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THE EFFECT OF THE DELAYED NEUROTOXIN DIISOPROPYLFLUOROPHOSPHATE (DFP) ON THE DISTRIBUTION OF GANGLIOSIDES IN THE HINDBRAIN OF THE CHICKEN (GALLUS DOMESTICUS)

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# THE EFFECT OF THE DELAYED NEUROTOXIN DIISOPROPYLFLUOROPHOSPHATE (DFP) ON THE DISTRIBUTION OF GANGLIOSIDES IN THE HINDBRAIN OF THE CHICKEN (GALLUS DOMESTICUS)

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ABSTRACT

THE EFFECT OF THE DELAYED NEUROTOXIN DIISOPROPYLFLUOROPHOSPHATE (DFP) ON THE DISTRIBUTION OF GANGLIOSIDES IN THE HINDBRAIN OF THE CHICKEN (GALLUS DOMESTICUS)

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The mechanism of action of compounds causing organophosphorus ester-induced delayed neurotoxicity (OPIDN) is still a mystery as is the function of gangliosides in the nervous system. To determine if there was a relationship between the development of OPIDN and the relative proportion of endogenous brain gangliosides, hens were injected subcutaneously with diisopropylfluorophosphate (DFP) at a dose of 1 mg/kg body weight. Birds were sacrificed at time intervals corresponding to different stages of ataxia. DFP was found to have no effect on the concentrations of protein, total lipid, lipid phosphorus, total cholesterol and ganglioside-bound sialic acid in the chicken hindbrain. The effect of DFP on the relative proportion of the major gangliosides in the hindbrain was also examined. DFP affected the relative proportion of GM4, GD3, GT1b and GQ1b within the hindbrain. Results suggest that changes in the ganglioside profile began to occur before the advent of neuronal degeneration.

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# INTRODUCTION

The study of organophosphorus-induced delayed neurotoxicity (OPIDN) began with the work of Maurice Smith and his co-workers (1930) at the National Institutes of Health. They sought the cause of a peculiar form of paralysis which affected thousands of people during the 1920s. It is now sixty years after their initial studies and the mechanism of OPIDN is still a mystery. More than 40,000 people have suffered from the paralysis caused by organophosphorus delayed neurotoxins (Abou-Donia and Lapadula, 1990). In 1978, the Environmental Protection Agency published guidelines in the Federal Register for testing organophosphorus compounds to determine their ability to cause OPIDN (U.S. Environmental Protection Agency, 1978). According to Abou-Donia and Lapadula (1990), there were more than 1,000 cases of OPIDN in humans during the 1980s.

Like the mechanism of delayed neurotoxicity, the function of gangliosides is also a mystery. Since their discovery by Ernst Klenk in the 1930s, gangliosides have been studied intensively. These lipids have received a great deal of attention because they have neuronotrophic and neuritogenic characteristics. The effect of delayed

neurotoxins on lipid components of nervous tissue was studied frequently during the 1960s and 1970s, but ganglioside concentrations were not examined.

Few studies have examined the effect of physical or chemical insults on the ganglioside profiles of the nervous system. This is unfortunate because much useful information concerning the function of gangliosides could be gathered from such studies. By knowing how a nerve degenerates after exposure to a toxin, one can learn useful information concerning the mechanism of action of the toxin and possible ways of reducing or preventing neuronal damage. In the following study, the degeneration caused by an organophosphorus delayed neurotoxin will be used as a model for studying the role of gangliosides in the nervous system.

# LITERATURE REVIEW

## GANGLIOSIDES

### Discovery-

Ernst Klenk discovered an unusual lipid during the late 1930s while studying the brains of patients who suffered from Tay-Sachs disease. This lipid, which Klenk called "substance X", was abnormally high in the brains of Tay-Sachs patients. Since Klenk thought "substance X" was concentrated in neuronal cells (ganglienzellen), he gave it the less mysterious name ganglioside (Yu, 1983; Wiegandt, 1985).

### Structure, classification and nomenclature-

It was more than twenty years after Klenk's discovery that the structures of the major gangliosides of the mammalian brain were determined. It is now known that gangliosides are sialic acid-containing glycosphingolipids. A ganglioside molecule consists of a hydrophilic sialosyloligosaccharide headgroup attached by a glycosidic bond to a hydrophobic ceramide tail (Figure 1). Sialic acid residues are attached either to a galactose of the oligosaccharide chain or to another sialic acid. The ceramide portion, consisting of sphingosine and a fatty acid bound in amide linkage, is

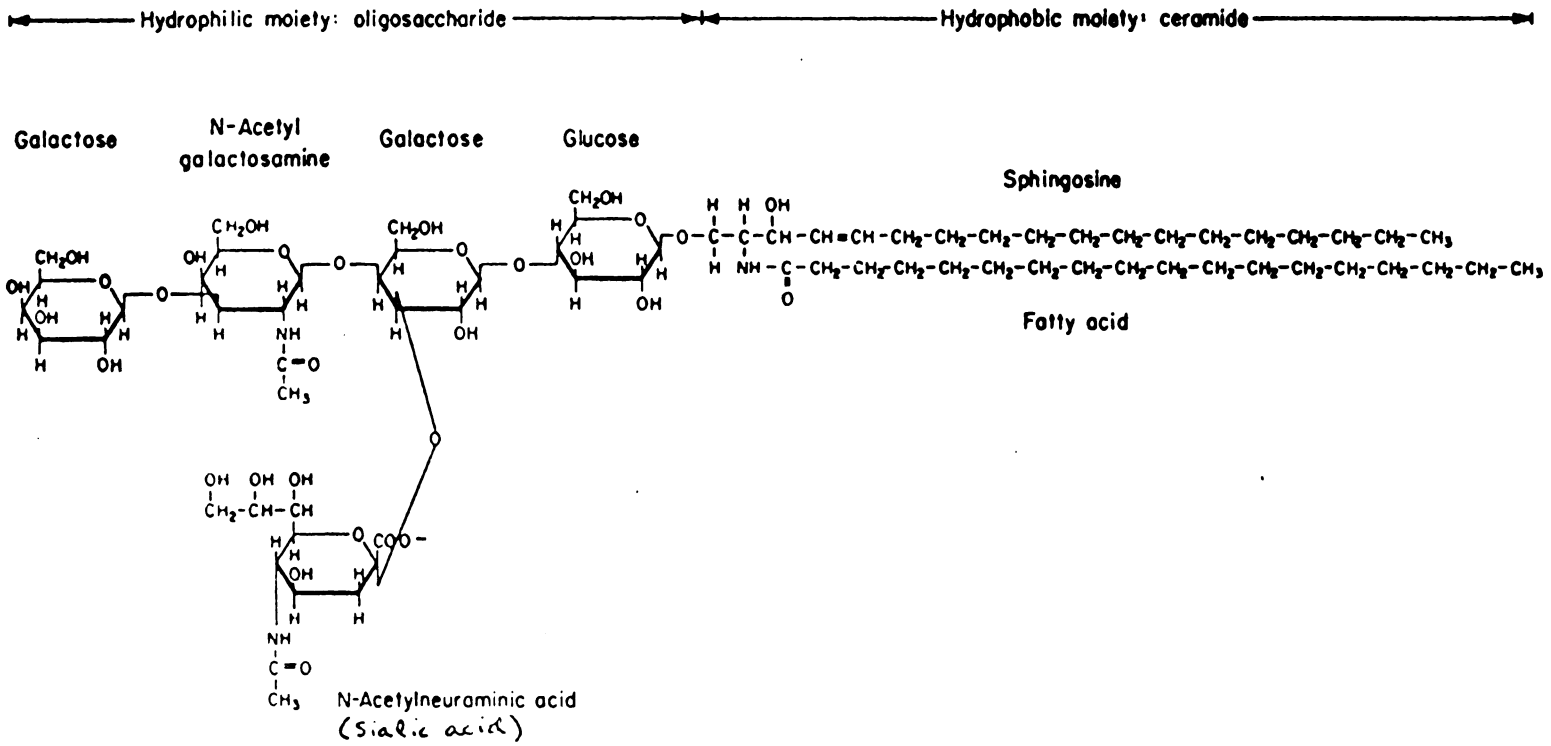


Figure 1. The structure of the ganglioside GM1 (From Rapport, 1981).

located in the plasma membrane. The sialosyloligosaccharide portion extends out from the membrane surface into the extracellular space and significantly contributes to the negative charge of the cell surface (Ando, 1983).

Gangliosides can be classified into four series (gala, neolacto, globo, and ganglio) based on the composition of the oligosaccharide chain (Table 1). The majority of the gangliosides of the brain belong to the ganglio-series. Gangliosides may differ from one another in the following ways (Rapport, 1981; Ledeen and Yu, 1982):

1. The number of sugar residues may vary.
2. The number and position of sialic acid residues may vary.
3. Glucosamine may substitute for galactosamine.
4. Fucose residues are sometimes present.
5. Glycolyl may substitute for acetyl groups on the sialic acid.
6. O-acetyl groups are sometimes present.
7. The structure of the ceramide may vary.

Gangliosides are normally named using the rules established by the IUPAC-IUB Commission on Biochemical Nomenclature (1977) or by a method developed by Svennerholm (1980). In this paper, the shorter notation of Svennerholm (1980) will be used. Figure 2 shows the chemical structures of some of the more common gangliosides of the central nervous system. Even though more than 70 different gangliosides have been detected in the mammalian brain, certain gangliosides

Table 1. Classification of gangliosides according to the structure of their carbohydrate core.

| Series | Structure |
|---|---|
| Gala | Gal-Cer |
| Neolacto | Gal-GlcNAc-Gal-Glc-Cer |
| Globo | GalNAc-Gal-Gal-Glc-Cer |
| Ganglio | Gal-GalNAc-Gal-Glc-Cer |

Gal=Galactose, Cer=Ceramide, Glc=Glucose, GlcNAc=N-Acetylglucosamine and, GalNAc=N-Acetylgalactosamine

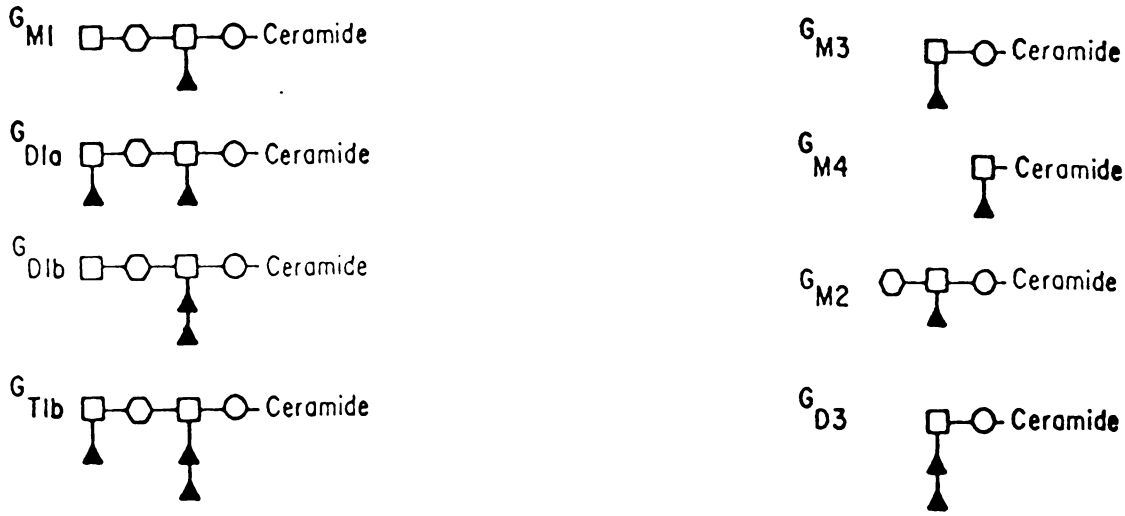


Figure 2. Chemical structure of selected gangliosides. The nomenclature of Svennerholm (1980) is used. According to this method, M, D, and T indicate 1, 2, and 3 sialic acid residues respectively (Q and P would indicate 4 and 5 such residues, respectively). G stands for ganglioside and the letters a and b are used to distinguish between positional isomers of sialic acid. Symbols: circle, glucose; square, galactose; hexagon, N-acetylgalactosamine; filled triangle, sialic acid (From Rapport, 1981).

predominate. For instance, the gangliosides GM1, GD1a, GD1b, GT1a and GT1b constitute over 80% of the total quantity of gangliosides in the mammalian brain (Mahadik and Karpiak, 1988).

Isolation and identification-

The partitioning method of Folch et al. (1957) is a popular method of isolating gangliosides. This method involves the extraction of gangliosides into a chloroform-methanol phase, followed by the partitioning of the gangliosides into an upper water-enriched phase. The gangliosides enter the water-enriched phase because of their hydrophilic sugar moieties. The problem with this method is that there is not complete recovery of the less polar gangliosides (Ando, 1983). Susuki (1965), Svennerholm and Fredman (1980), Ladisch and Gillard (1985) and others have modified this procedure to obtain better recovery of gangliosides.

Gangliosides can also be isolated without using methods which rely on partitioning. Lipids can be extracted from other membrane components by homogenizing the sample in various combinations of chloroform and methanol. Chloroform-methanol (2:1) is frequently used for lipid extraction because this combination is very efficient at dissociating the lipid-protein complexes that are found in membranes (Radin, 1969). Byrne and co-workers (1985) used mild acidification following

homogenization to dissociate gangliosides from lipophilic peptides. Svennerholm and Fredman (1980) found that the addition of water to chloroform and methanol enhanced the recovery of gangliosides. They found that a chloroform-methanol-water ratio of 4:8:3 worked most effectively.

After the lipids are extracted, the gangliosides must be separated from other lipids. This can be done quickly for a small amount of tissue by applying the lipid extract to a Unisil silicic acid column and eluting the gangliosides with a chloroform-methanol mixture (Irwin and Irwin, 1979). Because contaminants are often present in the ganglioside fraction after using this method, researchers have developed other methods of isolating gangliosides that are better able to remove these unwanted substances (Ando, 1983).

