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THE EFFECT OF VITAMINS A AND E ON NEUROBLASTOMA GROWTH AND DIFFERENTIATION

by

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ABSTRACT

The effects of dl-α-tocopherol and dl-α-tocopheryl succinate on neuroblastoma, N1E 115, cells were studied. Tocopherol had no growth-arresting properties, whereas its succinate ester derivative inhibited growth at concentrations $\geq$ 20 μM. The succinate derivative was taken up somewhat more readily than free tocopherol; however, for any equal uptake of both forms of vitamin E, only the succinate derivative could affect growth. Tocopheryl succinate was taken up without marked conversion to tocopherol. Cell fractionation revealed a distinct difference in the subcellular distribution between the natural form of vitamin E and its succinate derivative. Fluorescence polarization studies indicated no differences in the overall fluidity of the plasma membranes treated or not treated with either form of vitamin E. A marked augmentation of calcium uptake in neuroblastoma treated with 40μM dl-α-tocopheryl succinate was found, while dl-α-tocopherol, or dl-α-tocopheryl acetate had little or no effect. The data point to the functionality of the carboxyl group of the succinate derivative as a basis for the difference in potency of the two forms of vitamin E. The succinate derivative of vitamin E was effective in growth inhibition of neuroblastoma, although the mechanism of action appears to involve cytotoxicity rather than induction of differentiation.

The effects of retinoic acid on the morphology, the lipid metabolism and protein kinase activities of two related human neuroblastoma cell lines, SK-N-SH-F (SH-F) and SK-N-SH-N (SH-N) were studied. These cells differentiate into flat-fibroblast-like and neuronal-like phenotypes, respectively, when treated with retinoic acid. Incorporation studies with [^{3}H]oleic acid and [^{3}H]arachidonic acid showed a selective distribution within the lipid classes, the monounsaturated fatty acid favoring labelling of phosphatidylcholine and the polyunsaturated analogue favoring phosphatidylinositol and phosphatidylethanolamine both in terms of rate and amount. Pulse-chase experiments with both cell lines showed that turnover of arachidonic acid in lipid classes was most rapid in phosphatidylinositol, followed by phosphatidylethanolamine and phosphatidylcholine, and very low in phosphatidylserine. The redistribution of the arachidonic acid label following the chase was found to be markedly different in these variant cell lines. In SH-F the decrease of label in phospholipids was accompanied by an increase in the labelling of triglyceride. In SH-N, this redistribution of label in triglyceride was not observed. Following a six day exposure to retinoic acid, increased arachidonic acid turnover was observed in phosphatidylcholine and phosphatidylserine in SH-F and SH-N cell lines. No short-term effects of retinoic acid on phosphoinositide turnover, diglyceride levels or other aspects of lipid metabolism were observed.

The effect of retinoic acid on components of signal transduction pathways was investigated. Marked, but opposite, changes in protein kinase C activity were observed in both cell lines following retinoic acid treatment. SH-F cells displayed a substantial increase in protein kinase C activity; and SH-N cells, exhibited a

decrease in protein kinase C activity. To test the hypothesis that protein kinase C inhibition is required for the formation of the neuronal phenotype, the protein kinase C inhibitor, staurosporine, was used. When SH-F cells were treated by adding retinoic acid and staurosporine to the culture medium, a marked inhibition of the activity was noted. Under these conditions the usual flattened fibroblast-like phenotype was not formed, instead, these cells formed a neuronal-like phenotype similar to that produced by, retinoic acid-induced differentiation of SH-N. Examination of the expression of the protein kinase C isozymes showed that these neuroblastoma cell lines expressed PKC alpha only. Comparisons of the steady-state levels of mRNA revealed that the observed responses of protein kinase C activity in these variant cell lines was not brought about by perceptable alterations at the level of gene expression. Studies on the activities of adenylate cyclase and protein kinase A showed that these cell lines responded similarly with an increase in both activities following retinoic acid treatment. No evidence of cross-talk between the two major signalling systems was found in these neuroblastoma cell lines. As a role for protein kinase C is implicated in phenotype development a detailed study of the phenotypes produced by the retinoic acid-induced differentiation of these variant cell lines was carried out. Immunohistochemical data as well as electron microscopic examination confirm that SH-F and SH-N cells form distinctly different phenotypes following retinoic acid treatment. SH-N show typical markers and ultrastructural features indicative of neuronal differentiation, whereas, SH-F appear to lose neuronal markers and characteristics following retinoic acid induced differentiation. When the protein kinase C inhibitor, staurosporine, was added together with retinoic acid to these same SH-F cells, neuronal differentiation as revealed by the markers, similar to that produced by SH-N in the presence of retinoic acid was observed. The results presented herein show that retinoic acid has marked effects on signal transduction in these cells and strongly suggest a role for protein kinase C in phenotype development.

DEDICATION

TO JAMIE

whose support and encouragement made

this work possible

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
cAMP	cyclic adenosine 3',5'-monophosphate
cm	centimeter
CRABP	cytosolic retinoic acid binding protein
dbcAMP	dibutyryl cyclic adenosine 3',5'-monophosphate
DNA	deoxyribonucleic acid
HPLC	high performance liquid chromotography
mRNA	messenger ribonucleic acid
NCAM	neural cell adhesion molecule
NF	neurofilament protein
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-monophosphate
PIP_2	phosphatidylinositol,4,5-bisphosphate
PKC	protein kinase C
PS	phosphatidylserine
SPH	sphingomyelin
RA	retinoic acid

RAR	retinoic acid receptor
RNA	ribonucleic acid

GENERAL INTRODUCTION

Neuroblastoma are commonly used as in vitro models for the study of neoplastic differentiation and mechanisms underlying neuronal maturation (Reviewed by Abemayor and Sidell, 1989). This tumor is of neural crest origin and is one of the most common cancers occurring in childhood where an average of 525 cases per year are diagnosed in the United States (Woods and Tuchman, 1987). In the majority of cases neuroblastoma are highly malignant and the prognosis for patients with disseminated disease is poor, as these tumors do not respond well to current chemotherapeutic techniques (Finklestein, 1982). Very interestingly, however, in about 7 % of cases, including some exhibiting metastases in the liver, skin and bone marrow, regression is observed in which case tumors stop proliferating and undergo spontaneous differentiation (Evans et al., 1976; Russel and Rubenstein, 1989). Since, neither these tumors, nor patients, respond well to conventional therapies, the active pursuit of alternative strategies for the treatment of this cancer is ongoing. As neuroblastoma can occasionally undergo spontaneous maturation, an understanding of the mechanisms by which this differentiation is controlled, together with a search for new agents which may trigger this process, may prove beneficial and lead to improvements in current treatment protocols. Neuroblastoma, therefore, serve as excellent models with which to study tumor cell differentiation, and the mechanisms underlying neuronal development.

In recent years fat-soluble vitamins have received considerable attention as it

is thought that these agents may eventually provide effective alternatives to conventional chemotherapeutic techniques (Meyskens and Prasad, 1983). In vitro studies with a wide variety of cultured tumor cells have shown that some of these vitamins have the ability to reverse the transformed phenotype, inducing cells to differentiate. As a consequence they cease proliferation and exhibit specialized characteristics. While vitamins A, D and E have all been shown to exert such effects, (Meyskens and Prasad, 1983; Lotan, 1980), the focus of this thesis will be on the effects of derivatives of vitamin A and E only, with neuroblastoma as the model system. The overall goal is to contribute to the understanding of the mechanisms by which these agents bring about cell differentiation.

The mode of action with which the vitamin E derivative, tocopheryl succinate, causes growth arrest and possible differentiation in certain cell lines, is poorly understood. Numerous investigations, however, have confirmed its in vitro effects on growth inhibition, and indeed a few studies have alluded to a possible role in the differentiation of certain cultured tumor cell lines (reviewed by Prasad, 1988; Kline et al., 1990; Helson et al., 1983). Studies to gain insight on the mechanisms by which vitamin E derivatives exert these biological effects were pursued with the murine neuroblastoma, N1E 115, and results are reported in Chapter 1.

The vitamin A derivative, retinoic acid (RA), has received much more attention as an inducer of growth arrest over recent years and therefore more information on its mechanism of action has become available. This compound has been shown to induce differentiation in a wide variety of cultured cell types

including, embryonal carcinoma, leukemic cell lines, melanoma, and several lines of human neuroblastoma (Jetten and Jetten, 1979; McBurney et al., 1982; Ludwig et al., 1980; Breitman et al., 1980; Abemayor and Sidell, 1989). More recently it has been found that RA exerts it biological effect by interaction with nuclear receptors; the RA receptors (RAR) such as alpha, beta, and gamma, which have been found to belong to the steroid-thyroid hormone receptor gene family (Petkovich et al., 1987; Evans, 1988; Ragsdale et al., 1989; Giguere et al., 1987; Brand et al., 1988). This suggests that RA acts by a mechanism similar to that of steroid hormones. The role of the cytosolic RA binding protein, CRABP, is less clear, however; it has been hypothesized that it may mediate the transport of RA from the membrane to its site of action, and control levels of free RA, a function considered important in the maintenance of a gradient during embryonic development (Dolle et al., 1989). Much less is known about the events taking place after receptor mediated alterations of gene expression, which subsequently bring about this differentiation process.

The study of RA effects on neuroblastoma differentiation reported herein, will focus primarily on those events which take place following RA-mediated regulations of gene expression. The studies will address the question: which components of cellular signalling pathways are affected during RA-induced differentiation of neuroblastoma? Chapters 2 to 4 include investigations on the role of the vitamin A derivative in neuroblastoma differentiation. For these studies, two cell lines, spontaneously generated during repeated culture and derived from the parent human cell lines SK-N-SH, established by Biedler et al. (1973), were used. These two

variants, respond to RA by differentiation into distinctly different phenotypes (Sidell et al., 1986). The SK-N-SH-F (SH-F) cells, when exposed to RA undergo differentiation to form a flattened fibroblast-like cell type. The other variant SK-N-SH-N (SH-N), treated under identical conditions forms a neuronal phenotype, producing cells which form aggregates with elaborate neuritic processes (Sidell et al., 1986).

The related cell lines were used not only to investigate the effect of RA on important components of cellular signalling pathways, which may be involved in bringing about differentiation, but more interestingly, to study mechanisms regulating phenotype development. Since SH-F and SH-N cells were found to contain comparable levels of CRABP (Sidell et al., 1986), and appeared to respond similarly with respect to levels of the RAR during RA-induced differentiation (Proulx et al. unpublished results), it was conceived that signals controlling phenotype development in these divergent cell lines are likely initiated following the ligand-receptor mediated alteration of gene expression. Throughout these studies, the responses of the SH-F and SH-N sublines were compared in an effort to uncover differences in regulatory systems which may be implicated in phenotype development.

As most signalling events are initiated at the level of the cell membrane (Berridge, 1986; Whitfield et al., 1987), a study examining various aspects of lipid metabolism before and after RA-induced differentiation was pursued. To bring out the effects of RA on cellular signalling events, Chapter 3, will focus primarily on the role of protein kinase C in the differentiation of these cells. Protein kinase A and

adenylate cyclase were also studied and the possible interaction of these two important signal transduction pathways will be reported as well. As the results in Chapter 3 indicated that the protein kinase C (PKC) may play a role in phenotype development, a detailed study utilizing immunohistochemical techniques and electron microscopy was carried out to define the phenotypes and the differentiation process further. The phenotypes expressed by both variant cell lines before and after RA treatment, as well as that produced by SH-F in the presence of RA and the protein kinase C inhibitor, staurosporine, were characterized and are reported in Chapter 4. Salient features of these studies will be summarized in the conclusion.

CHAPTER 1 - STUDIES ON THE EFFECTS OF VITAMIN E ON NEUROBLASTOMA

1.1 INTRODUCTION

A. VITAMIN E AND ITS RELATIONSHIP TO CANCER

Over the past decade vitamin E has received considerable attention by cancer researchers as it is thought to protect against certain types of tumors (Birt, 1986; Bright-See and Newmark, 1983; Ellison and Londer, 1981; Newberne and Suphakarn, 1983; Newmark and Mergens, 1981; Slaga, 1983). Its anticarcinogenic role has been confirmed in epidemiological studies involving dietary supplementation with the vitamin (Menkes et al., 1984; Wald et al., 1984). Tocopherol has also been shown to inhibit chemically-induced carcinogenesis in many different animal models (Borek et al., 1986; Boutwell, 1983; Horvath and Ip, 1983; Ip, 1982; Odukoya et al., 1984). For example, skin tumorigenesis by 7,12-dimethylbenz(a)anthracene (DMBA) and 12-0-tetradecanoylphorbol-12-acetate was inhibited in mice treated topically with vitamin E prior to exposure to carcinogen (Perchellet et al., 1985). Results from epidemiological and animal studies therefore, strongly point to a protective role for dietary vitamin E against cancer.

This protection brought about by vitamin E may well act at several different levels, namely, at the level of cancer prevention thereby affecting factors responsible for the onset of carcinogenesis, and at the level of neoplastic cells, once they are formed, by inhibiting their growth or possibly reversing their transformed phenotype

to a differentiated state.

B. ROLE OF VITAMIN E IN CANCER PREVENTION

The importance of the antioxidant properties of vitamin E in cancer prevention is becoming more obvious as evidence accumulates. Lipid peroxidation, which is accelerated by radiation and heat, results in the formation of free radicals which can cause membrane and DNA damage in cells (Diplock, 1983; McCay et al., 1978). It is now well known that vitamin E can protect cells from the damaging effects of lipid peroxidation by acting as a free radical scavenger (Tamai et al., 1986; McCay et al. 1978). The antioxidant properties of this molecule are due to the free hydroxyl group on the phenolic ring, which may donate its hydrogen to a chain propogating free radical. Vitamin E itself, then becomes a free radical, however, this structure is stabilized by the resonance of the phenolic ring, preventing further lipid peroxidation (Witting, 1980). Vitamin E by acting as an antioxidant can also provide protection from nitrosating agents to which North Americans are exposed on a daily basis (Bright-See and Newmark, 1983). By acting as an electron donor, tocopherol can reduce the active NO_2^- compound to NO, the inactive form, such that it can no longer participate in nitrosation reactions (Crosby, 1983). N-Nitroso compounds have been shown to cause tumors in animals and treatment with vitamin E suppresses this effect (Newmark and Mergens, 1981). It would seem from such evidence that the anticarcinogenic effects of vitamin E can be explained for the most part by its ability to act as an antioxidant.

C. DIRECT ACTION OF VITAMIN E ON NEOPLASTIC CELLS

The mechanism whereby vitamin E may act on neoplastic cells directly to reverse the malignant process is less clear but has received considerable attention over the past ten years. The widespread interest in vitamin E and its succinate derivative is in the quest for a potential chemotherapeutic agent for the treatment of cancer. It is believed that this agent may be effective against certain types of tumors by its ability to inhibit cell growth and induce differentiation. A brief literature review on the effect of vitamin E on cultured tumor cells and its proposed mechanism of action will follow. The focus of this chapter will be on the ability of the esterified derivative, tocopheryl succinate, to effect growth inhibition and differentiation of cultured neuroblastoma. These studies will add insight as to the potential of this agent in cancer treatment.

D. EFFECT OF VITAMIN E ON CELL GROWTH AND DIFFERENTIATION

Many reports, emanating mostly from the same laboratory, have been published suggesting that vitamin E can inhibit cell growth. Indeed, some of these reports claim that cell differentiation is also induced by vitamin E (Reviewed by Prasad, 1988; and Prasad et al., 1986). In these studies, several different forms of vitamin E have been used on cultured tumor cells. The most common forms tested are dl-α-tocopherol (free alcohol), dl-α-tocopheryl hemisuccinate (tocopheryl succinate), dl-α-tocopheryl acetate, dl-α-tocopheryl nicotinate and trolox, which is the water soluble analogue lacking the hydrocarbon side chain, but still having

antioxidant properties. Of all derivatives tried, only tocopheryl succinate has given consistent results as reported by several independent laboratories (Prasad et al., 1986; Rama and Prasad, 1983; Helson et al., 1983; Kline et al., 1990). None of the other derivatives or the free alcohol, when used with appropriate solvent controls showed consistent results with respect to growth-arresting properties or differentiation of cultured tumor cells (Prasad et al., 1982; Kline et al., 1990). Growth inhibition by tocopheryl succinate on several cell types was first observed by Prasad and coworkers (Prasad et al., 1979). Since this earlier report, several laboratories have confirmed, based on cell counting and thymidine uptake studies, that the succinate ester of vitamin E can inhibit growth of melanoma (Sahu and Prasad, 1988; Rama and Prasad, 1983; Prasad, 1989), neuroblastoma (Helson et al., 1983; Rama and Prasad, 1983) glioma, (Rama and Prasad, 1983), C4#1 cells (avian retrovirus transformed immature lymphoid tumor cell lines) (Kline et al., 1990), and myeloid leukemia cells (Sakagami et al., 1981) in culture. Doses are effective in a very narrow range with higher concentrations displaying toxicity to cultured cells. The doses required for growth inhibition vary markedly with cell type, cell density and serum concentration of the medium.

The effects of vitamin E appear to be additive to the growth inhibitory effects of other agents such as, prostaglandin A_2 and sodium butyrate when present in neuroblastoma cell cultures (Rama and Prasad 1983, 1984). In these same cultures, tocopheryl succinate is also thought to enhance prostaglandin A_2-induced morphological differentiation (Rama and Prasad, 1984).

The claim that tocopheryl succinate causes or enhances differentiation is, in the majority of cases , not well substantiated. In most studies, differentiation is based solely on morphological observations (Rama and Prasad, 1983; 1984). So far, there is only one cell line, the B16 melanoma, described by Prasad et al. (1989), where morphological differentiation was supported by examination of a differentiation marker. In this case melanin production, which is a differentiated function of this cell type, was increased in concert with inhibition of cell growth and morphological differentiation. A more recent report has also shown that tocopheryl succinate treatment resulted in the decreased expression of the c-myc and H-ras oncogenes in this melanoma cell line (Prasad et al., 1990).

Studies with other cell types in which there was an attempt to use markers, such as cell surface antigens, together with morphological changes, have failed to indicate cell differentiation (Kline et al., 1990). Cell cycle studies with C4#1 cells showed that these cells were blocked within Go/G1 and early S phase, whereas a differentiated cell population would show most cells arrested with DNA content representative of the Go/G1 phase only.

With evidence available at the present time it appears that tocopheryl succinate behaves quite differently from tocopherol free alcohol or other vitamin E derivatives. While the natural form of vitamin E has no effect on growth, tocopheryl succinate is effective at inhibiting proliferation in most cell lines tested and becomes toxic when added at high concentrations. Although tocopheryl succinate appears to cause differentiation in B16 melanoma, this cell line seems to be the exception since

differentiation by this agent is not well documented in any other cell type.

E. CURRENT HYPOTHESES REGARDING MECHANISMS OF ACTION

The suggested mechanism that was put forth by Prasad and coworkers (Rama and Prasad, 1983; Prasad et al., 1986), attempted to explain how the succinate derivitive is effective in inhibiting cell growth while the natural form of this vitamin is ineffective. Since the free carboxyl group of tocopheryl succinate imparts increased water-solubility to the molecule, this feature would greatly increase monomer concentration in the medium and facilitate uptake by cultured cells. It was believed that once this agent is taken up by the cells, the succinate ester is cleaved and free tocopherol would then be capable of exerting its effect on cellular metabolism. It should be emphasized that the contention is: the effects observed for tocopheryl succinate are due to the action of the free alcohol which is released from the derivative as it becomes metabolized in the cell. Enhanced absorption of the succinate derivative would lead to a comparatively greater concentration of free tocopherol inside the cell. However, none of the aspects of the proposed mechanism has been tested and verified.

Prasad et al. (1983) have also proposed that the antioxidant function of vitamin E may be contributing to the growth-arresting properties. Accordingly, addition of antioxidants such as butylated hydroxyanisole (BHA) to cells was found to inhibit the proliferation of neuroblastoma and melanoma cells (Rama and Prasad, 1983). The manner in which these antioxidants would act to inhibit growth has yet to be explained. At any rate, other investigators were unable to reproduce this result

(Kline et al., 1990).

Certain fat-soluble vitamins, such as vitamins A and D, effective in causing cell differentiation, act specifically by binding to receptor proteins which initiate the chain of events leading to cell differentiation (Jetten and Jetten, 1979; Jetten, 1983; Lotan, 1980; Eisman et al., 1983). As it was reported that certain cell types may undergo differentiation in response to treatment with tocopheryl succinate, a specific receptor protein was sought to explain this effect.

The search for a tocopherol binding protein was first carried out by Prasad and coworkers (Prasad et al., 1981). These earlier studies with vitamin E (free alcohol), showed that this vitamin does, in fact, associate with certain proteins. Cultured neuroblastoma and glioma were labelled with [5-methyl-^{3}H] tocopherol. Cytosolic and nuclear extracts prepared from these cells were separated by gel filtration. Definite peaks showing tocopherol binding activity were obtained when radioactivity was measured in effluent fractions. In neuroblastoma, it was found that the cytosolic fraction contained five such proteins, the nuclear fraction contained one, and the post nuclear 100,000 X g pellet, also one.

Although the evidence that tocopherol binding proteins are present in these cells is convincing, their affinity and specificity has not been defined and consequently their significance still remains obscure. Because such a protein has been detected in the nuclear fraction, a role for this vitamin acting at the level of gene regulation was alluded to (Prasad et al., 1981). So far, however, little evidence has been found suggesting that vitamin E acts at the level of gene expression. Since

the highest proportion of free tocopherol is in the cytosolic fraction (Prasad et al., 1981), one is tempted to conclude that these binding proteins may simply function to help stabilize the distribution of this fat-soluble vitamin within the aqueous phase.

Many important primary events leading to cell proliferation and differentiation occur at the level of the plasma membrane (Berridge, 1987; Macara, 1985). The effects of tocopheryl succinate were therefore, examined on systems which operate at the level of the membrane.

One membrane enzyme system well known for its involvement in both cell proliferation and differentiation is the adenylate cyclase system, which is part of the protein kinase A pathway (Reviewed by Whitfield et al., 1987). Prasad and coworkers (1987; 1988) examined the effect of tocopheryl succinate on the adenylate cyclase system in B16 melanoma and in neuroblastoma cells, and found with the former, a significant fall in basal as well as in NaF-, and forskolin-stimulated adenylate cyclase activities after three days of tocopheryl succinate treatment (Sahu et al., 1987). However, in neuroblastoma, which does not differentiate following exposure to tocopheryl succinate, decreases in adenylate cyclase activities were also observed in the presence of NaF, forskolin, and prostaglandin A_2 (Sahu et al., 1988). These decreased adenylate cyclase activities, therefore, seem to be a typical response of cultured cells to tocopheryl succinate, whether they differentiate or not.

On the basis of morphological studies it was previously reported that tocopheryl succinate augments the differentiation effect of prostaglandin A_2, which is a stimulator of adenylate cyclase (Rama and Prasad, 1984 a; b). Biochemical

studies on adenylate cyclase however, revealed that tocopheryl succinate inhibits this enzyme and therefore acts in an antagonistic manner with prostaglandin A_2. How these two compounds may have antagonistic biochemical effects and synergistic biological effects, which are dependent on the biochemical effects, is somewhat confusing. Morphological results appear to to be in conflict with biochemical results.

Protein kinase A, which plays a key role in the same signalling pathway was also studied in the murine B16 melanoma cell line (Torelli et al., 1988). The increased protein kinase A activity observed in tocopheryl succinate-treated melanoma, resembles the biochemical change brought about by retinoic acid (RA) (Ludwig et al., 1980). When melanoma are differentiated with tocopheryl succinate, protein kinase A activity is increased approximately two-fold (Torelli et al., 1988). Although this protein kinase A response seems consistent with that obtained with RA, the meaning of these effects becomes very difficult to ascert. How this agent can have opposing effects on enzymes belonging to the same signalling pathway and how, at the same time, this signalling pathway can be determinantly involved in differentiation remains obscure. In view of these uncertainties, it is possible that these changes are all secondary and the entire mechanism of action of tocopheryl succinate lies in a different pathway.

F. POSSIBLE MECHANISM OF TOCOPHERYL SUCCINATE ACTION

Tocopherol is thought to form complexes with polyunsaturated fatty acid residues in the lipid bilayer (King and McCay, 1980; Diplock et al., 1972). Uptake of tocopherol and other chromanols by artificial and natural membranes in vitro was

reported to have a rigidifying effect as measured by flourescence spectrofluorometry techniques (Urano and Matsuo, 1987; Niki, 1986). This effect could be at the origin of the growth-arresting properties of vitamin E, because the cell cycle is intimately linked with fluidity changes in the membrane (DeLatt and Van der Saag, 1982).

The free carboxyl group of tocopheryl succinate is a potentially important functional group which sets this derivative apart from other forms of vitamin E. This group gives the molecule a unique amphipathic nature, a property which would be expected to influence its biological effects and increase its membrane distribution compared to the free alcohol analogue. Once incorporated in the membrane this molecule could well alter the binding and entry of divalent cations. It could even be hypothesized that tocopheryl succinate, because of its free carboxyl group could bind calcium and thereby facilitate calcium entry into the cell. By analogy, free fatty acids have been postulated to act as calcium ionophores when incorporated into membranes (Maenz and Forsyth, 1982). Alternatively, and quite to the contrary, the accumulation of excessive amounts of tocopheryl succinate in the membrane could result in calcium binding at this site and thus present a barrier to calcium entry. Knowing the importance of calcium as a second messenger, such changes could certainly alter important cellular events (Hennings et al., 1983; Berridge, 1987).

Few studies have so far attempted to address the possibility of increased calcium uptake due to vitamin E succinate treatment. One approach that has been reported by Kline et al. (1990) was to incubate tocopheryl succinate treated and control C4#1 cells in medium with varying calcium concentrations ranging from

0.4 mM to 2.0 mM. If this derivative acted by causing an increase in calcium transport in these cells, incubation in medium containing elevated calcium levels should have enhanced the cytostatic effect produced by tocopheryl succinate. Because no changes in cell growth were observed due to these conditions, it was concluded that tocopheryl succinate is not acting by affecting calcium transport. It should be noted that these studies monitor the effect of tocopheryl succinate on calcium transport in a very indirect way and are therefore by no means conclusive. A more direct means of measuring calcium uptake would have been more informative.

1.2 OBJECTIVE

Performed with the murine neuroblastoma cell line, N1E 115, the study that follows attempts to further elucidate the mechanism of tocopheryl succinate action by answering some of the following questions:

1) Do tocopherol and tocopheryl succinate have growth arresting effects?

2) Is the succinate ester more readily absorbed than the natural form of the vitamin?

3) Is tocopheryl succinate hydrolysed or does it exert its biological effect in the intact form?

4) What is the subcellular distribution of the succinate derivative in comparison to the free alcohol.

5) Do these vitamin E analogues effect membrane fluidity?

6) Does vitamin E and its succinate ester effect calcium uptake?

1.3 MATERIALS AND METHODS

A. CELL CULTURE

Murine neuroblastoma N1E 115 cells, kindly provided by Dr. M. Nirenberg (NIH, Bethesda, M.D.) were grown in Dulbecco's MEM containing 5% fetal calf serum, 5% newborn calf serum and 1 ml/100 ml medium of antibiotic-antimycotic solution Gibco Laboratories, St. Louis, MO). Cells were maintained at 37°C in a humidified atmosphere containing 10% CO_2.

B. EFFECT OF VITAMIN E ON CELL GROWTH

dl-α-Tocopherol and its succinate ester (dl-α-tocopheryl hemisuccinate) were dissolved in 99% or 75% ethanol respectively and added in required amounts to culture media such that the final ethanol concentration during growth of the cells did not exceed 1%. Control media contained ethanol in similar concentrations.

For cell count studies, cultures were initiated by inoculating 5×10^5 cells in 25 cm^2 flasks containing 5 ml of medium. After 24 hours, fresh media admixed with vitamin E in different concentrations, were added to replace the old and incubations were continued for 3 days. The cells were then trypsinized by treatment with a solution of 0.05% trypsin and 0.02% ethylenediaminetetraacetate (EDTA) in Hank's balanced salt solution and counted with a haemocytometer.

For thymidine uptake studies, cells (2×10^5) were inoculated in 35 mm dishes containing 2 ml of medium. After 24 hours, the old medium was replaced with new containing ethanol vehicle only or different concentrations of each vitamin E derivative. After 72 hours, 0.5 μCi of [methyl-^{3}H] thymidine was added and

incubations were continued for 2 hours. Attached cells were then washed twice with 10 mM phosphate buffer, pH 7.5 - 150 mM NaCl (PBS) solution and trypsinized. The cell suspensions were then transferred to scintillation vials containing 10 ml of Aquasol (New England Nuclear) and counted in a Beckman LS1801 spectometer.

C. UPTAKE OF VITAMIN E

For uptake studies, 5 x 10^5 cells were seeded in 100 mm x 15 mm tissue culture dishes. When cells approached confluence, dl-α-tocopherol and its succinate derivative were added in increasing concentrations using ethanol carrier as usual. After 48 hours attached cells were detached with a rubber policeman, counted, washed 3 times with phosphate-buffered saline (PBS), harvested by centrifugation (300 x g for 6 minutes) and extracted for lipids by the method of Bligh and Dyer (1959). Lipid extracts were dried under N_2 and redissolved in methanol. Tocopherol and tocopheryl succinate were separated and quantified by high pressure liquid chromatography (HPLC) essentially as described by Bieri et al (1979) using a Beckman HPLC apparatus, model 110B, and a C-18 column. The absorbance detector was fitted with a 280 nm interference filter. The solvent system used was methanol: water: trifluoroacetic acid (99:1:0.1 V/V/V). Tocopheryl acetate was used as internal standard for quantification of the tocopherol and tocopheryl succinate peaks (Bieri et al., 1979).

D. CELL FRACTIONATION

Cells were homogenized with a Kontes mini pressure cell at 100 p.s.i. for 3 minutes and fractionated into mitochondrial, plasma membrane, microsomal and

cytosolic fractions as described by Chakravarthy et al. (1985) who designed the method using N1E 115 cells. The purity of the fractions was monitored by assaying marker enzymes, namely, alkaline phosphatase for plasma membrane (Kamm et al., 1977), succinic dehydrogenase for mitochondria (King, 1967) and cytochrome C reductase for endoplasmic reticulum in the microsomal fraction (Hodges and Leonard, 1974). Protein was measured fluorometrically by the method of Sims and Carnegie, (1975).

E. FLUORESCENCE POLARIZATION MEASUREMENTS

Measurements were made essentially as described previously (Proulx and Szabo, 1985). To 2.5 ml of plasma membrane suspension (0.15 - 0.2 mg protein/ml), 10 μl of $1x10^{-4}$ M diphenylhexatriene in tetrahydrofuran were added to give a probe: phosphoacylglycerol ratio of 1:100. After thorough mixing the suspension was transferred to a 1 cm quartz cell in which mixing was continued by use of a small Teflon stirring bar. Polarization ratio measurements were made with an Aminco-Bowman spectrofluorometer fitted with Polaroid HNP'B polarization filters in the excitation and emission paths and interfaced with an analog-to-digital converter and a TRS-80 computer. Readings were taken at 10, 20 and 37°C. Excitation and emission wavelengths were 350 nm and 430 nm respectively and excitation and emission bandpasses were 11 nm. Fluorescence intensities were measured parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the vertically polarized excitation and data were expressed as fluorescence polarization ratio values defined as:

$$P = \frac{I_{\parallel}}{I_{\perp}\ (G)}$$

where G = HV/HH, a factor correcting for instrument anisotropy (Chen and Bowman, 1965). HV represents the fluorescence intensity with horizontal and vertical orientations of the filters placed in the excitation and emission positions respectively. HH is the fluorescence intensity when both filters are in the horizontal position. Corrections for background fluorescence and light scattering were made using membrane suspension blanks containing no probe. Depolarization due to light scattering in this protein concentration range was found to be negligeable.

F. CALCIUM UPTAKE STUDIES

Cells were seeded in 2 ml of their usual medium in Nunc eight well dishes at a density of approximately 1 X 10^5. After 24 hours the medium was replaced with fresh medium containing varying concentrations of the vitamin or appropriate solvent controls. Control and treated samples were seeded such that they could be assayed in the same multi-well dish. After incubation of cells for a further 48 hours in treatment medium, calcium uptake studies were carried out. Medium was aspirated from dishes and cells were washed three times in 10mM Hepes buffer (pH 7.5) containing 0.9% NaCl. The calcium solution, 0.1mM $CaCl_2$ with 2 μCi [^{45} calcium] per well was then added to each well. Kinetics were established by measuring calcium uptake versus time and the curve was found to be linear for at least 90 seconds. As 30 seconds was well within the linear range of the assay, for most experiments the incubation was stopped at this time point. The solution was

aspirated and the cells were washed three times in the same wash buffer. Cells were solubilized in 0.25 ml of 0.6 N NaOH and the solutions were then diluted in 2 ml of distilled water. An aliquot of 1 ml was removed for scintillation counting and another was taken for assay of protein by the method of Bradford (1976).

dl-α-Tocopherol, and dl-α-tocopheryl hemisuccinate, diphenyl-1 trans, 3 trans, 5 trans - hexatriene and Percoll were purchased from Sigma Chemicals Co., St. Louis, MO. ([Methyl-^{3}H] thymidine and Aquasol were purchased from NEN Research Products, Dorval, QUE. Tissue culture reagents were purchased from Gibco Laboratories, Grand Island, NY.

1.4 RESULTS

B. EFFECT OF VITAMIN E ON CELL GROWTH

The effects of tocopherol and its succinate derivative on the growth of neuroblastoma N1E 115 cells are shown in Figure 1.1. Whereas free tocopherol had no effect on viable cell count or on thymidine uptake, the succinate derivative decreased these parameters at concentrations of 20 X 10^{-6}M and greater. At concentrations greater than 50 μM, death of the cells occurred as could be assessed from their detachment from the substratum and their inability to exclude trypan blue.

C. UPTAKE OF VITAMIN E

Modification of the method of Bieri et al. (1979) by acidification of the solvent system resulted in the appearance of a well defined peak for tocopheryl succinate as well as a good separation of all three standards. Figure 1.2 illustrates a representative separation of tocopherol derivatives by HPLC performed on a lipid

Figure 1.1 - Effect of dl-α-tocopherol and dl-α-tocopheryl succinate on cell growth.

Cells were treated with dl-α-tocopherol (●) and dl-α-tocopherol succinate (□) at concentrations ranging from 0-250 μM for 72 hours after which time cells were harvested (A) viable cells were counted. Cells were incubated for another 2 hours following addition of 0.5 μCi [methyl-^{3}H] thymidine prior to harvest (B).

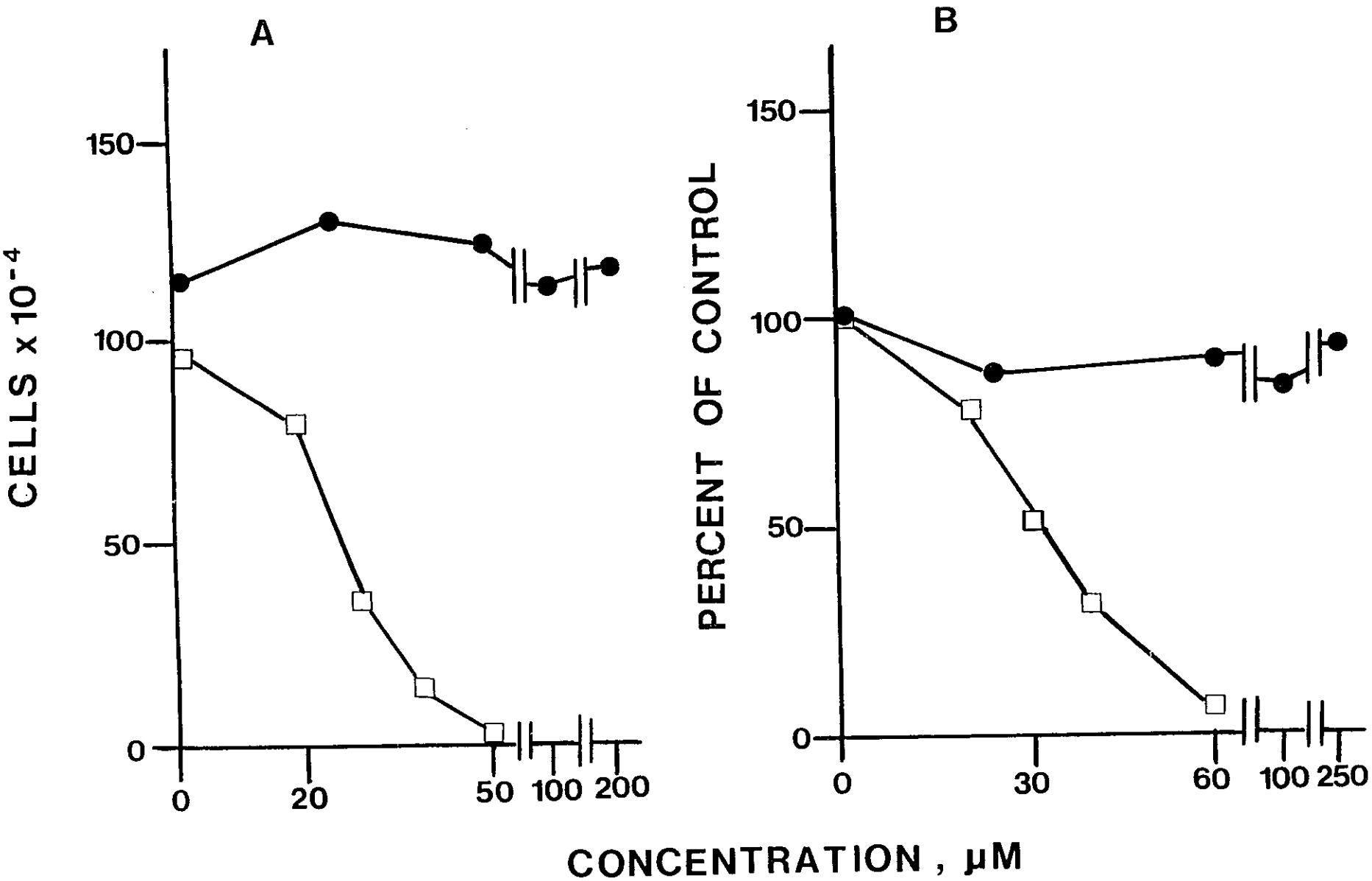
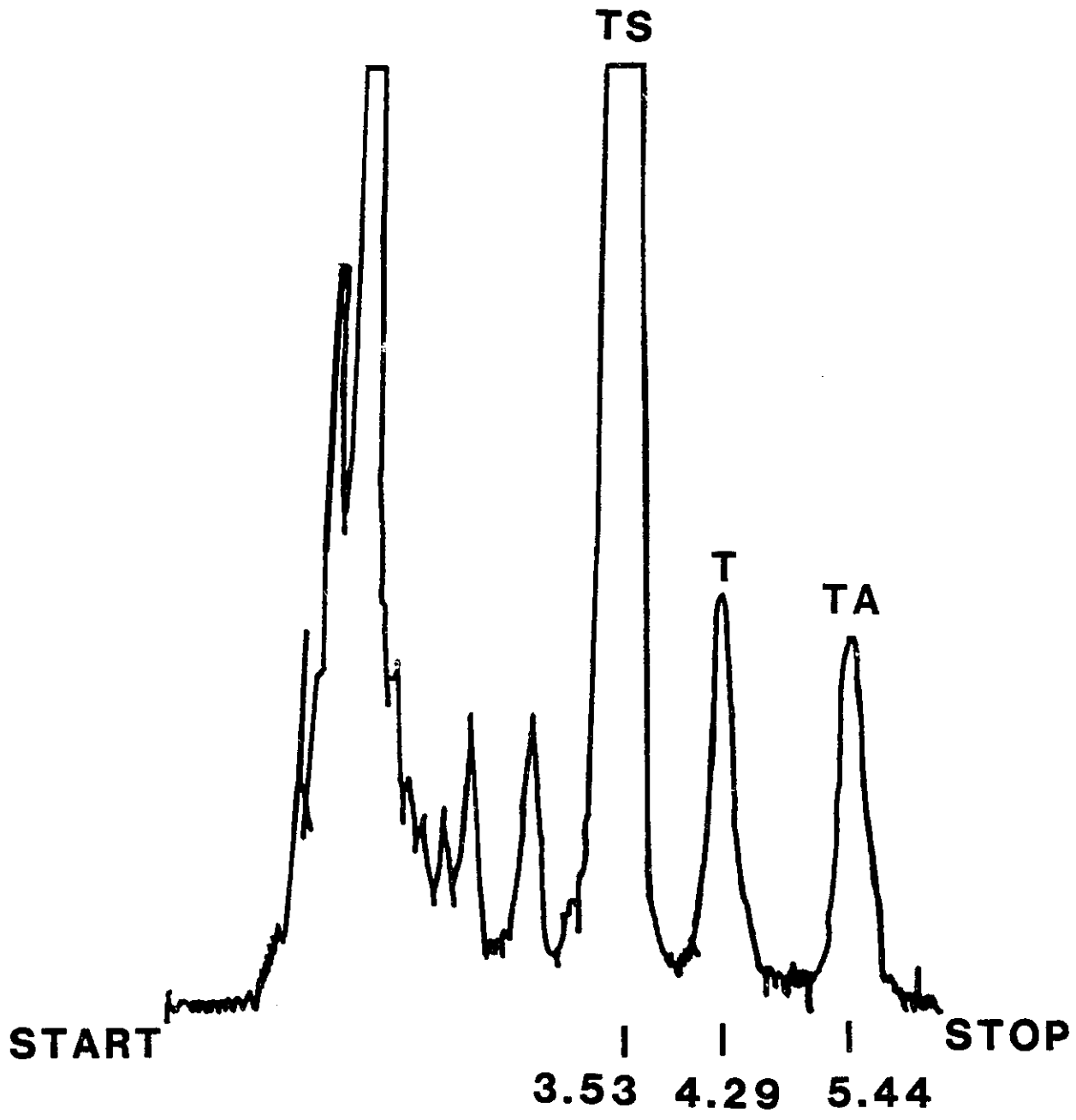

A
B
CELLS x 10⁻⁴
PERCENT OF CONTROL
150
100
50
0
0
20
50
100
200
0
30
60
100
250
CONCENTRATION , µM

Figure 1.2 - HPLC separation of vitamin E derivatives.

Cells were treated with 40 μM tocopheryl succinate for two days. One microgram of tocopheryl acetate was added as internal standard. Lipids of total cell homogenate were extracted by Bligh and Dyer (1956), and separated by HPLC. Retention times (minutes) are indicated for tocopheryl succinate (TS), tocopherol (T), and tocopheryl acetate (TA).


TS
T
TA
START
STOP
3.53
4.29
5.44
RETENTION TIME

extract of cells treated with 50 μM tocopheryl succinate. The data illustrated in Figure 1.3 indicate that the uptake of tocopheryl succinate was somewhat higher than that of tocopherol. Usually 5 % or less of the ester derivative was degraded to free tocopherol showing that the growth inhibitory effect is not due to conversion of tocopheryl succinate to tocopherol. The greater uptake of the succinate derivative was very consistant although on occasion could be considerably more pronounced, with twice as much ester being absorbed. It can be readily seen however from Figures 1.1 and 1.3, that for any equal uptake of both vitamin E forms, only the succinate ester had growth inhibitory properties.

D. CELL FRACTIONATION

Enrichments of each marker enzyme in its appropriate fraction as compared to homogenate were similar to those reported (Chakravarthy et al., 1985) i.e., eight- to nine-fold increase in specific activity of the alkaline phosphatase in the plasma membrane fraction and an impoverishment in the other fractions, four-fold increase in the specific activity of NADH cytochrome C reductase in the microsomal fraction and no significant enrichment in the other fractions and four- to five-fold increase in the specific activity of succinic dehydrogenase in the mitochondrial fraction and no enrichment in the other fractions. A typical marker enzyme profile for purified plasma membranes is shown in Table 1.1. These results are in good agreement with those published by Chakravarthy et al. (1985).

Results summarized in Table 1.2 show the distribution of tocopherol and tocopherol succinate among the different subcellular fractions. The most notable

Figure 1.3 - Uptake of Vitamin E.

Cells were treated with increasing concentrations of dl-α-tocopherol (●) and dl-α-tocopherol succinate (□). After 48 hours cells were harvested with a rubber policeman, washed three times and lipids were extracted. Lipid extracts were analysed by HPLC. Results are the averages of duplicate determinations from a single experiment and are representative of those obtained in 2 other similar experiments. Error bars represent range of technical variation.

