like Forssman antigen (282), that is, prior unintentional sensitization may occur for most species early in life.

IMMUNOLOGICAL ASPECTS OF PHA

Immunologists became interested in PHA following the early contributions relevant to the several, perhaps unrelated, properties of PHA. The biological actions of PHA (16-19) together with the characteristics of target cells (92-97) have been reviewed. These properties of PHA include the ability to agglutinate various cell types (previously discussed), its antigenicity (28, 283-286), its ability to indirectly induce lymphocytes to destroy target tissues in histocompatibility test systems *in vitro* (287-291), the relationship of specific antigens and PHA to lymphocyte synthesis or release of gamma globulin both *in vivo* (292-297) and *in vitro* (272-275) and, finally, the role of PHA in immunosuppression (20-23, 298-304).

That PHA itself may act as an antigen has been demonstrated by Marshall and Norins (283) who produced immune rabbit sera which inhibited the blastogenic effects of PHA when added to cultures of human peripheral lymphocytes. Astaldi (284) has also suggested that intravenous injection of PHA in man causes the formation of circulating antibodies capable of neutralizing the blastogenic action of PHA.

Lymphocytes from animals sensitized to specific antigens will aggregate with cells in culture which carry the specific antigens and cause their destruction (287, 289, 291). In addition, lymphocytes

from unsensitized animals can bring about destruction of target cells if PHA is present in the tissue culture medium (288, 290). PHA alone has been reported as producing no significant effect on the target cells in this system (289) and PHA together with lymphocytes only produces the usual transformation. This action does not require PHA-induced agglutination since specific antigens which do not produce lymphocyte aggregation still work in this system to cause the destruction of target cells (289). Cell destruction was usually demonstrated by measuring the release of radioactive isotope (^{14}C-thymidine or ^{51}Cr) into the incubation medium from prelabelled target cells.

It has been suggested that the above cytotoxic effects reflect the activated state of the immunocompetent lymphocytes after exposure to PHA or nonspecific tissue antigens because, first, there was a twofold increase in the cytotoxicity of these in vitro systems if PHA was present (289), and second, when other tissues less responsive to PHA (less immunocompetent) were used, the ability of the PHA-treated cells to destroy target cells was significantly lowered (139).

Thus, the cell culture system is considered to be a model for the study of cellular and humoral antigens (287-291). The mechanism by which the cytotoxicity in vitro is brought about by nonspecific stimulation of normal human lymphocytes is not known at present. It is apparent, however, that the cytotoxic effects reported for such systems are not direct effects of PHA and are possibly related to gamma globulin production.

The synthesis or release of gamma globulin has received much attention (72-75, 243-246). An early hypothesis of Hirschhorn et al. (54, 269)

was generated as a result of their observations of gamma globulin production *in vitro* after either PHA or specific antigenic stimulation of lymphocytes (82, 89, 90). These investigators suggested that both cases represented an immune stimulus. Other investigators (243, 244, 246), using radioelectrophoresis, radioimmune electrophoresis and controlled immunological precipitation methods have suggested that only a small amount of immunoglobulin is synthesized in culture systems of PHA-stimulated lymphocytes. Further, lymphocytes stimulated by pokeweed mitogen or PPD have been shown to synthesize greater relative amounts of immune proteins than PHA-stimulated lymphocytes (243). Convincing data is lacking to support any direct role of PHA in the regulation of antibody titer *in vivo*.

A number of reports in the literature indicate that administration of PHA *in vivo* increased the survival time of skin allografts (20, 21, 300, 301) or depressed the primary and secondary immune response to injected sheep erythrocytes (22, 129, 292). For these effects the administration of PHA had to be started prior to or simultaneously with the antigen (22). It has been noted that PHA prolongation of the survival time of tissue homotransplant is dose-dependent and can occur at dosages which do not markedly suppress antibody formation *in vivo* (21, 301).

Others report (303) that pretreatment of immunologically-competent donor cells with PHA *in vitro* can result in an inability to demonstrate an otherwise fatal graft-versus-host reaction in the recipients. Further, pretreatment of donors with PHA prior to injection of donor immunologically-competent cells into newborn animals resulted in suppression of the graft-versus-host reaction (23). Finally, the ability of lymph node cells to

transfer experimental autoimmune encephalomyelitis (EAE) to susceptible recipients was decreased by the action of PHA (304). When lymph node cells were first cultured for one to four hours with mitogenic concentrations of PHA before the cells were injected into recipients, the ability of the lymph node cells to transfer EAE was lost.

TOXICOLOGY AND PHARMACOLOGY OF PHA

Toxic properties in addition to the hemagglutination and mitogenic constituents in extracts of beans have also been attributed to different seeds of Leguminosae, including some members of Phaseolus vulgaris (10-12, 24-31).

The effect of PHA on the survival of tumor cells was first considered by Nungester and Van Halsema (10). They reacted crude PHA preparations with Flexner-Jobling carcinoma cells hoping that it would be selectively absorbed to the surface membrane of the cells. They were aware of the agglutinating activity of lectins and postulated that such substances could interfere with the growth of tumor cells in vivo. They reported that a crude PHA preparation isolated from white navy beans after the method of Li and Osgood (65), when added to suspensions of Flexner-Jobling rat carcinoma cells before inoculation, destroyed the ability of these cells to produce tumors in rats. Their PHA was ineffective when administered for ten days immediately following inoculation of rats with untreated human cells. Aware of these results, Leiner and Seto (11) injected 25 mg/kg of a soybean hemagglutinin into rats inoculated with Walker tumor. They observed both a delay in appearance and a reduction in the size and final weight of the tumor. Sometime later Nungester, Haines and Rising (30) examined a series of 70 crude

extracts from various strains of beans, peas and corn for antitumor activity in several tumor lines growing in vitro. Eight extracts, all from strains of beans, exhibited growth-inhibitory effects. Andrew and Gabourel (29) reported PHA-induced growth inhibition of three cell lines in tissue culture and a prolonged survival time for PHA-treated BDF_1 mice carrying an ascites form of the L-1210 lymphocyte leukemia. Using a pure PPHA preparation (44) they found that a concentration of 3 mcg/ml of culture medium produced a 50 percent inhibition of cell growth in vitro. They also demonstrated the ability of PPHA to increase the mean survival times of tumor-bearing mice up to 219 days after a single dose of 100 mcg PPHA/kg, administered one hour after tumor inoculation*.

Rubio and Unsgaard (305) showed that pretreatment of mice with small amounts of commercial PHA failed to protect them from the growth of injected ascites tumor cells. Robinson (35, 306) also investigated the effect of PHA administration on ascites tumor cell survival in mice. The lowest numbers of tumor cells recovered after seven days were from mice injected with PHA 72 hours prior to tumor transplantation. Administration of their PHA 120 hours before tumor inoculation was reported to have had no protective effect.

As early as 1929 Goddard and Mendel (53) injected a crude phytohemagglutinin preparation and found it was not toxic at a dose of 8 mg/kg in rabbits and of 600 mg/kg in mice. Later, Jaffe and Gaede (25) observed toxic effects when they fed animals raw beans. They then prepared two different extracts of the black kidney beans which were

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* These results will be discussed in greater detail as part of this thesis.

labelled Phaseolotoxin A and Fraction B, respectively. Using Phaseolotoxin A they killed approximately 50 percent of the mice (LD_{50}) when they injected them with 60 mg/kg of their bean extract. Phaseolotoxin A was shown to be a single protein. However, it was their Fraction B which demonstrated physical and biological properties most like those of the original PHA preparation of Rigas and Osgood (43). Approximately 200 mg/kg of Fraction B was required to determine its LD_{50} value. DeMuelenaera (27) and others (10-12, 24-26) have compared the toxicity and hemagglutinating activity for crude preparations of a number of legumes. DeMuelenaera found that intraperitoneal toxicity in rats occurred only from those preparations which had the higher hemagglutinating activity, although he did not consider that hemagglutination was necessarily the factor responsible for toxicity. Another proposed mechanism for the fatal effects following PHA administration was the inhibition of gastrointestinal tract proteases (26, 307). But it was considered unlikely that this could be the exclusive mechanism of toxicity since either intraperitoneal administration of bean extracts or the feeding of raw beans was lethal (24, 25). Recent reports (28, 31, 129, 295, 299, 308), all using commercial preparations of PHA, are conflicting as to LD_{50} values. This probably reflects variations in purity, concentration of preparation, frequency and route of administration, and species tested. Using a highly purified PPHA preparation (4, 44), Andrew and Gabourel (29) established that 100 mg PPHA/kg was the largest single dose that could be given intraperitoneally to BDF_1 mice without killing some animals within 48 hours. They determined the LD_{50} of this preparation to be 140 ± 10 mg PPHA/kg body weight of these animals.

Toxicity studies did not consider histological and/or pathological changes following PHA administration until recently (26-31, 126-129, 308-311). Several authors observed lesions in the liver (126, 127, 309) and intestinal wall (26, 27), and involution of lymphoid organs and leukopenias (26, 31). There are also descriptions of emboli and thrombi following intravenous administration (10, 13, 31). These reports of pathological sequellae following PHA administered parenterally are in contrast to those of lymphoid proliferation noted earlier (70, 126-129, 308) in the discussion of the mitogenicity of PHA in vivo. Again, the inconsistency of the results may reflect the preparation and the mode of administration.

Several attempts have been made to use PHA clinically. As a mitogen PHA has been considered for the role of a stimulator of erythropoiesis in the treatment of aplastic anemia (28, 34, 36, 37, 131, 133) and as a radioprotection agent for the bone marrow against the lethal effects of irradiation (31, 32, 311, 312). Its stimulatory action was considered when PHA was used to decrease the leukopenia associated with anti-metabolite cancer chemotherapy (35, 313). Assuming that PHA plays a role in immune mechanisms, it has been used alone and with immuno-suppressant agents for the prolongation of survival of homotransplants (20, 21, 33, 300, 301). The role of phytohemagglutinin as a therapeutic agent has not yet been clearly defined.

METHODS AND MATERIALS

CELL CULTURE TEST SYSTEMS

Growth inhibitory effects of PHA were studied on four rapidly-growing mammalian cell lines in tissue culture and on one transplantable tumor line growing *in vivo*.

Cell Culture In Vivo. Mouse lymphocytic leukemia L-1210* was used for cell culture studies *in vivo*. It was maintained in the ascites form in the peritoneal cavity of BDF_1 mice** (F_1 hybrid of DBA/2 male and C57BL female). The hosts were all females two to six months old. The L-1210 tumor arose from a DBA mouse treated topically with methylcholanthrene, and its growth characteristics for cultures *in vivo* (314, 315) and *in vitro* (316, 317) have been described.

In our laboratories an intraperitoneal inoculation of 5×10^5 cells produced a tumor with a doubling time of about 0.55 days. Mean survival time (MST) of the hosts after this inoculum was approximately seven days. The mice were maintained on an *ad libitum* diet of water and Purina laboratory chow, and kept in an air conditioned room at 75 ± 1° F.

Cell Culture In Vitro. Four established cell lines were grown *in vitro* as monolayer cultures. They were the L-fibroblast (318, 319), mouse lymphoma (ML-388) (320, 321), a steroid-resistant subline of

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* Obtained from the peritoneal cavity of a BDF_1 mouse sent to us by Dr. G. A. LePage, Chief of Biochemical Oncology, Stanford Research Institute, Palo Alto, California.

** Supplied by Simonsen Laboratories, Gilroy, California.

ML-388 (designated as ML-388F^r) (322), and a newly established rat thymus cell line (designated as RT*). The ML-388 cells have also been cultured in suspension in our laboratory.

All cell lines were maintained on the defined culture medium of Eagle (323). The Minimum Essential Medium of Eagle (MEM Eagle) consisted of a mixture of salts, vitamins, amino acids and glucose**. The basic medium was supplemented with 6 to 10 percent calf serum** (324), 0.005 percent sodium penicillin G, and 0.005 percent streptomycin sulfate for the L-fibroblasts. The other cell lines required the additional supplements of 10^{-4} M L-serine and 10^{-4} M sodium pyruvate. Medium and supplements for the suspension cultures were the same as those used for the monolayer growth of ML-388 cells, except that calcium chloride was omitted and the phosphates were increased tenfold**. All media received sodium bicarbonate to a final concentration of 0.22 percent and were adjusted to pH 7.3 $\pm$ 0.1. Media were sterilized by filtration through Millipore filters (HA 0.45μ) prior to addition of the sterile serum.

Doubling times (time for population to increase twofold) for these cell lines in our laboratory ranged from 20 to 24 hours. Stock cultures of all cell lines have been maintained in continuous culture and in the frozen state under liquid nitrogen.

The L-fibroblasts (Earles "L" cells) were derived from Clone 929 originally isolated by Sanford, Earle and Likely (319) from the parental

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* Derived by K. E. Fox and Leona Baker, 1964, Department of Pharmacology, University of Oregon Medical School.

** Medium (Cat. #F-11 and F-14) and serum (Cat. #617) supplied by Grand Island Biological Co., Grand Island, New York. Packages of powdered medium were sufficient to prepare 1 liter of 1X medium per package.

strain-L established earlier (318). The parent L-strain was derived from normal subcutaneous areolar and adipose tissue of a C3H/An mouse. The cells were brought to our laboratory in culture in 1964 from Stanford University and have been maintained as monolayer cultures.

Mouse Lymphoma ML-388 was cloned by Herzenberg and Roosa (321) from formerly-designated P-388 cells derived by Dawe and Potter (320). The designation was changed when the requirements of serine and pyruvate were determined for growth in vitro (321). The parental P-388 line was derived from a mouse lymphoma induced by methylcolanthrene in a female DBA/2 mouse and cultured in vitro after 29 passages in CDBA/$2F_1$ mice (320). ML-388* cells are known to be inhibited by hydrocortisone (1, 325). Dose-response studies of the effect of hydrocortisone on growth gave an IC_{50} (concentration required for 50 percent inhibition of growth) of about 6×10^{-8} M.

The ML-$388F^r$ stock cells were made resistant to 4×10^{-5} M hydrocortisone in our laboratory following the method of Aronow and Gabourel (322). This sub-line was derived from the mouse lymphoma ML-388 described above, and was carried here on 1×10^{-6} M steroid-supplemented growth medium.

Rat thymus cells (designated as RT) were isolated in this laboratory from a Sprague-Dawley rat. The primary culture was grown out, and contained both thymic and fibroblast-like cells. The thymic cells were selectively removed by gently washing the cell layer in the bottle with fresh medium. The fibroblastic cells adhered firmly to the glass, while

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*Received in 1965 from Dr. R. A. Roosa, Wistar Institute, Philadelphia, Pennsylvania.

a large percentage of the thymic cells were dislodged. This procedure was repeated through several passages until no fibroblast-like cells could be visualized. These RT cells have been maintained in monolayer culture and frozen in liquid nitrogen storage. Dose-response data on growth in the presence of hydrocortisone gave an IC_{50} of about 3×10^{-7} M.

Replicate Monolayer Cultures. Procedures used were similar to those described by Saltzman (326) and others (327-331). For the preparation of monolayer cultures, replicate cell inocula of 1 to 3×10^5 cells in 20 ml of sterile medium (MEM Eagle) were dispensed to 8 ounce prescription bottles, using an automatic sterile pipetting machine*. The bottles were then gassed with a mixture of five percent carbon dioxide in air and stoppered with silicone stoppers. Cultures were incubated at 37 ± 1° C. Under these conditions control cultures uniformly multiplied twenty to thirtyfold by five days after inoculation.

Suspension Cultures. The technics used in the preparation and maintenance of the suspension cultures have been reviewed (332-334). Agitation of the suspension cultures of ML-388 cells in our laboratory was achieved by the use of the Vibromixer**. Characteristics of this instrument are described elsewhere (335). The unique aspect of this instrument is that agitation is accomplished by a specially designed

*Filamatic Dispenser manufactured by National Instrument Co., Baltimore, Maryland.

**Manufactured by Chemap AG, Switzerland, and sold by Chemapec Inc., Hoboken, New Jersey.

perforated stirring disk vibrating at a frequency of 120 cycles/sec. Amplitude of the vibration from 0 to 4 mm is adjustable so that agitation can be changed from very gentle to violent pumping of the culture medium. Controlled agitation provides reduced shearing stress to the submerged cells. Several drive units can be operated at identical rates when current is supplied through the same rheostat. For sterile operation the stirring shaft connecting the disk to the drive unit passes through a diaphragm seal with a Neoprene membrane. The seal is then mounted through a rubber stopper placed in the central top opening of the culture vessel. The drive unit is easily disconnected from the stirrer shaft, permitting the vessel and stirring apparatus (exclusive of drive unit) to be autoclaved.

Culture vessels*, ranging from one to twelve liters in capacity, were never filled with more than one-half of their volume. The vessels, of standard manufacture, are pyrex, three-necked, round-bottomed boiling flasks, with the center necks of some flasks modified to accept stirring disks up to 65 mm in diameter.

Inocula of 5×10^4 to 1×10^5 cells per ml of medium multiplied approximately tenfold by six to seven days after initiation of control cultures.

EXPERIMENTAL PROCEDURES FOR MEASURING GROWTH INHIBITION

Determination of Cell Number. Monolayer cultures were inverted and the medium carefully poured off so that adherent cells were not

*Flasks (Cat. #29275) were purchased from Van Waters & Rogers, Portland, Oregon.

dislodged. For some procedures the adherent cells were gently rinsed once or twice with five to ten milliliters of fresh medium, basic salt solution (BSS*), or saline diluting fluid**. Bottles were drained for about ten minutes before the diluting fluid was added. Cells were scraped off the glass with a rubber policeman, pipetted up and down vigorously a few times to disperse them, and appropriate dilutions of the cell suspension prepared. Cell preparations from other sources such as suspension culture and ascites were also diluted appropriately for cell enumeration. This procedure was found, by phase microscopic examination, to give well-dispersed single cells with minimal signs of cell lysis.

Cell number was determined using a Model A Coulter electronic particle counter*** in accordance with prescribed technics (336, 339). Relative cell size was estimated in a few experiments by taking successive readings at threshold settings varying from 0 to 100, then plotting threshold curves against instrument readings or percent of total counts obtained at such settings. Shifts in distribution curves indicated shifts in relative size (340-342).

Dose-Response Procedures. Replicate cultures of the different cell lines were grown as monolayers as previously described. Three to six bottles were randomly selected from the group 16 to 24 hours after

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*Same composition as MEM Eagle except serum omitted.

**Saline diluting fluid consisted of 1.0 percent formaldehyde in 0.9 percent sodium chloride. Solution was first filtered through a Millipore filter, type HA 0.45μ, to remove any particulate material.

***Manufactured by Coulter Electronics, Inc., Hialeah, Florida.

inoculation and cell number was determined as previously noted. All remaining bottles received either PHA at various final concentrations or equal volumes of appropriate vehicle (as a control). Phytohemagglutinin* solutions were prepared by dissolving the drug in BSS or 0.9 percent saline. The solution was then sterilized by filtration through a Type HA 0.45μ Millipore filter and further dilutions made with sterile vehicle as necessary. Protein content of the various PHA solutions was determined colorimetrically using the phenol reagent of Folin and Ciocalteu according to the technic described by Lowry et al. (343). PHA at various concentrations was added as 0.1 to 0.3 ml of solution to 20 ml of culture medium. On the fifth day after inoculation (96 hours after drug addition) the number of cells in each of the remaining bottles was determined. A minimum of three bottles were counted separately to determine the effect of each treatment on cell growth. Results were expressed as percent of control growth versus PHA protein concentration or as percent inhibition versus PHA protein concentration. Addition of vehicle to cell cultures had no effect on cell growth when compared to untreated cultures.

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*Sources: PPHA 365-B-P6 supplied by Dr. D. A. Rigas, Department of Experimental Medicine, University of Oregon Medical School.

PHA (M form) (Cat. #536) obtained from General Biochemicals, Chagrin Falls, Ohio.

PHA-M and PHA-P (Code #0528 and #3110) obtained from Difco Laboratories, Detroit, Michigan.

PHA-BW Batch X-5 and Batch E-118 were obtained from Dr. G. Hitchings, Vice-president in Charge of Research, Burroughs Wellcombe & Co., Inc., Tuckahoe, New York.

Estimation of Mean Survival Time (MST) for Tumor-bearing Mice. Protein PHA (PPHA) was administered to normal BDF_1 mice by intraperitoneal injection over a dose range of 3 to 300 mg PPHA/kg body weight. Maximum permissible single doses and LD_{50} values were determined. All deaths attributed to PPHA administration occurred within the first 48 hours following injection.

Following determination of the maximum permissible dose of PPHA in vivo, the effect of this glycoprotein on the survival time of BDF_1 mice inoculated with the L-1210 tumor was determined. Twenty control mice and 30 experimental mice were inoculated intraperitoneally with 5×10^5 L-1210 cells. Twenty-four hours later ten of the twenty control mice were injected intraperitoneally with saline and the other ten with a solution of bovine serum albumin, also intraperitoneally. Ten of the thirty experimental mice were injected with PPHA one hour after tumor inoculation. Twenty-four hours after tumor inoculation the other 20 experimental mice were injected with PPHA, and 48 hours after tumor inoculation ten of these received a second injection. All treatments consisted of 100 mg PPHA/kg of body weight.

Chemical Analyses of Cell Cultures. Replicate monolayer cultures of L-fibroblasts were prepared as previously described (327). At various times after PPHA addition, cultures were removed from the incubator and the medium decanted. Most cells remained attached to the bottle and were gently rinsed once with BSS, then suspended in cold BSS with the aid of a rubber policeman. The BSS rinse solution was added to the decanted medium and this solution was either discarded or centrifuged

1100 x G for ten minutes to recover any free (unattached) cells. These recovered free cells were then resuspended in BSS. Small aliquots of the resulting cell suspensions were assayed for cell number and the remainder centrifuged in the cold to give a cell pellet (see Figure 1). On occasion it was necessary to pool the cells from several bottles (up to six) to obtain sufficient cellular material for chemical analysis.

The cell pellets (see Figure 2) were resuspended in and extracted twice with 6 to 8 percent ice-cold trichloroacetic acid (TCA) or perchloric acid (PCA) for 5 to 10 minutes at 3 to 5° C before centrifugation at 10,000 x G for ten minutes (344). The resulting cold-acid insoluble pellets were then extracted with 3 ml 6 percent PCA for 20 minutes at 90° C to hydrolyze the purine components of the nucleic acids. After chilling at 0 to 2° C in an ice bath, the material was centrifuged and the hot-acid soluble supernatant fraction was separated from the residue by centrifugation at 16,000 x G for ten minutes (344).

For some experiments samples were removed from suspension type cultures at various times after PHA. Aliquots were both assayed for cell number and centrifuged in the cold to give cell pellets which were processed according to the flow diagram in Figure 2.

Protein was determined on the hot-acid insoluble residue after alkaline digestion in 1 to 2 ml of 1N NaOH by the procedure of Oyama and Eagle (345). Ribose (R) was determined in an aliquot of the hot-acid soluble supernatant solution using the orcinol reaction of Volkin and Cohn (346). Deoxyribose (DOR) was determined in another aliquot utilizing the procedure described by Burton (347). Since DOR contributed a small amount of color to the orcinol reaction (10 µmole of DOR was

Figure 1

Flow diagram outlining procedures employed in obtaining cell suspensions from replicate monolayer cultures for both cell number determination and cell pellet preparation.