Ledeen and Yu (1982) add the lipid extract to a DEAE-Sephadex column and then elute the gangliosides with chloroform-methanol-sodium acetate. The elutant from the column is treated with a base to destroy any phospholipids that may be present. Dialysis is used to remove salts and other small molecular weight contaminants from the sample. There is a risk of losing some gangliosides from the dialysis tubing when a small sample is used. To prevent this loss, alternative methods such as a Sephadex G-50 column or a Sep-Pak C18 reverse phase cartridge can be used in place of the dialysis tubing. Finally, a Unisil silicic acid or iatrobead column is used to remove sulfatides, fatty acids and any

acidic lipids other than gangliosides that are still present in the sample.

Leakage of weakly ionized gangliosides and reduced recovery of total gangliosides may occur when a DEAE-Sephadex column is used. The DEAE-Sephadex column can be replaced with a DEAE-Toyopearl column to prevent these losses. Ganglioside recovery can be improved even further by using a phenyl-sepharose column (Ando et al., 1988). The recovery of gangliosides achieved using different extraction methods is shown in Table 2.

Column chromatography has been used to isolate individual gangliosides (Momoi et al., 1976). Gangliosides are first added to a DEAE-Sephadex column and then eluted according to the number of sialic acids they contain. This is done by adding different concentrations of sodium acetate in methanol to the column. The eluants from the DEAE-Sephadex column having the same number of sialic acid residues are then added to an iatrobead column. Different combinations of chloroform, methanol and water are used to elute the different species of gangliosides.

Thin layer chromatography is used to determine the ganglioside profile of the sample. Thin layer chromatography separates individual gangliosides according to their polarity. Less polar gangliosides travel faster, and as a result, farther up the plate than the more polar gangliosides. Chloroform-methanol-aqueous calcium chloride,

Table 2. Recovery of gangliosides from monkey brain total lipid extracts using different isolation methods.[1] Values expressed as mean ± standard deviation.

| Isolation method | Recovery (%) |
|---|---|
| Two-phase partitioning | |
| with chloroform/methanol/0.9% aq NaCl[2] | 75 ± 4 |
| with diisopropylether/n-butanol/ 50 mM aq NaCl[3] | 94 ± 8 |
| Ion-exchange chromatography | |
| DEAE-Sephadex[4] | 84 ± 9 |
| DEAE-Toyopearl[5] | 88 ± 14 |
| Chromatography using a hydrophobic resin followed by ion-exchange chromatography | |
| Phenyl-sepharose | 99 ± 7 |
| Phenyl-sepharose-DEAE-Toyopearl | 97 ± 3 |

[1]Table from Ando and co-workers (1988)
[2]Suzuki (1965)
[3]Ladisch and Gillard (1985)
[4]Ledeen and Yu (1982)
[5]Ando and Saito (1987)

chloroform-methanol-ammonia solution-water, and n-propanol-water are commonly used solvent systems. A salt is often added to the solvents to prevent interactions between the gangliosides and the silica gel on the plates (Ando, 1983).

To visualize the gangliosides, the plates are normally sprayed with the resorcinol-hydrochloric acid reagent developed by Svennerholm (1957). Using this reagent, Ando and co-workers (1987) found a lower detection limit of approximately 100 pmol sialic acid when gangliosides were separated on 20 centimeter long plates. Sprays containing 3,5-diaminobenzoic acid, thiobarbituric acid and periodate-resorcinol have also been used to visualize gangliosides. In certain cases, it is necessary to use non-destructive agents like rhodamine, iodine or water to reveal the gangliosides on the chromatography plates (Ledeen and Yu, 1982).

The resorcinol-hydrochloric acid reagent of Svennerholm (1957) can be used to determine the amount of sialic acid in a sample. Yu and Ledeen (1970) developed a different method of quantifying sialic acid which uses gas chromatography. Gas chromatography is more accurate than colorimetry because the values obtained are not affected by contaminants in the sample. Gas chromatography is also more informative than the colorimetric method because it is able to distinguish between different types of sialic acids (Ledeen and Yu, 1982).

Suzuki (1964) developed a method to quantify gangliosides which are separated by thin layer chromatography. According

to this method, the band associated with each ganglioside is scraped from the plate and the amount of sialic acid is determined from the scraping. This method cannot be used in all situations because the detection and quantification of minor gangliosides is difficult and a large sample is normally required. Densitometry is a useful alternative because it is faster, easier and it requires a smaller amount of sample than does Suzuki's method (Ando et al., 1978).

Thin layer chromatography has often been used to separate different types of gangliosides. The problem with this technique is that it is often difficult to separate minor gangliosides from the more prevalent gangliosides on chromatography plates (Ledeen and Yu, 1982). Ohashi (1979) developed two-dimensional thin layer chromatography to enhance resolving power. This method, especially when combined with autoradiography, is capable of detecting minor gangliosides which cannot be observed if the one-dimensional method is used (Ledeen and Yu, 1982).

Ganglioside standards are often co-chromatographed with the samples so that the gangliosides comprising each band can be identified. This is a difficult method to use when minor gangliosides are being studied. Enzyme-immunostaining has been used to identify minor gangliosides on thin layer plates (Hirabayashi et al., 1988), but in many instances the actual structure of the ganglioside must be determined. Structural characterization requires the determination of:

(1) carbohydrate composition; (2) carbohydrate sequence; (3) the type, quantity and position of sialic acid residues; and (4) the sphingoid and fatty acid composition of the ceramide (Yu, 1983; Wiegandt, 1985).

The oligosaccharide composition of a ganglioside can be determined by gas-liquid chromatography (GLC) of individual sugars. The sugars must first be converted to their methyl glycoside form. The methyl glycosides are then trimethylsilylated (Sweeley and Walker, 1964) or trifluoroacetylated (Ando and Yomakawa, 1971) to form compounds that can be analyzed by gas-liquid chromatography. The use of trifluoroacetylation is advantageous because the fluorine atoms provide for highly sensitive analysis when used in conjunction with an electron capture detector (Ando, 1983). Quantification is difficult using these methods because methanolysis produces more than one methyl glycoside for each sugar. Use of the alditol derivative avoids this problem, but the method causes partial destruction of sialic acid residues. It is therefore often necessary to use more than one method to analyze the oligosaccharide (Ledeen and Yu, 1982).

The carbohydrate sequence of the ganglioside can be determined by sequential reactions with specific glycosidases. These reactions are normally applied to the desialylated or partially desialylated ganglioside. Acid hydrolysis and acetolysis of the gangliosides followed by analysis of the

liberated sugars have both been used to determine carbohydrate structure. Mass spectrometry can also be used. This method is advantageous because it requires only a small quantity of sample. Electron-impact ionization, chemical ionization and fast atom bombardment mass spectrometry have all been used (Ando et al., 1988). Handa and Kushi (1988) developed a method whereby a sample can be analyzed by mass spectrometry without being eluted from the chromatography plate.

Glycosidic substitution is determined by periodate oxidation or permethylation followed by gas chromatography. Anomeric configuration of gangliosides is normally determined by using specific glycosyl hydrolases. The drawback of this method is that it is difficult to obtain pure enzyme preparations. Nuclear magnetic resonance spectroscopy is an effective alternative to the enzymatic method. It is a quick and nondestructive method that can be used not only in the analysis of anomeric configuration but also in the analysis of other structural features as well (Ando, 1983).

Distribution-

Almost every vertebrate tissue contains gangliosides, but their concentrations are especially high in the gray matter of the brain. At the cellular level, they are primarily located in plasma membranes. Gangliosides are estimated to constitute 5-10% of the total neuronal plasma lipids, but because they are mainly present in the outer half

of the lipid bilayer, their concentration at the surface of the cell can be as high as 10-20% of the total lipids (Ledeen, 1978). It is not known whether gangliosides are distributed evenly throughout the neuron, but there is evidence that the synaptic junctions may have a higher concentration of gangliosides than do the other parts of the neuron (Ando, 1983). Svennerholm (1980) found that the proportion of GD1b, GT1b and GQ1b was high in the synaptic junction.

Ganglioside distribution differs among cell types within the nervous system. For instance, astroglia have higher concentrations of gangliosides than neurons while oligodendroglia have lower concentrations of gangliosides than either of these cell types. Astroglia and neuronal perikarya have similar ganglioside profiles whereas oligodendroglia have a higher proportion of GM1 and GM4 (Ando, 1983).

The study of mice with genetic mutations has provided insight into the cellular distribution of gangliosides in the cerebellum. Mutant mice have been developed that lose specific populations of cerebellar neurons during their development. Studies of these mice have shown that GD1a is enriched in granule cells and GT1a is enriched in Purkinje cells. GD3 is associated with reactive glial cells and other cells with a high metabolic activity, e.g. undifferentiated or mitotically active neurons, Muller glia, oligodendroglia, neuroblastomas and astrocytomas. GT1b is enriched in both Purkinje and granule cells. GD1b is thought to be located in

the plasma membranes of myelinated axons. GQ1b, GM3 and GD1b do not appear to be enriched in Purkinje or granule cells (Seyfried et al., 1984).

Ganglioside distribution also differs among regions of the nervous system. For example, ganglioside profiles of the peripheral nervous system are different from those of the central nervous system. The peripheral nervous system has a large proportion of lacto- and globo-series gangliosides whereas the central nervous system has mainly gangliosides from the ganglio-series (Wiegandt, 1985). The concentration of gangliosides in the brain is higher than in the spinal cord while the peripheral nerve has a lower concentration of gangliosides than either of these regions.

Biosynthesis and degradation-

Gangliosides are synthesized in the Golgi apparatus of the neuronal perikarya by the stepwise addition of sugar residues to ceramide. The sugar residues, which are bound to nucleotides in the cytosol, are transported into the golgi apparatus by carrier proteins. The sugar residues are then transferred to the ceramide acceptor molecule by glycosyltransferases which are located in the Golgi cisternae. The newly formed gangliosides are transported by fast axonal transport via vesicles to the plasma membrane. After being inserted into the plasma membrane, a ganglioside may be modified by being internalized, glycosylated and then returned

to the plasma membrane as a more complex ganglioside (Tettamanti, 1988) (Figure 3).

There are three pathways, designated A, B and C, by which gangliosides may be synthesized (Figure 4). GM4 has a galactose instead of a glucose attached to the ceramide, so it is not formed via one of these pathways. Birds and reptiles use both pathways A and B to synthesize gangliosides, whereas mammals use mainly pathway A. Lower vertebrates, such as fish, use almost exclusively the C pathway to synthesize gangliosides (Rahmann, 1983).

Ganglioside profiles of the whole brain differ between species of animals. The brain of the adult chicken has a greater proportion of GD3 and a smaller proportion of GM1 than do the brains of other species (Dreyfus et al., 1975; Iwamori and Nagai, 1978). The ganglioside profiles of brain myelin also differ between different species (Table 3). The chicken has a higher concentration of gangliosides and a greater proportion of GM4 in the brain myelin than do other species (Cochran et al., 1981).

Gangliosides are broken down in the lysosomes by sequential hydrolysis reactions catalyzed by the following hydrolases: sialidase, galactosidase, hexosaminidase, glucosylceramide glucosidase, and ceramidase (Ando, 1983). The only exception to exclusive lysosomal degradation of gangliosides is the occurrence of sialidase in synaptosomal preparations as well as lysosomes (Schengrund and Rosenberg,

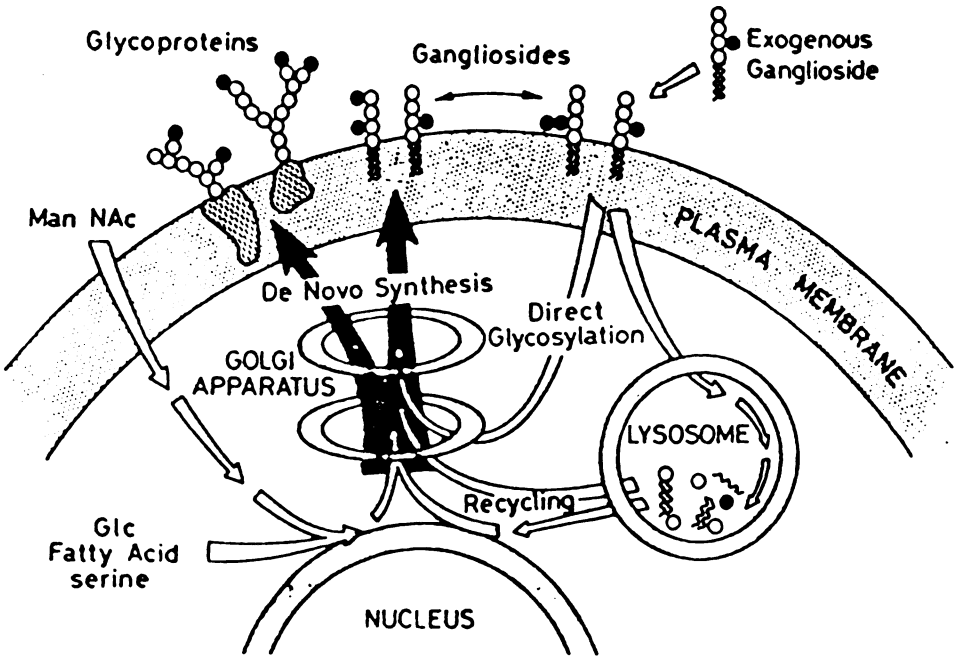


Figure 3. Schematic representation of the different routes of ganglioside biosynthesis (Tettamanti, 1988).

