An Electrophoretic Study of Fetal Mouse Brain Proteins after in vivo Exposure to Phenytoin and Disulfiram

by

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CHAPTER 1

INTRODUCTION

1.1 Fetal vulnerability to drugs and chemicals

In our daily lives we are exposed to a great variety of chemicals. In 1980 an estimated 55,000 chemicals were in use, and approximately 650 new chemicals enter the market-place every year (Schardein, 1985a). Many of these chemicals are synthesized in minute quantities for experimental purposes, but every year new chemicals are produced that achieve wide distribution, whether as therapeutic drugs, new plastics, or herbicides.

Many of these substances carry hidden hazards that become apparent after many years of use. In the industrial nations particularly, there have been a number of public campaigns and legal battles over substances that have been claimed to be harmful to people and to the environment. Organic mercury, DDT, and dioxin are examples of chemicals that have had restrictions placed on their use. Substances that are made available for therapeutic use are more carefully controlled, but there are historical examples of medicines that were thought to be safe and yet have proved to be toxic in unexpected ways.

Since the early 1960's it has been mandatory to test new drugs for possible ill effects on the fetus as well as the adult. Adult animal testing alone is no longer adequate because the developing organism has numerous molecular and cellular events that are unique to it. Consequently, a substance that has been demonstrated to be completely non-toxic to the mother may nevertheless lead to injury to the fetus.

Any agent that damages the fetus is called a **teratogen,** and the study of injury to the fetus is called teratology. These words stem from the Greek root "teras", meaning monstrosity. However, birth defects need not be gross. Schardein (1985b) defines a teratogen as "an agent that induces structural malformations, metabolic or physiological dysfunction, or psychological or behavioural alterations or deficits in the offspring, either at birth or in a defined postnatal period".

1.2 Thalidomide : The Spur for Experimental Teratology

Thalidomide ingestion by pregnant women in the late 1950's and early 1960's resulted in thousands of malformed infants, the majority with limb malformations, a few with more severe generalized malformations. The manufacturer of the drug, Chemie Grunenthal, had declared thalidomide totally free of toxicity, and had explicitly stated in an advertisement, that thalidomide "does not damage either mother or child". (Teft and Munro, 1976).

These claims were based on what would today be regarded as very inadequate animal tests. Although general toxicity studies were done using mice, rats, guinea pigs and rabbits, no teratogenicity testing was done before thalidomide was released to the marketplace (Schardein, 1985c). However, it should be remembered that Chemie Grunenthal had not in fact broken any laws. As Wilson (1979) pointed out, in the 1950's there were few official guidelines and no legal requirements regarding teratogenicity testing. He claimed that "even if appropriate animal tests had been run and the results been available prior to 1960, it is questionable whether they would have been applied in such a manner as to have avoided the thalidomide or some other occurrence". The reason for this is because "neither the concepts nor the technology of prior animal testing had evolved to the point where they could have been intelligently applied" to the estimation of teratogenic risk (Wilson, 1979).

Today, the "FDA Guidelines of 1966" have, with some modifications, been adopted by most of the regulatory agencies in the West. The legislation instituted by the FDA requires that no drug for human consumption, which may be administered to pregnant women, will be released for sale without first having been tested in at least two rodents and one other of the dog, rabbit, or cat (Shepard, 1979).

Animal studies give no guarantee that a drug which fulfills the legal requirements of animal testing for teratogenicity, will be innocuous to the human fetus. For instance, thalidomide is not a potent teratogen in the commonly used laboratory animals, and limb defects on the same pattern as those found in humans, have been observed only in a few breeds of rabbits and in primates. It is therefore not inconceivable that the extreme toxicity of thalidomide to the human fetus would not have been appreciated even if the FDA guidelines had been applied prior to its release. None of the known human teratogens has been identified using animal studies, although animal studies have been useful in elucidating the principles and mechanisms of teratogenesis (Chernoff and Lyons Jones, 1981).

The mechanism of thalidomide injury to the unborn remains as much of a mystery now as it was 25 years ago. A number of mechanisms of action have been proposed, but no hypotheses has yet been proved. Schardein (1985d) listed some of the possible mechanisms: folic acid antagonism, acylation, intercalation with nucleic acid, interference with glutamic acid metabolism, vitamin antagonism, chelation of essential bivalent cations, deranged nucleic acid and protein synthesis, immunosuppression, and formation of an arene oxide metabolite. The pathogenesis of thalidomide-induced malformations appears to be just as obscure as its mechanism of action. McCredie and Macbride (1973) proposed that the embryo suffers a peripheral neuropathy during thalidomide teratogenesis, and suggested that if this occurs at the stage when the spinal nerves are developing, then the result would be incomplete or

absent limb bones. Since then it has been shown that in fact skeletal morphology is independent of innervation (Strecker and Stephens, 1983).

The determination of the mechanism of action, and pathogenesis, of a suspected teratogen, may be seen as an important adjunct to the process of predictive teratogenicity testing using animals. The reason is that once the mechanism of action of a teratogen is known, then it may be possible to predict, on the basis of structural similarity, related biochemical action, or effect, which new drugs (and metabolites of these drugs) may also be teratogenic. In addition, once the pathogenesis is known, it may become more evident which of the developmental processes are critically sensitive to disruption by xenobiotics.

1.3 Teratogenic Susceptibility and the Stage of Development

The fetus is susceptible to drug injury in different ways during intrauterine life. During the preimplantation and presomite periods, drug ingestion by the mother may lead to death and abortion of the embryo. Exposure to teratogens during the critical periods of organogenesis will make normal patterns of growth vulnerable to disruption. Once fetal organogenesis is complete, there is less likelihood that exposure to a teratogen is likely to have a teratogenic outcome.

In experimental animals comprehensive information is available on the different stages of organogenesis. For instance, in the rat the period of active embryological differentiation occurs between day 8 and day 16 (Wilson, 1973). A teratogen must normally be administered to the pregnant animal during this period for specific structural malformations to occur in the offspring. It is generally held that the organ that is the most actively differentiating at the time of the exposure to the teratogen will be the most affected. For example, the eye and the heart of the rat achieve maximum

growth on day 9, and we therefore expect both organs to be the most vulnerable to injury at this time. The brain, which achieves optimal development about a day later, will also be vulnerable, whereas the urogenital system, which develops late in organogenesis, is likely to be unaffected.

Thalidomide produced malformations in children when taken on days 20-36 following conception, which is well within the period of most active organogenesis (which ends at about 12-14 weeks). Abnormal ears occured when thalidomide was taken on days 20-24, whereas phocomelia of the limbs was induced on days 27 to 33. Ingestion of the drug between day 27 and day 30 was associated most often with defective arms, whereas ingestion from day 30 to day 33 caused more leg deformities with less involvement of the arms (Shepard, 1979). These findings correlate with the appearance of the lower limb buds in the human embryo at about day 30.

It may be possible to establish a casual link between drug ingestion and birth defects by showing that maternal drug ingestion occurred at a specified time during pregnancy. For instance, Rivas and colleagues (1984) have made such an association in a single case report of a craniofacial cleft in a child. In the human the development of the frontonasal prominence occurs at the end of the sixth week. Rivas and colleagues were able to establish that the mother had taken a large single dose of diazepam on day 43 of gestation. On the strength of this temporal concordance, and despite epidemiological evidence to the contrary (Rosenberg et al., 1983), the authors proposed an aetiological relationship between diazepam ingestion by the mother concerned and the oral cleft in the child.

1.4 Mother and Fetus: Biological Distinctions

For some time it was believed that the placenta was a shield for the fetus, and that any toxic effects due to drug ingestion were experienced by the mother alone. This matter

has been completely re-evalued. "It is clearly evident that there really is no placental barrier per se: almost all chemicals given the pregnant animal (or woman) reach the fetus in significant concentrations soon after administration" (Schardein, 1985e). In fact, the placenta and fetus have some drug metabolizing capacity and may even generate toxic intermediates.

The way in which mother and fetus respond to drugs may differ markedly. Thalidomide, at dosages that achieve therapeutic efficacy, is the best example of a substance that is relatively non-toxic in adults, but is a powerful teratogen. Likewise, inorganic cadmium may cause widespread morphological and neurological damage in immature animals. Inorganic cadmium does not enter the mature central nervous system, but does enter the central nervous system of developing animals with relative ease. (Newland et al., 1986). In the brain of the chicken embryo, naloxone does not reverse the inhibition of adenylate cyclase by morphine between days 6 to 8 (Sakellaridas and Vernadakis, 1986), indicating that there is a transitory form of the enzyme in the chick which is not found in the adult.

That chemical substances should often have different effects on the mother and the fetus is to be expected. While mother and fetus have close genetic kinship, the patterns of gene expression are under independent control. Adult and fetal cellular phenotypes may differ profoundly, with many fetal tissues made up partly of undifferentiated cells. The result is that the fetus will produce proteins that are qualitatively and quantitatively different from those of the adult organism.

The biosynthetic enzymes are highly active during development. In the central nervous system the activities of enzymes of purine biosynthesis are apparently related to the level of nucleic acid synthesis. Dominguiez and Ordonez (1982) found that early in the development of the rat central nervous system, the levels of two folate-dependent enzymes of **de novo** purine biosynthesis are more than nine times above adult levels. Ornithine decarboxylase is another enzyme that is developmentally

regulated. Slotkin and co-workers (1984) have shown that this enzyme is present at high levels during phases of differentiation and cell growth as well as replication, and as cells mature or growth ceases the levels decline.

Qualitative differences in protein biosynthesis are evident at the level of tissue and organ. Fetal and adult blood proteins differ in the type of haemoglobin that is expressed. Kastern and colleagues (1986) report that in the human fetus the level of $alpha_1$-microglobulin peaks at 15-17 weeks and then rapidly decreases until birth. There are differences in myocardial function among fetal, newborn and adult animals. The immature heart operates near the plateau of the cardiac function curve, which Mahoney and Jones (1986) claim is due to there being more junctional sarcoplasmic reticulum in fetal hearts.

These fetal cellular phenotypes must mature in a scheduled way. If any step in this process is inhibited or delayed then subsequent steps may be delayed or will not take place at all. Fetal development is sequential and every stage is under temporal constraint. The fetus differs not only from the adult because it has different proteins and tissues, but also because it is vulnerable in a temporal sense. The mother is not vulnerable in the same way and drug injury that is not too severe may be overcome by synthesizing cellular components anew or by regenerating damaged tissue. Although we may expect the fetus to have some capacity for repair, any disruption of the schedule of its developmental programme is likely to result in structural and functional shortcomings at birth, which we then refer to as teratogenic damage.

1.5 Development of the Brain. Molecular and Cellular Aspects

1.5.1 Phase of Maximal Growth

In the mouse embryo the central nervous system is the first organ to begin development. At $7^1/_2$ days, when the neuronal groove, neural plate and head process

stages have been completed, the primary parts of the brain can already be identified (Rugh, 1968). By day 14 the mouse brain is typically that of a mammal. The period from day $7^1/_2$ to day 14 is therefore one of rapid and extensive organogenesis. It is during this "growth spurt" that developmental processes in the brain are particularly sensitive to disruption.