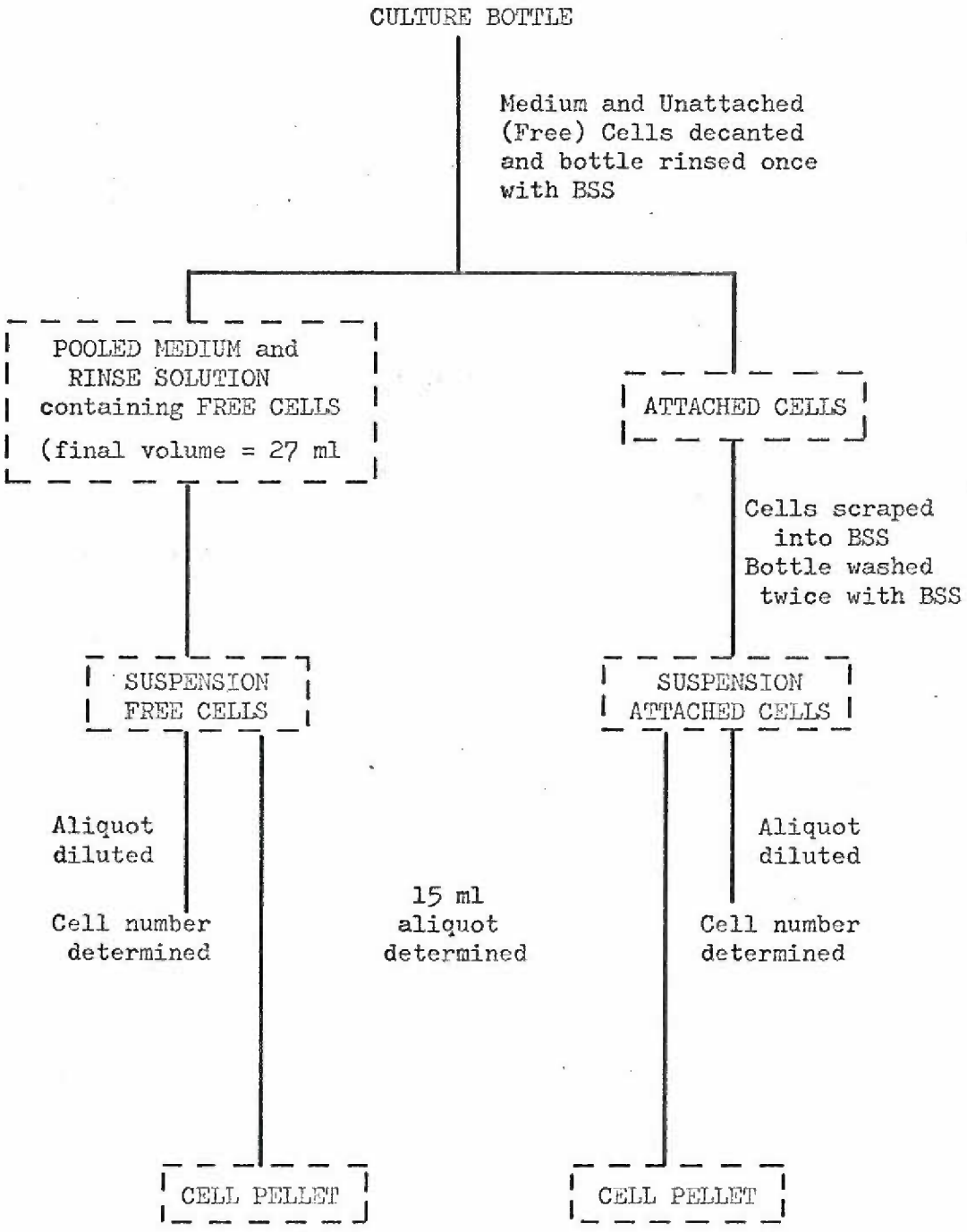
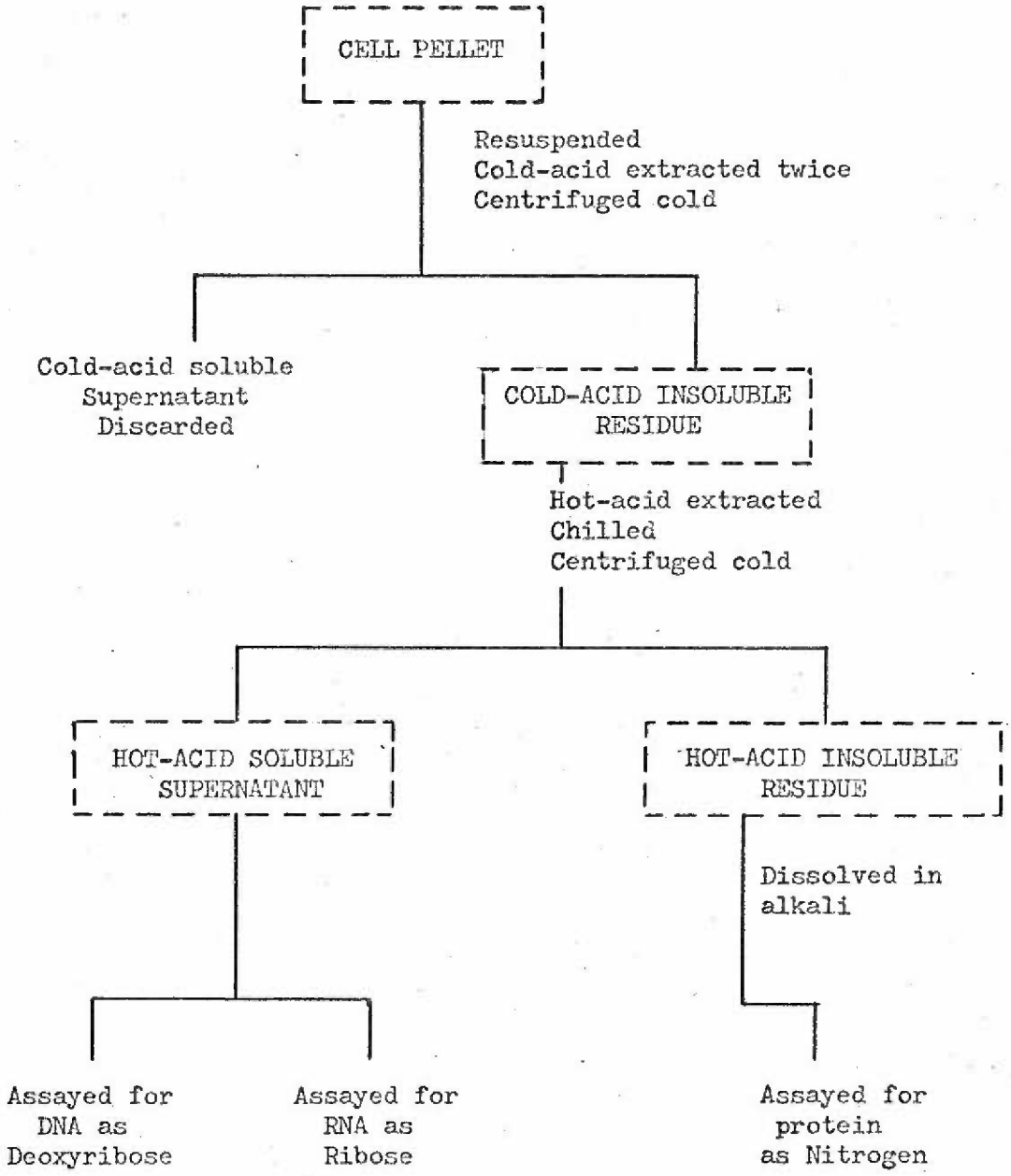

CULTURE BOTTLE
Medium and Unattached (Free) Cells decanted and bottle rinsed once with BSS
POOLED MEDIUM and RINSE SOLUTION containing FREE CELLS
(final volume = 27 ml
ATTACHED CELLS
Cells scraped into BSS
Bottle washed twice with BSS
SUSPENSION FREE CELLS
SUSPENSION ATTACHED CELLS
Aliquot diluted
Cell number determined
15 ml aliquot determined
Aliquot diluted
Cell number determined
CELL PELLET
CELL PELLET

Figure 2

Flow diagram outlining the procedures employed in preparation of cell pellets for chemical analysis.


CELL PELLET
Resuspended
Cold-acid extracted twice
Centrifuged cold
Cold-acid soluble
Supernatant
Discarded
COLD-ACID INSOLUBLE
RESIDUE
Hot-acid extracted
Chilled
Centrifuged cold
HOT-ACID SOLUBLE
SUPERNATANT
HOT-ACID INSOLUBLE
RESIDUE
Dissolved in
alkali
Assayed for
DNA as
Deoxyribose
Assayed for
RNA as
Ribose
Assayed for
protein
as Nitrogen

equivalent to 0.66 µmole of R) the ribose determination was corrected by an appropriate amount (328, 348, 349). All colorimetric determinations were usually performed only once per sample, but six or more samples were assayed for each time and treatment. Results were expressed both in terms of accumulation per bottle and as amount per cell.

Measurements of Radioactive Labelled Precursors. Carbon-14 (^{14}C) radioactivity was determined in a Packard Tri-carb Liquid Scintillation Spectrometer*. One milliliter of Nuclear Chicago Solubilizer (NCS)** was used to digest cold-acid insoluble residues before the scintillator was added, or to facilitate solution of aqueous samples (up to 0.1 ml) in the toluene scintillation solution (final volume of 16 ml) (350). This scintillation solution consisted of 4.0 gm 2,5-diphenyloxazole (PPO) and 0.1 gm 2,2-p-phenylenebis (5-phenyloxazole) (POPOP) per liter of toluene, reagent grade. Quenching for all samples was determined by the channels ratio technic (351) and necessary corrections were applied.

Measurement of Prelabelled DNA. For one series of experiments the cellular DNA was prelabelled with ^{14}C by growing the cells in two Roux flasks for two days in a medium containing 15 µc of thymidine-2-^{14}C*** (53.8 µc/µM) per 70 ml of medium. At the end of the labelling period

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*Model 3214, Packard Instrument Co., Inc., Downers Grove, Illinois.

**Nuclear Chicago Corporation, Des Plaines, Illinois.

***All radioactive precursors obtained from New England Nuclear Corporation, Boston, Massachusetts.

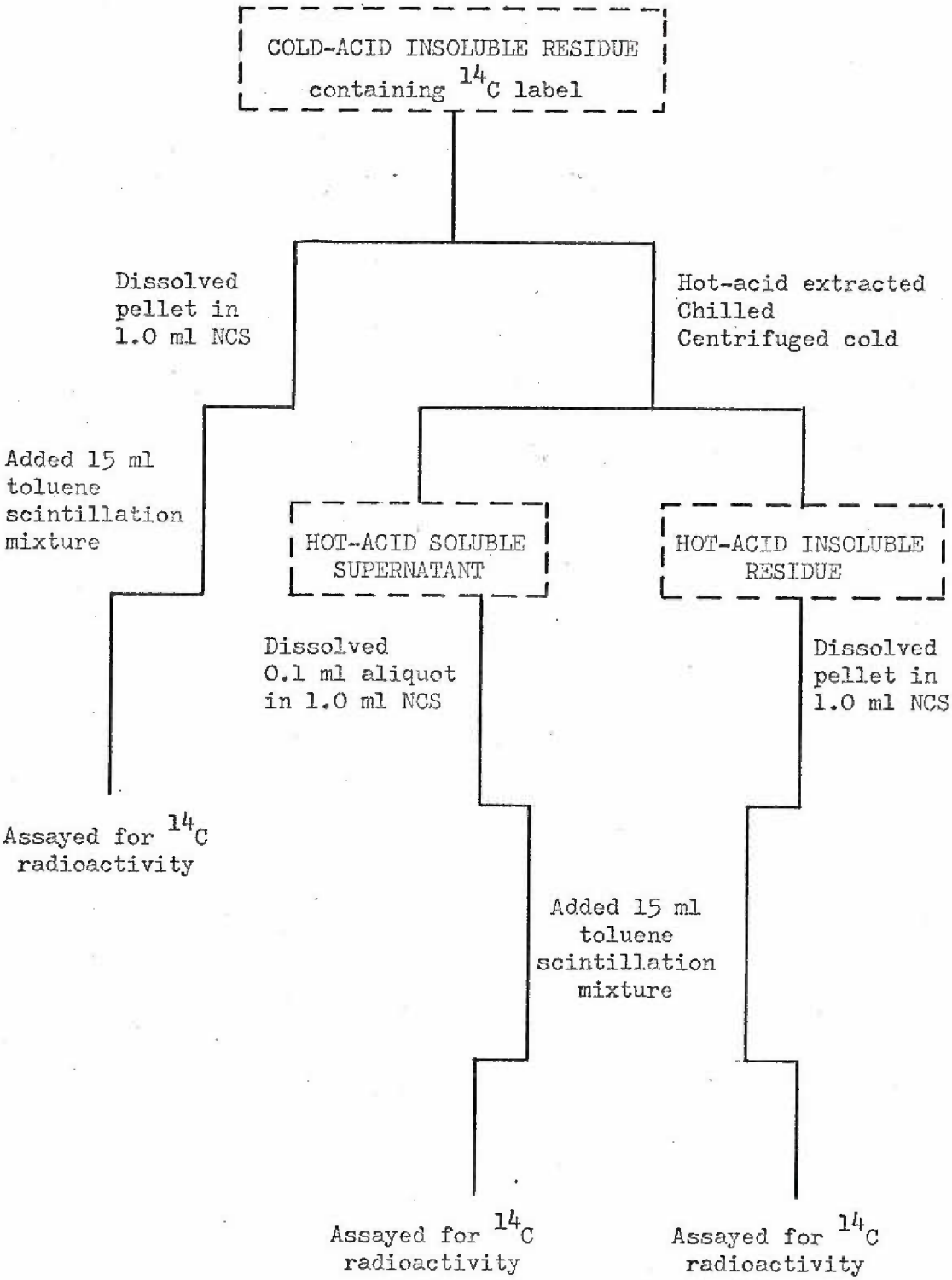
the medium was decanted and the adherent cells gently washed twice with 10 to 15 ml of fresh (nonradioactive) medium. The cells were then scraped into nonradioactive medium and a cell suspension made for the preparation of 100 to 150 replicate cultures as previously described.

At various times after drug addition to the replicate cultures, groups of bottles were selected randomly. Cells were collected for chemical analysis in the manner described in Figures 1 and 2. Cold-acid insoluble fractions were assayed for radioactivity in one of two ways as outlined in Figure 3. In some experiments the cold-acid insoluble residue was dissolved in 1.0 ml of NCS solubilizer, 15 ml of toluene scintillation solution added, and the radioactivity determined. In other experiments the cold-acid insoluble residue was hydrolyzed in hot acid. The products were chilled and separated by centrifugation as outlined in Figure 2. Both chemical analysis and radioactivity were measured on the resultant supernatants and residues. The hot-acid insoluble residue was prepared for radioactivity determinations in the same manner as previously described for cold-acid insoluble residues (Figure 3.) For the hot-acid soluble supernatants, 0.1 ml aliquots were dissolved in 1.0 ml of NCS solubilizer, 15 ml of toluene scintillation fluid was added, and radioactivity was determined as outlined in Figure 3. Results were expressed as counts per minute (cpm) per cell and as cpm per mμmole of DOR.

Aliquots of decanted medium were centrifuged at 11,000 x G for ten minutes to remove any cells present. The cell-free medium was then assayed for ^{14}C radioactivity by direct addition of 0.1 ml to the NCS-toluene-scintillation mixture. Results were expressed as total cpm in the medium.

Figure 3

Flow diagram outlining the procedures employed in assaying cold-acid insoluble residues, hot-acid soluble supernatants and hot-acid insoluble residues for ^{14}C radioactivity.


COLD-ACID INSOLUBLE RESIDUE
containing ^{14}C label
Dissolved
pellet in
1.0 ml NCS
Hot-acid extracted
Chilled
Centrifuged cold
Added 15 ml
toluene
scintillation
mixture
HOT-ACID SOLUBLE
SUPERNATANT
HOT-ACID INSOLUBLE
RESIDUE
Dissolved
0.1 ml aliquot
in 1.0 ml NCS
Dissolved
pellet in
1.0 ml NCS
Assayed for ^{14}C
radioactivity
Added 15 ml
toluene
scintillation
mixture
Assayed for ^{14}C
radioactivity
Assayed for ^{14}C
radioactivity

Precursor Uptake-Pulse Labelling. Replicate cultures were established as previously described and 24 hours later PHA or vehicle was added. At various times after drug addition the appropriate labelled precursor was added and incubated either 30 or 60 minutes at 37° C. The incorporation of radioactive precursors (thymidine-2-^{14}C, uridine-2-^{14}C and L-leucine-1-^{14}C) into cold-acid insoluble material under the conditions specified was used as a measure of DNA, RNA and protein synthesis, respectively.

Incorporation of label was terminated by the addition of one to two milliliters of 1 percent nonradioactive precursor. Cells were harvested as outlined in Figure 1 to the cold-acid insoluble residue stage as outlined in Figure 2. The radioactivity of the cold-acid insoluble residue was measured directly by solubilization of the residue in NCS solubilizer or this residue was subjected to hot-acid hydrolysis and a 0.1 ml aliquot of the supernatant material was added to the NCS-toluene system as outlined in Figure 3. Results were expressed as cpm per cell and as the specific activity of the labelled macromolecule (i.e., cpm per mμmole DOR, cpm per mμmole R, or cpm per mcg protein nitrogen).

Morphology. Both ML-388 and L-fibroblast cells in monolayer culture were examined for the effect of PHA on gross morphology and on cellular ultrastructure (328). For gross morphology studies the cells were grown on microscope slides in eight ounce french square bottles inoculated in the same manner as replicate cultures in eight ounce prescription bottles. Twenty-four hours later drug or vehicle was

added and the glass slides were removed at varying intervals after treatment. Those slides not used for viability testing were rapidly air-dried. The adhered cells were then fixed in methanol and stained with hematoxylin and eosin before dehydration in a graded series of alcohols, and mounted in Permount. Photomicrographs were prepared from the stained preparations. In addition, cells from monolayer cultures were examined under phase microscopy for direct visualization of viable cells.

Cells harvested from monolayer cultures were centrifuged 760 x G for five minutes to form a loose cell pellet for ultrastructure analysis. These cell pellets were fixed in phosphate-buffered glutaraldehyde, then post-fixed with phosphate-buffered osmium before dehydration in a graded series of alcohols, all at room temperature. The cells were then embedded in Araldite. Thin sections of the embedded material were cut with a Porter-Blum MT-2 microtome and stained with Reynolds lead citrate (352) for 30 seconds, then aqueous uranyl acetate for five minutes and, finally, Reynolds lead acetate again for 30 seconds. Sections were examined in an RCA EMU 3-G or Phillips EM200 electron microscope, and photomicrographs were prepared.*

Cell Viability. Cell viability was measured by the trypan blue exclusion test (332). The principle of this procedure is that changes in the permeability of cells, as in degeneration, permit the cells to stain readily, while viable cells retain the ability to exclude the dye. The extent of trypan blue staining was measured for cells growing

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*Embedding, sectioning, staining and preparation of electron photomicrographs were performed by Miss Betsy Adkison, Research Assistant, Department of Pathology, University of Oregon Medical School.

directly on microscope slides as well as for cells harvested for cell number enumeration.

Both total cell counts and counts of stained cells were performed. Slide preparations were removed from the culture bottles, flooded for five minutes with 0.4 percent trypan blue solution, rinsed with 0.9 percent saline and rapidly air-dried. The adherent cells were fixed in methanol, counterstained lightly with eosin in the same manner as the hematoxylin and eosin permanent slide preparations. These were examined with the light microscope and scored for the percent of trypan blue positive cells per 500 total cells counted.

RESULTS

GROWTH EFFECTS OF PHA

Cells Grown in Vitro. Growth characteristics for L-fibroblasts in monolayer culture are presented in Table 2. These are typical of all other cell lines used in this study. These cells multiply at such a rate that their number increases 19 to 21 times over a five-day period.

Dose-response curves for the growth-inhibitory effects of PHA on four rapidly-growing cell lines (RT, ML-388, ML-388F^r and L-fibroblasts) are shown in Figure 4. The concentrations of PHA required to inhibit growth to 50 percent of control growth (IC_{50}) for the different cell lines ranged from 2.3 to 4.1 mcg PPHA/ml of medium and did not differ significantly between cell lines at the five percent level as noted in Table 3. Further, resistance to hydrocortisone (ML-388F^r cell line) did not provide protection from the growth inhibitory effects of PHA. Additional data was obtained from the experiment described in Figure 4 and Table 3 which will not be presented. This data shows that cell numbers determined five days after exposure to the highest concentration of PPHA (100 mcg/ml of medium) were never less than those at the time of PPHA addition. This would argue against nonspecific cell lysis as a primary mechanism for the growth inhibitory effect.

When the effect of PPHA on cell viability was investigated directly (Table 4) the percentage of cells stained with trypan blue seldom exceeded five percent. The difference seen in staining between PHA-treated and saline-control cells was constant but small.

When cell suspensions prepared for cell number analysis were examined under the phase microscope no differences in the amount of clumping in

TABLE 2.

Multiplication of L-Fibroblast Cells in Monolayer Culture.

Cell Classification	Cell Number x 10^{-5} per bottle at various times after inoculation (Mean ± s.e.m.)				
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Attached Cells (n = 8)	1.86 ± 0.12	4.08 ± 0.48	9.84 ± 1.18	19.8 ± 2.63	38.2 ± 3.65
Free Cells (n = 5)	1.03 ± 0.15	1.20 ± 0.31	1.90 ± 0.35	8.36 ± 1.01	10.2 ± 2.25
Total Cells (n = 5) (attached plus free)	2.87 ± 0.23	5.75 ± 0.70	12.3 ± 1.86	31.0 ± 4.09	57.4 ± 6.70
Percent Free Cells: $\frac{\text{(Free cell number}}{\text{(Total cell number}} \times 100$)	36	21	15	27	28

Eight ounce prescription bottles were inoculated with 20 ml of growth medium containing 1 to 3 x 10^5 L-fibroblast cells. At various times after culture inoculation at least six bottles were randomly selected for cell number determination. The medium was carefully poured from each bottle and saved for free cell counts. The remaining cells (attached) were then gently washed once in the bottles with BSS and this wash solution added to the original medium for the determination of free cell number (see Methods). The attached cells were then suspended in BSS with a rubber policeman, and cell numbers determined (see Methods). Total cell values were obtained from the sum of the values for free cells and attached cells. Pooled results for five to eight experiments are given above as the mean and standard error (± s.e.m.). Mean doubling time (hours required for twofold increase in cell number per bottle) for attached fibroblasts in these experiments was estimated to be 21.2 ± 0.34 hours.