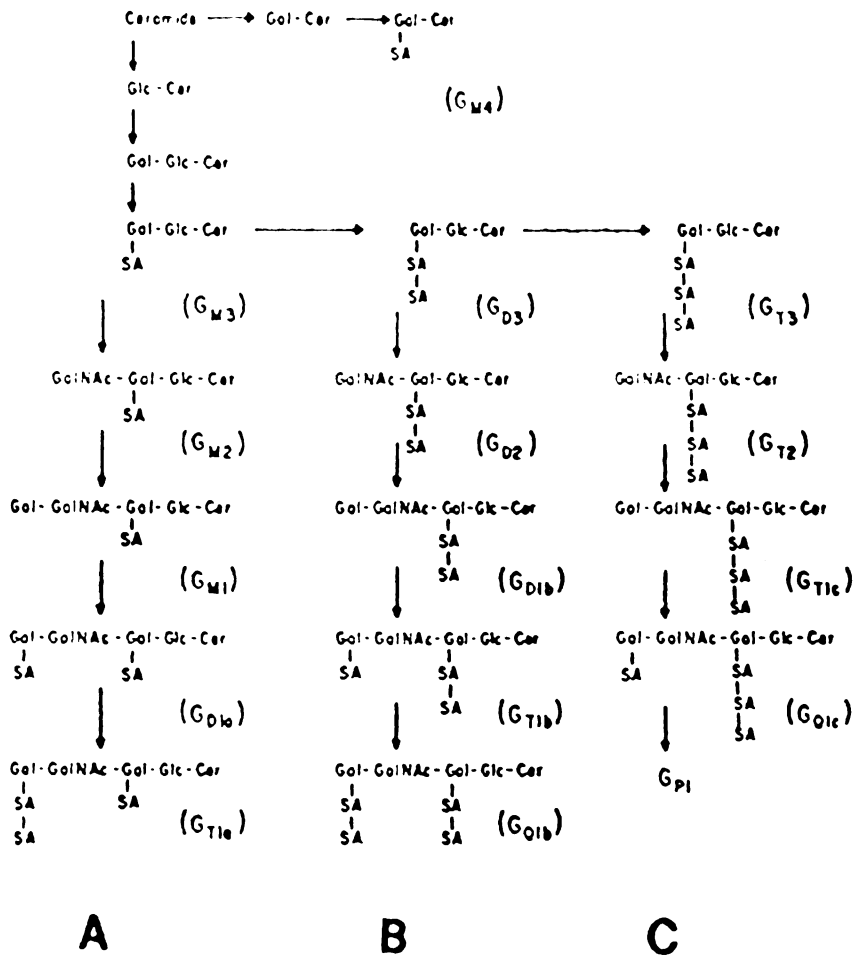


Figure 4. The pathways of ganglioside synthesis. Abbreviations are: Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; and SA, sialic acid (From Yu, 1983).

Table 3. Distribution patterns of myelin gangliosides of the brain. Values are expressed as a percentage of total ganglioside sialic acid. Table from Cochran et al. (1982).

| | CHICKEN | HUMAN | RAT | MOUSE |
|---|---|---|---|---|
| GM4 | 31.8 | 20.3 | 4.6 | 4.7 |
| GM3 | 2.9 | 3.5 | 1.4 | 0.3 |
| GM2 | 2.0 | 3.2 | 2.0 | 3.6 |
| GM1 | 33.8 | 31.7 | 63.7 | 59.6 |
| GD3 | 5.8 | 1.8 | 3.0 | 4.2 |
| GD1a | 11.4 | 7.8 | 5.8 | 8.0 |
| GT1a+GD2 | - | 1.2 | 2.6 | 4.8 |
| GD1b | 5.7 | 19.5 | 6.6 | 8.0 |
| GT1b | 5.0 | 8.9 | 6.2 | 4.2 |
| GQ1b | 1.6 | 2.1 | 4.1 | 2.6 |

1970). The break-down products from these reactions can be recycled by transferral from the lysosomes to the Golgi apparatus (Tettamanti, 1988) (Figure 2). Some inherited disorders are characterized by an overabundance of a ganglioside due to a deficiency in the activity of one of these enzymes. For example, in GM1 gangliosidosis there is a build-up of GM1 due to a defect in B-galactosidase and in Tay-Sachs disease there is a build-up of GM2 due to a defect in B-acetylhexosaminidase (Ando, 1983).

Changes during brain development-

During the development of the brain, there are changes in the concentrations of individual gangliosides. These changes have been shown to parallel structural changes. For instance, the levels of GD3 and GM3 increase during proliferation of neuronal and glial precursor cells; GQ1b increases during cell migration and arborization; GD1a and GT1b increase during the formation of synapses; GM1 and GM4 increase during myelination (Seybold and Rahmann, 1985; Mahadik and Karpiak, 1988; Skaper et al., 1989).

There is not a constant increase in ganglioside concentrations in the brain of a chicken as it ages. Dreyfus and co-workers (1975) found that the quantity of gangliosides in the chicken brain increases significantly just prior to hatching and then afterwards increases gradually to the adult concentration. The accumulation of gangliosides after

hatching parallels brain growth, but the increase prior to hatching does not. Other important changes are occurring during development. For instance, glycosyltransferases and sialyltransferases are highest during early post-hatching development and decrease during the adult stage (Mahadik and Karpiak, 1988).

Ganglioside patterns within the brain also change after parturition and hatching. There is an increase in the proportions of GM1 and GD1a and a slight decrease in the proportions of GD1b and GT1b in the brain of a chicken as it ages (Dreyfus et al., 1975). This pattern is not universal since brains of humans (Suzuki, 1965) and rats (Vanier et al., 1971) exhibit changes with age opposite to those observed in the chicken.

Certain gangliosides are expressed mainly during an animal's early development. The polysialogangliosides formed by the C pathway are important components in the brain of a young chicken, but are negligible in the adult. This is evidence of phylogenetic recapitulation since there is a decrease in polysialogangliosides during phylogeny to higher order species (Hilbig et al., 1981). The disappearance of the polysialogangliosides may also be related to the transition from a heterothermic to homeothermic state of development. The brain ganglioside profiles of different species of birds have been found to differ depending on whether their postnatal development is nidifugous or nidicolous (Seybold and Rahmann,

1985).

Biological functions-

Gangliosides may mediate the response of the membrane to environmental factors by affecting the dynamics and fluidity of the membrane. The relationship between gangliosides and membrane fluidity is the basis for Rahmann's hypothesis (1983) that gangliosides are involved in synaptic transmission. According to this hypothesis, calcium ions complex with gangliosides at the synapse before an action potential occurs. At this time, the gangliosides are clustered together making the membrane rigid and impermeable to the ions. An action potential causes a local change in ion concentration outside the membrane which displaces calcium ions from the gangliosides resulting in ganglioside dispersal. This dispersal increases the fluidity of the membrane resulting in an increased permeability to calcium ions. The resulting influx of calcium triggers the release of a transmitter into the synaptic cleft.

Gangliosides may be involved in the transfer of information through the plasma membrane. They may accomplish this function by influencing the production of secondary messengers or the generation of ion fluxes through the plasma membrane. Gangliosides have been found to influence the following membrane-associated proteins: adenylate cyclase, sodium-potassium ATPase, protein kinase, cyclic nucleotide

phosphodiesterase and tyrosine kinase (Fishman, 1988). Gangliosides may influence the activities of these enzymes by modifying the microenvironment of the membrane around them (Yates, 1986). Gangliosides may also have an effect on sodium and calcium channels (Spiegel, 1988).

Gangliosides may function as receptors in the central nervous system (CNS). Gangliosides have many characteristics that make them ideal receptors. For instance, they are located in the outer surface of the plasma membrane, they have a variety of structures and they carry a charge (Wiegandt, 1985). They are thought to bind the following ligands: viruses (sendai and influenza), bacteria and bacterial toxins (enterotoxin, cholera, tetanus and botulinum), fibronectin, interferon, neurotransmitters (serotonin), glycoproteins, and calcium ions (Fishman, 1988).

Gangliosides themselves may also function as ligands. There is evidence for the existence of a ganglioside-binding protein on the surface of cells. It is thought that when a ganglioside binds to this protein, it alters either the activity of a kinase or ionic flux. Altering kinase activity will in turn alter the rate of protein phosphorylation (Schengrund, 1990). Gangliosides have been found to both stimulate and inhibit the phosphorylation of proteins (Goldenring et al., 1985; Chan, 1987). Altering the rate of phosphorylation could affect the rate of cell growth or affect cellular functions (Schengrund, 1990).

The discovery that changes in the levels of gangliosides during development parallel CNS differentiation provided evidence that gangliosides play an important role in neuritogenesis. This hypothesis was supported by Purpura and Baker (1977) who showed that GM1-gangliosidosis caused affected neurons to display meganeurites and other types of aberrant sprouting from regions of the cell that had elevated levels of stored gangliosides. Kasarskis and co-workers (1981) provided additional support for this hypothesis when they produced long-lasting morphological and behavioral abnormalities by administering GM1 antibodies to developing animals.

These early studies led many researchers to add gangliosides to cell cultures and administer them to animals with the hope of stimulating neuritogenesis. Exogenously administered gangliosides are able to cross the blood-brain barrier, concentrate in the plasma membranes and function like endogenous gangliosides (Tettamanti, 1988). Exogenous gangliosides were found to enhance neurite formation in a wide variety of cultured cells, including neuroblastoma, pheochromocytoma, dorsal root ganglion and fetal brain of the rat and chick. Gangliosides were also found to accelerate reinnervation *in vivo* (Yates, 1986; Gorio, 1986; Sabel, 1988; Mahadik and Karpiak, 1988; Schengrund, 1990).

In animals, early post-lesion treatment with GM1 has been shown to improve neuronal cell survival of dopaminergic,

serotonergic, and cholinergic neurons (Skaper et al., 1989). The mechanism by which gangliosides produce their neuronotrophic effects is unknown. According to Mahadik and Karpiak (1988), gangliosides may be able to prevent additional damage following an initial neuronal insult by:

(1) enhancing the neuron's responsiveness to neuronotrophic signals. It is hypothesized that the increase in trophic activity following a brain injury may be insufficient to prevent additional damage to the neuron. In vitro studies show that GM1 enhances the effect of neuronotrophic factors (NTFs) on NTF-responsive neuronal cells. Antibodies to GM1 have been shown to block NGF-induced neuritogenesis (Skaper et al., 1988).

(2) protecting the neurons from imbalances between excitatory and inhibitory stimulation. Neural injuries are characterized by imbalances in neurotransmitter activities which may be harmful to the neuron. For example, it has been found that excitatory amino acid transmitters have neuronotoxic effects following ischemic brain damage (Mahadik and Karpiak, 1988). GM1 has been found to protect the brain against various biochemical and functional deficits during cerebral ischemia (Skaper et al., 1989).

(3) protecting the structure and function of neuronal membranes. Neuronal injury may affect the structure of neuronal membranes by altering their lipid content. Changes in membrane lipids will affect membrane fluidity and stability

(Mahadik and Karpiak, 1988). Exogenously administered GM1 has been found to reduce toluene-induced increases in fluidity of synaptosomal membranes (von Euler et al., 1990). A reduction in membrane lipids may lead to increases of free fatty acids which are thought to be pathogenic. Gangliosides may prevent increases in fatty acids by preventing calcium influx. Preventing the influx of calcium into the cell will prevent the activation of phospholipases which release fatty acids from phospholipids (Mahadik and Karpiak, 1988). Calcium will also not be able to enhance the activities of calcium-dependent proteases and kinases which have been associated with neuronal degeneration (El-Fawal et al., 1990).

Neuronal injury may adversely affect the function of plasma membranes. Alterations of membrane lipids and destabilization of the plasma membranes will adversely affect the activities of membrane-bound enzymes like sodium-potassium ATPase and magnesium ATPase. Exogenous GM1 may prevent the loss of enzyme activity by stabilizing the membrane or by interacting with the enzyme directly. The failure of membrane function may also lead to cellular ionic imbalances and edema. Gangliosides may reduce ionic imbalances by stabilizing the membrane and/or by protecting the activity of an important ion pump like sodium-potassium ATPase (Mahadik and Karpiak, 1988).

Effect of injury on endogenous gangliosides-

The heavy metals lead and mercury alter the pattern of gangliosides in the central nervous system. Young rats given a diet containing 1% lead showed an increased proportion of GD1a and decreased proportions of GD1b and GT1b (Stephens and Gerber, 1981). After chronic exposure to mercury, the basal ganglia of monkeys showed an increase in GD2, GD1b, GT1b and GQ1b, and a decrease in GM2, GM1 and GD1a (Ando, 1983). Following pentylenetetrazol-induced convulsions in rabbits, there was a relative decrease in GT1b and GD1b, and increases in GD1a and GM1 (Kostic, 1981). Rats administered a single pharmacological dose of alcohol had a reduction in the levels of GM1, GM3, GD1a, GD1b and GT1b in their brains (Klemm and Foster, 1986).

Few studies have examined the effects of trauma on the synthesis and composition of gangliosides. After Sbaschnig-Agler and co-workers (1984) injected a radiolabeled ganglioside precursor into the crushed eye of a goldfish, there was an eight-fold increase in the amount of radiolabeled ganglioside within the visual pathway. Yates and Thompson (1978) found a 64% increase in the amount of gangliosides within a rabbit sciatic nerve by three weeks after transection. There was an increase in GM2, GM3 and GD3 and a decrease in GT1b, GD1b and GD1a. Seifert and Fink (1983) electrolytically lesioned the entorhinal cortex of a rat which resulted in the partial deafferentiation of the dentate gyrus.

The lesion stimulated axonal sprouting and synapse formation which resulted in an increase in the biosynthesis of gangliosides, with GD2 having the most notable increase.

Ganglioside metabolism is altered by the presence of neural tumors. There is an increase in polysialogangliosides and decrease in monosialogangliosides as a result of increases in cell density. There are also significant changes in ganglioside distribution in the following central nervous system diseases: Creutzfeld-Jacob disease, multiple sclerosis, and amyotrophic lateral sclerosis (Ando, 1983). The central nervous system is not the only location where there are changes in ganglioside profiles following an insult. Bouchon and co-workers (1985) found a reduced proportion of GM3 in pathological thyroids.

# ORGANOPHOSPHORUS ESTER-INDUCED DELAYED NEUROTOXICITY

## General uses of organophosphorus esters-

There is a high potential for exposure to organophosphorus esters due to their wide range of uses. In industry they are used as plasticizers, gasoline additives, stabilizers in lubricating and hydraulic oils, and as flame retardants. In agriculture, they are used as insecticides, anthelmintics and defoliants. Organophosphorus esters function as biocides by inhibiting acetylcholinesterase. In addition to the effects caused by the inhibition of this enzyme, some organophosphorus esters also cause a delayed neurotoxic effect known as organophosphorus ester-induced delayed neurotoxicity (OPIDN) (Metcalf, 1982).