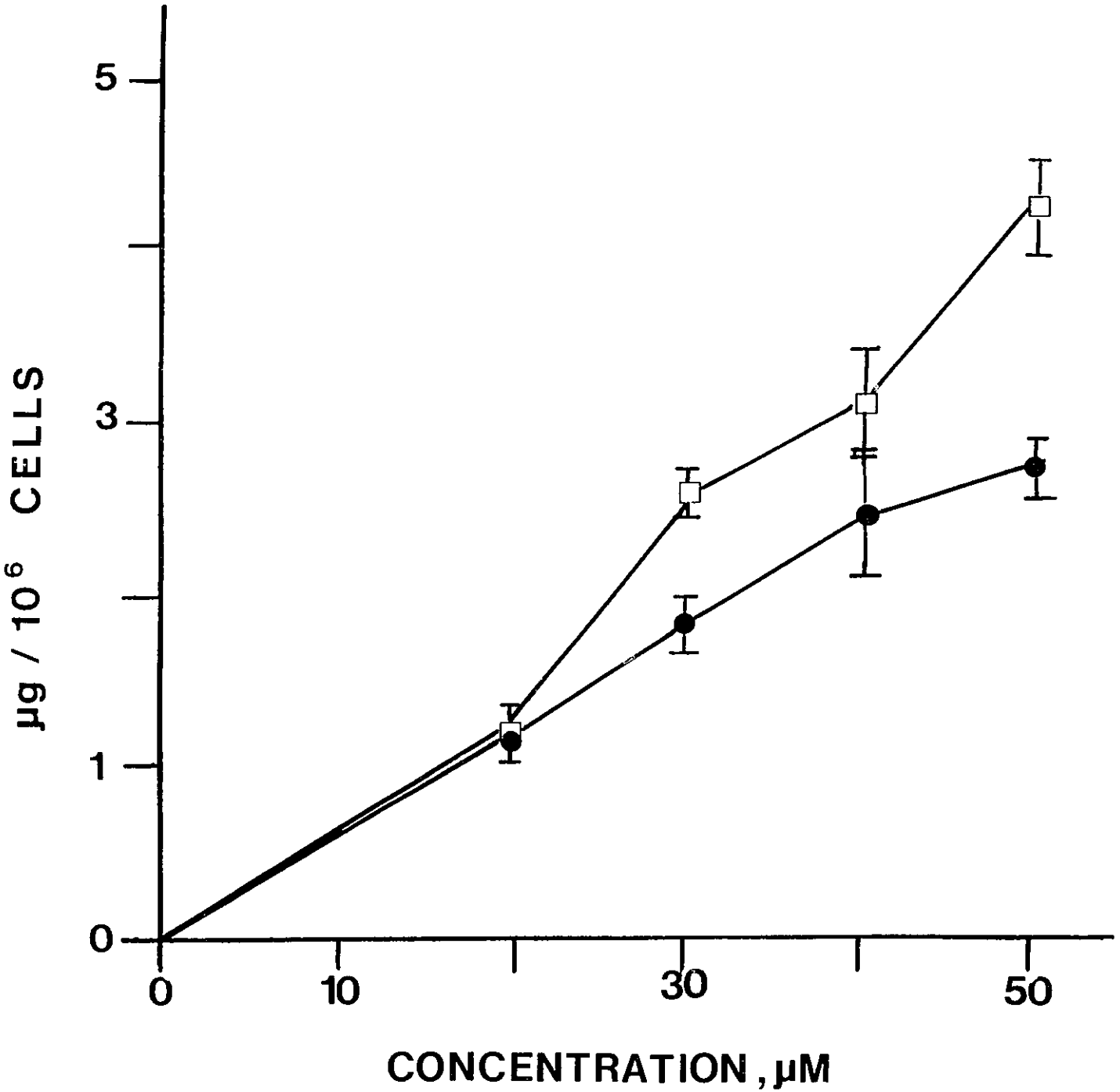

5
3
1
0
µg / 10⁶ CELLS
0
10
30
50
CONCENTRATION , µM

TABLE 1.1

Purification of Plasma Membranes

	Total Homogenate		Plasma Membranes		Enrichment Factor
Protein		(100)		(4.75)	
Alkaline Phosphatase	36.3 ± 3.8	(100)	314. ± 48	(38.0)	8.7
NADH Cytochrome C Reductase	0.522 ± .033	(100)	0.717 ± .056	(7.5)	1.3
Succinate Dehydrogenase	0.145 ± .039	(100)	0.180 ± .021	(9.4)	1.2

Percent recoveries of proteins and markers are shown in parentheses. Values are average specific activities ± standard error for 3-4 determinations. Specific activities are nmoles/mg/min for alkaline phosphatase and ∆ O.D. units per mg protein for NADH cytochrome C reductase and succinate dehydrogenase.

TABLE 1.2

Subcellular Distribution of Tocopherol and Tocopherol Succinate[a]

Fraction	Tocopherol Succinate,[b] µg/mg protein	Tocopherol,[b] µg/mg protein
Total homogenate	3.3 ± 1.0	2.2 ± 0.4
Plasma membrane	6.7 ± 1.3	2.2 ± 0.3
Mitochondria	12.5 ± 1.3	5.1 ± 1.0
Microsomal fraction	9.7 ± 3.2	2.8 ± 0.1
	Percent of total recovered[c]	**Percent of total recovered[c]**
Nuclear pellet	26.6 ± 2.3	28.7 ± 2.2
Plasma membrane	12.0 ± 1.3	8.6 ± 0.4
Other organelles	45.1 ± 5.7	25.5 ± 6.8
Cytosol	16.3 ± 4.6	37.2 ± 1.9

[a] Cells were treated with 25 µM dl-α-tocopherol and dl-α-tocopherol succinate for 48 hrs. Samples were washed 3 times and fractionation was carried out according to Chakravarthy and co-workers (1985). Other organelles include all membrane and organelle fractions other than nuclei and plasma membranes.
[b] Values represent averages of 2-4 experiments ± mean deviations.
[c] Values represent averages of 3 experiments ± mean deviations. Total recoveries in subcellular fractions compared with homogenate varied from 90 to 113%.

feature displayed in these results is that, although most of either agent is recovered in membrane and organelle fractions as would be expected from a previous report on the cellular storage sites of vitamin E (Yogeeswaran and Mbawike, 1986), the membrane and organelle content of the succinate derivative is, on a mg of protein basis or on the basis of percentage recovered in each fraction, approximately two to three times as high as that of tocopherol. On the other hand the concentration of tocopherol in the cytosol, probably distributed in the oil droplets and/or associated with cytosolic proteins therein, is proportionally higher than that of the tocopherol succinate.

E. FLUORESCENCE POLARIZATION MEASUREMENTS

Results summarized in Table 1.3 indicate that although plasma membrane concentrates more tocopherol succinate than tocopherol, the fluidity of the membrane is not significantly affected by either vitamin E form.

F. CALCIUM UPTAKE STUDIES

The time course for calcium uptake is shown in Figure 1.4. At a calcium concentration of 0.1 mM, linear kinetics were established for 90 seconds. Calcium uptake in treated samples versus controls was measured at 30 seconds which was well within the linear range of this curve.

Table 1.4 shows that statistically significant differences in calcium uptake were obtained with tocopherol and tocopheryl succinate. It should be noted however, that although the increased calcium uptake at 60 μM tocopherol is significant, the change is very small, i.e. 25% increase over controls. Tocopheryl succinate, in

TABLE 1.3

Effect of Tocopherol and Tocopherol

Succinate on Fluorescence Polarization Ratio[a]

Temperature	Control	Tocopherol	Tocopherol Succinate
10°C	2.37	2.35	2.23
20°C	2.19	2.17	2.13
37°C	1.85	1.85	1.86

[a] Membranes were prepared from cells incubated with 40 μM dl-α-tocopherol or dl-α-tocopherol succinate for 48 hrs. Values are the average of 2 determinations and are representative of 3 experiments yielding similar results. Variation was less than 10%.

Figure 1.4 - Time course for calcium uptake by N1E 115 cells.

Cells were grown for 48 hours in Dulbecco's minimum essential medium after which calcium uptake was measured at indicated times. Each time point is the average of at least 10 determinations. Error bars represent standard deviation.

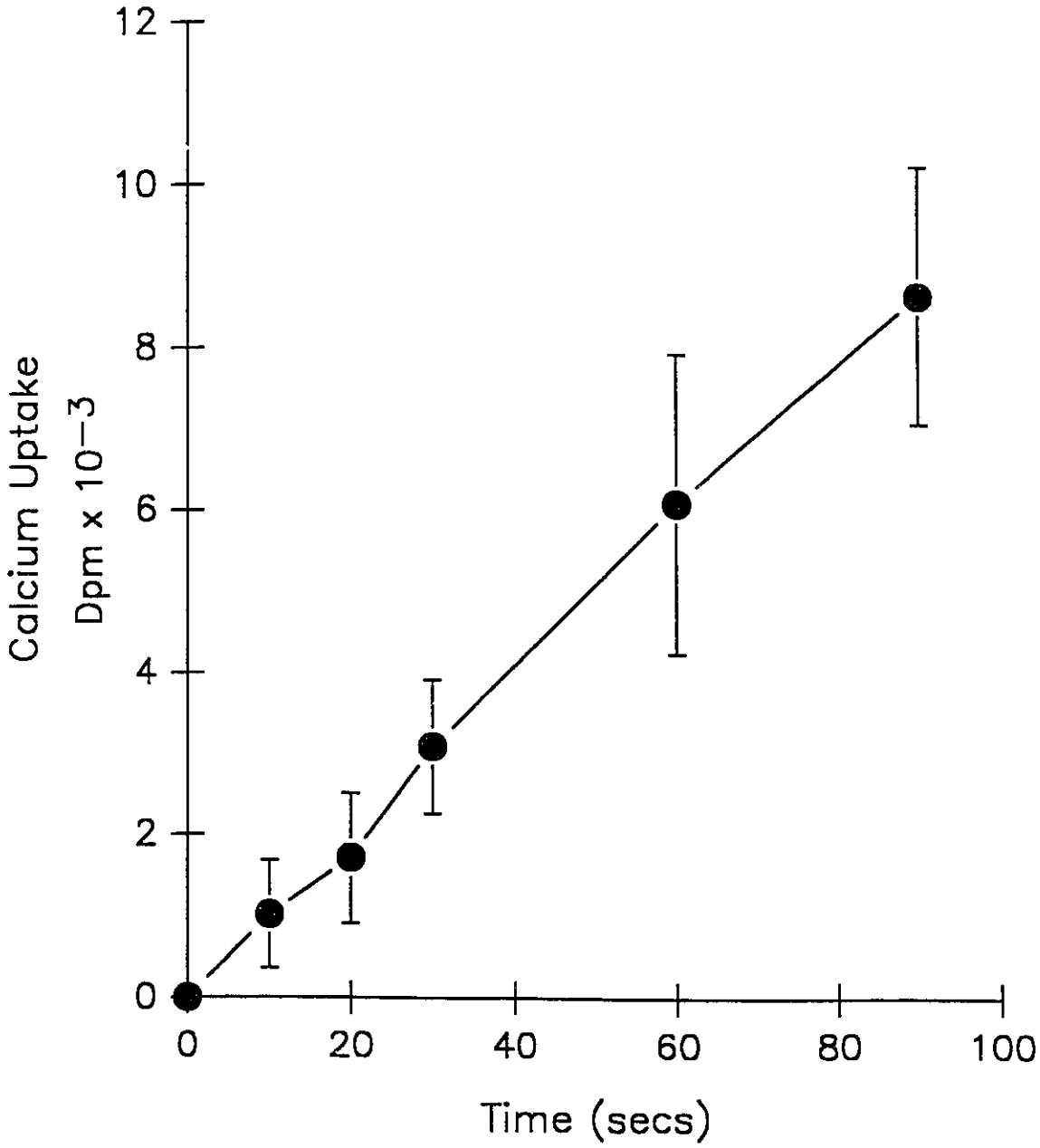

12
10
8
6
4
2
0
Calcium Uptake
Dpm x 10−3
0
20
40
60
80
100
Time (secs)

TABLE 1.4

Calcium Uptake by N1E 115 Cells (nmole/mg/min)

Treatment	dl-α-Tocopherol	dl-α-Tocopheryl-hemisuccinate	dl-α-Tocopheryl-acetate
Control	0.15 ± 0.02	0.26 ± 0.04	0.26 ± 0.03
20 μm	0.16 ± 0.01	0.28 ± 0.03*	0.24 ± 0.03
40 μm	0.16 ± 0.02	0.32 ± 0.05*	0.23 ± 0.02
60 μm	0.19 ± 0.02*	0.45 ± 0.03*	0.25 ± 0.03

In each determination values represent mean ± standard deviation, n=8.
Means with astericks are significantly different from control by one way ANOVA followed by Neuman-Keuls analysis. $P \leq 0.01$
The data in each column, dl-α-tocopherol, dl-α-tocopheryl hemisuccinate and dl-α-tocopheryl acetate are the results of separate experiments.

contrast, caused significant changes in calcium uptake even at the lowest concentration, 20 μM. At 60 μM a much greater increase of 72 % was observed. No significant differences were detected with dl-α-tocopheryl acetate.

1.5 DISCUSSION

In agreement with work previously published with other neuroblastoma cell lines (Prasad et al., 1979; Helson et al., 1983) the results illustrated in Figure 1.1 show that only tocopherol succinate has growth arresting properties. The natural form of vitamin E has no such activity even when added to cultures at high concentrations.

Uptake studies shown in Figure 1.3 confirm that the succinate derivative, probably due to its enhanced solubility, is taken up more readily than the natural form of the vitamin.

HPLC analyses clearly showed that cells treated with tocopheryl succinate retained this compound in its intact form. No significant conversion of tocopheryl succinate to the tocopherol free alcohol is observed suggesting that cultured cells are ill equipped to hydrolyse this vitamin E derivative. These very simple observations have several important implications and call into question the hypothesis put forward by Prasad and coworkers (Rama and Prasad, 1983; Prasad et al., 1986) regarding the mechanism of action of this compound.

Although tocopheryl succinate is taken up more readily by cells, this increased uptake alone cannot account for the observed growth-arresting properties of the esterified derivative. According to the results shown in Figure 1.1, at concentrations in the range of 50 μM, tocopherol was totally ineffective in inhibiting cell growth.

At this concentration, the amount of tocopherol taken up by the cells and membrane fractions would be expected to be at least as great as an amount of vitamin E succinate derivative capable of inhibiting growth.

Another important implication from these uptake studies is the point that the active agent within these cells causing growth arrest is tocopheryl succinate and not free tocopherol. Previous experiments which were carried out to test the effectiveness of antioxidants to inhibit cell growth led Prasad and coworkers to propose that this property could contribute to the growth inhibition of the succinate derivative (Rama and Prasad, 1983). As it is known that vitamin E must be in the free alcohol form to act as an antioxidant, it is clear that the esterified analogue must not be exerting its biological effect by this mechanism.

The results in Table 1.2 showing different subcellular distributions of these two forms of vitamin E further emphasize that the succinate derivative is an entity distinct from the natural form of vitamin E. One essential difference between tocopherol and its succinate derivative is in the amphipathicity of the molecules, the succinate being much more water-dispersable by virtue of the ionized carboxyl group at physiological pH. One should expect also, that this property would favor its greater retention by membranes as our results indicate. The data in Table 1.2 show that the largest proportion of tocopheryl succinate is found in cellular membranes (57 %) while the largest proportion of free tocopherol (37 %) is found in the cytosolic fraction.

The plasma membrane is the site where growth factors and intercellular

signalling pathways intervene (Berridge, 1987). Tocopheryl succinate accumulation in the membrane, therefore, could have an impact on signal transduction. Relevant to this is our investigation on calcium uptake. Calcium is now recognized as an important second messenger involved in stimulus-response coupling in numerous cellular processes (Berridge, 1986; Rasmussen et al., 1986). As resting cytosolic calcium levels are maintained at submicromolar concentrations, the majority of cellular calcium (99.9%) is bound in internal stores or on the surface of the cell membrane (Campbell, 1987). Our studies, which employed a direct approach for measuring calcium uptake by neuroblastoma suggest that the succinate derivative of vitamin E and to a much lesser extent, free tocopherol, enhances calcium uptake by these cells. It should be emphasized that results from these studies simply provide a general overview of calcium uptake which is the summation of several different complex processes. One may generalize that this increased uptake of calcium could be a function of two distinct mechanisms: a) tocopheryl succinate treatment could augment calcium transport into the cell resulting in increased intracellular calcium concentrations; and/or b) the presence of these compounds, particularly the succinate ester on the membrane could result in an increased calcium binding at the cell surface due to an accumulation of carboxyl groups from tocopheryl succinate. Either of these effects could have serious consequences on cellular function. Recent studies in our laboratory, (Schwartz and Proulx, unpublished results) using the fluorescent probe, fura-2, have revealed that neither free tocopherol nor the succinate ester cause changes in steady-state intracellular calcium concentrations when cells are

treated several days with these vitamin E derivatives. Also, when non-treated cells are exposed to these derivatives and calcium uptake is measured with the fluorescent dye, no enhanced uptake is seen. Therefore, an ionophoric function of tocopherol succinate seems very unlikely. These results rule out the former possibility suggesting that the observed changes in calcium uptake are due to increased calcium influx into the cell. The more pronounced effect of tocopheryl succinate on calcium uptake is likely due to a retention of calcium on membranes due to the free carboxyl group.

The cell cycle of neuroblastoma appears to be linked to changes in membrane fluidity (DeLatt and Van der Saag, 1982). The addition of tocopherol in vitro to membrane preparations has a rigidifying effect (Urano and Matsuo, 1987; Niki 1986), and it was thought that decreases in fluidity resulting from greater uptake of tocopherol succinate by membranes, as compared to free tocopherol might explain the growth inhibiting properties of the former and the lack of effect of the latter. However, no changes in fluidity were detected. Quite possibly, the quantities of tocopherol and tocopherol succinate absorbed by the plasma membrane were insufficient to change its average fluidity as evidenced by the fluorescence polarization data obtained. It may be added that other in vivo studies have failed to show a change in fluidity as a result of vitamin E administration (Molinar, 1980). As another possibility one might suggest that, unlike lipid vesicles in vitro, membranes in vivo are able to effectively adapt to changes in fluidity by modification of their lipid composition. In this particular context, it would be interesting to verify if lipid compositional changes do result from vitamin E treatment of the cells.

SUMMARY - MECHANISM OF ACTION

The precise mechanism of action of tocopheryl succinate is not well understood however these investigations have helped clarify certain points. The apparent accumulation of tocopheryl succinate and the inability of cells to metabolize this compound precludes antioxidant mechanisms.

The plasma membrane is a major site where signal transduction occurs and cell growth is regulated (Whitfield et al., 1987). As this compound is concentrated in the membrane it seems likely that this is at least one of the sites of action of this derivative. One may hypothesize that the tocopheryl succinate molecule becomes inserted in the lipid bilayer in a way similar to free tocopherol. Unlike tocopherol, however, a free carboxyl group would protrude at the membrane surface. Although no fluidity effects are apparant, this free carboxyl group may have several effects which could greatly impair normal function of the membrane. Tocopheryl succinate insertion in the lipid bilayer could cause an alteration of surface charge which could effect the binding of growth factors and other agonists to the plasma membrane. Increased binding of divalent cations such as calcium, as our data with calcium uptake suggest, could interfere with the normal activity of membrane bound enzymes especially those involved in signal transduction. The results of Sahu et al. (1987) with adenylate cyclase, lend some support to this hypothesis. This membrane bound enzyme requires co-ordinated interaction with several proteins to function normally. Tocopheryl succinate which decreases the activity of this enzyme in several different cell lines could be perturbing this system at the level of the catalytic subunit, the G-

proteins and/or at the level of the receptors. The membranes of mitochondria, Golgi and endoplasmic reticulum, where this amphipathic molecule is highly enriched, are also areas where the succinate derivative may be hindering the activity of membrane bound enzymes. Such effects on these organelles could certainly contribute to the observed toxicity of this unique vitamin E derivative. Further study on membrane effects of tocopheryl succinate should help elucidate this mechanism.

1.6 CONCLUSION

As a chemopreventive agent, the benefits of the natural form of vitamin E, which possesses antioxidant properties cannot be disputed. However, the claim that vitamin E has the ability to reverse the transformed phenotype back to a differentiated state seems rather doubtful. As attested by our results, as well as those of others, (Prasad et al., 1986; Rama and Prasad, 1983; Helson et al., 1983; Kline et al., 1990), tocopherol has no growth arresting ability and certainly does not induce cell differentiation. The succinate derivative does however, cause inhibition of proliferation but our data indicate that this could be due to cytoxicity as this compound is not metabolized and accumulates in membranes. With neuroblastoma as a model, the current body of evidence shows that the succinate ester is unable to induce differentiation, therefore, as a novel alternative to conventional chemotherapeutic techniques, vitamin E has very limited potential. The search for suitable fat soluble vitamins in an effort to provide innovative treatment strategies for cancer is definitely needed but the utilization of vitamin E or its derivatives for this purpose does not show promise. Attention should be directed towards vitamin

A and its derivatives as the ability of certain forms of this vitamin to induce differentiation in a wide variety of cell types is well documented (Jetten, 1983; Lotan, 1980) and studies with these vitamins will prove to be more beneficial. The following chapters will focus on the effect of retinoic acid on neuroblastoma differentiation.

CHAPTER 2 - EFFECTS OF RETINOIC ACID ON THE LIPID METABOLISM OF NEUROBLASTOMA CELLS

2.1 INTRODUCTION

A. IMPORTANCE AND RELEVANCE OF LIPID METABOLISM IN CELL GROWTH AND DIFFERENTIATION

The plasma membrane is the site where signals received from the external environment are processed. Hormones or growth factors exert their biological effects by interacting with receptors at the cell surface causing perturbations which are transduced across the membrane and transmitted to effector sites within the cell by the generation of second messengers (Berridge, 1986). The production of these signalling compounds have been shown to result directly from, or to be influenced indirectly by membrane lipid composition and metabolism. Examples of second messengers, the release of which is caused directly by alterations in lipid metabolism are those generated in the phosphoinositide cycle (Berridge, 1986). Here, the receptor-mediated activation of phospholipase C results in the hydrolysis of inositol phospholipids, thereby producing inositol phosphates and diglyceride. In some cases the phospholipase C hydrolysis of other phospholipids is implicated, resulting in the production of diglyceride as the sole second messenger (Rando 1988). It is in this manner that the turnover of lipids, especially the inositol phospholipids, is involved in the control of cell growth and differentiation (Berridge, 1987).

Although cell differentiation by retinoic acid (RA) is known to be mediated

by nuclear retinoic acid receptors which belong to the steroid and thyroid hormone receptor superfamily (Petkovich et al., 1987; Evans, 1988; Brand et al., 1988), the participation of non-nuclear mechanisms has not been ruled out completely. Interestingly, it has recently been reported that neuroblastoma differentiation by RA is preceded by an early decrease in phosphoinositide turnover. Within one minute of RA treatment of the neuroblastoma cell line, LAN 1, a marked decrease of inositol 1,4,5 trisphosphate and diglyceride content was found (Ponzoni and Lanciotti, 1990). This agent, therefore, may be mediating its effect, in part, by alterations in lipid metabolism.

The fatty acid composition of membranes has been found to greatly alter activities of membrane proteins, namely those involved in transport, and those possessing various catalytic functions (Balcar et al., 1980; Yorek et al., 1984; Merrill et al., 1987). Of particular interest here is the fact that fluidity, controlled by fatty acid composition, markedly influences the transport of calcium in membranes, and it is well established that the flux of divalent cations, such as that of calcium, plays a key role in cellular signalling (Whitfield et al., 1987; Macara, 1985). Therefore, any changes in the activity of these proteins, due to lipid environment, may influence important cellular processes including cell differentiation. Some of the best examples demonstrating the effects of membrane lipids on transport function are seen in studies employing brush border membrane vesicles. Due to their highly specialized function in digestion and absorption, membranes of this type are particularly enriched with enzymatic and transport activities (Reviewed by Proulx,

1991). When the fluidity of brush border membrane vesicles was enhanced by incorporation of unsaturated fatty acids, calcium uptake was found to be increased in several different systems (Fontaine et al., 1981; Bikle et al., 1984; Merrill et al., 1986; Maenz and Forsyth, 1982). Other studies with membranes prepared from the same source have shown that unsaturated fatty acids resulted in inhibition of Na+/H+ exchange, and Na+ gradient- dependent glucose and alanine transport (Tiruppathi et al., 1988).

Activities of signal transduction systems within the membrane, similar to other transport and enzyme activities, may also be influenced indirectly by changes in the composition of bilayer phospholipids since proteins generating second messengers can be affected by the lipid environment. A good example of this phenomenon is seen with the adenylate cyclase system, the activity of which has been shown to be regulated by the polyunsaturated fatty acid content of the membrane phospholipids. Accordingly, Murphy (1984) modified the polyunsaturated fatty acid content of N1E 115 neuroblastoma lipids by supplementing the growth medium with linoleic acid. These changes in lipid composition were correlated to a two- to five-fold increase in basal cAMP levels (Murphy, 1984). Cessation of polyunsaturated fatty acid supplementation caused a corresponding decrease in phospholipid polyunsaturated fatty acid content and a subsequent return to normal levels of cAMP production (Murphy, 1985; Murphy, 1986). Similar reports documenting the effects of the lipid environment on adenylate cyclase activity in brain slices and cultured fibroblasts have been presented (Baba et al., 1984; Anderson and Jaworski, 1977). These results

demonstrate that the activity of a membrane-bound enzyme, known to play a key role in cell growth and differentiation, can be significantly influenced by lipid composition.

Studies monitoring the fluidity changes on the membranes of neuroblastoma have shown that differentiation of these cells is correlated with definite changes in membrane fluidity (DeLatt and Van der Saag, 1982). One may speculate that changes in lipid metabolism, in co-ordination with nuclear events, may operate in a synchronized manner to successfully bring cells to the end-point of differentiation.

Changes in lipid metabolism may not only influence developmental processes by fluidity changes, but specific acyl groups can affect the activities of proteins directly, and the fatty acids themselves may act as substrates to produce bioactive agents which may influence cell differentiation. The metabolism and turnover of arachidonic acid is one such example. The release of arachidonic acid from cellular phospholipids is mediated primarily by phospholipase A2, which cleaves off the acyl chain from the second ester position of the glycerol backbone. Arachidonic acid may also be released by the sequential action of phospholipase C and lipase. Alternatively, phospholipase D hydrolysis followed by a phosphatidic acid-specific phospholipase A2 may further contribute to the cellular pool of free arachidonic acid (Reviewed by Irvine, 1982). The free fatty acid may either be recycled to membrane phospholipids by the action of an acyltransferase or serve as a substrate for eicosanoid synthesis. Liberation of free arachidonic acid from the phospholipid pools is thought to be the rate-limiting factor in the biosynthesis of eicosanoids (Irvine, 1982). These oxygenated compounds are produced by peroxidation of arachidonate

mediated by enzymes of the cyclo-oxygenase or lipoxygenase pathways. Prostaglandins are produced by the former while leukotrienes are synthesized by the latter (Needleman et al., 1986).

Important roles for eicosanoids have been implicated in neuronal differentiation in several systems. The release of arachidonic acid and subsequent eicosanoid production is thought to be regulated by nerve growth factor in PC12 cells and dorsal root ganglion neurons (DeGeorge and Carbonetto, 1987; DeGeorge et al., 1988). Direct correlations of nerve fibre growth with arachidonic acid release from phospholipids, coupled with production of oxygenated metabolites, suggests a causal link between nerve growth factor stimulated phospholipid metabolism and nerve fibre outgrowth. This relationship was further substantiated when lipoxygenase inhibitors, added together with nerve growth factor, prevented this morphological change (DeGeorge et al., 1988).

Prostaglandins have been shown to influence neuronal development by stimulating synapse formation and nerve fibre outgrowth in neuroblastoma hybrid cells (Nirenberg et al., 1983). Prostaglandin E has also been shown to increase cAMP production which, in certain neuroblastoma cell lines, can cause differentiation (Prasad and Hsie, 1971).

Other than serving for eicosanoid production, free arachidonic acid has been shown to be an activator of the PKC isozymes, alpha and gamma. PKC activity is measured in vitro by the transfer of phosphate from ATP to histone. In such an in vitro assay, PKC is activated by addition of phosphatidylserine (PS), diglyceride and

calcium to the reaction mixture. With the isozyme, alpha, however, activation can be achieved by addition of arachidonic acid and calcium. For this isozyme therefore, it appears that arachidonic acid can substitute for diglyceride and PS. The gamma isozyme, found exclusively in tissues of the central nervous system, is also activated by calcium, PS and diglyceride, but it has recently been found that arachidonic acid alone can replace calcium, PS and diglyceride as an activator for this species of PKC (Kikkawa et al., 1989). Free arachidonic acid, therefore, may serve as an activator of PKC in vivo, revealing yet another mechanism by which release of this fatty acid may mediate cellular events.

Numerous studies have been carried out on the modulation of fatty acid metabolism in the neuroblastoma cell line, N1E 115. Fatty acids, which are synthesized in the cytosol, act as substrates for phosphoglyceride and triglyceride synthesis. The enzymes involved in the biosynthesis of phospholipids are tightly bound to membranes of the endoplasmic reticulum. The biosynthetic products become incorporated in the bilayer of this organelle, where they are subsequently transported in the form of vesicles to the Golgi, and finally inserted into the plasma membrane (Lehninger, 1982). More recently, however, it has been demonstrated that lipid synthesis may also take place directly on the plasma membrane. Subcellular fractionation studies (Chakravarthy et al., 1986) have demonstrated that purified plasma membranes contain significant levels of choline phosphotransferase, an enzyme required for de novo synthesis of phosphatidylcholine, as well as lysophosphatidylcholine acyltransferase, an enzyme required for the exchange of acyl

chains in phospholipids. To further illustrate the presence of two biosynthetic pools, cells were prelabelled for 16 hours in the presence of [^{14}C]linoleic acid. A subcellular fractionation was carried out and the specific activities of phospholipids were compared among the various fractions. The specific activities of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) were found to be at least two-fold greater in lipids extracted from the plasma membrane in comparison to those obtained from microsomal fractions. These results, with N1E 115 cells, indicate that there are at least two sites of lipid metabolism, which draw substrate from two distinct pools, one which is at the plasma membrane, and another which is in the lumen of the endoplasmic reticulum (Chakravarthy et al., 1986).

The incorporation of fatty acids into phospholipids and triglycerides is a highly regulated process. Precursor fatty acids taken up from the medium can be subject to modification before incorporation into cellular lipids. Detailed studies on fatty acyl chain modification were carried out by the same group, also with N1E 115 neuroblastoma cells (Cook et al. 1987). Following the incubation of cells with various radiolabelled free fatty acids, lipids were extracted and the fatty acid composition within the lipid classes were analyzed. For example, when cultures were incubated with [^{14}C]linoleic acid, a significant proportion of radioactivity was incorporated into phosphoglycerides as arachidonic acid. This indicates that the labelled precursor had undergone processing involving two desaturation steps as well as chain elongation prior to esterification in complex lipids. These studies also demonstrated that the

incorporation of fatty acids in various lipid classes was a highly selective process. Accordingly, the uptake and distribution pattern of polyunsaturated fatty acids within the lipid classes are clearly distinct from those of monounsaturated fatty acids (Cook et al., 1982; 1983). Incorporation studies with labelled arachidonic acid resulted in the highest specific activities in PE and PI, whereas monounsaturated fatty acids were distributed in a relatively uniform manner throughout the lipid classes.

The processing and incorporation patterns of polyunsaturated fatty acids were also found to be influenced by the presence of other acyl chains in the medium. For example, when cultures were incubated with [^{14}C]linoleic acid alone, or together with saturated fatty acid, most of the labelled fatty acid was incorporated into phospholipids. If, on the other hand, labelled diunsaturated fatty acid was added with an excess of arachidonic acid, two significant changes were observed. Firstly, there was an acceleration in the rate of processing by desaturation and chain elongation of linoleic acid, and secondly, a much larger percentage of labelled fatty acid was diverted to the triglyceride fraction. The levels of labelled arachidonic acid ([^{14}C]linoleic acid-derived), as well as that of other polyunsaturated fatty acids, which were incorporated in phospholipid pools, seemed to be limited and carefully controlled in these cultured neuroblastoma cells. It has been proposed that triglyceride may act as an expandable reservoir for excessive levels of arachidonic acid and other polyunsaturated acids, thus limiting the quantities esterified in phospholipids and restricting the amounts reaching cellular membranes (Cook et al., 1987).

There could be several reasons for the regulation of polyunsaturated fatty acid incorporation in these bilayers: due to their high degree of unsaturation, these fatty acids are more subject to free radical attack and lipid peroxidation, therefore excessive amounts could result in loss of membrane integrity. Increased levels of polyunsaturated fatty acids in the membrane will also enhance fluidity which, as described above, may further alter protein function. In excess, they could destabilize the membrane and cause its lysis. It appears therefore, that the incorporation of various fatty acyl chains into the different lipid classes is a process which is subject to complex mechanisms of regulation.

While extensive studies have been carried out on fatty acid metabolism in quiescent, untreated neuroblastoma cultures, few studies have been designed to investigate differences in arachidonic acid metabolism before and after RA-induced differentiation. Clearly, the influence of lipids on cell growth and differentiation extends far beyond the realm of the well known hydrolysis of inositol phospholipids. Accordingly, the turnover of arachidonic acid in phospholipids, its release and its conversion to eicosanoids appears to be linked to important developmental processes especially those of neuronal differentiation.

2.2 OBJECTIVES

A. LIPID METABOLISM IN SH-F AND SH-N NEUROBLASTOMA CELLS

As no previous experiments on lipid metabolism had been carried out with these variant cell lines, the following studies were designed to investigate and compare aspects of their lipid metabolism. The phospholipid composition of both

cell lines was first analyzed, and in order to determine if polyunsaturated fatty acids display a selective incorporation pattern within the lipid classes, the uptake of [^{3}H]arachidonic acid and the distribution of its label within the lipid classes was compared to the uptake and incorporation patterns of a radioactive monounsaturated fatty acid, [^{3}H]oleic acid. Following labelling of SH-F and SH-N cells with [^{3}H]arachidonic acid, the redistribution of label within the lipid classes was also studied as a function of time under chase conditions.

B. EFFECT OF RETINOIC ACID ON LIPID METABOLISM IN SH-F AND SH-N CELLS

Since it had been previously shown that RA treatment resulted in a rapid decrease of inositide metabolism/turnover in certain neuroblastoma cells (Ponzoni and Lanciotti, 1990), it was decided to pursue studies in search of rapid effects of RA on lipid metabolism of SH-F and SH-N sublines. Experiments were thus carried out to measure the short term effects of RA on diglyceride levels and phosphoinositide turnover, as well as fatty acid incorporation in the various lipid classes.

As studies have shown that arachidonic acid redistribution appears to be an important event in neuronal differentiation (DeGeorge and Carbonetto, 1987; DeGeorge et al., 1988), the release and turnover of this fatty acid was examined and compared in untreated growing cells and those induced to differentiate with RA.

2.3 MATERIALS AND METHODS

All tissue culture products were purchased from GIBCO Laboratories, St. Louis, MO. All-trans-retinoic acid (RA), arachidonic acid, lipid standards, silica gel,

and fatty acid-free bovine serum albumin were purchased from Sigma Chemical Company, St. Louis, MO. Radiochemicals were purchased from DuPont (NEN Products) Lachine, Quebec. Solvents and other reagents were purchased from BDH Inc., Toronto, Ontario.

A. CELL CULTURES

The SK-N-SH-F, (SH-F) and SK-N-SH-N,(SH-N) cell lines used in our experiments are descendants of the cell line SK-N-SH originally established by Biedler et al. (1973) and were kindly supplied by Dr. Neil Sidell, Division of Surgical Oncology, UCLA, School of Medicine. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 1 ml/100 ml medium of antibiotic/antimycotic solution purchased from GIBCO. All cells used were not maintained beyond 15 passages and were grown at 37°C in a humidified 5% CO_2 atmosphere. Cell cultures were treated with all-trans RA, each time freshly dissolved in ethanol, and added to yield concentrations of 3×10^{-6}M as previously described by Sidell et al. (1986). Control cultures were treated with corresponding concentrations of ethanol (<1% final concentration). Media were changed every third day.

B. FATTY ACID INCORPORATION STUDIES

Cells were seeded at a density of approximately 2×10^6 per 100 X 20 mm dish 24 hours prior to each experiment. At time 0, 1 μCi of either [^{3}H]arachidonic acid or [^{3}H]oleic acid was added to RPMI 1640 medium containing 5% fetal calf serum and 5% newborn calf serum. Each labelled fatty acid was diluted with the same

unlabelled fatty acid such that the final concentration was 1 μM. At time points of 1, 2, 4, 8, 12, 24 hours, medium was aspirated and dishes were washed twice with serum-free medium containing 0.05% fatty acid-free bovine serum albumin. Cells were harvested by scraping in 1.5 ml methanol followed by a second 1.5 ml methanol rinse. Both fractions were combined and stored at -20°C for lipid extraction.

C. ARACHIDONIC ACID CHASE

Cells were seeded as described above. Cultures were pulsed with 1 μCi [^{3}H]arachidonic acid and diluted such that the final concentration of fatty acid was 1 μM. After a 16-hour incorporation period the labelled medium was removed and dishes were washed twice with 5 ml serum-free medium containing 0.05% fatty acid free bovine serum albumin. Chases were carried out in RPMI 1640 medium containing 5% fetal calf serum and 5% newborn calf serum, supplemented with 100 μM unlabelled arachidonic acid. Cells were harvested in methanol at time points of 0, 1, 3, 6, 8, and 24 hours.

D. EFFECTS OF RETINOIC ACID ON LIPID METABOLISM AND TURNOVER

Short-term effects of RA on phosphoinositide turnover were investigated with the same arachidonic acid chase protocol described in Section C above. Two groups of cells, treated versus control, were prelabelled for 16 hours with [^{3}H]arachidonic acid. At time 0 of the chase, RA was added to the medium of the treatment group and vehicle only was added to that of the controls. Lipids were extracted at time points of 0, 2.5, 4.5, 6.0 and 8.0 hours during chase. The effect of RA was also

followed for shorter time points. Cells were again pulsed with [^{3}H]arachidonic acid for 16 hours and chased with unlabelled medium at time points of 0, 15 seconds, 30 seconds, 1 minute, 5 minutes, and 10 minutes. The reaction was stopped by aspiration of medium and addition of methanol to the adherent cells. For each time point radioactivity was measured in the phosphoinositide and the diglyceride fractions.

The direct effects of RA on lipid metabolism were also investigated in a series of short-term incorporation studies with [^{3}H]oleic acid and [^{3}H]arachidonic acid. Incorporations were conducted as described in Section B above; however, RA was added at time 0 for the treatment group and vehicle only was added for the control group. At time points of 1, 2, 4, 8, 12, and 24 hours, cells were harvested and lipids were analyzed. Levels of incorporation of fatty acids were measured in phospholipids and neutral lipids at each time point.

To examine long-term RA effects on arachidonic acid turnover and label redistribution, cells were treated for 6 days with 3 X 10^{-6} M RA prior to experiment. In all experiments RA-treated samples were matched with solvent-treated controls which were grown under identical conditions. The same chase protocol as described in Section C above was used, with minor changes. After a 16-hour incorporation period, cells were washed and chase medium containing 100 μM unlabelled fatty acid was added. For the treated group, RA was included in the chase medium; whereas solvent only was added to that of the control group.

E. LIPID ANALYSIS

Lipids were extracted according to the method of Bligh and Dyer (1959) and separated by thin layer chromatography. In one-dimensional analysis, extracts were separated on activated silica gel G plates developed with chloroform/methanol/water (65:25:4, v/v/v) in the case of phospholipids, and petroleum ether/diethyl ether/formic acid (55:45:1.4 v/v/v) in the case of neutral lipids. Lipids were revealed with iodine vapours and identified by cochromatography with authentic lipid standards. Silica gel in zones corresponding to individual lipid classes was scraped, collected in vials, suspended in a scintillation mixture that elutes the lipids, and radioactivity was counted in an LS 1801 Beckman spectrometer, all as described (Froissart et al., 1989). In all experiments, lipids were also analyzed, at least once, with two dimensional thin layer chromatography according to the method of Kramer et al. (1983). Briefly, 20 X 20 cm glass plates were coated with silica gel H and prewashed by developing in chloroform/methanol (2:1, v/v). Following a one hour activation of plates at 110°C, lipids were spotted in the lower left hand corner. The plates were developed in the first dimension with chloroform/methanol/28% aqueous ammonia (65:25:5, v/v/v) and in the second dimension with chloroform/methanol/acetone/acetic acid/water (50:20:10:15:5, v/v/v/v/v). Lipids were visualized with iodine vapours and identified by cochromatography with lipid standards. Silica gel in areas corresponding to individual phospholipids was collected and counted as stated before.

To quantify the phospholipid composition, total lipid extracts from several

culture plates were separated by two dimensional thin layer chromatography. Spots were visualized by iodine vapours, and the silica gel therein was scraped and collected. Lipid phosphorus was determined by the method of Vaskovskey et al. (1975).

For the analysis of lipid composition before and after RA-induced differentiation, a more sensitive technique was also employed. This method consisted of labelling cells with [^{32}P]orthophosphate, (50 μCi per 10 ml medium) for three days which was sufficient time for complete label equilibration among the different phospholipid classes as shown in preliminary experiments. On day two of phosphorus incorporation, 16 hours prior to harvest of the cells, 1 μCi of [^{3}H]arachidonic acid was also added such that the incorporation of [^{32}P] and [^{3}H] could be monitored simultaneously. On day three, cells were harvested, lipids were extracted and separated by two-dimensional thin layer chromatography as described above. Spots corresponding to phospholipids were visualized by iodine vapours, and silica gel corresponding to individual phospholipids was scraped, and radioactivity measured.

2.4 RESULTS

A. PHOSPHOLIPID COMPOSITION OF NEUROBLASTOMA

The phospholipid composition of the two variant cell lines as determined by the phosphorus analysis and equilibrium [^{32}P] incorporation, is presented in Table 2.1. Phosphatidylcholine (PC) constituted the major fraction of membrane phospholipids contributing 57 to 63% of total lipid phosphorus. Phosphatidylethanolamine (PE)

TABLE 2.1

Phospholipid Composition of Neuroblastoma

Lipid Fraction	SH-F		SH-N	
	nmolP/mg Prot.	Percentage Distribution	nmolP/mg Prot.	Percentage Distribution
PE	25.0 (23.2)	22.0 ± 0.4	21.0 (22.9)	27.6 ± 0.6
PC	65.0 (60.4)	63.1 ± 1.1	54.0 (58.8)	57.0 ± 1.0
PI	7.8 (7.2)	10.2 ± 1.0	5.4 (5.9)	9.9 ± 1.0
PS	6.6 (6.1)	4.5 ± 0.1	5.2 (5.7)	5.0 ± 0.1
SPH	2.4 (2.2)	1.5 ± 0.1	2.2 (2.4)	1.9 ± 0.1
LYSO-PC	0.8 (0.7)	0.7 ± 0.3	4.0 (4.4)	0.7 ± 0.1

Values are means of 3 separate samples for SH-F, and 2 samples for SH-N (percentage shown in parenthesis). Variation was less than 10% for phosphoglycerides and within 30% for sphingomyelin (SPH), and lysophosphatidylcholine (LYSO-PC). Percentage distributions are calculated from equilibrium [^{32}P]orthophosphate incorporations, values are the average of 3 experiments, ± S.E.

made up the second largest fraction corresponding to 22 to 27% of the total. All other phospholipids, PI, PS, sphingomyelin (SPH), and lyso PC, contributed a total of 10% to 20%. No major differences in phospholipid composition were evident between the SH-F and SH-N cell lines.

B. FATTY ACID INCORPORATIONS

The incorporation of two fatty acids, [^{3}H]arachidonic acid and [^{3}H]oleic acid, was compared in both cell lines. The aim of these studies was to reveal: a) if arachidonic acid has an incorporation pattern distinct from oleic acid among the lipid classes; and b) if differences in this aspect of lipid metabolism are to be found when SH-F and SH-N are compared. The results of all incorporations are summarized in Tables 2.2 and 2.3, where they are shown as changes in distribution of label with time. There was little variation on these distributions from experiment to experiment, although the actual counts incorporated did vary substantially. Figures 2.1 to 2.4 illustrate the averages of data from two to three experiments in which incorporations were similar and are qualitatively representative of all incorporations obtained.