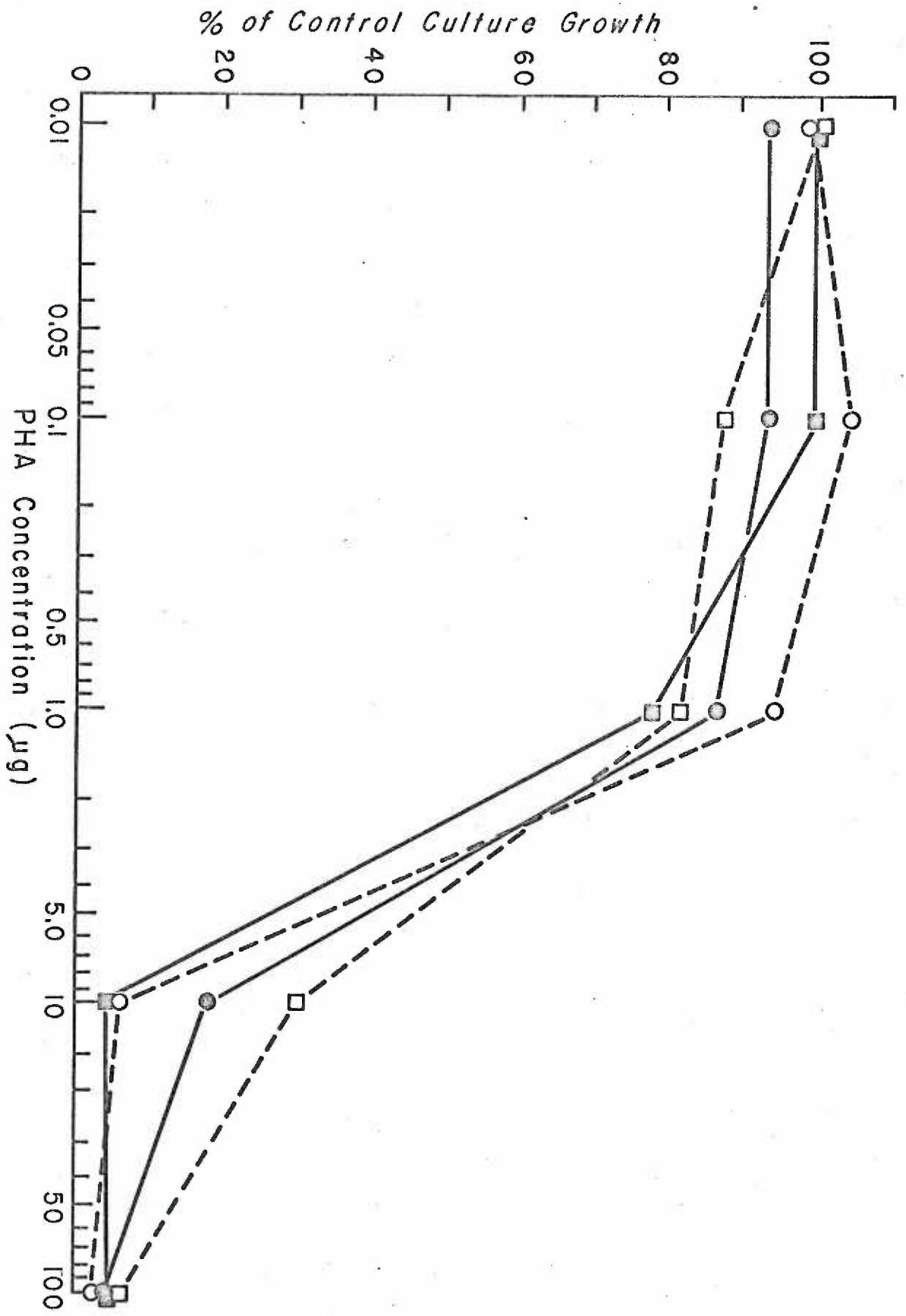

% of Control Culture Growth
0
20
40
60
80
100
PHA Concentration (µg)
0.01
0.05
0.1
0.5
1.0
5.0
10
50
100

Figure 4.

Effect of varying concentrations of PHA on growth of different mammalian cell lines in vitro.

Growth inhibition in the presence of various concentrations of PPHA preparation or vehicle (control) was measured for four different cell lines grown as replicate monolayer cultures in eight ounce prescription bottles. Bottles were inoculated with 20 ml of nutrient medium containing 1 to 3 x 10^5 cells. Twenty-four hours later preparation PPHA was added to each culture to give a final concentration ranging from 0.01 to 100 mcg/ml of medium.

Growth was estimated 96 hours later by determining the number of attached cells per bottle as described in the Methods. Each point in the Figure represents the average cell number determined from a minimum of three bottles. Note that the open circles represent RT cells, the solid squares and solid circles ML-388 and ML-388F^r cells, respectively, and the open squares, L-fibroblasts.

Results are expressed as percent of control growth versus PPHA concentration. The standard error of the mean was no more than four percent for each point plotted in the Figure. There was no significant difference($P < 0.05$) between cell lines for response to PPHA as determined by the Student's t-test (two-tail).

TABLE 3.

Comparison of Relative Sensitivity of Four Cell Lines to PHA

Cell Line	$IC_{50} \pm$ s.e.m.
L-fibroblast	4.1 ± 0.6 µg/ml
ML-388F^r	3.5 ± 0.5 µg/ml
ML-388	2.3 ± 0.4 µg/ml
RT	3.2 ± 0.4 µg/ml

IC_{50} values (concentration required for 50% inhibition of growth compared to control) were estimated graphically from the dose-response data given in Figure 4 for four rapidly-growing mammalian cell lines. Values did not differ significantly ($P < 0.05$) as determined by Student's t-test (two-tail).

TABLE 4.

Effect of PHA on L-Fibroblast Viability

Treatment	Trypan Blue Exclusion Test - Percent Positive Stained at Various Times (hours) after PHA				
	0	24	48	72	96
Saline-control	0.2	4.7	4.2	2.3	0.8
PHA-treated (30 mcg PPHA/ml)	---	5.4	7.6	2.5	1.9

L-fibroblasts were grown as monolayer cultures on microscope slides and cells were examined for their ability to exclude trypan blue as an index of viability, as described in the Methods. The number of cells which retained the dye were determined (positive stained). The results presented here are representative values from a typical experiment. The values were for 500 total cells counted per sample.

PHA-treated or control cells was observed. Thus, cell suspensions were uniform and errors due to cells clumping were few.

Further studies on the effect of PHA on cell multiplication in vitro for fixed doses of PPHA were performed by determining cell numbers at various times after exposure. This was done in two culture systems: L-fibroblasts in monolayer culture and ML-388 cells in suspension culture. The results are presented in Figures 5 through 8 and in Table 5.

Results given in Figures 5 and 7 show that for L-fibroblasts in monolayer culture, cell number was depressed as early as 24 hours after exposure to PPHA at a concentration of 30 mcg/ml of medium. This inhibition increased to approximately 50 percent by 48 hours after PPHA addition.

The number of free cells in PPHA-treated cultures (Figure 6) did not appear to change significantly throughout the four-day period following addition of the growth-inhibitory protein. In contrast, after a 24 hour lag period the number of free cells in the medium of rapidly-growing control cultures appeared to multiply at the same rate as the number of attached cells per culture.

The data in Table 5 show that the growth-inhibitory effect of PPHA for rapidly-growing mammalian cells is related to dose and to length of exposure.

In the second test system the effects of 20 mcg of PHA-BW/ml of medium on the proliferation of Mouse Lymphoma cells in suspension culture were investigated (Figure 8). The effect of PHA addition was an immediate cessation of growth whereas the control cultures continued in logarithmic phase growth for at least 72 hours.

Figure 5.

Growth inhibitory effect of a fixed dose of PHA on L-fibroblasts: Effect on number of attached cells.

Cells were grown as replicate monolayer cultures in eight ounce prescription bottles. Preparation PPHA or saline (as control) was added 16 to 24 hours after cell inoculation to give a final concentration of 30 mcg PPHA/ml of culture medium. At various times after PPHA addition cells growing attached to the glass were harvested from six bottles each of control and treated cells, and cell number per bottle determined as described in the Methods. Data presented in this Figure represent the mean and standard errors for cell number per bottle from eight such experiments.

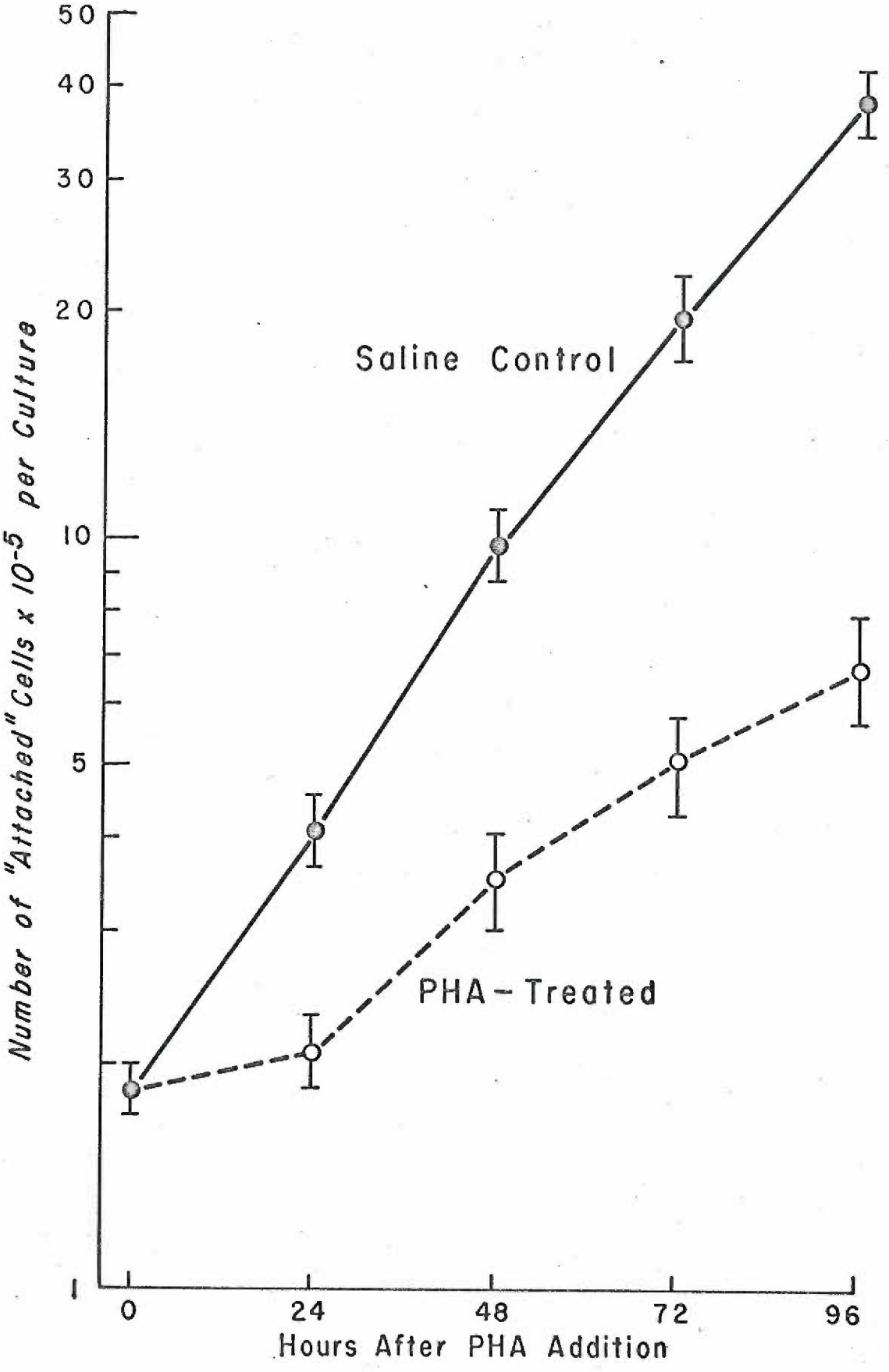

50
40
30
20
10
5
1
Number of "Attached" Cells x 10-5 per Culture
Saline Control
PHA-Treated
0
24
48
72
96
Hours After PHA Addition

Figure 6.

Growth inhibitory effect of a fixed dose of PHA on L-fibroblast: Effect on number of free cells.

Experimental conditions were the same as those described for Figure 5. Free cells were those growing unattached to the glass and collected either when the medium was decanted from the culture bottles or when the attached cells were gently rinsed once with BSS. The rinse solution and medium were pooled before aliquots were removed for cell number determination as described in the Methods. Data presented in this Figure represent the mean and standard errors for cell numbers per bottle from eight such experiments.

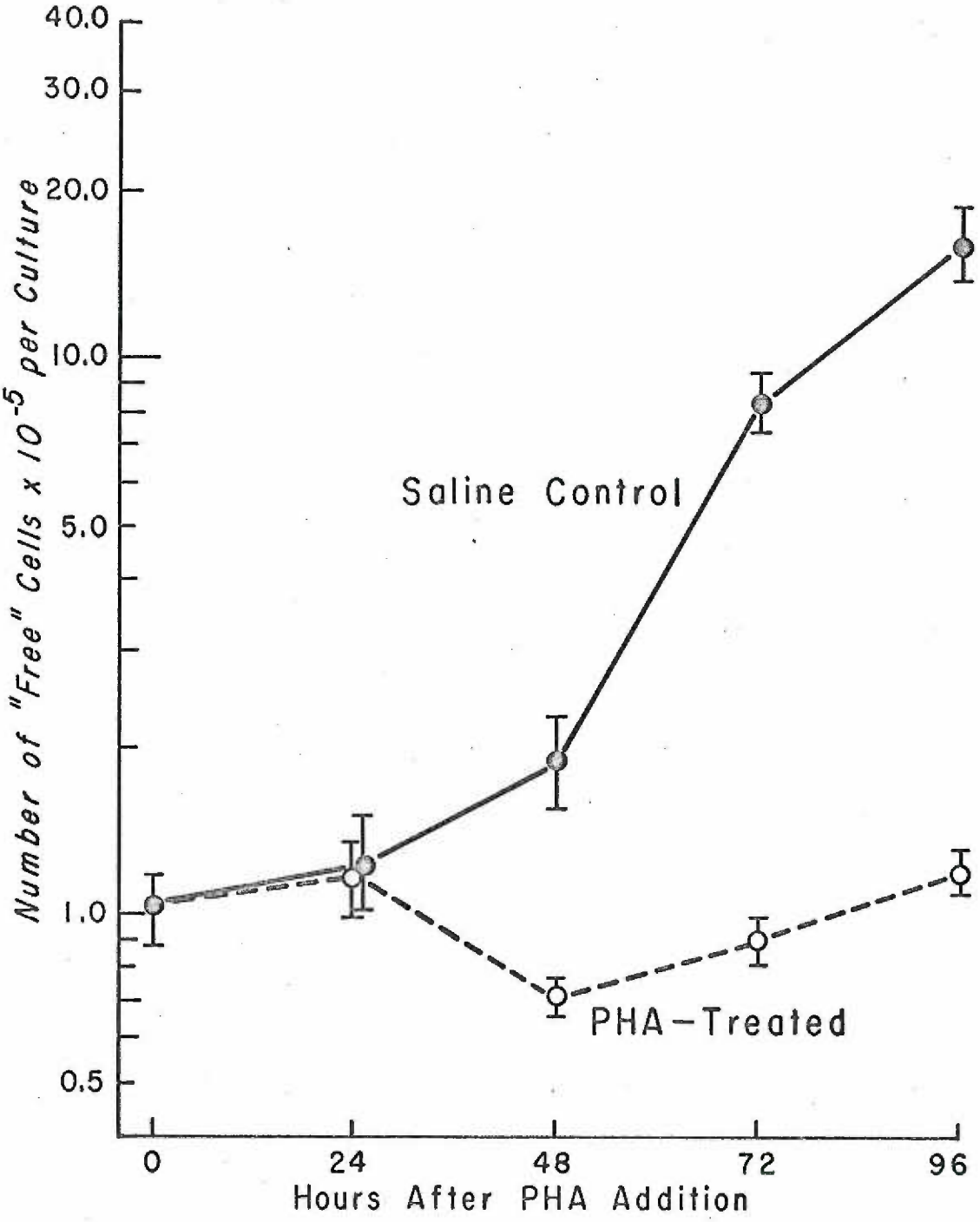

40.0
30.0
20.0
10.0
5.0
1.0
0.5
Number of "Free" Cells x 10^{-5} per Culture
Saline Control
PHA-Treated
0
24
48
72
96
Hours After PHA Addition

Figure 7.

Growth inhibitory effect of a fixed dose of PHA on L-fibroblasts: Effect on total cell number.

The experimental conditions were the same as those described in Figures 5 and 6. The values for total cells represent the sum of the values for free and attached cells per bottle determined at various times after PPHA addition to the drug-treated cultures. Values plotted represent mean and standard errors for the pooled data of eight typical experiments.

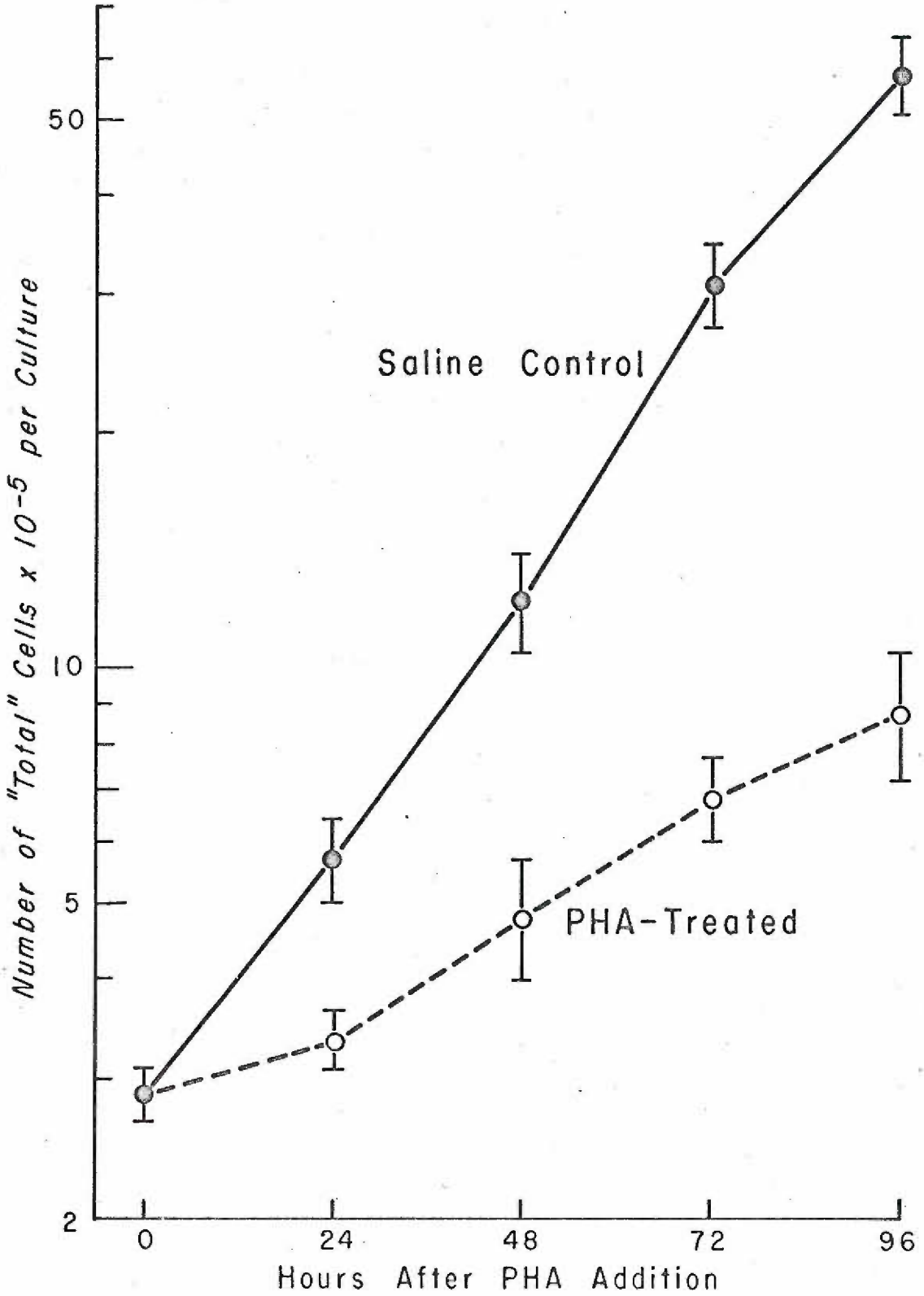

Number of "Total" Cells x 10-5 per Culture
50
10
5
2
Saline Control
PHA-Treated
0
24
48
72
96
Hours After PHA Addition

Figure 8.

Growth inhibitory effect of PHA on ML-388 cells grown in suspension culture.

Mouse Lymphoma ML-388 cells were grown in log-phase in two identical suspension cultures. Although the absolute number of cells per flask differed slightly, growth rates (doubling times) in the two cultures were shown to be identical for at least 48 hours prior to the addition of PHA. One culture received preparation PHA-BW (Batch X5) to give a final concentration of 20 mcg PHA-BW/ml of medium and appropriate vehicle (saline) was added to the other culture. Growth was estimated by triplicate determinations of cell number at various intervals after PHA-BW addition as described in the Methods. Results plotted represent the mean and standard error for five typical experiments from which the data was pooled. The doubling time of the control cultures was not materially affected by the addition of vehicle.

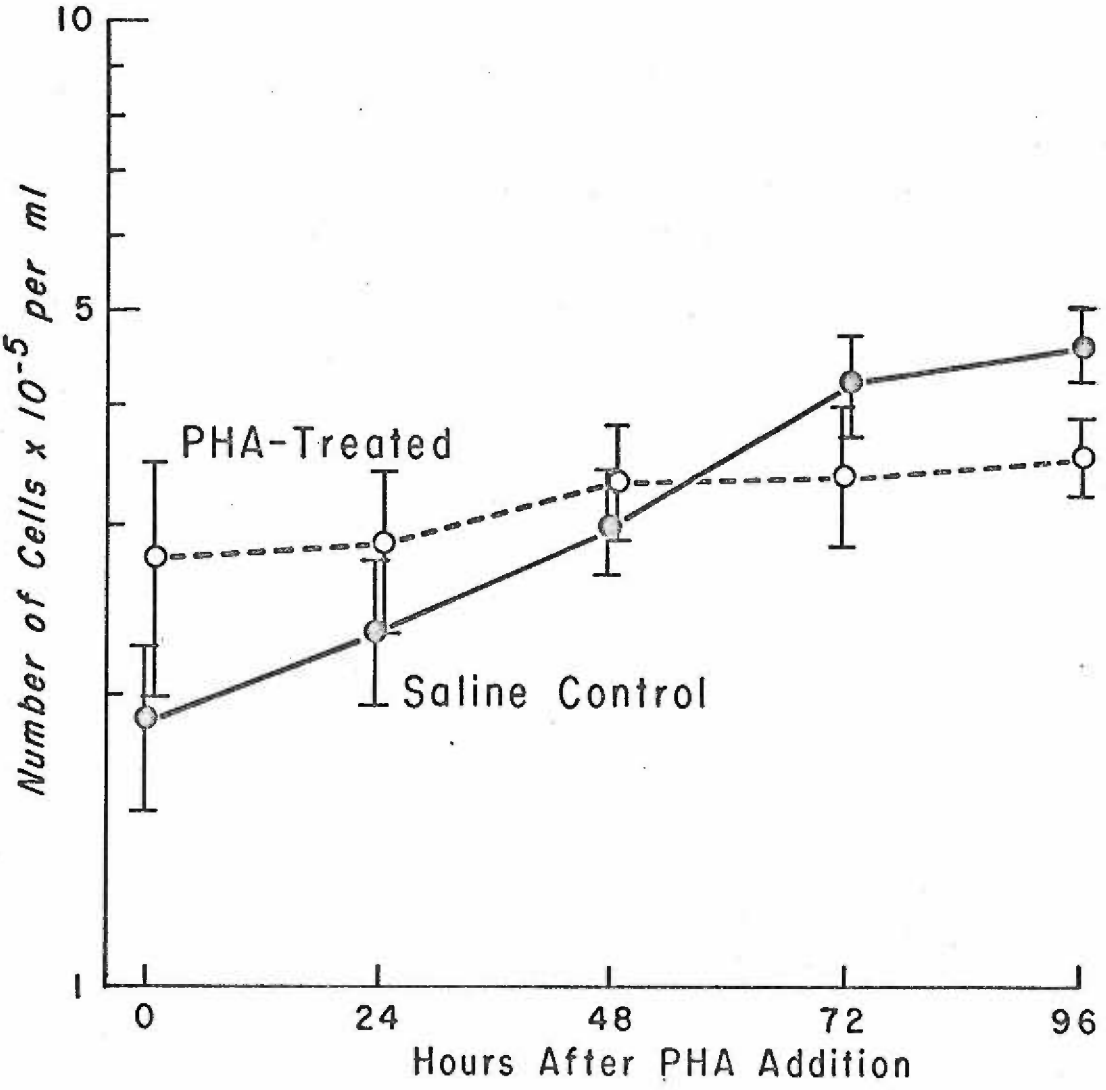

10
5
1
Number of Cells x 10^{-5} per ml
PHA-Treated
Saline Control
0
24
48
72
96
Hours After PHA Addition

TABLE 5.