Diisopropylfluorophosphate (DFP) (Figure 5) was first synthesized during World War II by McCombie and Saunders (1946), but due to security reasons their results were not published until after the war. DFP has never been used in warfare, agriculture or industry, but it has been used in research. It has frequently been used in cholinesterase inhibition studies because: (1) it is very effective at inactivating acetylcholinesterase and certain other esterases; (2) it has a high lipid solubility which allows it to enter the central nervous system and (3) it is relatively specific (Taylor, 1980).

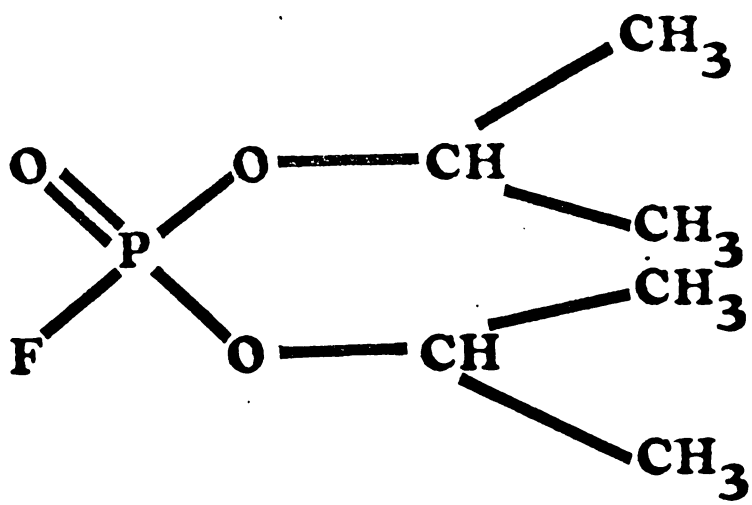


Figure 5. The structure of DFP

Toxicokinetics and metabolism of DFP-

When DFP is administered intravenously at a dose of 0.1 mg/kg body weight, it binds to serum proteins and is distributed to the lungs. It accumulates in the liver and kidney following the administration of larger doses (Abou-Donia, 1984). The penetration of DFP into the brain is slow and dose dependent. DFP appears to bind to the tissues it encounters first with less of the toxin reaching the more isolated tissues like the brain (Ramachandran, 1967). When DFP is injected into a femoral artery, it distributes in a proximal to distal gradient in the sciatic nerve (Howland et al., 1980).

Aromatic esters usually undergo metabolic activation to metabolites that are more neurotoxic, whereas aliphatic esters like DFP are direct acting neurotoxins. The liver is the main site of detoxification following intraperitoneal injection of DFP. After being absorbed by the liver, it is bound to microsomal esterases and hydrolyzed to a product more readily excreted. Intravenous injection of DFP into guinea pigs results in the formation of O,O-diisopropylphosphate. O,O-diisopropylphosphate is mainly excreted in the urine and to a lesser degree in the bile. Small amounts of DFP are excreted without being broken down. Large toxic doses of DFP result in increased biliary excretion of DFP and its metabolites with reduced urinary excretion (Abou-Donia, 1984).

Clinical signs of OPIDN-

In humans, the clinical signs of delayed neurotoxicity are observed 8 to 14 days after the initial exposure to the toxin. The first symptoms of OPIDN are tingling, numbness and weakness in the distal portion of the lower limbs. The weakness and ataxia eventually lead to paralysis of the lower limbs. The upper limbs are also affected in severe cases (Johnson, 1982).

Chickens administered an oral dose of 1 ml tri-ortho-cresyl phosphate (TOCP)/kg body weight exhibit symptoms of OPIDN 8-10 days after exposure. At this time there is an unwillingness to walk with a preference for squatting. The birds tire easily and after exertion they walk with a broadening and stumbling gait. On the following day the weakness and clumsiness are more pronounced, the feet slap heavily on the floor and the legs are spread widely to maintain balance. During the subsequent 4 or 5 days the legs become progressively more weakened until the bird is unable to stand. During this same period the leg and tail reflexes become progressively reduced until they are virtually non-existent. The wings are sometimes weakened but not to the same degree as the legs (Cavanagh, 1954).

Human exposure to delayed neurotoxins-

The clinical signs of OPIDN were first noted during the late 19th century in tuberculosis patients who were treated

with an uncharacterized mixture of esters called phosphocreosote. OPIDN was not studied in humans until TOCP, a chemical once widely used in industry, was identified as the cause of a peculiar form of paralysis that affected thousands of people in the United States during the 1920s. This paralysis was caused by the consumption of an alcoholic extract of Jamaican ginger adulterated with TOCP. "Ginger Jake" paralysis affected approximately 20,000 people during the prohibition era. TOCP was also responsible for the paralysis of 10,000 people in Morocco in 1959 after engine oil containing TOCP was accidentally mixed with cooking oil (Metcalf, 1982).

Organophosphorus esters are popular as insecticides because they have a high acute toxicity, they are not persistent in the environment, and they are relatively inexpensive to manufacture. These characteristics made them logical replacements for organochlorine insecticides. However, organophosphorus esters are not "perfect" insecticides because certain of these compounds have caused delayed neurotoxicity in humans (Baron, 1981). The compounds mipafox and leptophos were the most highly publicized of the OPIDN-causing pesticides. Their synthesis was halted after several workers in manufacturing plants developed delayed neurotoxicity. A total of seven organophosphorus pesticides have been associated with delayed neurotoxicity in humans (Cherniack, 1988).

Initiation of OPIDN-

The mechanism of organophosphorus ester-induced delayed neurotoxicity is unknown. OPIDN is most likely initiated by the binding of the toxin, or an active metabolite of the toxin, to a protein called neuropathy target esterase (NTE). NTE is a membrane-bound protein found mainly in neuronal tissues whose physiological function is unknown (Johnson, 1975). Dudek and Richardson (1982) found the following NTE activities in various tissues of the adult hen (values are expressed as percentage of brain NTE activity): spinal cord (21%), peripheral nerve (1.7%), gastrocnemius muscle (0%), pectoralis muscle (0%), heart (14%), liver (0%), kidney (0%), spleen (70%), spleen lymphocytes (26%), and blood lymphocytes (24%). The NTE activity in the hen brain was determined by Dudek and Richardson (1982) to be 2426 $\pm$ 104 nmoles/min/gm wet weight (mean $\pm$ S.E.M.). Subcellular fractionation of hen brain homogenates revealed that NTE activity is low or absent in nuclear, mitochondrial, and myelin fractions and enriched in synaptosomal and axonal preparations (Richardson et al., 1979).

The NTE activity within a tissue is determined by using one of its substrates. Because no physiological substrates of NTE have been discovered, the synthetic compound phenyl valerate is used. Phenyl valerate is also hydrolyzed by enzymes other than NTE so a differential assay is needed to separate the activity of NTE from the activity of the other

enzymes. The activity of NTE is determined by a colorimetric assay which measures the amount of phenol released following the hydrolysis of phenyl valerate. The activity of the enzyme is determined after preincubating the tissue with: (1) paraoxon and (2) paraoxon plus the delayed neurotoxin, mipafox. Paraoxon is used in the assay to saturate all the esterases, other than NTE, which hydrolyze phenyl valerate. By subtracting the enzyme activity found in (2) from that found in (1), it is possible to determine the amount of phenol formed solely from the hydrolysis of phenyl valerate by NTE (Johnson, 1982).

Normally, the total NTE activity must be inhibited by 70-80% for OPIDN to occur. Sprague and Bickford (1981) administered repeated subneurotoxic doses of DFP to chickens and produced delayed neurotoxicity in birds by inhibiting the total NTE activity by less than 50%. Carbamates and certain phosphinyl and sulphonyl compounds inhibit NTE by more than 70%, yet they do not cause delayed neurotoxicity. These compounds do not cause OPIDN because, unlike delayed neurotoxins, they are unable to undergo the process of aging. Aging occurs when an "R" group of the phosphorylated protein is cleaved from the phosphorus, leaving behind a negatively charged protein (Figure 6). The aging of NTE following its phosphorylation by DFP is shown in Figure 7. The non-delayed neurotoxins which are capable of binding to NTE do not have hydrolyzable bonds and therefore cannot undergo the process

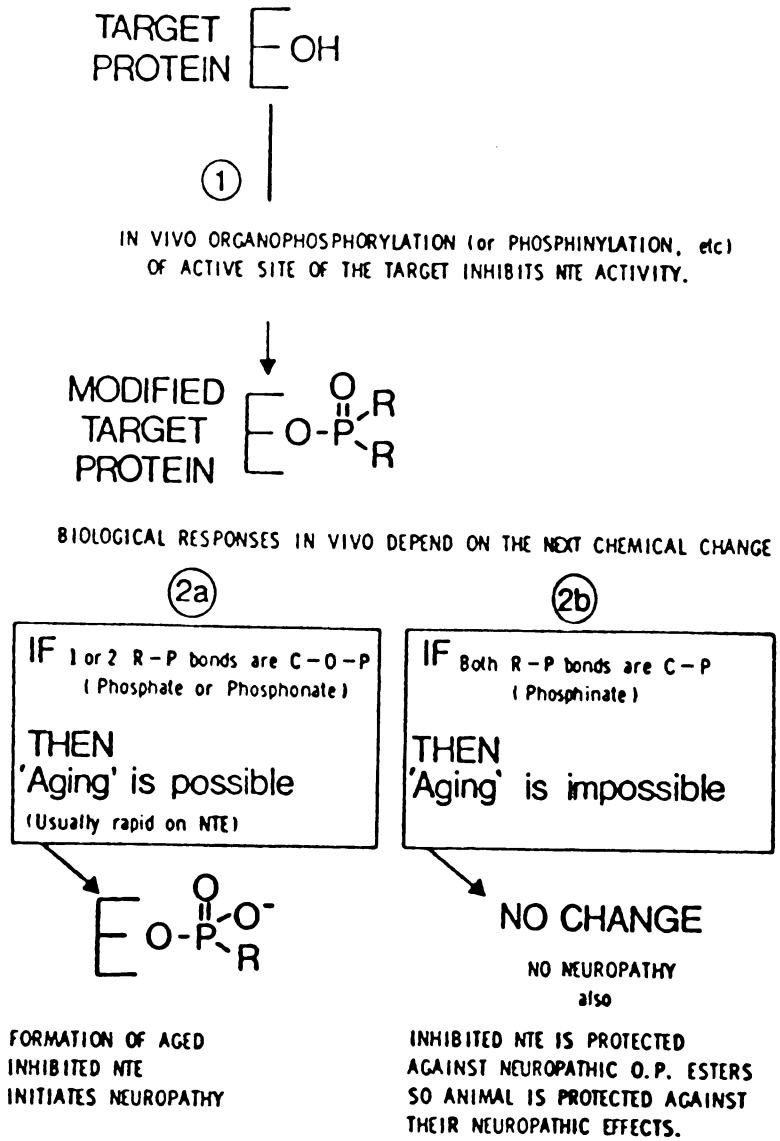


Figure 6. The consequences (2a and 2b) resulting from the binding of certain organophosphorus compounds to NTE (From Johnson, 1982).

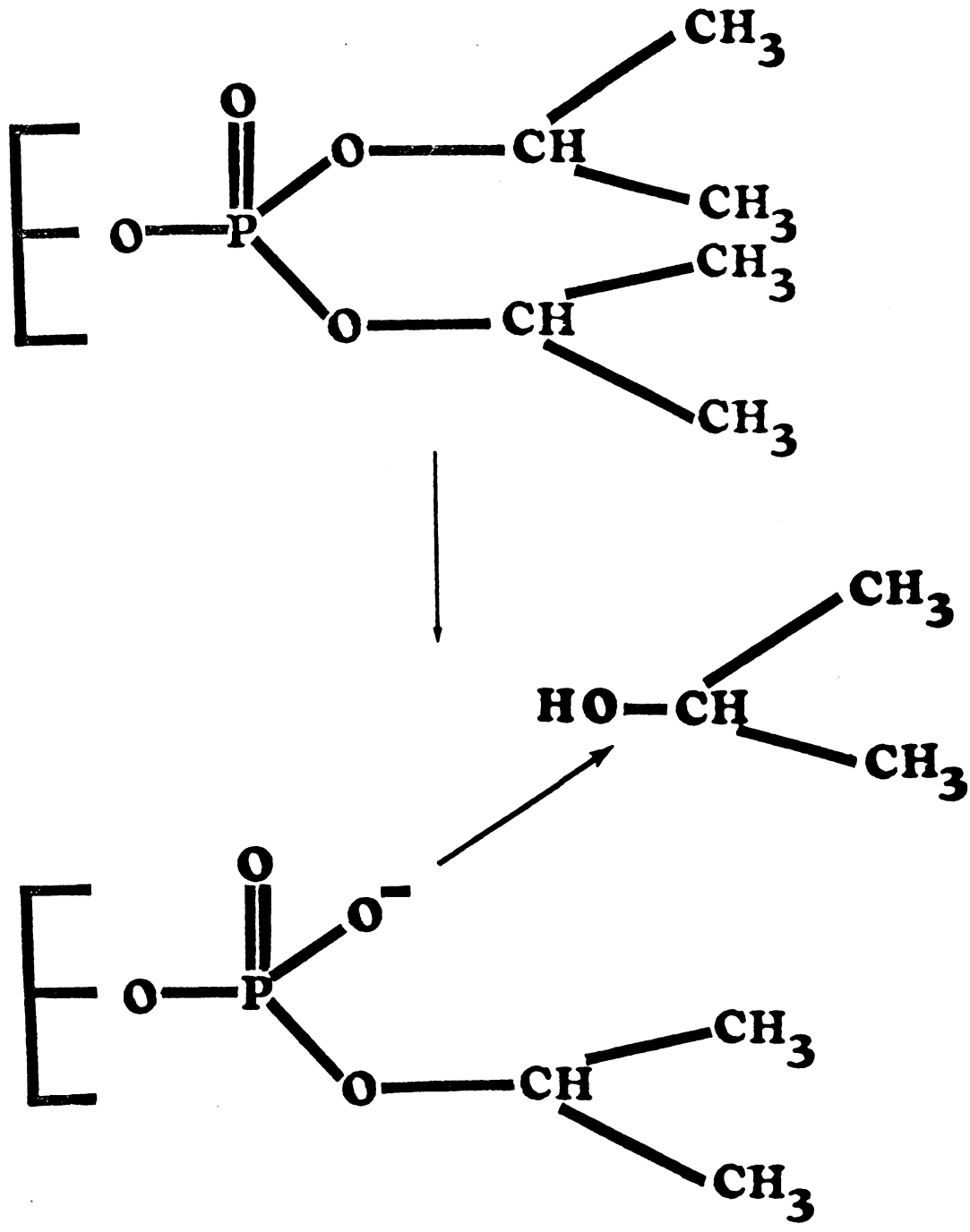


Figure 7. The aging of NTE following phosphorylation by diisopropylfluorophosphate.

of aging (Johnson, 1982). The R group is thought to be transferred to another site on the NTE molecule (Williams, 1983). The aging of DFP has a half-life of about 2-4 minutes (Clothier and Johnson, 1979).