As may be expected the rate of protein synthesis during the "growth spurt" is high. Gilbert and Johnson (1974), using cell suspensions derived from mouse brain, found that protein synthesis activity was very high on the 13th gestational day, and that between the 13th day of gestation and adulthood there was a 25-30 fold decrease in the rate of protein synthesis. In a later study done on intact fetal rat brain, Johnson (1985) established that the high rate of protein synthesis is accompanied by a lower rate of degradation, as compared with the neonatal rat brain. Qualitative protein changes are likewise extensive during fetal development, and the brain protein complement constantly changes as the brain matures (Hoshida and Takahashi, 1980).

During the "growth spurt" the fetal brain may be particularly vulnerable to drugs that interact with any of the numerous components of the complex machinery of protein biosynthesis. For example, xenobiotics may influence protein biosynthesis by intercalating with DNA bases, or with RNA; by binding to the ribosome; by disrupting translation or post-translational modifications. Disruption of genes that regulate the expression of other genes is likely to have profound effects. An enzyme inhibitor may lead to the loss of that protein only, whereas drugs that destroy the cell membrane may lead to the deletion of a whole population of cells. Teratogens therefore have a variety of potential targets, and some of them may simultaneously disrupt a number of different cellular components.

1.5.2 Neuronal Outgrowth

The great complexity of the brain arises not only from its cellular diversity, but also from the extent of the interconnections between neurones. There are an estimated 10^{11} neurones in the human brain, each of which makes 10^4 connections with each other neurone (Black, 1986). These connections are not random but are uniquely specified by genetic and epigenetic factors. Despite this complexity however, detailed knowledge of brain biology has been obtained from the study of the simple and accessible segmented nervous systems of lower animals.

Neurones differentiate from neuronal precursor cells as a family of closely related but not identical cells. As it differentiates, each neurone undergoes unique interactions with other neurones or with neuroepithelium. These interactions involve a series of sequential cell recognition events between each neurone and its substrate. If a cell that forms part of the substrate is experimentally ablated, then the neurone will not show affinity for any other cell (Thomas et al., 1984). The distinctive neuronal morphology is therefore generated by the way in which the developing neurone interacts with neighbouring cells.

Cell recognition during neuronal outgrowth requires the temporal and spatial expression of many different cell-surface molecules. One of these membrane proteins is the neuronal cell adhesion molecule (N-CAM) (Rutishauser, 1984). N-CAM is transiently expressed at sites that provide selective adhesion for neurones. Changes in the carbohydrate content of N-CAM can produce a hierarchy of binding affinities (Sunshine et al., 1987), and it is this differential affinity among neurones that determines their unique morphology (Edelman 1985; Edelman, 1986). The process of neuronal differentiation is therefore a fine interplay between intrinsic cellular programs and signals from the extracellular environment.

Immunological techniques have confirmed the heterogeneity of neurones in the mouse central nervous system, and cell surface markers have been described to distinguish between subclasses of neurones (Schnitzer et al., 1984). Developmentally distinct classes of oligodendrocytes have also been distinguished, and astrocytes and subclasses of astrocytes have been recognised by several markers (Schnitzer et al., 1984). In view of the large variety of cell types, and the complexity of their interconnections, it is to be expected that the brain has a large number of sites where potentially harmful drugs may act. The developing brain experiences complex spatial and temporal biological events. In the fetal brain we may therefore expect a wide spectrum of teratogenic manifestations, ranging from gross structural damage to mild behavioural maladaptations.

1.5.3 Postnatal development

Of all the organs the brain takes the longest to mature. Brain development in many species is not completed until well after birth. In altricial species, especially, morphogenesis extends well into the post-natal period (Brunjes, 1983). In the mouse, as well as the rat, axonal and dendritic growth, synaptogenesis, gliogenesis, and myelination takes place within the first weeks after birth (Knaus et al., 1986; Burgoyne et al., 1981).

There are numerous examples of postnatal maturation of the brain. In the hypothalamic-pituitary of the rat oestradiol receptors reach maximum concentration on the 15th day after birth (MacLusky et al., 1979). Likewise, a neuronal cell-surface sialidase isolated from the rat attains maximum expression on the 20th day after birth (Moran et al., 1986). Desialylation is at a maximum during the time of extensive synaptogenesis, and the amount of sialic acid is thought to regulate cellular plasticity. Oohira and co-workers (1986) report that the glycosaminoglycan content of the rat

brain reaches a plateau by 10 days after birth. Glycosaminoglycans constitute the extracellular matrices of various tissues, and changing levels of the different glycosaminoglycans during the process of organ development are thought to have an important role in morphogenesis.

During the development of the fetal brain into a mature organ, the various cells of the brain undergo transitions from proliferating, undifferentiated precursor cells to non-proliferating, terminally differentiated cells. This process extends well into the postnatal period and is reflected in changes in protein composition. An analysis by Burgoyne and co-workers (1981) of 59 polypeptides from fractions of rat cerebral cortex revealed that three polypeptides increased in amount during postnatal development. Yoshida and Takahashi (1980) did a detailed electrophoretic study of the proteins of rat cerebral mantle, cerebellum, and brain stem. At every developmental stage the electrophoretic profile was different, although in fetal animals the three regions were very similar in composition. During the postnatal period there was a remarkable increase in the number of polypeptides in all three regions, suggesting that proteins become more heterogeneous during postnatal development. Non-histone proteins and histones from rat neuronal and glial cells undergo increased acetylation and phosphorylation up to 30 days after birth (Serra et al., 1986). Heizmann and co-workers (1982) demonstrated that many single-stranded DNA-binding proteins undergo conspicuous developmental fluctuations, especially in the perinatal period and the first postnatal week.

The extensive and protracted postnatal modulations of gene expression may be crucial to the successful maturation of the organism. If this is so then the rodent brain will be vulnerable to teratogens for an extended period of time. Schardein (1985a), in his definition of a teratogen, has included the postnatal period as a time during which developmental processes may be disrupted. Conceptually, injury to the brain in the

postnatal period is the same as prenatal injury, if the outcome is structural or functional deficits in adulthood.

1.6 Experimental Approaches to Drug Injury to the Fetal Brain

1.6.1 Macroscopic Examination

Injury to the fetus may be assessed in experimental animals by noting the number of resorptions and intrauterine deaths, as well as litter size, birth weight, sex ratios, malformations to limbs and organs, and intrauterine position (Cornwall et al., 1984). Fetal brain weight was used by Patsalos and Wiggins (1982) to assign relative teratogenic risk to a number of potential central nervous system teratogens. Likewise, exencephaly, enlarged cerebral ventricles and open eyes in experimental animals have been used to assess the degree of central nervous system teratogenicity of a number of drugs (Sullivan and McElhatton, 1977). Johnson and colleagues (1985) found that procarbazine administration to pregnant rats leads to microencephaly in the offspring. Depending on the dosage and the day of administration, the various regions of the brain were affected differently. The neocortex suffered the greatest size reduction; the hippocampus, cerebellum, and the encephalon - midbrain were also reduced in size, while the corpus striatum and pons-medulla were spared. Of course, a teratogen may not result in discernible physical damage to the fetus, but may nevertheless cause behavioural deficits after birth (Voorhees et al., 1979).

1.6.2 Ultrathin Sectioning of the Brain

Histological staining in conjunction with light microscopy has been used on ultrathin sections of fetal brain, in order to detect any lesions that were possibly caused by drugs that had been administered to the mother. Bergman and co-workers (1980) obtained ultrathin sections from fetal mouse brain, and demonstrated that maternal exposure to phenobarbitone resulted in a reduction in the number of Purkinje and

pyramidal cells; Yanai and Bergman (1981) demonstrated the same effect by using granule cells. In neonatal mice, phenobarbitone exposure has been found to reduce the number of dendritic spines of Purkinje cells (Yanai and Iser, 1981). Hannah and co-workers (1982), using sections of the rat cerebellum, have demonstrated that chlorpromazine exposure also leads to a reduction in Purkinje cell number in the developing brain.

Transmission electron microscopy may be used to study intracellular changes in developing brain. For example, by studying ultrathin brain sections from mice, Fishman and co-workers (1983) found that phenobarbitone exposure leads to degeneration of mitochondria and also to the disintegration of the myelin sheath.

1.6.3 Tissue Culture

Brain slices may be placed in a nutrient medium and the cells monitored in the living state by light microscopy. Drugs at defined concentrations may be added directly to the medium. Alternatively, explants of sections of the brain may be used; they can be kept alive in a nutrient medium for as long as 28 days. For instance, Blank and colleagues (1982) were able to show that neonatal mouse cerebellar cultures that were exposed to phenytoin underwent cortical degeneration, while explants derived from mature animals were more resistant to phenytoin.

Tissue culture more commonly involves maintaining cells in their dissociated state. Different brain cell types may be obtained in a virtually pure form, and drug effects on these cultures may be determined. Bergey and coworkers (1980) used fetal mouse spinal cord neurones, and found that phenobarbital produced dosage-dependent decreases in neurone counts. A similar effect was found with pentobarbitone on nerve and glial cells obtained from chick embryos (Roth-Schechter and Mandel, 1976). Phenytoin, too, has a cytotoxic effect on neurones in culture, as Swaiman and

colleagues (1982) were able to show, using cells obtained from the cerebral cortex of fetal mice. Pure cultures of dissociated cells also lend themselves to detailed cellular and biochemical analysis, although it appears that this avenue of teratological research has been relatively neglected (see 1.6.4).

1.6.4 Molecular studies

As yet very little is known about the molecular events that accompany drug injury to the fetus. Even less is known about the molecular teratology of the brain, and it is probably because of its complexity that this organ has been the subject of so few biochemical investigations. The few molecular studies that have been done merely emphasise how little is known about those critical biochemical events that are perturbed by teratogens.

This state of affairs is partly due to the limitations of a holistic approach, where the starting material is often extremely complex, and from which a promising line of biochemical attack must be taken. The alternative is a reductionistic approach, which involved the dismantling of cellular components of interest and then reassembling them in a highly simplified but still functional system. Such an approach gives the investigator more versatility in formulating and proving hypotheses; unfortunately, until more is known about which the cellular components of interest are, the holistic approach will continue to be used.

Bergey and coworkers (1980) investigated the effect of phenobarbitone on the enzyme choline acetyltransferase. Cell cultures of fetal rat spinal cord were used, and this enzymatic work was part of a three-pronged approach that included cellular and histological observations. Although phenobarbitone was found to produce dose-dependent decreases in the enzyme, this finding does not tell us more about phenobarbitone as a teratogen, beyond the fact that it is potentially harmful. Any

attempt to determine the underlying molecular processes will require extensive and specific knowledge of the cellular physiology and biochemistry of the particular cell-type. It is reasonable to suppose that the more restricted the experimental context is, the easier it will be to establish a direct link between macroscopic damage and molecular events, and the easier it will be to assess the potential usefulness of a screening procedure.