Dose-time Relationship of Growth Inhibitory Effect of PHA on L-fibroblasts Grown *In Vitro*

Hours after PPHA Addition	Percent Inhibition of Growth at Various PPHA Concentrations per ml of Culture Medium		
	3 mcg/ml (n = 3)	10 mcg/ml (n = 6)	30 mcg/ml (n = 6)
24	-	35.5 ± 4.8	47.6 ± 2.0
48	-	56.0 ± 3.5	63.3 ± 1.9
72	-	64.6 ± 2.9	73.0 ± 1.5
96	57.0 ± 4.0	74.4 ± 1.9	81.3 ± 1.5

L-fibroblasts were grown in replicate cultures and exposed to various concentrations of PPHA as indicated. At various times after PPHA addition, cell number was determined (see Methods) and the results were expressed as percent inhibition as compared to control cultures receiving an equivalent volume of the appropriate vehicle.

Results presented are the mean and standard error of three to six bottles per concentration.

Cells Grown in Vivo. The effect of PHA on cell growth in vivo was also examined for the L-1210 transplantable lymphocytic leukemia growing in the ascites form in the peritoneal cavity of BDF_1 mice. First, toxicity studies of PHA in normal mice were performed for intraperitoneal administration over a dose range of 3 to 300 mg PPHA/kg of body weight (see Methods). The maximum permissible single dose which did not kill any mice was 100 mg PPHA/kg when given intraperitoneally. A single dose of 150 mg PPHA/kg killed 15 of 21 mice so injected. A single dose of 200 mg PPHA/kg killed all mice. The 100 mg PPHA/kg dose, given to nontumor-bearing mice on two consecutive days, resulted in a 57 percent survival rate. All deaths attributed to PPHA administration occurred within the first 48 hours following administration. Animal weight studies revealed that normal BDF_1 mice with a single intraperitoneal dose of 100 mg PPHA/kg experienced a weight loss of one to three grams during the first 48 hours following PPHA administration. During the next five days their weights returned to pretreatment values.

The effects of PPHA to increase the mean survival times (MST) of mice inoculated with the L-1210 tumor are seen in Figure 9 and Table 6. The tumor had a doubling time of 0.55 days under the conditions used in these studies. Thus a prolongation of 0.55 days in the MST was equivalent to a 50 percent inhibition of growth.

The effect of intraperitoneal administration of PPHA on subcutaneous inoculated tumors of L-1210 cells in BDF_1 mice was also tested. No increase in MST was observed in these animals.

Figure 9.

Effect of PHA administration on the mean survival time of BDF_1 mice inoculated with Mouse Leukemia L-1210 tumor cells.

Twenty control and thirty experimental mice were inoculated intraperitoneally with approximately 5×10^5 L-1210 cells. Twenty-four hours after tumor inoculation ten control mice were injected intraperitoneally with 0.1 percent solution of bovine serum albumin, and ten received no treatment (see Methods).

Normal survival times for control tumor-bearing mice with or without bovine serum albumin are represented here by solid circles. All such mice died within a twenty-four hour period. Open circles represent tumor-bearing mice which received a single dose of PPHA twenty-four hours after tumor inoculation. Open squares represent tumor-bearing mice which received a single dose of PPHA one hour after tumor inoculation. Solid squares represent tumor-bearing mice which received one dose of PPHA at twenty-four hours, and a second dose of PPHA at forty-eight hours after tumor inoculation. All PHA treatments were intraperitoneal, with a dosage of 100 mg PPHA/kg of body weight.

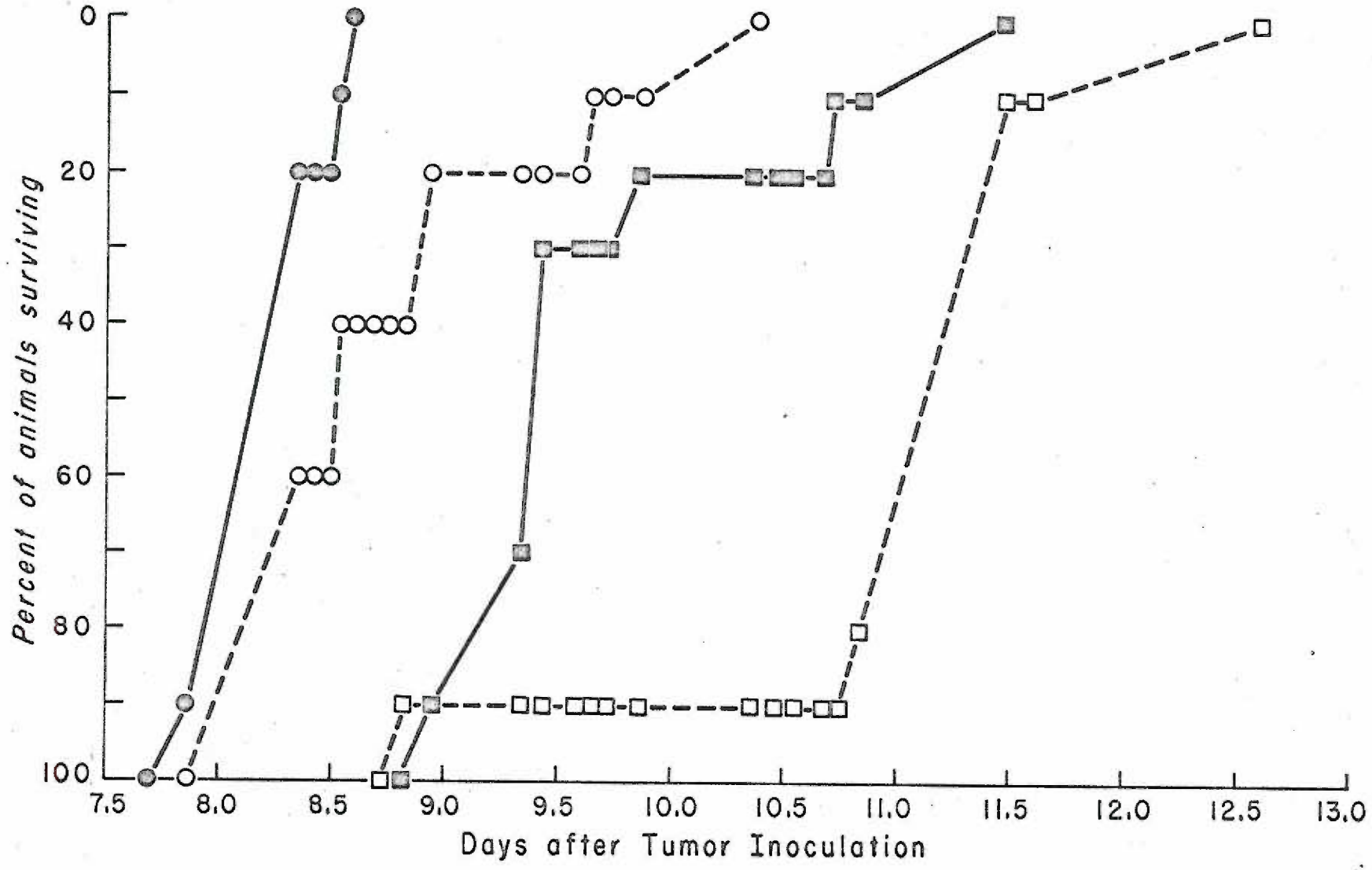

Percent of animals surviving
0
20
40
60
80
100
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
11.5
12.0
12.5
13.0
Days after Tumor Inoculation

TABLE 6.

Effect of PHA on Mean Survival Times of BDF_1 Mice Inoculated with L-1210 Tumor Cells

PHA Treatment	Mean Survival Times (MST) (± s.e.m.)	Difference in MST from Control	P Value*
None	8.34 ± .06 days	-	-
1 dose 24 hours post tumor	8.83 ± .22 days	0.49 days	< 0.05
2 doses 24 and 48 hrs post tumor	9.73 ± .28 days	1.39 days	< 0.01
1 dose 1 hour post tumor	11.24 ± .30 days	2.90 days	< 0.01

MST values were estimated graphically from data given in Figure 9 for ten animals per group as the mean and standard error (s.e.m.)

*Student's t-test (two-tail) was performed to determine the level of significance for the MST differences.

Characteristics of PHA preparations. To investigate the possibility that most commercial PHA preparations (although varying in purity) were also capable of inhibiting growth, the relative potency of a variety of preparations was determined for Mouse Lymphoma (ML-388) cells growing in vitro in replicate monolayer cultures. The effects of various concentrations of the PHA preparations on the rapidly-growing ML-388 cells are indicated in Table 7. The IC_{50} values for preparation PPHA were shown to vary somewhat from experiment to experiment (see Tables 3, 7, 8 and 9). These variations have been shown to be due, at least in part, to variations in cell inoculum size between experiments (Table 8). A dose-response determination for a freshly-prepared solution of PPHA was performed simultaneously with any other preparation tested (Table 7). This was done to minimize possible fluctuations which might occur because of slight differences in inoculum size or other undetermined factors.

A comparison of the proper preservation conditions for preparation PPHA was shown by dose-response assays to be storage of the lyophilized material in a screw-capped vial at -10 to -20° C rather than as sterile solutions at $4 \pm 1°$ C (355). The effect of storage in solution is shown in Table 9. Potency is seen to decrease by threefold within six months.

BIOCHEMICAL ANALYSIS OF GROWTH INHIBITION

Effect of PHA on prelabelled DNA dilution. To investigate further the early effects of PHA on cellular proliferation, experiments were conducted with monolayer cultures of L-fibroblasts that had their DNA prelabelled with ^{14}C-thymidine. Radioactivity per cell, specific activity (cpm/mμmoles deoxyribose) and appearance of radioactivity into the medium were followed with time after exposure to PPHA (30 mcg/ml of

TABLE 7.

Relative Potencies of Different
Commercial PHA Preparations

Preparation	IC_{50} Value	Relative Potency
PPHA	4.0 ± 0.2	0.28
PHA-M	14.5 ± 1.1	
PPHA	5.9 ± 0.2	0.37
PHA (M form)	16.0 ± 1.8	
PPHA	2.2 ± 0.2	0.43
PHA-P	5.1 ± 0.5	
PPHA	2.9 ± 0.2	0.58
PHA-BW (Batch X-5)	5.0 ± 0.1	
PPHA	5.1 ± 0.8	1.55
PHA-BW (Batch E-118)	3.3 ± 0.5	

Mouse Lymphoma (ML-388) cells were grown in replicate monolayer cultures for standardized dose-response experiments as described in the Methods and in the legend to Figure 4. The IC_{50} values were estimated graphically for simultaneously conducted analyses using purified protein phytohemagglutinin (PPHA) and each commercial PHA preparation evaluated (see Materials). Results were expressed as the mean and standard error for three cultures per dose for each preparation. Relative potencies are with respect to PPHA equal to 1.00.

TABLE 8.

Relationship of Inoculum Size to Determination of IC_{50} Values for Preparation PPHA

Experiment Number	Cell Number per Bottle at Time of PPHA Addition	IC_{50} Value ($\pm$ s.e.m.)
083166	$6.40 \pm 0.10 \times 10^4$	2.2 ± 0.2
081765	$9.50 \pm 0.11 \times 10^4$	3.5 ± 0.5
040667	$1.21 \pm 0.05 \times 10^5$	2.9 ± 0.2
081168	$1.60 \pm 0.11 \times 10^5$	3.2 ± 0.4
021767	$2.31 \pm 0.12 \times 10^5$	2.8 ± 0.6
082966	$2.68 \pm 0.02 \times 10^5$	4.0 ± 0.2
022168	$3.04 \pm 0.06 \times 10^5$	5.1 ± 0.8
092166	$3.47 \pm 0.11 \times 10^5$	7.6 ± 0.6
091466	$3.94 \pm 0.14 \times 10^5$	5.9 ± 0.2

ML-388 cells were grown in replicate monolayer cultures for dose-response analyses as described in the Methods and in the legend to Figure 4. Cell number per bottle for a minimum of three bottles was determined for each experiment prior to the addition of various concentrations of preparation PPHA. Ninety-six hours later cell numbers per bottle were again estimated and the IC_{50} values were determined graphically. Results were expressed as the mean and standard error for at least three bottles per PPHA concentration tested. The apparent positive correlation was 0.492 and it was significant ($P < 0.10$) as determined by the Student's t-test (one-tail).

TABLE 9.

Storage Properties of PPHA Solutions

	IC_{50} Value (± s.e.m.)
Freshly prepared	1.9 ± 0.3
3 months in storage	3.6 ± 0.5
6 months in storage	5.8 ± 0.5

Preparation PPHA solutions (10 mg/ml), stored sterile for various times at 4 ± 1° C, were added to replicate cultures of Mouse Lymphoma (ML-388) cells and dose-response curves were obtained (see Methods and Figure 4). IC_{50} values for each solution were determined graphically.

medium) or an equal volume of saline-vehicle (as control). The results of these experiments are given in Figures 10 and 11 and Table 10. The data in Figure 10 shows that the decrease in the radioactivity per cell with time for rapidly-growing control cells occurred at a more rapid rate than for the PPHA-treated cells. The difference appeared significant by 24 hours after PPHA addition. The rate of dilution of radioactivity per mμmole of deoxyribose (Figure 11) by newly-formed non-radioactive DNA was also substantially reduced in PPHA-treated cells by 24 hours as compared to saline-treated cells. At the same time the radioactivity measurements of the cell-free medium showed that the control cells and the PPHA-treated cells were not significantly different in their ability to retain the ^{14}C-label up to 72 hours after treatment (Table 10).

Effect of PHA on precursor uptake. The effects of PHA on nucleic acid and protein synthesis were studied by measuring the incorporation of ^{14}C-labelled precursors into cold-acid insoluble material in the presence and absence of PPHA. L-fibroblasts grown in replicate monolayer cultures were exposed to 30 mcg PPHA/ml of medium or an equal volume of saline as control. This concentration of PPHA had been demonstrated to produce approximately 63 percent inhibition of growth within 48 hours (Table 5).

The data from the one-hour "pulse" incorporations of radioactive-labelled precursor was expressed on a per culture basis and on a per cell basis for ^{14}C-leucine (Figures 12 and 13), ^{14}C-uridine (Figures 14 and 15) and ^{14}C-thymidine (Figures 16, 17 and 18). Figures 12, 14 and 16 show that incorporation of leucine, uridine and thymidine into protein, RNA and DNA, respectively, has been inhibited by 24 hours after treatment

Figure 10.

Effect of PHA on radioactivity of L-fibroblasts prelabelled with ^{14}C-thymidine.

Preparation PPHA (or an equivalent volume of saline) was added during early logarithmic phase growth to replicate monolayer cultures of L-fibroblasts containing 20 ml of medium. Final PPHA concentration was 30 mcg/ml of medium. The DNA of these cells had been prelabelled with ^{14}C-thymidine as described in the Methods.

At various times after PPHA addition, cell number and radioactivity in the cold-acid insoluble material extracted from the cells was determined.

Each value presented in the Figure represents the mean and standard error for at least six replicate cultures.

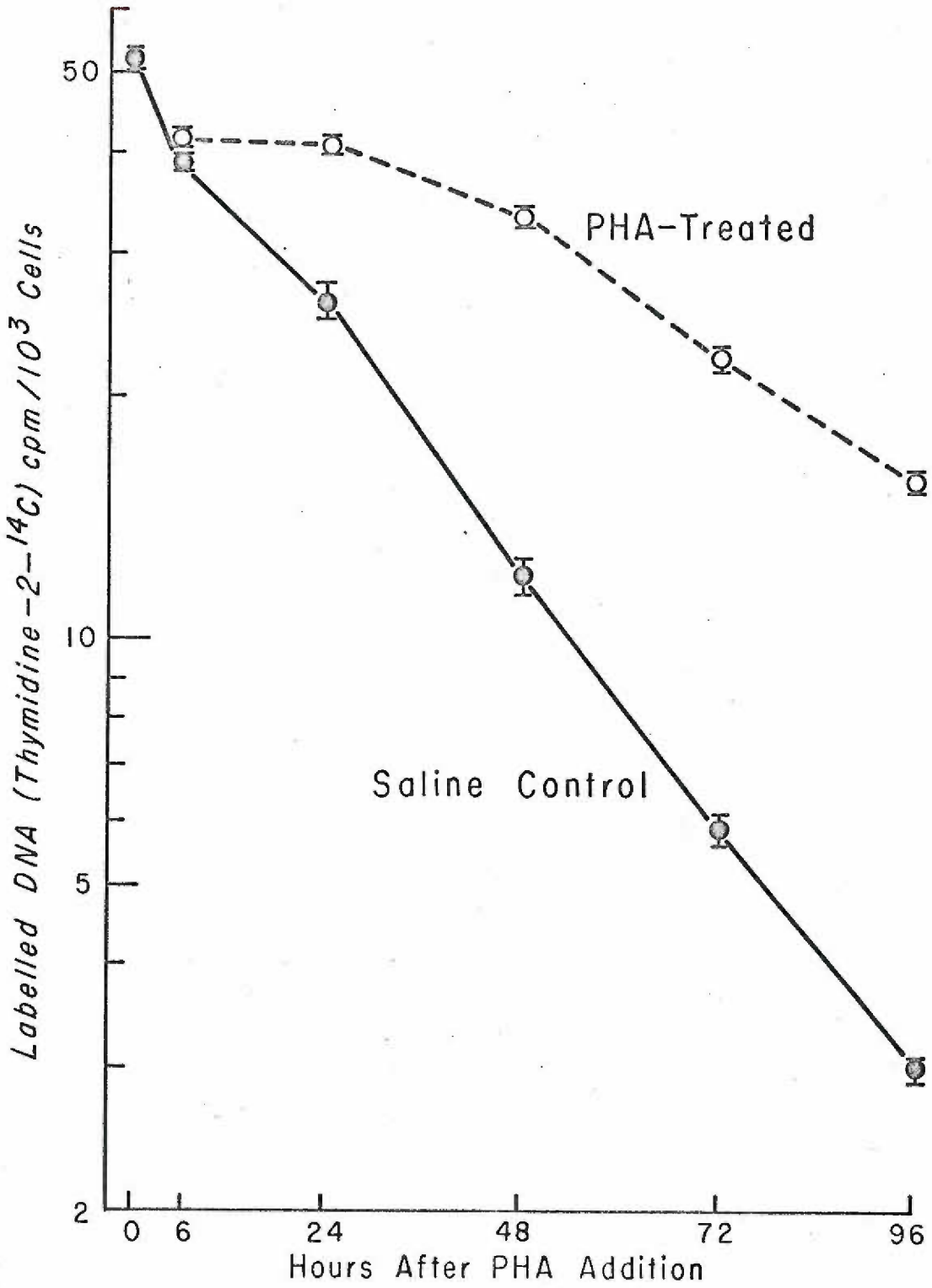

50
10
5
2
Labelled DNA (Thymidine-2-^{14}C) cpm/10^3 Cells
PHA-Treated
Saline Control
0
6
24
48
72
96
Hours After PHA Addition

Figure 11.

Effect of PHA on specific activity of DNA in L-fibroblasts prelabelled with ^{14}C-thymidine.

The concentration of PPHA and the culture conditions have been described in the legend to Figure 10. At various times after PPHA addition the cellular content of both DNA (deoxyribose) and radioactivity were determined as described in the Methods, and specific activity (cpm/mμmole deoxyribose) was calculated. Each value presented in the Figure represents the mean and standard error for at least six replicate cultures.

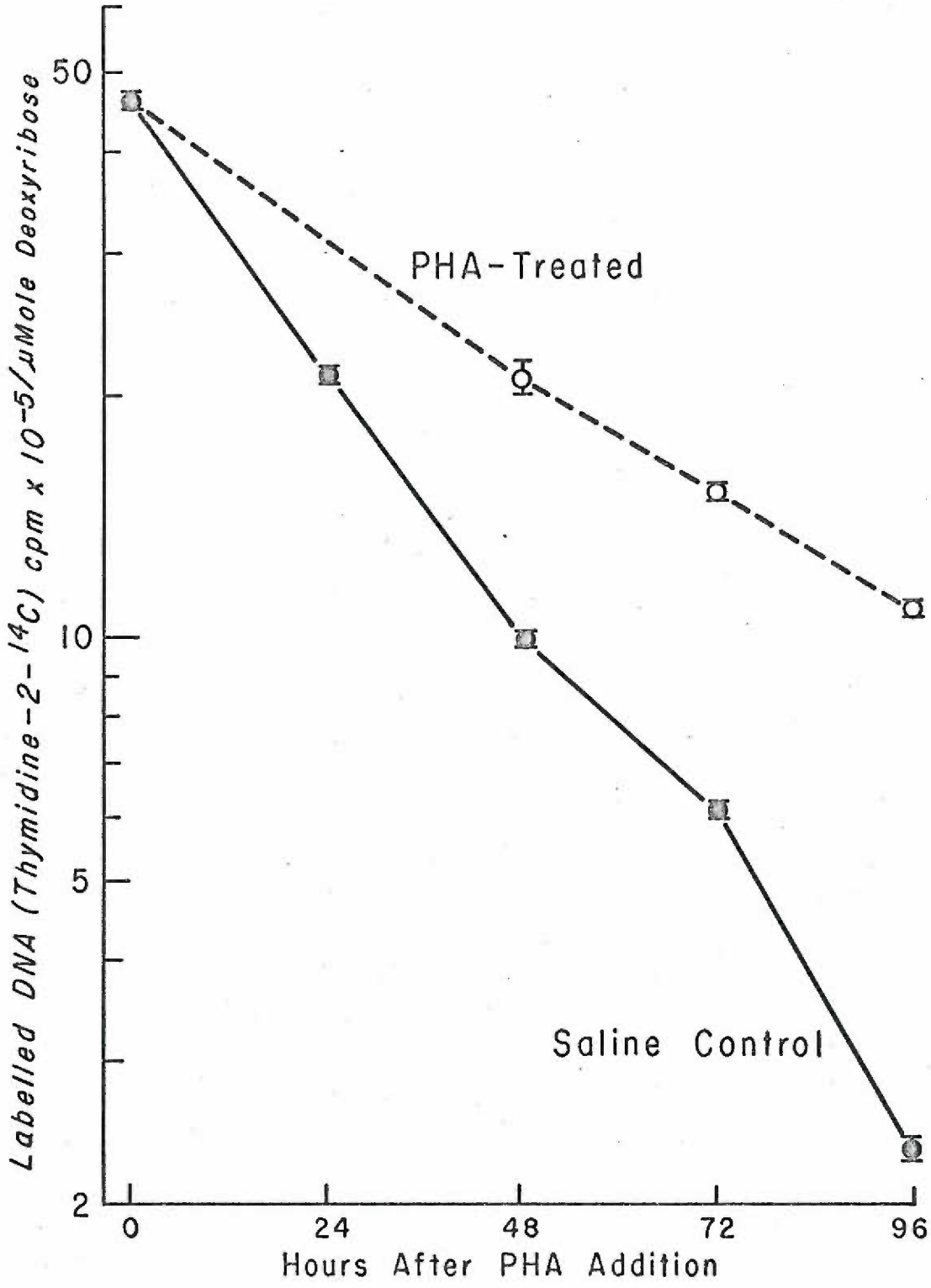

Labelled DNA (Thymidine-2-^{14}C) cpm x 10^{-5}/μMole Deoxyribose
50
10
5
2
PHA-Treated
Saline Control
0
24
48
72
96
Hours After PHA Addition

TABLE 10.

Effect of PHA on Appearance of Radioactivity in the Culture Medium by L-Fibroblasts Prelabeled with ^{14}C-thymidine.