The results show definite differences in the manner of incorporation of oleic acid and arachidonic acid in cellular lipids (Table 2.2). In SH-F, after 24 hours of oleic acid incorporation, the following distribution of label was observed; 49% in PC, 10% in PE, 7% in PI, and 4.2% in PS. With time, the relative amount of labelling in triglyceride, which was substantial initially, decreased to approximately 22%. This change was accompanied by increases in PC and PE; however about 10% of the

TABLE 2.2

Percent Incorporation of Fatty Acids in Lipids of SH-F

Time (hr)	PS		PI		PC		PE		TG	
	OA	AA	OA	AA	OA	AA	OA	AA	OA	AA
1	2.0	3.30	9.6	26.1	44.7	37.8	5.1	13.3	41.5	12.8
2	2.2	3.20	3.9	24.5	46.6	35.3	5.8	18.6	42.7	9.8
4	2.2	3.4	6.0	29.0	42.4	31.3	6.1	18.6	40.0	10.6
8	2.7	3.80	5.7	26.7	47.1	28.7	6.7	22.1	39.4	9.1
12	3.1	3.10	6.7	26.4	48.4	29.2	7.6	27.9	29.5	8.2
24	4.2	3.00	7.0	27.2	49.4	23.3	10.6	34.1	22.2	6.8
	(± 0.07)	(± 0.02)	(± 0.11)	(± 0.53)	(± 5.5)	(± 0.65)	(± 1.4)	(± 0.71)	(± 0.50)	(± 0.35)

OA (oleic acid), n = 5, variation less than 20%. AA (arachidonic acid), n = 3, variation less than 15%.
Examples of the typical variations (± S.E.) are shown in parenthesis for 24 hrs.

counts were not recovered in these two fractions and probably represented loss to the medium or oxidation to water-soluble products. Arachidonic acid, showed a distinctly different pattern of incorporation. By 12 hours, the label of this fatty acid was distributed about evenly in PC, PE and PI. After 24 hours, the PC fraction contained 23%, of the label, while PE contained 34%, and PI, 27%. The percentage incorporation of arachidonic acid in PS was similar to that of oleic acid. Quite interestingly, the proportion in PE increased with time to exceed the radioactivity recovered in PC and triglyceride, a phenomenon which was not seen with oleic acid. Furthermore, the counts recovered in triglyceride in the case of arachidonic acid, were modest compared to those of oleic acid. The percentage incorporation of oleic acid and arachidonic acid within the phospholipid pools of SH-N were similar to those determined for SH-F (Table 2.3). Also the redistributions of label with time, within the different lipid classes and typifying each labelled fatty acid used, were the same in this cell line as in the other cell line. In both SH-F and SH-N, free fatty acid accounted for 1% or less of the counts with either oleic acid or arachidonic acid as precursors, and diglyceride for 2 % or less in the former case and under 1% in the latter. Recoveries of labelled oleic acid and arachidonic acid in sphingomyelin were 0.4% and 2.0% respectively, and 0.7% and 2.0% respectively, in lyso-PC. After one hour of incubation recovery of counts in the main lipids was approximately 82%. In this case the remainder of radioactivity was found mainly in uncharacterized neutral lipid. This phenomenon was not seen at later times.

TABLE 2.3

Percent Incorporation of Fatty Acids in Lipids of SH-N

Time (hr)	PS		PI		PC		PE		TG	
	OA	AA	OA	AA	OA	AA	OA	AA	OA	AA
1	2.10	3.81	6.00	22.0	32.0	33.0	6.60	8.6	34.6	14.0
2	2.10	4.52	5.60	23.0	33.0	37.0	6.00	12.2	43.2	16.0
4	2.10	3.70	5.30	23.0	35.0	34.0	6.20	16.2	48.0	16.0
8	2.60	2.82	5.40	26.0	38.5	29.0	6.70	20.0	43.3	15.7
12	2.80	3.70	5.20	25.0	38.3	26.0	7.40	25.0	35.8	14.5
24	4.15	2.45	5.60	25.0	42.0	23.0	9.60	33.0	34.5	12.5
		(± 0.40)		(± 1.5)		(± 0.1)		(± 1.0)		(± 0.7)

OA, n = 1, AA, n = 2 with variatic · less than 10% except for PS where variation was within 25%.
Examples of typical variations (± range) are shown in parenthesis for 24 hrs.

Table 2.4 shows the specific activities for fatty acids incorporated in phospholipids of SH-N and SH-F following a 24 hour incubation. These values, measured in the different phospholipid classes, show that arachidonic acid incorporation is highly selective in both cell lines. The specific activity of PI is about ten-fold higher than that of PC, while that of PE is three- to four-fold greater than PC. When these incorporations were compared to those measured for oleic acid, distinct differences were noticed. Although, the specific activities of phospholipids labelled with oleic acid were found to be more uniform in comparison to those observed for arachidonic acid, definite trends were also apparent. In both SH-F and SH-N, the specific activities were approximately two-fold greater in PC than those measured for PE. As PC makes up greater than 50 % of total phospholipids the largest percentage of total radioactivity in cells labelled with [^{3}H]oleic acid is recovered in this fraction. Again, in both cell lines, the lowest specific activities were measured in PE.

The rates of uptake of [^{3}H]fatty acids in SH-F cells are seen in Figures 2.1 and 2.2. The same data are expressed as changes in specific activity in Figures 2.3 and 2.4. From the total uptake values it is seen that incorporation of labelled arachidonic acid proceeds more rapidly than that of [^{3}H]oleic acid. Accordingly, 60% of the total arachidonic acid uptake had taken place by two hours whereas only 14% of the total oleic acid was incorporated in this time.

As seen in Figure 2.1, incorporation of [^{3}H]oleic acid in triglycerides was extensive and maximal by 12 hours, whereas that in phospholipids continued to

TABLE 2.4

Specific Activity of Fatty Acid Incorporation

in Phospholipids after 24 hrs

Lipid Fraction	SH-F dpm/nmol		SH-N dpm/nmol	
	OA	AA	OA	AA
PE	1,138 ± 90	4,860 ± 174	1,640 ± 10	5,716 ± 43
PC	2,069 ± 221	1,290 ± 58	2,953 ± 230	1,551 ± 42
PI	1,333 ± 203	12,254 ± 393	3,672 ± 33	16,981 ± 800
PS	1,451 ± 74	1,449 ± 12	2,659 ± 180	1,621 ± 465

Values represent incorporation (dpm) per nmol phosphorus ± S.E., after a 24 hour incorporation for SH-F, OA, n = 2 ± range; AA, n = 3.
For SH-N, AA and OA, n = 2, with variation shown as range.

Figure 2.1 - [^{3}H]Oleic acid incorporation in lipid fractions in SH-F cells.

Cells were incubated in 1 μCi [^{3}H]Oleic acid, final concentration 1 μM, for 24 hours. At indicated time points cells were harvested and lipids were analyzed as described in the Methods. Points represent the average recovery $\pm$ range, of 2 separate experiments.

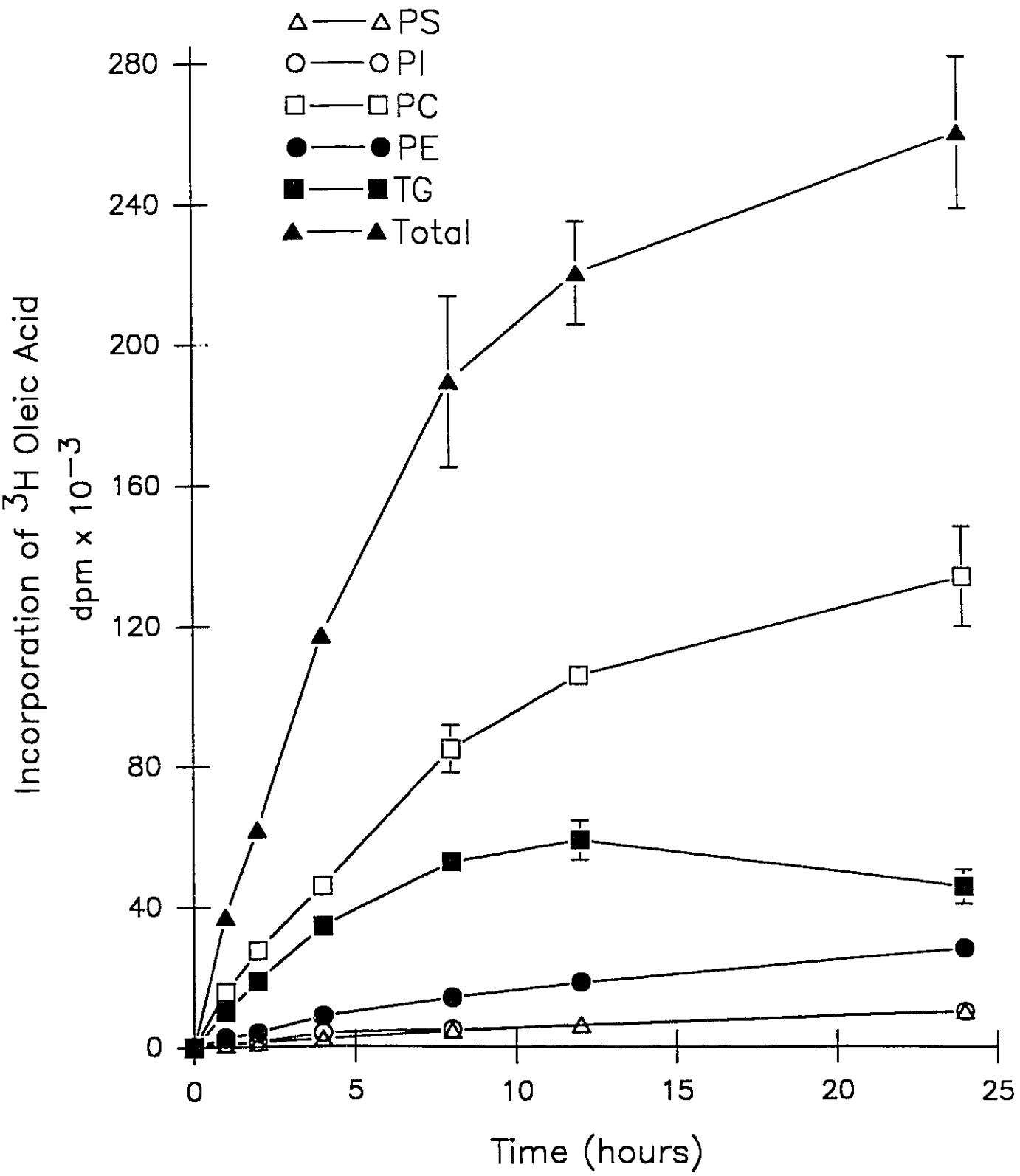

PS
PI
PC
PE
TG
Total
280
240
200
160
120
80
40
0
0
5
10
15
20
25
Incorporation of ^{3}H Oleic Acid
dpm x 10^{-3}
Time (hours)

Figure 2.2 [^{3}H]Arachidonic acid incorporation in lipid fractions in SH-F cells.

Cells were incubated in 1 μCi [^{3}H]arachidonic acid, final concentration 1 μM, for 24 hours. Values represent average recovery $\pm$ S.E of 3 experiments.

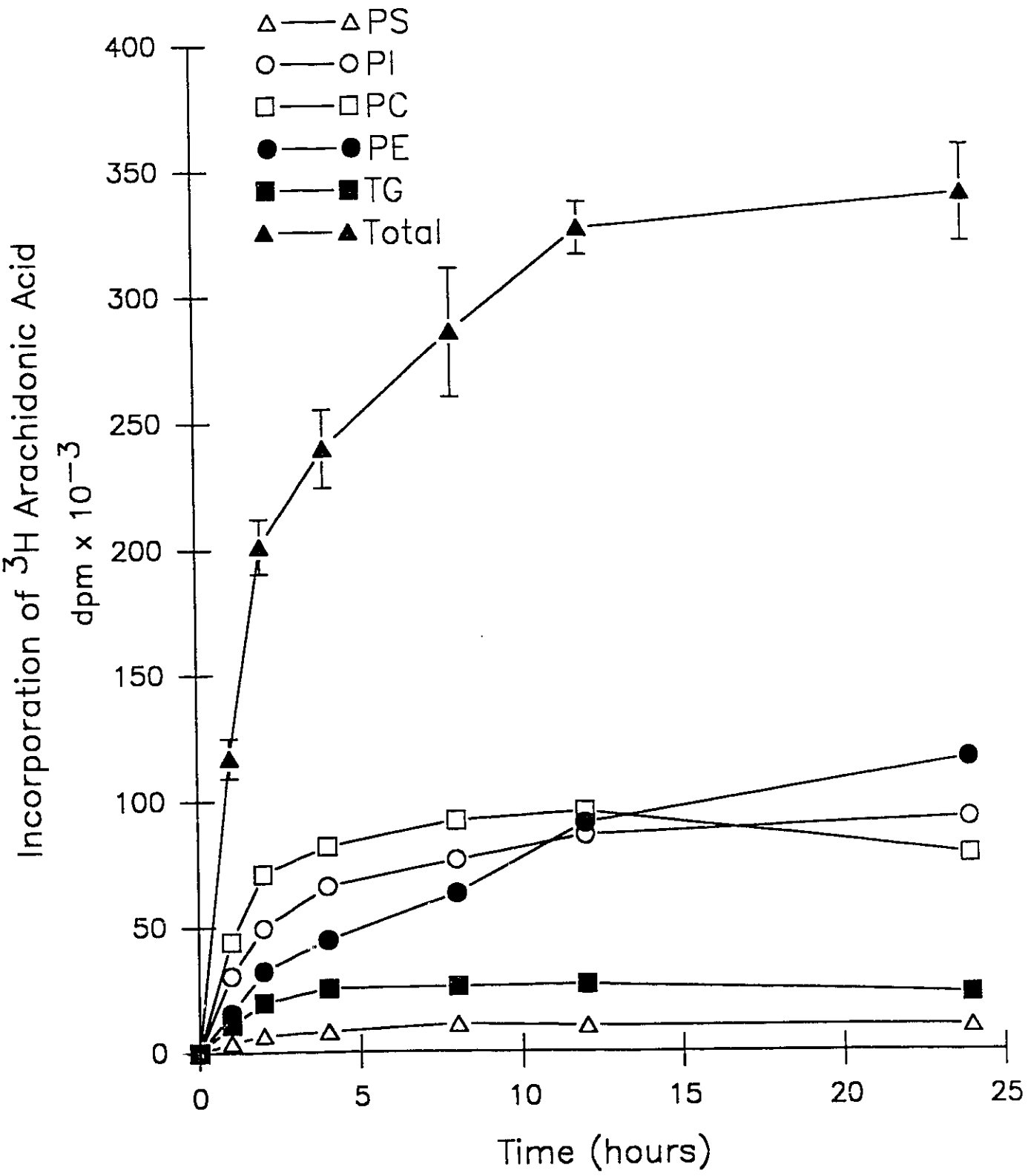

PS
PI
PC
PE
TG
Total
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
Time (hours)
Incorporation of ^{3}H Arachidonic Acid
dpm x 10^{-3}

Figure 2.3 - [^{3}H]Oleic acid incorporation in lipid fractions in SH-F cells.

Cells were incubated in 1 μCi [^{3}H]Oleic acid, final concentration 1 μM, for 24 hours. At indicated time points cells were harvested and lipids were analyzed as described in the Methods. Points represent the average specific acitivity (DPM/nmol phosphorus $\pm$ range, of 2 experiments.

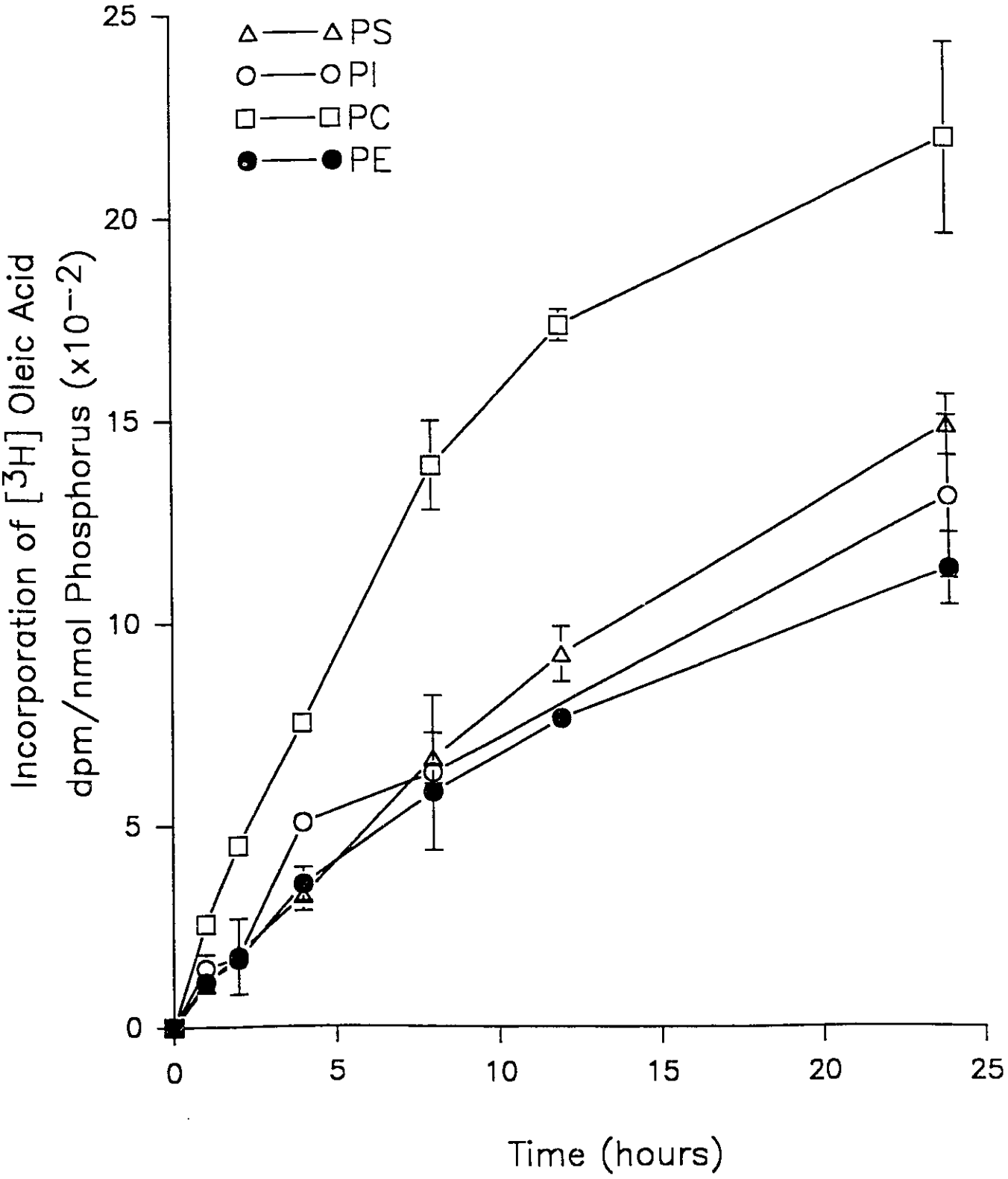
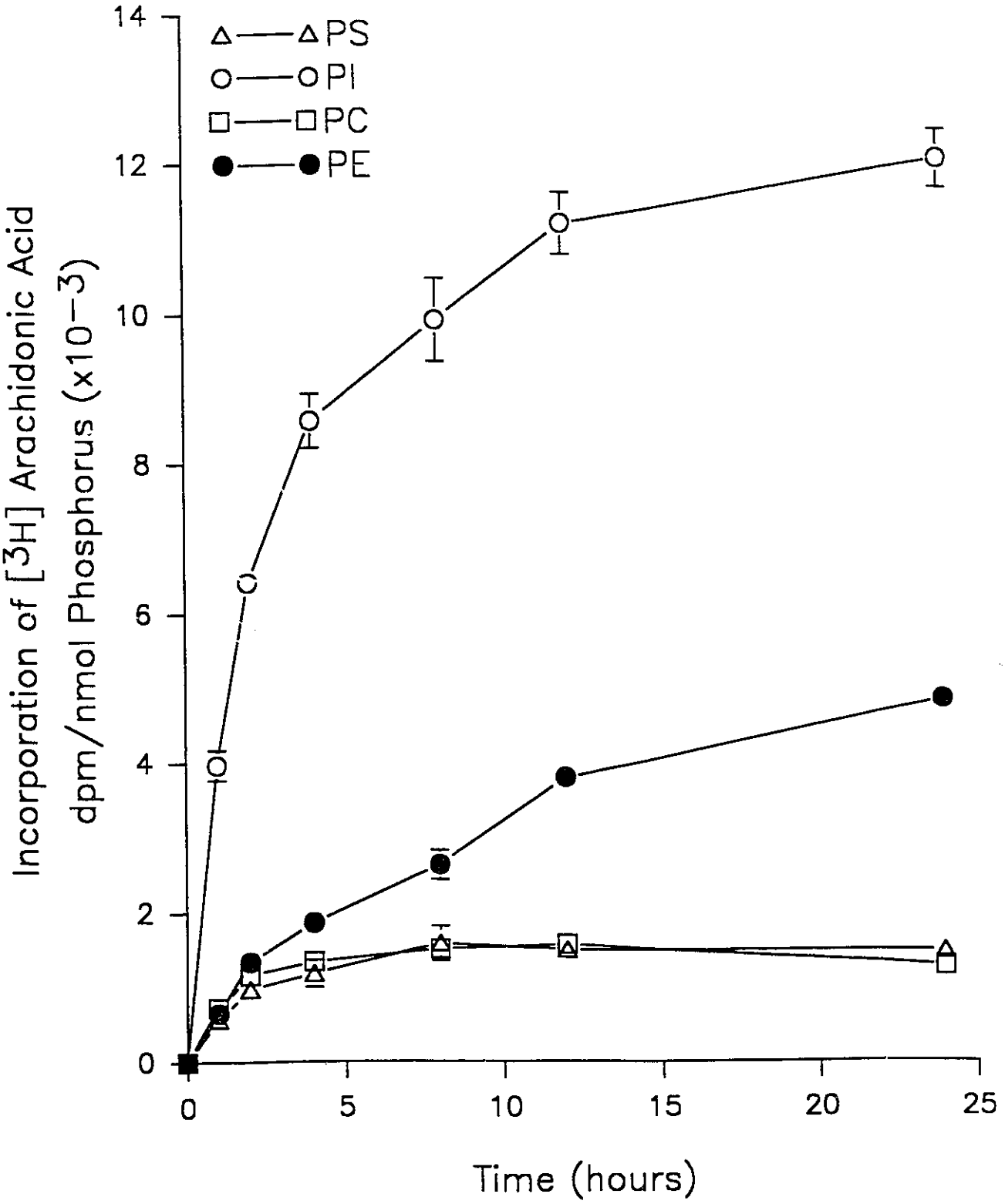

PS
PI
PC
PE
Incorporation of [3H] Oleic Acid
dpm/nmol Phosphorus (x10−2)
Time (hours)
0
5
10
15
20
25

Figure 2.4 [^{3}H]Arachidonic acid incorporation in lipid fractions in SH-F cells.

Cells were incubated in 1 μCi [^{3}H]arachidonic acid, final concentration 1 μM, for 24 hours. At indicated time points cells were harvested and lipid analyzed as described in the Methods. Values represent average specific activities (DPM/nmol phosphorus) $\pm$ S.E. of 3 experiments.


PS
PI
PC
PE
Incorporation of [3H] Arachidonic Acid
dpm/nmol Phosphorus (x10−3)
Time (hours)
0
2
4
6
8
10
12
14
0
5
10
15
20
25

increase beyond this time, albeit at a slower rate. The rate of uptake of this fatty acid was greatest in PC and after 24 hours, the radioactivity distribution in the different phospholipid classes broadly reflected their pool sizes. Nonetheless, labelling of PC, as incorporations neared equilibrium, was proportionally greater than its pool size. This can be further appreciated from Figure 2.3 in which the PC fraction displays the highest specific activity throughout the entire course of the experiment. It is apparent from these results that, not only is PC labelled more rapidly but to a greater extent. There is consequently some degree of selection in the incorporation pattern which favors the choline phosphatide. This selection could involve a de novo synthesis process or could be indicative of a larger proportion of acyl groups in the PC fracton capable of exchange with exogenously supplied [^{3}H]oleic acid.

Incorporation of [^{3}H]arachidonic acid appeared to be even more selective than, and different from that obtained with oleic acid (Figure 2.2). The extent and rate of incorporation into triglyceride was comparatively very low. Although initially the rate of incorporation into PC appeared greatest, it was also very high in PI. After 24 hours, near equilibrium values indicated a higher labelling in the PE and PI fractions than in PC. If indeed the incorporation of the labelled fatty acid proceeds via an acyl exchange mechanism, results in Figure 2.4 indicate that relative to the total acyl pool of each phospholipid, a greater proportion of the acyl chains were exchanged for labelled arachidonic acid in the PI and PE fractions compared to PC and PS. If a de novo synthesis is involved, again a selective process favoring

enrichment of the PI and PE fractions with arachidonic acid would have to be invoked.

Incorporation of fatty acids in SH-N gave rate patterns similar to those obtained for SH-F (data not shown), in which arachidonic acid incorporation in lipid classes was again, selective and favored PI and PE (Table 2.3).

C. TURNOVER AND REDISTRIBUTION OF [^{3}H]ARACHIDONIC ACID LABEL

The results for the arachidonic acid chase in SH-F are presented in Figure 2.5. A steady decrease in labelling of phospholipids is evident concomitant with an increase in labelling of triglyceride. The rates of turnover of PE and PC were approximately equal and both were exceeded by the rate of fall in PI. No significant turnover occurred in PS. Results summarized in Figure 2.6 indicate that relative to the lipid phosphorus (and therefore to total acyl groups) in each phospholipid fraction, PI is losing its label 5.4 times as fast as PE which in turn is losing its label twice as fast as PC.

Pulse chase experiments with SH-N were carried out under precisely the same conditions as those conducted with SH-F, however, certain differences were evident. (Figure 2.7) The loss of arachidonic acid from the phospholipid classes in SH-N cells was not accompanied by a corresponding increase in triglyceride levels. Triglyceride levels decreased slightly in the first 6 hours. As in SH-F, the relative rates of label loss were slowest in PC and PS fractions and most rapid in PI and PE (Figure 2.8).

Figure 2.5 - Arachidonic acid redistribution in SH-F cells.

Cells were pulsed with 1 μCi [^{3}H]arachidonic acid, final concentration 1 μM, for 16 hours. Samples were washed and then chased with medium containing 100 μM unlabelled arachidonic acid. Samples were harvested and lipids analyzed at time points indicated. Values represent mean recoveries $\pm$ S.E. (n=12).

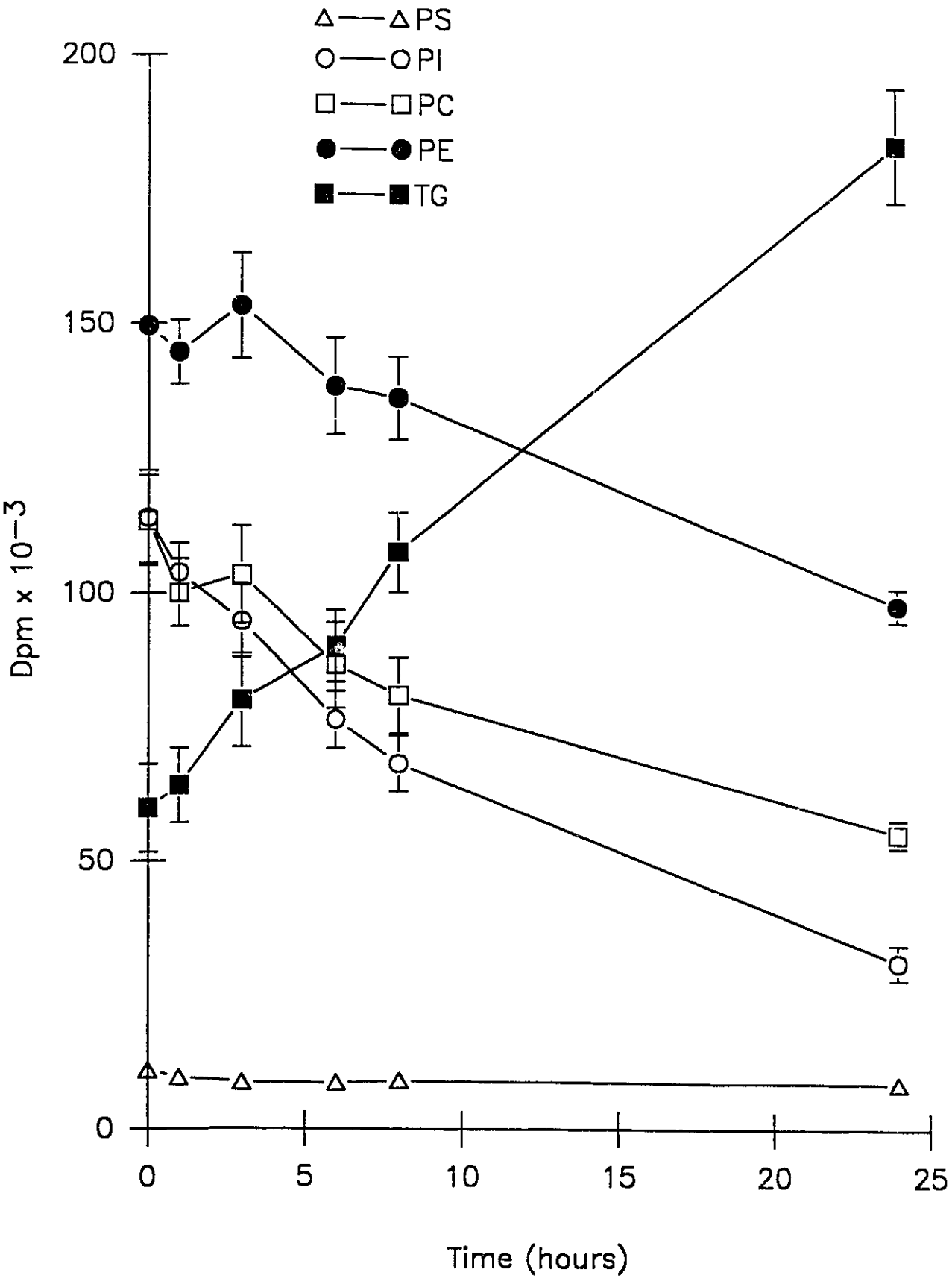

PS
PI
PC
PE
TG
200
150
100
50
0
Dpm x 10⁻³
0
5
10
15
20
25
Time (hours)

Figure 2.6 - Arachidonic acid redistribution in SH-F cells.

Cells were pulsed with 1 μCi [^{3}H]arachidonic acid, final concentration 1μM, for 16 hours. Samples were washed and then chased medium containing 100 μM unlabelled arachidonic acid. Samples were harvested and lipids analyzed at time points indicated. Values represent mean specific acitivity (DPM/nmol phosphorus) $\pm$ S.E. (n = 12).

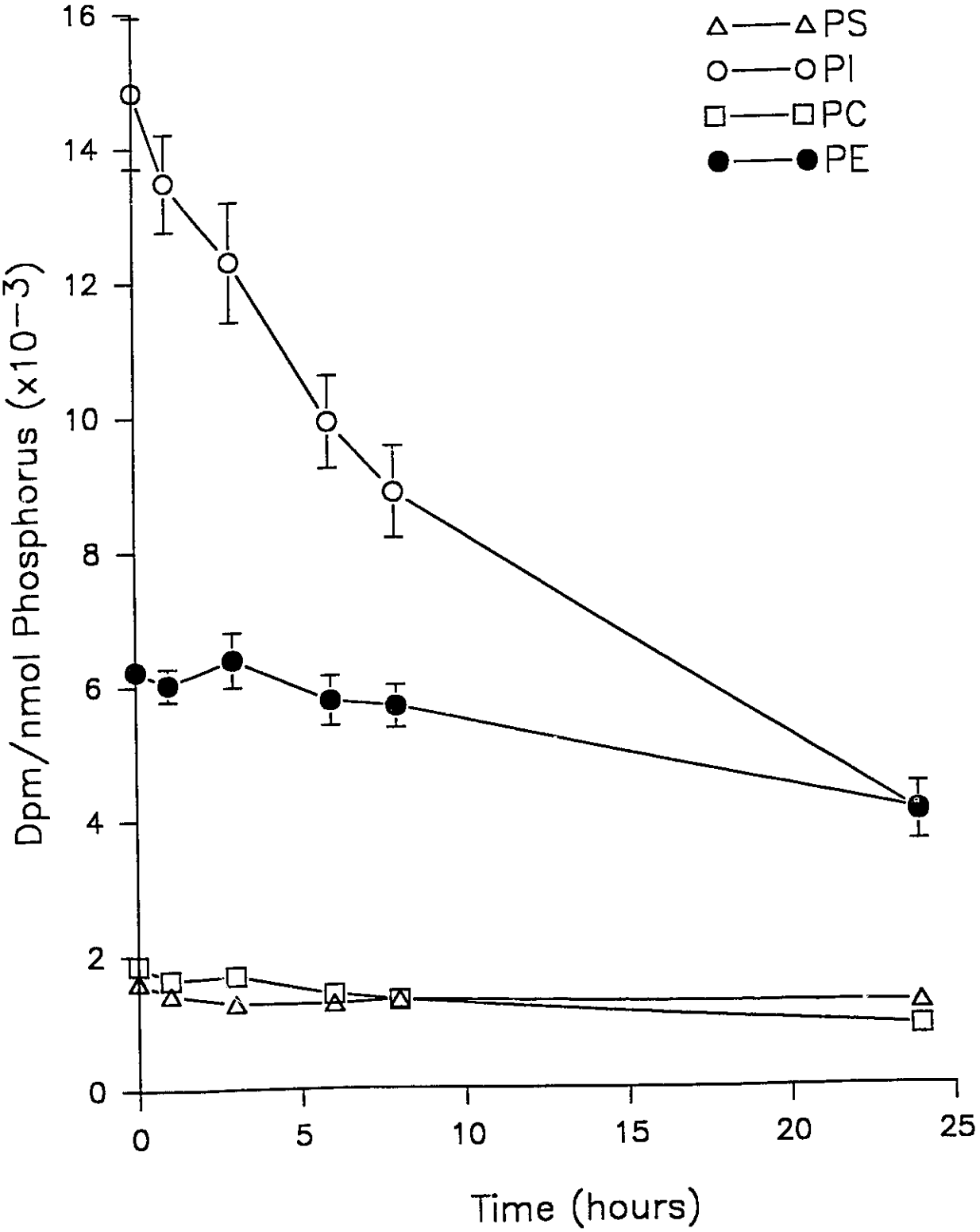

PS
PI
PC
PE
Dpm/nmol Phosphorus (x10−3)
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
Time (hours)

Figure 2.7 - Arachidonic acid redistribution in SH-N cells.

Experiments were conducted as described for SH-F. Cells were harvested and lipids analyzed at time points indicated. Values represent mean recoveries ± S.E. (n=6).

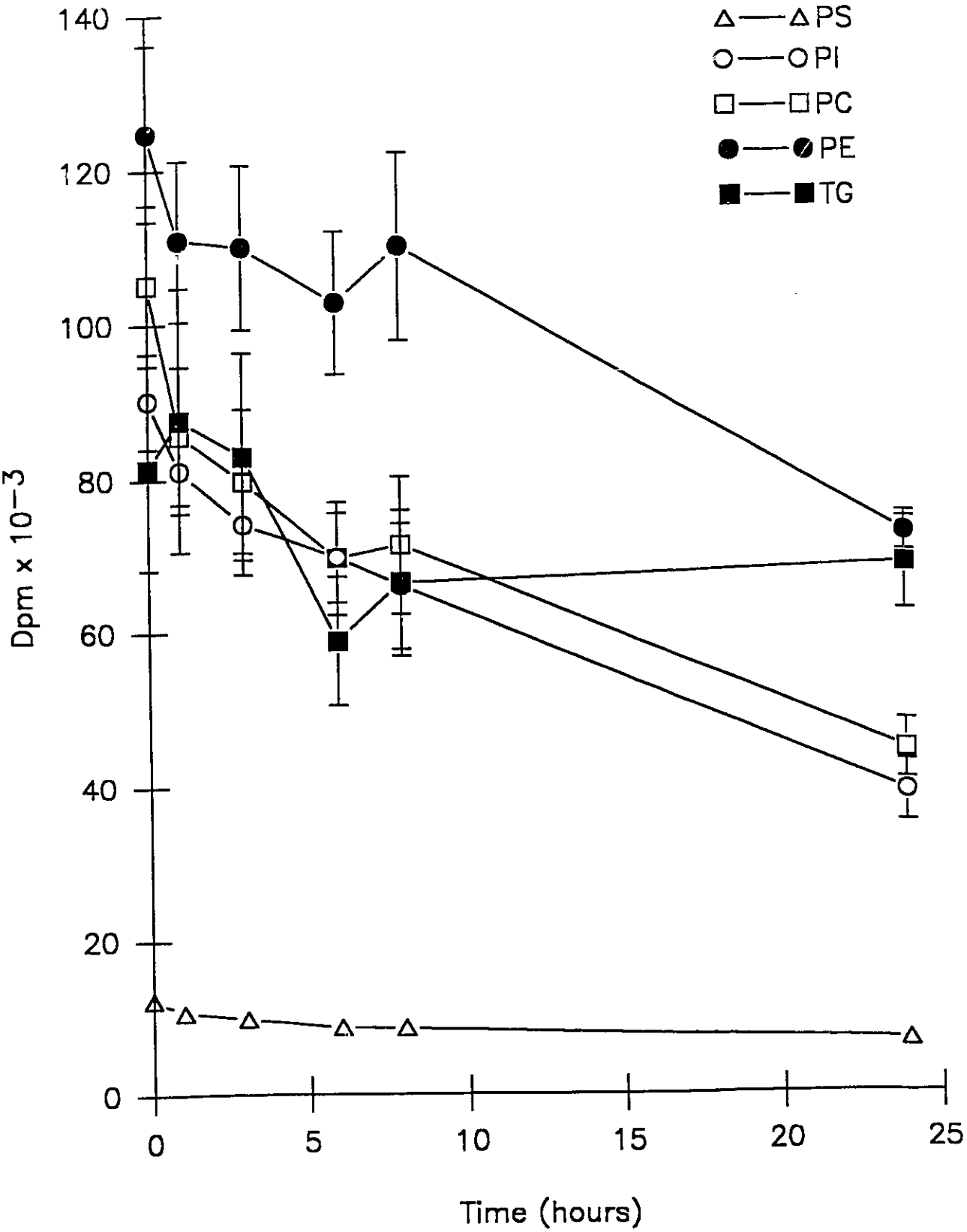
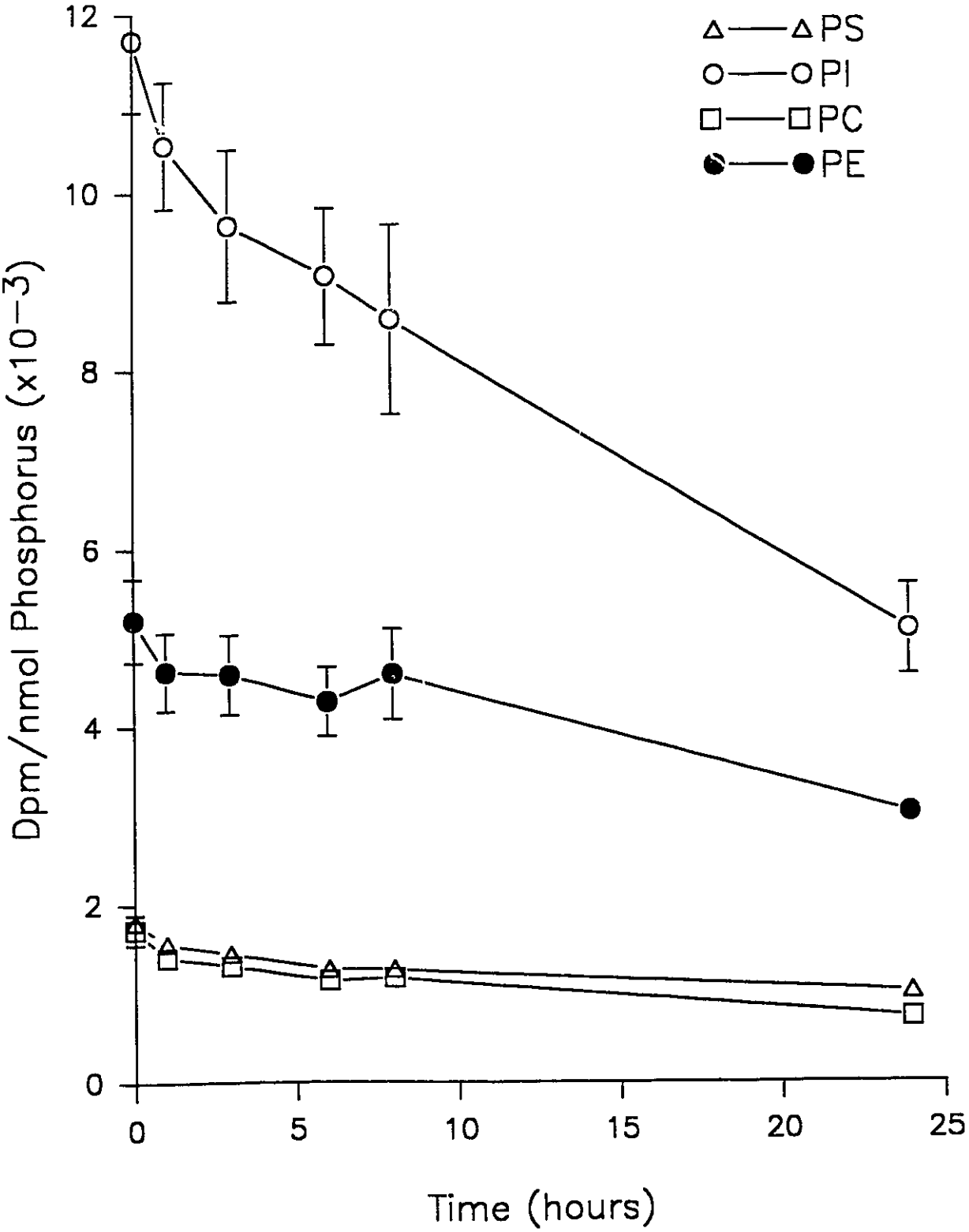

PS
PI
PC
PE
TG
140
120
100
80
60
40
20
0
Dpm x 10−3
0
5
10
15
20
25
Time (hours)

Figure 2.8 - Arachidonic acid redistribution in SH-N cells.

Experiments were conducted as described for SH-F. Cells were harvested and lipids analyzed at time points indicated. Values represent mean specific activity (DPM/nmol phosphorus) $\pm$ S.E. (n=6).


PS
PI
PC
PE
Dpm/nmol Phosphorus (x10−3)
Time (hours)
0
2
4
6
8
10
12
0
5
10
15
20
25

In summary, although the results for the arachidonic acid chase for SH-F and SH-N differed markedly with respect to the redistribution of label into triglyceride, the rates of loss of labelled fatty acid from phospholipids were very similar. Relative to the pool sizes, arachidonate was lost most rapidly from PI, followed by PE, and was lost most slowly from PS and PC. These chase studies are a direct reflection of results obtained for incorporations (Figures 2.3 and 2.4), where, relative to pool size, PI incorporated the label at the highest rate, followed by PE, leaving PC and PS taking up this fatty acid at the slowest rates.