The use of a biosynthetic pathway to detect drugs that are harmful to the fetal brain has been adopted by Slotkin and coworkers (1985). They chose the enzyme ornithine decarboxylase, which catalyses the initial step in the synthesis of polyamines; polyamines in turn play a regulatory role in macromolecular synthesis during replication, differentiation and growth. It is known that drugs, hormones and environmental conditions may perturb the ornithine decarboxylase/polyamine system, and Slotkin and his co-workers postulated that such a perturbation may account for the widespread disruption of fetal tissue that some teratogens cause. They proposed therefore that ornithine decarboxylase levels or activities may be used as a biochemical marker for teratogenic events.

Such an approach does not solve the difficulty of distinguishing between primary and secondary events. Since cellular biosynthetic pathways are closely linked, it is probable that some of the widespread perturbations attributed to ornithine decarboxylase suppression or inhibition are partly the result of the disruption of a host of other pathways. For this reason the use of ornithine decarboxylase as a biochemical marker may be of limited value both in screening for new drugs of teratogenic potential and for the determination of the mechanism of action of a known teratogen.

Another difficulty in the molecular approach is to distinguish toxic from teratogenic mechanisms. For instance, Garrett and Tabakoff (1986) found that rats that had been

exposed to phenobarbitone **in utero** had 17% fewer benzodiazepine receptors at birth, compared to controls. However, the chronic administration of phenobarbitone to adult mice also reduces the number of benzodiazepine receptors in the brain. Moreover, in the **in utero** experiment, if the drug exposure was stopped at birth, then the benzodiazepine receptor number returned to normal. It may well be that every xenobiotic has widespread effects within cells, with some of these effects being toxic, and that only at a specific dosage and at a specific point in time will a teratogen disrupt a critically susceptible biochemical pathway in such a way as to produce a teratogenic outcome at birth.

1.7 The Experimental Mouse

Every human teratogen was uncovered either through case reports or epidemiological research, and there are limitations to the predictive value of animal experimentation. A short program of animal experimentation is unlikely to direct suspicion to a drug that may only after many years of human use be discovered to have subtle teratogenic manifestations. Part of the difficulty of using animals to screen potential human teratogens is that "almost all the putative teratogens in the human have teratogenic activity at fractional dosages compared with those demonstrated in the laboratory in various species" (Schardein, 1985f).

Nevertheless, animals generally are far more susceptible to other human teratogens than they are to thalidomide, and animal testing must be performed satisfactorily before a new drug may be registered. In terms of their close phylogenic relationship to humans, the primates should be the most suitable animals to use for testing; unfortunately they are expensive to use and their birth rate is low. Rodents are the most extensively used animals and Tuchman-Duplessis (1972) claimed that no chemical exists that is teratogenic in the human that has not produced malformations

in rodents. There is the ever-present danger that negative results in rodents may be used to predict that an agent will lack teratogenic effect in humans; for this reason a non-rodent animal must also be used in teratogenicity testing of a new drug.

In the present study mice were used because of the many advantages they have over other experimental animals. They are readily available and easy to feed, raise, mate and handle. Because they are small, they are inexpensive, but nevertheless are big enough to manipulate without difficulty. The duration of pregnancy is short, fertility rate are high, and litters are large. Although mice show good resistance to the toxic effects of most drugs, they are nevertheless fairly susceptible to teratogens. A drawback is that mice have a fairly high spontaneous malformation rate (0.5% in Swiss albino colony) which means that fairly large control groups are needed (Tuchman-Duplessis, 1972). Various in-bred strains are available, with interstrain differences in susceptibility to teratogens (Hansen and Hodes, 1983). Because the mouse has been extensively used in experimental teratology and as a general laboratory animal, there is a large body of accumulated knowledge on this animal.

1.8 Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis makes it possible to examine a large number of different protein molecules simultaneously. The technique is regularly used to give a display of the protein complement of a particular tissue, and is ideally suited to the examination of the effects that experimental manipulations may have on protein expression. A solubilized tissue sample is subjected first to isoelectric focussing and then to sodium dodecyl sulphate polyacrylamide gel electrophoresis. In the first dimension proteins are separated by charge and in the second dimension by mass. Within these parameters a thousand or more proteins may be resolved from a complex

mixture. Test and control gels may be compared for qualitative and quantitative changes to these proteins.

Two-dimensional gel electrophoresis has been used to visualise the modulation of protein synthesis by xenobiotics. In a study using regenerating mouse liver, isoproterenol and phenobarbitone were shown to change nuclear protein concentration, including some of the histones (Pipkin et al., 1985). In toxicological studies, two-dimensional gel electrophoresis has been used on various tissues. The widely used organic solvent dimethylformamide was used to induce minor protein changes in the serum of rats when the solvent was injected intraperitoneally; two proteins, IgG and x_1-acid glycoprotein were tentatively identified, but no suggestion was offered regarding the mechanism of this effect (Marshall et al., 1985). When Aroclor 1254, a mixture of chlorinated biphenyls, was injected intraperitoneally into mice, 31 protein changes were found in a liver extract following two-dimensional gel electrophoresis (Anderson et al., 1986). One of the proteins that underwent a change was cytochrome b_5, an electron transport protein, and the authors speculated that each of the protein changes may play an important part in the mechanism of toxicity.

The effect of drugs on brain tissue has been the subject of few studies using two-dimensional gel electrophoresis. When administered chronically, desmethylimipramine resulted in a reduction in the concentration of two proteins in the parietal cortex and the hippocampus of rats, and also lead to the increase in concentration of a single protein (Heydorn et al., 1984). In contrast, chronic treatment with reserpine produced effects on these three proteins in the hippocampus which were quantitatively opposite to those obtained after chronic desmethylimipramine administration. Since both agents affect noradrenergic reactivity, the authors postulated that one of these proteins is tyrosine hydroxylase, an enzyme involved in noradrenergic neurotransmission.

Although there have been two-dimensional electrophoretic studies on fetal brain tissue (for instance, Yoshida and Tokahashi, 1980), the emphasis in most of this work has been on developmental changes in protein expression, and not on the effects that drugs have on fetal brain protein complement. Klose and co-workers (1977) did an early study using two-dimensional gel electrophoresis to determine the effects of various teratogens on whole embryos. No protein changes were found and that line of research was not continued.

In this study two-dimensional gel electrophoresis is extensively used, in the belief that the usefulness of this technique to experimental teratology has not been fully evaluated. It is reasonable to suppose that a central nervous system teratogen administered during critical periods of susceptibility will led to perturbations of orderly brain development, and that these perturbations will be reflected as changes to the protein complement. The total brain protein complement of mice that have been exposed to drugs **in utero** will therefore be analysed, in the hope that any inductions or deletions of proteins as a result of drug exposure may provide a clue to the molecular events underlying drug injury to the fetus.

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CHAPTER 2

METHODOLOGY

2.1 Manipulation of experimental animals

2.1.1 Choice of Mouse

C_3H mice were used to evaluate the effects of selected drugs on proteins of the fetal brain. The C_3H strain (full designation C_3H/HeAfHg/ICR) is known to be moderately susceptible to teratogens (Hansen and Hodes, 1983). Other advantages were availability and the fact that details of breeding and drug administration have already been established (Pillans et al., 1988).

It was essential to select an inbred strain, because individual animals from such strains have low genetic variability. Among inbred animals the range of responses to drug injury between individuals is kept to the minimum (Kalter and Warkany, 1961) and this makes it easier to differentiate between test and control in the response to drug injury. Low genetic variability is achieved by repeated matings of genetically closely related individuals. Usually an inbred strain is accomplished by more than 20 generations of brother-sister matings.

A feature of the C_3H strain that was potentially disadvantageous was a high spontaneous abnormality rate (Heinecke, 1972). This detriment was overcome by using large numbers of control animals.

2.1.2 Conditions of Housing

Animals were kept in an environment that was carefully regulated to be optimal for their maintenance and breeding. This included a light-dark cycle of 12 hours, air temperature at a constant 21^{o}C, constant humidity, and 12 to 15 air changes per hour.

Animals were housed in plastic cages that were cleaned twice a week and autoclaved approximately every two weeks. Fresh bedding (consisting of wood chips) was sterilized before being placed in cages. Food and water was allowed **ad libitum**. The food consisted of 13 mm mice cubes. At one stage food was autoclaved after delivery, but this practice was discontinued when it was noticed that fertility rates had drastically declined; low fertility rates possibly resulted from the destruction by heat of important nutrients in the feed.

Females were housed communally in large cages, while males were kept individually in small cages of approximately 450 cm^2 floor area.

2.1.3 Mating Protocol

Animals were supplied from breeding stocks once they had achieved reproductive maturity (6-8 weeks). Only virgin females were used for matings. Males were regularly weighed to ensure that they fell within a 24,5 - 27,5 g weight optimum. Stocks were renewed every 8 weeks.

Animals were mated one male to one female. The females were placed in the cages with the males at 17h00, and left there overnight. Mice are nocturnal breeders, and oestrus begins in 75% of mice 4-6 hours after dark (Hafez, 1970).

In the morning females were separated from the males and inspected for the presence of a copulation plug. The presence of a plug is a reliable indicator that mating had occurred. Midnight on the night of mating was taken as the time of conception and designated Day 0.

The pregnancy rate normally fell in the range 15-20%, but sometimes increased or decreased markedly, for reasons unknown (apart from low pregnancy rates attributed to feed that had been autoclaved). The mating success of each male was recorded.

Males with a low mating frequency, or males that were overweight, were not used for mating.

2.1.4 Drug Administration

Drugs were administered to pregnant females on day $8^1/_2$ or day $9^1/_2$ post conception, which coincides with the period of rapid fetal brain development (Nishimura and Shiota, 1977). It is believed that the fetal brain is most vulnerable to central nervous system teratogens during the period of active organogenesis (see Section 1.3).

Drugs were administered by means of gastric intubation. Animals were carefully immobilized to prevent them from wriggling, and a curved needle with a bulbous tip was carefully introduced into the stomach. At no stage was the the intubation process hastened or forced. Great care was taken to avoid causing the animal any pain, and if an animal resisted all intubation attempts, it was released and not used again. The volume that was introduced into the stomach was kept to a maximum of 100 µl.

2.1.5 Removal of the Fetal Brain

On day $18^1/_2$ post conception (p.c.) the pregnant mice were reweighed and then sacrificed by cervical dislocation. The pregnancies were not allowed to continue to term, because newborn mice with abnormalities are usually cannibalized by the mother. Laparotomy was performed and the uterine horns exteriorized and extended. The fetuses were carefully dissected out, and the number of live, dead and resorbed fetuses were recorded. Each fetus was blotted dry, weighed, and examined macroscopically for abnormalities. The fetal brains were dissected out, weighed, and placed in cryotubes. They were then stored at -196^{o}C.

2.2 Sample Solubilization

After retrieval from storage, the frozen tissue sample was dropped into solubilizing fluid. Thawing was hastened by agitation, and homogenization was begun immediately to minimize the action of proteases. The volume of solubilizing fluid was adjusted to 20 µl per mg brain weight.

Solubilization was undertaken with "UKS solution" (Damerval et al., 1986), consisting of 9,5 M urea, 5mM K_2CO_3, 1,25% sodium dodecyl sulphate, 0,5% dithiothreitol, 0,1 mM phenylmethylsulphonyl fluoride (PMSF), 2% Ampholines pH 3,5 to 10 (LKB Bromma), and 6% Triton X-100. K_2CO_3 was first used for protein solubilization by Horst and co-workers (1980) to augment the solubilizing properties of 9,5M urea on membrane proteins.