Hours after PPHA addition	Cpm per milliliter of medium		
	Saline-control	PPHA-treated	P Value*
0	544 ± 35	---	---
24	503 ± 53	562 ± 26	< 0.40
48	558 ± 37	624 ± 27	< 0.40
72	594 ± 21	642 ± 17	< 0.20
96	1552 ± 139	846 ± 30	< 0.01

Data presented here was derived from the experiment described in Figure 10.

At various times after PPHA addition and termination of label incorporation, the medium was decanted from the bottles and the attached cells of each bottle were rinsed once with 5 ml of BSS. The medium and rinse from each bottle were pooled (total volume 25 ml), free cells were removed by centrifugation, and the radioactivity in an aliquot of cell-free medium was determined (see Methods). The results presented represent the mean and standard error values for five bottles each of treated and control cultures.

*Values were determined by Student's t-test (two-tail).

Figure 12.

Effect of PHA on ^{14}C-leucine incorporation by L-fibroblasts (per culture).

Preparation PPHA (or an equivalent volume of saline) was added to replicate monolayer cultures of L -fibroblasts containing 20 ml medium during early logarithmic phase growth (see Methods). Final PPHA concentration was 30 µg/ml of medium.

At various times after PPHA addition the original medium was replaced with fresh medium containing 3.99×10^{-4} M leucine in both treated and control cultures. Then, 0.476 µc L-leucine-1-^{14}C (31.0 µc/µM) was immediately added to each culture. One hour later label incorporation was terminated by chilling and the addition of 1.0 ml 1.0 percent L-leucine per culture. The cold-acid insoluble cell residue was extracted, collected and assayed for radioactivity as described in the Methods.

Each value presented in the Figure represents the mean and standard error for at least six replicate culture.

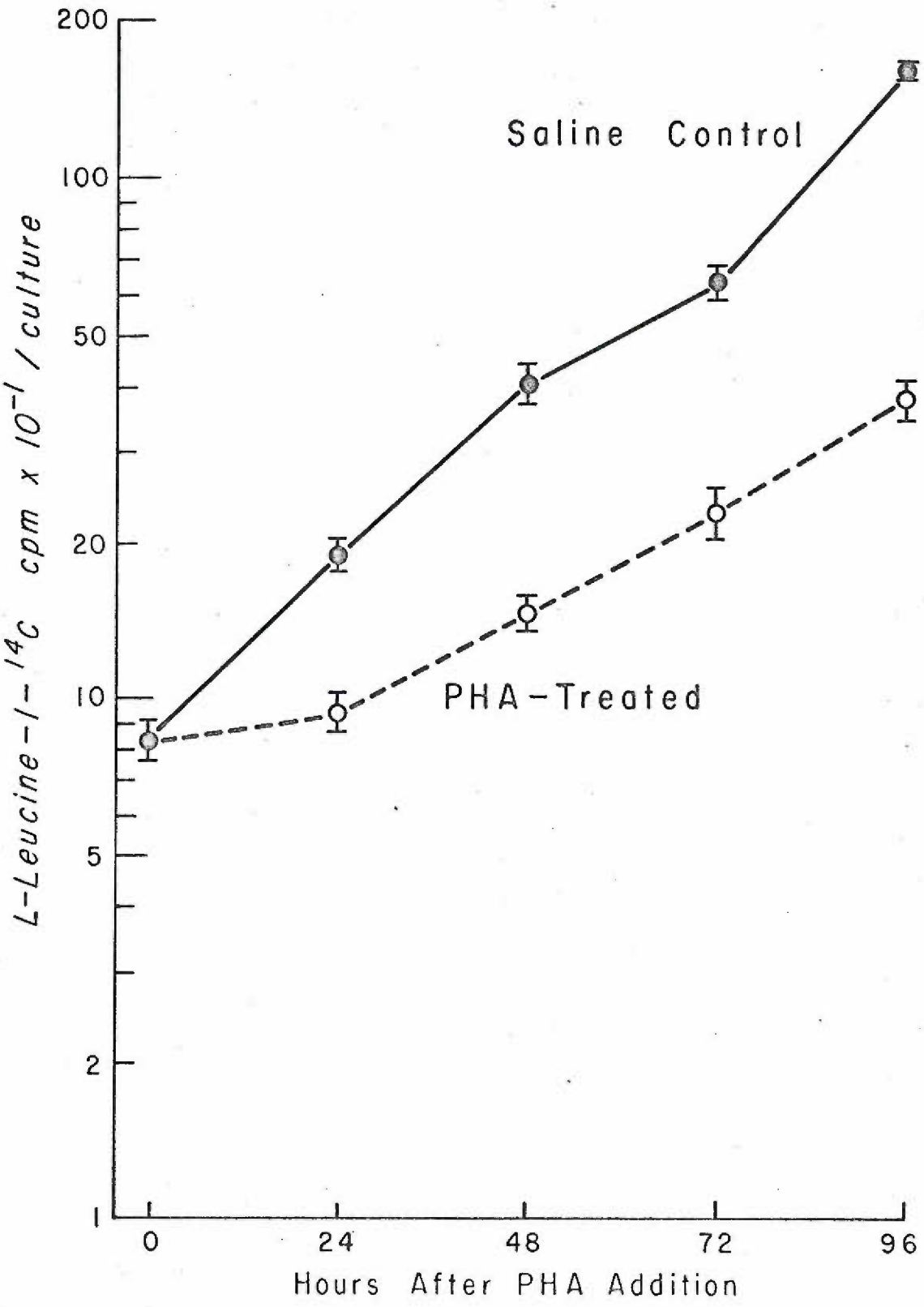

200
100
50
20
10
5
2
1
L-Leucine-1-^{14}C cpm x 10^{-1} / culture
Saline Control
PHA-Treated
0
24
48
72
96
Hours After PHA Addition

Figure 13.

Effect of PHA on ^{14}C-leucine incorporation by L-fibroblasts (per cell).

Cell number per bottle was also obtained for the experiments described in Figure 12 (see Methods) and incorporation was calculated on a per cell basis for each culture. Each value given in this Figure represents the mean and standard error for at least six replicate cultures.

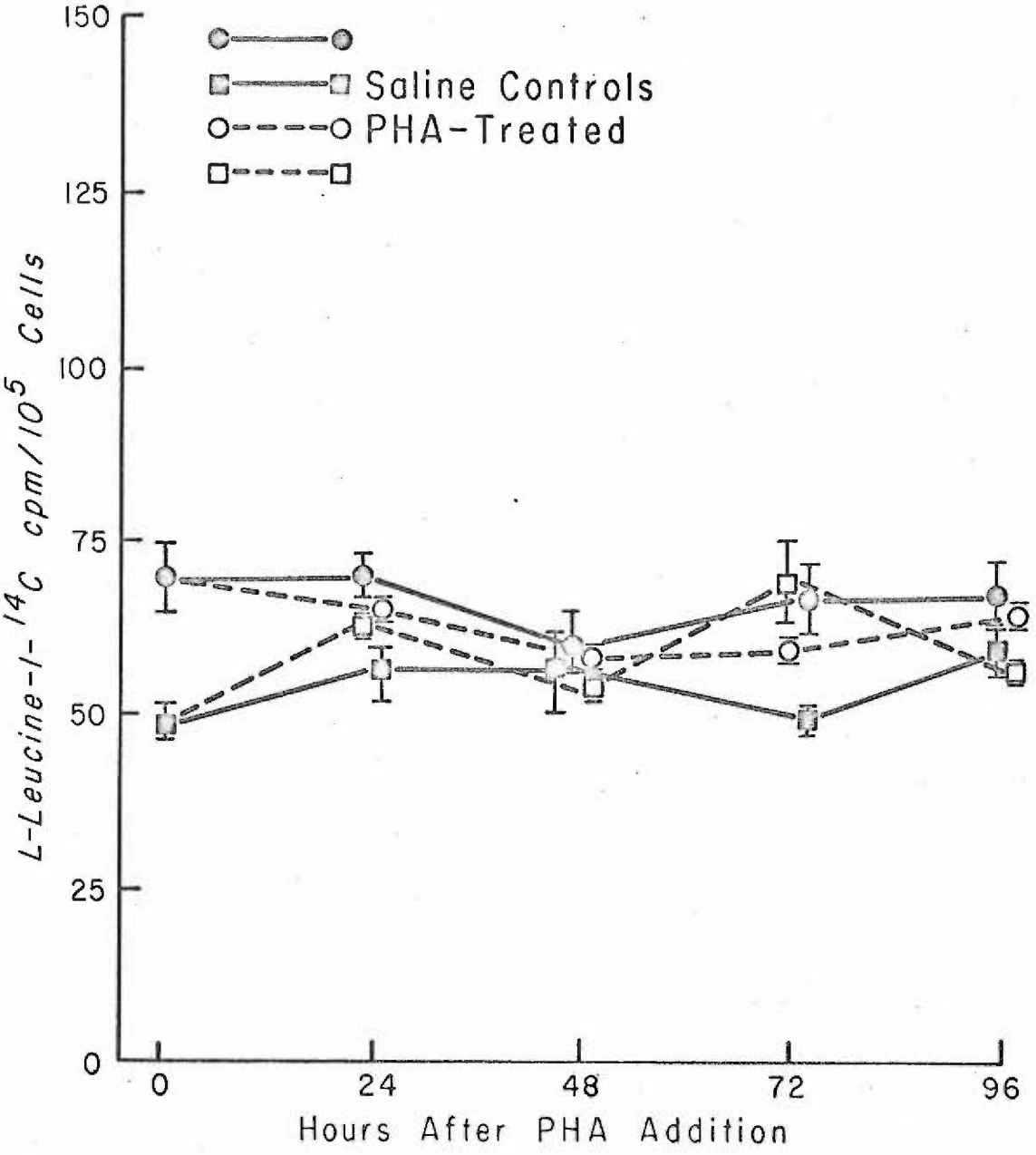

150
125
100
75
50
25
0
Saline Controls
PHA-Treated
L-Leucine-1-14C cpm/10^5 Cells
0
24
48
72
96
Hours After PHA Addition

Figure 14.

Effect of PHA on ^{14}C-uridine incorporation by L-fibroblasts (per culture).

Preparation PPHA (or an equivalent volume of saline) was added during early logarithmic phase growth to replicate monolayer cultures of L-fibroblasts containing 20 ml of medium (see Methods). Final PPHA concentration was 30 mcg/ml of medium.

At various times after PPHA addition the cells were exposed to 0.476 μc uridine-2-^{14}C (8.37 μc/μM) per culture. One hour later label incorporation was terminated by chilling and the addition of 1.0 ml of 1.0 percent uridine per culture. The cold-acid insoluble cell residue was extracted, collected and assayed for radioactivity as described in the Methods.

Each value presented in the Figure represents the mean and standard error for at least six replicate cultures.

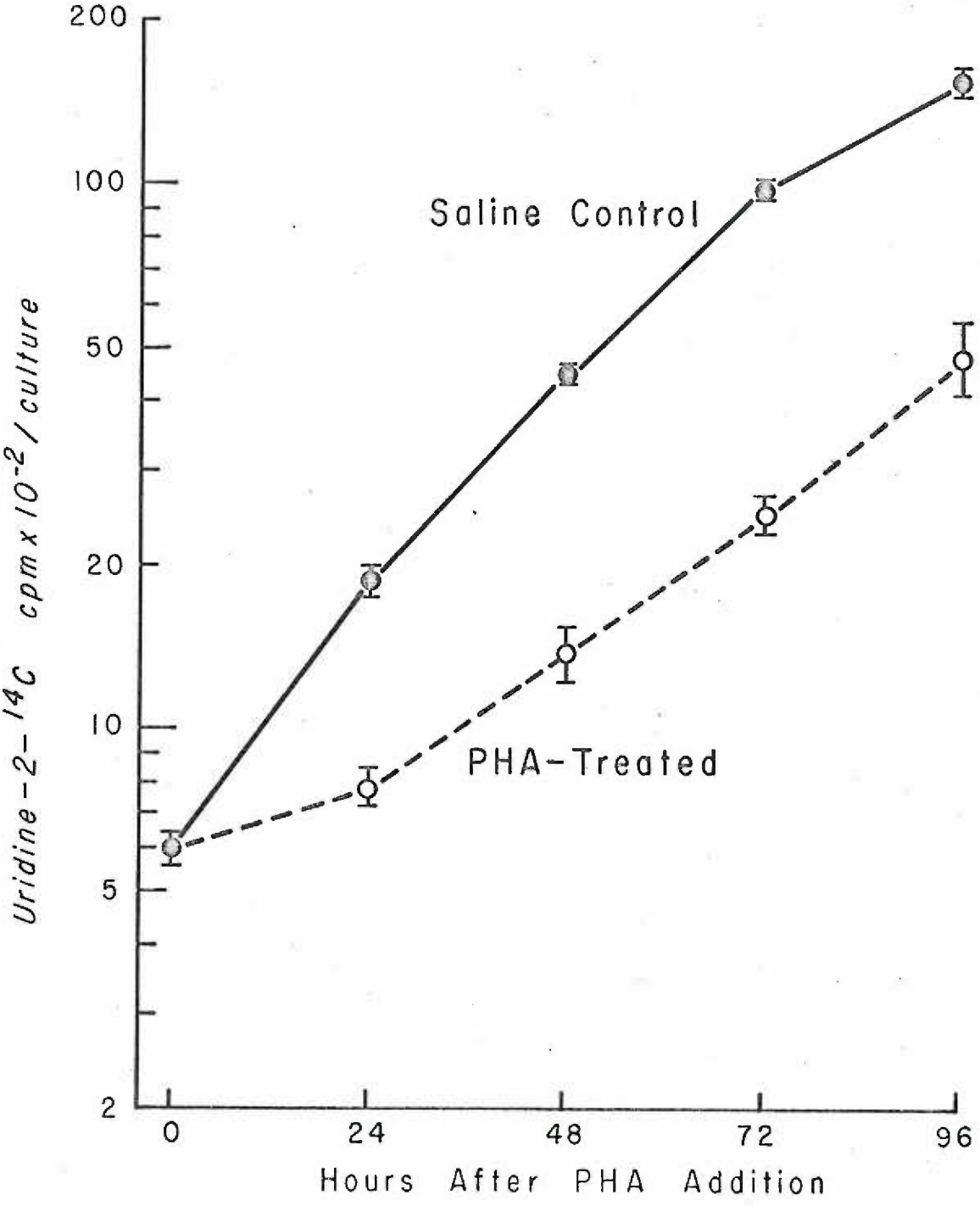

200
100
50
20
10
5
2
Uridine-2-^{14}C cpm x 10^{-2} / culture
Saline Control
PHA-Treated
0
24
48
72
96
Hours After PHA Addition

Figure 15.

Effect of PHA on ^{14}C-uridine incorporation by L-fibroblasts (per cell).

Cell number per bottle was also obtained for the experiments described in Figure 14 (see Methods), and incorporation was calculated on a per cell basis for each culture. Each value given in this Figure represents the mean and standard error for at least six replicate cultures.

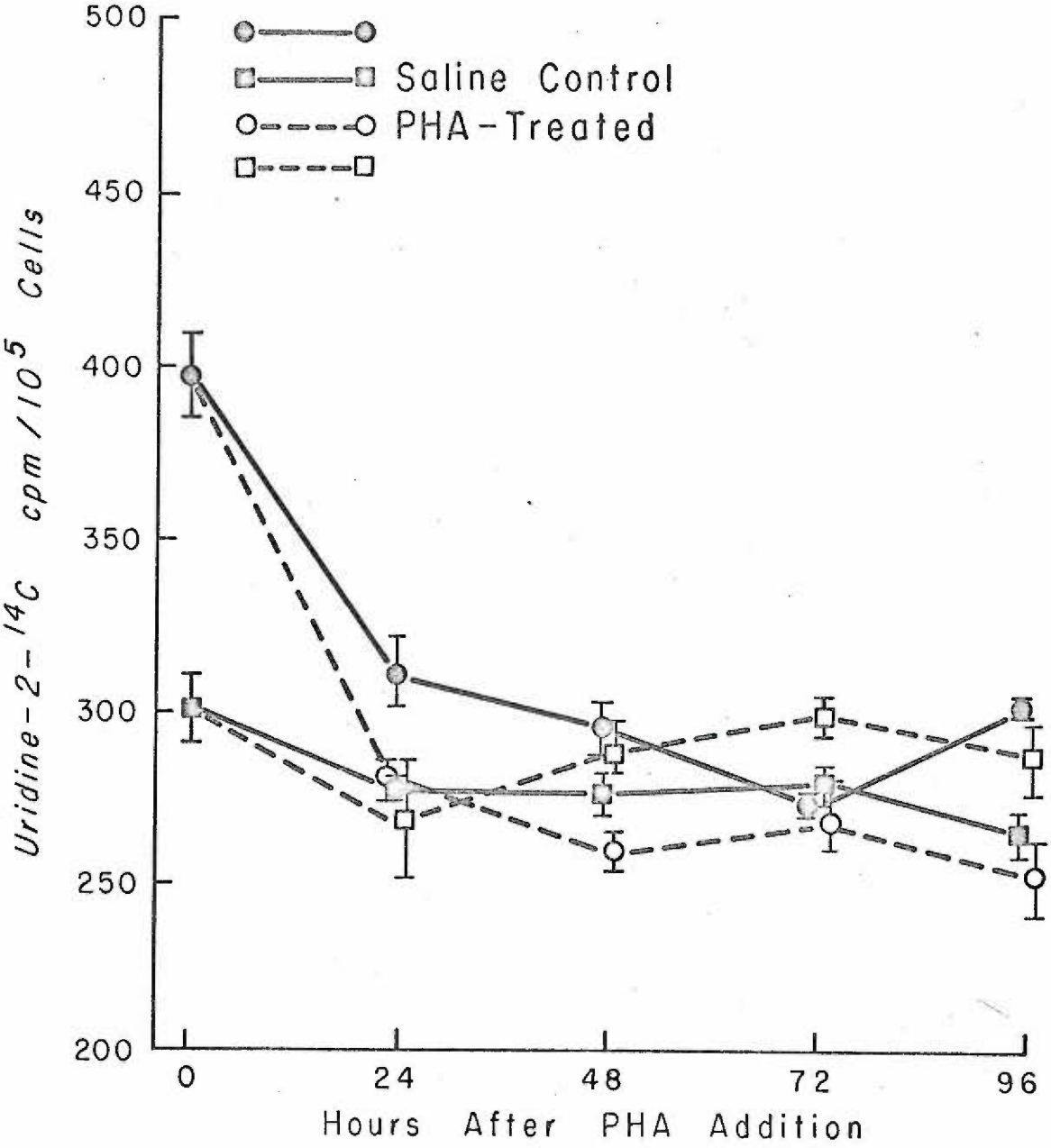

500
450
400
350
300
250
200
Saline Control
PHA-Treated
Uridine-2-^{14}C cpm / 10^5 Cells
0
24
48
72
96
Hours After PHA Addition

Figure 16.

Effect of PHA on ^{14}C-thymidine incorporation by L-fibroblasts (per culture).

Preparation PPHA (or an equivalent volume of saline) was added during early logarithmic phase growth to replicate monolayer cultures of L-fibroblasts containing 20 ml of medium (see Methods). Final PPHA concentration was 30 mcg/ml of medium.

At various times after PPHA addition the cells were exposed to 0.476 µc thymidine-2-^{14}C (8.43 µc/µM) per culture. One hour later label incorporation was terminated by chilling and the addition of 1.0 ml of 1.0 percent thymidine per culture. The cold-acid insoluble cell residue was extracted, collected and assayed for radioactivity as described in the Methods.

Each value presented in the Figure represents the mean and standard error for at least six replicate cultures.

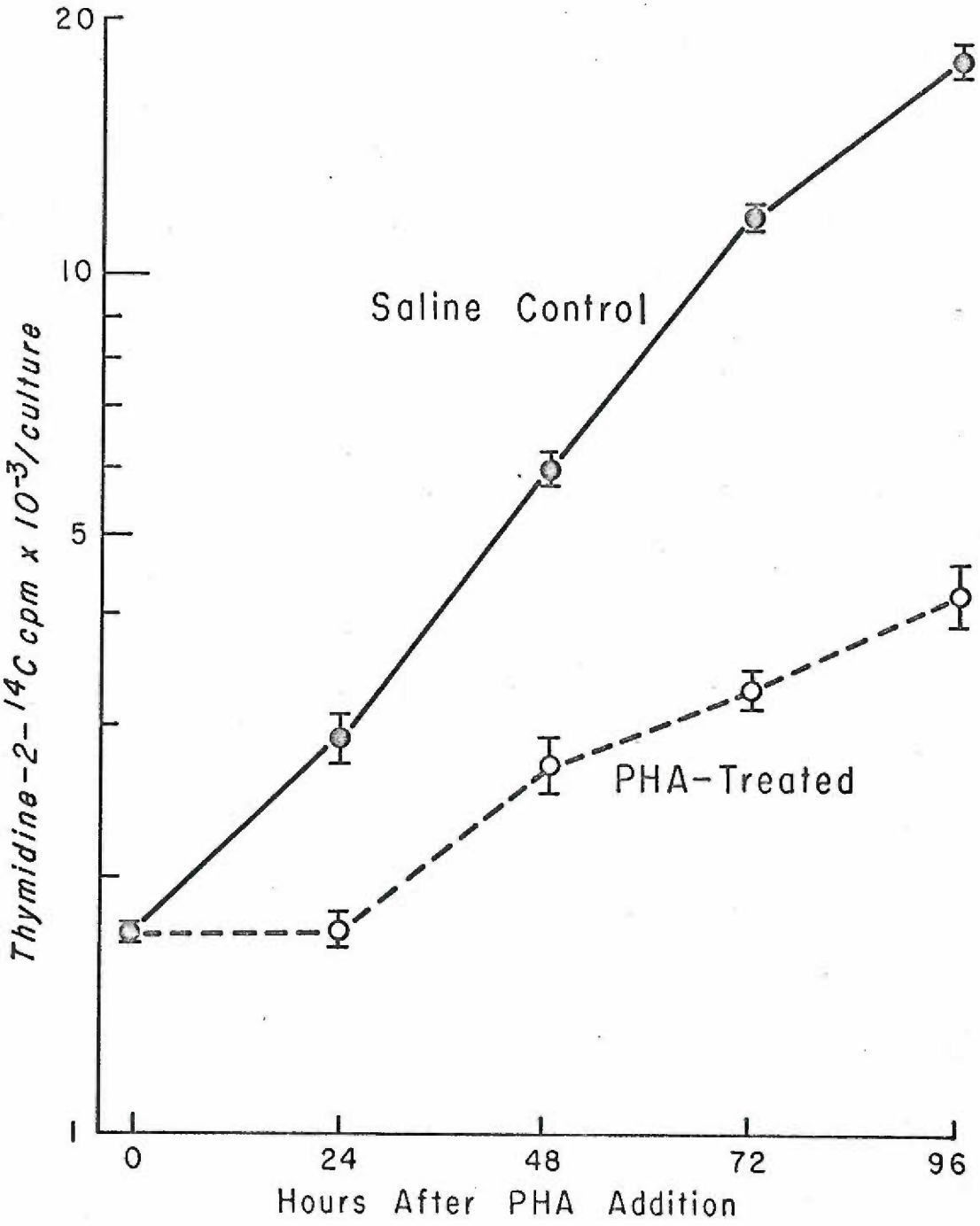

20
10
5
1
Thymidine-2-^{14}C cpm x 10^{-3}/culture
Saline Control
PHA-Treated
0
24
48
72
96
Hours After PHA Addition

Figure 17.

Effect of PHA on ^{14}C-thymidine incorporation by L-fibroblasts (per cell).

Cell number per bottle was also obtained for the experiments described in Figure 16 (see Methods), and incorporation was calculated on a per cell basis for each culture. Each value represents the mean and standard error for at least six replicate cultures.