D. EFFECTS OF RETINOIC ACID ON LIPID METABOLISM AND TURNOVER

Once the pattern of arachidonic acid turnover and redistribution was established in untreated cells, the direct effects of RA on membrane lipid turnover were examined. Previous studies had shown that short-term treatment of neuroblastoma with RA resulted in a marked decrease in phosphoinositide turnover (Ponzoni and Lanciotti, 1990). Accordingly, such direct effects of RA were therefore followed up with the SH-F cell line. To measure phosphoinositide turnover, cells were prelabelled for 16 hours with [^{3}H]arachidonic acid. This label was chosen as it permitted assessment of the turnover of the diglyceride moiety of PI as well as that of the entire lipid itself. It also allowed some evaluation of the turnover of position two of this lipid which is often affected by agonist interactions with the membrane. Parallel studies with [^{3}H]myoinositol label were being concurrently performed by another member of our laboratory (Proulx, P. unpublished observations). Turnover

was measured under chase conditions, in which radioactivity recovered in the diglyceride and phosphatidylinositol fractions were measured at time points of 0, 1.0, 2.5, 4.5, 6.0 and 8.0 hours. The results presented in Table 2.5 show that RA had no significant effects on turnover of phosphoinositides, as no differences in the distribution of radioactivity in PI or diglyceride were observed in comparison to controls. A similar study was undertaken to further pursue the possibility of a direct effect of RA on inositol phospholipid turnover, however, shorter time points, similar to those used by Ponzoni and Lanciotti (1990) were used. When label redistribution was determined at 0 time, 15 seconds, 30 seconds, 1 minute, 5 minutes and 10 minutes, no differences were found in phosphoinositide turnover due to the presence of RA (Table 2.6). It should be emphasised that although the values reported in Tables 2.5 and 2.6 highlight only two lipids of particular interest in signal transduction metabolism, none of the other lipids examined showed significant differences in rate of turnover as a result of RA treatment. The results of these experiments, therefore, do not support the premise, that alterations of phosphoinositide turnover are involved in the mediation of the effect of RA on neuroblastoma differentiation.

As negative results were obtained with respect to the direct effect of RA on PI turnover, the possibility that RA may exert some early influence on membrane lipid metabolism was explored in short-term incorporation studies. The incorporation of [^{3}H]arachidonic acid and [^{3}H]oleic acid in the various lipid classes was compared in cells with and without RA in their culture media. When RA was added simultaneously with the label at time 0, no differences in label distribution in

TABLE 2.5

Effect of Short-Term Retinoic Acid

Treatment on Phosphoinositide Turnover in the SH-F Cell Line

Time (hr)	Diglyceride		PI	
	CTL	RA	CTL	RA
0	1.3 ± 0.10	1.3 ± 0.10	27.9 ± 0.1	27.9 ± 0.1
1.0	0.9 ± 0.03	1.1 ± 0.05	29.6 ± 0.2	29.5 ± 0.3
2.5	1.6 ± 0.08	1.6 ± 0.06	25.1 ± 0.1	24.9 ± 0.3
4.5	1.5 ± 0.07	1.5 ± 0.02	23.2 ± 1.0	22.7 ± 0.2
6.0	1.5 ± 0.02	1.4 ± 0.09	14.0 ± 3.0	14.9 ± 2.6
8.0	1.3 ± 0.04	1.0 ± 0.02	16.3 ± 4.0	19.0 ± 2.4

Values represent percentage distribution in lipid fraction, ± range, n = 2.

TABLE 2.6

Effect of Short-Term Retinoic Acid

Treatment on Phosphoinositide Turnover in the SH-F Cells

Time	Diglyceride		PI	
	CTL	RA	CTL	RA
0	0.63 ± 0.04	0.65 ± 0.05	23.0 ± 0.4	24.9 ± 0.1
15 sec	0.72 ± 0.10	0.55 ± 0.10	22.7 ± 0.4	20.8 ± 1.8
30 sec	0.61 ± 0.09	0.55 ± 0.23	18.8 ± 2.2	22.6 ± 1.8
1 min	0.76 ± 0.09	0.82 ± 0.01	19.2 ± 0.1	20.9 ± 1.3
5 min	0.72 ± 0.04	0.75 ± 0.06	20.4 ± 0.5	20.3 ± 1.4
10 min	0.64 ± 0.01	0.76 ± 0.09	20.1 ± 1.2	21.2 ± 0.5

Values represent percentage distribution in lipid fraction, ± range, n = 2.

phosphoglycerides or neutral lipids were observed due to short-term treatment (Table 2.7). The results of these studies, therefore, do not support a role for changes in membrane lipid metabolism/turnover in the mediation of the early effects of RA on cell differentiation.

Long term RA treatment (six days), however, was found to result in significant effects on arachidonic acid turnover. The pattern of labelling measured after 16 hours of incorporation, revealed distinct differences between control and RA-treated cells. This effect was most pronounced in SH-F cells (Figure 2.9). In untreated cells, PE received the largest proportion of the phospholipid label, which was approximately 36%. After differentiation induced by RA, most of the [^{3}H]arachidonic acid was found in PC, which received approximately 42% of the label. RA treatment therefore, resulted in a significant 14% and 26% decrease in labelling of PE and PI respectively, with a corresponding 58% increase in the levels recovered in PC. No differences were seen in the [^{3}H]arachidonic acid incorporation in PS after RA treatment.

A similar trend was seen with SH-N although the effect was less apparent (Figure 2.10). There were no differences between PE and PS incorporations, however, again, significant changes were evident with PC and PI. A 30% decrease in PI was accompanied by a 23% increase in PC. After a 16 hour [^{3}H]arachidonic acid incorporation therefore, the distribution pattern in both SH-F and SH-N, is markedly different following RA induced differentiation.

TABLE 2.7

Effect of Short Term Retinoic Acid Treatment on Fatty Acid Incorporation in SH-F Cells

[^{3}H] Arachidonic Acid												
Time (hr)	PC		PE		PI		DG		FA		TG	
	CTL	RA	CTL	RA	CTL	RA	CTL	RA	CTL	RA	CTL	RA
1	23	23	35	34	29	31	1.0	1.0	0.2	0.2	4.6	4.6
2	23	22	35	36	32	31	0.9	0.9	0.3	0.2	4.2	4.0
4	22	22	36	38	32	31	0.9	0.9	0.3	0.2	4.0	3.6
8	21	21	38	38	31	31	0.8	0.8	0.2	0.2	3.3	3.7
24	18	18	41	41	32	32	0.9	0.8	0.1	0.1	3.4	3.5
[^{3}H] Oleic Acid												
Time	PC		PE		PI		DG		FA		TG	
	CTL	RA	CTL	RA	CTL	RA	CTL	RA	CTL	RA	CTL	RA
2	45	--	5.6	7.5	0.6	0.6	4.8	4.9	1.3	1.0	41	38
4	39	38	6.6	6.7	2.0	1.4	2.8	2.9	0.9	0.8	49	51
8	44	40	6.5	6.5	1.7	1.8	2.5	2.0	1.0	0.8	48	48
12	43	42	7.2	7.2	2.6	2.9	2.1	1.8	1.0	0.6	44	46
24	44	47	8.9	9.2	3.9	3.9	1.3	1.3	1.0	0.8	40	37

Retinoic Acid was added in the RA-treated group at time 0. Lipids were extracted and analysed at the given time points.
AA, (n = 2) and 0A (n = 1), variation was less than 10%. Values represent percent of total radioactivity found in each fraction.

Figure 2.9 - Comparison of [^{3}H]Arachidonic acid and [^{32}P]orthophosphate incorporation in Retinoic acid-treated and control, SH-F cells.

Cells exposed to RA for 6 days were compared to solvent-treated controls. Samples were incubated with 1 μCi [^{3}H]arachidonic acid, final concentration 1 μM. Incorporation was allowed to proceed for 16 hours at which time cells were harvested and lipids analyzed as described in the Methods. In 3 samples, [^{32}P]orthophosphate and [^{3}H]arachidonic acid were added to the same dishes, 72 and 16 hours prior to harvest (respectively), such that both isotopes could be followed simultaneously. For [^{3}H]arachidonic acid incorporation n =11, for [^{32}P]orthophosphate incorporation n = 3. Error bars represent S.D. Columns labelled with asterisks indicate statistically significant differences based on two- tailed paired t analysis, $p < 0.01$. Statistical analysis was not made with phosphorus incorporation.

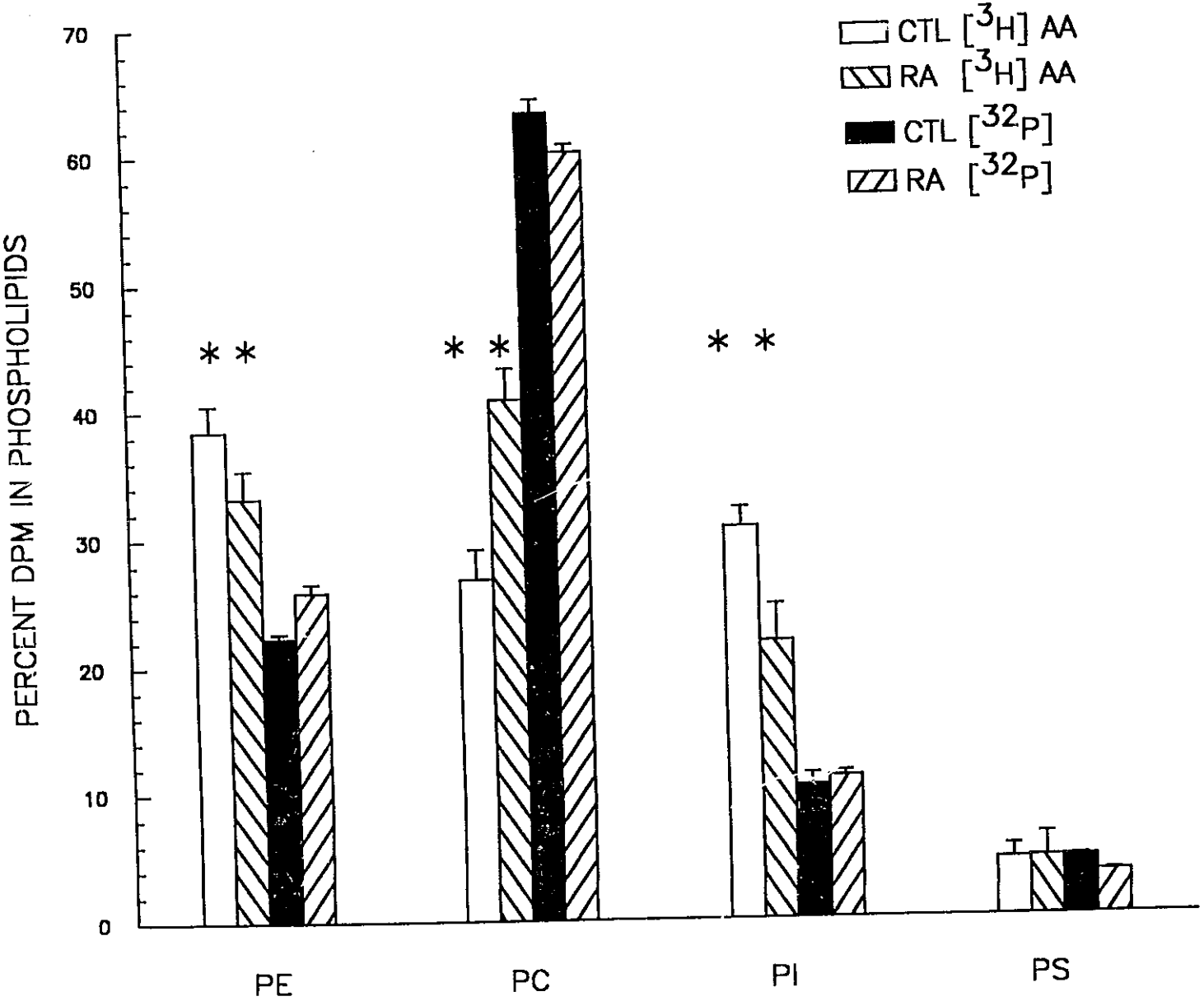
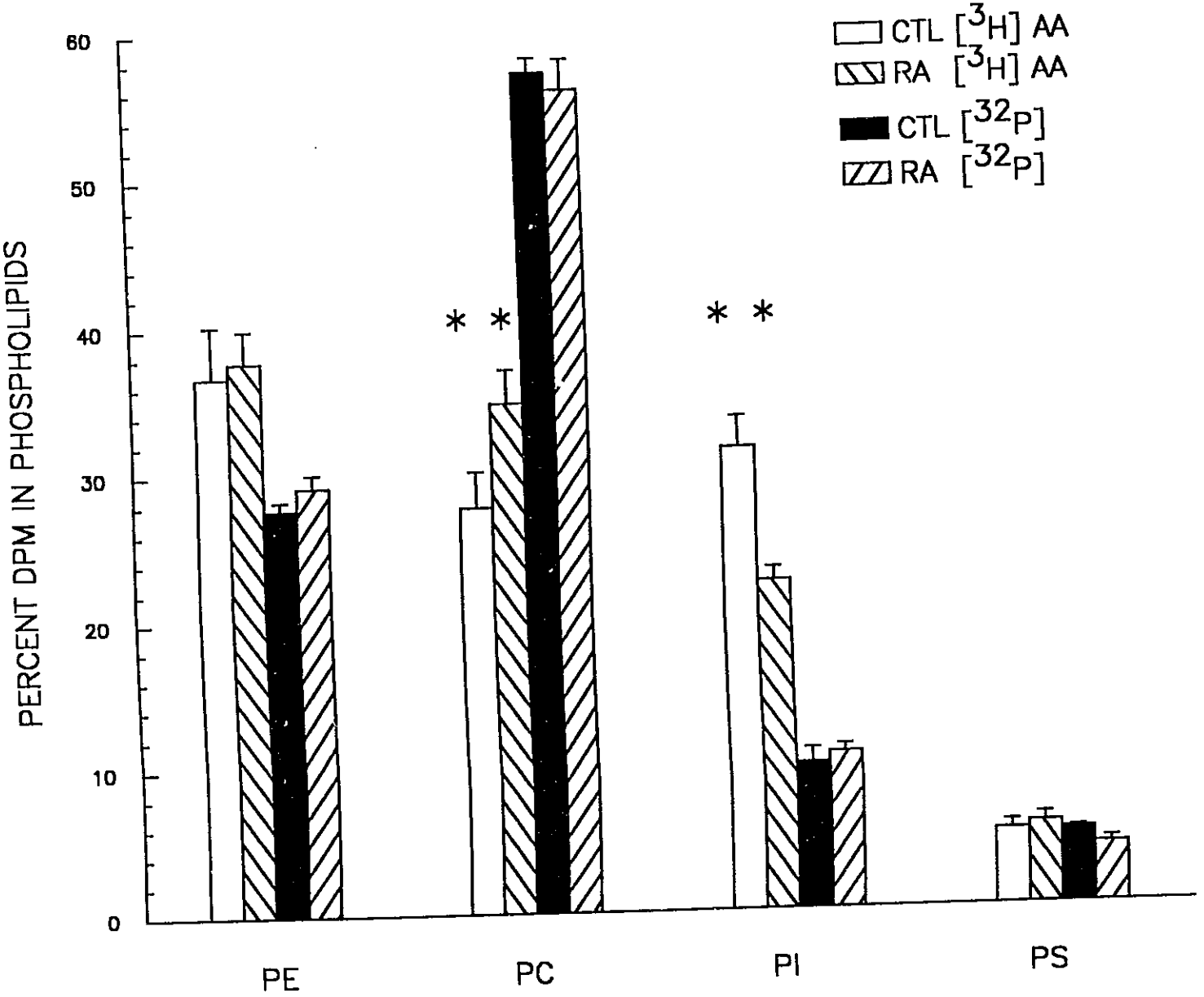

CTL [3H] AA
RA [3H] AA
CTL [32P]
RA [32P]
PERCENT DPM IN PHOSPHOLIPIDS
70
60
50
40
30
20
10
0
* *
* *
* *
PE
PC
PI
PS

Figure 2.10 - Comparison of [^{3}H]Arachidonic acid and [^{32}P]orthophosphate incorporation in retinoic acid-treated and control,SH-N cells.

Experimental conditions were as described for SH-F. For [^{3}H]arachidonic acid incorporation, n = 9; for [^{32}P]orthophosphate incorporation, n = 3. Error bars represent S.D. Bars labelled with asterisks indicate statistically significant differences based on two- tailed paired t analysis, $p < 0.01$. No statistical analysis was made with phosphorus incorporation.


CTL [3H] AA
RA [3H] AA
CTL [32P]
RA [32P]
PERCENT DPM IN PHOSPHOLIPIDS
60
50
40
30
20
10
0
* *
* *
PE
PC
PI
PS

A 16 hour arachidonic acid incorporation study was also carried out with cells previously labelled to equilibrium with [^{32}P]orthophosphate to enable estimation of relative phospholipid levels before and after differentiation. The results of these studies, included in Figures 2.9 and 2.10, show that no major differences in phospholipid composition are evident following RA-induced differentiation. These data indicate, for example, that the lower incorporation of arachidonic acid in PI is not due to a decreased level of this phospholipid in the membrane as phosphorus levels remain the same. Similarly, the increased fatty acid incorporation observed in PC following RA treatment is not likely a result of enhanced de novo synthesis of this phospholipid, as phosphorus levels incorporated in this phospholipid class remain the same before and after differentiation. The various changes in arachidonic acid incorporation in the phospholipid fractions must therefore, be due to alterations in the metabolism of fatty acyl chains in the lipid classes.

The effects of long-term treatment with RA on the redistribution of the [^{3}H]arachidonic acid label in SH-F and SH-N are reported in Table 2.8. Recovery of counts was less than 1.0 % in the diglyceride fraction, and less than 0.5 % in the free fatty acid fraction, and were therefore not shown. Significant differences in turnover rates were found in the PC and PS fractions in both SH-N and SH-F. After 24 hours in untreated cells, PC retained 62.9% of its [^{3}H]arachidonic acid, whereas in RA-treated cells, PC retained only 39.4% of label incorporated at time 0. It appears therefore, that the incorporation and release of arachidonic acid from PC is enhanced following RA treatment in both cell lines. Similarly, in control cells PS

TABLE 2.8
Effects of Long Term Retinoic Acid Treatment on Arachidonic Acid Redistribution in SH-F and SH-N

Time (hrs)		SH-F		SH-N	
		CTL	RA	CTL	RA
PE	1	97.0 ± 6.0	97.6 ± 4.4	90.0 ± 8.1	99.3 ± 8.8
	3	101.0 ± 11	106.0 ± 5.3	94.0 ± 12	93.3 ± 13
	6	88.0 ± 10	90.1 ± 6.3	91.0 ± 11	102.0 ± 15
	8	96.0 ± 10	101.0 ± 7.0	91.0 ± 11	94.7 ± 17
	24	75.0 ± 8.0	84.8 ± 12.0	74.0 ± 9.0	76.0 ± 14
PC	1	91.5 ± 4.3	83.1 ± 17	85.0 ± 7.0	88.8 ± 9.1
	3	83.3 ± 18	86.3 ± 7.7	84.5 ± 19	73.0 ± 14
	6	68.6 ± 7.8	69.5 ± 7.9	73.2 ± 11	72.2 ± 18
	8	73.9 ± 13	65.8 ± 6.8	64.3 ± 12	63.3 ± 14
	24	62.9 ± 10	**39.4 ± 7.6	53.7 ± 8.4	**36.5 ± 7.5
PI	1	92.6 ± 6.3	91.8 ± 15	86.6 ± 3.1	85.5 ± 17
	3	83.8 ± 10	94.4 ± 14	80.0 ± 10	83.2 ± 13
	6	62.1 ± 6.7	82.6 ± 14	74.2 ± 12	83.3 ± 6.2
	8	61.9 ± 12	72.3 ± 8.7	67.2 ± 17	76.2 ± 12
	24	41.6 ± 10	42.6 ± 2.8	44.2 ± 8.4	39.5 ± 10
PS	1	101.0 ± 3.8	89.4 ± 18	88.0 ± 5.4	86.3 ± 11
	3	91.7 ± 11	84.4 ± 12	84.2 ± 16	71.5 ± 20
	6	75.8 ± 6.2	73.3 ± 9.9	78.3 ± 9.8	80.8 ± 17
	8	80.6 ± 10	68.4 ± 16	75.4 ± 16	69.7 ± 13
	24	79.6 ± 13	**48.0 ± 10	82.0 ± 28	* 43.0 ± 12
TG	1	98.0 ± 7.3	84.6 ± 5.9	89.7 ± 3.7	80.2 ± 16
	3	112.0 ± 12	101.0 ± 18	93.2 ± 11	82.7 ± 21
	6	131.0 ± 16	108.0 ± 30	82.0 ± 14	83.6 ± 21
	8	154.0 ± 9.9	149.0 ± 73	77.0 ± 17	85.5 ± 23
	24	277.0 ± 19	192.0 ± 99	85.0 ± 32	88.2 ± 19
		For SH-F: CTL: PE, n=20; PC, PI n=8; PS, n=6; TG, n=2; RA: PE, PC, PI, n=8; PS, n=6; TG, n=4		For SH-N: CTL: PE n-12; PC, PI, PS, TG, n=6; RA: PE, PC, PI, PS, TG, n=6	

Cells were pulsed with [^{3}H]AA for 16 hours. Chase proceeded in RPMI medium supplemented with 100 μM unlabelled AA for 24 hrs. Percent dpm remaining in lipid classes was measured at time points indicated. Values represent average ± S.D. Time 0 = 100%. Based on independent two-tailed t analysis: *significantly different from control ≤ .05; and, ** p ≤ .01

retained 79.6% of arachidonic acid label whereas RA-treated cells retained much less i.e., 48%. The same effect was evident, and statistically significant in SH-N. No significant differences were found in arachidonic acid turnover and redistribution in PI, PE, and triglyceride, where RA-treated cells followed similar patterns of label loss as in vehicle-only controls.

The results of these studies, measuring the effect of long-term RA treatment show definite differences in the metabolism of arachidonic acid. Differentiation of these two variant neuroblastoma cell lines therefore, results in significant changes in lipid composition.

2.5 DISCUSSION

Although the main goal of this chapter was to study the effect of RA on lipid metabolism, a detailed study of fatty acid incorporation was necessary to establish some understanding of this process within these cells. Two commonly used fatty acids served for this study. Arachidonic acid was selected because its metabolism is usually very active in cells and leads to prostaglandin and leukotriene synthesis, and because it had been previously examined in the neuroblastoma cell line, SK-N-BE, (Spinedi et al., 1990) Furthermore, a possible involvement of this fatty acid in neuronal differentiation had been suggested (DeGeorge et al., 1988). Oleic acid was also chosen because like arachidonic acid, it incorporates mainly in the second acyl position of phosphoglycerides, but displays incorporation patterns among the lipids which resemble those of saturated fatty acids (Cook et al., 1982; 1983). Oleic acid could therefore serve as a useful basis for comparison with results obtained with

arachidonic acid.

On the basis of lipid phosphorus analysis, no differences in phospholipid composition were apparent between SH-F and SH-N cell lines. Their phospholipid compositions are similar to those reported for other neuroblastoma such as SK-N-BE (Spinedi et al., 1990) and N1E 115 (Cook et al., 1982).

A. INCORPORATION OF [^{3}H]FATTY ACIDS IN UNTREATED SH-F AND SH-N CELLS

The curves showing the time course for fatty acid uptake in lipid classes also reveal marked differences in the rates of incorporation of these two precursors (Figures 2.1 to 2.4). The uptake of arachidonic acid appears to be much more rapid than that of oleic acid. Their could be several reasons for this observation: serum, which is present in the medium throughout these experiments may contain much higher levels of oleic acid than arachidonic acid, thereby diluting the label and causing the apparent slower incorporation rate of this monounsaturated fatty acid. However, ongoing experiments in our laboratory have shown that serum has no marked effects on comparative rates of oleic and arachidonic acid incorporations in neuroblastoma cells. Another possibility is that fatty acids may differ in their rates of transport or diffusion through the plasma membrane and their subsequent translocation to the sites of lipid biosynthesis. Furthermore, the more rapid uptake of arachidonic acid may be accounted for by the activity of enzymes involved in the incorporation of fatty acids in lipids, particularly the phospholipids, whereby their kinetic parameters may favor more rapid incorporation. For example, certain

acyltransferases may have higher affinities for arachidonic acid than saturated or monounsaturated fatty acids. In fact, it had been suggested that the preference for arachidonic acid may be due to the existence of separate acytransferases which utilize only this fatty acid as substrate (Irvine 1982). Moreover, the fact that arachidonic acid is an active metabolite, serving as substrate for other metabolic functions such as those of eicosanoid biosynthesis, could account for its more rapid incorporation.

The results for the incorporation of [^{3}H]oleic acid in phospholipid classes show that the percentage label recovered in each fraction reflects somewhat the phospholipid composition of these cells. As PC comprises about 60% of the total phospholipid, the largest proportion of label, 42 to 49% is recovered after 24 hours in this fraction. However, at earlier time points up to 40% of the label may also be found in the triglyceride fraction, a result which is not obtained with arachidonic acid. Again, PI, which makes up about 10% of the phospholipids received about 5 to 7% of label of oleic acid (Table 2.2 and 2.3). Following a 24 hour incorporation, the specific activity of the label in PC was found to be about twice that measured in PE, in both SH-F and SH-N cell lines. Therefore, although oleic acid seems to be less discriminating than arachidonic acid in its labelling pattern, there is some selective process involved favoring enrichment in the PC fraction.

The uptake of the saturated fatty acid, palmitic acid, was also investigated, (Proulx, P. unpublished observations). In SH-F cells, the neutral lipid fraction received more than 20% of [^{3}H]palmitic acid by 24 hours. In this same time period, PC had incorporated about 56% of the total label, while 13% was recovered in PE,

and 5% or less in PS and PI. The incorporation pattern obtained with palmitic acid closely resembles that found for oleic acid in the SH-F cell line (Table 2.2). These results confirm previous studies (Cook et al., 1982), that the uptake profile of monoenoic fatty acids among the lipid classes, is very similar to that of saturated fatty acids, both of which are remarkably different from the incorporation patterns obtained with polyenoic fatty acids such as arachidonic acid.

The incorporation of arachidonic acid in phospholipids does not reflect the lipid composition of these cells, but is highly selective. The highest specific activity is seen in the PI fraction (Table 2.4), where it is some seven- to nine-fold greater than that calculated for PC. PE also displays enhanced arachidonic acid incorporations, as specific activities are approximately three- to four-fold greater than those determined for PC. It has been suggested that elevated arachidonic acid incorporation in phosphoinositides is due to increased affinities of the acyltransferase for arachidonoyl CoA when lyso-phosphatidylinositol is the fatty acyl acceptor (Irvine 1982). The selective incorporations of oleic and arachidonic acids could also be explained on the basis of specificities of acyltransferases towards the acyl group. Furthermore, the possibility that enzymes may be operating at different membranous sites in the cell where the compositions of the lipid acceptors could be very different from that of the total lipid composition could account for these effects. For example, it is possible that an arachidonoyl transferase may be located at a site where lyso PI and lyso PE concentrations exceed those of lyso PC. The selectivity in this case would be due to topographical distributions of the acyltransferase and its

lyso substrates. These results are in agreement with those of Cook et al. (1982) who reported that polyenoic fatty acids show selectivity in their incorporations, such that highest specific activities are measured in PI and PE.

The levels of triglyceride can be estimated based on the determination of the number of ester bonds present in the triglyceride fraction (Renkonen 1962), however, the sensitivity of this method is limited and therefore does not lend itself very well to small lipid samples such as those obtained from single culture plates containing 1.0 to 2.0 X 10^6 cells. Throughout these experiments therefore, the incorporation of fatty acids in the triglycerides was expressed as counts, or percent of total radioactivity recovered in this fraction, rather than specific activity. Triglyceride, after 12 hours, represented approximately 10% or less of total arachidonic acid incorporation. This is about one third of the recovery obtained with oleic acid and palmitic acid in this same fraction following a 12 hour incubation. Such a comparatively low uptake was expected, as triglyceride, which is the principal storage lipid, is normally less enriched in arachidonic acid than phospholipids (Irvine 1982).

B. ARACHIDONIC ACID CHASE IN UNTREATED SH-F AND SH-N CELLS

Arachidonic acid chase studies showed that there are differences in the rates at which arachidonic acid is lost from phospholipids. PI lost its arachidonic acid label most rapidly whereas PE and PC lost their labels at comparative rates. Changes in specific activities indicated however, that the loss of arachidonic acid relative to the total acyl content of PE proceeded at twice the rate found for PC and

approximately 30% of the rate found for PI. Results obtained for chase studies appeared to be a direct reflection of those obtained for arachidonic acid incorporation. It seems, therefore, that under conditions in which cells are confluent and little growth occurs, there was little net synthesis in which arachidonic acid incorporation is involved. These results are compatible with a complete breakdown and resynthesis process, or a renewal involving acyl exchange, or with turnover involving both processes.

Studies by Chakravarthy et al. (1986) have shown that the rate measured for the loss of arachidonic acid from phospholipids is a product of both complete phospholipid breakdown and partial turnover involving a deacylation / reacylation cycle in phospholipid classes. Both of these processes were found to contribute to the measured release of arachidonic acid from phospholipids in murine neuroblastoma. Dual labelling studies, (Proulx, P. unpublished observations) have shown that rates of loss of radioactivity in the fatty acyl moiety exceeded those of the phospholipid head group thereby demonstrating the exchange of fatty acyl chains among phospholipids. The results obtained with SH-F cells closely resemble those reported for N1E 115. Accordingly, the label from arachidonic acid was steadily transferred from phospholipids to triglycerides. As in N1E 115, it appears that in SH-F, triglyceride may act as a reservoir for acyl chains released from phospholipids. This redistribution of arachidonic acid in triglyceride could be attributable to the high levels of arachidonic acid in the chase medium. Previous studies by Cook et al. (1987) have shown that incubation in high concentrations of arachidonic acid results

in an increased accumulation of this fatty acid in triglyceride as a means to control levels of polyunsaturated fatty acids being incorporated into the membranes. However, when SH-N were studied under identical conditions, the redistribution of arachidonic acid from phospholipids was not accompanied by a corresponding increase in triglyceride. It appears, therefore, that these variant cell lines differ in certain aspects of arachidonic acid metabolism. In SH-N, no increase in free fatty acid was found, therefore arachidonic acid may have been diverted to an alternative metabolic route such cyclo-oxygenase or lipoxygenase pathways. However, no corresponding increases in any of the lipid fractions were observed, therefore eicosanoid biosynthesis certainly could not account for the entire arachidonic acid redistribution in these cells. Other possible fates of this fatty acid could be its conversion to water-soluble degradation products as well as complete oxidation to ^3H_2O. As changes in lipid content were not analyzed in the medium, the possible release of arachidonic acid or its metabolites may also explain the redistribution in the SH-N cell line. Further investigations regarding the metabolism of arachidonic acid in these cells are ongoing in our laboratory.

C. EFFECTS OF RETINOIC ACID ON LIPID METABOLISM

Both fatty acid incorporation and chase studies were employed to examine the effects of RA on lipid turnover. While normally one would expect that chase results would be a reflection of incorporation, an agent may affect enzymes which are involved in one process but not the other. For example, RA could possibly affect the activity of phospholipases but not the acyltransferases and thereby cause

modifications of the acyl chain composition of membrane phospholipids. In such a case incorporation and chase studies might present very different images. Lipid turnover was therefore examined using both methods.

C.1 EFFECT OF SHORT-TERM RETINOIC ACID TREATMENT

It has recently been shown that short term treatment of the neuroblastoma cell line, LAN-1, caused a rapid decrease in PI turnover (Ponzoni and Lanciotti 1990). Levels of diglyceride were found to decrease by more than 50% following 1 minute of exposure of cultures to RA. This decrease persisted for up to 25 minutes. Similarly, levels of inositol phosphates were found to be decreased by 60% following short exposure of cells to RA. To follow up these studies, investigations on early effects of RA on phosphoinositide metabolism were carried out in our laboratory. The turnover of this active lipid pool was measured in different ways. The levels of diglyceride, one of the products of phospholipase C activation, was measured directly in short-term chase studies in which lipids had been labelled with [^{3}H]arachidonic acid. Phosphoinositide turnover was also monitored with the use of labelled arachidonic acid as precursor since it incorporates very well in the inositide fraction. Studies measuring turnover of inositol phospholipids labelled with myo-[^{3}H]inositol were pursued, (Proulx, P. unpublished observations). In the latter case, relative proportions of PI, phosphatidylinositol-4-monophosphate (PIP), and phosphatidylinositol,4,5-bisphosphate (PIP_2) were determined following short term chases in the presence of RA for 0, 5 minutes, 30 minutes, 1 hour, 4, 8, and 24 hours, (n=4). The results of these investigations, measuring PI turnover by three different

approaches, showed no changes following short-term exposure to RA with respect to: 1) the levels of labelled diglyceride, 2) the turnover in PI and the polyphosphoinositides. It appears, therefore, that retinoic acid has no immediate effects on membrane lipid turnover in the SH-F cell line. Therefore, although RA treatment may have resulted in early effects on lipid metabolism in LAN-1 cells, these results could not be confirmed by the present investigations with SH-F cells. As RA-resistant LAN-1 variants did not exhibit this early response, Ponzoni and Lanciotti (1990) reported that the inhibition of PI turnover is highly correlated with the ability of RA to induce differentiation in neuroblastoma cell lines. Previous investigations by Sidell et al. (1986), as well as the results reported presently (Chapter 4), clearly show that RA treatment of SH-F and SH-N cell lines results in differentiation. However, our studies investigating early effects of RA on PI turnover provide no indication that this mechanism plays any role in the differentiation process induced by RA. We therefore conclude that early effects on PI metabolism are not required for the mediation of RA-induced differentiation in neuroblastoma. It should be noted, however, that changes in PI turnover could be involved in later events leading to differentiation. Further investigations pursuing changes in the metabolism of phosphoinositides such as PI, PIP, PIP_2, and diglyceride levels following long-term RA treatment (3 to 7 days), would serve to further elucidate this question.

C.2 EFFECT OF LONG-TERM RETINOIC ACID TREATMENT ON ARACHIDONIC ACID METABOLISM IN SH-F AND SH-N CELLS

Differences in distribution of arachidonic acid label among phospholipids following long term RA treatment were found after 16 hours of fatty acid incorporation (Figures 2.9 and 2.10). Cells treated with RA showed significantly higher percentage incorporations of arachidonic acid in PC and significantly lower levels in PI. Dual label incorporation studies (data included in figures 2.9 and 2.10) showed that the phospholipid composition of both SH-F and SH-N remain similar following RA-induced differentiation. These differences in fatty acid incorporation, therefore, cannot be attributed to changes in pool sizes of these phospholipids. From these studies it seems more likely that the differences in arachidonic acid uptake, observed following RA treatment, are a result of changes in rates of acyl chain exchange within the phospholipid classes.

To determine the effect of RA-induced differentiation on arachidonic acid redistribution, chase experiments were conducted. While the overall pattern of label redistribution was not altered in cells differentiated with RA in comparison to that of untreated controls, significant differences were detected in the rate of arachidonic acid label loss from specific phospholipids. The turnover of arachidonic acid in PC appears to be markedly enhanced in cells induced to differentiate with RA. The increased rate of incorporation in PC corresponds well with the increased rate of loss of this fatty acid during the 24 hour chase period. The results of incorporation and chase experiments taken together provide highly consistent evidence that RA-induced

differentiation results in an increase in arachidonic acid turnover in the major membrane phospholipid, PC.

RA-induced changes in fatty acid metabolism in phospholipids, such as PI, and PS, become more difficult to explain. For example, Figures 2.9 and 2.10 show, that after a 16 hour incorporation of arachidonic acid, the radioactivity in PI is significantly reduced in both cell lines following RA treatment. This reduction cannot be attributed to lower levels of PI in the membranes, as phosphorus incorporation studies indicate that relative proportions remain the same following differentiation. To add further to the complexity, the rate of loss of labelled arachidonic acid from PI is not significantly different in RA-treated and control groups. Several reasons for such discrepancies can be considered. RA treatment could decrease the activities of enzymes involved in synthesis of specific lipids, such as PI specific acyltransferases, without effecting the activities of enzymes involved in the breakdown of lipids. The converse of this argument could explain the increased loss of arachidonic acid in PS, which is not accompanied by any changes in incorporation rates. Topographical factors might also be involved. For example, the synthesis and hydrolysis enzymes for a particular lipid may be located at different sites. Retinoic treatment could be affecting mechanisms which are responsible for the distribution (transport) of lipid and/or other substrate or cofactors to these sites. For example, in RA treated cells, the synthesis of PS in the endoplasmic reticulum may be normal but the transport of this lipid to the plasma membrane or other site where breakdown normally occurs could be enhanced. To resolve this question

future studies with subcellular fractions involving actual measurements of synthesis and breakdown enzymes would be useful. Studies by DeGeorge et al. (1988) have shown that nerve growth factor-induced differentiation of PC 12 cells resulted in an increase in the release of arachidonic acid from phospholipids with a concomitant increase in eicosanoid production. Differentiation of PC 12 cells therefore, was accompanied by an increased capacity for eicosanoid production. Few studies have pursued eicosanoid production in neuroblastoma; however, similar to what is found for nerve growth factor-induced differentiation of PC12 cells, long-term RA treatment of neuroblastoma is also accompanied by an accelerated turnover of arachidonic acid in certain phospholipids, namely, PS and PC.

The results of these studies show that RA-induced differentiation of neuroblastoma result in definite changes in lipid metabolism. The short term effects of RA were investigated using both oleic and arachidonic acid as precursors. Neither chase experiments measuring the turnover of phosphoinositides, nor data obtained from incorporation studies, gave any indication of immediate or early changes in lipid metabolism as a result or RA treatment. Any lipid effects of RA, therefore, appear to be genomically controlled and are seen as long-term changes resulting from long-term treatment. The mechanisms by which RA exerts these changes are presently unknown: alterations in levels or activities of enzymes involved in deacylation/reacylation, such as acyltransferases, or those involved in breakdown such as phospholipase A2, are all potential candidates. It should be emphasized however, that these effects are apparent only after long term treatment of cells (greater than

48 hours) suggesting that RA must bind to its receptor and exert its effects at the nuclear level before any changes in lipid metabolism may be observed. Future experimentation measuring the steady-state levels of mRNA of some of the important enzymes involved in lipid turnover, such as the phospholipase A2, could further elucidate the mechanisms of RA action.

The significance of these changes with respect to cell differentiation remains to be determined, however previous studies with neuroblastoma cell lines suggest that changes in fatty acid metabolism may have important implications in the growth and maturation of cells. Differences in fatty acyl chain metabolism may alter fluidity of the membrane following RA-induced differentiation and with respect to this, studies with neuroblastoma have indicated definite changes in membrane fluidity accompanying cell differentiation (DeLatt and Van der Saag, 1982). Alterations in acyl chain composition leading to fluidity changes have been shown to have significant effects on transport as well as enzyme activity in the plasma membrane. For example, calcium transport, (Fontaine et al., 1981; Bikle et al., 1984; Merrill et al., 1986; Maenz and Forsyth, 1982; Reviewed by Proulx, 1991) as well as adenylate cyclase, (Murphy 1984; 1985; 1986; Baba et al., 1984), are two important systems where activities have been shown to be significantly altered by membrane acyl chain composition. Both of these systems have been shown to play key roles in important cellular processes such as differentiation (Berridge, 1986; 1987; Whitfield et al., 1987). Changes in membrane fatty acid metabolism in the neuroblastoma cell lines, SH-F and SH-N, may, therefore, contribute to later effects accompanying and perhaps

mediating aspects of RA-induced differentiation.

2.6 CONCLUSION

The phospholipid composition as well as the oleic acid and arachidonic acid incorporation patterns have been characterized in both SH-N and SH-F cell lines. Oleic acid is distributed more uniformly among phospholipid classes, whereas arachidonic acid incorporation is highly selective. The release and redistribution of arachidonic acid have been characterized by pulse-chase studies. Distinct differences in the redistribution of this fatty acid are observed between SH-F and SH-N.

Our results do not confirm short term effects of RA on lipid metabolism previously reported for the neuroblastoma cell line, LAN-1, (Ponzoni and Lanciotti, 1990). Long term RA treatment, however, significantly influenced lipid metabolism in these cells. Alterations in the incorporation of arachidonic acid in phospholipid classes are significantly different in both cell lines. The liberation of arachidonic acid from PC and PS is also markedly accelerated in SH-F and SH-N following differentiation. Long term treatment with RA , therefore, results in alterations of the lipid metabolism in these neuroblastoma cell lines.

CHAPTER 3 - EFFECTS OF RETINOIC ACID ON SIGNAL TRANSDUCTION PATHWAYS IN NEUROBLASTOMA

3.1 INTRODUCTION

The goal of the experiments to be described was to characterize some of the changes that take place in signal transduction during RA-induced differentiation of two variant neuroblastoma cell lines. The main focus in this study was on protein kinase C (PKC), a component of the signalling pathway initiated by receptor mediated hydrolysis of inositol phospholipids and/or phosphatidylcholine by phospholipase C (Nishizuka, 1989). As the PKC is thought to interact with other pathways, attention was also given to protein kinase A and adenylate cyclase which are components of a second major signalling pathway.

Recent studies have shown that certain cultured human neuroblastoma cells derived from the parent line SK-N-SH, can differentiate into several different phenotypes (Ross et al., 1983; Ross and Biedler, 1985; Tsokos et al., 1987). Differentiating agents such as RA, acting on two such descendants of the SK-N-SH cell line give distinct phenotypic responses after RA treatment (Sidell et al., 1986). One descendant of SK-N-SH, the (SH-F) subline, forms large flattened fibroblast-like cells, while the other variant, the SH-N subline, shows a typical neuronal-like differentiation (Sidell et al., 1986). In view of their capacity to express different phenotypes, neuroblastoma, as exemplified by the SK-N-SH sublines, when treated with agents such as RA, represent simple models which lend themselves very well to

the study of primitive neuronal development and cell differentiation.

A. PROTEIN KINASE C AND CELL DIFFERENTIATION

Several lines of evidence indicate that PKC may be involved in cell differentiation (Serra et al., 1986; Heikkila et al., 1987; Adem et al., 1987). Kraft and Anderson (1983) observed that F9 cells induced to differentiate with RA displayed significant increases in cytosolic PKC activity after five days of treatment. Niles and Lowey (1989) observed a five-fold increase in PKC activity after two days of RA treatment in B16 melanoma. Dimethylsulfoxide and RA, agents which induce myelocytic differentiation in HL60 cells, caused a marked elevation of PKC activity (Zylber-Katz and Glazer, 1985; Zylber-Katz et al., 1986). Treatment with the phorbol ester, 13-tetradecanoyl-phorbol-13-acetate, however, resulted in macrophage differentiation and disappearance of PKC (Zylber-Katz and Glazer, 1985). Balazovich et al. (1987) observed very pronounced PKC changes in Friend murine erythroleukemia cells induced to differentiate with dimethylsulfoxide or hypoxanthine. Within 15 minutes a dramatic translocation as well as a gradual decline in total cellular PKC over a five-day period was observed. These several studies taken together, show definite correlations between cell differentiation and divergent changes in PKC activity. They also provide at least scant evidence, indicating that the direction in which PKC changes occur may have a marked influence on the type of differentiation obtained. Although few studies of this type have been conducted with neuroblastoma cells, evidence exists indicating that PKC changes also accompany their differentiation. Phorbol esters, which are well known PKC

activators, have been shown to decrease proliferation and induce morphological and biochemical changes characteristic of human SH-SY5Y neuroblastoma differentiation (Pahlman et al., 1981; Pahlman et al., 1984) As little is known regarding the PKC response in RA-induced neuroblastoma differentiation, it was of interest to characterize this response with the two neuroblastoma variants differentiating to alternative cell types.

B. USE OF PROTEIN KINASE C INHIBITORS

Although many PKC inhibitors such as 1-5(isoquinolinylsulfonyl-2methyl)piperazine (H-7), (Hidaka et al., 1984) and sphingosine (Hannun et al., 1986) exist, there are no known inhibitors that are entirely specific to the protein kinase C. Staurosporine, a microbial alkaloid, isolated from Streptomyces staurospores has recently been found to be the most potent PKC inhibitor described so far (Wolf and Baggliolini, 1988; Tamaoki et al. 1986). As well as being highly potent in vitro, (Tamaoki et al., 1986) this agent has also been shown to affect cellular, PKC-mediated events in vivo, (Okazaki et al., 1988; Schachtele et al., 1988), and cause the morphological differentiation of the NB-1 human neuroblastoma cell line (Morioka et al. 1985). Staurosporine exerts its effect on the catalytic moiety of the PKC by competing for ATP (Hashimoto and Hagino, 1989). As this catalytic moiety is homologous with other protein kinases (Nishizuka, 1988), it is not surprising that some of these kinases might also be inhibited by this agent. The inhibitory properties of staurosporine, however, are most effective against PKC and to a lesser extent, against the protein kinase A and the protein tyrosine kinases (Smith et al.,

1988). If lowest possible effective doses are used, and caution is exercised in interpretation of the data, staurosporine may serve as a useful tool for the in vivo study of the role of PKC in cellular processes in general, as well as elucidating the role of PKC in differentiation.