Development of OPIDN-

It is not known how the aging of NTE leads to the formation of lesions in the nervous system. The increased negative charge of the membrane due to the aged NTE or the transfer of the R group may disrupt an important function of the membrane resulting in neuronal damage (Johnson, 1982). It is possible that the aged NTE mimics the activity of another phosphorylated protein. This mimicry could harm the axon because of the slow turnover rate of phosphorylated NTE as compared to other phosphorylated proteins (Richardson, 1984).

Bouldin and Cavanagh (1979b) studied the early stages of axonal degeneration in nerve fibers from cats dosed with DFP. They found unique varicosities in nerve fibers before degeneration occurred. These swellings were associated with intra-axonal and/or intra-myelinic vacuoles. They suggested that these vacuoles were due to a breakdown in control mechanisms regulating intracellular and extracellular ionic gradients. A net influx of ions into the axon or the myelin would lead to an influx of extracellular fluid resulting in the development of vacuoles.

Studies in the past have examined the effect of delayed neurotoxins on lipid metabolism in the peripheral nervous system. In the sciatic nerve of the hen, there was an increase in cholesterol levels and a decrease in triglyceride content following exposure to TOCP. The levels of phospholipids, diglycerides, cholesterol esters, and proteolipids, as well as the activity of phospholipase were not altered (Morazain and Rosenberg, 1970). The sciatic nerves of chickens exposed to TOCP aerosols had reduced levels of cerebrosides, triglycerides and lysolecithins and increased levels of cholesterol esters, mono- and diglycerides, lecithin and ceramide. There were also changes in the fatty acid compositions of cholesterol esters, sphingomyelin, cerebrosides and phosphatidylserine in the sciatic nerves of the chickens (Berry and Cevallos, 1966).

Very few studies have examined the effect of delayed neurotoxins on lipid metabolism in the central nervous system. In cerebral slices from chickens dosed with TOCP, there was no change in the distribution of neutral lipids or phospholipids. There was also no change in whole brain levels of proteolipids (Morazain and Rosenberg, 1970). Mipafox had no effect on whole brain levels of phospholipid, sphingomyelin, cholesterol, cerebroside or total lipids in chickens. In the spinal cord myelin from chickens dosed with sumithion, there was an increase in the concentrations of cholesterol and cholesterol ester and a decrease in the

concentrations of cerebroside, sulphatide and gangliosides. Sumithion did not cause a significant change in total lipid or phospholipid concentrations (Nag and Ghosh, 1984). No studies to date have examined the effect of delayed neurotoxins on ganglioside profiles.

Other important discoveries have been made that may help to explain the development of OPIDN. Seifert and Casida (1982) have evidence that microtubules and microtubule-associated proteins are the target sites for delayed neurotoxins. El-Fawal and co-workers (1989) found that the calcium channel blocker verapamil modified the effects of the delayed neurotoxin phenyl saligenin phosphate. El-Fawal and co-workers (1990) also found an elevation of calcium-activated neutral protease activity in neuronal tissue and muscle during the period between the inhibition of NTE and the development of OPIDN. Moretto and co-workers (1987) found increasing impairment of retrograde transport in the sciatic nerve from days four through seven after exposure to the delayed neurotoxin, dibutyl dichlorovinylphosphate (DBDCVP). Delayed neurotoxins may also accelerate anterograde axonal transport (Reichert and Abou-Donia, 1980; Carrington et al., 1989).

The phosphorylation of numerous brain and spinal cord proteins that are $Ca^{+2}$- and calmodulin-dependent was enhanced after the oral administration of TOCP to chickens (Patton et al., 1985). The primary proteins affected were cytoskeletal

proteins (alpha- and beta-tubulin, MAP-2, and neurofilament triplet proteins) that are phosphorylated by $Ca^{+2}$/calmodulin kinase II (CaM kinase II). CaM kinase II, instead of NTE, may therefore be the initial target for OPIDN (Abou-Donia and Lapadula, 1990).

Expression of OPIDN-

Neuronal degeneration occurs in the peripheral nerves, spinal cord and the lower brainstem. In the peripheral nervous system, both the sensory and motor nerves are affected. The long myelinated axons with large diameters are affected most severely (Cavanagh, 1954). The initial degeneration occurs at the distal portion, but not the extreme terminus, of the axons. Degeneration of the myelin occurs secondary to the axonal degeneration (Bouldin and Cavanagh, 1979 a, b).

In the central nervous system, degeneration occurs in the cervical spinal and medullary tracts. The fasciculus gracilis, dorsal and ventral spinocerebellar tracts, spinal lemniscus, glossopharyngeal and vagus nerve are all affected. Terminal degeneration occurs in the following brainstem nuclei: lateral cervical, gracile-cuneate, external cuneate, inferior olivary nuclei, nucleus tractus solitarius, and the reticular formation. Mossy fiber degeneration occurs in the granular layer of the cerebellar folia I-Vb (Tanaka et al., 1990). In the spinal cord of chickens dosed with TOCP, the

lumbar portion of the medial pontine-spinal tract is also affected (Tanaka and Bursian, 1989).

Variability of response-

There is a difference in species sensitivity to the delayed neurotoxic effects of OPIDN. Sensitive species include the baboon, man, chicken, squirrel monkey, water buffalo, horse, cow, sheep, pig, dog, cat, slow loris, duck, pheasant, turkey, ferret and partridge. The less sensitive or insensitive species include the rat, mouse, rabbit, guinea pig, hamster, gerbil and Japanese quail. The adult White Leghorn hen (Gallus domesticus) is used as the test animal for OPIDN because its response to delayed neurotoxicity is similar to that of humans (Metcalf, 1982).

There is also an age difference in sensitivity to the delayed neurotoxic effects of compounds which cause OPIDN (Barnes and Denz, 1953; Bondy et al., 1960). Johnson and Barnes (1970) injected chicks at ages up to 49 days with DFP at doses of 2-5 mg/kg body weight without the chicks developing delayed neurotoxicity. The chicks were more susceptible to this dosage at 60-100 days of age and highly susceptible from 100-130 days of age. Kittens also did not develop OPIDN after being administered large doses of TOCP (Taylor, 1967). Children also appear to be less sensitive than adults to OPIDN (Cherniack, 1988).

The NTE activity of the chick and rat can be inhibited

by more than 70% without either animal exhibiting ataxia. Because the initiation steps of OPIDN are similar in the rat and chicken, the difference in species susceptibility to ataxia is likely due to events which occur after phosphorylation of the enzyme (Johnson, 1975). The difference in susceptibility to OPIDN may be due to neuroanatomical differences between species (Morazain and Rosenberg, 1970; Johnson, 1975; Baron, 1981).

It is also possible that young and nonsusceptible animals differ from susceptible animals in their ability to repair the neuronal damage caused by the aged NTE (Richardson, 1984). Richardson (1984) hypothesizes that inhibition and aging of NTE above a critical level should lead to pathogenesis irregardless of species and age. Part of the basis for this hypothesis is Novak and Padillas' (1986) research showing the similarity of NTE found in the hen and rat. The inhibition of NTE activity in the rat and chicken results in peripheral nerve and spino-cerebellar degeneration in both animals, but only the chicken exhibits ataxia (Veronesi, 1984). Veronesi (1984) found clumps of axonal sprouts in the peripheral nervous system of rats dosed with TOCP. The hypothesis is also supported indirectly by research which shows that young animals have a greater potential for repair than do older animals (Richardson, 1984).

## MATERIALS AND METHODS

Husbandry-

Thirty 85-week-old White Leghorn (Gallus domesticus) hens were obtained from the Michigan State University Poultry Science Research and Teaching Center and housed individually in cages measuring 41 cm long x 21 cm wide x 51 cm high in an environmentally controlled room. The hens were provided with a commercial layer ration (Purina Layena) and water ad libitum and the photoperiod was maintained at 16h light : 8h dark. The temperature and the relative humidity of the room were 21 degrees celsius and 30%, respectively. The birds were allowed to adjust to room conditions at least two weeks prior to the administration of the test chemical.

### EXPERIMENT 1

Fourteen hens were assigned randomly to four groups. Group 1 consisted of four hens which were injected subcutaneously over the right breast muscle with di-isopropylfluorophosphate (DFP) (Sigma Chemical Company) at a dose of 1 mg/kg body weight in a volume of 1 ml/kg body weight. Dimethyl sulfoxide (DMSO) was used as the vehicle. The birds were given a 1 ml intraperitoneal injection of

atropine sulfate (15 mg/kg body weight) immediately prior to, and when necessary after dosing to protect against the acute cholinergic effects of DFP. The birds were killed 4 days after dosing. Just prior to being killed, the degree of ataxia exhibited by the hens was assessed using the 8-point scale of Cavanagh and co-workers (1961). On this scale, 0 indicates no ataxia, 1-2 indicates mild ataxia, 3-4 indicates moderate ataxia, 5-6 indicates severe ataxia, and 7-8 indicates paralysis.

The other three groups in this experiment served as controls. Group 2 (four birds) consisted of untreated birds, group 3 (two birds) were vehicle controls and therefore received 1 ml DMSO/kg body weight while group 4 (four birds) were administered by intraperitoneal injection 10 mg parathion [O,O-diethyl-O-(4-nitrophenyl) phosphorothioate] /kg body weight. Parathion was used as the negative control because both DFP and paraoxon (the neuroactive metabolite of parathion) are cholinesterase inhibitors but paraoxon does not cause OPIDN (Soliman et al., 1982). Birds in the parathion and DMSO groups were killed four days after injection.

## EXPERIMENT 2

Thirteen birds were administered DFP at a dose of 1 mg/kg body weight using the same procedure described for Experiment 1. Four birds were killed on days 7 and 14 post-dosing while five birds were killed 21 days after the administration of

DFP. The administration of DFP to each group was staggered so that the birds could be killed on the same day. Three untreated birds were also killed at this time and used as controls. The degree of ataxia was determined for all birds just prior to sacrifice according to the method developed by Cavanagh and co-workers (1961). The number of birds in each of the treatment groups used in the two experiments is shown in Table 4.

Table 4. Number of birds in the treatment groups of Experiments 1 and 2.

| | Untreated | DMSO | Parathion | DFP | | | |
|---|---|---|---|---|---|---|---|
| | | | | 4D* | 7D | 14D | 21D |
| EXP 1 | 4 | 2 | 4 | 4 | | | |
| EXP 2 | 3 | | | | 4 | 4 | 5 |

*Refers to the number of days post-DFP administration.

Brain removal-

After the birds in the two experiments were killed, their brains were quickly removed and the hindbrain, consisting of the cerebellum and brainstem, was separated from the rest of the brain. The hindbrain was then weighed and frozen for subsequent analysis. The hindbrain was the only region of the brain assessed because OPIDN-induced

degeneration is limited to this area (Tanaka et al., 1990).

Summary of tissue analysis-

Gangliosides were isolated using a modification of a method described by Ledeen and Yu (1982). At various points in this procedure, aliquots were taken for protein, total cholesterol (cholesterol and cholesterol esters) and lipid phosphorus quantification. Total lipid was measured gravimetrically.

Lipid extraction and protein quantification-

The hindbrain of each bird was homogenized in 60 mls of chloroform-methanol-water (2:1:0.1) in a 100 ml beaker. Aliquots were taken for protein estimation using the method described by Lowry and co-workers (1951). The beakers containing the homogenates were placed in an oscillating water bath at 37 degrees celsius for 30 minutes. The contents of each beaker were then poured into a scintered glass filter (60 mls; 10-15 microns) attached to a 125 ml filtering flask. A vacuum was applied to the flask and the filtrate from each sample was poured into a separate 250 ml Erlenmeyer flask and covered. The pellet remaining in the filter was scraped back into the original beaker and 60 mls of chloroform-methanol-water (1:1:0.1) were added to it. After mixing, the samples were again placed in a water bath for 30 minutes and then filtered. The filtrate from this step was combined with the

first filtrate. The above steps were repeated using chloroform-methanol-water (1:2:0.1) and the filtrate combined with the two previous filtrates.

Lipid, cholesterol and lipid phosphorus quantification-

The combined filtrates were placed in a previously weighed 250 ml round bottom flask. The solvent was evaporated from the samples at 40 degrees celsius using a rotary evaporator and the amount of lipid present was measured gravimetrically. The samples were then dissolved in 10 mls of chloroform-methanol (1:1). Aliquots of the samples were taken for the estimation of total cholesterol using a colorimetric cholesterol kit (Sigma Chemical Company) and for the quantification of lipid phosphorus using Dodge and Phillips' (1967) modification of the method developed by Bartlett (1959).

DEAE-Sephadex chromatography and base treatment-

The samples were added to columns containing 4 mls of activated DEAE-Sephadex A-25. The DEAE-Sephadex was activated by placing 2.2 gms of resin in a beaker and mixing it with 30 mls methanol-chloroform-0.8 M aqueous sodium acetate, 60:30:8 (solvent A). After the resin settled, the supernatant was removed by aspiration. This step was repeated three times with fresh solvent A and the resin was allowed to stand overnight in the same solvent. The supernatant fluid was then

removed and the resin was washed three times with 30 ml portions of methanol-chloroform-water, 60:30:8 (solvent B). The resin was made into a slurry by adding 10 mls of solvent B. The slurry was transferred to a column containing a glass-wool plug. After the resin settled, it was washed with an additional 60-80 mls of solvent B to assure that all the sodium acetate was removed. After the sample was added to the column, 10 mls of chloroform-methanol (1:1) were added to elute the neutral and zwitterionic lipids. The acidic fraction was isolated by adding 20 mls of 0.8 M sodium acetate in methanol to each column and collecting the eluates in 250 ml round bottom flasks. The acidic fraction was evaporated to dryness at 40 degrees celsius with a rotary evaporator. To eliminate acidic phospholipids (serine phospholipids, inositol phosphoglycerides, etc.), the samples were dissolved in 20 mls of 0.1 N sodium hydroxide in methanol and left standing overnight. The samples were then neutralized with 1 M acetic acid. The solutions were evaporated to dryness with a rotary evaporator and then placed in a dessicator. A vacuum was applied to the dessicator and run for 30 minutes. After dessication, the samples were dissolved in 10 mls of distilled water.