Until fairly recently, the use sodium dodecyl sulphate in tissue solubilization was avoided because of suspected interference with isoelectric focussing. However, Wilson and co-workers (1977) used 1% sodium dodecyl sulphate successfully in the solubilization and two-dimensional resolution of proteins from a eukaryotic sample. O'Farrell (1975) reported that small amounts of sodium dodecyl sulphate may be used without detriment because during isoelectric focussing much of the detergent separates from the protein and forms mixed micelles with Triton X-100. These micelles migrate to the acidic end of the gel and in this way interference by the highly charged molecule with the pH gradient is avoided.

Homogenization, using a Teflon pestle in a glass barrel, was continued until all solid bits of tissue had been fragmented. The sample was then sonicated with a probe. Three five-second bursts were usually sufficient to produce a uniform sample consistency. Pauses were allowed for local heating to disperse. It is particularly

important to avoid heating a urea solution, since heating may lead to isocyanate formation and the carbamylation of proteins (O'Farrell, 1975).

After sonication the sample was centrifuged at 35 000 g for 15 minutes to remove cellular debris. To prevent the crystallization of urea, the temperature during centrifugation was maintained at 20^{o}C. The supernatant was collected and stored at -196^{o}C.

The total protein concentration of the supernatant was 5 mg/ml. The Folin phenol method of protein determination (Lowry et al., 1951) was not used as originally described, due to the presence in the protein sample of interfering substances. A modification was therefore used which included a deoxycholate-TCA protein precipitation step (Peterson, 1977). Once the supernatant containing the interfering substance was discarded, the Folin phenol method of protein determination was used on the re-dissolved protein.

2.3 Isoelectric focussing

2.3.1 Principles

The first stage of the two-dimensional gel electrophoresis of a complex mixture of proteins is separation on the basis of charge. Every protein has a native charge, which is the sum of the positive and negative charges on the side groups of the amino acids that constitute the protein. If two different proteins cannot be resolved because they have the same charge, then they may be separated in the second dimension on the basis of differences in molecular weight.

Whether an amino acid side group is charged or not depends on the pH of the immediate environment. For example, a highly acidic environment will make protons available for the ionization of groups of high pKa (amino groups), and a highly alkaline

environment will remove protons from groups of low pKa (carboxyl groups). The number of charges on a particular protein, and whether they are negative or positive, may therefore be manipulated by changing the pH of the environment. When the pH of the environment is such that the protein molecule has no nett charge, then that particular pH is the isoelectric point (pI) of the protein.

In an isoelectric focussing gel, the protein (or in this case, protein mixture) migrates through a pre-existing pH gradient under the impetus of an electric field. As the migration proceeds, the protein moves into an environment of either increasing pH or decreasing pH, and the state of ionization of the amino acid side groups changes. Should it happen that a point is reached where the protein has no nett charge, then migration will cease; the protein is at its isoelectric point.

The pH gradient is established during pre-electrophoresis. pH gradients may be generated using a mixture of ordinary buffers that will have the required spread of pKs; these are "natural" gradients (An der Lan and Chrambach, 1981). More usually pH gradients are generated from synthetic elements (ampholytes) that provide both an increase in resolving power and heightened stability of the gradient. Ampholines (LKB Bromma) are made from a mixture of oligoamines that are reacted with unsaturated compounds, mainly acrylic acid, giving a highly complex mixture of aliphatic oligo-amino oligo-carboxylic acids (Righetti et al., 1986). These acids can be designed in such a way as to give a wide and continuous pH range, as well as having buffering and conductance properties.

The principle that makes a pH gradient possible rests on the fact that when a single ampholyte species is dissolved in water the pH value is changed towards the isoionic point of the ampholyte (isoionic point is the pH value at which the net charge of the ampholyte is zero). So, when two or more ampholytes with different isoionic points are dissolved in water, the pH value obtained will fall within the range defined by the

isoionic points of the ampholytes (Schafter-Nielsen, 1986). When the current is switched on, ampholytes with isoionic points above the pH of the gel (positively charged) will migrate towards the cathode, and those with isoionic points below the pH of the gel (negatively charged) will migrate towards the anode. As the ampholytes migrate the local pH of the gel will change, since the local pH is defined by the isoionic points of the ampholytes themselves. Eventually the pH will form an even gradient from ampholytes with high isoionic points to those of low isoionic points. A steady state is reached due to the fact that the electrophoretic migration of the constituents of the electrode reservoirs is exactly balanced by their influx due to passive diffusion.

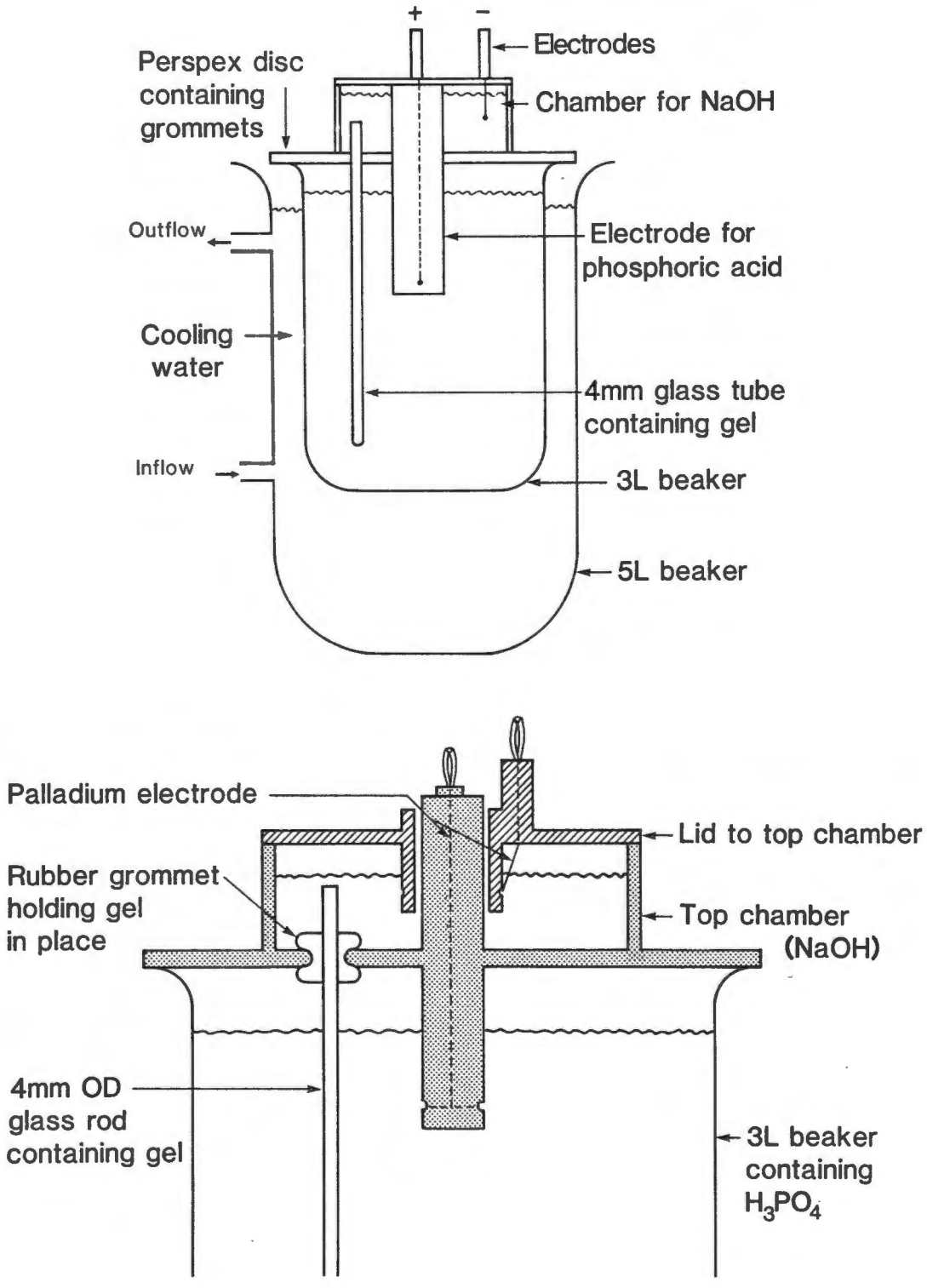
The polyacrylamide gel in the isoelectric focussing tube must be "non-restrictive" to enable proteins to reach their pI. The migration velocities of proteins decrease as they approach their pI (charge approaches zero), and at low velocities the frictional effects of the matrix are exaggerated. A highly restrictive gel may therefore halt migration before the isoelectric point is reached.

2.3.2 Apparatus

In this study, large, non-standard gels were used. Large gels offer at least two advantages over the standard "O'Farrell" gels; more proteins can be loaded onto large gels, and large gels have a greater separation area (Colbert and Young, l986).

To produce large gels, standard electrophoretic equipment had to be modified or scaled up. Compared to the standard isoelectric focussing tube, the tubes used in this study were both longer and thinner (225 mm long; 2,5 mm internal diameter). The tubes were cut from lengths of Pyrex capillary tubing. Although the longer, thinner gels were difficult to manipulate, they offered the advantage of more efficient heat dispersion (small cross-sectional area), and made possible the application of higher voltages over a longer period of time.

FIGURE 1 : APPARATUS FOR ISOELECTRIC FOCUSSING



Perspex components were made to specification by the Department of Biomedical Engineering, University of Cape Town Medical School (Fig. 1). The glass tubes passed through the floor of the upper chamber, and rubber grommets were used to hold them in place and to seal the chamber. The perspex top chamber holding the glass tubes and both electrodes was lowered into a three litre glass beaker containing the lower electrode solution. The three litre beaker was in turn lowered into a five litre beaker, and the space between the two beakers was used for the circulation of cooling water. A high volume of tap water was used for cooling; chilled water was tried but made no discernible difference to the quality of the gels.

Because platinum is so expensive, palladium was used for electrodes. The apparatus was connected to the power source (Hoefer PS1200) with wiring and plugs supplied by the Department of Biomedical Engineering.

2.3.3 Methodology

The glass tubes used in isoelectric focussing were carefully treated to ensure optimal binding between the surface of the glass and the gel, and to ensure reproducibility. Between runs the tubes were stored in chromic acid. On the day before isoelectric focussing they were rinsed thoroughly with distilled water, and any remaining acid was neutralized by dipping the tubes into a methanolic alkaline solution. The tubes were once more thoroughly rinsed in distilled water, and then sialinized with a 1% solution of dimethylchlorosilane in toluene. This process coats the surface of the glass with methoxy-silane groups, which reduces the adherence between the gel and the glass. Sialinization was essential because the narrow diameter and the length of the glass tubes made it impossible to otherwise remove the gels after electrophoresis. On two occasions all the gels slipped out of the tubes during electrophoresis. It is not known whether the cleaning and sialinization methodology was responsible for this.