C. THE PROTEIN KINASE C GENE FAMILY

It has recently been discovered that the PKC consists of a family of related enzymes. Three homologous species, alpha,beta and gamma have been located on human chromosomes 17,16,and 19 respectively (Coussens et al., 1986). The gene encoding PKC beta can give rise to two separate mRNA species, beta 1 and beta 2, by alternative splicing (Coussens et al., 1987). These subspecies have been cloned from rat, human, and bovine brain cDNA libraries. A further group of related isozymes have been identified by cDNA cloning. Species delta, epsilon, and zeta have been cloned from a rat brain library (Ono et al., 1988; Ono et al. 1987).

The PKC isozymes show highly distinctive tissue distributions (Nishizuka, 1989). PKC gamma is found in central nervous tissue only, concentrated mainly in the hippocampus, cerebral cortex, and amygdaloid complex. Studies have shown that expression of PKC gamma in rat brain increases post-natally approaching maximum levels three weeks after birth. The expression of this isozyme appears to be developmentally regulated (Yoshida et al., 1988). Beta 1 and beta 2 are found in a wide variety of tissues such as brain, liver, kidney, spleen, lung, heart and testis. Distinctive localization patterns of beta 1 and beta 2 have been found in brain (Saito et al., 1988). PKC alpha is expressed universally (Kikkawa et al., 1989). Very little

is known about tissue localization of delta, epsilon, and zeta although it has been suggested that in rats, delta is found in many tissues while epsilon has been found mainly in brain. No studies of tissue distribution of expression have been performed for the zeta isozyme (Parker et al., 1989).

Kinetic properties of these kinases are highly variable. Activation of PKC alpha and gamma may be brought about, not only in the presence of phosphatidylserine, diglyceride and calcium, but also by arachidonic acid and calcium. PKC beta has been found to be less sensitive to calcium than alpha and gamma and cannot be activated by arachidonic acid. The more recently discovered isozymes appear to be calcium-independent (Nishizuka, 1989). Further diversity with respect to substrate specificity and susceptibility to proteolysis by calpain (Nishizuka, 1989; Kikkawa et al., 1989) suggest that these isozymes, when present together in cells, probably fulfill distinctive functions.

The observation that expression of PKC gamma rises during brain maturation suggests that PKC isozyme mRNA levels may be altered during cell differentiation. Immunochemical evidence shows that increases in abundance of PKC isozymes accompanies RA- and dimethylsulfoxide-induced differentiation of HL60 cells (Makowske et al., 1988). Few studies exploring isozyme expression have been carried out with respect to neuroblastoma differentiation, leaving several questions unanswered: Which of the PKC isozymes are expressed? Does RA affect the steady state levels of mRNA for these isozymes, and could such changes be involved in phenotype development?

D. INVOLVEMENT OF THE OTHER SECOND MESSENGER PATHWAY

Although PKC changes may be important in cell differentiation it seems highly unlikely that this component would be the sole mediator of RA-induced differentiation in neuroblastoma cells. One may expect that this process is brought about by a concerted and co-ordinated response of several signalling pathways. Considerable evidence exists that the pathway initiated by receptor-mediated stimulation of adenylate cyclase may also be involved in cell differentiation.

E. INVOLVEMENT OF cAMP AND ADENYLATE CYCLASE

In neuroblastoma it has been demonstrated that the addition of cAMP derivatives, agents that activate adenylate cyclase such as forskolin, or inhibitors of phosphodiesterases result in reduced growth rates and increased neuritogenesis (Abemayor and Sidell, 1989). Conditions which induce neuronal differentiation, such as serum deprivation, have been shown to increase levels of cAMP concomitant with neurite outgrowth and increased acetylcholinesterase activity (Liu et al., 1980).

F. PROTEIN KINASE A

Protein kinase A is thought to initiate all cellular responses to cAMP and is best characterized for its role in hormone-stimulated glycogen metabolism. Three species of the catalytic subunit have been cloned to date (Taylor et al., 1990). The regulatory subunit functions to bind and inactivate the catalytic domain in the absence of cAMP. Two categories of regulatory subunit exist, RI and RII, both of which have alpha and beta isoforms. The alpha isoforms are expressed in most cells whereas the beta isoforms are characterized as having tissue specific expression, such

as in neuronal cells (Taylor et al., 1990).

Retinoic acid as well as other differentiating agents are known to increase the levels of the protein kinase A (Ludwig et al., 1980; Fontana et al., 1986; Plet et al.,1982). While few investigations have been carried out with neuroblastoma, several studies with other cell lines have been reported. Upon RA treatment, B16 melanoma show increased protein kinase A activity (Ludwig et al., 1980). RA-induced differentiation of HL60 cells to the mature myeloid phenotype results in increased protein kinase A activity before phenotypic changes become evident (Fontana et al., 1986). F9 embryonal carcinoma were found to respond similarly by a progressive increase in enzymatic activity in the cytosolic fraction and a transient increase in the membrane fraction (Plet et al., 1982). More recently with the same cell line, work by Plet et al. (1986) showed that the RA-induced increase in protein kinase A is correlated to the presence of cytosolic retinoic acid binding protein (CRABP). Studies employing differentiation-defective sublines have shown that cell lines without CRABP did not exhibit elevated protein kinase A levels. Differentiation-defective sublines which contained functional CRABP but harboured alternate genetic lesions demonstrated the usual increase of protein kinase A activity in response to RA treatment although differentiation did not take place. These authors suggested that the observed elevation of the cAMP-dependent kinase is correlated to the presence of CRABP, and may or may not be causative in the differentiation of these cells.

As numerous cell types show changes in protein kinase A activities in response to RA treatment, it seems likely that this pathway may also be altered in RA-induced differentiation of neuroblastoma.

G. CROSS-TALK BETWEEN THE TWO SIGNALLING PATHWAYS

While both of these pathways may play important roles in cell differentiation it seems likely that co-ordinated interaction must take place. Phorbol ester treatment has been found to augment cAMP levels in several cell types. In non-neoplastic T51B rat liver cells this treatment had no effect on basal adenylate cyclase but caused a 1.5-fold amplication of cAMP production mediated by forskolin and ß-adrenergic agents (Aasheim et al., 1989). Similar augmentations in cAMP accumulation by phorbol ester have been found in other cell lines such as pinealocytes (Sugden et al., 1985), smooth muscle, synaptoneurosomes, lymphoma cells, pheochromocytoma PC12, and primary cultures of myoblasts (cited in Hollingsworth and Daly, 1987). Opposite results have been obtained in a neuroblastoma - brain explant hybrid, the NCB-20 cell line. In these cells, phorbol ester had no effect on basal or forskolin-stimulated cAMP accumulation; however, ß-adrenergic receptor-mediated cAMP production was inhibited by this agent. As all ß-adrenergic agonists tested exhibited attenuated cAMP production in the presence of phorbol ester, it was surmised that this compound likely exerted its effect by phosphorylation of a common component such as the stimulatory G-protein (Hollingsworth and Daly, 1987). Phorbol esters have also been shown to reduce receptor-mediated responsiveness in cell lines such as those of glioma and fibroblasts (cited in Hollingsworth and Daly, 1987). The main body of evidence suggests that the predominant effect of these PKC activators is on receptor-mediated cAMP production, although this response is highly variable depending on the cell type used. The disparity of effects of phorbol esters on cAMP

accumulation is most likely due to differences in the components of the adenylate cyclase system in different cell types.

Mounting evidence suggests, therefore, that cross-talk exists between these two important signal transduction pathways. It is presently uncertain whether such interactions occur in neuroblastoma cell lines.

3.2 OBJECTIVES

The overall goal of this chapter is to characterize some of the changes that take place in signal transduction pathways during RA induced differentiation. More specifically, these investigations have been carried out to fulfill the following objectives:

1a) To characterize changes in PKC activity accompanying RA-induced differentiation of two neuroblastoma variants.

b) To determine if cells giving rise to alternative phenotypes exhibit alternative PKC responses following RA treatment.

2) To intervene in the cellular response to RA treatment by use of the PKC inhibitor staurosporine and to study the effect of PKC inhibition on phenotype development as revealed by morphological changes.

3) To ascertain which PKC isozyme(s) are expressed in these neuroblastoma cell lines and to compare the steady state levels of PKC mRNA's before and after RA-induced differentiation.

4) To characterize activity changes of protein kinase A and adenylate cyclase following RA-induced differentiation of SH-F and SH-N.

5) To explore the possibility of interaction between these pathways by examining:

a) the effect of phorbol ester on cAMP production and adenylate cyclase activity.

b) the effect of cAMP analogues on PKC activity.

3.3 MATERIALS AND METHODS

A. MATERIALS

All tissue culture products were purchased from GIBCO Laboratories, St. Louis, MO. All-trans retinoic acid, (RA), phenylmethylsulfonylfluoride (PMSF), ß-mercaptoethanol, DEAE-Sephacel anion exchanger, Triton X-100, histone (type IIIS), phosphatidylserine, 12-tetradecanoyl-phorbol-13-acetate, 1,2-dioctanoyl-rac-glycerol, dithiothreitol, Nonidet P-40, phosphocreatine, phosphocreatine kinase, Ficoll, polyvinylpyrrolidine, agarose, and calf thymus DNA were purchased from Sigma Chemical Company, St.Louis,MO. γ-[^{32}P]-ATP was purchased from ICN Biomedicals, St.Laurent, Quebec. [^{14}C-cAMP, [^{3}H]adenine, α-[^{32}P]-ATP, [^{3}-H]-cAMP from New England Nuclear, Boston, MA. Staurosporine was purchased from Boehringer-Mannheim, Canada, Dorval, Quebec.

Hybond N, nylon membranes, Hybond map paper, Multiprime kits, restriction enzymes, and [^{32}-P]-dCTP (3,000 Ci/mmol) were purchased from Amersham Corporation, Oakville, Ontario. A Geneclean kit was purchased from Bio/Can Scientific Inc., Mississauga, Ontario, Canada. Sephadex G50M was purchased from Pharmacia Ltd., Dorval, P.Q. All other chemicals were purchased from BDH Inc.,Toronto, Ontario and were of the finest grade possible.

B.1 CELL CULTURES

Cells were maintained as described in Chapter 2.

B.2 TREATMENT OF CELL CULTURES

To induce differentiation, cells were treated with RA according to the method of Sidell et al. (1986), described in Chapter 2. Staurosporine was dissolved in dimethylsulfoxide to give a concentration of 2 X 10^{-3}M and kept at 4 °C. From this stock solution, a second stock solution (1 X 10^{-5}M), was made by dilution in ethanol and stored at -20°C. This second stock was then further diluted as required in experiments. Cells were seeded in the usual medium and grown for 24 hours, after which time, a replacement with medium containing appropriate concentrations of staurosporine or vehicle was made.

C. KINASE ASSAYS

C.1 PREPARATION OF PROTEIN KINASE C EXTRACTS

Cytosolic, particulate and total cellular PKC-containing extracts were prepared as follows: for each sample to be analysed, two confluent (or near confluent) 150 X 20 mm dishes were used. Medium was always changed within 24 hours prior to an experiment. With the neuroblastoma cell lines studied, linear kinetics were not obtainable without some purification of the cytosolic extract for PKC. The method used was essentially as described by McCaffrey et al. (1984), with some modification. After the end of the culture period, following two washings of the dishes with 10mM phosphate-buffered saline (PBS), pH 7.4, cells were suspended with the aid of a rubber policeman in 1 ml scraping buffer consisting of 20mM Tris-HCl, 2mM EDTA,

0.5mM EGTA, 1mM PMSF, 50mM beta-mercaptoethanol, pH 7.5. Samples were disrupted by two 20-sec bursts with the small probe of a (Ultrasonics Ltd., Model A350G) sonicator at a setting of 5, and centrifuged at 105,000 X g for 60 minutes. The supernatant containing the cytosolic fraction was then partially purified by column chromatography with DEAE-Sephacel (McCaffrey et al., 1984). The particulate, PKC-containing fraction was obtained by centrifugation, as stated above, and the 105,000 X g pellet was resuspended in 2 ml of scraping buffer containing 1% Triton X-100. The suspension was kept on ice with occasional stirring, and after 45 minutes, was centrifuged again at 105,000 X g for 30 minutes. The resultant supernatant was retrieved and subjected to purification by the same anion exchange column chromatography procedure. Total cellular PKC was prepared by collecting cells in scraping buffer containing 1% Triton X-100. The cells were homogenized as stated above in detergent-containing buffer and allowed to stand on ice for 30 minutes prior to centrifugation. After centrifugation, the supernatant containing the total cellular PKC was applied to the anion exchange column as before.

C.2 PROTEIN KINASE ASSAYS

PKC activity was determined as described by Kleine et al. (1986). Briefly, 40 μl of the column chromotography-purified EDTA extract (5 μg protein) was added in a final volume of 270 μl of assay mixture also containing 5 μmoles Tris-HCl, to give pH 7.5, 1.25 μmoles $MgCl_2$, 50 μg histone type III-S, and 2.5 nmoles γ [^{32}-P] - ATP (5 X 10^5 cpm/nmole), 10 μg phosphatidylserine and 1 μg dioctanoylglycerol. The final calcium concentration was 1mM. The reaction was allowed to proceed for

3 minutes at 25°C and was stopped with 20% trichloroacetic acid. Phosphorylated histones were recovered and counted as described (Kleine et al., 1986). PKC activity was measured in triplicate and is expressed throughout as pmoles of [^{32}P] incorporated into histone III-S in the presence of added lipids (total protein kinase activity) minus the amount incorporated in their absence (protein kinases other than PKC).

Total protein kinase A was measured according to the method of Prashad et al. (1987). Briefly, the assay consisted of a measurement of [^{32}P] incorporation into histone in the presence of γ-[^{32}P]ATP with and without the addition of cAMP. The difference due to the cyclic nucleotide was ascribed to protein kinase A. A linear time course was obtained for 5 minutes using 20 μg of cell extract protein. Protein was determined by the method of Bradford (1979) with bovine serum albumin as standard.

Except when stated otherwise, the data for PKC activity are averages of triplicate determinations for a single experiment representative of at least two other experiments yielding qualitatively similar results. The data for protein kinase A activity are the averages from two experiments with triplicate analysis of the samples. All values for this enzyme varied within 20 percent from experiment to experiment and the technical error was within 5 percent.

D. MEASUREMENT OF STEADY-STATE LEVELS OF mRNA FOR THE PROTEIN KINASE C ISOZYMES

D.1 ISOLATION OF RNA

The two variant neuroblastoma cell lines were treated for 7 days with 3 μM RA or vehicle control. RNA was extracted from cultured cells by the LiCl/Urea procedure of Auffray and Rougeon (1979). RNA recoveries were monitored by A260/A280 ratios and purity was determined by gel electrophoresis (see section D.2). Polyadenylated RNA (poly(A)+ RNA was isolated using Amersham hybond map paper (oligo dT paper) according to the protocol provided by Amersham. Approximately 5% of poly(A)+ RNA was recovered from total RNA as measured by A260/A280 readings. Analyses of steady state levels of mRNA were determined entirely with poly(A)+ RNA.

D.2 GEL ELECTROPHORESIS OF RNA

RNA was separated by electrophoresis through 1.5% agarose gels containing formaldehyde (Lehrach et al., 1977). RNA samples for electrophoresis were prepared as described by Maniatis et al.(1982). The gels contained 20 mM morpholinopropane sulfonic acid (MOPS) (pH 7.0), 5mM sodium acetate, 1mM EDTA, 2.2 M formaldehyde. The running buffer comprised 20mM MOPS (pH 7.0), 5mM sodium acetate, 1 mM EDTA and 1.1 M formaldehyde. Following electrophoresis the gels were washed in water for 30 minutes and stained with ethidium bromide (1 μg/ml). Gels were photographed under 306 nm UV light using an orange filter and Ilford FP4 film.

D.3 NORTHERN TRANSFER

Poly(A)+ RNA samples were electrophoresed on denaturing agarose gels as described in (secton D.2) without staining with ethidium bromide. After a 60 minute wash in distilled water, RNA was transferred to nylon membranes according to the method of Thomas (1980) for nitrocellulose filters. The filters were prehybridized at 42°C for 2 hours in buffer containing 50% formamide, 0.75 M sodium chloride, 0.075 M sodium citrate, 5 times concentrated Denhardt's solution (0.1% albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), and 100 μg/ml calf thymus DNA. At least 4 ml of solution per 100 cm^2 membrane was used. Hybridization was carried out for at least 12 hours by adding [^{32}P]-labelled plasmid to the prehybridization solution (about 10 to 30 X 10^6 cpm per 10 ml). The membranes were washed twice for 15 minutes in 0.30M sodium chloride, 0.030 sodium citrate , 0.1% SDS at room temperature and once for 30 minutes in 0.015 sodium chloride, 0.0015 sodium citrate, 0.1% SDS at 55°C. A volume of 250 ml of wash buffer was used per 100 cm^2 of membrane. Membranes were wrapped in Saran wrap and autoradiographed with Kodak XAR-5 film between Kodak X-Omatic intensifying screens.

D.4 SLOT BLOTS

Hybond N Paper (Amersham) was cut to the size of the Schleicher and Schuell Inc. Minifold apparatus. Membranes were prewetted with a solution containing 0.30 sodium chloride, 0.030 sodium citrate and assembled into the Minifold apparatus as per the manufacturer's instructions. RNA samples were prepared by adding 3 volumes of a denaturing solution containing 500 μl formamide

(deionized), 162 μl formaldehyde (37%), 100 μl 10 times concentrated MOPS buffer and heated for 5 minutes at 65°C. Four volumes of a solution of 3.0M sodium chloride, 0.30 sodium citrate were added and the samples were applied to the slots under vacuum. The membrane was baked at 80°C for 2 hours under vacuum. Blots were prehybridized, hybridized and washed by the same method as used for Northern analysis.

D.5 DNA CLONES

Clones for the human PKC alpha, beta (homologous to beta I and II), and gamma isozymes were purchased from American Type Culture Collection, Rockville, Maryland. The PKC inserts were cloned into the Eco R1 site of plasmids pUC12 for alpha and beta and pUC13 for gamma. The clone for rat α-tubulin was obtained from Dr. P. A. Sharp, Centre for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachesetts.

D.6 PREPARATION OF PLASMID DNA

Large scale preparation of plasmid DNA was carried out with the use of cesium chloride - ethidium bromide gradients as described in Maniatis et al., 1982. After butanol extraction of the sample recovered from gradient, the plasmid DNA was precipitated with the addition of 3 volumes of water and 10 volumes of cold 95% ethanol.

D.7 ISOLATION OF DNA INSERTS

Inserts were removed by digestion with restriction enzyme Eco R1. The resultant digests were electrophoresed on a 0.8% agarose gel with lambda DNA

digested with Hind III as a standard. Inserts were excised from the gel and DNA was recovered using the Geneclean Kit following the manufacturer's instructions. The quantity of DNA was estimated by electrophoresing 10% of the sample on a 0.8% agarose gel and staining with ethidium bromide to visualize bands. Lambda DNA, digested with Hind III, was used as a control.

D.8 LABELLING OF DNA INSERTS

The PKC inserts were labelled with alpha [^{32}P]-dCTP by the random priming method of Feinberg and Vogelstein (1984) using the multiprime kit purchased from Amersham according to the protocol provided by the manufacturer. The labelled inserts were separated from the labelled nucleotides by purification over a Sephadex G50M spin column as described by Maniatis et al.(1982). These labelled probes for PKC alpha, beta and gamma were then used for Northern and slot blots.

E. SYNTHESIS OF cAMP - [^{3}H]ADENINE PRELABELLING ASSAY

Cells were seeded in 100 X 20 mm dishes such that at the time of experiment they were approximately 50% confluent. Prelabelling was carried out for 2 hours prior to each experiment by the addition of 0.2 μCi [^{3}H]adenine in medium containing 0.2% fetal calf serum. For short time course assays dishes were transferred to a shaking water bath at 37°C. Cells were incubated for 10 minutes with 0.1mM 1-methyl-3-isobutyl xanthine to inhibit cyclic nucleotide phosphodiesterase activity. Basal cAMP synthesis was measured using inhibitor alone. Cyclic AMP synthesis was stimulated by addition of forskolin for 5 minutes such that the final concentration in the reaction mixture was 10 μM.

After appropriate incubation periods, medium was aspirated, and dishes were washed once with cold phosphate buffered saline. Cells were lysed with 0.5 ml buffer containing 150 mM NaCl, 10mM phosphate (pH 7.2), 1 mM EDTA, 0.1mM cAMP, 0.1mM 1-methyl-3-isobutyl xanthine, 1% Nonidet P-40 and 0.001 μCi [^{14}C]cAMP to measure recovery of [^{3}H]cAMP. The lysate was transferred to a centrifuge tube and the dishes were rinsed with a second 0.5 ml of lysis buffer. The combined lysate was centrifuged at 1000 X g for 10 min and the supernatant containing the nucleotides was reserved for further separation.

The method used for separation of ATP and cAMP is based on the method by Salomon et al. (1974) modified by Franks and Malamud (1976). The cell lysate was applied to a 5.0 X 0.5 cm column containing Dowex (AG 50W-X4) resin after which the column was washed with 2 ml of H_2O. The eluate contained the labelled ATP pool and was collected in scintillation vials for counting. The Dowex columns were then placed over 3.0 X 0.5 cm column of neutral alumina and washed again with 4 ml of H_2O. This second eluate contained the [^{3}H]cAMP which ran directly into the alumina column. The labelled cAMP was eluted from the latter column by washing with 5 ml Tris-HCl (50mM, pH 7.5). This eluate was collected in scintillation vials, 10 ml of scintillation cocktail, Formula 998 (New England Nuclear, Boston, MA.) was then added to each and the samples containing the labelled cAMP were counted. Protein was determined in replicate dishes by the method of Bradford (1979).

F. ASSAY OF ADENYLATE CYCLASE

Assay of adenylate cyclase was essentially as previously described (Franks et al., 1987). Cells were washed twice with PBS and harvested with the aid of a rubber policeman in 10 ml ice cold PBS. This suspension was centrifuged at 500 X g for 6 minutes and washed once with 5 ml PBS. After a second centrifugation, cells were resuspended in 2.5 ml of homogenization buffer containing 50mM Tris HCl (pH 7.4), 330 mM sucrose, lmM $MgCl_2$ and 1 mM dithiothreitol. Cells were homogenized in a motor driven teflon/glass homogenizer (40 strokes at 10,000 rpm) and the homogenate was centrifuged at 20,000 X g for 20 minutes to obtain a crude membrane preparation. The pellet was resuspended in 0.5 ml homogenization buffer with a Dounce homogenizer. 50 μg of crude membrane protein was used for the adenylate cyclase assay.

Adenylate cyclase activity was measured in a reaction mixture containing 50mM Tris-HCl buffer (pH7.4), 10 mM $MgCl_2$, 2mM dithiothreitol, 2mM cAMP + 0.01 μCi [^{3}H]cAMP, 0.5mM ATP + 0.5 μCi α-[^{32}P]ATP , 0.015% bovine serum albumin, and ATP-regenerating system consisting of 5 mM phosphocreatine and 0.4 mg/ml phosphocreatine kinase. The final assay volume including enzyme protein was 100 μl. The assay was initiated by addition of enzyme and incubation was for 6 minutes at 37°C. The reaction was terminated by dilution with 0.6ml H_2O and addition of 0.1 ml of 10 mM ATP. Nucleotides were separated by column chromatography as described for prelabelling using [^{3}H]cAMP instead of [^{14}C]cAMP to monitor recovery. Protein was determined by the method of Bradford (1979).

3.4 RESULTS

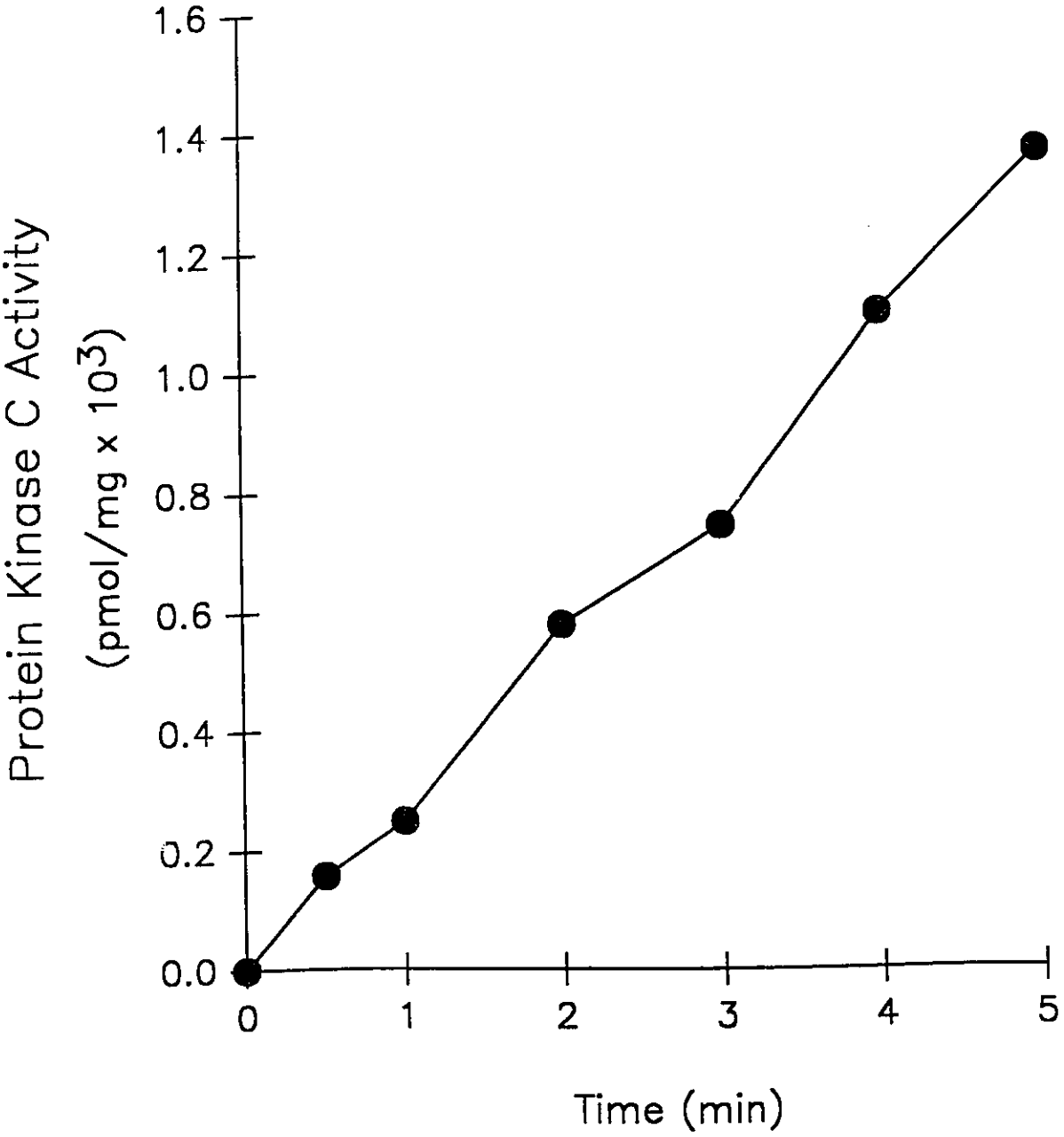
A. PROTEIN KINASE C ASSAYS

Linear kinetics could not be obtained using crude cytosolic PKC extracts. Figure 3.1 shows that following semi-purification of crude extracts on DEAE-sephacel columns, PKC activity produced a linear time course for at least 5 minutes when 5 μg of protein was used and standard conditions prevailed. The enzyme was therefore routinely assayed at 3 minutes which was well within the linear range of this curve.

As an indication of the percentage of the total protein kinase activity represented by PKC the following data are provided, calculated from two representative experiments. When assayed prior to semi-purification on DEAE-Sephacel column, the supernatant PKC specific kinase activity of untreated cells was 150 ± 30 pmol/mg/min, which represented 26% of total protein kinase activity. Following RA treatment as specified in the methods, the PKC specific activity in the supernatant fraction was estimated as 420 ± 50 pmol/mg/min accounting for 57% of the total protein kinase activity. Following semi-purification on the DEAE-Sephacel columns PKC constituted a major portion of the Histone IIIS protein kinase activity measured. For example, the supernatant PKC activity of untreated cells was estimated as 570 ± 150 pmol/mg/min which accounted for 74% of the total kinase activity. With RA treatment the PKC activity was 1,310 ± 230 pmol/mg/min and represented 83% of the total protein kinase measured. In detergent-extracted particulate fractions the PKC comprised 16 to 34% of the total protein kinase activity.

Figure 3.1 - Timecourse for Protein Kinase C Activity.

Crude cytosolic extract was semi-purified on DEAE Sephacel columns and assayed for PKC as described in the Methods. Points represent the average of 3 determinations with an error of less than 5%.


Protein Kinase C Activity
(pmol/mg x 10^3)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Time (min)

B. PROTEIN KINASE C RESPONSES TO RETINOIC ACID TREATMENT OF SH-F CELLS

Results summarized in Table 3.1 show the changes in PKC activity of the various fractions after 7 days of RA treatment. Although technical error was normally within 5%, specific activities were quite variable from one experiment to the next. The observed effects, however, were qualitatively, highly consistent. The cytosolic, EDTA-extractable PKC activity displayed a marked 2.3-fold increase after 7 days of RA treatment. This RA effect was also found in the particulate fraction which showed on the average, a 3.2-fold increase in PKC activity over controls. The distribution of PKC between supernatant and particulate fraction was however not significantly altered by RA treatment. Measurement of total cellular PKC, again, gave the very pronounced increase in activity reflecting that of the cytosolic and particulate fractions. The results from Table 3.1 clearly indicate that cells treated for a minimum of 7 days display a striking increase in PKC activity.

The RA concentration of 3 X 10^{-6} M reported previously (Sidell et al., 1986) was used throughout this study because it gave the most consistent results. The morphological effects as well as the changes in PKC activity were also observed at lower RA concentrations (results not shown). A similar 2.5-fold increase accompanied by the typical morphological changes was observed at 10^{-7} M RA. At 10^{-8} M a 25% increase was detectable with some flattening of the cells.

TABLE 3.1

In Vivo Effects of Retinoic Acid on Protein Kinase C Activity of SH-F Cells

Cellular Fractions	Control pmol/mg/min	Retinoic Acid pmol/mg/min
Cytosolic *n=6	488 ± 147 (94.4 ± 2.6)	1,150 ± 252 (96.4 ± 2.5)
Particulate *n=3	45 ± 4.9 (5.6 ± 2.6)	140 ± 11 (3.8 ± 2.6)
Total *n=6	211 ± 36	1,070 ± 119

Values are the means ± S.E. with triplicate determinations of samples semi-purified by DEAE chromatography. The number of experiments is indicated by n.
*P < .01 as determined by paired t test analysis.
Values in parenthesis represent the percent distribution of the PKC between membrane and cytosol calculated from 3 experiments with triplicate determinations.

In order to determine how early this change in PKC activity was occurring, a time course of the RA treatment was carried out. Figure 3.2 shows that the increase in activity could be detected as early as 2 days after treatment and increased steadily up to 6 to 7 days after which time it levelled off. No changes prior to 48 hours were detectable.

To investigate whether or not RA might have a direct effect on the enzyme, this agent was added at concentrations ranging from 10^{-9} to 10^{-4} M in vitro. Table 3.2 shows that there were no significant changes in PKC activity at any concentration up to 10^{-5} M however, at 10^{-4} M a consistent 25% increase over control activity was measured. This concentration of RA was far greater however, than that required to engender changes in PKC activity levels in vivo.

C. EFFECT OF RETINOIC ACID ON PROTEIN KINASE C OF SH-N CELLS

After the PKC responses to RA treatment were established in the SH-F cell line, the behavior of the subline, differentiating into the neuronal phenotype, became a point of interest. Table 3.3 reveals that both cytosolic and membrane PKC were decreased substantially, i.e., by about 50-60% compared to control. The data show that neuroblastoma giving distinct phenotypic responses to RA treatment, also respond differently with respect to PKC activity.

D. EFFECT OF STAUROSPORINE ON NEUROBLASTOMA DIFFERENTIATION

To gain more insight on the role of PKC in cell differentiation, the use of the

Figure 3.2 - Time course of retinoic acid treatment on total protein kinase C activity of SH-F cells.

Values are expressed as means $\pm$ SD of triplicate determinations, representative of 2 experiments showing similar results.

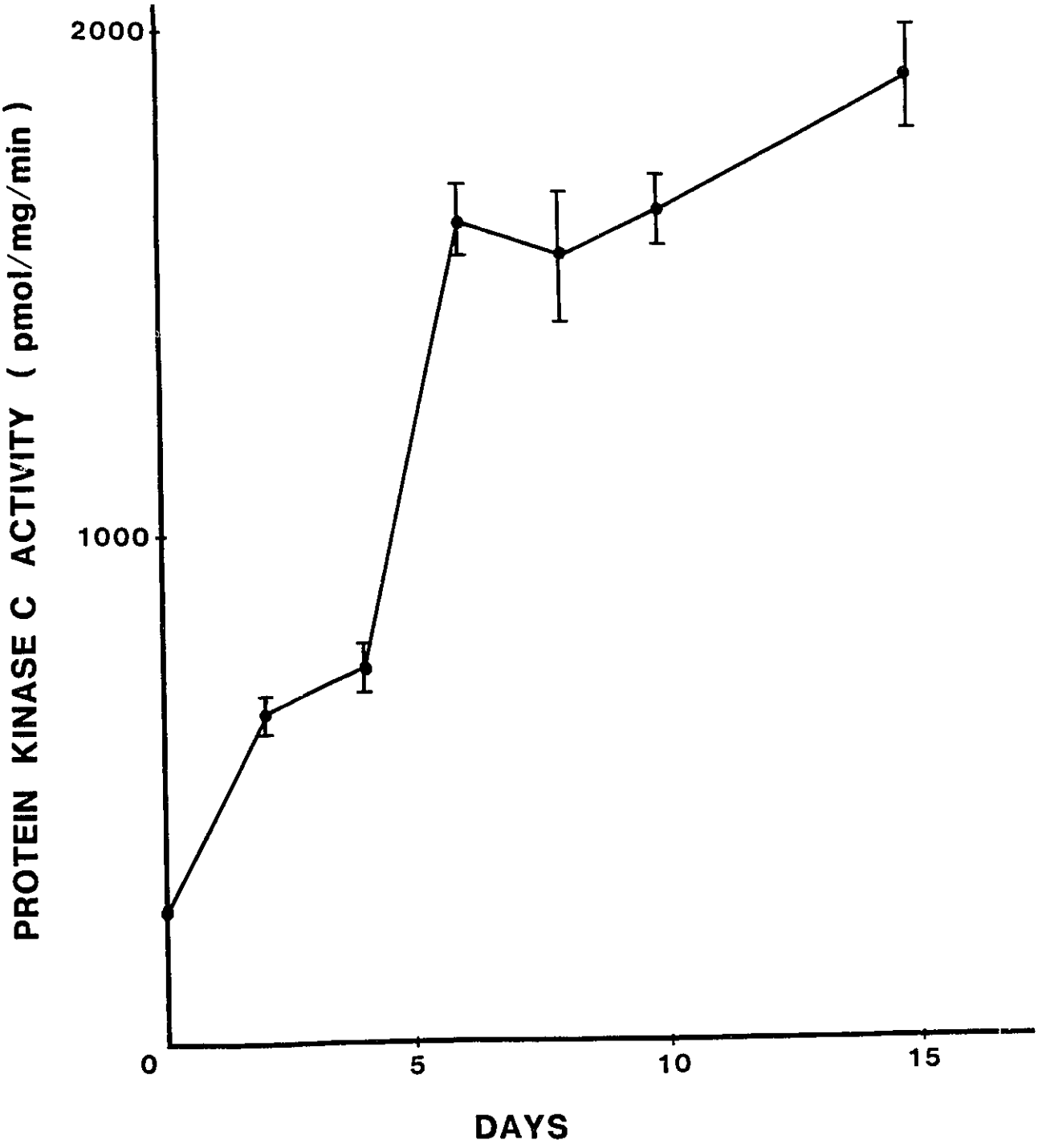

2000
1000
0
5
10
15
PROTEIN KINASE C ACTIVITY (pmol/mg/min)
DAYS

TABLE 3.2

Response of Protein Kinase C to Retinoic Acid Added to DEAE-Sephacel Semi-purified Cytosolic Fractions of SH-F Cells

Retinoic Acid Concentration (M)	Protein Kinase C Activity (Percent of Control)
10^{-9}	102 ± 3
10^{-6}	115 ± 1
10^{-5}	118 ± 1
10^{-4}	127 ± 1

Values are means ± S.E. of triplicate determinations from one experiment, representative of three yielding similar results. For controls average PKC activity was estimated as 1.040 nmol/mg/min ± 0.086.

TABLE 3.3

In Vivo Effects of Retinoic Acid on Protein Kinase C

Activity of SH-N Cells

Cellular Fraction	Control pmol/mg/min	Retinoic Acid Treated pmol/mg/min
Cytosolic	10,720 ± 4,014	5,186 ± 1,447
*n=6	(93 ± 1)	(93 ± 5)
Particulate	5,083 ± 486	1,832 ± 845
n=2	(7 ± 1)	(7 ± 5)

Cytosolic PKC activities are represented as mean ± S.E.
*Significant difference between control and experimental groups as determined by paired t analysis. $P \leq .05$
Particulate fraction values represent mean activities ± range
Values in parenthesis represent the percent distribution of the PKC between particulate and cytosolic fractions, calculated from two experiments.
Total extracts were not assayed with SH-N cells.
n refers to the number of experiments with triplicate analyses of each sample

PKC inhibitor, staurosporine, was made. Before undertaking the morphological studies that follow, we established conditions under which the action of this drug in vivo was effective without having any major effect on protein kinase A. Figure 3.3 shows dose response curves demonstrating the effect of staurosporine on PKC and protein kinase A. It is evident that PKC is far more sensitive to lower concentrations of inhibitor than protein kinase A. At 10 nM, the former enzyme is inhibited by greater than 50% while the latter is only slightly so. Again at 15nM the PKC is inhibited by 70% while the protein kinase A still retains 80% of the activity found in control cells. It was concluded from this that if low concentrations of staurosporine are used, in the range 10-15nM, a substantial decrease corresponding to a 50-70% inhibition of PKC can be induced without causing much change in protein kinase A. Table 3.4 shows that although a striking 189% increase in PKC is seen with RA, staurosporine at 10 or 15 nM not only prevented this increase but caused a sizeable drop in PKC activity. Assessment of cell viability by trypan blue exclusion revealed that staurosporine may be used at concentrations of up to 20nM without adversely affecting cell viability.

Results illustrated on Figure 3.4 reveal the effects of staurosporine on morphological differentiation of SH-F and SH-N. Consistent with previous studies (Sidell et al., 1986), SH-F treated with RA differentiates to form flattened fibroblast-like cells (compare Figures 3.4a and 3.4c). As the results from Table 3.1 show, these flattened cells have an elevated PKC activity. In contrast, the SH-N cells, descendents of the same parent line, when treated with RA differentiate to

Figure 3.3 - Effect of staurosporine on protein kinase activity in vivo.

SH-F cells were treated with increasing concentrations of staurosporine for two days, after which time, EDTA-extractable PKC, and protein kinase A, as described in the Methods, was measured. Variation did not exceed 10% for PKC and 15% for protein kinase A. Points represent the average of two experiments determined in triplicate. (▫) PKC, (●) PKA.

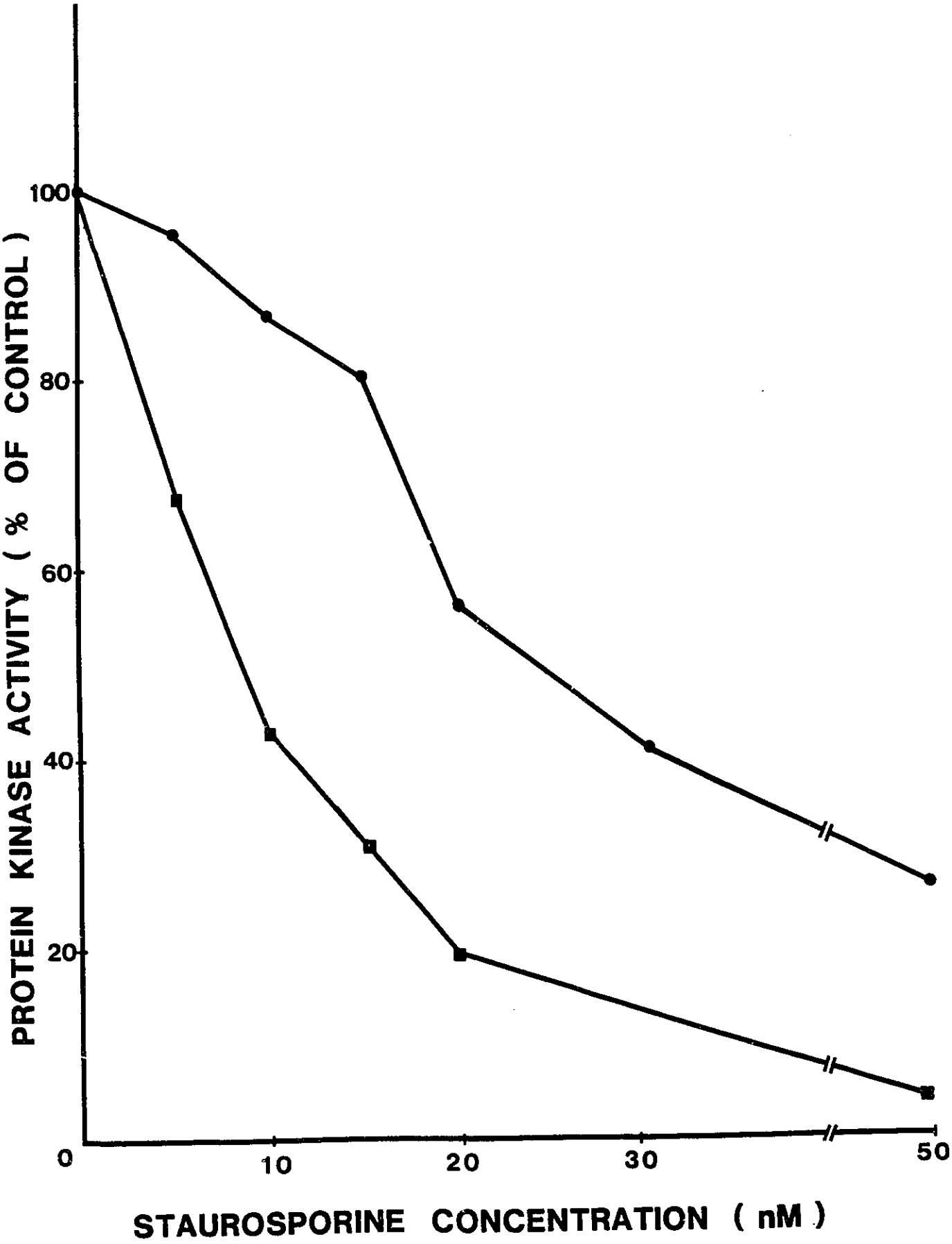

PROTEIN KINASE ACTIVITY (% OF CONTROL)
100
80
60
40
20
0
10
20
30
50
STAUROSPORINE CONCENTRATION (nM)

TABLE 3.4

In Vivo Effects of Staurosporine on

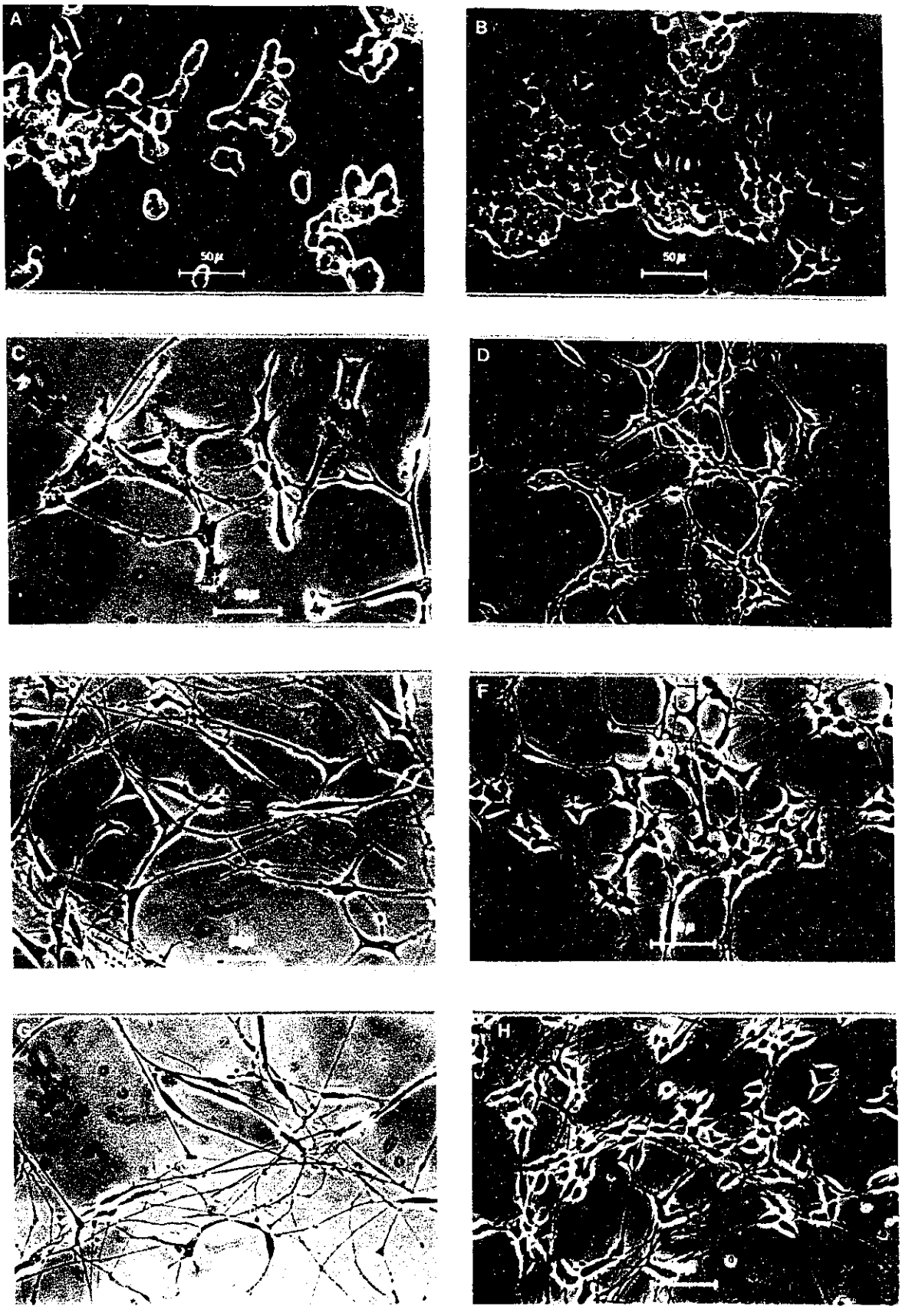
Total Protein Kinase C Activity of SH-F Cells

Sample	Activity (pmol/mg/min)	% of Control
Control	1294 ± 34	100
RA-Treated	2451 ± 56	189
RA-Treated + 10 nM SSP	74 ± 74*	6
RA-Treated + 15 nM SSP	30 ± 30*	2

Cells were treated with either 3 x 10^{-6}M RA or 3 x 10^{-6}M RA + staurosporine for 6 days. Values represent means ± deviation from mean of 2 separate experiments with triplicate determinations of each sample. *No PKC activity was detectable in the second determination.