Dialysis-

Dialysis tubing was used to remove salts and other small molecular weight contaminants from the samples. The first

step of this procedure required tying two knots at one end of a 12" strip of dialysis tubing that had been prewashed in boiling water for one hour. Each sample was added to the open end of a tube with a funnel and pipet. The empty flasks were rinsed two to three times with a small amount of distilled water and the rinsates were added to the tubes. The open end of the tubes were knotted and the dialysis sacs were placed in a 4000 ml beaker of distilled water. The beaker was covered with plastic wrap and placed on a stirrer in a coldroom. The distilled water was changed after 24 hours in order to increase the concentration gradient and thereby allow more contaminants to diffuse from the dialysis tubing into the surrounding fluid. After an additional 24 hours in the coldroom, the samples were poured into 120 ml beakers, covered with cheesecloth, and placed in a freeze evaporator for two days.

Iatrobead chromatography-

After lyophilization, the samples were dissolved in 4 mls of chloroform-methanol (1:1) after which an additional 6 mls of chloroform were added. The samples were added to columns containing pre-washed iatrobeads (Iatron Industries, Inc.). The following method was used to remove contaminants from the iatrobeads: two gms of iatrobeads were placed in a 50 ml beaker. The beads were washed four times with 30 ml portions of methanol-chloroform-2.5 N ammonium hydroxide (60:30:8).

The beads were then washed four times with 30 ml portions of methanol-chloroform-water (60:30:8). After the contaminants were removed from the iatrobeads, 10 mls of the methanol-chloroform-water mixture were added to the iatrobeads and the slurry was poured into a column containing a glasswool plug. After the silicic acid settled, it was washed with 20 mls of chloroform-methanol (1:1) and then with 20 mls of chloroform-methanol (95:5). After the sample was added to the column, 25 mls of chloroform-methanol (4:1) were added to elute sulfatides and fatty acids. Ninety mls of chloroform-methanol (1:1) were added to the column to elute the gangliosides.

Lipid-bound sialic acid quantification-

The solvents were removed from the samples using a rotary evaporator. Each sample was dissolved in approximately 2.5 mls of chloroform-methanol (1:1) and placed in a glass tube with a teflon-lined screw top. The flasks were rinsed with a small volume of chloroform-methanol (1:1) and the rinsates were added to the tubes. The samples were taken to dryness with a nitrogen evaporator and then dissolved in 1 ml of chloroform-methanol (1:1). Aliquots were taken for lipid-bound sialic acid quantification using the procedure described by Svennerholm (1957) and modified by Miettinen and Takki-Lukkainen (1959). The absorbance of the solutions was read at 620 nms with a Staser II Spectrophotometer.

Thin layer chromatography-

Twenty microliters of each sample were applied as 0.5 cm strips on a 20 by 20 cm silica gel 60 HPTLC plate with a 10 microliter Hamilton syringe. The plate was placed in a paper-lined chamber containing 155 mls of chloroform-methanol-0.5% aqueous calcium chloride (55:45:10). Before being placed into the solution, the plate was allowed to equilibrate in the tank for twenty minutes. The plate was removed from the chamber when the solvent had risen to a point 5 cms from the top of the plate.

After the plate was dry, it was sprayed with the resorcinol-HCl reagent described by Svennerholm (1957). This reagent was made by adding 0.5 mls of 0.1 M copper sulfate to 80 mls of concentrated hydrochloric acid followed by the addition of 10 mls of a 2% resorcinol solution and 9.5 mls distilled water. The reagent was allowed to stand at room temperature for four hours before being used. After spraying, the plate was dried with a stream of warm air, covered with a glass plate and heated at 110 degrees celsius for 20 minutes. Gangliosides appeared as purple bands and were identified by comparing them to a bovine standard (ICN Biochemicals) co-chromatographed with the samples. The developed plates were stored in a freezer to prevent the color of the bands from fading. The relative proportion of individual gangliosides within the samples was determined using a scanning densitometer.

Statistics-

The treatment groups within each experiment were tested for homogeneous variance using Bartlett's test. The treatment groups were found to have homogeneous variances for all of the measured variables. For both experiments, the control group was compared to the other groups using Dunnett's test. All statistical computations were made according to the procedures described by Gill (1978). Computations were made using Toxstat (Gulley et al., 1985) software.

The Student's t-test was used to determine if data from the two experiments could be analyzed together. It was important to combine the data from the two experiments so that the ganglioside profile at 4 days post-dosing could be compared with the ganglioside profiles at 7, 14 and 21 days post-dosing. It was determined from the Student's t-test that the untreated control groups from Experiments 1 and 2 could be combined for all variables tested.

## RESULTS

### Clinical assessment of DFP-treated birds-

As shown in Table 5, only one bird was showing signs indicative of OPIDN at 4 days post-DFP administration. By 7 days post-dosing, three of four birds were showing signs of mild ataxia and by 14 days post-dosing all birds were showing signs of moderate to severe ataxia. By 21 days post-DFP administration, the birds were exhibiting signs of paralysis.

Table 5. Clinical assessment of adult chickens administered a single subcutaneous dose of DFP (1 mg/kg body weight).

| Days Post-DFP | Number of Birds* | Degree of Ataxia** |
|---|---|---|
| 4 | 1/4 | 3.0 |
| 7 | 3/4 | 2.0 ± 0.65*** |
| 14 | 4/4 | 4.8 ± 0.57 |
| 21 | 5/5 | 7.2 ± 0.51 |

* Number of birds exhibiting ataxia/number of birds in the treatment group.
** None (0); mild (1-2); moderate (3-4); severe (5-6); paralysis (7-8).
***Data expressed as mean ± standard error of the mean.

### Experiment 1-

The concentrations of protein, total lipid, total cholesterol, lipid phosphorus and ganglioside-bound sialic acid in the hindbrains of control and DFP-treated chickens at

7, 14 and 21 days post-dosing are shown in Table 6. The treatment groups did not differ significantly from one another with respect to any of these variables.

The relative percent distribution of gangliosides in the hindbrains of control and DFP-treated chickens at 7, 14 and 21 days post-dosing is shown in Table 7. The value of GM4 on day 7 was significantly lower than the control value. The value of GM4 increased after day 7 such that its values on days 14 and 21 were not significantly different from the control value. The decrease in the relative level of GM4 on day 7 is depicted in the representative chromatogram shown in Figure 8. The relative level of GQ1b also changed with time. The value of GQ1b on day 7 was similar to the control value. However, from day 7 to day 21 the value of GQ1b increased linearly such that its value on day 21 was significantly higher than the control value. The line fit to these points had a correlation coefficient of 0.99 and a slope of +0.47.

Experiment 2-

The wet weight concentrations of protein, total lipid, total cholesterol, lipid phosphorus and ganglioside-bound sialic acid in the hindbrains of control, DMSO-treated, parathion-treated and DFP-treated chickens at 4 days post-dosing are shown in Table 8. The treatment groups did not differ significantly from one another with respect to any

Table 6. Concentrations of protein, total lipid, total cholesterol, lipid phosphorus and ganglioside-bound sialic acid per gram wet weight (gww) in the hindbrains of control and DFP-treated chickens at 7, 14 and 21 days post-dosing.

| | Control | DFP-Treated | | |
|---|---|---|---|---|
| | | Days Post-Dosing | | |
| | | 7 | 14 | 21 |
| Protein (mg/gww) | 69.1* ±10.3 | 81.0 ±8.9 | 74.0 ±8.9 | 76.6 ±7.9 |
| Total Lipid (mg/gww) | 288 ± 32 | 305 ± 28 | 282 ± 28 | 270 ± 25 |
| Total Chol. (mg/gww) | 17.9 ±1.5 | 21.5 ±1.3 | 20.6 ±1.3 | 19.4 ±1.1 |
| Lipid Phos. (ug/gww) | 2430 ±340 | 2448 ±294 | 2808 ±294 | 2901 ±263 |
| Sialic Acid (ug/gww) | 221 ± 28 | 210 ± 24 | 274 ± 24 | 263 ± 22 |

* Data expressed as mean ± standard error of the mean.

Table 7. Percent distribution of ganglioside-bound sialic acid in the hindbrains of control and DFP-treated chickens at 7, 14 and 21 days post-dosing.

| Ganglioside | Control | DFP-Treated | | |
|---|---|---|---|---|
| | | Days Post-Dosing | | |
| | | 7 | 14 | 21 |
| GM4 | 6.59* ±1.37 | 1.69** ±1.19 | 3.08 ±1.19 | 3.20 ±1.06 |
| GM1 | 3.39 ±1.05 | 2.82 ±0.91 | 3.46 ±0.91 | 2.87 ±0.82 |
| GD3 | 13.76 ±3.32 | 19.82 ±2.87 | 15.36 ±2.87 | 12.38 ±2.57 |
| GD1a | 12.89 ±1.37 | 17.35 ±1.19 | 14.40 ±1.19 | 15.51 ±1.06 |
| GT1a +GD2 | 5.44 ±0.79 | 6.00 ±0.68 | 6.75 ±0.68 | 4.66 ±0.61 |
| GD1b | 10.78 ±1.21 | 10.22 ±1.05 | 10.01 ±1.05 | 8.51 ±0.94 |
| GT1b | 36.24 ±4.56 | 31.17 ±3.95 | 33.70 ±3.95 | 35.33 ±3.53 |
| GQ1b | 10.72 ±2.05 | 10.93 ±1.78 | 13.25 ±1.78 | 17.54** ±1.59 |

* Data expressed as mean ± standard error of the mean.
**Significantly different from control value at $p<0.05$.

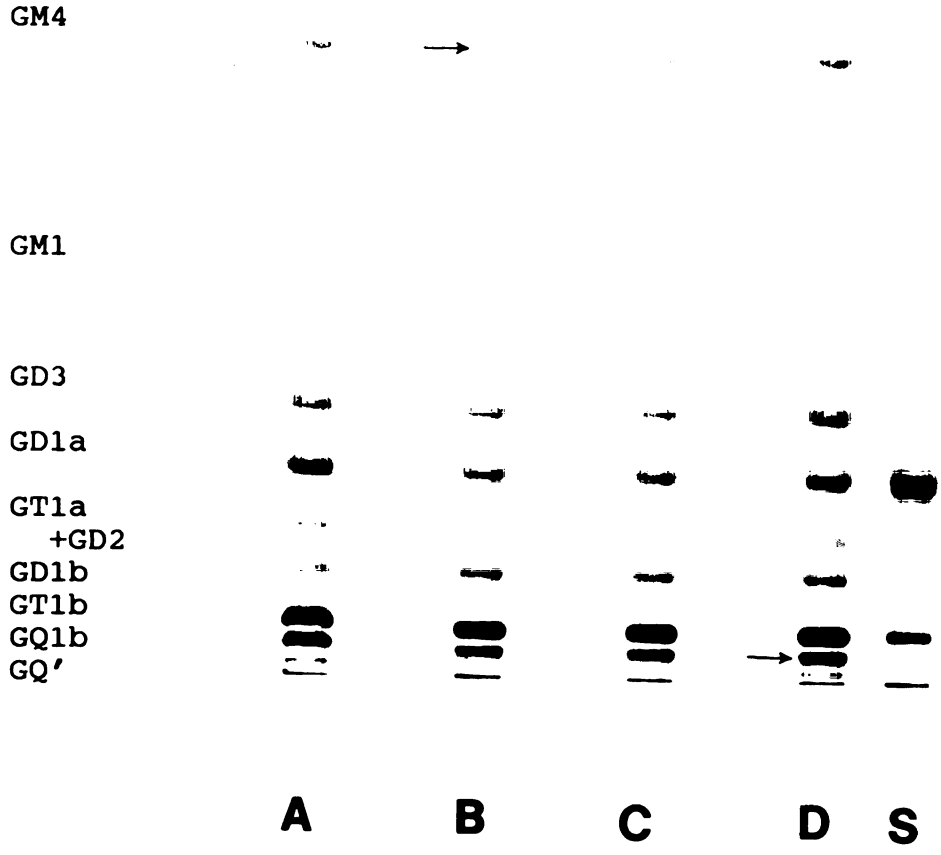


Figure 8. Thin-layer chromatogram of ganglioside extracts from the hindbrains of (A) control and DFP-treated chickens at (B) 7, (C) 14 and (D) 21 days post-DFP administration. The bovine standard is represented by (S). The arrows indicate gangliosides which were found to differ significantly from controls.

Table 8. Concentrations of protein, total lipid, total cholesterol, lipid phosphorus and ganglioside-bound sialic acid per gram wet weight (gww) in the hindbrains of control, DMSO-treated (vehicle control), parathion-treated (negative control), and DFP-treated (test group) chickens at 4 days post-dosing.

| | Control | DMSO | Parathion | DFP |
|---|---|---|---|---|
| Protein (mg/gww) | 88.8* ±2.7 | 89.3 ±3.8 | 87.3 ±2.7 | 88.9 ±2.7 |
| Total Lipid (mg/gww) | 288 ± 15 | 300 ± 21 | 306 ± 15 | 269 ± 15 |
| Total Chol. (mg/gww) | 19.8 ±0.7 | 18.8 ±1.0 | 19.1 ±0.7 | 19.6 ±0.7 |
| Lipid Phos. (ug/gww) | 2461 ± 65 | 2388 ± 92 | 2520 ± 65 | 2225 ± 65 |
| Sialic Acid (ug/gww) | 326 ± 37 | 313 ± 52 | 374 ± 37 | 448 ± 37 |

* Data expressed as mean ± standard error of the mean.

of these variables.