The gel mixture used was essentially that developed by O'Farrell (1975). To have enough gel mixture to fill all the tubes of the apparatus the following recipe was used: To 8,25 g urea was added 2 ml of acrylamide stock, 3 ml of 10% Triton X-100, and 3 ml of water to which had been added 0,75 ml Ampholines (0,6 ml pH 5-7, 0,15 ml pH 3-10). Acrylamide stock of 30% incorporated 1,4% bisacrylamide i.e. 30%T, 1.4%C. The amount of bisacrylamide determines the density of cross-linkage between acrylamide polymers, and is therefore one of the critical parameters in achieving good resolution. In the present system less bisacrylamide was used than was used by O'Farrell in order to improve the resolution of high molecular weight proteins.

The gel mixture was vigorously swirled in order to dissolve the urea. Due to the possibility of carbamate ion formation, heating of the mixture was avoided. Once all the urea was dissolved, 15 µl of freshly made 10% ammonium persulphate was added. The gel mixture was placed under a partial vacuum for 2 minutes to allow dissolved gasses to boil off (oxygen inhibits polymerization). Finally, 10,5 µl of TEMED (N,N,N^1, N^1 -tetramethyl ethylenediamine) was added, and the mixture was briefly swirled.

The narrow internal diameter of the glass tubes and their hydrophobic surfaces (due to sialinization) made it impossible to fill the tubes using a needle and syringe. Instead, the gel mixture was drawn up into the tubes using a rubber bulb, and the filled tubes were then stoppered with rubber bungs. Care was taken to avoid the entrapment of any air bubbles inside the gel. The tubes were filled smoothly with one movement to ensure that polymerization was uniform. To keep the tubes steady and vertical they were pushed through the rubber grommets. The gel mixture was brought to about 10 mm of the top of the tubes, and a small volume of 8M urea was layered onto the top of the gel mixture to act as an air seal.

Gels were allowed to polymerize for 1,5 hours. During this time the lid was kept on the top chamber to prevent crystallization of the 8M urea used as a seal. After 1,5 hours

the urea was removed with a syringe, and replaced with 20 µl of Lysis Buffer (O'Farrell, l975) per gel, which was then overlayered with a small volume of water. The UKS solution, which was used in the sample solubilization, was not used in place of Lysis Buffer, since it is important in isoelectric focussing to keep the use of sodium dodecyl sulphate to a minimum. Gels were allowed to polymerize for a further 1,5 hours.

The bottom tank was filled with three litres of 0,01M H_3PO_4. The buffer for the top chamber (0,03M NaOH) was prepared by boiling off dissolved CO_2 in a partial vacuum. At the end of the polymerization time, the bungs were removed from the bottom of the tubes, and the Lysis Buffer from the top of the tubes. 20 µl of fresh Lysis Buffer was applied to the top of the gel, and the tubes were then filled to the top with NaOH. The perspex disc holding the tubes (Fig. 1) was placed on top of the bottom tank, and any air bubbles trapped at the bottom of the tubes were removed. The top chamber was filled with NaOH, and the apparatus connected to the power source.

Before isoelectric focussing of protein samples could commence it was necessary to establish the pH gradient. During the process of pre-electrophoresis the voltage was increased in a step-wise fashion, as in O'Farrell (1975): a) 200 volts for 15 minutes; (b) 300 volts for 30 minutes; and (c) 400 volts for 30 minutes. Cooling was provided by a continuous flow of tap water at room temperature. During the course of pre-electrophoresis the current typically decreased from 1,7 mA to 1,1 mA.

Once the pH gradient had been established, the apparatus was switched off and the NaOH in the top chamber was poured off. The Lysis Buffer was removed from the surface of the gel. It was essential that all the fluid was removed since any remaining fluid would dilute the protein sample which would affect resolution and reproducibility. Protein samples were loaded on by means of a syringe, and a volume of 40 µl (200 µg protein) was used throughout. It was found that 200 µg protein was enough to make the detection of a large number of spots possible, without causing excessive streaking

due to overloading. The protein sample was overlayered with 10 µl of 9M urea. Freshly degassed 0,03M NaOH was used to top up the tubes and to fill the top chamber.

Electrophoresis of the proteins was commenced as soon as possible after the establishment of the pH gradient. Gels were run at 10 000 Vh, comprising 23 hours at 400V, and one hour at 800V. The purpose of the final hour of isoelectric focussing was to achieve fine focussing of protein bands; it was never extended beyond one hour because of risk of obtaining distorted protein bands due to heat build-up. Water recirculation took place throughout the 24 hours of electrophoresis.

After electrophoresis was concluded, the apparatus was disassembled and the glass tubes were pushed out of the grommets. Gels were extruded from the tubes by water under pressure from a syringe. The gels were dropped into glass test tubes each containing 7,5 ml of SDS Sample Buffer (O'Farrell, l975), consisting of 10% glycerol, 2,3% sodium dodecyl sulphate, 0,0625M Tris HCl (pH6,8), and 5% 2-mercaptoethanol. The tubes were stoppered and rocked back and forth a number of times to facilitate the diffusion into the gel of the reducing and precipitating agents contained in the buffer. This critical process must be done as soon as possible after the conclusion of electrophoresis in order to minimize any protein diffusion.

The test tubes containing the gels were stored in a freezer at approximately -20^{o}C. Gels were stored in this manner for up to a week without loss of resolution. Storage of polyacrylamide gels in liquid nitrogen was not practised because of the frequency of gel fragmentation.

2.4 SDS Polyacrylamide gel electrophoresis

2.4.1 Principles

A polyacrylamide gel may be thought of as an open-meshed three-dimensional lattice which has ideal anti-convection properties as well as a poor affinity for sample molecules. The lattice structure arises from the polymerization of acrylamide monomer into long chains and the cross-linking of these by the bi-functional compound N_1,N^1-methylene bisacrylamide. The density, or pore size, of the lattice can be varied by changes in the concentration of acrylamide monomer as well as changes in the amount of bisacrylamide. Protein molecules migrating through the lattice are subject to frictional forces and are selectively retarded (and hence separated) according to molecular size.

Gradient gels were used because of the advantages they offer in terms of versatility and resolving power. In a gradient gel the concentration of acrylamide varies (in this case, linearly) from one end of the gel to the other. The gel therefore has a range of pore sizes, and will accept a wider range of molecular sizes than a gel of uniform pore size. Gradient gels also offer enhanced resolution; as the proteins migrate into areas of decreasing pore size, the advancing edge of the migrating protein zone is retarded more than the trailing edge, resulting in a marked sharpening of protein bands (Hames, 1981a).

A discontinuous (or multiphasic) buffer system was used. In the discontinuous system, the buffer within the gel differs from the buffer in the electrode reservoirs. For example, Tris/HCl was used in the gel, and Tris/Glycine in the reservoirs. The pH was also "discontinuous" - the pH of the reservoir buffer was 8,3 while the pH of the stacking gel was 6,8 and the pH of the resolving gel was 8,8.

The advantages in having a discontinuous buffer system can be seen by examining what happens to the relative ionic mobilities during electrophoresis. At the pH of the stacking gel (pH 6,8), glycine is poorly dissociated and its mobility is low. Chloride ions, on the other hand, have a high mobility at this pH, whilst the mobilities of proteins are intermediate. The chloride ions therefore migrate away from the glycine, creating a "moving boundary", behind which there is a steep voltage gradient. The proteins, having a higher velocity than the glycine, are swept behind the moving boundary, and in the zone of steep voltage gradient they become concentrated into very thin zones, or "stacks". This "stacking" of the proteins is largely independent of the initial protein concentration (Hames, 1981b), and is a direct outcome of having a discontinuous buffer system.

When the moving boundary reaches the resolving gel, the pH increases to 8,8. The glycine becomes more dissociated and overtakes the migrating proteins. At the same time, the gel pore size decreases markedly, and the proteins are retarded by molecular sieving. They now become "unstacked". While they migrate in a zone of uniform voltage and pH value, they are separated according to their intrinsic sizes (Hames, 1981c).

Proteins that are resolved by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis are always dissociated. Secondary and tertiary protein structure is disrupted by sodium dodecyl sulphate and mercaptoethanol. Sodium dodecyl sulphate avidly binds proteins (1,4g of sodium dodecyl sulphate per gram of polypeptide), resulting in a high density of negative charges on the polypeptide. The intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound detergent. Sodium dodecyl sulphate-polypeptide complexes therefore have essentially identical charge densities and migrate in the gel according to size rather than charge. It is impossible to draw conclusions about protein

conformation as a result of sodium dodecyl sulphate polyacrylamide gel electrophoresis, but it is relatively simple to determine the molecular weight of a polypeptide, which is an important criterion for identification.

2.4.2. Apparatus for Casting and Running Slab Gels

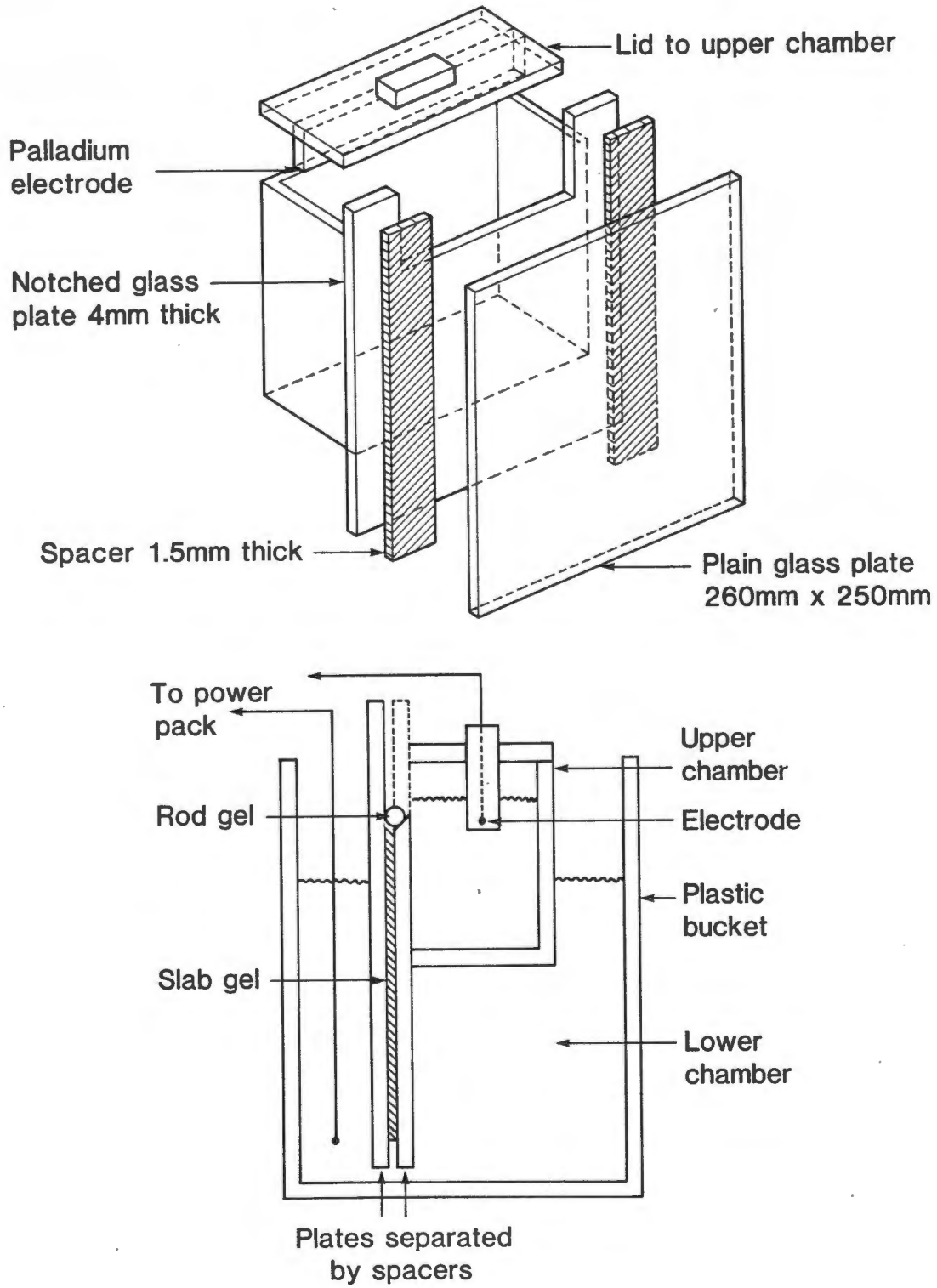
Slab gels with an area of approximately 400 cm^2 were cast, which is more than twice the area of a standard gel. The slab gel apparatus was designed after O'Farrell (1975), and put together by the Department of Biomedical Engineering, University of Cape Town Medical School (Figure 2).