Figure 3.4 - Effect of retinoic acid and staurosporine on the morphology of two neuroblastoma SK-N-SH sublines.

The cells were examined under phase contrast. SH-F(a) and SH-N(b) cells treated with vehicle only: SH-F(c) and SH-N(d) treated for 7 days with 3 x 10^{-6} M RA: SH-F(e) and SH-N(f) treated for 7 days with 15nM staurosporine. SH-F(g) and SH-N(h) treated for 7 days with 15mM staurosporine and 3 x 10^{-6} M RA.


A
50μ
B
50μ
C
D
E
F
G
H

form a neuronal cell type (Figures 3.4b and 3.4d). In this cell line differentiation is accompanied by a decrease in PKC activity (Table 3.3). Addition of 15nM staurosporine alone (Figure 3.4e), or together with RA to the SH-F variant, (Figure 3.4g), results in a different morphology. Such treated cells no longer resemble the RA-differentiated SH-F cells seen in figure 3.4c. Their bodies, from which protrude more elaborate extensions, are much smaller and are no longer flat. SH-F cells treated with RA and staurosporine, where the PKC activity is decreased, are closer in appearance to the SH-N cells that differentiate to produce the neuronal phenotype as opposed to the enlarged, flattened cells produced by RA alone. If SH-N cells are treated with 15nM staurosporine, a morphology similar to that of RA-differentiated cells is observed (Figure 3.4f). When RA and 15 nM staurosporine are used together, again, the same neuronal cell type is seen (Figure 3.4h). These responses show that a similar morphology can be produced by treatment of SH-N with the PKC inhibitor as with RA. Both of these agents cause a decrease in PKC activity in this cell line.

These studies, in which the PKC response to RA is deliberately altered by use of staurosporine, show that in SH-N, the differentiated morphology can be mimicked by staurosporine, and staurosporine and RA together. Conversely, in the cells in which PKC is normally elevated in response to RA, treatment with staurosporine which prevents the increase in PKC, can prevent the formation of the flattened fibroblast-like cell type and produce instead a phenotype that is similar to the neuronal cell type seen in the related SH-N cell line.

E. MEASUREMENT OF STEADY STATE LEVELS OF mRNA OF PROTEIN KINASE C ISOZYMES

Since it is known that PKC is a family of enzymes (Coussens et al., 1986) the following experiments were carried out to determine if the observed PKC activity changes accompanying RA differentiation are a result of alterations in the expression of the mRNA for the PKC. RNA was extracted in two separate experiments, each yielding two samples from control and RA-treated cells.

The message for PKC alpha was not detectable on Northern blots prepared with total RNA therefore isolation of Poly A+ RNA was necessary. Northern and slot blots were screened with probes for human PKC alpha, beta (c),(recognizing both beta I and II) and gamma. Screening of these blots at high stringency showed hybridization with PKC alpha only. It appears therefore, that both SH-F and SH-N cell lines express only the PKC alpha before and after differentiation. This is consistent with the fact that this tumor originates from the peripheral nervous system (Beidler et al., 1973).

Size verification of mRNA for PKC alpha was determined by Northern analysis (results not shown). Based on distance of migration of 18S and 28S ribosomal RNA, which were used as standards, a band of 8,000 base pairs was detected. This corresponds to the mRNA for the larger species of PKC alpha. The hybridization patterns for PKC alpha has been previously shown to give three species of mRNA i.e., at 8.1, 3.8 and 3.0 kb (Coussens et al., 1986). Northern blots published by Coussens et al. (1986) show that the 8.0 kb species is the most abundant

while the mRNA's for PKC 3.8 and 3.1 species are present at much lower levels. Due to the low levels of PKC alpha message slot blots were used for measurement of relative amounts of mRNA as Northern blots had high levels of background hybridization. Figure 3.5 shows that there were no obvious differences in intensity of these slots. Density scanning of slot blots also showed no marked differences in levels of PKC alpha message. Although striking changes in enzyme activity are observed following RA induced differentiation of SH-F and SH-N, these changes do not appear to be reflected in the expression of the PKC alpha gene.

F. EFFECT OF RETINOIC ACID ON PROTEIN KINASE A

Although PKC is more sensitive to inhibition by staurosporine, the data clearly show that protein kinase A is also affected by this compound. While the inhibition of the latter protein kinase is quite small, i.e., 20% inhibition at 15 nM, one cannot rule out the possibility that this may also contribute to the observed staurosporine effect. It was therefore decided to examine the effect of RA on protein kinase A in these neuroblastoma. A linear time course for at least 5 minutes with 25 μg of protein was obtained when a crude cell extract was used in the assay mixture. For activity comparisons with RA treated and control cells, the extracts were assayed for 3 minutes with 20 μg of protein per tube. Table 3.5 shows that following 7 days of RA treatment there is a significant increase in protein kinase A activity in both the SH-N and SH-F cell lines. This augmentation is, on the average, similar in both cell lines where an approximate 1.7-fold increase of activity is seen. This effect was detectable following two days of treatment and remained constant for at least seven days.

Figure 3.5 - Slot blot analysis for relative amounts of PKC alpha mRNA.

Slot blots 1 and 2 represent RNA from 2 separate experiments. Each slot contains 5μg of RNA from separate groups of cells. Blot #1 was probed with the insert for PKC alpha with 3.5 X 10^7 cpm and exposed for 8 days. Blot #2 was probed with the same insert as blot one with 1.0 X 10^7 cpm and exposed for 4 days. The slots and scan units (% of maximum; where band with maximum density was designated as 100%) are labelled as follows:
slot blot #1: A)SH-F, control 70; B)SH-F, control 78; C)SH-F, RA treated 81; D)SH-F, RA treated 86; E)SH-N, control 100; F)SH-N, RA treated 80.
slot blot #2: A)SH-F, control 73; B)SH-F, control 75; C)SH-F, RA treated 80; D)SH-F, RA treated 87; E) SH-N, control 81; F)SH-N, control 98; G)SH-N, RA treated 100.

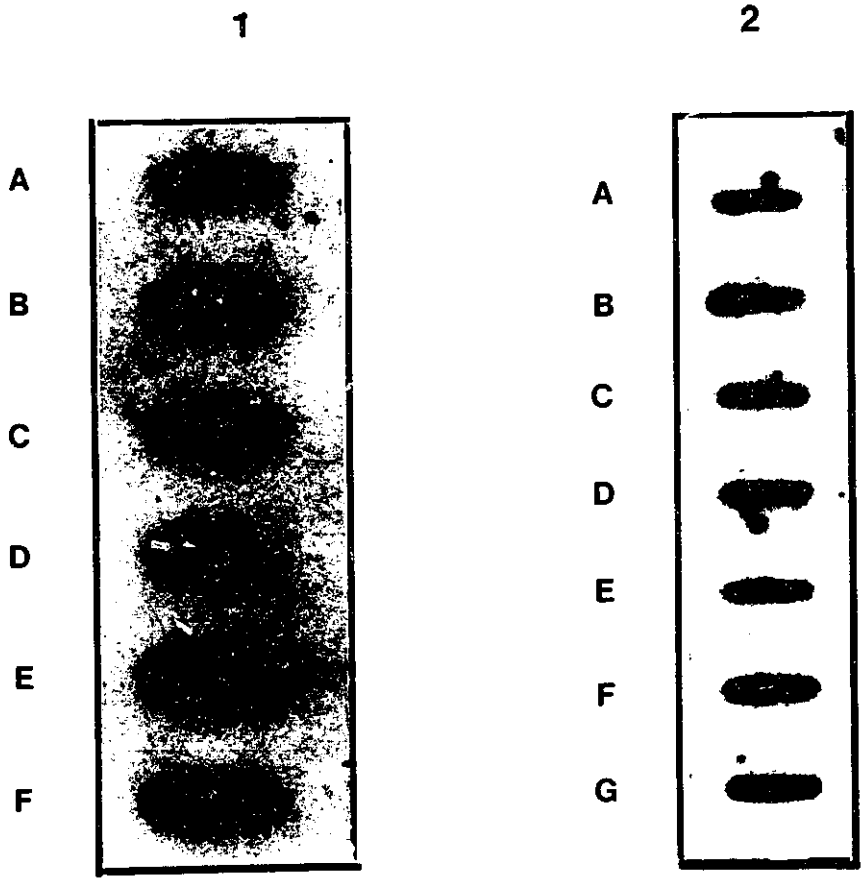

1
2
A
B
C
D
E
F
A
B
C
D
E
F
G

TABLE 3.5

In Vivo Effects of Retinoic Acid

on Total Protein Kinase A Activity

Cell Line	Control pmol/mg/min	Retinoic Acid Treated pmol/mg/min
SH-F n=7	20.5 ± 1.8	34.8 ± 3.0
SH-N n=7	24.2 ± 1.7	41.5 ± 6.0

Cells were treated with 3 x 10^{-6} M RA or vehicle for 7 days.
Values represent means ± S.E. with n representing the number of experiments. Control and RA-treated protein kinase A activities were compared by t analysis and were found to be significant for both SH-F and SH-N. $P \leq .01$.

G. EFFECT OF RETINOIC ACID ON ADENYLATE CYCLASE ACTIVITY

As the results presented above show that RA enhances protein kinase A activity, it was decided to investigate to what extent adenylate cyclase, which produces cAMP, the activator of the protein kinase A, is affected during differentiation of these neuroblastoma. Preliminary experiments showed that 50 μg of crude membrane protein added to the incubation mixture, containing either NaF or forskolin, produced a linear curve for at least 10 minutes. A similar curve was obtained with forskolin-stimulated adenylate cyclase. This enzyme was routinely assayed for 6 minutes using 50 μg of protein per tube. Table 3.6 reveals that both SH-F and SH-N when treated for seven days with retinoic acid show enhanced adenylate cyclase activity. This increase is apparant when basal adenylate cyclase activity is measured or when the enzyme is stimulated with NaF or forskolin. Although both cell lines show augmentation, this effect is most marked in the neuronal cell type where basal, NaF-and forskolin-stimulated adenylate cyclase activities rise by more than two-fold. These results are consistent with those observed with protein kinase A, where an enhancement of this signalling pathway is seen.

H. STUDIES OF POSSIBLE INTERACTION OF THE TWO SIGNALLING PATHWAYS DURING RETINOIC ACID-INDUCED DIFFERENTIATION OF NEUROBLASTOMA

H.1 EFFECT OF PHORBOL ESTER ON ADENYLATE CYCLASE

The prelabelling assay which enables the measurement cAMP synthesis from

TABLE 3.6

Effect of RA on Adenylate Cyclase

Activity in SH-F and SH-N

		Activity (pmol/mg/6 min)		
Cell Line	**Treatment**	**Basal**	**NaF**	**FSK**
SH-F	CTL	155 ± 27	564 ± 45	1,283 ± 226
	RA	304 ± 2	737 ± 14	1,895 ± 273
SH-N	CTL	343 ± 42	1,351 ± 157	3,588 ± 121
	RA	793 ± 97	3,361 ± 576	7,461 ± 791

Values are averages of 2 separate experiments with triplicate determinations ± range.
Cells were treated with either RA or vehicle control (CTL) for 7 days prior to assay.

a prelabelled ATP pool in intact cells was employed for two reasons: a) it allows one to study the effect of phorbol ester on cAMP production without disrupting the cells; and b) positive phorbol ester effects have been reported in rat T51B cells (Aasham et al., 1989) using this same assay. It was found that under the conditions of the assay the ATP pool in these cells was approximately 1,200,000 cpm per mg protein and remained constant in control and phorbol ester-treated samples. As no stimulation of cAMP production with ß-adrenergic agonists such as isoproterenol was observed in these neuroblastoma cells the effect of phorbol ester was measured on basal and forskolin-stimulated cAMP production only. The timing of treatment was determined in preliminary experiments and showed that PKC translocation (activation) occurred within 15 minutes and persisted for at least 30 minutes following exposure to phorbol esters. By 60 minutes no PKC activity was detectable in membrane or cytosolic fractions. The results in Table 3.7 show that there is an approximate 2.5 fold stimulation of cAMP production in the presence of forskolin with both control and phorbol ester-treated cells. Based on paired t analysis, no significant differences were detected in basal or forskolin-stimulated cAMP levels with phorbol ester treatment.

As this result was negative, it was decided to re-examine the effect of phorbol ester on adenylate cyclase activity directly. Consistent with the results obtained with the prelabelling assay, Table 3.8 shows that this agent has no effect on basal, NaF-, or forskolin-stimulated adenylate cyclase activity. This confirms the result obtained by measuring cAMP production in intact cells.

TABLE 3.7

Effect of Phorbol Ester on cAMP Production in SH-F Cells (cpm/mg protein)

Treatment	Basal	FSK 10 μM
Control	1927 ± 135	4692 ± 268
TPA	2022 ± 247	5660 ± 393

Cells were prelabelled for 2 hours with ^{3}H-adenine. 200nm phorbol ester was added for 15 minutes at 37°C prior to assay. Values represent means ± S.E. of 6 separate samples. Based on paired t analysis there are no significant differences in basal or forskolin (FSK) stimulated cAMP levels between control or phorbol ester (TPA) treated cells.

TABLE 3.8

Effect of TPA on Adenylate Cyclase Activity

Sample	Adenylate Cyclase Activity (pmol cAMP/mg/min)		
	Basal	**NaF**	**FSK**
Control	252	611	1933
TPA	326	602	1821

Cells were treated with 200nm TPA for 15 minutes at 37°C prior to homogenization.
Values are the results of triplicate determinations from one experiment.
Technical error was less than 5%.

H.2 EFFECT OF cAMP ANALOGUES ON PROTEIN KINASE C

As our results do not indicate an effect of PKC activation on adenylate cyclase it was decided to pursue the concept of cross-talk further as it seemed logical that these important signalling pathways should interact. The same question was approached from a different angle, that is, by testing the effect of cAMP analogues on PKC activity. Preliminary experiments have shown that the addition of 2mM dbcAMP to SH-F cells does cause an inhibition of growth. Changes in cAMP levels could therefore be indirectly affecting PKC activity. Table 3.9 shows, that there is no significant difference in PKC activity with SH-F cells in the presence of 2mM dbcAMP or 8-Bromo-cAMP. With SH-N, the PKC activities also show very little change in the presence of cAMP analogues.

The results presented in Tables 3.7 to 3.9 show that under the conditions tested no indication of interaction between these two major signalling pathways is detectable in these neuroblastoma cell lines. The significance of this will be addressed in the discussion.

3.5 DISCUSSION

A. PROTEIN KINASE C RESPONSE TO RETINOIC ACID TREATMENT IN SH-F CELLS

The large increase in PKC activity in both particulate and cytosolic fractions when cells are treated for seven to ten days with RA, resembles that obtained by Kraft and Anderson (1983), who reported that RA treatment of embryonal carcinoma cells resulted in a marked increase in cytosolic PKC activity detectable five days after

TABLE 3.9

Effect of cAMP on Protein Kinase C

Cell Line	PKC Activity (nmol/mg/min)		
	Control	2 mM dbcAMP	1 mM 8-Bromo-cAMP
SH-F	2.82 ± 0.5	2.65 ± 0.36	3.95 ± 0.6
SH-N	9.79	12.2	12.1

Cells were treated for 7 days with the cyclic AMP analogues.
Values for SH-F are the average of 4 separate experiments with 3 determinations ± S.E.
No significant differences were detected using paired t analysis.
SH-N is the average of 3 determinations of one experiment in which the technical error was within 7%.

treatment. A similar result was also reported by Niles and Loewy (1989) with B-16 melanoma cells. In this case cells treated with 10 μM RA showed a two-fold increase in total PKC activity by 24 hours and a five-fold increase by 48 hours. These time course results would suggest a delayed effect on PKC which consequently may not be involved in controlling the early events of differentiation; however, our data with staurosporine suggest that the changes in PKC activity are important to phenotype development since inhibition of this enzyme results in a considerable change of morphology and type of differentiation.

It is also clear that the RA effects are not mediated by a redistribution of PKC to favor the membrane fraction. Results of Table 3.1 indicate that the percent of activity in the membrane fraction does not increase as a result of this treatment. This suggests that the increment in PKC activity in this particular cell type was due to either, a direct effect of RA on the enzyme or to some other mechanism resulting in a general augmentation of enzyme activity, such as elevated levels of the protein. One may add that it has been previously demonstrated that RA may directly stimulate PKC in the absence of phosphatidylserine (Okkubo et al., 1984). From the data summarized in Table 3.2, it becomes apparent that a direct effect of RA could not explain the observed increase in PKC activity. The increase in PKC activity therefore must be brought about by increased levels of enzyme, posttranslational modifications and/or changes in levels of endogenous activators or inhibitors.

B. EFFECTS OF RETINOIC ACID ON PROTEIN KINASE C IN SH-N CELLS

The effect of RA on the other neuroblastoma subline, SH-N, was also tested to determine if cell lines differentiating into the neuronal phenotype show an alternative PKC response. Although responses to RA treatment will also be defined in terms of cell type specific marker expression (Chapter 4), on the basis of changes in PKC and morphology at least, SH-N did exhibit a completely different response to this vitamin A derivative. In contrast to the SH-F variant, a marked decrease in total PKC activity was observed. This type of phenomenon is not totally unusual. Indeed, HL60 cells, when treated with RA, differentiate into the myelocytic lineage. This change is accompanied by an increase in PKC activity. The same HL60 cells, when treated with phorbol ester, show a rapid disappearance of the PKC concomitant with the appearance of the adherent macrophage type cells (Katz and Glazer, 1985; Katz et al., 1986). The results of Katz and Glazer (1985) corroborate our results demonstrating that the same, or related cells, differentiating into different phenotypes also produce different PKC responses. The data presented lead to the obvious question as to what the function of the PKC in RA-induced neuroblastoma differentiation may be.

C. EFFECT OF STAUROSPORINE ON NEUROBLASTOMA DIFFERENTIATION

To gain insight as to the role of PKC, we used the fungal alkaloid, staurosporine, which has been shown to be a potent inhibitor of this enzyme in vitro

(Tamaoki et al., 1986) as well as in vivo (Figure 3.2a). A concentration of 15nM was chosen for our morphological studies because it gave a substantial decrease in PKC activity and only a slight inhibition of the protein kinase A without affecting cell viability. Observed effects of this inhibitor therefore, are likely due to its action on the PKC. We cannot, however, completely rule out the possibility that even a small decrease in the protein kinase A may have an effect on cell differentiation and morphology, or that staurosporine may be acting by mechanisms other than inhibition of PKC, since this inhibitor affects other protein kinase activities including tyrosine kinases.

To determine if PKC played a key role in the differentiation of the SH-F cell line in which PKC was increased with RA-induced differentiation, cells were treated with RA as well as with staurosporine. The premise of this experiment was to inhibit PKC and observe changes, if any, in RA induced differentiation. If PKC played an important role in the regulation of morphological differentiation then either, a) differentiation would not have taken place, or b) the same phenotype would not have been produced. The data in Table 3.4 verify that staurosporine does in fact keep PKC activity well below control levels even in the presence of RA. The morphological results were very striking. Instead of the flattened cells that are normally produced with RA treatment, staurosporine alone or together with the vitamin A derivative caused the appearance of a different morphology resembling the neuronal phenotype, normally produced only by the SH-N variant. The results suggest that PKC activity must be inhibited for morphological maturation to the

neuronal phenotype, whereas development of the fibroblast-like phenotype in the SH-F subline requires PKC activity, at least in some aspects of the differentiation process. It must be emphasized, however, that levels of PKC are significantly higher in SH-N cells than in SH-F cells even after RA treatment and consequently absolute levels of this enzyme are presumably not relevant to phenotype expression.

Within the past year much evidence has been reported that is in good agreement with the results presented herein and strongly uphold the hypothesis that PKC inhibition is required for neuronal differentiation. 1-5(Isoquinolinylsulfonyl-2-methyl)piperazine (H-7), a well known PKC inhibitor, has been shown to cause neurite outgrowth and increased acetylcholinesterase activity in Neuro-2A cells (Felipo et al., 1990). Similar results have been reported with other neuroblastoma cell lines such as SK-N-BE2, Lan-1, and Lan-5 (Parodi et al., 1990). Other PKC inhibitors such as gangliosides have been found to exert similar effects on Neuro-2A cells (Minana et al., 1990). Treatment of PC12 cells with staurosporine induced neurite outgrowth at a much more rapid rate than with nerve growth factor or dbcAMP. Interestingly, it was found that, unlike neuritogenesis caused by nerve growth factor, the effect of staurosporine was not blocked by actinomycin D, which means that changes at the level of the regulation of gene transcription were not required (Hashimoto and Hagino, 1989). The SH-N cell line also undergoes neuritogenesis more rapidly in response to SSP than with RA, suggesting that SSP must be by-passing early steps required to bring about RA-induced differentiation.

The use of phorbol ester as an agent to reduce PKC levels has produced

contradictory results. In additional preliminary experiments with SH-N or SH-F cells, it was found that this inhibitor did not reproduce the morphological effects of staurosporine. Similarly, phorbol ester did not mimick the differentiation normally caused by H-7 in Neuro-2A cells (Minana et al., 1990). In other neuroblastoma however, such as SH-SY5Y, both phorbol ester and H-7 promote neuritogenesis (Heikkila et al., 1989). Clearly, biological variation exists. In some cell types phorbol ester and H-7 act similarly, whereas in other cell types they do not. In consideration of these data it is important to realize that phorbol ester does not act in the same inhibitory manner as H-7 or staurosporine. Heikkila et al. (1989) describes phorbol ester-induced differentiation of SH-SY5Y involving two phases: 1) PKC activation; and 2) PKC down regulation. PKC inhibitors do not cause activation prior to down regulation. One may speculate that following phorbol ester treatment, events occurring during the activation phase prior to down regulation may inhibit neuritogenesis in certain neuroblastoma cell lines.

D. EXPRESSION OF PKC ISOZYMES

As striking changes in enzyme activity accompany RA-induced differentiation, it was of interest to characterize this system further in order to define which of the PKC isozymes are expressed in these cells. Our data show that neuroblastoma cell lines, SH-F and SH-N, express PKC alpha only. There was no hybridization with beta(c) and gamma DNA probes. These cells were not screened for the novel PKC isozymes: delta, epsilon, and zeta, as no cDNA's from human libraries are presently available (Ono et al., 1987; Kikkawa et al., 1989). The expression of a single isozyme

in cultured tumor cells is not uncommon as rat C6 glioma and NIH 3T3 cells also express PKC alpha only (Young et al., 1987). PKC alpha is found universally (Kikkawa et al., 1989) therefore the presence of this isozyme in these cell lines was expected. PKC gamma is expressed solely in the brain and spinal chord being at highest levels in the cerebrum (Nishizuka, 1989). Being of periferal origin, (Biedler et al., 1973) these neuroblastoma cell lines were not expected to express gamma. PKC beta I and II are highly expressed in brain and lymphoid cells (Nishizuka, 1989; Wada et al., 1989); however, no hybridization to beta was detected in our cell lines. This agrees with the results of Wada et al. (1989) who found no hybridization with PKC beta in the neuroblastoma cell line, Neuro-2A. This cell line, however, being of murine origin, was also found to express the novel isozymes PKC epsilon, and zeta.

As determined by slot blot analysis, no changes in steady state levels of PKC alpha mRNA were detected after RA treatment of SH-N or SH-F. It should be stated, however, that low levels of this message in these cell lines necessitated the use of high levels of probe and long development times of five days or more. This resulted in extremely high levels of background in northern blots and may have contributed to the higher intensity of slot blots. Such an artifact may have obscured changes in expression of a mRNA, such as PKC alpha, which is expressed at low levels. The use of a more sensitive technique may have been more conclusive in these experiments. Wada et al. (1989) report down regulation of the PKC isozymes after Neuro-2A differentiation induced by 8-Br-cAMP and the ganglioside, GM1,

based on Northern hybridization. Due to low levels of PKC alpha mRNA, Northern blots presented by this group had extremely high levels of background hybridization and bands were difficult to discern. As we encountered similar difficulties with PKC message, Northern analysis was used for size verification only, and levels of mRNA were measured by slot blots. Although nonspecific hybridization may have contributed significantly to the intensity of these signals, this method provided clear bands which were readily quantified by densiometric scanning. Based on these studies, large differences in PKC expression following RA treatment can be discounted. However, fine decreases in mRNA levels cannot be precluded at this time.

E. REGULATION OF PROTEIN KINASE C

As evidence accumulates it appears that the regulation of the PKC is a very complex process. Aside from the well documented mechanism of PKC activation through receptor-mediated release of calcium, diglyceride and other lipids, such as arachidonic acid, (Nishizuka, 1989) activity changes can be affected at many other levels. While this does not seem probable with the neuroblastoma used presently, changes at the level of gene expression have been reported in other cases such as in the vitamin D induced differentiation of HL60 cells (Obeid et al., 1990). Modulation of protein levels provides for yet another level of regulation where changes in synthesis or degradation can alter the amount of PKC protein. The PKC is an interesting example of an enzyme for which proteolytic degradation appears to play a key role in its regulation (Young et al. 1989). More recently it was found that

proteolysis of PKC by the calcium neutral protease, calpain I, not only altered the levels of the protein but was also a requirement for the terminal differentiation of MEL cells (Melloni et al., 1989).

Posttranslational modification adds a further dimension to the control of PKC activity. It has recently been found that activators of phospholipase C or PKC lead to an enhanced phosphorylation state of the kinase itself. Autophosphorylation of the PKC at multiple sites on serine and threonine residues has been correlated to increases in protein kinase activity (Mitchell et al., 1989). It appears that changes in the phosphorylation state of this enzyme are highly regulated thereby providing a further mechanism of control of PKC activity.

Recently several peptide PKC inhibitors have been reported. The best characterized so far is the 13,690 dalton protein kinase C inhibitor - 1 (PKCI-1) protein which has been detected in bovine, murine, avian and human tissues (McDonald et al., 1987; Pearson et al., 1990). This protein does not function as a phosphatase, or compete with any of the known cofactors for the PKC. Rather this peptide binds PKC with high specificity and has been shown to inhibit PKC specific processes. In bovine brain it was reported that there is sufficient PKCI-1 to inhibit the total cellular PKC activity (Pearson et al., 1990). As this protein has not been found to inhibit any other kinase, it, and perhaps several others, may play an important physiological role in the the regulation of PKC activity. Once cDNA's and antibodies become available for these proteins it would be of interest to determine if their expression is elevated during neuronal differentiation. Changes in the levels

of these endogenous inhibitors could certainly contribute to the observed inhibition of PKC activity in SH-N cells.

Natural PKC inhibitors other than proteins have also been reported. Many cationic amphipathic compounds have been shown to act as potent inhibitors and likely play important roles in controlling PKC activity (Rando, 1988). An example of this are the sphingoid bases, such as sphingosine, which are natural inhibitors of protein kinase C. Their mechanism of action is thought to involve interaction with the regulatory domain of the PKC and competitive inhibition with the positive activators of this enzyme (Rando, 1988; Merrill et al., 1986; Wilson et al., 1986; Hannun et al., 1986).

The regulation of protein kinase C activity within cells, therefore, is an extremely complex process and can be altered at many levels: a) steady-state levels of mRNA, b) protein synthesis or c) degradation, d) posttranslational modification, e) peptide modulators, f) receptor mediated activation of phospholipase as well as g) other changes in the lipid environment. Excluding changes in the levels of mRNA, in the system described herein, any one or several of the mechanisms described above could be responsible for the changes in PKC activity observed in these cells.

F. EFFECT OF RETINOIC ACID ON PROTEIN KINASE A

Few studies have been carried out in search of changes in PKA activity in RA-induced neuroblastoma differentiation. In the present studies both SH-F and SH-N variants show significant increases in cytosolic protein kinase A activity after at least 24 hours of RA treatment. This response is similar to that seen in B16 melanoma

(Ludwig et al., 1980), and F9 embryonal carcinoma (Plet et al., 1982). The role of the protein kinase A in this process remains elusive, however, it has been shown that increases in protein kinase A activity in response to RA treatment are correlated to the levels of CRABP (Plet et al. 1987). Although the neuroblastoma variants described above differentiate into alternative phenotypes, their levels of CRABP remain similar (Sidell et al., 1986). Acordingly, these variants show similar changes with respect to protein kinase A activities. Whether increases in this kinase are required to mediate the RA effects through CRABP is presently unknown, although several important functions have been attributed to protein kinase A.

Protein kinase A is thought to function in neurotransmitter and neuropeptide synthesis at several levels. This kinase regulates the activity of the catecholamine-synthesizing enzyme, tyrosine hydroxylase by phosphorylation, leading to its activation (Meligeni et al., 1982). In other systems, such as neuropeptide biosynthesis, protein kinase A has been shown to play important roles in gene expression. The somatostatin gene is positively regulated by cAMP (Montminy and Bilezikjian, 1987). It has been demonstrated that transcription of this gene requires the phosphorylation of a nucleoprotein by protein kinase A. The phosphorylated protein then binds a conserved 30 nucleotide sequence known as the cAMP response element and initiates the transcription of the somatostatin gene (Montminy and Bilezikjian, 1987). Protein kinase A may also be acting at the level of gene expression in RA-induced differentiation. Studies with antisense RNA to block the expression of either the catalytic or regulatory subunits of protein kinase A during RA treatment would help

define the role of this enzyme in RA-induced neuroblastoma differentiation.

G. EFFECT OF RETINOIC ACID ON ADENYLATE CYCLASE

Because elevated protein kinase A activities were observed upon RA-induced differentiation, and this kinase is the only known receptor for cAMP (Taylor et al., 1990) it was of interest to measure activity of adenylate cyclase in this system. As is seen with protein kinase A, data clearly showed that adenylate cyclase activity is augmented in both cell variants following RA-induced differentiation. Changes in sensitivity of this enzyme do not appear to be the case since forskolin- and NaF-stimulated as well as basal adenylate cyclase activities are all enhanced. These results suggest that the entire enzyme complex is augmented, perhaps by increased levels of the enzyme. The results with adenylate cyclase and protein kinase A suggest that this pathway is 'stepped up' following RA- induced differentiation.

An increase in adenylate cyclase activity does not appear to be a universal phenomenon in the differentiation of neuroblastoma. While addition of cAMP elevating agents, such as dbcAMP, was found to cause decreased proliferation and increase morphological differentiation, this same effect can be brought about by agents which do not alter intracellular cAMP levels (Lando et al., 1990). In fact, a LAN-5 neuroblastoma subline with reduced sensitivity to RA exhibited little change in cAMP levels following RA treatment. It appears therefore, that neuroblastoma differentiation can take place by multiple biochemical pathways, depending on the differentiating agent tested, as well as the cell line under investigation.

H. CROSS-TALK BETWEEN THE SIGNAL TRANSDUCTION PATHWAYS

As both pathways studied in this chapter are important in mediating cellular events, it seemed logical that such pathways operate in a co-ordinated fashion. Cross-talk, however, could not be demonstrated in these neurotumor cells. Unlike in rat T51B ceils (Aasheim et al., 1989), phorbol ester had no effect on forskolin-stimulated adenylate cyclase activity. Hollingsworth and Daly (1987) employed a neuroblastoma - brain explant hybrid for their studies. In this neuronal system, phorbol ester also had no effect on basal or forskolin-stimulated adenylate cyclase activity; however, ß-adrenergic receptor stimulation was attenuated by phorbol ester. As our cell lines did not exhibit functional beta receptors this interaction could not be demonstrated.

No significant changes in PKC activity were detected following treatment of these cells with cAMP analogues. This suggests that cAMP mediated events do not effect the activity of the PKC in these cultured cells.

Although the results reported here show no evidence of cross-talk between these two signal transduction pathways, this phenomenon has been demonstrated in other systems (Aasheim et al. 1989; Hollingsworth et al., 1987; Rozengurt et al., 1987; Sugden et al., 1985). One reason that no interaction was detected could be due, in part, to the fact that these experiments are carried out with tumor cells. Tumor cells are transformed and therefore harbour one or more metabolic defects that may interfere with cross-talk between these pathways.

3.6 CONCLUSIONS

In fulfillment of the objectives set out for this chapter the following conclusions can be made:

1) Cytosolic and particulate PKC activity is increased in the non-neuronal variant upon RA induced differentiation.

2) Cytosolic and particulate PKC activity is decreased upon RA induced differentiation to the neuronal phenotype

3) Staurosporine, which inhibits PKC, prevents formation of the enlarged flattened cells in SH-F and causes formation of the neuronal phenotype in both variant cell lines.

4) Both cell lines express PKC isozymes alpha, whose steady state levels of mRNA appears to remain unchanged before and after differentiation.

5) Both SH-F and SH-N respond similarly to RA treatment with respect to adenylate cyclase and protein kinase A activities. An increase in both enzyme activities occurs following RA-induced differentiation.

6) No interaction between these pathways could be demonstrated in these neuroblastoma cell lines.

On the basis of the results presently reported, one can correlate different PKC responses with alternative phenotypes. This correlation in itself strongly implicates a function for the PKC in phenotype development in neuronal differentiation. Our hypothesis that, 'PKC inhibition is instrumental in neuronal differentiation', was tested with the use of a potent PKC inhibitor, staurosporine. The results described

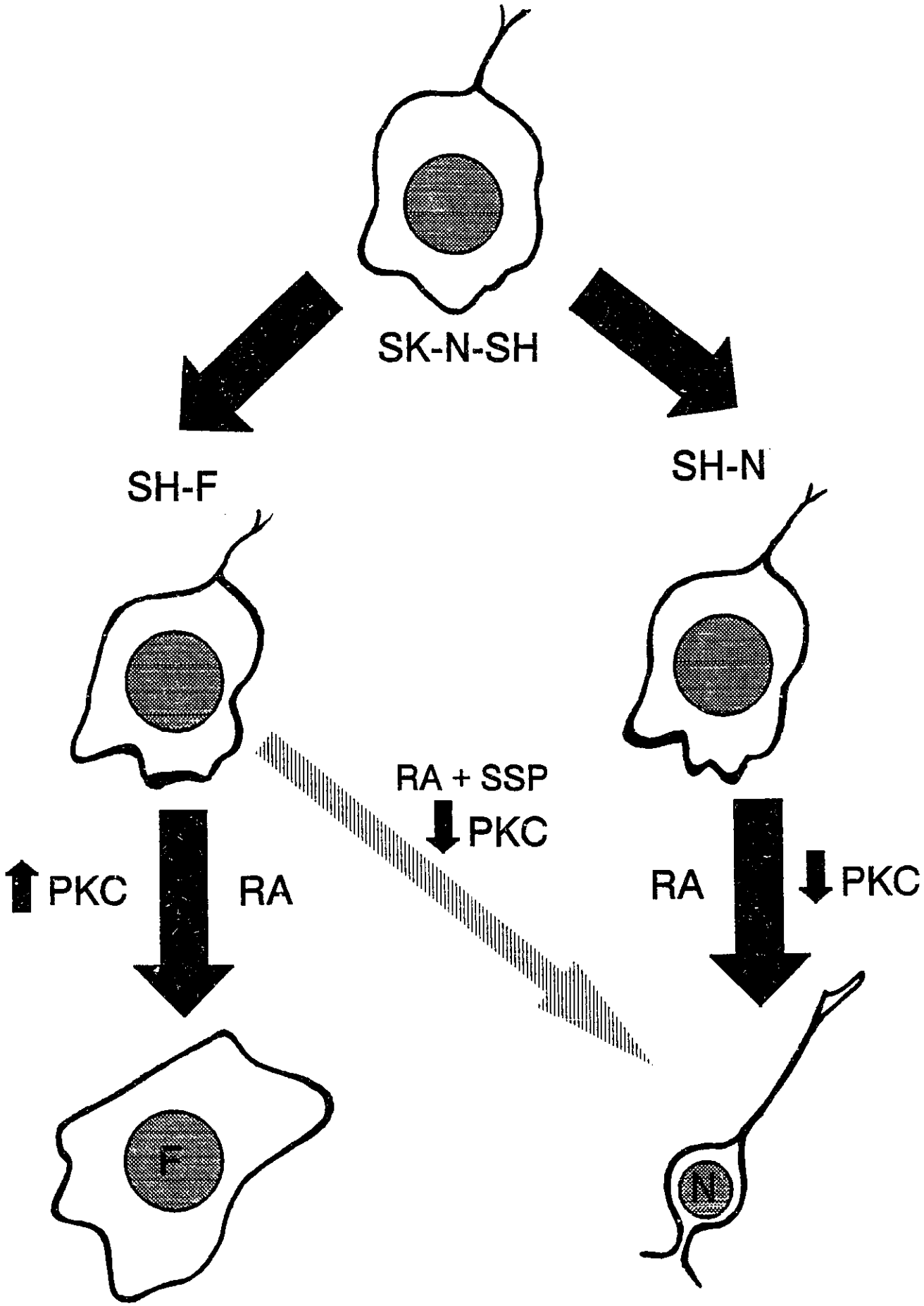
in this chapter together with the recent results reported by others with other cell lines (cited above) provide strong support for this hypothesis. The treatment of SH-F with staurosporine, which prevented the increase in PKC activity, appeared to change the direction of the differentiation pathway of this variant.

Whereas recent studies strongly implicate a role for the nuclear retinoic acid receptors in the mechanism of RA induced differentiation, (Petkovich et al., 1987), staurosporine does not appear to require transcription to exert its effect. (Hashimoto and Hagino 1989) This agent, therefore, may initiate differentiation much faster than RA. As staurosporine likely 'short circuits' the RA effect by acting on the PKC directly, its action becomes apparant much earlier than those of RA. The proposed hypothesis arising from the results of these studies are summarized in Figure 3.6.

Although the data presented offer strong support for the hypothesis that PKC is involved in phenotype development, assessment of phenotype is based entirely on morphological studies. Morphology is insufficient to confirm the resultant phenotypes, in the following chapter therefore, we characterize these phenotypes based on cell-type specific markers and ultrastructural features. These data will provide further evidence in favor of the proposed hypothesis.

Figure 3.6 - Proposed hypothesis for the role of PKC in phenotype development.

SH-F and SH-N variant cell lines arise from a common precursor cell. In response to RA, SH-F produces a flattened fibroblast-like cell, in which PKC activity is increased. In contrast, SH-N forms a neuronal-like phenotype, in which PKC activities are depressed following RA treatment. Treatment of SH-F with RA together with a PKC inhibitor, staurosporine, results in the acquisition of a phenotype resembling that of differentiated SH-N. This suggests that PKC inhibition is involved in development of a neuronal cell type.


SK-N-SH
SH-F
SH-N
RA + SSP
PKC
PKC
RA
RA
PKC
F
N

CHAPTER 4 - PHENOTYPE CHARACTERIZATION OF DIFFERENTIATED SH-F AND SH-N CELL LINES

4.1 INTRODUCTION

Neuroblastoma is a tumor which originates from the neural crest (Machin, 1982). The cells of this embryonic structure begin their migration during closure of the neural tube. The fate of these neuroblasts is dependent on the pathway of their migration and the environment produced by surrounding cells (Theiry et al., 1985; Edelman, 1986). These cells give rise to most peripheral neurons, glia, melanocytes, Schwann cells, and ectomesenchyme which includes meninges, as well as skeletal and connective tissues of the head and face (LeDouarin et al., 1982; Thiery et al., 1985). Studies with neuroblastoma cell lines have demonstrated that they may undergo differentiation in several different pathways in culture, reminiscent of those seen in neural crest development in vivo (Tsokos et al., 1987). For example, cultured cell lines such as those derived from SK-N-SH, and SK-N-BE, demonstrate both neuronal as well as melanocytic and schwannian differentiation. More interestingly however, karyotype analysis and studies with clonal sublines have demonstrated that these differentiated phenotypes may originate from the same precursor cell (Ross et al., 1983; Rettig et al., 1987; Biedler et al., 1987). Studies with cell type specific markers have confirmed the occurrence of both neuronal and those described as 'non-neuronal', or 'substrate adherent' cells, from the same clonal subline (Ciccarone et al., 1989; Ross et al., 1987). It has therefore been suggested that some cultured

neuroblastoma may be multipotent.

Characterization of multiple phenotypes produced by these cultured cells can be carried out in several ways. Ultrastructural examination can reveal certain morphological characteristics typical of specific cell types. For example, neuronal cell types can be identified by the characteristic cytoskeletal arrangements in processes, and the presence of neurosecretory granules (Peters et al., 1976). Immunohistochemical methods can be exploited to identify specific marker proteins, which cannot be readily recognized by electron microscopy, and thereby contribute further data for phenotype identification. Antibodies against certain markers are routinely used to identify cell types.

The S100 protein is a soluble, acidic, calcium-binding polypeptide which may exist as a homo- or heterodimer, consisting of alpha and/or beta subunits of about 10.5 kD (Moore, 1965; Isobe et al., 1981). This protein is expressed at high levels in Schwann and glial cells where it may account for two percent of the soluble protein, and has consequently been employed as a marker for identification of Schwann and glial cells (Kahn et al., 1983). The S100 protein has been previously used to follow the differentiation of certain neuroblastoma cell lines which do not form the characteristic neuronal phenotypes. Accordingly, the differentiation of the CHP-126, and the GOTO cell lines, which form flattened, substrate adherent cell types, was found to be accompanied by an induction of S100 expression (Tsunamoto et al., 1987; Tsokos et al., 1987). This protein may therefore serve as a useful marker to follow schwannian or glial differentiation in cultured neuroblastoma.