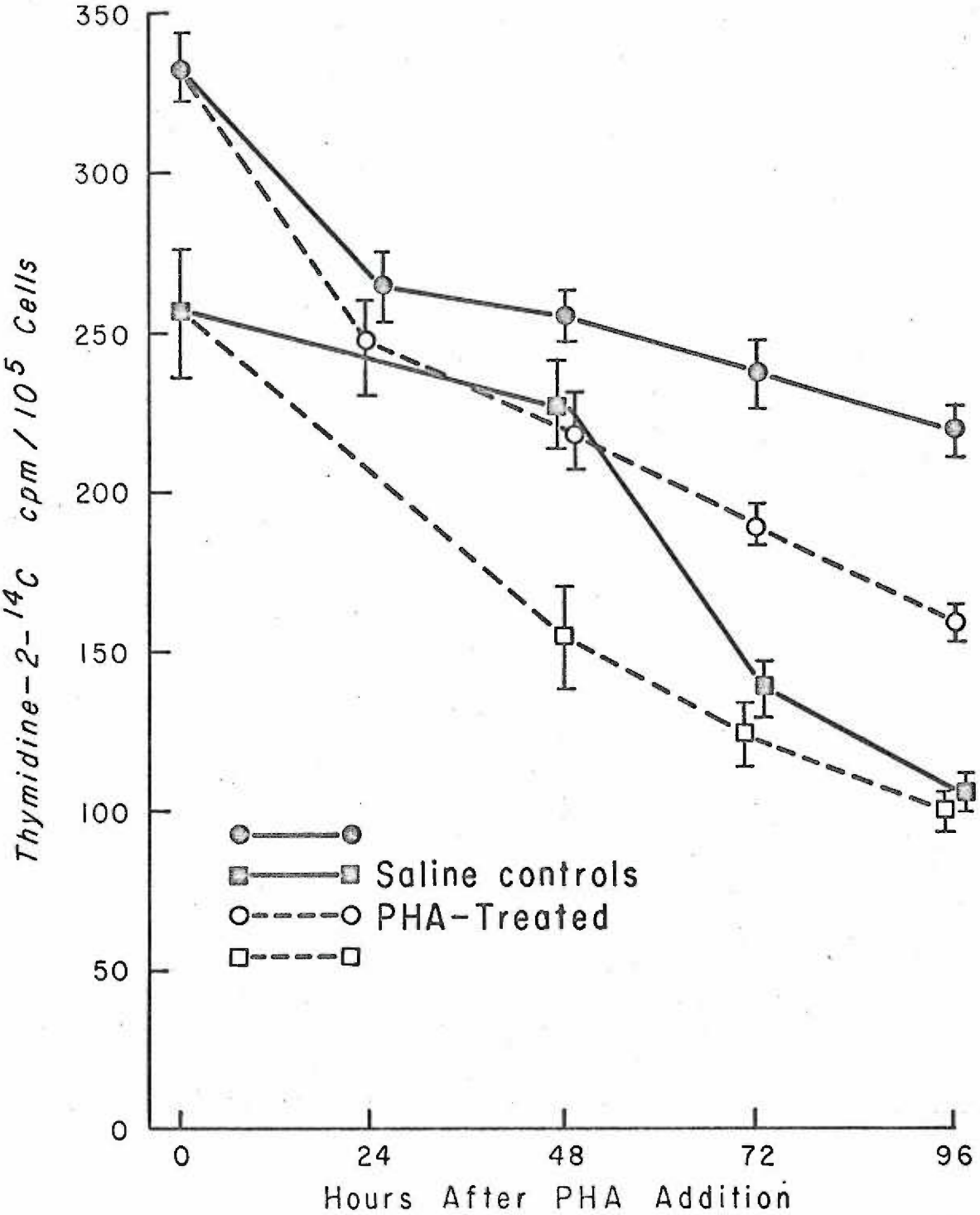
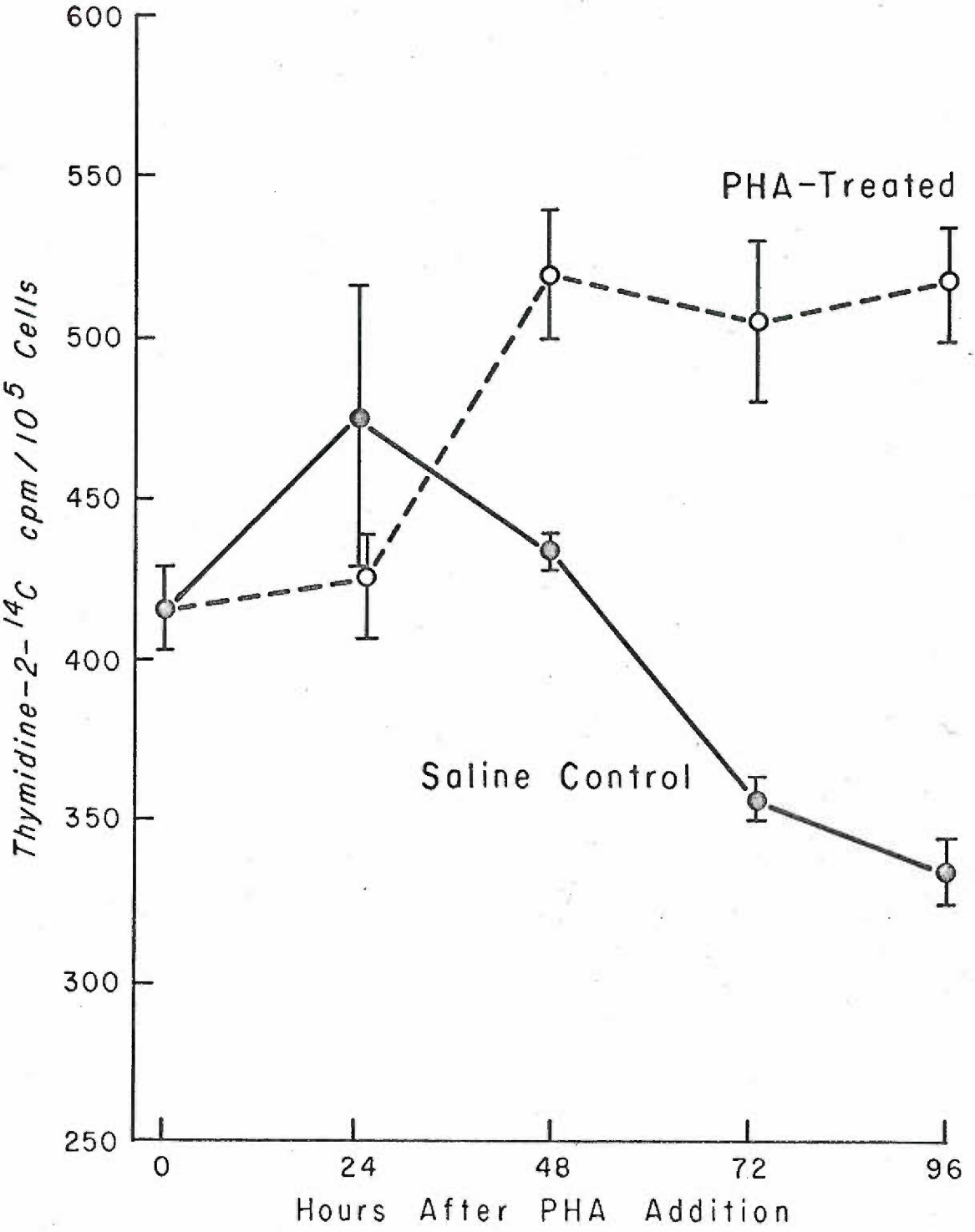

350
300
250
200
150
100
50
0
Thymidine-2-^{14}C cpm / 10^5 Cells
0
24
48
72
96
Hours After PHA Addition
Saline controls
PHA-Treated

Figure 18.

Effect of PHA on ^{14}C-thymidine incorporation by L-fibroblasts (per cell).

This experiment was conducted in the same manner as those described in the legends to Figures 16 and 17 except that the specific activity of the label was 9.1 μc/μM.

Note the difference in results from those presented in Figure 17.


600
550
500
450
400
350
300
250
Thymidine-2-^{14}C cpm / 10^5 Cells
PHA-Treated
Saline Control
0
24
48
72
96
Hours After PHA Addition

with PPHA on a per culture basis. In contrast, incorporation of ^{14}C-leucine and ^{14}C-uridine on a per cell basis continues at control rates for up to 96 hours after PPHA addition (Figures 13 and 15).

While the slopes of the plots for ^{14}C-thymidine incorporation per saline-treated control cell are similar in both Figures 17 and 18, the slopes of the curves representing incorporation per PPHA-treated cell differ significantly between the two Figures. The data in Figure 17 for two experiments suggest that there was no effect of PPHA exposure on ^{14}C-thymidine incorporation. In contrast, the data for the single experiment in Figure 18 indicates a different result. Possible reasons for this discrepancy will be considered further in the Discussion.

Effect of PHA on cellular content of DNA, RNA and protein. The effects of PPHA (30 mcg/ml of medium) on the net accumulation of cellular constituents by L-fibroblasts on a per cell basis are shown in Table 11 for assays performed 48 hours after PPHA addition. The data are presented in terms of amounts of RNA-ribose, DNA-deoxyribose and protein nitrogen per 10^5 cells. These data show that there was an increase in the accumulation of DNA, RNA and protein on a per cell basis by 48 hours after exposure to PPHA, as compared with vehicle-treated cultures. Similar results were seen for the effect of PHA on the cellular content of free cells. Although the absolute values for free cells are greater than those for attached cells, the ratios for the values of treated versus control cells are similar.

To examine more precisely the effects of PPHA on cellular composition of DNA, content per cell was assayed at various times after cells

TABLE 11.

Effects of PHA on RNA, DNA and Protein Content of L-Fibroblasts

Cell Treatment	per 10^5 Attached Cells			per 10^5 Free Cells		
	Ribose (mμ moles)	Deoxyribose (mμ moles)	Protein N (μg)	Ribose (mμ moles)	Deoxyribose mμ moles)	Protein N (μg)
Saline control	2.7 ± 0.2	4.8 ± 0.3	3.9 ± 0.4	5.9 ± 0.7	6.9 ± 0.6	7.2 ± 1.2
PHA-treated	4.2 ± 0.5*	8.8 ± 1.0**	11.4 ± 1.8**	12.0 ± 1.3*	12.0 ± 0.9**	26.4 ± 4.2*
Ratio $\frac{\text{treated}}{\text{control}}$	(1.56)	(1.83)	(2.92)	(2.03)	(1.74)	(3.47)

* $P < 0.02$ as determined by Student's t-test (two-tail).
** $P < 0.01$ as determined by Student's t-test (two-tail).

Preparation PPHA (or an equivalent volume of saline) was added during early logarithmic phase growth to replicate monolayer cultures of L-fibroblasts (see Methods). Final PPHA concentration was 30 mcg/ml of medium. Forty-eight hours after PPHA addition, free and attached cells were harvested separately from control and treated cultures. Cells were pooled from four to six bottles in each case and the combined samples were assayed for cell number, RNA, DNA and protein content as described in the Methods. Data presented in this Table represent the mean and standard error for content per cell from six similar pulse label experiments given in Figures 12 through 17. Values for PHA treatment were significantly different from control values. Percent inhibition of growth as determined by cell counts at 48 hours was 63 percent for attached and free cells (Figures 5 and 6).

were exposed to the growth-inhibiting protein. Effects of PPHA (30 mcg/ml of medium) are shown in Table 12. The amount of deoxyribose per cell was found to be greater in cells grown in the presence of PPHA than in cells from saline-treated cultures. Cellular content of DNA was increased significantly in the PPHA-treated group as compared to the control group by 48 hours after PPHA addition and this level was maintained without significant change up to 96 hours. These results (Table 12) confirm those shown in Table 11 for DNA, that is, in the presence of PPHA the levels of DNA per cell are significantly higher than those found in rapidly-growing control cultures. The decline in deoxyribose per cell seen in control cultures of L-fibroblasts as logarithmic phase growth proceeds (Figure 12) has been quite regularly observed and has been discussed by others (326, 356-359).

Another way of looking at the data was to calculate the specific activities (cpm/mμmole ribose, cpm/mμmole deoxyribose and cpm/mcg protein nitrogen) from the data in Figures 13, 15 and 17, as well as Table 11, for the labelled precursor incorporation studies (Table 13). These calculations demonstrate again that by 48 hours after PPHA addition there was a significant difference between the values for control and treated cultures.

Both PHA-treated and control cells incorporated the ^{14}C-labeled precursors at the same rates during the one hour "pulse" periods, as previously noted (Figures 13, 15 and 17). However, by 48 hours after PPHA addition the levels of RNA, DNA and protein per treated cell were approximately twice those of control cells (Table 11). Therefore, the differences in specific activities observed in Table 13 reflect a

TABLE 12.

Effects of PHA on DNA Content of L-Fibroblasts at Various Times after Drug Addition

Hours after PPHA addition	Deoxyribose (mμ moles) per 10^5 attached cells	
	Saline-control	PHA-treated
0	5.7 ± 0.1	--
24	4.7 ± 0.6	5.8
48	5.0 ± 0.4	7.0 ± 0.4
72	4.7 ± 0.8	7.2 ± 0.5*
96	2.2 ± 0.1	7.9 ± 0.9*

Preparation PPHA (or an equivalent volume of saline) was added during early logarithmic phase growth to replicate monolayer cultures of L-fibroblasts (see Methods). Final PPHA concentration was 30 mcg/ml of medium. At various times after PPHA addition cells growing attached to glass were harvested from control and treated cultures. Cells were pooled from four to six bottles in each case and the combined samples were assayed for cell number and DNA (deoxyribose) as described in the Methods. Data presented in this Table represent the mean content per cell in duplicate samples of control and treated cultures from two experiments.

*These values were significantly different from control values ($P < 0.02$) as determined by Student's t-test (two-tail).

TABLE 13.

Effect of PHA on Labelled Precursor Incorporation into RNA, DNA and Protein of L-Fibroblasts Growing Attached to Glass

Cell Treatment	^{14}C-uridine cpm/mμmole Ribose/hour	^{14}C-thymidine cpm/mμmole Deoxyribose/hour	^{14}C-leucine cpm/mcg Protein N/hour
Saline-Control	108 ± 2	73 ± 14	16 ± 2
PHA-Treated	62 ± 3*	25 ± 4**	6 ± 1*
Ratio $\frac{\text{Treated}}{\text{Control}}$	(0.574)	(0.342)	(0.410)

* $P < 0.01$ as determined by Student's t-test (two-tail).
** $P < 0.05$ as determined by Student's t-test (two-tail).

Preparation PPHA (or an equivalent volume of saline as control) was added during early logarithmic phase growth to replicate monolayer cultures of L-fibroblasts (see Methods). Final PPHA concentration was 30 mcg/ml of medium. Forty-eight hours after PPHA addition the cells were exposed to a ^{14}C-labelled precursor for one hour before incorporation was terminated, as described in the legends to Figures 10, 12 and 14.

Determinations of radioactivity and chemical content of DNA, RNA and protein were performed as described in the Methods. Results are expressed as the amount of radioactivity (cpm) per cell constituent measured (protein nitrogen, ribose and deoxyribose) per hour for duplicate assays from two experiments for each labelled precursor given as Figures 13, 15 and 17 and Table 11, respectively.

Values for PHA treatment were significantly different from control values.

dilution effect by the relative pool sizes of previously synthesized material. Since the PPHA-treated cells had the larger amounts of nonlabelled material, they had lower specific activity values as compared to control cells.

To test whether PHA had an effect on size (cellular volume) of mammalian cells in logarithmic phase growth in vitro, cell size distribution curves were determined using the Coulter electronic particle counter (see Methods). Samples were collected from typical experiments testing the effect of PHA upon the proliferation rates of ML-388 cells grown in suspension culture and L-fibroblasts grown in replicate monolayer cultures. No significant differences could be detected between curves for cells from saline control cultures and those for cells grown up to 72 hours after addition of PPHA (30 mcg/ml of medium). The absence of striking differences in cell size was confirmed by the photomicrographic evidence in Figures 19 and 20.

MORPHOLOGICAL ANALYSIS OF GROWTH INHIBITION

Effect of PHA on cell morphology and distribution. Photomicrographs of normal L-fibroblasts and cells from PHA-treated cultures (30 mcg PPHA/ml of medium) 48 hours after PPHA addition are given in Figures 19 and 20 at two different magnifications (100X and 400X, respectively). It can be readily seen that the morphology of the PPHA-treated fibroblasts (Figures 19A, 20A and 20B) is characterized by predominately round cells whose nuclei tend to be darkly stained with hematoxylin. Mitotic figures were rarely seen in the treated-cell populations. Very large or "giant" cells (mono- or poly-nucleated

Figure 19.

Effect of PHA on morphology of mouse fibroblasts in monolayer culture after 48 hours (low magnification)

L-fibroblasts were grown on microscope slides as described in the Methods.

The day after inoculation, preparation PPHA to a final concentration of 30 mcg/ml of medium (A) or saline vehicle (B) was added. Forty-eight hours later the slides were removed, fixed in methanol, and stained with hematoxylin and eosin (see Methods). Both photomicrographs are 100 X magnification.

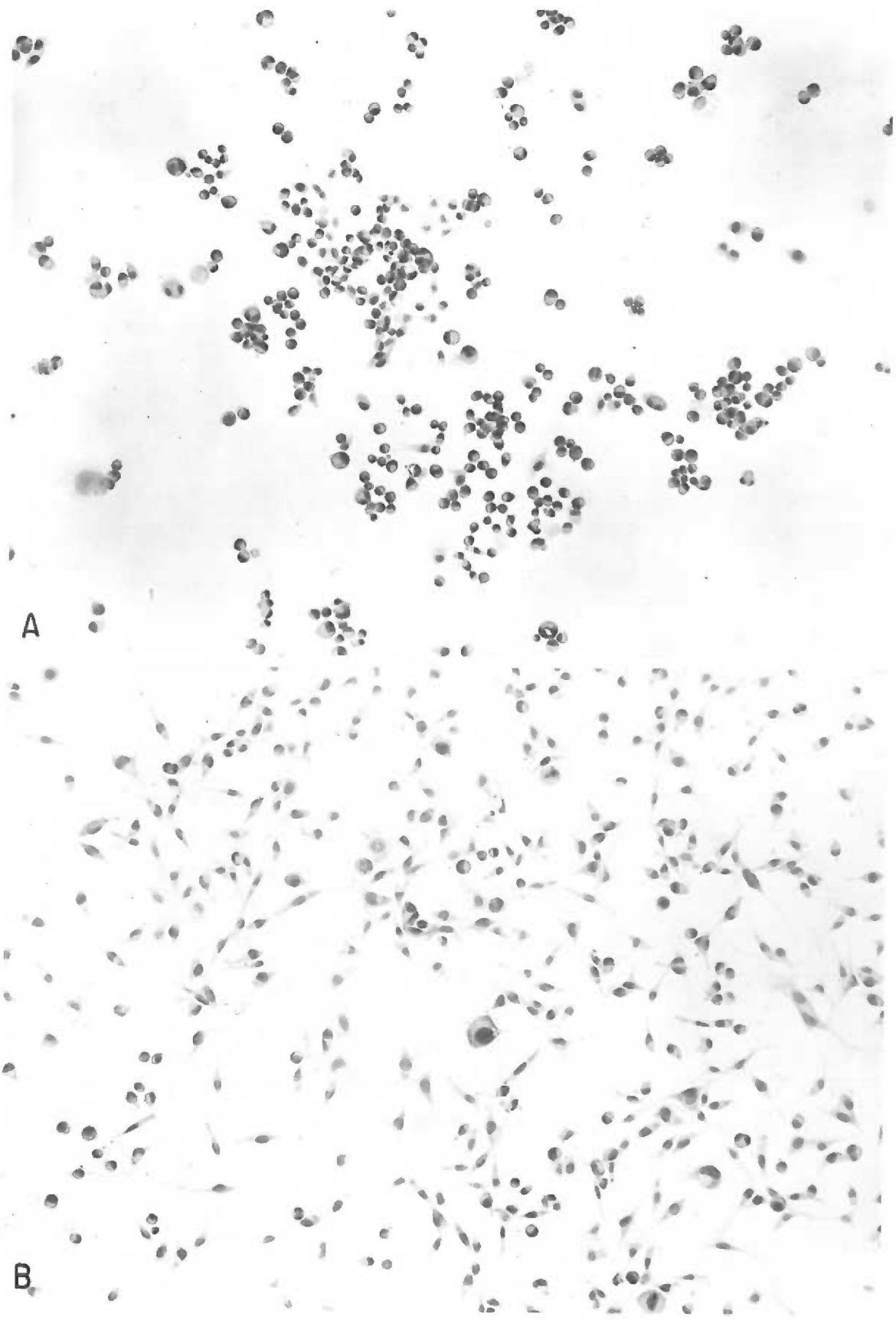

A
B

Figure 20.

Effect of PHA on morphology of mouse fibroblasts in monolayer culture after 48 hours (high magnification).

The cells, culture conditions and cell staining procedures were the same as those described in the legend to Figure 19.

Forty-eight hours after PPHA (A and B) or saline (C and D) the slides were removed, fixed and stained (see Methods).

All photomicrographs are 400 X magnification.

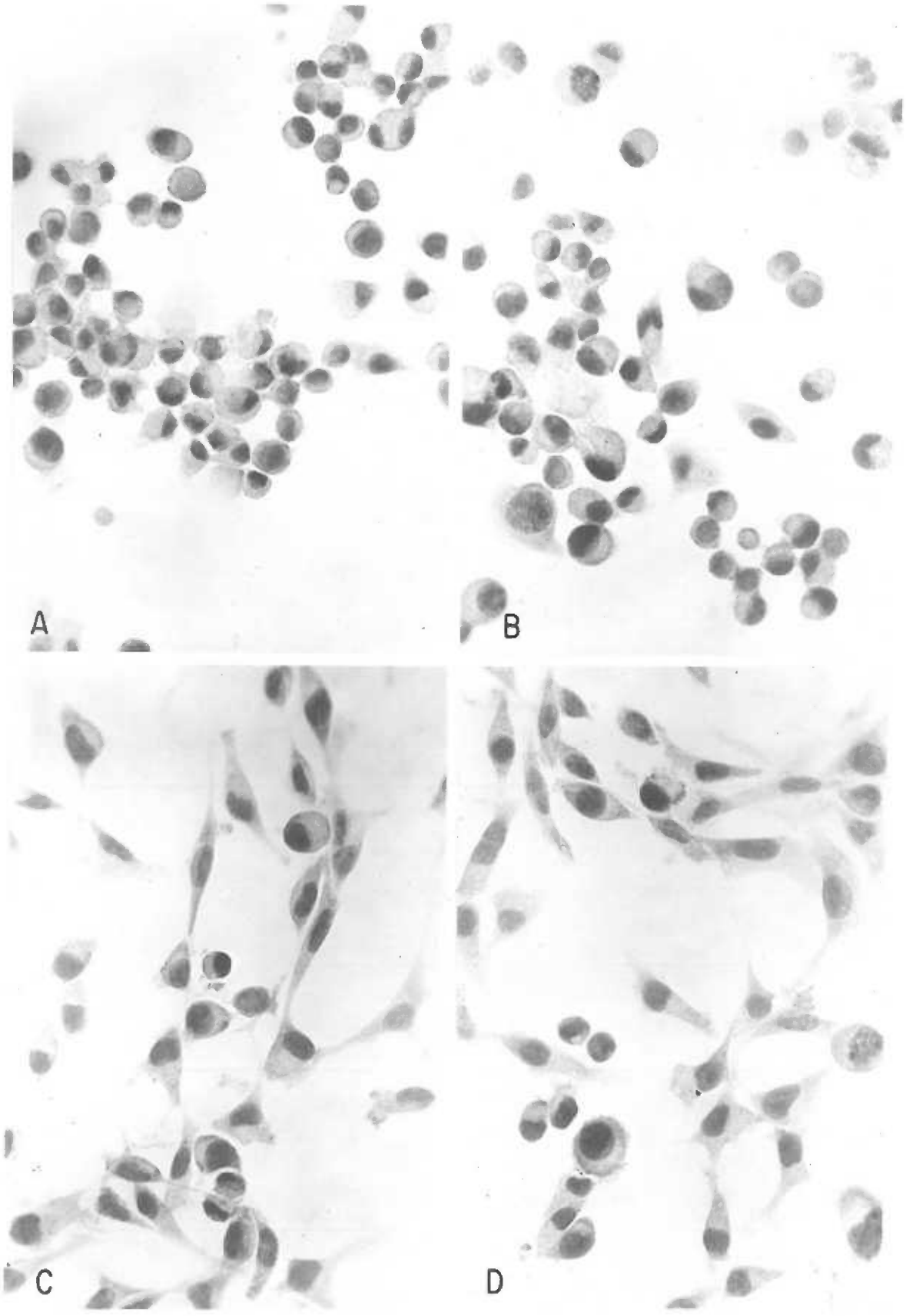

A
B
C
D

Figure 21.