It was essential to achieve a uniform gel thickness. Glass plates 4 mm thick were used to provide the necessary flatness. Thicker glass (a laminated glass) was not used due to potential problems with heat dissipation. A bevelled edge was cut into one of the plates to hold the rod gel in position (as in Wilson et al., 1977).

To assemble the apparatus, plastic spacers 1,5 mm thick were clamped between the glass plates along the sides and the bottom edge. The joints between spacers were sealed with a film of Celloseal (Hoefer). Before pouring the gel, the space between the plates was filled with water to show up any leaks.

Gradient gels were made using a standard two-chambered gradient mixer (Hames, 1981d). A linear gradient is obtained if the two chambers are of equal cross-sectional area, and if the two volumes of acrylamide gel solution are equal. To prevent mixing of the solution once it had flowed into the gap between the plates a low flow rate from the mixer was maintained.

FIGURE 2 : APPARATUS FOR SLAB GELS



2.4.3 Slab Gel Methodology

Electrophoresis grade acrylamide (BDH Electron) was used in the stacking and resolving gels. The stock solution (T30%, C1,6%) was filtered to remove any undissolved acrylamide, and when not in use was stored at 2-4^{o}C. Fresh stock was made up every 6-8 weeks.

An acrylamide gradient of 6-17% was utilized to resolve the proteins in the fetal brain sample. 64 ml of the running gel mixture was made up. The recipe for the mixture closely follows O'Farrell (1975)(See Table 2.1). There were, however, a number of modifications. Buffer A was made up with 50% glycerol (not 75% as in O'Farrell) because of solubility difficulties. No sodium dodecyl sulphate was included in the resolving gel (or in the stacking gel) because it was found that protein patterns were sharper when sodium dodecyl sulphate was not included. This is a paradoxical result without a ready explanation (Bloemendal and Jansen, l986). Ammonium persulphate was made up freshly on the day of use.

The stock acrylamide and Buffer A (or Buffer B) were pipetted into a 50 ml measuring cylinder, and the volume was brought up to 32 ml with distilled water. The gel preparation was then mixed by inverting the measuring cylinder, as it was after the addition of the ammonium persulphate and the TEMED. The 17% acrylamide mixture was poured into the front chamber of the gradient mixer immediately after adding the TEMED Great care was taken to follow a uniform procedure to ensure reproducibility in the gel gradient. The mixture was allowed to trickle into the gap between the plates at a rate of about 4 ml per minute. The gap was filled up to about 3 cm of the bevelled edge. A few drops of water were carefully pipetted onto the top of the gel to act as an air seal. After about 1,5 hours the gel was checked for the presence of a clear phase transition between gel and water, an indication that polymerization had occurred.

The stacking gel mixture was prepared (see Table 2.1). The water layer was removed from the top of the resolving gel, and the stacking gel mixture was poured from the measuring cylinder onto the top of the resolving gel, up to the bevelled edge. A few drops of water were pipetted onto the unpolymerized stacking gel to act as an air seal. Any gel mixture that was left over in the measuring cylinder was set aside to serve as an indication of polymerization.

Once the stacking gel had polymerized, the clamps were removed from the bottom of the glass plates and the plastic spacers along the bottom edge were pushed out. Tank Buffer consisting of 0,03% Tris, 1,44% glycine, and 0,01% sodium dodecyl sulphate was poured into the bottom tank and the apparatus containing the gel was lowered into the tank buffer. Any air bubbles in the space at the bottom of the gel were removed. The bottom tank was filled up until the buffer reached the top of the resolving gel (the buffer had a cooling function).

A frozen rod gel was removed from storage and allowed to thaw gently . 30 ml of fresh SDS Sample Buffer (see section 2.3.3) was brought to the boil. When the rod gel had thawed, the beaker containing the SDS Sample Buffer was removed from the flame and the rod gel was dropped into the hot solution, where it was allowed to equilibrate for 5 minutes.

Preparations were now ready to anneal the rod gel to the slab gel. The thin layer of water was removed from the surface of the stacking gel. This was replaced with a thin layer of molten 1% agarose (LKB Bromma, electrophoresis grade) in SDS Sample Buffer. The rod gel was lifted from the hot SDS Sample Buffer, and using a folded sheet of Parafilm, was manoeuvred onto the layer of molten agarose. The bevelled edge of the glass plate prevented the rod gel from slipping off, and made it possible to envelop the entire rod gel with agarose, annealing it into position. It was extremely important that no air remained trapped between the rod gel and the slab gel.

Table 2.1 Recipe for Resolving and Stacking Gel

	RESOLVING		
	HIGH 17%	LOW 6%	STACKING 3.6%
Stock acrylamide (ml)	18,2	6,5	2,4
Buffer A (ml)	8,0		
Buffer B (ml)		8,0	
Stacking buffer (ml)			5,0
Water up to (ml)	32,0	32,0	20,0
10% Ammonium persulphate (µl)	106,0	132,0	80,0
TEMED (µl)	12,0	12,0	10,0

Buffer A **1,5 M Tris/HCl pH 8,8 50% Glycerol**
Buffer B **1,5 M Tris/HCl pH 8,8 5% Glycerol**
Stacking Buffer **0,5 M Tris/HCl pH 6,8 5% Glycerol**

Once the agarose had set, the top chamber was filled with Tank Buffer. This was done slowly to avoid creating turbulence that could have disturbed the seating of the rod gel. A few drops of 0,1% bromophenol blue were added to the top chamber to provide a visible migration front during electrophoresis.

Molecular weight standards (see Appendix) were pipetted into a pre-formed notch in the agarose, or were run separately on slab gels that were identical to the slab gels used in the second dimension. For the sake of simplicity molecular weight standards were not run with every test gel.

Electrophoresis was now ready to begin. The power pack was set at 30mA per gel on "Constant Current" although it was also possible to do the run on "Constant Voltage", beginning at 100V. Electrophoresis lasted for 14 hours. Cooling was not necessary. At the end of the run the clamps were removed and the glass plates prised apart. The stacking gel was separated from the resolving gel and discarded. The resolving gel was immediately placed in a staining solution to avoid excessive surface drying. Gloves were always worn when handling gels. All resolving gels were stained with Coomassie blue as a matter of course.

2.5 Visualization of protein patterns

2.5.1 Coomassie blue staining

Coomassie blue dyes provide a quick and inexpensive method for the visualization of protein patterns on polyacrylamide gels. Although Coomassie blue is by no means the most sensitive stain available, in this study it was routinely used to stain every gel. The advantage this offered was that unsatisfactory gels could be identified before subsequent, more time-consuming, visualization procedures were embarked upon.

To make up the staining solution, 0,25% Coomassie blue R was dissolved in 40% methanol and 10% trichloroacetic acid (Neuhoff et al., 1985). This is only one of a host of different Coomassie blue staining procedures, and was selected for speed and simplicity. The R form of Coomassie blue (Colour Index 42660), obtainable from Sigma Chemical Company, is considered to be superior to other forms of the dye (Wilson, 1979). A high percentage of trichloroacetic acid and methanol in the staining mixture ensure that the proteins remain precipitated during staining; methanol also assists in the penetration of the dye into the gel.

Staining was allowed to proceed for an hour with constant agitation. After one hour the staining solution was discarded and the gel destained with a 5% methanol and 7% acetic acid aqueous solution. The destaining solution was changed every 30 minutes. After approximately three hours the gel was destained to the point where all the major spots could clearly be seen.

2.5.2 Silver Staining

The visualization of proteins on gels by means of silver staining is clearly superior to any of the detection methods using Coomassie blue. Literature reports claim that in terms of sensitivity, silver staining has the potential to give a fifty to one hundred fold improvement over Coomassie blue staining (Merril et al., 1981; Oakley et al., 1980; Switzer et al., 1979). Nielsen and Brown (1984) claimed that the detection on a gel of 1 ng of protein is feasible. There have in fact been claims of detection limits of sensitivity well below 1 ng protein. For example, Morrissey (1981) managed to detect 42 pg of protein, and Ohsawa and Ebata (1983) claimed a detection limit of 10 fg protein. Silver staining has been refined to the point where it is now comparable in sensitivity to autoradiography of ^{35}S-methione-labelled proteins (Sammons et al., 1981), and ^{14}C-labelled proteins (Switzer et al., 1979; Merril et al., 1981).

In this study, there was a four-fold increase in sensitivity of silver staining over Coomassie blue staining, in terms of the number of countable protein spots. Approximately 1250 different proteins were detected on wet gels immediately following silver staining.

Methodology: Numerous silver staining protocols have been published. Many of these propose minor improvements over previously published methods. Generally, silver staining methods may be divided into two categories: those that rely on an alkaline environment (Oakley et al., 1980) and those that rely on a weak acid environment (Sammons et al., 1981; Merril and Pratt, 1986). The method chosen for the detection of fetal mouse brain proteins was that of Guevara and co-workers (1982). It is a development of the procedure of Oakley and co-workers (1980) in that it utilizes ammoniacal silver. It was found to be extremely sensitive and had the additional attraction of giving low background staining.

A detailed method description is followed by a discussion of some of the steps:

Step 1 After electrophoresis, gels were placed in a 20% ethanol solution containing 5% v/v acetic acid, and 2,5% w/v sulfosalicylic acid (400 ml per gel) for at least 12 hours with one solution change.

Step 2 Gels were washed in 20% ethanol (Merck) 3 times, 400 ml per gel, for 20 minutes each.

Step 3 Gels were placed in a 20% ethanol solution (400 ml per gel) containing 5,9 mmol silver nitrate, 31 mmol ammonium hydroxide, and 2,8 mmol sodium hydroxide, and were equilibrated for 1 hour.