The relative percent distribution of gangliosides in the hindbrains of control, DMSO-treated, parathion-treated and DFP-treated chickens at 4 days post-dosing is shown in Table 9. The ganglioside profiles for these four treatment groups were not significantly different. A representative chromatogram of these four treatment groups is shown in Figure 9.

Because there was a linear increase in the value of GQ1b from days 7 to 21 post-DFP exposure, the changes in the values of the other gangliosides during this same period were examined. Two gangliosides, GD3 and GT1b, were also found to undergo a linear change from day 7 to day 21. The line fit to the values of GD3 during this period had a correlation coefficient of 0.99 and a slope of -0.53. The line fit to the values of GT1b during the same period had a correlation coefficient of 0.99 and a slope of +0.30. A plot of the relative proportions of the gangliosides in the hindbrains of control and DFP-treated chickens at 7, 14 and 21 days post-DFP administration is shown in Figure 10.

Combined data from Experiments 1 and 2-

According to the results of the Student's t-test, the untreated control groups from the two experiments were not significantly different. The data from the two experiments were therefore combined (Table 10) so that possible trends in

Table 9. Percent distribution of ganglioside-bound sialic acid in the hindbrains of control, parathion-treated (negative control), DMSO-treated (vehicle control) and DFP-treated (test group) chickens at 4 days post-dosing.

| Ganglioside | Control | DMSO | Parathion | DFP |
|---|---|---|---|---|
| GM4 | 4.22* ±1.01 | 3.47 ±1.43 | 2.66 ±1.01 | 5.11 ±1.01 |
| GM1 | 5.72 ±0.70 | 4.42 ±0.99 | 4.90 ±0.70 | 5.76 ±0.70 |
| GD3 | 19.42 ±1.57 | 19.59 ±2.22 | 18.00 ±1.57 | 20.96 ±1.57 |
| GD1a | 17.17 ±1.11 | 18.01 ±1.01 | 18.33 ±1.11 | 18.74 ±1.11 |
| GT1a +GD2 | 5.76 ±0.51 | 5.85 ±0.70 | 5.74 ±0.51 | 6.15 ±0.51 |
| GD1b | 8.81 ±0.49 | 9.55 ±0.70 | 8.88 ±0.49 | 9.91 ±0.49 |
| GT1b | 27.27 ±2.06 | 27.29 ±2.92 | 29.41 ±2.06 | 25.24 ±2.06 |
| GQ1b | 11.65 ±1.57 | 11.82 ±2.22 | 12.10 ±1.57 | 8.15 ±1.57 |

* Data expressed as mean ± standard error of the mean.

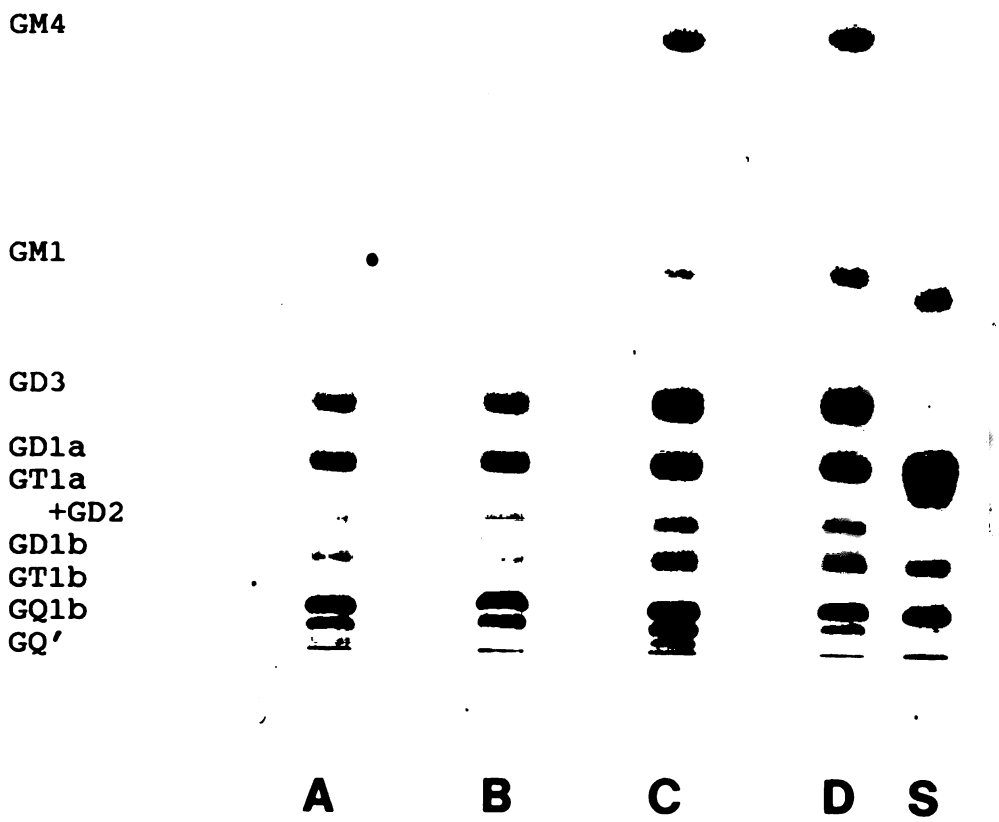


Figure 9. Thin-layer chromatogram of ganglioside extracts from the hindbrains of (A) control, (B) parathion-treated, (C) DMSO-treated and (D) DFP-treated chickens at 4 days post-dosing. The bovine standard is represented by (S).

Figure 10. The relative percent distribution of GM4, GM1, GD3, GD1a, GT1a + GD2, GD1b, GT1b and GQ1b in the hindbrains of control chickens (day 0) and DFP-treated chickens at 7, 14 and 21 days post-DFP administration.

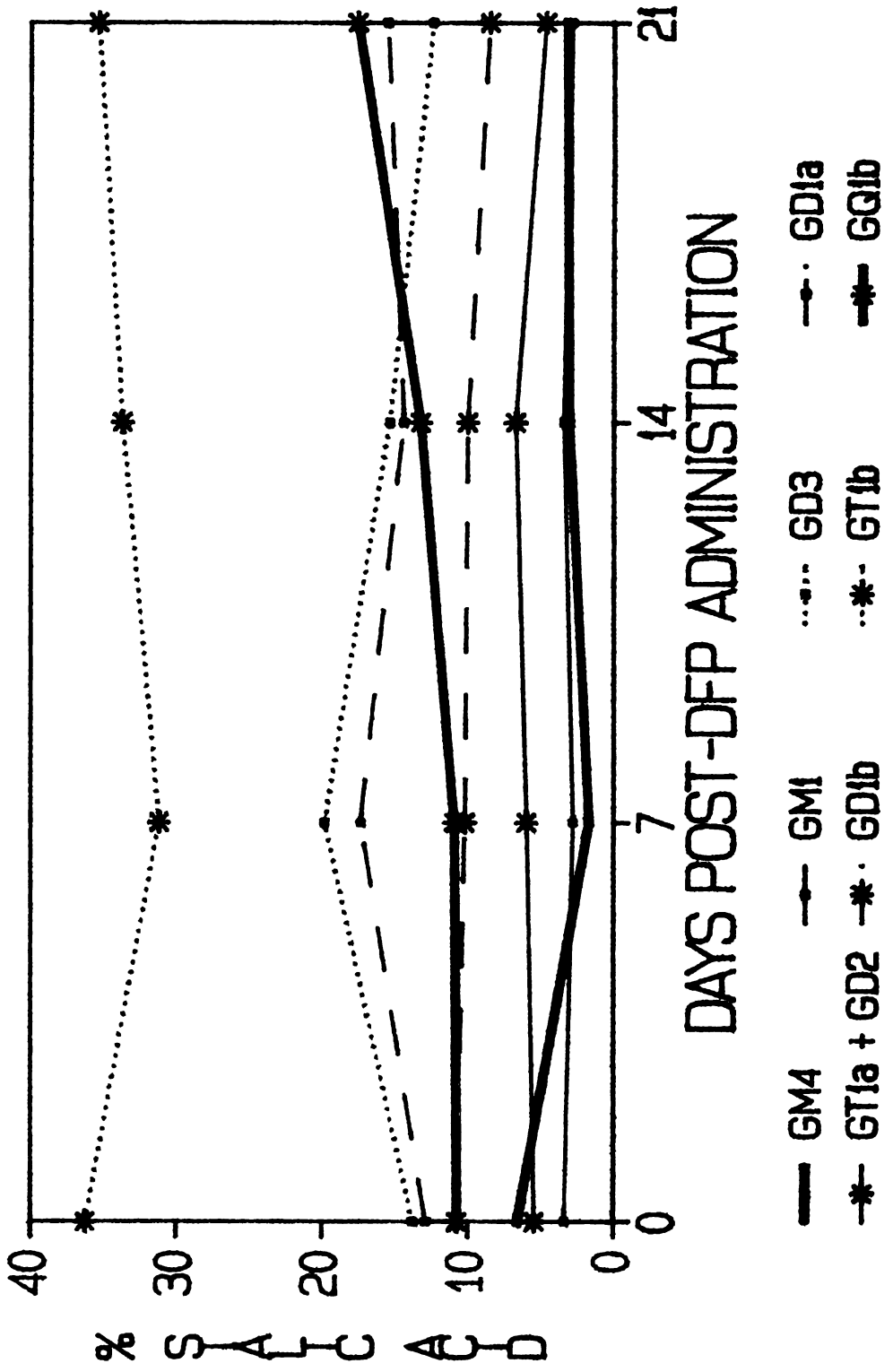
% SIALIC ACID
40
30
20
10
0
0
7
14
21
DAYS POST-DFP ADMINISTRATION
GM4
GM1
GD3
GD1a
GT1a + GD2
GD1b
GT1b
GQ1b

Figure 10.

Table 10. Percent distribution of ganglioside-bound sialic acid in the hindbrains of control and DFP-treated chickens at 4, 7, 14 and 21 days post-dosing.

| | Control | DFP-Treated | | | |
|---|---|---|---|---|---|
| | | Days Post-Dosing | | | |
| | | 4 | 7 | 14 | 21 |
| GM4 | 4.84* ±0.75 | 5.11 ±1.13 | 1.69 ±1.13 | 3.08 ±1.13 | 3.20 ±1.01 |
| GM1 | 4.66 ±0.32 | 5.76 ±0.72 | 2.82 ±0.72 | 3.46 ±0.72 | 2.87 ±0.58 |
| GD3 | 17.57 ±1.61 | 20.96 ±2.42 | 19.82 ±2.42 | 15.36 ±2.42 | 12.38 ±2.16 |
| GD1a | 15.93 ±0.85 | 18.74 ±1.66 | 17.35 ±1.66 | 14.40 ±1.66 | 15.51 ±1.33 |
| GT1a +GD2 | 5.67 ±0.45 | 6.15 ±0.67 | 6.00 ±0.67 | 6.75 ±0.67 | 4.66 ±0.36 |
| GD1b | 9.63 ±0.57 | 9.91 ±0.85 | 10.22 ±0.85 | 10.01 ±0.85 | 8.51 ±0.58 |
| GT1b | 30.26 ±2.24 | 25.24 ±3.35 | 31.17 ±3.35 | 33.70 ±3.35 | 35.33 ±3.00 |
| GQ1b | 11.38 ±1.08 | 8.15 ±1.62 | 10.93 ±1.62 | 13.25 ±1.62 | 17.54 ±1.45 |

* Data expressed as mean ± standard error of the mean.

the distribution of gangliosides from day 4 to day 7 could be discerned. An interesting discovery concerned the three gangliosides GD3, GT1b and GQ1b, which were found in Experiment 1 to undergo a linear change from day 7 to day 21 post-DFP administration. A linear change in the relative distribution of GD3, GQ1b and GT1b occurred from day 4 to day 21 post-DFP administration. The line fit to the mean relative values of GD3 during this period had a correlation coefficient of 1.00 and a slope of -0.52. The line fit to the mean relative values of GQ1b during this period had a correlation coefficient of 0.99 and a slope of +0.52 which is equal, but opposite in direction, to the slope of GD3. The line fit to the mean relative values of GT1b from day 4 to day 21 post-dosing had a correlation coefficient of 0.90 and a slope of +0.52 which is equal to the slope found for GQ1b for this same time period. A plot of the relative proportion of GD3, GT1b and GQ1b from day 4 to day 21 post-DFP administration is shown in Figure 11.

Figure 11. The relative percent distribution of GD3, GT1b and GQ1b in the hindbrains of control chickens (day 0) and DFP-treated chickens at 4, 7, 14 and 21 days post-DFP administration.