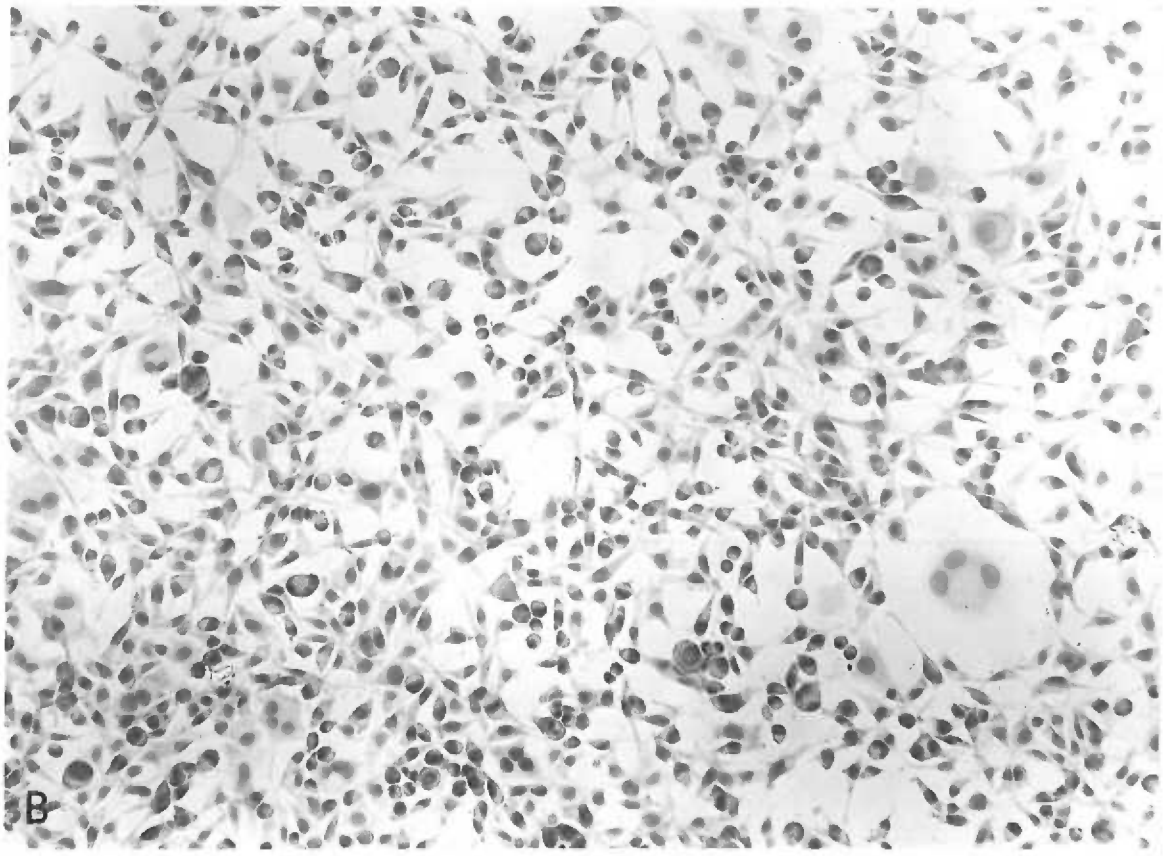
Effect of PHA on morphology of mouse fibroblasts in monolayer culture after 96 hours (low magnification)

The cells, culture conditions and cell staining procedures were the same as those described in the legend to Figure 19.

Ninety-six hours after PPHA (A) or saline (B) the slides were removed, fixed and stained (see Methods).

Both photomicrographs are 100 X magnification.


A


B

with extensive cytoplasm) were seen occasionally as well as binucleated cells. The distribution of cell growth on the slides characteristic of fibroblasts exposed to PPHA was an irregular or patchy arrangement of groups of cells.

In contrast, the morphology of normal rapidly-growing (saline control) L-fibroblasts (Figures 19B, 20C and 20D) displayed typical spindle-shaped forms as their predominant characteristic. The basophyllic elements of the nuclei showed only a moderate degree of staining. Occasional "giant" cells were seen along with binucleate forms. Mitotic figures were frequently visualized. The growth pattern of the normal cells was one of uniform distribution.

The results of the effects of PPHA (30 mcg/ml of medium) on the morphology and distribution of L-fibroblasts growing in replicate monolayer cultures by 96 hours after PPHA addition are consistent with the growth-inhibitory effects of PHA noted previously (Figure 21). A comparison of the photomicrographs of PHA-treated and saline control cells at 48 hours (Figures 19A and 19B) with those at 96 hours after PPHA addition (Figure 21A and 21B) reveals a striking difference in cell number. The number and distribution of PHA-treated cells seen at 96 hours (Figure 21A) does not appear to be different from that seen at 48 hours after drug addition (Figure 19A), and these results are consistent with growth inhibition. In contrast, a comparison of the data in Figure 19B with that in Figure 21B for control cultures reveals a marked increase in cell number with even distribution, and these results are consistent with rapid growth.

Examination of photomicrographs of cells exposed to PPHA for 96 hours (Figures 21A and 21B) reveals more pronounced differences in individual cell morphology than seen after a 48-hour exposure. At this time there was an obvious heterogeneity of cell types, the most prominent being small round cells characterized by pyknotic (densely-stained) eccentric nuclei. Other cell forms seen included a few typical spindle-shaped fibroblasts, a number of "giant" cells and medium-sized round forms with pyknotic nuclei. While the number of normal (saline control) fibroblasts appear to be markedly increased at 96 hours (Figure 21B) the cells retained the morphology described for control cells at 48 hours after saline addition (Figure 19B). Some "giant" cells and a predominance of typical spindle-shaped fibroblasts with pale to moderately-stained nuclei were seen.

Effect of PHA on cellular ultrastructure. The ultrastructure of L-fibroblasts and ML-388 cells grown in the presence and absence of PHA was examined to provide greater detail on morphological effects. The cells were harvested after exposure to PPHA (30 mcg/ml) or vehicle for 48 hours. Approximately 100 electron photomicrographs were prepared for this study (see Methods) and the most representative ones are reproduced as Figures 22 through 26. The most prominent feature of the PHA-treated cells is observed in the cytoplasm. There is an increase in electron dense material which is associated with the intermediate reticulum (IR) of ML-388 cells (Figure 23) and with the mitochondrial cristae of L-fibroblasts (Figures 25 and 26). A comparison of the appearance of nuclear chromatin between control cells (Figures 22 and 24) and PHA-treated cells (Figures 23 and 25) suggests that less densely-aggregated

chromatin (heterochromatin) occurs in the nuclei of cells exposed to PPHA. At higher magnification (Figure 26) some virus-like particles (VLP) were observed in both PHA-treated and saline control cells.

Figure 22.

Ultrastructure of a normal mouse lymphoma cell grown in monolayer culture.

Mouse Lymphoma ML-388 cells were grown for 72 hours in culture before being harvested and prepared for electron microscopy (see Methods).

The cytoplasm of normal cells contains numerous ribosomes, moderate numbers of mitochondria (M) and some granular endoplasmic reticulum (ER). Golgi apparatus components (G) are evident, as are residual bodies (RB). Nuclei (N) are large with prominent nucleoli (NC) and chromatin margination. X 22,800.

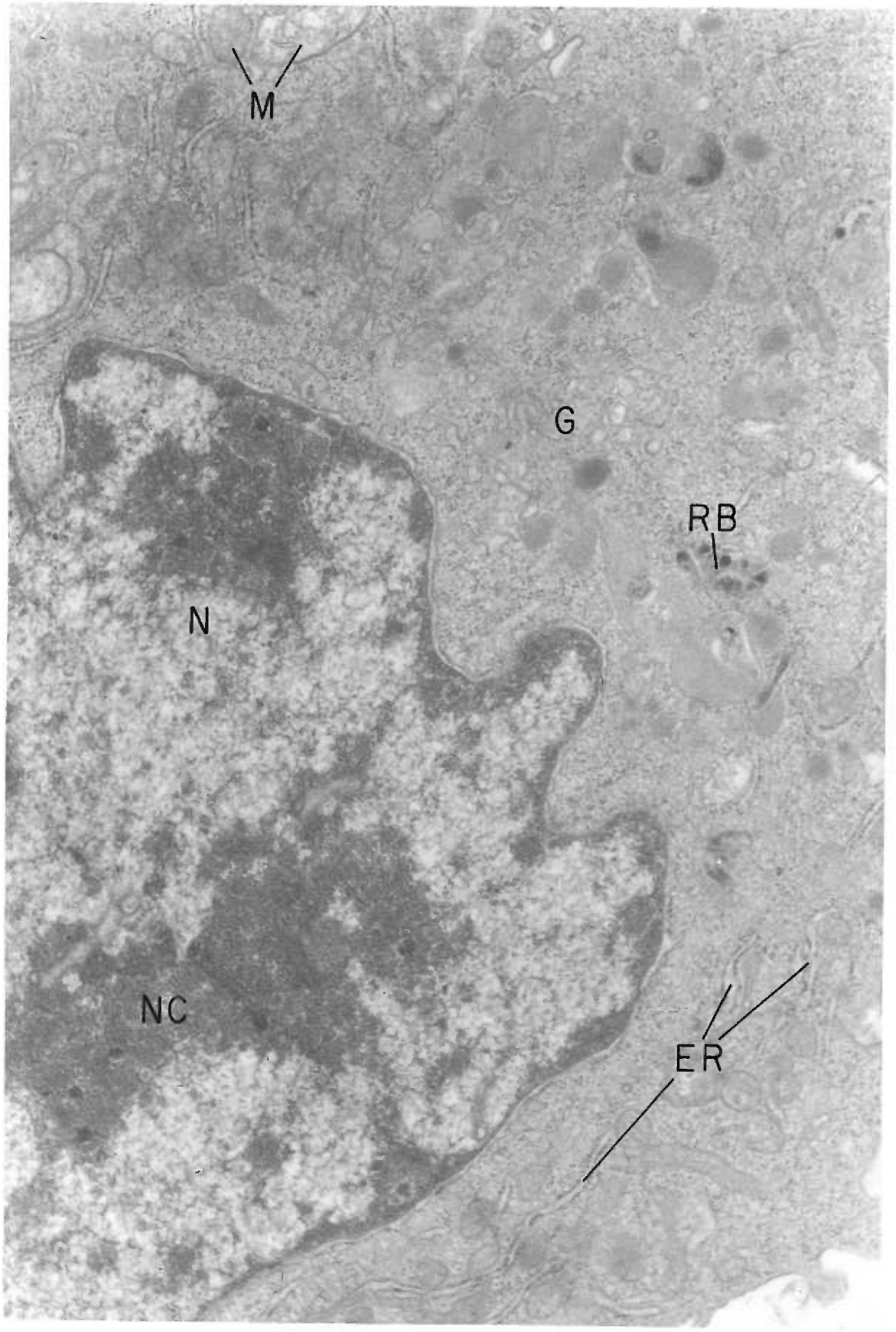

M
G
RB
N
NC
ER

Figure 23.

Effect of PHA on ultrastructure of a mouse lymphoma cell grown in monolayer culture.

Mouse Lymphoma ML-388 cells were grown 48 hours in the presence of 30 mcg PPHA/ml of medium (72 hours in culture) before being harvested and prepared for electron microscopy as described in the Methods.

The drug-treated cell is characterized by hypertrophy of a pleomorphic membrane enclosed system considered to be related to the Golgi membrane system, multivesicular bodies and lysosomes. The membrane system is classified here as intermediate reticulum (IR). Mitochondria (M) are not especially numerous, and Golgi apparatus components are inconspicuous in this section. The general cytoplasm contains numerous ribosomes and sparse granular endoplasmic reticulum (ER). Lysosomal residual bodies (RB) occur in moderate numbers. Chromatin appears generally in the pale, homogeneous euchromatin form in the nucleus (N) although some dense chromatin is located along the nuclear margins. X 22,800.

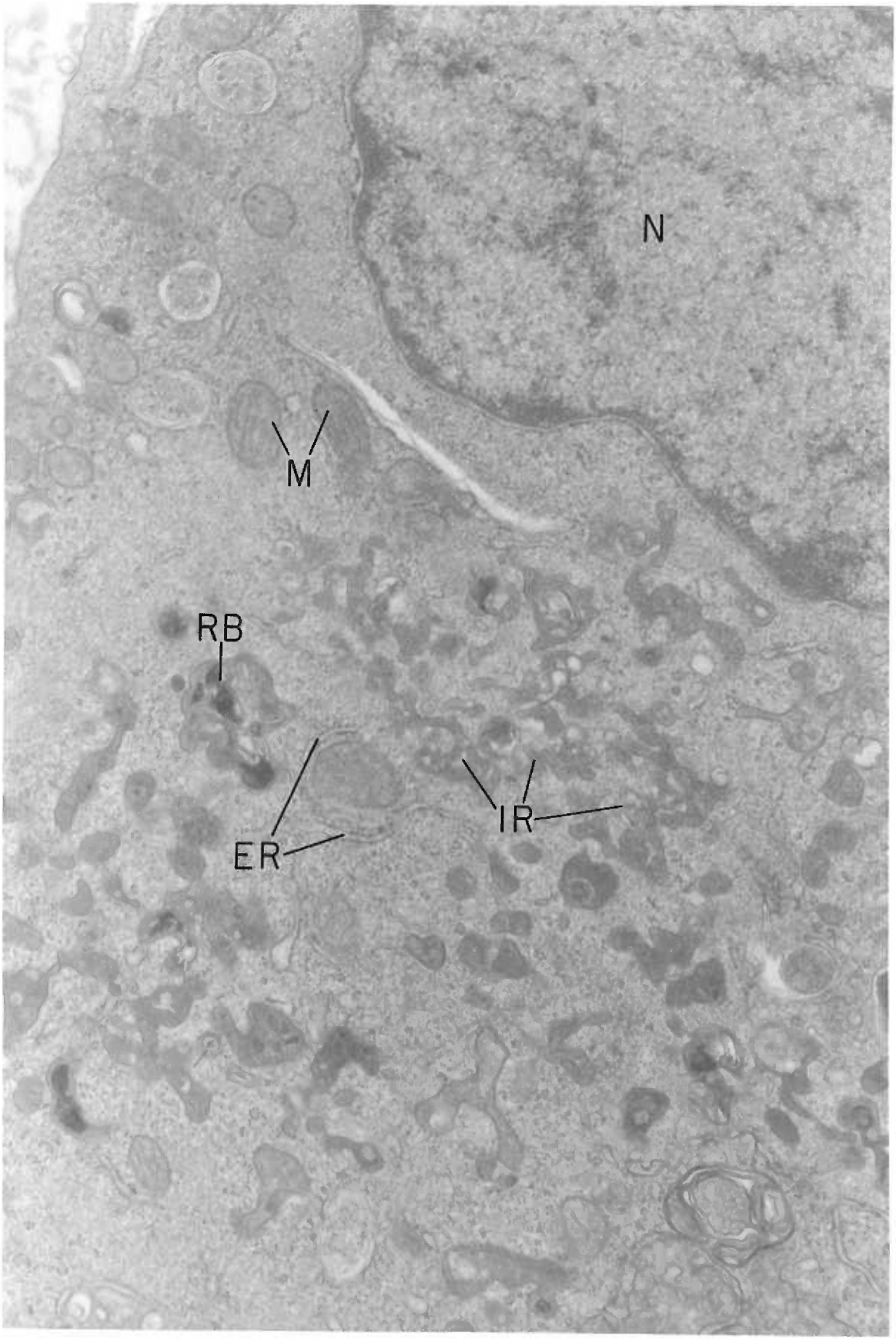

N
M
RB
ER
IR

Figure 24.

Ultrastructure of a normal L-fibroblast cell grown in monolayer culture.

L-fibroblast cells were grown for 72 hours in culture before being harvested and prepared for electron microscopy (see Methods).

The cytoplasm of normal L-fibroblasts contains numerous ribosomes, moderate numbers of typical mitochondria (M) and some granular endoplasmic reticulum (ER). Golgi apparatus components (G) are numerous and both lysosomal residual bodies (RB) and lipid particles (LP) are evident. Nuclei (N) are large with prominent nucleoli (NC). The nuclear chromatin appears evenly distributed between the euchromatin and heterochromatin forms. X 22,800.

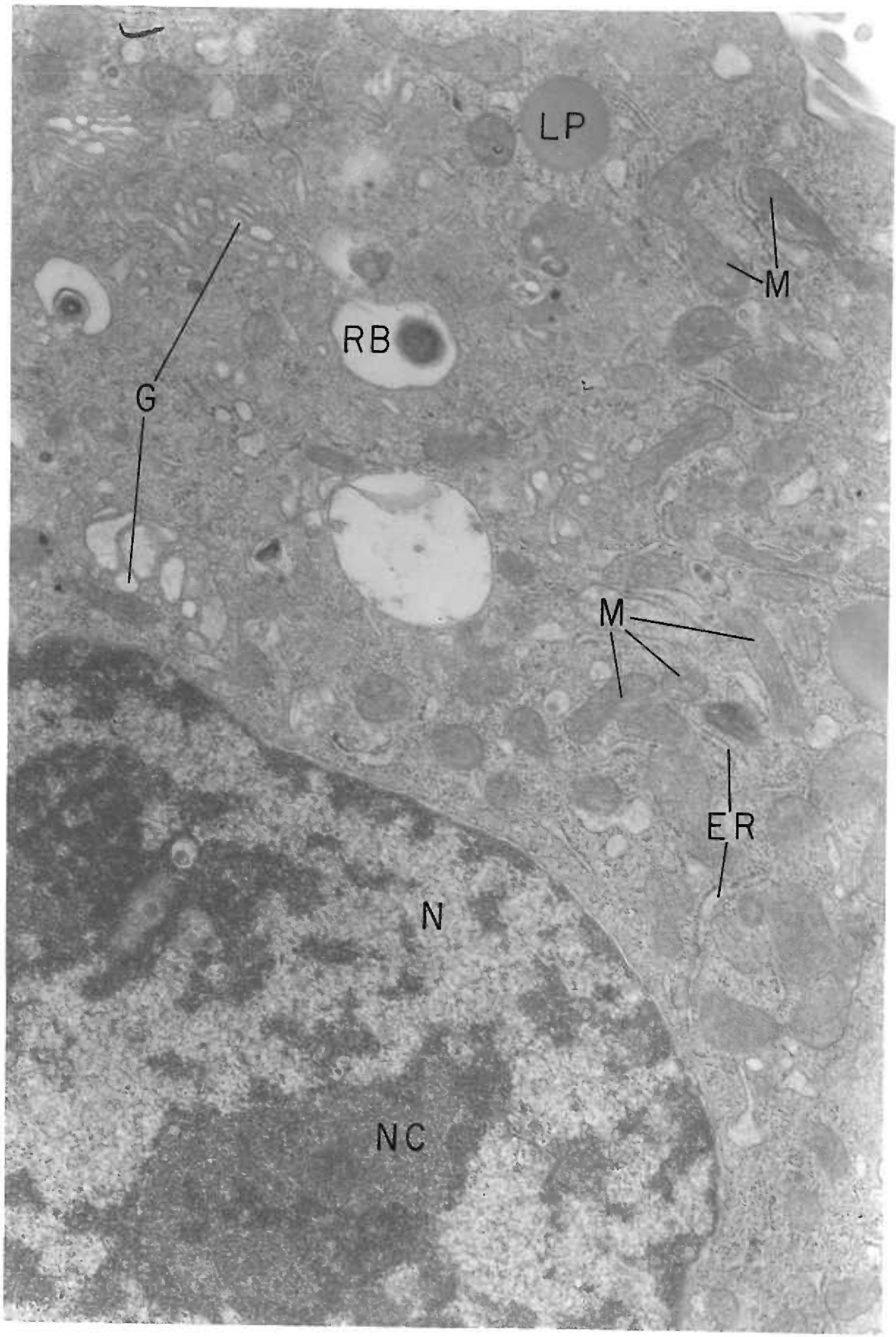

LP
M
RB
G
M
ER
N
NC

Figure 25.

Effect of PHA on ultrastructure of an L-fibroblast cell grown in monolayer culture (low magnification).

L-fibroblast cells were grown 48 hours in the presence of 30 mcg PPHA/ml of medium (72 hours in culture) before being harvested and prepared for electron microscopy as described in the Methods.

The drug-treated cell is characterized by numerous bizarre mitochondria (M) having electron-dense material between their cristae. Golgi apparatus components (G) are evident, as are lysosomal residual bodies (RB) and granular endoplasmic reticulum (ER). Chromatin of the nucleus (N) appears generally in the euchromatic form except along the nuclear margin where the heterochromatin form predominates. X 22,000.