Synaptophysin is a 38,000 kD protein found in presynaptic vesicles and transiently on the plasma membranes near synapses (Weidenmann and Franke, 1985). This protein is present in vesicles of neurons of the brain, spinal chord, neuromuscular junctions, and the adrenal medulla. Synaptophysin is an integral membrane protein containing four membrane spanning regions (Sudhof et al., 1987). Due to its presence in the zones of synapses and axon termini, as well as its function in neurotransmitter storage and release, synaptophysin may not only serve as a neuronal marker, but also as an indicator of synapse formation.

The neural cell adhesion molecule (NCAM), represents a group of morphoregulatory molecules derived from the alternative splicing of a single gene (Barbas et al., 1988). The timing and tissue specific expression of these glycoproteins is precisely controlled during development, and their influence on the fate of embryonic cells, such as those of the neural crest, is well documented (reviewed by Theory et al., 1985; Edelman, 1988). Prior to migration, neural crest cells produce this surface molecule but as the expression of this molecule is lost, cell migration begins. Once cells, such as those of the dorsal root ganglia, reach their destination, this surface glycoprotein is re-expressed and differentiation ensues (Theiry et al., 1982). In adult tissue, this molecule continues to be produced in neurons and astrocytes (Chuong and Edelman 1984). It has also been shown that following transformation of embryonic chicken retinal cells, the expression of this glycoprotein on the cell surface is reduced (Brackenbury et al., 1984). Due to its crucial role in development, as well as its altered expression in transformed cells, NCAM may serve

as a useful differentiation marker, as well as contribute to phenotype characterization of tumor cells of neural crest origin.

Intermediate filaments are routinely used to study cell differentiation, as cells having different embryonic origins express characteristic neurofilament proteins. Intermediate filaments are subdivided into 5 classes, (1) cytokeratins, found in epithelial cells, (2) neurofilament proteins, found in neurons, (3) glial fibrillary acidic protein, found in astrocytes, (4) desmin, found in muscle, and (5) vimentin, found in mesenchyme (Traub, 1985). Vimentin is also expressed transiently during development by a variety of cell types. For example, in murine embryogenesis, during development of neuroectoderm, most cells of the neural tube express vimentin which precedes the expression of neurofilament polypeptides. The latter proteins then appear in maturing neurons, however, these two intermediate filament types may be co-expressed for a very short time. As maturation proceeds, these cells synthesize primarily neurofilaments proteins (Reviewed by Traub, 1985). More recently, three novel intermediate filament proteins have been identified in neuronal cell types. A vimentin-like intermediate filament protein, periferin, has been described in some peripheral neurons (Leonard et al., 1988). Alpha-internexin, a polypeptide resembling neurofilament proteins (Fliegner et al., 1990), and nestin, an intermediate filament subunit that has been found in neuroepithelial stem cells (Lendahl et al., 1990). Neurofilament (NF) proteins, characteristically expressed in neurons, exist as a triplet consisting of polypeptides of approximately 68, 160 and 200 kD (Leim et al., 1978). These proteins are organized such that NF68 forms the core,

about which NF160 and 200 are arranged. NF68 is essential for assembly of the three neurofilament proteins. NF68 forms the backbone, while NF200 is thought to form a cross-linking function between neighbouring neurofilaments, and NF160 is more closely associated with the NF68 polypeptide (Scott et al., 1985). These proteins also undergo posttranslational modification, primarily phosphorylation (Julien and Mushynski, 1982; 1983). The addition of phosphate groups is thought to function as a targeting mechanism for axonal transport of these proteins. Unphosphorylated forms are found primarily in the cell body, whereas the phosphorylated forms are found in the stationary cytoskeletal structure of the axons (reviewed by Hollenbeck, 1989). NF200 and 160 are the most highly modified, where NF200 may be phosphorylated up to fifty times (reviewed by Matus, 1988).

The expression of these proteins are differentially regulated during development, such that NF68 and NF160 are normally expressed simultaneously very early in development, while NF200 is not expressed in all cells and appears much later (Scott et al., 1985). As these proteins are the intermediate filaments of neurons, they may serve as markers for cells of this lineage. Also, knowing that the temporal expression of these proteins is highly regulated, and neurofilament polypeptides, particularly NF200, serve as indicators of the degree of differentiation. They are therefore used extensively as markers for phenotype identification and as indicators of differentiation in cultured cells.

Immunohistochemical data, taken together with ultrastructural examination, can provide reliable methods for phenotype identification of cultured cells. With the

exploitation of some of these methods, clonal sublines from the neuroblastoma cell line SK-N-SH have been characterized and classified prior to any treatment with differentiating agents by Biedler and coworkers (Ciccarone et al., 1989; Ross et al., 1987). Cells were initially categorized based on their features in tissue culture, such as substrate adherence, nuclear to cytoplasmic (volume) ratio and the presence of processes, all of which are known to vary greatly depending upon the phenotype produced. Variants were also assessed based on cell-type specific markers, such as neurofilament proteins. Accordingly, cells were divided in three groups consisting of neuronal (N), substrate adherent (S), and intermediate (I), phenotypes. Cells classified as the 'N' type are characterized by weak substrate adherence, numerous processes, a large nuclear to cytoplasmic ratio, the presence of one or more neurofilament proteins, as well as the presence of neurotransmitter enzymes. The 'S' type, or also referred to as "non-neuronal", is described as highly substrate adherent, small nuclear to cytoplasmic ratio, absence of processes and neurofilament proteins, absence of neurotransmitter enzymes and the presence of an elaborate cytoplasmic and perinuclear network of vimentin. In some cases the 'S' cell type could be further subdivided as a) melanocytic , based on presence of melanosomes and/or tyrosinase activity, b) Schwann/glial, based on expression of 2',3'-cyclic nucleotide-3'phosphohydrolase and, c) meningeal, based on cell surface markers. Cells expressing markers associated with both neuronal and "non-neuronal" cell types were classified as intermediate (I). Extracellular matrix secretion was also used for classification as it had been previously shown that each lineage produced a

characteristic pattern. Studies by Tsokos et al. (1987) have shown that neuronal cells do not secrete a matrix in culture (hence the weak substrate adherence), melanocytes secrete fibronectin only, and Schwann cell types secrete large amounts of fibronectin, laminin and collagen Type IV. This observation is consistent with the stronger substrate adherence exhibited by 'S' type cells.

The cell lines under present investigation are descendants of the same SK-N-SH parent cell line described above. Detailed studies of variants derived from this cell line have been carried out by Biedler and coworkers, however, the main focus of their work was on the characterization of untreated neuroblastoma in culture, with particular attention to phenotypes arising following spontaneous differentiation. No detailed studies of phenotypic changes induced by RA on descendants of this cell line have yet been reported. The SH-N and SH-F variants were first described by Sidell et al. (1986). These are undifferentiated, rapidly proliferating cells that exhibit round morphologies, however, following RA-induced differentiation, these sublines show two distinct responses. As seen in chapter 3, the SH-F variant forms a large fibroblast-like phenotype, whereas the SH-N variant displayed a typical neuronal-like morphology. Karyotype analysis has verified that these two cell lines originate from a common precursor cell (Sidell et al., 1986). Evidence that these variants express markedly different phenotypes following RA treatment is seen in glycoprotein profiles, Coomassie-blue labelled proteins and ganglioside patterns. The results of Sidell et al. (1986) suggest that the flattened cell type is not likely to be a less differentiated form of the neuronal cell type. As distinctly different protein profiles

are seen with these variant cell lines, it was suggested that SH-F and SH-N are undergoing differentiation by different pathways. Further studies by Sidell et al. (1986) had revealed no major differences in cellular retinoic acid binding protein (CRABP) with respect to binding capacity and affinity. Preliminary studies in our laboratory have also revealed similar responses with the nuclear retinoic acid receptors following differentiation of both cell lines. Differences in phenotype development, therefore, could not be accounted for on the basis of CRABP or the retinoic acid receptors.

While Sidell et al. (1986) have shown that these cell lines undergo differentiation by alternate pathways, the phenotypes produced by these variants have not been characterized with respect to cell type-specific markers or ultrastructural features. Studies such as this are required to further characterize the bidirectional differentiation following RA treatment in these cultured neuroblastoma cell lines.

4.2 OBJECTIVE

In Chapter 3, differentiation was defined based on gross morphological changes only, therefore to further confirm these observations, experiments will be reported which further characterize the phenotypes produced by the RA-induced differentiation of these two variant cell lines. Assessments will be made based on their features in culture, cell type specific markers, and ultrastructural characteristics. As these divergent phenotypes induced by RA have not been previously described, some aspects of the classification scheme presented by Biedler and coworkers (Ciccarone et al., 1989; Ross et al., 1987) will be utilized in addition to a detailed

ultrastructural examination. More importantly, however, these results will provide further support for the hypothesis put forth in Chapter 3, that the inhibition of PKC results in the neuronal differentiation of SH-F cells. It will be demonstrated that the addition of the PKC inhibitor, staurosporine, together with RA to SH-F cells results in a phenotypic change favoring the expression of neuronal characteristics. The phenotype produced by SH-F in the presence of RA and staurosporine, therefore, will be characterized and compared to that produced by RA alone.

4.3 MATERIALS AND METHODS

A. MATERIALS

All tissue culture products and fine chemicals were purchased as described in chapter 3. Primary antibodies and antisera (working dilutions given in parenthesis) were purchased from the following suppliers: mouse monoclonal IgG anti-NF68 (1:20), and anti-NF200 (1:300), were obtained from Sanbio, Uden, Holland; mouse monoclonal IgG anti-NF160 (1:5), and anti-synaptophysin (1:20), from Boehringer Mannheim, Dorval,P.Q.; polyclonal rabbit IgG anti-vimentin (1:50), and S100 (1:100), from Euro Diagnostics, Appledoon, Holland. Secondary antibodies: Swine antirabbit IgG (1:400), rabbit peroxidase-antiperoxidase (1:300), fluorescein isothiocyanate conjugated swine anti-rabbit IgG (1:200), and fluorescein isothiocyanate-labelled rabbit anti-mouse IgG (1:20) were all purchased from Dako Corp., Santa Barbara, CA; biotin/avidin system was applied as an ABC kit from Vector Laboratories, Burlingame, CA. The NCAM antibody was a gift from Dr. D.G. Parry, Department of Physiology, University of Ottawa, Ottawa, Ontario.

B. METHODS

B.1 CELL CULTURE

Cells were grown and maintained as described in Chapter 3, except that they were seeded in small Petri dishes, on coverslips coated with 5 μg laminin. For cells treated with RA and staurosporine, these agents were added simultaneously at concentrations of 3 X 10^{-6}M, and 15 X 10^{-9}M, respectively.

B.2 IMMUNOHISTOCHEMISTRY

PEROXIDASE METHOD

Cells, grown on coverslips, were fixed for 3 minutes in 4% phosphate-buffered formalin solution, pH 7.4. Endogenous peroxidase was blocked by treating with 3% hydrogen peroxide:methanol (1:4) at room temperature for 15-30 minutes. Following three washes in phosphate-buffered saline (PBS), pH 7.4, nonspecific reactions were blocked by covering samples with pre-immune serum (1:20) for 20 minutes at room temperature. Following three washes in PBS, samples were treated with one of the primary antisera listed above, and incubated overnight at 4°C. Negative controls (secondary antibody only) were treated with normal serum under identical conditions. This was followed by the application of the peroxidase-antiperoxidase method (Sternberger, 1979). Briefly, after three washes in PBS, a second incubation with swine antirabbit (1:400) IgG was allowed to proceed for 30 minutes at 21°C. This was followed by a further three washes in PBS, after which, a third antibody, rabbit antiperoxidase IgG conjugated with horse radish peroxidase (1:400), was added to the sample and incubated at 21°C for 30 minutes. After three washes with PBS, 1 ml of

a solution containing 0.5 mg/ml 3,3'diaminobenzidine hydrochloride and 0.02% hydrogen peroxide in PBS was added. The reaction was developed for five minutes after which time coverslips were washed thrice in PBS, stained with Cresyl Echt Violet for 10 to 20 minutes, dehydrated in alcohol and mounted. In some cases nickel chloride intensification was used to enhance the sensitivity of the peroxidase method. Instead of the usual 0.5 mg per ml 3,3'diaminobenzidine hydrochloride, the solution contained 6 mg per ml nickel ammonium sulfate, with 0.2 mg per ml 3,3' diaminobenzidine hydrochloride and a final concentration of 0.06% hydrogen peroxide. After a five to ten minute development time the reaction was stopped by three washes in PBS, and mounted as described above.

For primary mouse monoclonal antibodies, samples were treated essentially as described above, however, the avidin/biotin peroxidase system was used, as part of an ABC kit (Vector Laboratories), and applied according to the supplier's instructions.

IMMUNOFLUORESCENCE

For detection of intermediate filaments, coverslips were fixed for five minutes in PLP fixative, a solution containing 10 mM sodium periodate, 75mM lysine, 2% paraformaldehyde, 1% Triton X-100, in PBS, for ten minutes (Muramoto and Kadin, 1987). For NCAM detection, samples were fixed for 45 minutes at 21°C in Lana's fixative which contains 4 % paraformaldehyde (w/v), and 14 % picric acid (v/v), in PBS, pH 7.1. After fixation, samples were washed three times in PBS. Primary antiserum was applied to the sample as described above. After three five-minute

washes in PBS, the secondary flourescein-isothiocyanate conjugated anti-mouse or anti-rabbit antibody was added to the coverslip which was then incubated at 37°C for 45 minutes. Following three five-minute washes in PBS, the coverslips were mounted in 50% glycerol and examined.

B.3 ELECTRON MICROSCOPY

Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for two hours. After initial fixation, coverslips were washed three times in 0.1 sodium cacodylate buffer, for 15 minutes each wash. Samples were postfixed on ice, in 1 % osmium tetroxide (OsO_4). After dehydration in increasing concentrations of methanol and acetone, samples were embedded in Epon-Araldite. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with a Philips 301 transmission electron microscope. At least two samples were examined for each treatment.

4.4 RESULTS

Results for cell type specific markers as well as general observations are summarized in Table 4.1. Based on day to day manipulations of cultured cells, the following observations were made. Untreated SH-N were moderately adherent, following exposure to RA however, these cells developed long processes and reduced substrate adherence as treatment time progressed. In comparison to RA-treated SH-F, the neuronal variant appeared to have a higher nuclear to cytoplasmic volume ratio. The SH-F subline responded in an opposite manner. Untreated cells were very round and weakly adherent when grown on glass; following RA-induced differentiation, the majority of the population developed as enlarged flattened cells

TABLE 4.1

Summary: Phenotype - Marker Expression

Cell line treatment	Morphology			Intermediate Filaments				S100	Synapto	NCAM
	Processes	nucleus cytoplasm	Subst. Adher.	VIM	NF68	NF160	NF200			
SH-F CTL	(±)	High	(±)	++	+++	+	--	+++	N.T.	+
SHF RA	--	Low	+++	+++	--*	--*	--	++	--*	--*
SHF RA + SSP	+++	High	+	+	+++	++	N.T.	(±)	N.T.	+++
SH-N CTL	+	High	+	++	+++	++	--	+++	(±)	++
SH-N RA	+++	High	(±)	+	++	+++	--	++	+++	+++

CTL - solvent treated control; RA - 7 day treatment with retinoic acid; RA + SSP retinoic acid and staurosporine together.
Antibodies tested = VIM-vimentin; NF68, NF160, NF200 - neurofilament proteins; Synapto - synaptophysin; NCAM - neural cell adhesion molecule.
+ positive; - negative; --* all flattened cells are negative, very small percentage of round cells are positive; N.T. - not tested.
Nuclear to cytoplasmic ratio was a qualitative assessment, based on the appearance of cells.
For each sample 2 to 5 coverslips were examined.

with a low nuclear to cytoplasmic ratio. This cell type was strongly adherent to glass coverslips. It should be noted that while most SH-F cells had a fibroblast-like appearance, a small percentage, about 1% or less, remained round or extended processes similar to those observed in SH-N. When SH-F cells were treated with staurosporine together with RA, virtually all cells assumed a neuronal-like phenotype. These cells developed very long processes with much smaller bodies, resulting in relatively larger nuclear to cytoplasmic ratios, and loss of substrate adherence.

A. CELL TYPE SPECIFIC MARKERS

Studies with the CHP-126 neuroblastoma cell line, have shown that RA-induced differentiation to a fibroblast-like morphology resulted in an induction of S100 protein (Tsokos et al., 1987). As one of the variants in this study also formed such a phenotype, it was decided to test for the expression of this marker. As shown on Table 4.1, all cells were strongly positive for this antigen regardless of cell type or differentiation state. SH-F, treated with RA and staurosporine together, appeared to express less; however, a small number of positive cells were still found.

Neurofilament protein (NF) expression, was studied with antibodies against NF68, NF160, and NF200 (Table 4.1). Untreated SH-N cells were found to be positive for NF68 and NF160. Following RA-induced differentiation both of these markers were retained in SH-N. No staining with NF200 was detectable in any of these samples. SH-F cells expressed both NF68 and NF160 prior to RA treatment. Following RA-induced differentiation, however, the majority of cells had lost these neuronal markers. All of the typical fibroblast-like, flattened cells were negative for

NF68, and NF160. In contrast, when staurosporine was added together with RA, all cells exhibited positive staining for both neurofilament proteins. The expression pattern obtained for neurofilament proteins paralleled that of NCAM, where RA-induced differentiation of SH-F cells resulted in a loss of the neuronal marker, whereas treatment of SH-F with RA and staurosporine together resulted in a positive reaction in all cells (Table 4.1).

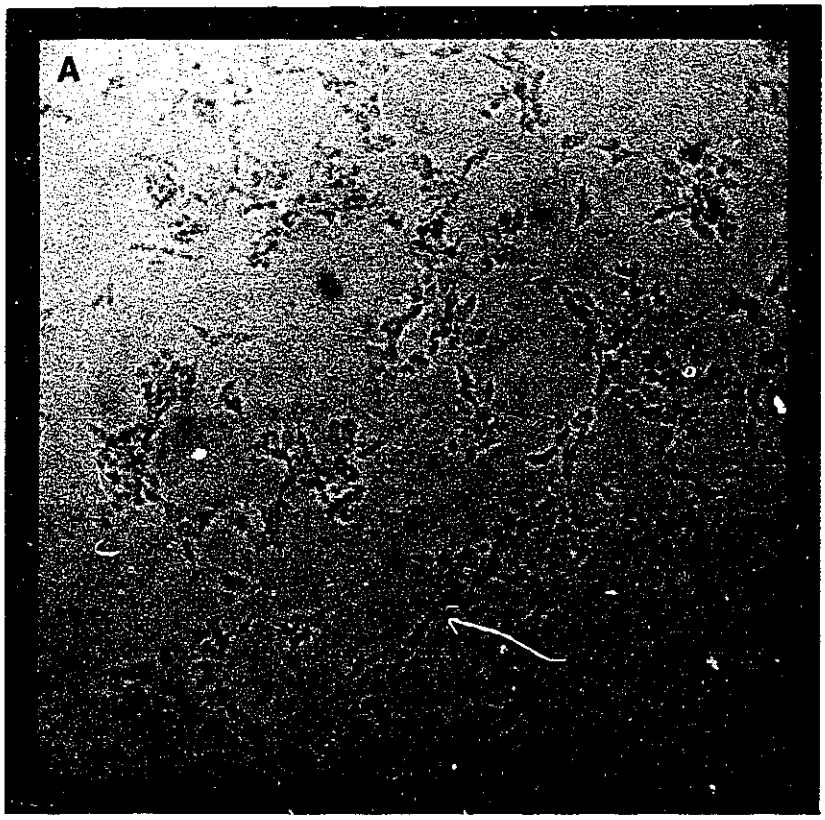
The expression of vimentin was also investigated as its presence had been previously confirmed in cultured neuroblastoma (Browning and Roisen, 1984; Shea et al., 1988). Although all cells were found to express this intermediate filament, the flattened cell type generated this marker more abundantly (Table 4.1).

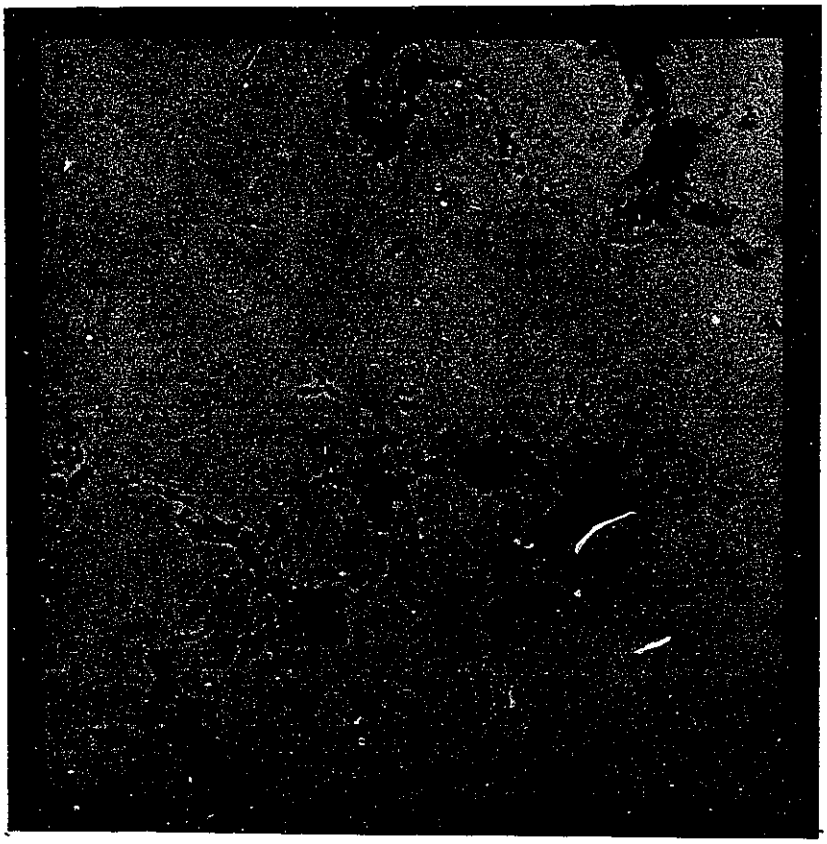
Synaptophysin was examined to assess the degree of differentiation of the neuronal phenotype produced by the SH-N cell line (Figure 4.1). In untreated cells rather faint immunostaining was seen for this protein (Figure 4.1a); however, upon a seven day exposure to RA, a striking increase in synaptophysin expression was seen (Figure 4.1b). RA-treated SH-F cells were also tested for this antigen and all flattened cells were found to be negative. The difference in marker expression in the SH-F and SH-N cell line is an indication of their divergent phenotypes. Upon RA treatment, the neuronal marker is induced in the SH-N cell line, whereas RA-treated SH-F are essentially negative for this protein.

A second marker,the neural cell adhesion molecule (NCAM), the expression of which has been extensively characterized in neural crest development, (Thiery et al., 1985; Edelman, 1988) was used to follow phenotypic changes in these cells. The

Figure 4.1 Synaptophysin in SH-N cells.

Cells were treated with antiserum to synaptophysin with nickel chloride intensification. A) Undifferentiated SH-N cells show very faint staining; B) SH-N cells, induced to differentiate by a 7 day RA treatment show a strong positive reaction for synaptophysin. An increased expression of this marker following RA-induced differentiation is clearly evident.


A



typical surface staining for NCAM is clearly visible in all cells demonstrating a positive reaction to this antibody (Figure 4.2). In SH-N, both control and RA-treated cells exhibited positive staining, however it appeared that the intensity of this reaction was greater in the differentiated cells (Figure 4.2b and 4.2d). Untreated SH-F cells expressed this surface glycoprotein as well (Figure 4.2a); however, following RA-induced flattening of these cells, this marker was lost (Figure 4.2c). As a small percentage of these cells exhibited a round or neuronal-like morphology, some positive cells were present. When RA and staurosporine were added simultaneously to SH-F cells, such that all cells assumed a neuronal-like morphology, a strong positive reaction was seen (Figure 4.2e). As with SH-N, the intensity appeared to be somewhat enhanced in comparison to controls.

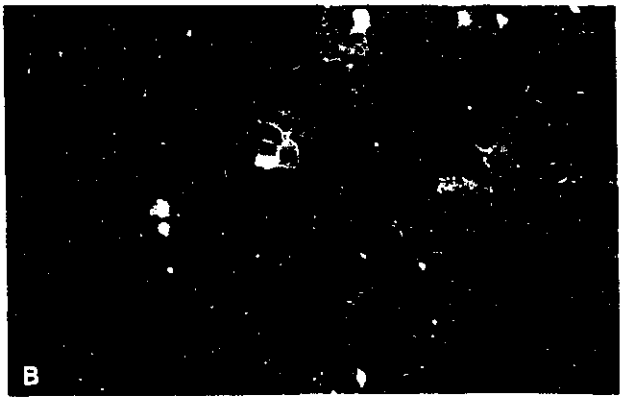
In summary, cell type-specific antigens demonstrate that RA-treated SH-N cells display neuronal differentiation, i.e., presence of neurofilament markers, retention of NCAM and the induction of synaptophysin, a protein expressed exclusively in neurons prior to synapse formation. In contrast, upon RA treatment, SH-F cells appeared to lose these same markers. The enlarged, flattened cells did not express neurofilament proteins, NCAM, or synaptophysin. Interestingly, when the PKC inhibitor, staurosporine, was added together with RA, these same cells, retained the neuronal markers, NF68, NF160 and NCAM. Staurosporine treatment, therefore, not only resulted in a striking morphological change, but also caused a marked alteration in the response of SH-F cells to RA treatment.

To further characterize the phenotypes induced by RA in SH-N and SH-F

Figure 4.2 Immunofluorescent reaction for NCAM in SH-F and SH-N cells.

A) Control SH-F cells, B) control SH-N cells. Both untreated cell lines were positive for NCAM. C) RA-treated SH-F cells; the majority of these cells were enlarged, flattened cells which could be readily seen by background fluorescence (arrow), and all were negative for NCAM. Relatively few neuronal-like cells, showing positive immunostaining were seen, and were purposefully captured in the same field to facilitate comparison. D) SH-N cells induced to differentiate with RA displayed a positive reaction for NCAM. E) SH-F cells treated with both staurosporine and RA. All cells showed a strong positive reaction for this antigen. Note the characteristic surface staining. (The same exposure time was used for each slide)
Magnification 400 X.


A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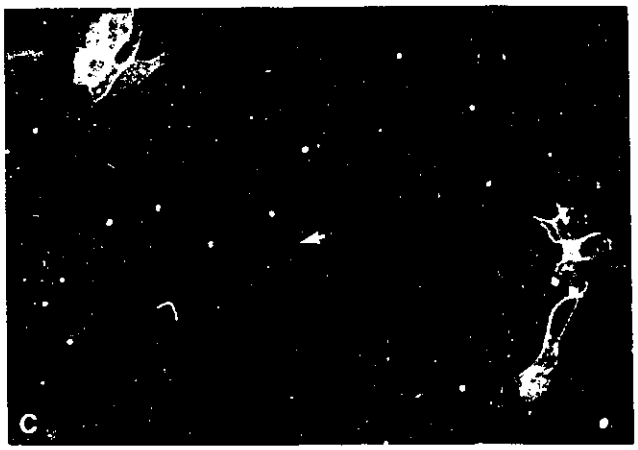
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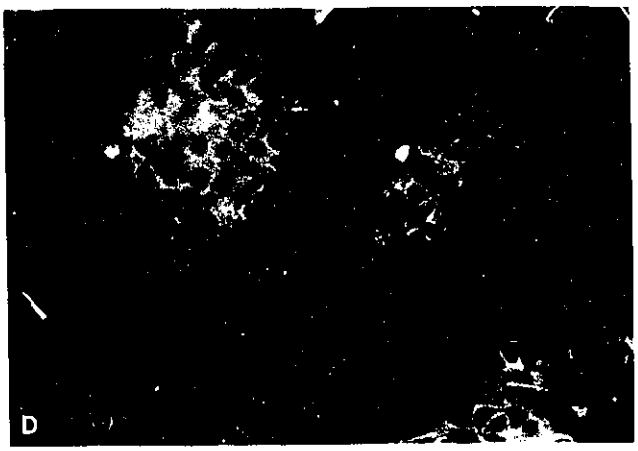

D


E

cells, and more importantly to provide further support for the neuronal specialization elicited by staurosporine in RA-treated SH-F cell lines, studies examining the ultrastructural features resulting from treatment of those agents were carried out.

B. ELECTRON MICROSCOPIC EXAMINATION OF NEUROBLASTOMA

B.1 UNTREATED SH-N CELLS

Untreated SH-N cells appeared small and round displaying numerous mitoses. Typical of undifferentiated cells, these untreated neuroblastoma had high nuclear to cytoplasmic volume ratios and contained few organelles discernable as mitochondria mainly (Figure 4.3). Few, poorly developed Golgi bodies, small numbers of neurosecretory granules and lipid droplets were seen. Some cells had very short processes containing microtubules and occasional neurosecretory granules and very few other organelles. Cells had numerous ribosomes, rough endoplasmic reticulum, and intermediate filaments dispersed throughout the cytoplasm. (Figure 4.4)

B.2 RETINOIC ACID-TREATED SH-N CELLS

Following RA treatment striking morphological changes were apparent (Figures 4.5 and 4.6). Typical of differentiated cells, mitoses were absent and their cytoplasms contained multiple organelles. Cells showed markedly increased numbers of neurosecretory granules localized in the perinuclear region and in cellular processes, and an increased number of well developed Golgi bodies (Figure 4.6a). Intermediate filaments were often present in collections in close proximity to the nuclei (Figure 4.6b). Microtubules appeared to be increased in number and were very well aligned in the cellular processes which were elongated and interconnected

Figure 4.3 Electron micrograph of undifferentiated SH-N cells.

Low magnification shows general morphology of cells exhibiting high nuclear to cytoplasmic ratios, and displaying very little evidence of neuronal differentiation. Nucleus (N); Magnification 5,760 X.

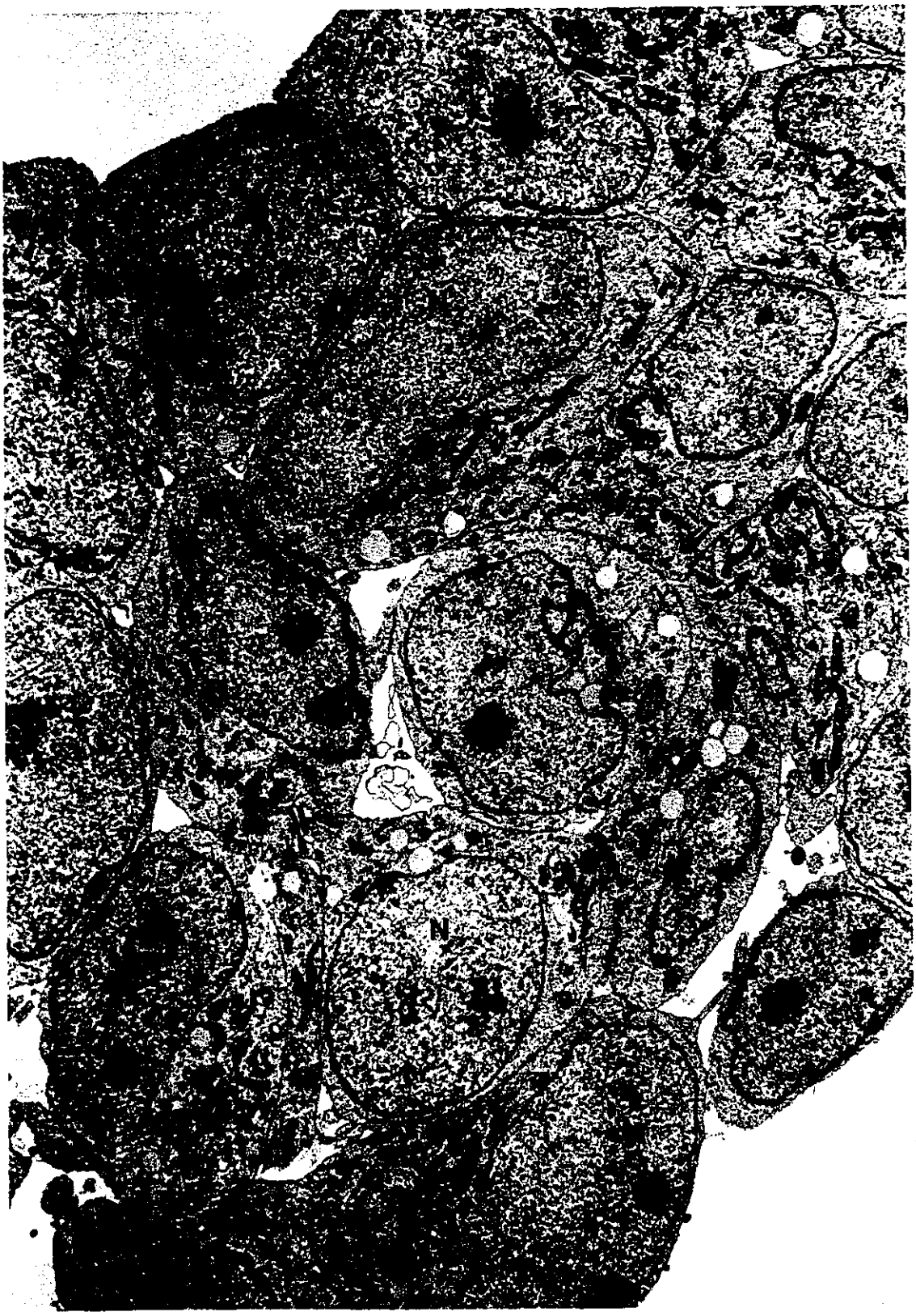

N

Figure 4.4 Undifferentiated SH-N cells showing detail of cytoplasm.

These cells contain few organelles and inclusions. Accordingly, lipid droplets (L), mitochondria (M), neurosecretory granules (solid arrow), endoplasmic reticulum (open arrow) was sparse, whereas numerous ribosomes were found scattered throughout the cytoplasm (arrowhead). Nucleus (N); Magnification 26,400 X.


M
L

Figure 4.5 SH-N cells following a 7 day treatment with retinoic acid.

A marked change in morphology was seen where cells exhibited numerous neuritic processes containing well ordered microtubules (MT). Neurosecretory granules were frequently seen (arrowhead), and processes exhibited numerous swellings that were filled with collections of many normal and degenerating organelles (large open arrow). Inset, lower left corner, shows multiple processes exhibiting terminal swellings (large open arrow) and accumulation of membranous organelles such as mitochondria (solid arrow), neurosecretory granules, smooth endoplasmic reticulum (small open arrow). Magnification 20,800 X.

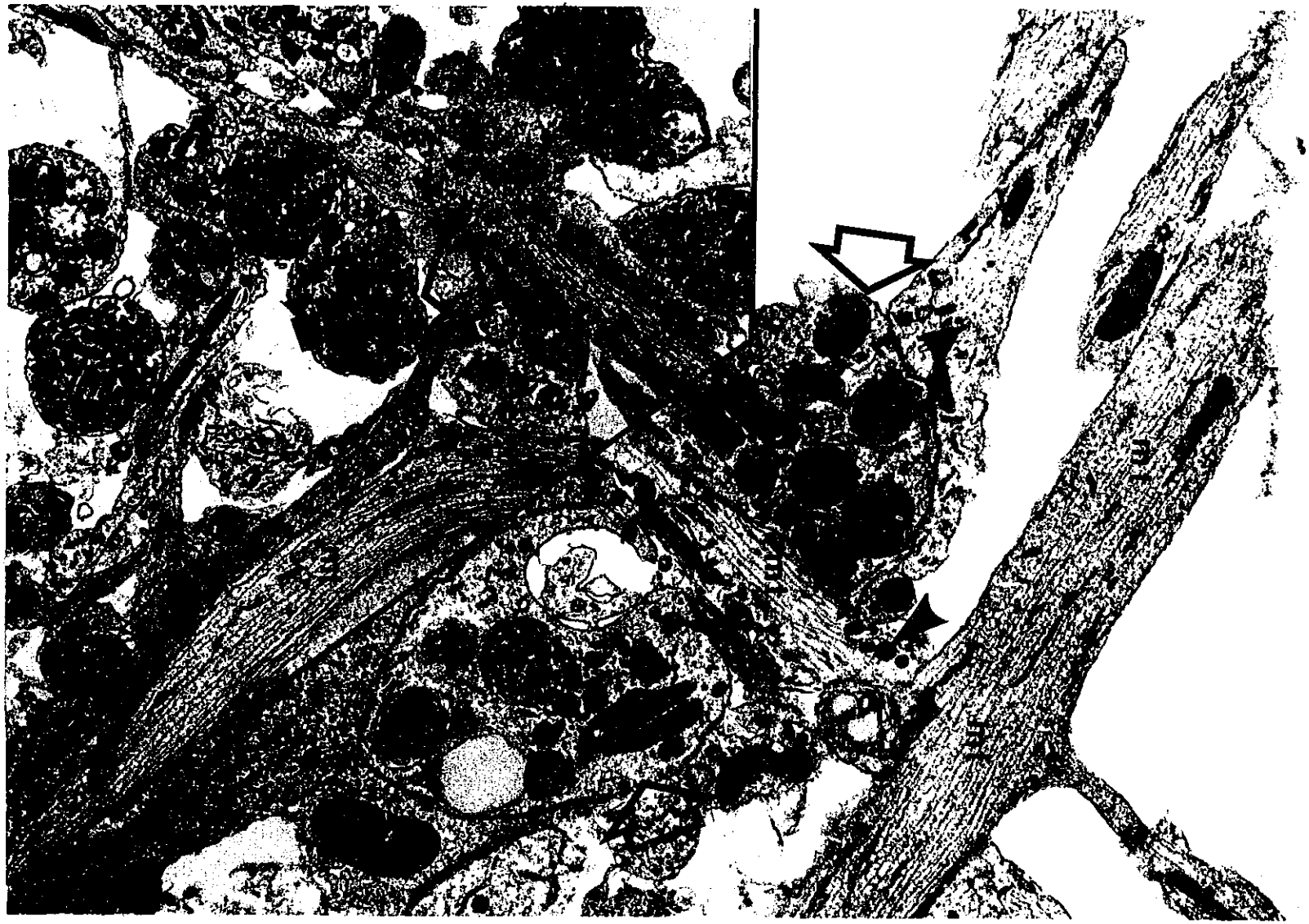
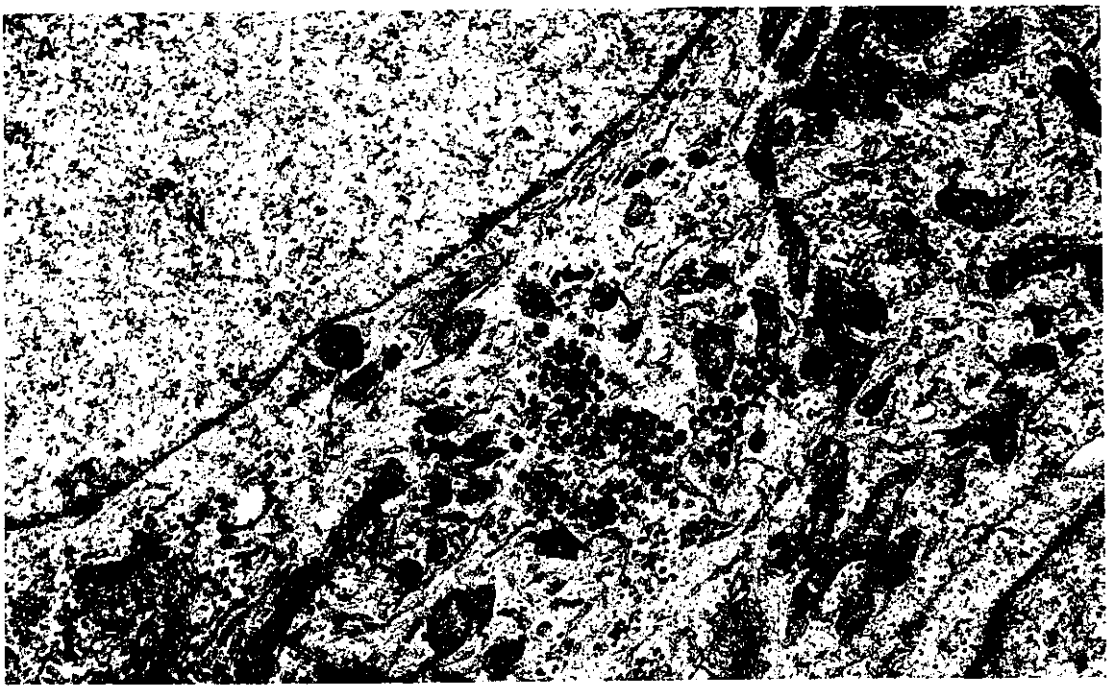
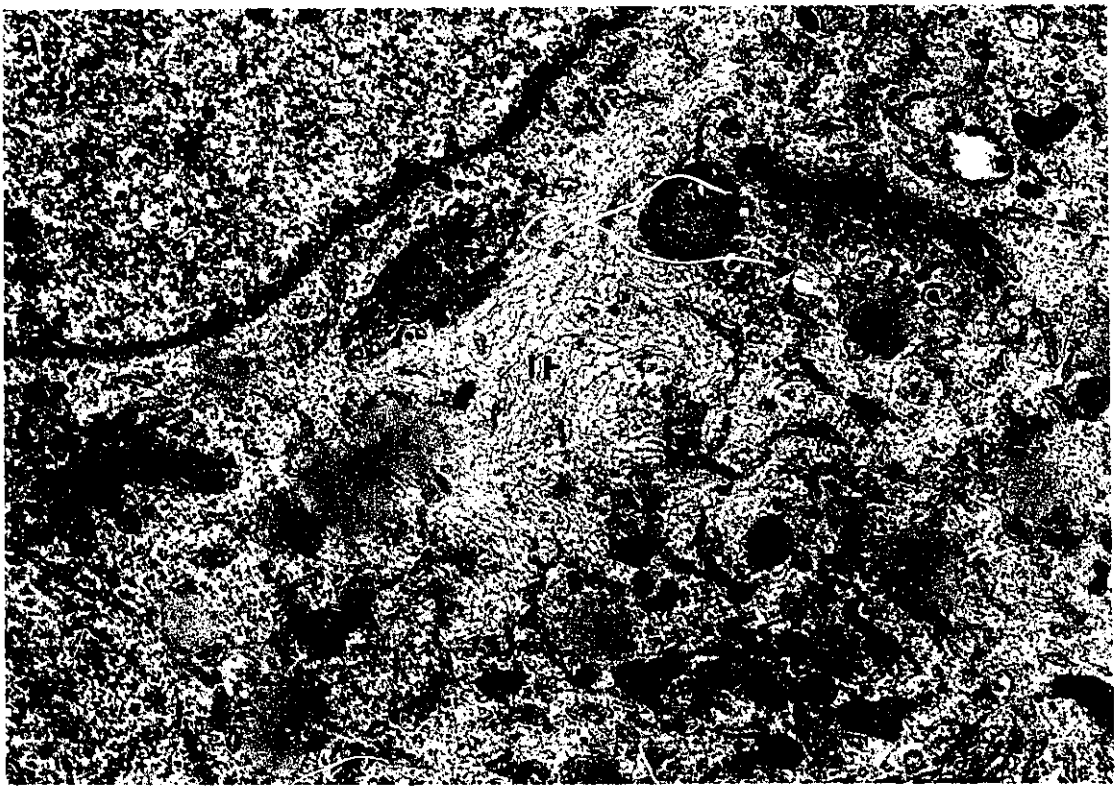


Figure 4.6 Micrographs showing detail of cytoplasm in differentiated SH-N.