Ammoniacal silver hydroxide solution was prepared by adding 4,2 ml 14,8M ammonium hydroxide, 0,226 g sodium hydroxide and 80 ml absolute ethanol to 296 ml

high purity, distilled water. Silver nitrate (2,0g) was then dissolved in 20ml water and added dropwise to the ammoniacal-ethanol solution, with vigorous agitation.

Step 4 Gels were rinsed in water; then step 2 was repeated.

Step 5 Gels were placed in a 20% ethanol solution (400 ml per gel) containing 0,01% citric acid and 0,037% formaldehyde "developing solution". Images appeared within 5 minutes and development was allowed to proceed for up to 30 minutes.

Step 6 Development was terminated by placing the gels in 500 ml 20% ethanol and 0,5% acetic acid for 30 seconds.

Step 7 Gels were washed in running tap water for 45 to 60 minutes.

Promising gels were stained twice to develop some of the fainter spots. This was accompanied by the overdevelopment of major spots, but did lead to the visualization of protein spots that failed to show up after the first staining. Quantitation of the spots by means of densitomitry was not contemplated because of the ease of recognizing pattern changes visually, and because densitometry is inadequate in the analysis of very faint spots (Hochstrasser et al., 1986).

Washing of Gels To obtain a low background staining, gels were extensively washed. 20% ethanol prevents the diffusion of proteins out of the gel, and also assists in the removal of the low molecular weight components that increase background staining; these include Tris, glycine, sodium dodecyl sulphate, glycerol, carrier ampholytes, and sulfhydryl reagents viz., dithiothreitol and 2-mercaptoethanol (Guevara et al., 1982). Electrophoresis grade agarose was used to anneal the rod gels to the slab gels, since impurities in the agarose will contribute to a high background staining (Amess et al., 1985). At every stage of silver staining high purity water (MilliQ - Millipore), with a resistivity of 10 megaohm.cm., was used.

Some brands of ethanol were unsuitable, giving uneven development with a rapid, intensely black staining. The problem was traced to the presence of aldehyde in the ethanol which caused premature reduction of the silver, and its precipitation out of the solution, resulting in high surface staining. Ethanol supplied by Merck S.A. was found to be free of this problem.

Colour Formation The silver staining method of Guevara and co-workers (1982) gave spots that were predominantly brown, orange and yellow, with some black and reddish spots. Sammons and colleagues(1981) described a modified silver staining procedure for enhancing colour differences, with the view to exploring the possibility that protein subclasses may be identified by colour alone. This possibility has not been realized, however, but colour differences do appear to reflect differences in the amino acid composition of proteins. For example, Chuba and Palchaudhuri (1986) found that proteins without cysteine do not stain on a gel at all. They were not however, able to correlate colour with mole percentage of cysteine in various proteins, and there must be additional factors that have a bearing on colour formation. If silver ions do show more affinity for some amino acids than others, then that would explain the poor correlation of protein concentration with grain density between different proteins (Yuksel and Gracy, l985).

Mechanism Silver staining is initiated by the formation of a metal ion complex with available moieties on the protein, followed by the reduction of the complexed ionic silver to metallic silver. The electron-donating properties of the biochemical groups are not sufficient for the reduction of the silver ions, and all the silver staining procedures need either additional photo- or chemical-reduction agents to produce a metallic silver image (Merril and Pratt, 1986). In the method of Guevara and co-workers (1982), an alkaline pH is maintained at protein clusters by ionic retention of ammoniacal silver ions and hydroxyl ions. The electrons for the reduction of

ammoniacal silver ions to metallic silver is provided by the oxidation of formaldehyde to formic acid. Citrate is an anti-oxidant, and attenuates the rate of formaldehyde oxidation, thereby preventing the deposition of black "dust" on the surface and throughout the gel.

2.5.3 Fluorography following Reductive Methylation

The detection of radiolabelled proteins on a gel by means of an X-ray plate is an effective way of visualizing constituents at very low detection limits. In this study fluorography was attempted in order to achieve even greater sensitivity than silver staining. The advantage of fluorography is that an image may be developed for as long as necessary in order to visualize areas of the gel that contain a host of very faint spots. However, success was limited, and the difficulties that were encountered will be discussed below.

It was not practical to radiolabel proteins by utilizing the metabolic incorporation of labelled amino acids by the living animal. Feeding animals with labelled amino acids is prohibitively expensive due to the dilution of the sample by non-target tissue in the mother and the fetus. A more realistic strategy was to transfer the radiolabel by purely chemical means onto proteins that have been solubilized. Unfortunately, however, chemical labelling of proteins is less efficient than labelling by the utilization of metabolic processes, and entails the modification of the native protein.

Reductive methylation using cyanoborohydride is an established method for the transfer of radioactive alkyl moieties onto free amino groups of proteins (Means and Feeney, 1968). This reaction, which occurs in the presence of a reducing agent, takes place specifically at the E-amino group of lysyl residues, and the amino groups at the N-terminus of the peptide. As many as 8,5% of amino acids of proteins have lysyl

residues; reductive methylation has therefore the potential for the effective labelling of proteins.

The radiolabel 50mCi of tritiated sodium cyanoborohydride with a specific activity of 195,2mCi/mg was obtained from Amersham. The vial contained 0,2561 mg of tritiated material, and was diluted tenfold with non-tritiated sodium cyanoborohydride, giving a total of 2,561 mg. A stock solution of 6 mg/ml was made up by adding 426,8 ml of buffer to the vial. The buffer was 50 mM sodium phosphate pH 7,0. The stock was divided into aliquots and frozen at -20^{o}C.

The reaction. The carbon atom for the methylation reaction is derived from formaldehyde, while the reducing hydrogens are supplied by sodium cyanoborohydride:

The reaction of formaldehyde with a free amino group produces a Schiff base; this reaction passes through two cycles. The secondary amine is a minor product of the reaction, and is less stable than the tertiary amine, a dimethyl derivative (Means, 1977). The reaction was buffered with a phosphate buffer, but borate, EDTA, and Hepes buffers are also suitable (Jentoft and Dearborn, 1979). Ammonia ions, or primary and secondary amine buffers (like Tris) cannot be used because they undergo alkylation themselves.

Tissue solubilization Some protein solubilizing reagents inhibit the reductive methylation reaction (Jentoft and Dearborn, 1979). For example, sulfhydryl compounds form adducts with formaldehyde thus lowering the available reagent. There is no significant inhibition of the methylation reaction in the presence of 8M urea, but 1% sodium dodecyl sulphate has a marked effect on the transfer of the label onto protein. Inhibitory effects that are pronounced after two hours of reductive methylation are invariably much less so after 24 hours. To achieve efficient labelling at the same time as achieving efficient solubilization, it was necessary to have the methylation reaction over an extended period of time.

The solubilization fluid consisted of 8M urea, 0,5% sodium dodecyl sulphate and 5% 2-mercaptoethanol, used at 20 ul/mg of brain tissue. After homogenization and sonication (protein concentration 5mg/ml), the sample was diluted 1:5 with Na^+ phosphate buffer (pH 7,0). To 600 ul of sample was added 80 ul HCHO and 80 ul of sodium cyanoborohydride. The mixture was shaken overnight.

Removal of unreacted label. After reductive methylation all unreacted label had to be separated from the sample to prevent free label contaminating the gel. In addition, to achieve high resolution separation of proteins during isoelectric focussing, the sample had to be desalted. Two methods were attempted:

(a) Precipitation of proteins. Trichloroacetic acid is commonly used (for example, see Jones and Vidaver, 1981). The reaction mixture was brought up to 10% with ice-cold trichloroacetic acid, thoroughly mixed, and placed in an ice-water mixture for 10 minutes. After centrifugation at 10 000xg for 5 minutes, the supernatant was discarded and the pellet washed twice with ethanol: ether (1:1). However, resolubilization of the pellet was impossible, even after dispersal in a solubilizing solution with sonication. Resolubilizing strategies included the use of Lysis Buffer, Lysis Buffer plus 1% sodium dodecyl sulphate, and 9,5M urea plus CHAPS

([Cholamidopropyldimethylammonia]-1-propanesulphonate). The failure to achieve resolubilization was attributed to the formation of insoluble beta-sheets during precipitation (Prof. W Brandt, personal communication). The use of acetone in the place of trichloroacetic acid did not help.

(b) Dialysis is the most frequently used method for removing unreacted label and for desalting samples (see for example, MacKeen et al., 1979) and was utilized in the present study. Spectrapor membrane tubing (10 mm wide) was used, with a molecular weight cut-off of 12 000 - 14 000 daltons (pore size 4). The sample was dialised against distilled water for 12 hours at 4^{o}C. Once dialysis was complete, the contents of the membrane tubing was mixed with an equal volume of UKS solution (see section 2.2). The sample was then applied to the isoelectric focussing gel.

Fluorography After electrophoresis gels were stained with Coomassie blue, and extensively destained with 5% methanol and 7% acetic acid in water (to wash out all trichloroacetic acid). To impregnate the gels with a scintillator, the gels were immersed in Amplify (Amersham) for 30 minutes with constant agitation. The gels were removed and dried onto cellophane using a gel dryer. Once dry, the cellophane was pulled off and the dried gel was firmly pressed down on pre-flashed X-ray plate (Kodak X-omat) and clamped into position. The cartridge was kept at -20^{o}C for 7 days, after which the x-ray plate was developed.

The results, however, were disappointing, and fluorography as a means of visualization was not pursued. After fluorography even the major spots were slightly fuzzy, and some of the fainter spots were visible merely as smudges. The fuzziness may have been due to the fact that labelling involved protein modification, although the problem may have been due to shortcomings in the scintillator (Hames, 1981e). Silver staining gave superior spot quality and in addition was much quicker to perform and also cheaper than fluorography.

2.6 Storage and Documentation

Gels that were stained with Coomassie blue were dried on a commercially available gel-dryer (Hoefer). Although the gels were considerably larger than standard gels, the dimensions had been chosen to make the large gels compatible with the available gel dryer. Coomassie blue-stained gels were dried onto a stiff plastic backing (Cronar clear base, 0,1mm). Due to heat and pressure during the drying process, the acrylamide gels annealed to the plastic, forming a permanent bond. The stiff plastic prevented gels from curling up or deteriorating in any way, and also facilitated storage. Silver stained gels were not dried, because drying appeared to intensify the background stain. Silver stained gels were stored as wet gels, in 20% ethanol, at 2-4^{o}C. Gels could be stored in this manner for up to six months.

Important gels were photographed. Because of the large number of extremely faint spots, a conventional SLR camera with a 35 mm negative was not used. Instead, photography was done by specialist photographers (Creative Colour Laboratory (Pty) Ltd), using an extra-large negative (100x125 mm). It was felt that this arrangement did justice to the exceptional resolving power of the two-dimensional electrophoretic system.

2.7 Statistical Analysis

Differences between the offspring of control and treated females were tested by one of two methods. Differences in fetal weight and fetal brain weight were tested by the Student t-test. Differences in the frequencies of resorptions, intra-uterine deaths and malformations were tested by chi-squared analysis (Underhill, 1978). A 5% level of significance was chosen for all analysis.