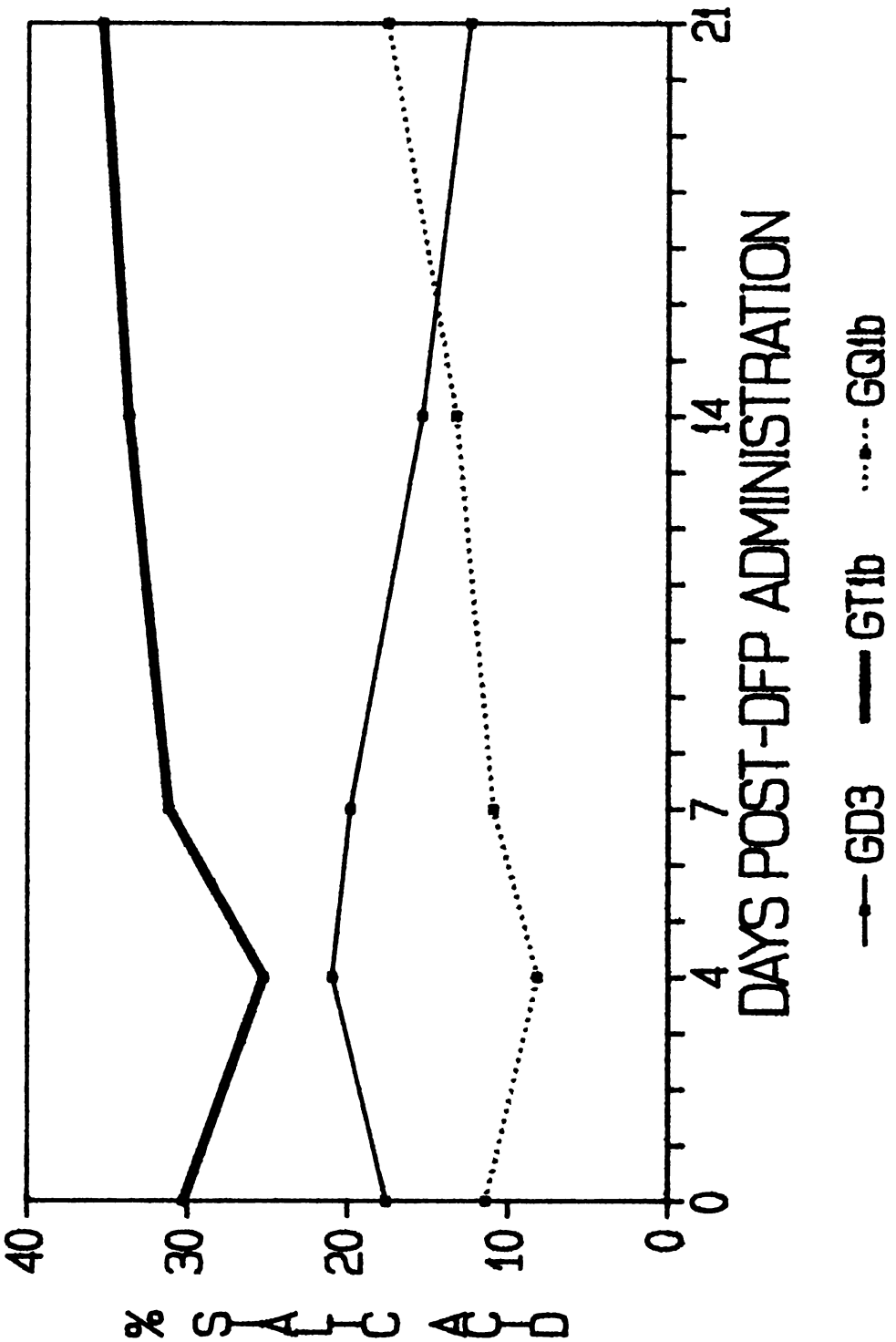
% SIALIC ACID
40
30
20
10
0
0
4
7
14
21
DAYS POST-DFP ADMINISTRATION
GD3
GT1b
GQ1b

Figure 11.

# DISCUSSION

## Ganglioside profile of the chicken hindbrain-

The relative distribution of gangliosides within the hindbrain of the chicken differed from the pattern found for the whole brain by Dreyfus and co-workers (1975) and Hilbig and co-workers (1981). The hindbrain had a larger proportion of GT1b and GQ1b and a smaller proportion of GD1a and GM1 than the whole brain. The cerebella of other species also have a larger proportion of GT1b and GQ1b when compared to whole brain levels. Ando and co-workers (1978) found a high proportion of GT1b and GQ1b in the cerebellum of the rat, mouse, guinea pig and human. Birds such as quail and finches were found to have a higher proportion of multi-sialylated gangliosides such as GQ1b and GP in their cerebella than in other regions of their brains (Seybold and Rahmann, 1985).

## Effect of parathion on lipid and protein levels-

The parathion treatment group was compared to the control group with respect to all the variables examined in this study. This comparison was made to determine if the anticholinesterase action of parathion altered the concentrations of any of the examined brain components.

Results of this comparison were used to assess whether the changes caused by DFP were due to its anticholinesterase effects or its delayed neurotoxic effects. Because parathion is not a delayed neurotoxin (Soliman et al., 1982), it was only examined at 4 days post-dosing. Changes which begin to occur at 7 days post-dosing would be due to the delayed neurotoxic effects of the compound. The parathion group was not significantly different from the control group with respect to any of the variables analyzed.

Effect of DFP on lipid and protein levels-

During the 1960s and the 1970s many studies examined the effect of delayed neurotoxins on the lipid content of the peripheral nervous system (Majno and Karnovsky, 1961; Berry and Cevallos, 1966; Williams et al., 1966; Joel et al., 1967; Morazain and Rosenberg, 1970), but few studies examined the effect of these toxins on the central nervous system. In the chicken, Williams and co-workers (1966) studied the effect of TOCP on the spinal cord, Joel and co-workers (1967) studied the effect of mipafox on the brain and the spinal cord and Morazain and Rosenberg (1970) studied the effect of TOCP on cerebral slices. The results of these studies indicated no changes in the levels of phospholipids, sphingomyelin, cholesterol, diglycerides, triglycerides or total lipid due to exposure to compounds causing OPIDN. Results of the present study support these findings. DFP had no effect on

total cholesterol, lipid phosphorus or total lipid levels in the hindbrain of chickens. DFP also had no effect on protein levels.

No previous studies have examined the effect of OPIDN-causing compounds on ganglioside levels. The present study indicated that DFP does not significantly alter ganglioside-bound sialic acid levels. At the beginning of the study, it was hypothesized that ganglioside-bound sialic acid levels would be affected by organophosphorus compounds. This hypothesis was based in part on Nag and Ghoshs' (1984) work which showed a decrease in ganglioside levels in the spinal cord myelin of pigeons administered the organophosphorus compound sumithion. In addition, Berry and Cevallos (1966) found an increase in ceramide, a constituent of gangliosides, in the sciatic nerves of chickens following exposure to TOCP aerosols. It cannot be ruled out that the extraneous tissue and high variability in ganglioside-bound sialic acid levels may have masked significant changes.

The problem of extraneous tissue "hiding" important changes could be avoided by examining the effect of a delayed neurotoxin on the ganglioside profile of a single nerve. This could be accomplished by examining the effect of a delayed neurotoxin on the distal portion of the sciatic nerve. The method for isolating gangliosides developed by Ledeen and Yu (1982) could be used in this new study, although this method cannot be recommended when large numbers

of samples are analyzed due to the large expenditure of time required to isolate the gangliosides.

Effect of DFP on individual gangliosides-

This study was designed to determine if the relative proportion of gangliosides was altered following the administration of an OPIDN-causing compound. Thin layer chromatography was thought to be sensitive enough to detect these changes even though only a small percentage of the fibers in the hindbrain degenerate. The confidence in this procedure was due in part to the nature of the fibers which undergo degeneration. The fibers which undergo degeneration during OPIDN are the largest and contain the most myelin (Morazain and Rosenberg, 1970), and therefore contain a large proportion of gangliosides.

DFP was found to affect the ganglioside pattern within the chicken hindbrain. A significant reduction was found in the relative proportion of GM4 at 7 days post-DFP administration. In this study, day 7 post-DFP administration was characterized by mild ataxia. Work by Tanaka and co-workers (1990) showed a slight degeneration in the hindbrain when the birds began to show signs of ataxia. The reduction in the level of GM4 may be occurring within the myelin since this is the site in the chicken brain where GM4 is concentrated (Cochran et al., 1981). There are two studies which suggest that organophosphorus compounds affect

the lipid content of myelin. Nag and Ghosh (1984) found a reduction in gangliosides, cerebrosides and sulphatides in the spinal cord myelin of pigeons exposed to sumithion. Berry and Cevallos (1966) found a reduction in the level of the myelin lipid cerebroside, in the sciatic nerve of chickens exposed to TOCP aerosols.

The relative proportions of GD3, GQ1b and GT1b were also affected by exposure to DFP. The linear changes in these gangliosides began to occur at 4 days post-dosing. GT1b and GQ1b increased at the same rate as GD3 decreased. The decrease in the concentration of GD3 may be due to its conversion to GT1b and GQ1b via the B-pathway (See Figure 4) of ganglioside synthesis. The increase in GQ1b and GT1b may further reduce the levels of GD3 since these two gangliosides have been found to inhibit GD3 synthase (Klein et al., 1988). No significant changes were seen in the levels of GM1, GT1a+GD2, GD1a or GD1b.

A goal of this study was to determine if there were changes in the levels of lipids in DFP-exposed birds prior to neuronal degeneration. The linear changes in the values of GD3, GQ1b and GT1b began to occur by day 4 post-dosing which was characterized by no ataxia. No degeneration has been found in the hindbrain at this time (Tanaka et al., 1990). Results of this study suggest that the changes in the relative proportion of gangliosides began to occur during the developmental stage of OPIDN, before the advent of neuronal

degeneration.

The functions of the gangliosides which were affected by DFP are unknown. It is known that GD3 is associated with reactive glial cells and with other cells with high metabolic activity. GD3 has been hypothesized to have a function related to membrane permeability. GT1b and GQ1b are found in glial cells and neurons but they do not appear to be concentrated in a specific cell type (Seyfried et al., 1984). During development of the brain, GD3 increases during the proliferation of neuronal and glial precursor cells, GQ1b increases during cell migration and arborization whereas GT1b increases during the formation of synapses (Seybold and Rahmann, 1985). GQ1b has been found to enhance neurite outgrowth and cell proliferation in cell cultures of neuroblastoma cell lines (GOTO and NB-1) at the ngm level (Tsuji et al., 1983).

Cellular location of ganglioside changes-

The changes in the relative proportion of gangliosides may be due to changes in the quantity of a certain cell type or it may be due to transformations within already existing cells. Cavanagh (1954) found an increase in microglia and astroglia cells after TOCP-treated hens had begun to show signs of ataxia. In the present study, changes in the ganglioside pattern occurred before the hens showed signs of ataxia. The ganglioside transformation may be occurring

within the glial cells, even though glial cells are thought to be unable to synthesize gangliosides. It has been hypothesized that gangliosides are transported from the neuronal perikarya to the glial cells via a transport protein (Byrne et al., 1988).

DFP may instead be affecting the gangliosides residing in the plasma membranes of the axons. Gangliosides of the plasma membrane can originate from three different processes. Gangliosides can be made in part from de novo synthesis and in part from recycling of ganglioside components which are broken down in the lysosomes. Gangliosides of the plasma membrane may also be internalized and made into more complex gangliosides by direct glycosylation (Tettamanti, 1988). DFP could be affecting any of these processes.

The changes in ganglioside patterns may also be caused by interactions between cells. Intra-axonal and intra-myelinic vacuoles have been found in degenerating nerves in chickens dosed with TOCP (Bouldin and Cavanagh, 1979b). These vacuoles displace a large amount of axoplasm which could alter the shape of the plasma membrane and its interaction with surrounding cells. The spinal cord of rats dosed with TOCP had vacuoles which severely compressed the axons of affected nerves against their myelin sheaths (Veronesi, 1984). In the peripheral nervous system, the gap between the axon and the Schwann cell membranes was closed

following the administration of TOCP to cats (Bischoff, 1967). This close juxtaposition of the two membranes may affect the distribution of gangliosides. Research has shown a pronounced change in ganglioside patterns when certain glial and neuronal cell lines are cultured together (Mandel et al., 1980). In addition, increases in cell density has been found to increase the concentration of polysialogangliosides and decrease the concentration of monosialogangliosides (Ando, 1983).

Gangliosides and susceptibility to OPIDN-

According to Richardson (1984), the susceptibility of axons to OPIDN differs depending on the: (1) region of the nervous system, (2) species of animal and, (3) age of the animal. The distribution of gangliosides differs according to all three of these factors. Gangliosides may therefore be one of the factors responsible for the difference in susceptibility of certain axons to OPIDN.

The age and species differences in susceptibility to OPIDN may be due to differences in the ability of the axons to repair or adapt to the damage caused by OPIDN (Richardson, 1984; Johnson, 1990). Gangliosides are thought to play an important role in the maintenance and repair of axons (Mahadik and Karpiak, 1988). For example, the administration of gangliosides to chickens dosed with TOCP reduced the degree of ataxia exhibited by the birds (Berry et

al., 1986). The chick and rat may have ganglioside profiles which, when compared to the adult chicken, are better able to: (a) repair damage to axons or, (b) prevent further damage after the initial injury. It is interesting that in the present study there was an increase in the levels of GT1b and GQ1b. These two gangliosides have been associated with synapse formation and arborization, respectively, during brain development.

Gangliosides and the mechanism of OPIDN-

OPIDN is initiated by the binding of the toxin, or an active metabolite of the toxin, to a plasma membrane protein called neuropathy target esterase (NTE). After the toxin is bound to NTE it ages. Aging occurs when an "R" group of the phosphorylated protein is cleaved from the phosphorus, leaving behind a negatively charged protein. Axonopathy develops 8 to 14 days after the initiation of OPIDN (Johnson, 1982). The increased concentration of negative charges due to the aged NTE may adversely interact with the negatively charged sialic acid moieties of the gangliosides within the plasma membrane. The aged NTE may also be indirectly altering ganglioside levels by disrupting the plasma membrane.

Very little is known about the period between the initiation of OPIDN and its expression. It is known that there is an increase in the activity of calcium-activated

neutral protease (CANP) in the chicken brain within 4 days and in the sciatic nerve within 2 days after the administration of the delayed neurotoxin TOCP. The increase of CANP was prevented when the calcium channel blocker, verapamil, was administered with TOCP (El-Fawal et al., 1990). These findings suggest that OPIDN enhances the amount of free calcium in the cell.

The changes in the relative proportion of gangliosides found in this study may influence the entrance of calcium ions into the cell. The arrangement of gangliosides within the plasma membrane is thought to control the influx of calcium into the cell during synaptic transmission (Rahmann, 1983). The B-subunit of cholera toxin has been found to react with GM1 to influence the influx of calcium through calcium channels (Spiegel, 1988). It is important to note that the gangliosides which are increasing are the more sialylated ones. These changes in ganglioside distribution may be a neuronal mechanism of stabilizing the membrane or preventing additional calcium from entering the cell. Further work needs to be done in this area before any specific hypotheses can be established.

# CONCLUSIONS

In this study no changes were found in the levels of protein, total lipid, total cholesterol, lipid phosphorus or ganglioside-bound sialic acid levels in the hindbrains of chickens administered the delayed neurotoxin, diisopropylfluorophosphate. Considering the small percentage of fibers in the hindbrain which are affected by OPIDN, it may be that this method was not sensitive enough to show small changes in the levels of these variables. DFP was found to alter the ganglioside profile of the chicken hindbrain. Results suggest that the change in the ganglioside profile following exposure to DFP began to occur before the advent of axonal degeneration.

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# BIBLIOGRAPHY


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