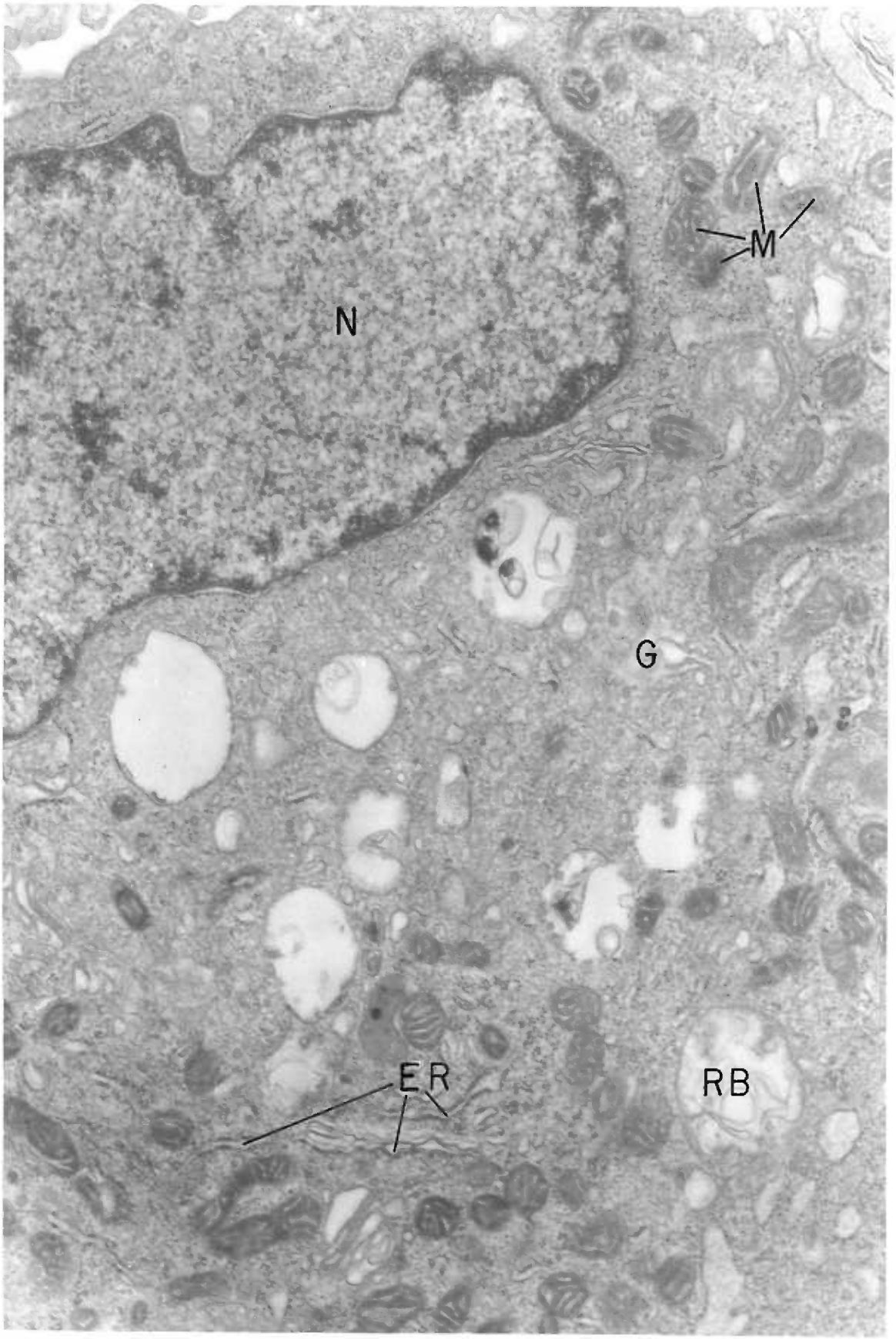
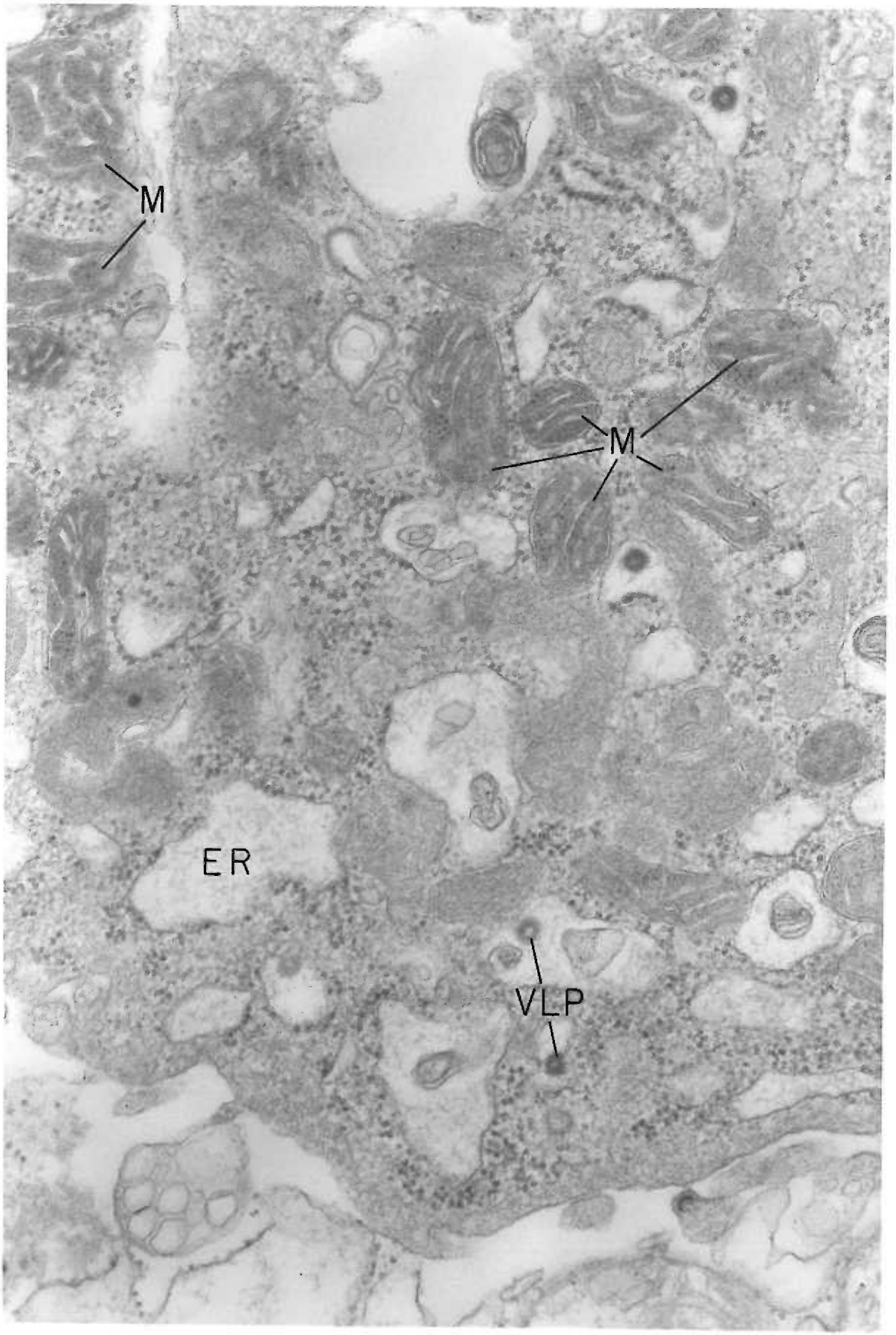

N
M
G
ER
RB

Figure 26.

Effect of PHA on ultrastructure of an L-fibroblast cell grown in monolayer culture (high magnification).

L-fibroblasts were grown, treated, harvested and prepared for electron microscopy as described in the legend to Figure 25 and in the Methods.

In this higher-magnification micrograph electron-dense material is especially evident between the mitochondrial cristae. The cytoplasm is characterized by these unusual mitochondria (M). The granular endoplasmic reticulum (ER) is evident. A number of virus-like particles (VLP) are seen in both PPHA-treated and normal cells at this magnification. X 67,250.


M
M
ER
VLP

DISCUSSION

The mitogenic action of PHA on normal nongrowing peripheral lymphocytes has been known for a number of years (2-4, 16-18). I became aware of the growth-inhibitory action of PHA when it was tested in our laboratory as a possible agent for reversing the growth-inhibitory effects of cortisol on a rapidly-growing cell line (ML-388) *in vitro*.

After the initial observation with ML-388 cells was made, it was decided to ascertain whether similar growth-inhibitory effects for purified protein phytohemagglutinin (preparation PPHA) could be observed in other rapidly-growing mammalian cell lines *in vitro* and *in vivo*. Cell lines selected for these studies included rat thymus (RT), Mouse Lymphoma (ML-388), a steroid-resistant subline (ML-388F^r) and L-fibroblasts, all growing *in vitro*, and the lymphocytic leukemia (L-1210) cell line growing in the peritoneal cavity of BDF_1 mice.

The tissue culture test system selected for most of the experiments employed replicate monolayer cultures of L-fibroblasts or Mouse Lymphoma (ML-388) cells. These cell lines, as well as the others grown in this test system, multiplied asynchronously and had a doubling time of 20-24 hours.

Table 2 provides some data for the growth characteristics of L-fibroblasts in monolayer culture which are typical of all of the cell lines used for this study. While the majority of the cells grew attached to the glass surface of the bottle (attached cells), some 20 to 30 percent of the total number of cells per culture could be recovered from the growth medium (free cells). The percent of free cells as compared to total cell number was relatively constant throughout the five-day culture period so that measurement of attached cell number accurately

reflected the growth rate of control cultures. Free cells probably represented a heterogeneous population of viable and dead cells. Fibroglasts grown in monolayer are known to become loosely attached to the glass during mitosis and are easily dislodged (360).

That measurement of attached cell number accurately reflects the growth of cultures exposed to PPHA is supported by data presented in Figures 5, 6 and 7. These data show that PPHA does not induce detachment of cells from the glass (Figure 6) and that the slope of the growth curves for attached cells and total cells (free plus attached cells) are identical for both PPHA-treated and saline-treated cultures (Figures 5 and 7).

Data in Figure 4 and Table 3 show that preparation PPHA inhibited the growth of all cell lines tested and did not exert a selective growth-inhibitory effect on Mouse Lymphoma (ML-388) cells as reported for hydrocortisone by Gabourel and Aronow (1). Further, resistance to anti-inflammatory steroids (a property of ML-388F^{r} cells) failed to protect the Mouse Lymphoma line from the growth-inhibitory effect of PPHA. These results suggest that PPHA and hydrocortisone depress cell proliferation by different mechanisms. The concentration of PPHA required for inhibition of growth in these rapidly-dividing cells is of the same order of magnitude (Table 3) as that reported by Rigas and others (Table 1) to stimulate mitosis in nongrowing lymphocytes.

The data in Table 5 summarizes the effect of PPHA on cell multiplication. Not only was there a dose-response relationship, but growth inhibition was shown also to increase with time after a fixed dose of PPHA.

Two explanations for the observed effects on cell number, other than a specific growth-inhibitory action of PPHA, were possible and had to be ruled out. The first stemmed from the reported leukoagglutinating properties of PHA (7-13 and 55-64) and involved the possible clumping of cells. Clumping could lead to erroneously low values for cell number when determined by the usual electronic particle counting method. To rule out this possibility, cell suspensions were also subjected to direct visual examination using phase contrast microscopy. Such examination showed no clumping for freshly prepared cell suspensions as normally used for cell number determination. PPHA-treated cells did form clumps, which were difficult to disperse, after standing two or three hours without agitation or after centrifugation for 10 to 15 minutes at approximately 1000 x G. All cell number determinations reported here were determined on freshly prepared suspensions prior to centrifugation.

Nonspecific cell death and subsequent lysis was also a possible explanation for the observed decrease in cell number in PHA-treated cultures. This possibility was tested using the trypan blue exclusion test (Table 4). Although the data show that the dye is consistently taken up slightly more by cells exposed to PPHA as compared to controls, the effect is so small that it is not considered to be significant (332, 354).

The possibility of nonspecific cell lysis was also ruled out by monitoring cells containing ^{14}C-labeled DNA growing in the presence and absence of PPHA. Cell lysis, if it occurred, should have caused the release of labeled DNA into the medium, and higher levels of radioactivity should have been found in the medium of PPHA-treated cultures as compared

to controls. Table 10 shows that there was no difference in the amount of radioactivity found in the nutrient medium of cells exposed to PPHA or saline vehicle at varying times up to 72 hours. These results were taken as evidence that lysis did not occur and that PHA was exerting a specific growth-inhibitory effect to cause the observed depression of cell number.

Experiments in which L-fibroblasts containing ^{14}C-labeled DNA were grown in the presence and absence of PPHA also provided direct evidence of a growth-inhibitory effect for this protein (Figures 10 and 11). The specific activity of DNA (cpm/mμmole deoxyribose) was shown to decrease at a slower rate in PPHA-treated cultures as compared to control cultures (Figure 11). This result was compatible with the slower growth rate observed in PPHA-treated cultures and was indicative of a reduction in the rate of dilution of the radioactive DNA by newly synthesized nonradioactive DNA. Cellular radioactivity (cpm/10^5 cells) was also decreased at a slower rate for PPHA-treated cells than for control cells (Figure 10).

Mouse Lymphoma cells growing in suspension culture were also found to be sensitive to the growth-inhibitory effects of PHA by 24 hours after exposure to this protein (Figure 8). The inhibition increased with time in this cell culture system as in the monolayer culture system. This test system offered an advantage over the monolayer culture system in that it was capable of providing large numbers of cells which could be harvested quickly and easily. Centrifugation of cells was required as part of the work-up procedure, however, and unreliable cell counts due to clumping in all but original cell suspensions plagued these experiments.

In addition, there were limitations on the number of such cultures per experiment. Usually only one treated and one control culture provided aliquots for analysis at various times after PHA or saline addition. Thus, statistical evaluation was more difficult than for the monolayer system which utilized 100 to 150 replicate cultures. Consequently, only preliminary studies were performed with the suspension system.

Growth inhibition had been seen earlier by Nungester and his associates (10, 30) with crude bean extracts. Pretreatment of tumor cell lines in vitro destroyed the ability of treated cells to produce tumors when inoculated into rats. Growth of all three cell lines was inhibited by their impure preparations. In contrast, Ioachim (120) failed to observe growth-inhibitory effects when PHA-M and PHA-P were added to L-fibroblasts in cell culture. No careful analysis of growth was performed but an increase in mitotic index in PHA-treated cultures was reported. While his data does show a twofold to threefold increase in mitotic index for five different PHA-P treated cell lines as compared to controls, the values for the number of mitotic figures per 500 cells appear low and statistical significance is doubtful. For example, the number of mitoses given for L-fibroblasts at 24, 48 and 72 hours after PHA-P or diluent as control were 6, 3 and 1 for control cultures and 9, 5 and 1 for PHA-P treated cultures.

The growth inhibitory effects observed for mammalian cells in vitro suggested that PPHA might be effective in the treatment of rapidly-growing tumors in vivo. PPHA was therefore examined for its ability to prolong survival time of BDF_1 mice carrying the L-1210 lymphocytic leukemia. It was decided to use maximum doses of PPHA compatible with

host survival in the initial experiments. A survey of the literature indicated that no relevant data was avilable concerning the toxicity of purified PPHA given parenterally to mice. Scanty data was available only for crude or partially purified preparations (24-27, 31, 38, 53). Toxicity studies were therefore undertaken and it was found that doses of 200 mg/kg killed all mice tested; doses of 140 $\pm$ 10 mg/kg killed 50 percent of mice tested; and single doses of 100 mg/kg did not kill any of the mice tested (29). It was subsequently shown that a dose of 100 mg PPHA/kg given either one hour or 24 hours after tumor inoculation produced a significant prolongation in survival time with the effect being greatest when PPHA was given early (Table 6, Figure 9). When two such doses of PPHA were given, the first 24 hours after tumor inoculation and the second, 48 hours after tumor inoculation, the prolongation of life was intermediate between the two previously-described treatments. No prolongation of life was observed for tumor-bearing animals when PPHA was given subcutaneously. This line of endeavor was not pursued further because it became obvious that the effectiveness of PPHA *in vivo* was severely limited by its toxicity (26-29, 31, 38), its antigenicity (283-286) and its immunosuppressant action (20-23, 292-298, 300-304).

These results obtained for PPHA on L-1210 lymphocytic leukemia (29) agree with those reported by Robinson (35, 306) who observed a decreased growth rate of an ascites tumor in the peritoneal cavities of mice treated parenterally with PHA-BW. In contrast, Rubio and Unsgaard (305) have reported that PHA-M was not effective in reducing tumor growth rates. They used only a single small dose of PHA-M administered prior to tumor inoculation.

Since the data presented in this thesis was obtained using an extensively purified and characterized preparation of PHA (PPHA), and since some of the results presented here conflict with those reported in the literature, it was decided to compare various commercial preparations with PPHA for growth-inhibitory effects. Such a comparison is shown in Table 7. Although they varied in potency, all preparations tested with growth-inhibitory to ML-388 cells growing in monolayer culture. Data from various dose-response experiments (Table 8) suggested that there was a correlation between the cell number per bottle determined at the time of PPHA addition and the IC_{50} value estimated for PPHA four days later. Although an attempt was made to hold inoculum size relatively constant (approximately 2×10^5 cells) variations did occur, and it was necessary to determine a dose-response curve for PPHA simultaneously with each preparation tested in order to make a valid comparison as in Table 7.

Studies were also undertaken on the effect of PPHA storage in solution. Results given in Table 9 indicate that PPHA solutions should be prepared fresh for each experiment. This procedure was followed for all experiments reported in this thesis.

Once the growth-inhibitory effects of PPHA had been established, attention was given to the biochemical alterations associated with these effects. Since nucleic acid and protein synthesis are essential for the replication process, emphasis was placed in these areas. The effects of PPHA on the incorporation of labelled precursors into DNA, RNA and protein and on the cellular levels of these components were investigated.

Rates of incorporation into cold-acid soluble material were determined for ^{14}C-leucine, ^{14}C-uridine and ^{14}C-thymidine at various times

after exposure of cells to PPHA or saline vehicle, and the data was presented on a per culture basis (Figures 12, 14, 16, respectively). These results showed that incorporation of all three precursors was inhibited at the same time and to the same extent as cell growth, determined by cell number measurements. This observation was confirmed when rate of incorporation was expressed on a per cell basis. The incorporation rates per cell for ^{14}C-leucine, ^{14}C-uridine and ^{14}C-thymidine did not differ significantly between PPHA-treated or control cells (Figures 13, 15 and 17, respectively.)

A third experiment on ^{14}C-thymidine incorporation (Figure 18) gave somewhat different results from those of the two experiments depicted in Figure 17. The rate of ^{14}C-thymidine incorporation for PPHA-treated cells in Figure 18 did not decrease with time as it did for control cells. There is no obvious explanation for this difference, and one can only speculate that the nutrient environment for slower-growing PPHA-treated cultures described in Figure 18 was such as to permit continued rapid thymidine uptake for a more prolonged period than was possible for the more densely-populated, rapidly-growing control cultures.

Chemical assay of cellular constituents of cells grown in the presence and absence of PPHA (Table 11) showed that although cell division was markedly inhibited in PPHA-treated cultures, protein (nitrogen), RNA (ribose), and DNA (deoxyribose) accumulation continued at rates sufficient to significantly increase the amount of these constituents on a per cell basis. Precursor incorporation studies mentioned previously confirm the fact that synthesis of these macromolecules continued at control rates in cells exposed to PPHA.

A more detailed time study was carried out for the cell levels of DNA (deoxyribose) (Table 12). These data show that the cellular level of DNA for PPHA-treated cells increased approximately 44 percent over control values within 48 hours and remained at this level for the duration of the experiment (additional 48 hours). DNA levels for control cells remained relatively constant for the first 72 hours and then dropped off sharply. A similar drop in cell DNA content has been reported for several established cell lines by other investigators (329, 356-359). This decrease in DNA content appears to increase with time and probably results from depletion of essential materials in the nutrient medium.

It is of interest to note that levels of DNA per cell can vary over wide ranges (more than a factor of two) suggesting that these cells may change their ploidy when placed under any stress (drug treatment or starvation). Several investigators (361, 362) have noted high variation in chromosome counts for these cells. It is also of interest that free cells contained more DNA than attached cells, both in control and PPHA-treated cultures (Table 11). Curiously, free cells from control cultures have DNA levels approximately equal to that of attached cells from PPHA-treated cultures. As previously mentioned (360) control cells undergoing mitosis tend to be easily dislodged from the glass surface, suggesting that the levels of DNA seen in this population of cells may be approaching pre-mitotic levels. DNA levels are still higher by about 74 percent for free cells from PPHA-treated cultures than for either attached cells from PPHA-treated cultures or free cells in control cultures. This would again suggest changes in ploidy.

It has been reported (363, 364) that any agent which primarily interferes with cell multiplication without affecting synthesis of proteins and nucleic acids will lead to the formation of cells with increased amounts of cellular constituents. When cells increase their ploidy under such conditions they do so either by the process of endoreduplication whereby there is replication of the modal chromosome number without accompanying spindle formation and cytokinesis, or by the process of cell and/or nuclear fusion with the formation of hybrids. Determinations of chromosome numbers (365) and the application of hybrid isolation technics (362, 366) could distinguish between these processes. Littlefield (366) was unsuccessful when he attempted to increase the frequency of hybrid formation in mouse fibroblasts exposed to an admittedly crude preparation of PHA.

The third phase of these investigations concerned morphological alterations associated with PPHA-induced growth inhibition. In contrast to their normal cytology (367), L-fibroblasts treated with PPHA for 48 or 96 hours exhibited under light microscopy an altered morphology characterized by scattered growth of predominantly small round cells with eccentric pyknotic nuclei (Figures 19, 20, 21). Ioachim (120) has also reported such changes in cellular morphology for a number of cell lines including L-fibroblasts as early as 24 hours after exposure to PHA-M or PHA-P.

The ultrastructure analysis of Mouse Lymphoma (ML-388) and L-fibroblast cells revealed increased amounts of electron-dense material in the cytoplasm of both cell lines studied and suggested a shift from predominately aggregated to dispersed chromatin in the nucleus. The

effect of PPHA on the ultrastructure of ML-388 cells (Figures 22 and 23) was seen as an increase in both residual bodies and myelinated bodies as well as the appearance of large amounts of intermediate reticulum. These structures seem to resemble the varied lysosome structures described by other authors for cells actively engaged in intracellular secretory (368) and digestive processes (369, 370). PHA has been reported by Hirschhorn and others (258, 269, 270, 272) to increase the lysosomal activity of peripheral lymphocytes transformed by PHA.

The effect of PPHA on the ultrastructure of L-fibroblasts (Figures 24, 25, 26) was characterized by the appearance of bizarre mitochondria with altered cristae configuration and increased electron density. These conformational changes in mitochondria appear to resemble those described by Hackenbrock (371) as well as by Green and his associates (372-375), who reported that dramatic reversible ultrastructural transformations can occur in mitochondrial cristae between an orthodox and a condensed conformation. According to Green et al. (375) the condensed state can be induced by electron transfer and by hydrolysis of ATP while it is discharged by monovalent or divalent ions and uncoupling reagents. The mitochondria in the PPHA-treated fibroblasts may resemble this condensed conformation but there is, as yet, no direct evidence that PHA affects the functional state of mitochondria.

Although it has been reported (364) that most serially propagated cell lines maintained by usual techniques in media containing antibiotics are contaminated by mycoplasma (PPLO), none were seen in the electron photomicrographs. In addition, there are two types of biochemical evidence arguing against PPLO contamination in L-fibroblasts used in

this laboratory. L-fibroblasts used in the experiments reported here were able to incorporate uridine and thymidine at substantial rates (Figures 15 and 17); whereas, L-fibroblasts contaminated with PPLO have been shown to be inhibited in their ability to incorporate these precursors (376). It has also been shown that noninfected L-fibroblasts containing labelled DNA release substantial amounts of radioactivity into the medium when infected with PPLO (377). Such an effect was not observed for L-fibroblasts grown in this laboratory (Table 10).

Virus-like particles (VLP) were observed in both control cultures and those treated with PPHA. Others (378) have also reported the presence of VLP in Earle's L-strain cells but no biological activity has been associated with the particles.

There are many reports in the literature for other growth-inhibitory agents such as nitrogen mustard (328), other alkylating agents (363), vinblastine sulfate (379-382), colchicine (383) and x-irradiation (384-386) which affect mitosis without altering protein or nucleic acid biosynthesis directly. These agents act at any time from early prophase to late metaphase, to produce characteristic nondividing giant cells with elevated premitotic amounts of DNA (382). The effects of PPHA described here are comparable to those reported for the growth-inhibitory agents mentioned above, except for the lack of noticeable cell enlargement.

Im summary, my results indicate that rapidly-growing mammalian cells exposed to PPHA show a decreased rate of cellular proliferation, no change in the rate of DNA, RNA and protein synthesis, a marked elevation in cellular levels of DNA, RNA and protein and an enhanced staining capacity

of the nuclei, indicative of an increased amount of chromatin. These results suggest that PHA inhibits growth by interfering with the regulatory mechanisms controlling the cellular growth cycle.

SUMMARY AND CONCLUSIONS

I have shown that the addition of PHA to rapidly-growing mammalian cells results in a decreased rate of cellular proliferation without clumping, killing or lysing the cells. In addition, it was shown that RNA, DNA and protein synthesis on a per cell basis continued at control rates in the presence of PHA while the levels per cell of these components were markedly increased. An increased staining capacity of the nuclei of PHA-treated cells was indicative of increased chromatin. These results suggest that PHA inhibits cell multiplication by interfering with the regulatory mechanisms controlling the cellular growth cycle.

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