Cytoplasms of differentiated SH-N were highly enriched with organelles.
A) Perinuclear collections of neurosecretory granules (arrow) were common among these cells. Golgi bodies were numerous (G). Nucleus (N)
Magnification 16,000 X.
B) Aggregates of intermediate filaments (IF) were often seen in close proximity to the nucleus (N). Golgi apparatus (G), lipid droplets (L), and neurosecretory granules (arrows) were numerous.
Magnification 26,400 X.





with one another. These well developed microtubule arrays are characteristic features of neuritic processes (Peters et al. 1976). Synaptic-like contacts between cells were commonly found but no pre-synaptic specialization and post synaptic densities were seen. Terminal neuritic swellings showed accumulation of organelles in the processes of these differentiated cells (Figure 4.5 inset). This is an abnormal feature reminiscent of dystrophic changes found in well differentiated cells of some neuronal tumors such as neuroblastomas, medulloblastomas, and gangliocytomas (Russell and Rubinstein, 1989). An example analogous to the dystrophic change seen in these cultured cells is presented in a section of a neuroblastoma biopsy specimen depicted in Figure 4.7.

B.3 UNTREATED SH-F CELLS

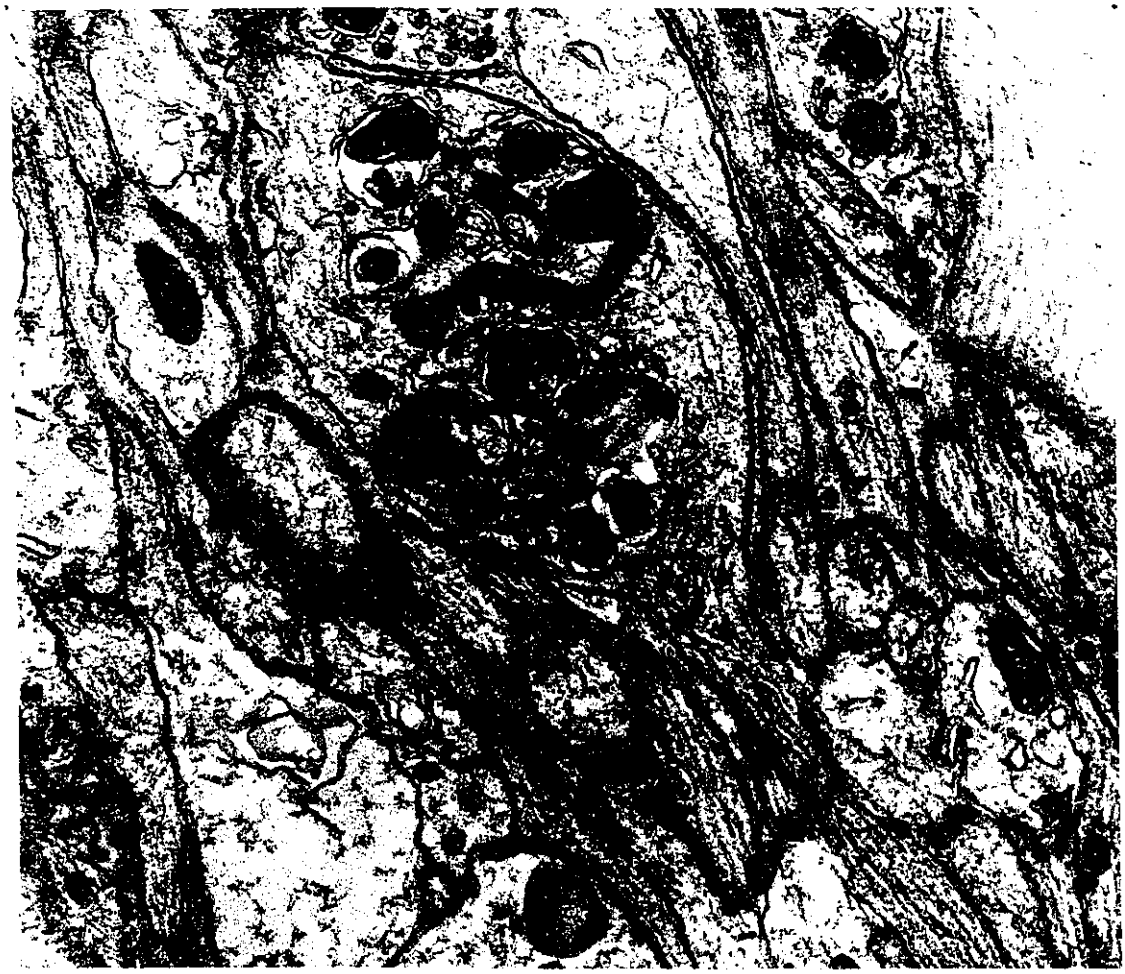
Untreated SH-F cells, display similar ultrastructural features as those seen for untreated SH-N. Cells were round, showing relatively high nuclear to cytoplasmic ratios (Figure 4.8a). Cells exhibited short, predominantly rough endoplasmic reticulum, numerous free ribosomes, few Golgi bodies, and a small number of lipid droplets. Similar to untreated SH-N, these cells show little specialization, however, a few neurosecretory granules as well as short processes containing intermediate filaments could be seen (Figure 4.8b and 4.9).

B.4 RETINOIC ACID-TREATED SH-F CELLS

Following RA treatment the majority of cells displayed the typical morphology depicted in Figure 4.10. No mitoses were seen and cells contained voluminous cytoplasms such that the nuclear to cytoplasmic ratios were low. Comparison of

Figure 4.7 Micrograph of a section from a human neuroblastoma biopsy specimen.

The figure shows a typical example of swollen dystrophic neuritic processes. These swellings contain large collections of cellular organelles. A) Swelling shown lengthwise. B) Higher magnifications showing accumulations of neurosecretory granules (solid arrows) and synaptic vesicles (open arrows). Magnification for A and B was 25,480 X.



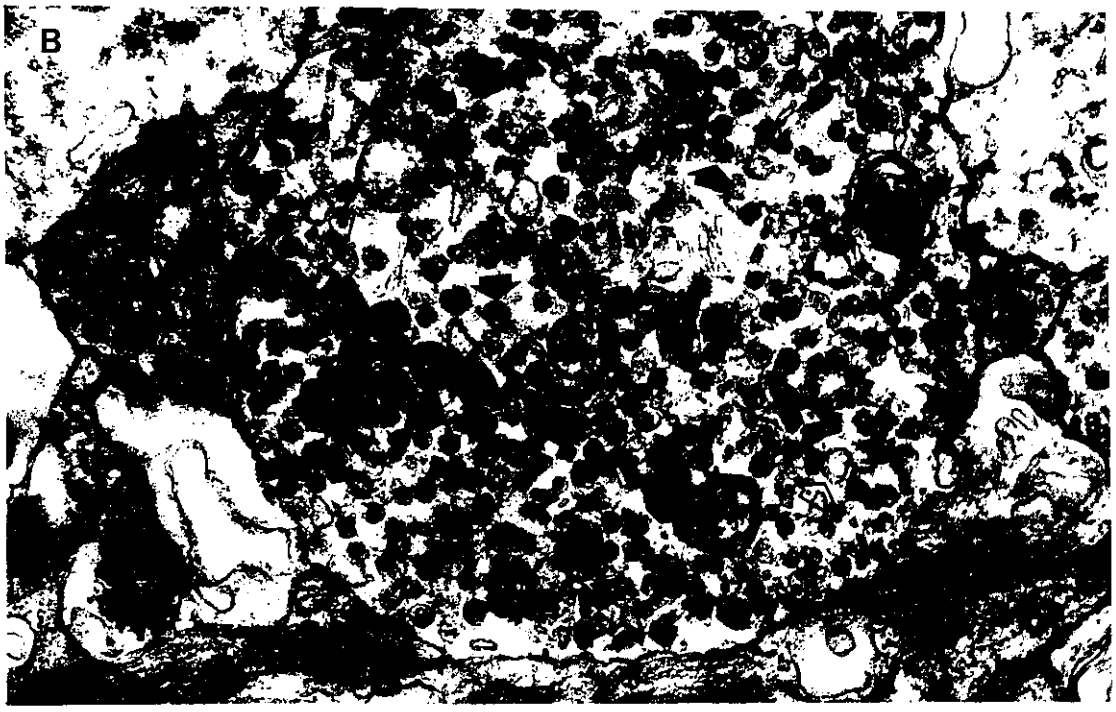
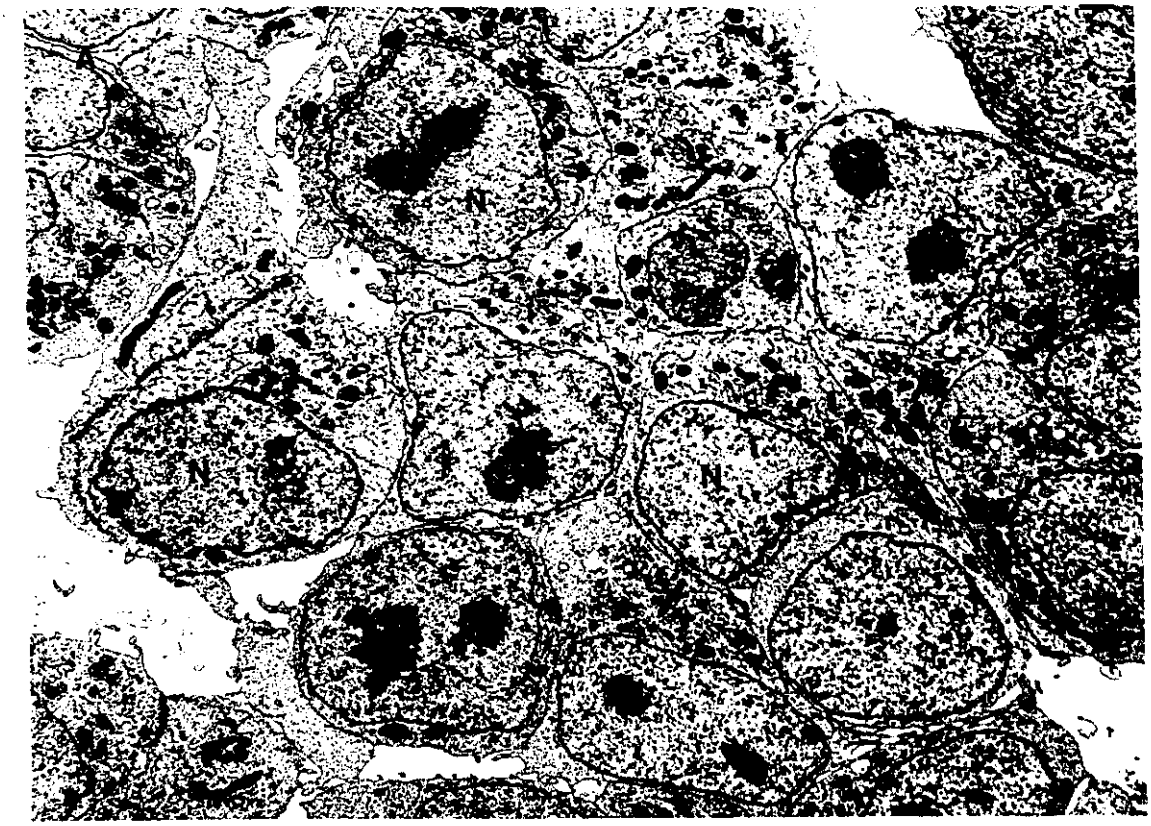

B

Figure 4.8 Electron micrograph of undifferentiated SH-F.

A) Low magnification depicting general morphology of the cells. As seen with SH-N, these cells are round, having large nuclear to cytoplasmic ratios and very few processes. Nucleus (N) Magnification 4,400 X.
B) Although cells had sparse cytoplasms with scanty organelles, occasional short processes containing intermediate filaments (IF) and a few scattered neuroscretory granules (arrow) were seen.
Mitochondria (M); Magnification 16,000 X.


N
N
N

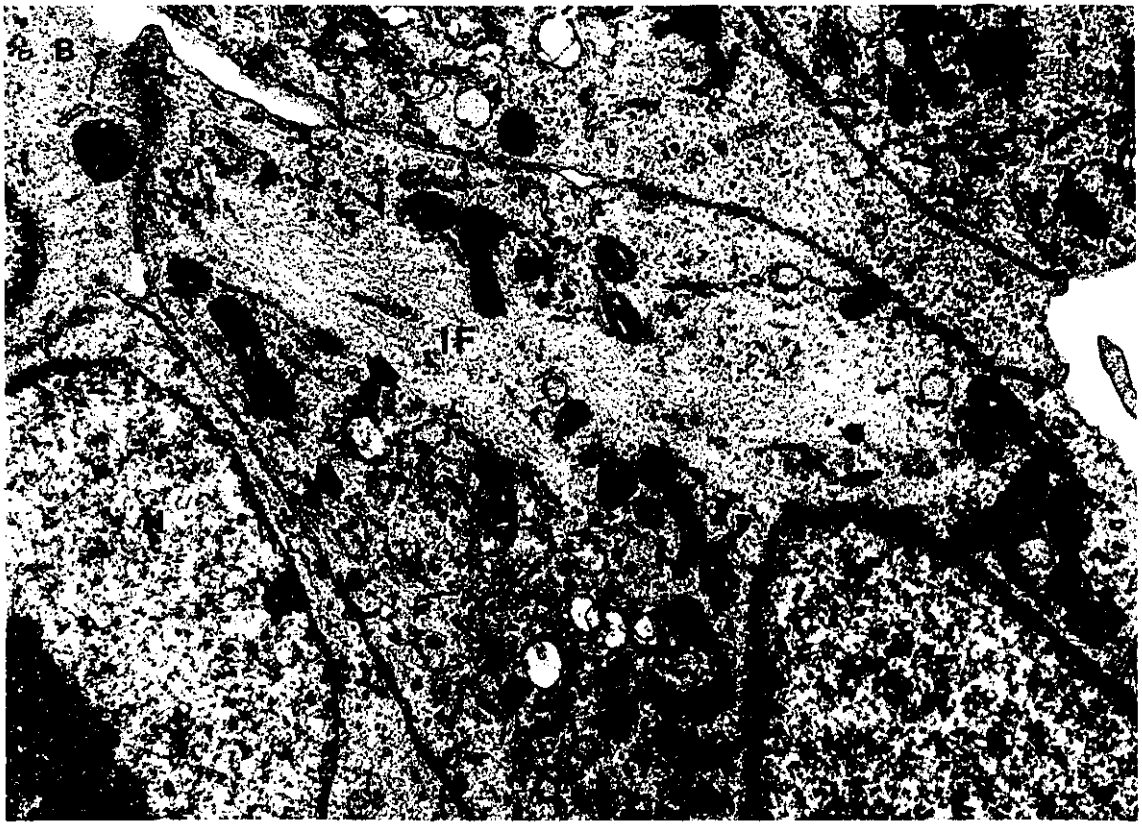

B
IF

Figure 4.9 Micrograph showing detail of cytoplasm of undifferentiated SH-F cells.

Cells contained a few neurosecretory granules (arrows), golgi apparatus (G), and endoplasmic reticulum (open arrow). Intermediate filaments were scanty and ribosomes (arrowhead) were seen dispersed throughout the cytoplasm. Mitochondria (M); Lipid droplet (L); Magnification 33,600 X.

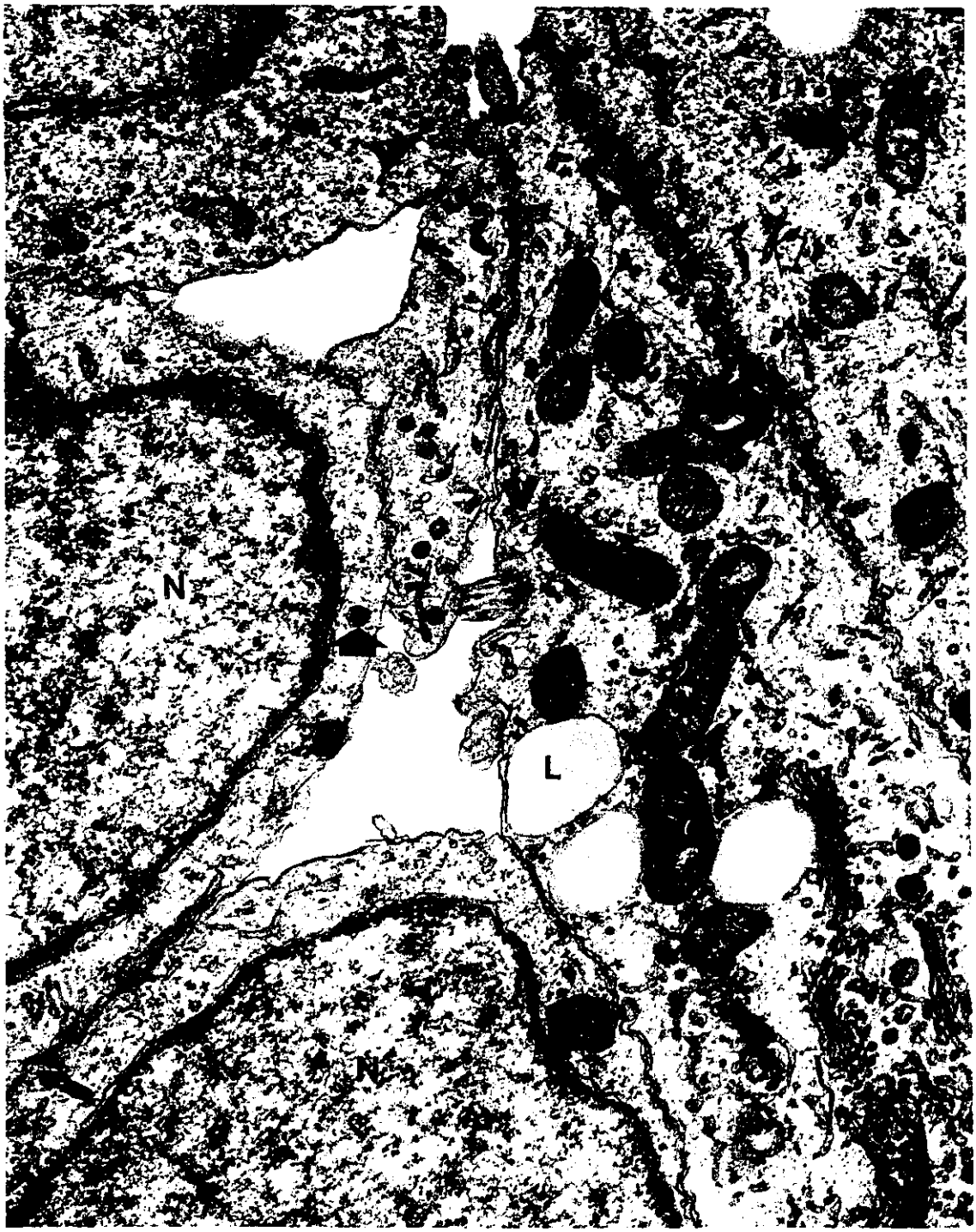
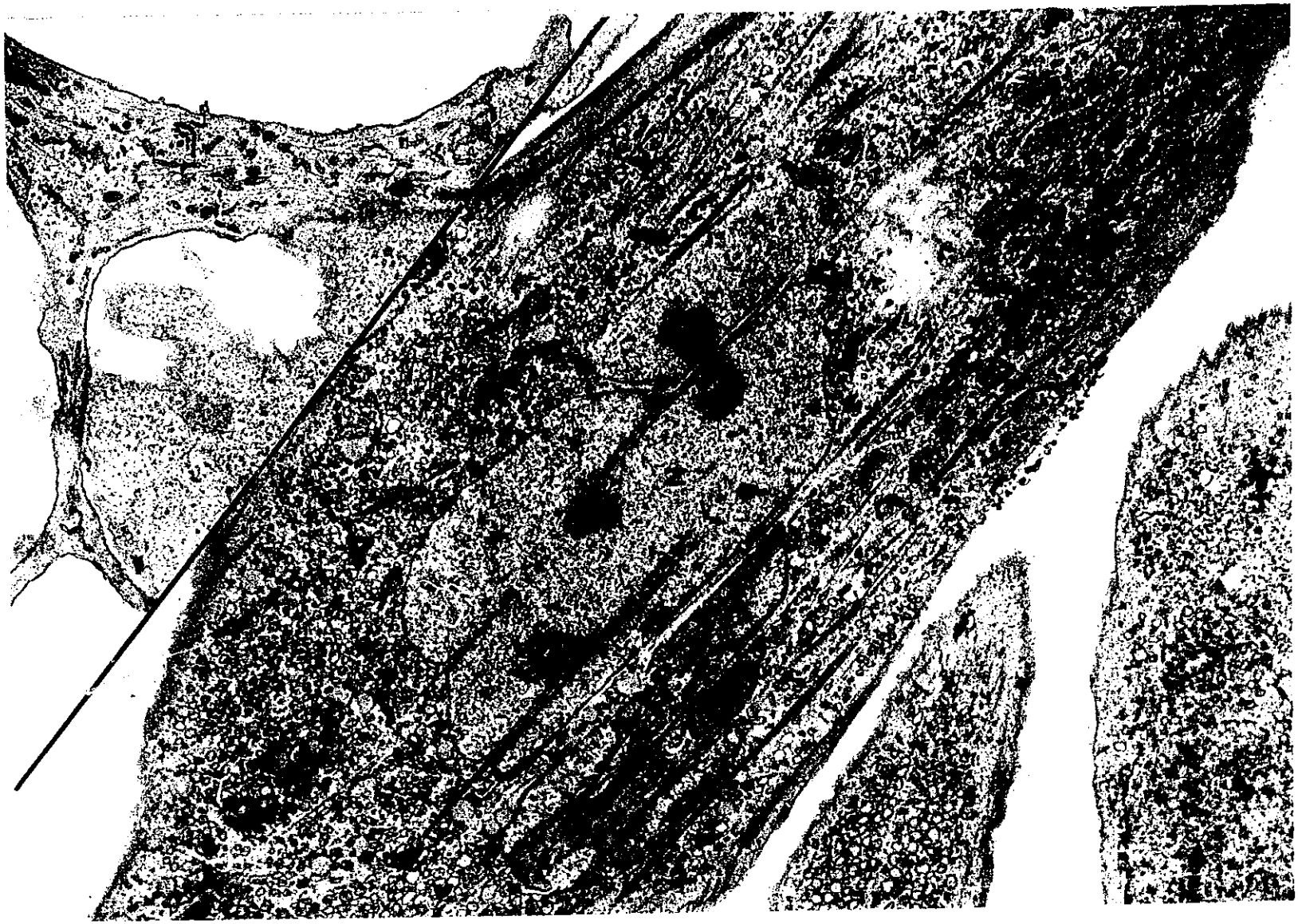

N
L
N

Figure 4.10 Micrograph of retinoic acid-treated SH-F cells.

The typical morphology of the enlarged, flattened cell is seen. Cells display enlarged cytoplasms highly enriched in organelles such as dilated vesicles (arrow). The nucleus (N) contains many folds. Although the majority of cells possess this morphology, on occasion cells are seen which show short processes (inset, lower left) Magnification 5,760 X.



differentiated SH-F to undifferentiated SH-N and SH-F cells (Figures 4.3 and 4.8a respectively), which were shown at similar magnifications demonstrates the striking enlargment of these cells following RA treatment (compare to Figures 4.3 and 4.8a). Nuclei were enlarged showing many deep infoldings of their membranes. Typical of differentiated cells, RA-treated SH-F were highly enriched with organelles containing large numbers of lipid droplets and numerous well developed Golgi complexes (detail of cytoplasm shown in Figure 4.11). Tight filamentous bundles were seen throughout the cytoplasm. Cells contained large amounts of dilated vesicles filled with homogenous or finely flocculent proteinaceous material. Such an abundance of vesicles and lipid droplets was not seen in differentiated SH-N cells, which further distinguishes the fine-structure of these variant sublines. Although the phenotype cannot be precisely identified at this time, RA-treated SH-F cells appeared to be much more differentiated than controls. It should be emphasized however, that the majority of cells showed no neuronal specialization, i.e. no processes with characteristic microtubule arrays and no neurosecretory granules. Perinuclear collections of intermediate filaments, similar to those seen in differentiated SH-N, were not found in these flattened cells. Occasionally, few cells were seen which were round with short processes, an example is shown in Figure 4.10 (inset).

B.5 STAUROSPORINE AND RETINOIC ACID TREATED SH-F CELLS

SH-F cells treated with staurosporine and RA together (Figure 4.12), displayed a morphology strikingly different from that of the enlarged, flattened cell type observed with RA alone. Unlike RA-treated SH-F cells, these cells contained

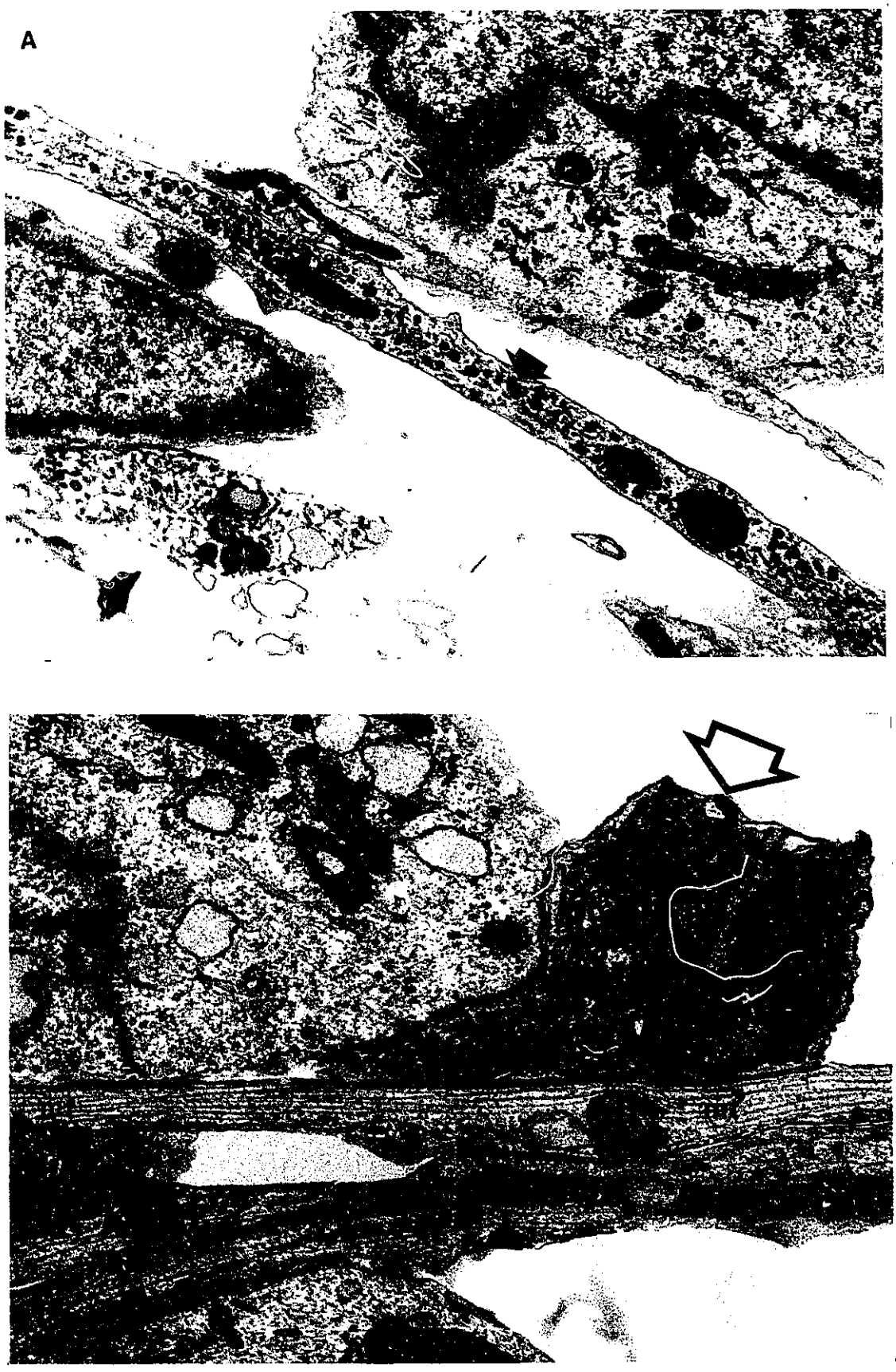
Figure 4.11 Electron micrograph showing details of the cytoplasm seen in retinoic acid-treated SH-F cells.

In differentiated cells the cytoplasm is crowded with organelles, numerous Golgi apparatus (G), lipid droplets (L), mitochondria (M), dilated vesicles (V), and thick aggregates of filaments (solid arrow). This cell type did not contain neurosecretory granules. Magnification 33,600 X.


L

Figure 4.12 Electron micrograph of SH-F cells treated with staurosporine and retinoic acid.

A) These cells exhibited a typical neuronal phenotype, characterized by extremely long neuritic processes which contained large amounts of neurosecretory granules (arrow).
Magnification 16,000 X.
B) Upon higher magnification of 26,400 X, these processes exhibited typical well ordered arrays of microtubules (mt), as well as neuritic swellings containing abundant stacks of smooth endoplasmic reticulum (open arrow). This is similar to the dystrophic abnormalitiess observed in differentiated SH-N cells (Figure 4.5), and those found in human neuroblastoma tumors (for example Figure 4.7).


A

much less lipid and fewer enlarged vesicles. Instead, much smaller, elongated cells, exhibiting characteristic features of neuronal differentiation were seen. Very long extensions containing the well ordered arrays of microtubules typically found in neuritic processes were seen. The neurites were highly enriched with neurosecretory granules (Figure 4.12a). Some of these cells exhibited the same dystrophic changes that were also seen in RA-treated SH-N. Similarly, synapse-like contacts were seen but no synaptic specialization could be found in these cells. When SH-F cells are treated with RA and staurosporine together, therefore, a striking phenotypic change indicative of neuronal differentiation is observed.

In summary, upon RA treatment, SH-N cells displayed neuronal differentiation, demonstrated by the presence of neuronal markers such as neurofilament proteins, induction of synaptophysin and NCAM, as well as characteristic ultrastructural features such as neurosecretory granules, elaborate processes with well-ordered microtubules and synapse-like connections. In contrast, RA-induced differentiation of SH-F cells, resulted in the loss of these neuronal markers. Electron microscopic examination also did not reveal any neuronal features in these flattened cells, instead, enlarged cells showing elaborate cytoplasmic differentiation were seen. The cell type that RA-treated SH-F represents, or is destined to represent remains to be defined. In contrast, when SH-F cells were treated with staurosporine and RA together, a neuronal phenotype was produced. This statement is based on the presence of markers such as NCAM, and neurofilament proteins (none of which was found when SH-F cells were treated with

RA alone), as well as specialized neuronal features such as neurosecretory granules, well ordered microtubules in neuritic processes, and synapse-like connections.

4.5 DISCUSSION

To characterize the phenotypes expressed by the differentiated SH-F and SH-N cells, some aspects of the classification scheme employed by Cicarrone et al. (1989) were used, as well as some additional markers such as synaptophysin and NCAM. Electron microscopy was carried out to provide further support for immunohistochemical findings.

The expression of the S100 protein has been previously used as a marker for Schwann cell differentiation in certain neuroblastoma cell lines (Tsokos et al., 1987; Tsunamoto et al., 1987). The results obtained in this study, however, showed that all cells tested, reacted positively to this antibody regardless of the differentiation state or phenotype expression. This indicates, at least for the SH-F and SH-N cell lines, that S100 protein is neither a suitable marker for differentiation, nor an indicator of phenotype, as cells showing markedly different morphologies, and distinct marker profiles, reacted similarly with this antibody.

Synaptophysin expression is clearly enhanced in RA-treated SH-N cells and was essentially absent in the RA-treated SH-F cell line, that forms the flat type phenotype. As this is a protein synthesized and incorporated in synaptic vesicles and presynaptic membranes, (Weidenmann and Franke, 1985), it serves as a clear indicator of neuronal differentiation in the SH-N cell line. The lack of expression of this protein in SH-F, indicates a divergence in marker expression in these two

variant cell lines following RA-induced differentiation.

The NCAM antigen, is a surface glycoprotein, the expression of which is precisely controlled in morphogenesis and is thought to play a key role in development (Theiry et al., 1985). The presence of this protein on the surface of the neuroblastoma cell line, Neuro-2A, has previously been documented (Pollerberg et al. 1985). In the present studies it appears that there is an increase in the surface expression of this marker, following RA-induced differentiation of SH-N, and staurosporine and RA-treated SH-F, however, this point is difficult to ascertain without quantitative measurement. Such an augmentation would be consistent with previous findings by Brackenbury et al. (1984) demonstrating reduced expression in transformed cells. As the changes in levels of this antigen do not appear to be striking, the use of NCAM as a differentiation marker per se, is of limited value in these neuroblastoma cell lines. A more interesting point arising from this study however, is the disappearance of NCAM from the surface of the fibroblast-like, RA-treated SH-F cells. In contrast, cells exhibiting neuronal differentiation following RA treatment, retained or appeared to exhibit enhanced expression of NCAM on their membranes. In view of the fact that this glycoprotein is a regulator of developmental pathways, the finding that the expression of this morphoregulatory molecule is completely different, not only in RA-treated SH-F and SH-N, but also before and after the addition of staurosporine in SH-F cultures, provides further evidence for the phenotypic diversion in these cell types. It should also be noted that the presence of this marker correlates well with that of the neurofilament proteins

in these variant cell lines.

Earlier work had suggested that the major intermediate filament protein found in cultured neuroblastoma was vimentin, which was present before and after differentiation (Browning and Roisen, 1984). This intermediate filament is usually found in mesenchymal tissue and does not occur in differentiated neurons in vivo (Traub, 1985). Other studies investigating intermediate filament expression in the murine neuroblastoma cell line, Neuro-2A cells, revealed that upon dbcAMP-induced differentiation, neurofilament expression was enhanced with concomitant decrease in the expression of vimentin (Shea et al., 1988). Studies of Biedler and coworkers (Cicarrone et al. 1989), have also shown that the substrate adherent neuroblastoma cell lines express vimentin at high levels, however this protein is not expressed or is present at much lower levels in cells exhibiting a neuronal phenotype. In our studies, this antigen was found in all cells tested, however, the flattened cell type (RA-treated SH-F) had large amounts of this intermediate filament throughout the entire cytosol, while the neuronal cell types demonstrated positive immuno-staining in the perinuclear region only.

The presence of vimentin in these cells may be attributable to two reasons. Vimentin is usually expressed temporarily by neuro-ectodermal cells prior to the appearance of neurofilament proteins. In development, cells may co-express these intermediate filament proteins for a very short period of time. The presence of this antigen may indicate the early origin of the tumor from which these cells were derived, but more likely it is a result of the growth of these cells in culture. Previous

studies have shown that vimentin is often re-expressed once cells are removed from the native tissue and grown in culture, where the usual tissue cell-cell contact is lost. The loss of tissue organization seems to correlate with the appearance of this protein in many cell types that are not of mesenchymal origin (Traub, 1985).

More recently, the presence of neurofilament proteins has been demonstrated in neuroblastoma cell lines (Shea et al., 1988; Cicarrone et al., 1989; Beidler et al., 1987). The murine neuroblastoma Neuro-2A cell line, was found to express all three neurofilament triplet polypeptides. Their expression was markedly increased following dbcAMP induced differentiation (Shea et al., 1988; Breen and Anderton, 1990). In the neuroblastoma cell lines under current investigation, NF68 and NF160 were present before differentiation but NF200 was not detected. Following RA-induced differentiation of SH-F both of these proteins were lost. However, when the SH-F cells were induced to differentiate to a neuronal phenotype by the addition of staurosporine and RA simultaneously, these neurofilament proteins continued to be expressed. The same pattern was observed for RA-treated SH-N, where the expression of NF68 and NF160 were maintained following differentiation. No NF200 was detected in any of these cells. Although it is known that neurofilament proteins usually occur as a triplet (Liem et al. 1978), the two proteins, NF68 and NF160 appear much earlier in development and are found to be expressed together. NF200 has been shown to appear later and is not necessarily present in all neurons (Scott et al., 1985). Consistent with these results, are our findings showing simultaneous expression of NF68 and NF160. Again, the fact that NF200 was not detected could

be attributed to the early possible origin of the tumor from which these cells were isolated.

Ultrastructural observations were consistent with results obtained by immunohistochemistry. Cells that contained neurofilament proteins, synaptophysin and NCAM, also showed morphologies characteristic of neuronal cells. The RA treated SH-F, that lost the neuronal markers such as neurofilament proteins and NCAM, did not display any typical neuronal features.

Following a 7-day treatment with RA, SH-N variants had undergone typical changes characteristic of neuronal differentiation. Soma developed from relatively small cytoplasms with scant organelles to cytoplasms highly enriched with organelles and increased neuronal specialization. Characteristic of these changes are the appearance of well developed Golgi bodies, numerous neurosecretory granules and numerous processes containing well-ordered microtubules. These cells also displayed perinuclear aggregates of intermediate filaments. Immunohistochemical results revealed positive staining with vimentin and NF160 in this neuronal cell type. This aggregate, therefore, is probably composed of a combination of these filaments the display of which is in striking resemblance to the filamentous aggregates reported for nerve growth factor -induced differentiation of PC12 cells (Lee et al., 1983). Fluorescent antibodies revealed collections of neurofilament subunits resembling 'ball-like' structures in the perinuclear region. The published Electron micrographs of these PC 12 cells contain intermediate filament aggregates which closely resemble those seen in RA-treated SH-N. (Figure 4.6b) Lee et al. (1983) suggested that such

collections of neurofilaments are likely a result of defective assembly and axonal transport of these proteins. Such putative defects of intermediate filament assembly may be common features in tumor cells induced to differentiate in culture.

Further abnormalities found in these cells were the dystrophic changes in the processes showing accumulation of organelles. Such changes are commonly found in numerous pathological conditions of the central nervous system, such as exposure to neurotoxins, defects related to aging, and differentiated cells of neuronal tumors (Russel and Rubenstein, 1989). The development of these abnormalities is thought to be related to defects in axonal transport (Traub, 1985; Russel and Rubenstein 1989). While varicosities containing dense core vesicles have been described in another well studied neuroblastoma cell line, LAN-5 (Robson and Sidell, 1985), no significant accumulation of organelles have been reported in the processes of these cells.

Although differentiated SH-N displayed increased synaptophysin expression, a protein required for synapse formation (Weidenmann and Franke 1985), such specializations were not found in these cell cultures. Many examples were found where synapse-like contacts were present but neither the characteristic collection of vesicles in presynaptic termini, nor the apparent membrane thickenings typical of synapses (Peters et al. 1976), were seen among any of these cells. Synapse formation, the hallmark of neuronal differentiation, is very rarely found in cultured tumor cells. Only a few exceptions have been reported (Spoerri, 1983; McBurney et al., 1988).

RA-treated SH-F cells displayed a strikingly different morphology from SH-N

cells. No indication of neuronal specialization was apparent in these cells. Accordingly, treatment with retinoid was accompanied by a loss of neuronal markers such as neurofilament proteins and NCAM following RA treatment. Addition of RA, however, clearly resulted in differentiation of non-neuronal cells, as attested by an increased cytoplasmic volume, heavily populated with organelles. Enlarged, dilated vesicles therein suggested that there was synthesis of compounds destined for secretion, however, the precise nature of the substance is unknown. These cells also seem to synthesize generous amounts of lipid. This abundance of enlarged vesicles was not seen in RA-treated SH-N, or SH-F treated with RA and staurosporine together. Further peculiarities of the flattened cells are the elaborate filamentous structures, the identity of which are unknown. Although heavy cytoplasmic staining with vimentin was found, the possibility that these filamentous aggregates could be composed of microfilaments has not been ruled out. While the differentiated SH-F cell type cannot be precisely identified at this time, it is appropriate to conclude that: a) RA treatment results in differentiation of this cell; and b) the resultant differentiated cells are strikingly different from those depicted in Figure 4.5 for RA treated SH-N. Therefore, divergent phenotypes arising from the same precursor cell are clearly demonstrated. Examination of these same SH-F cells treated with staurosporine together with RA, again, revealed a distinctly different phenotype.

Retinoic acid and staurosporine-induced differentiation of SH-F resulted in the development of a typical neuronal cell type as opposed to this enlarged flattened cell described above. Immunohistochemical findings, such as the presence of

neurofilament proteins, also indicate that this is a neuronal phenotype. Ultrastructural examination revealed elaborate processes with the typical well-ordered microtubule arrays and large numbers of neurosecretory granules were seen in most cells. Dystrophic-like swellings of the processes, as reported for RA-treated SH-N, were also observed. It may be added that in the present study, staurosporine was added together with RA because the purpose here was to show that even under conditions which normally cause flattening of SH-F cells, the PKC inhibitor could change the phenotype of this cell line. It must be emphasized that staurosporine alone (Chapter 3) causes neuronal type differentiation of both SH-F and SH-N sublines, although characterization of the differentiation process under this condition was not studied in detail. The results as a whole indicate a striking difference in the RA-induced differentiation of SH-F cell line, with and without staurosporine, and are supportive of the hypothesis that inhibition of the PKC by the addition of staurosporine promotes neuronal differentiation in both sublines.

4.6 CONCLUSION

Based on the above data, certain generalizations can be made regarding the phentoypes expressed by these cells. According to the classification put forth by Biedler and coworkers, for variant neuroblastoma cell lines,(Cicarrone et al. 1989) (Ross et al. 1987), SH-N induced to differentiate with RA, and SH-F treated with staurosporine and RA together, fit the description of a differentiated form of the 'N' type or neuronal cell type. This is based on the observation of extensive processes, neurosecretory granules, reduced substrate adherence and presence of neuronal

markers such as neurofilament proteins. The RA-treated SH-F cells, which form the flattened fibroblast-like cell type, do not fit the description of the 'neuronal' type cell. These cells were strongly substrate adherent, the majority of which had no processes, displayed relatively small nuclear to cytoplasmic ratios, and lacked the neurofilament proteins, synaptophysin and NCAM. They more closely resembled a differentiated form of the 'S'type cell, however, the exact subset to which these cells belong is still uncertain at this time. The recent detection of a melanocyte marker in undifferentiated SH-F (Maeda et al., 1990) may be an indication of the destined phenotype of this subline, but further work would be required to establish whether RA-induced differentiation accentuates the melanocytic properties.

The goal of this study was to characterize and compare the phenotypes produced by a) RA-induced differentiation of SH-F and SH-N cells; and b) the phenotypes produced by RA-treated SH-F cells with and without staurosporine. Not only do our results show that RA treatment resulted in differentiation of these cells, but immunohistochemical data and electron microscopic examination, also confirm that markedly different phenotypes are produced by SH-F and SH-N cells in response to RA. SH-N showed characteristics of neuronal differentiation, whereas SH-F, in contrast, more closely resembled a differentiated form of the 'S' type cell previously described by Biedler et al. (1987). The addition of staurosporine and RA together to this same SH-F cell line, however, resulted in a distinct change in the resultant marker expression and morphology, whereby these cells now displayed neuronal specialization. Inhibition of PKC by the simultaneous addition of the inhibitor,

staurosporine, to RA-treated SH-F cells, markedly altered the resultant phenotype causing a striking increase in the expression of neuronal characteristics. These results provide further support for the hypothesis presented in the previous chapter, that PKC inhibition promotes neuronal differentiation.

GENERAL CONCLUSION

The results of the studies reported herein clearly show that derivatives of fat-soluble vitamins can have marked effects on the growth and differentiation of neuroblastoma. The vitamin A derivative, RA, however, appears to cause more profound effects than the succinate ester of vitamin E.

Neither vitamin E, nor its succinate derivative, demonstrated the ability to cause differentiation of these cells. Instead the succinate ester exhibited cytotoxic effects which are not likely exclusive to rapidly growing cells. Therefore, the potential of this agent, in providing a novel approach for treatment of tumors such as neuroblastoma, whereby the cells would be reprogrammed to cease proliferation, and undergo differentiation, seems very limited. Attention was therefore directed to the vitamin A derivative, RA.

Retinoic acid, on the other hand, as well as staurosporine, have the ability to intervene in signal transduction pathways by affecting PKC and components of the protein kinase A system. Such effects reprogram cells and induce them to cease proliferation and undergo differentiation. Further research investigating the use of such agents is warranted in the quest of developmenting novel techniques for the treatment of certain types of tumors, including neuroblastoma.

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CURRICULUM VITAE

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PUBLICATIONS

Papers Published

Slack,R.S. and Proulx,P.
Effects of Retinoic Acid and Staurosporine on Protein Kinase C Activity and the Morphology of two related Human Neuroblastoma Cell Lines
Biochim. Biophys. Acta 1053(1): 89-96 (1990)

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Abstracts

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FEBS, 20th Annual Meeting, Budapest, Hungary (1990)

Slack,R.S. and Proulx,P.
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Effects of Retinoic Acid on Protein Kinase C
F.A.S.E.B., 73rd Annual Meeting, New Orleans (1989)

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