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CHAPTER 3

MORPHOLOGIC ANOMALIES TO THE FETAL MOUSE, AND MOLECULAR CHANGES TO FETAL MOUSE BRAIN AFTER *IN VIVO* PHENYTOIN EXPOSURE

3.1 Introduction

3.1.1 The Fetal Hydantoin Syndrome

Phenytoin has been in use as an anticonvulsant since 1938, and during the past 50 years has been the single most effective drug for the treatment of most forms of epilepsy. The numerous adverse side effects of phenytoin have been carefully documented, but until recently there was little information about its teratogenic potential. Although phenytoin has been the drug of choice for pregnant epileptic women, it has been only over the last 15 years that the consensus has emerged that phenytoin, over and above the effects of the epilepsy itself, is potentially harmful to the fetus. There now appears to be little doubt that the ingestion of phenytoin during critical stages of pregnancy increases the risk to women of giving birth to a dysmorphogenic or retarded child.

Meadow, in 1968, was one of the first to postulate an association between birth abnormalities and the use of phenytoin during pregnancy. He noticed a high incidence of cleft lip and cleft palate in children born to epileptic mothers on phenytoin. Other abnormalities included unusual facies and skulls, congenital heart lesions, and abnormalities of the peripheral skeleton. In a more recent study, Hanson and Smith (1975) examined five unrelated infants born to epileptic women who had been treated with phenytoin during pregnancy. They found a common multisystem pattern of abnormalities which they named the Fetal Hydantoin Syndrome. This syndrome includes craniofacial abnormalities, limb defects, and growth and mental deficiency. Hanson and Smith carefully documented all dysmorphic features, including those that were not necessarily medically or cosmetically significant. The craniofacial features

include short nose with low nasal bridge, inner epicanthic folds, ptosis, strabismus, hypertelorism, low-set ears, wide mouths, wide fontanels, and prominent lips. Some subjects had a short or webbed neck with or without a low hairline (features also noted by Meadow), and coarse hair. There were skeletal abnormalities of the ribs, sternum or spine, and appendicular defects including hypoplasia of the nails and distal phalanges, finger-like thumb, abnormal palmar creases, and five or more digital arches.

Since these early reports, the existence or otherwise of the Fetal Hydantoin Syndrome has been carefully examined. Shapiro and colleagues (1977) judged that the association between hydantoin ingestion and birth abnormalities was unconvincing, and attributed the abnormalities described by Hanson and Smith to the epilepsy itself. In their reply, Hanson and Smith (1977) granted that epilepsy was a confounding factor, but reaffirmed their conviction that hydantoins are teratogenic. They were unable to find a single infant of an epileptic mother **not** treated with a hydantoin during pregnancy, that showed the full pattern of dysmorphogenesis which they refer to as the Fetal Hydantoin Syndrome.

Although there is still some debate about the teratogenicity of phenytoin, it is becoming increasingly clear that infants born to mothers with seizure disorders treated with phenytoin during pregnancy are at an increased risk for congenital defects (Hanson, 1986). The full pattern of abnormalities sufficient to be recognised as the Fetal Hydantoin Syndrome is probably present in no more than 5 to 10% of exposed infants (Hanson et al., 1976). However, careful examination (including radiographic examination) for indications of altered growth and morphogenesis, may identify subtle prenatal hydantoin effects in an additional 30% or more of cases (Hanson, 1986).

3.1.2 The Fetal Hydantoin Syndrome and the Brain.

There is not enough epidemiological data to decide whether phenytoin is a central nervous system teratogen. What information there is has been derived from the examination of infants born to women who took a number of different anticonvulsants during pregnancy. It is therefore impossible to separate out the teratogenic effects potentially due to phenytoin from those due to other anticonvulsants.

The rate of central nervous system malformations in infants exposed to anticonvulsants before birth is not high. Schardein (1983), from a composite study of 2168 infants whose mothers were on a number of different anticonvulsants during pregnancy, gives a malformation rate of 0,8%. This figure is in fact **lower** than the frequency of central nervous system abnormalities for children not exposed to anticonvulsants (1%). Schardein records a number of severe malformations among the 2168 infants in the composite study: one case of spina bifida, 5 of anencephaly, 4 of hydrocephaly, 4 of microcephaly, and 4 of myelomeningocele. Unfortunately it is impossible to decide from the data whether the nature or incidence of the malformations differ between infants exposed to anticonvulsants during pregnancy and those not exposed.

Many of the central nervous system malformations associated with infants exposed to anticonvulsants **in utero** may be secondary effects arising from malformations to the skull and the spine. Phenytoin has been strongly implicated in the abnormal development of the skull and the head. For example, Hanson and Smith (1975) included abnormal fontanels in the Fetal Hydantoin Syndrome, and Meadow (1968) noted abnormally shaped skulls. Lewin (1973) reported a malformed child born to a woman receiving both phenytoin and phenobarbitone during pregnancy, among other abnormalities the child had an encephalocele. It is therefore not impossible that cases

of exencephaly in fetuses exposed to phenytoin **in utero** arise from a prolonged delay in the closure of the skull.

Microencephaly, accompanied by mental retardation, was recorded by McIntyre (1966) in two infants whose mothers had taken a hydantoin and another anticonvulsant in the 1st and 2nd trimesters. Likewise, Rating and Jager (1980) found postnatal microencephaly in children whose mothers had used phenytoin and phenobarbitone during pregnancy. On the basis of only two reports it is impossible to decide whether the ingestion of phenytoin on its own during critical stages of gestation is likely to increase the frequency of microencephaly in the offspring. It is also unclear whether microencephaly in children exposed **in utero** to anticonvulsants results primarily from growth suppression of the brain itself, or secondarily from growth suppression of the skull. Since reduced head size has been found in infants exposed **in utero** to anticonvulsants, microencephaly may be a secondary outcome to a suppression of the development of the bones of the head.

Mental deficiency is a feature of the Fetal Hydantoin Syndrome, and includes both delayed motor development and intellectual impairment (Hanson and Smith, 1975). In addition, adaptive and personal skills are delayed (Seeler et al., 1979). Behavioural and intellectual impairment in these children suggest that phenytoin may have subtle teratogenic effects on the brain itself; the more catastrophic brain abnormalities may result from phenytoin effects on bone development.

3.1.3 Phenytoin and the Fetal Brain : Experimental Approaches

Literature reports dealing with the effects of phenytoin exposure on fetal neural tissue cover a range of experimental techniques, and include, **inter alia**, tissue culture, macroscopic anatomical studies, and behavioural studies.

Primary dissociated cell cultures from the cerebral cortex of 16-day fetal mice were used by Swaiman and co-workers (1982) to investigate the teratogenicity of phenytoin. After 7 days of exposure to phenytoin (15-50 µg/ml) there was a significant decrease in the number of neurones. Blank and co-workers (1982) found that phenytoin, when applied to developing neonatal mouse cerebellar cultures in concentrations from 9 to 46 µg/ml, induced cerebellar cortical degeneration in a dose-dependent manner, with Purkinje cells the most susceptible.

Using mouse embryo cultures, Bruckner and co-workers (1983) studied aspects of early embryogenesis. Embryos with one to seven somites were exposed **in vitro** to 15 to 135 µg/ml of phenytoin for up to 42 hours; a dose-dependent increase in the frequency of abnormal embryos was found. Abnormalities included open neural tubes in the cranial regions and craniofacial deformities. A defect in the normal closure of the neural tube is considered to be a major factor in the development of exencephaly (Bruckner et al., 1983). These findings support the suggestion (made in section 3.1.2) that some of the severe brain abnormalities may result from closure defects.

Hicks and co-workers (1983) found that phenytoin administered to fetal mice on day 10 post conception, leads to altered DNA and protein synthesis in primary palates, which may explain the increased occurrence of cleft lip and cleft palate in fetuses exposed to phenytoin **in utero**. The same results were obtained for DNA and protein synthesis in limb buds. Interference with embryonic macromolecular synthesis by phenytoin may account for other teratogenic effects, such as open neural tubes and dysmorphology of the cranium.

Flint and Orton (1984), in their "teratogen assay", exposed cultured cells from rat embryo midbrain and limb buds to a range of substances, including phenytoin. Teratogenic potential was judged from the extent to which differentiation of embryo

midbrain cells was inhibited. Phenytoin strongly inhibited cell differentiation. This inhibition took place at concentrations lower than those causing cytotoxicity - which gives a convenient distinction between a teratogenic and cytotoxic manifestation.

Morphologic examinations of the fetal brain after **in vivo** phenytoin exposure have confirmed that phenytoin may be a central nervous system teratogen. Sullivan and MacElhatton (1977), as part of a detailed anatomical examination of fetal mice after **in vivo** exposure to anticonvulsants, found that phenytoin exposure was linked to increased incidence of exencephaly and enlarged cerebral ventricles. Harbison and Becker (1969) found, among other abnormalities, hydrocephalus and exencephaly (infrequently). Beyond suggesting that phenytoin is in fact a central nervous system teratogen, this experimental approach has not proved to be particularly fruitful. One reason is that the brain, although highly complex, has few external features that may be used as parameters in a teratology study.

Behavioural deficits may be extensively studied in young or adult animals that have been exposed to teratogens pre-natally. For example, Mullenix and co-workers (1983) detected behavioural changes in rats that were exposed to phenytoin **in utero**. From a study of two different parameters - performance in a residential maze, and a variety of motor acts - it appears that prenatal phenytoin exposure may result in animals maintaining immature activity levels into adulthood.

The results of these experiments show that a variety of changes may be induced in fetal neural tissue, obtained from the mouse and the rat, following phenytoin exposure. In the present study molecular manifestations of phenytoin teratogenicity were sought, since it was hoped that these would provide an insight at a fundamental level into the events that lead to injury to the fetal central nervous system.

3.1.4 Some Possible Mechanisms of Phenytoin Teratogenicity

As with thalidomide, the mechanisms of phenytoin teratogenicity remain unknown, although a number of hypotheses have been advanced.

The so-called folic acid antagonism hypothesis arose from the observation that folic acid levels are lower in the blood and the spinal fluid, of people on phenytoin medication (Berg et al., 1988). Folic acid is of critical importance as a co-factor in many metabolic processes. According to Reynolds (1973), subnormal values of folic acid occur in 91% of women taking anticonvulsants and they may be even further lowered during pregnancy. Several folic acid antagonists are known human teratogens (Schardein, 1976). In some species of experimental animals folic acid antagonists and analogues readily induce congenital defects (Schardein, 1976). For example, rodents kept on a folic acid-deficient diet or exposed to folic acid antagonists will produce abnormal offspring (Schardein, 1976). There is therefore a definite association between the reduction by phenytoin of folic acid levels and a teratogenic effect.

Various experiments have shown however, that the link between phenytoin induced folic acid depletion and a teratogenic outcome is tenuous. Netzloff and Rennert (1976) measured oxygen consumption along with folic acid levels in mouse embryonic cells following treatment with phenytoin. Both variables were reduced, compared with levels in the controls, and similar findings followed treatment with the folic acid antagonist, 9-methylpteroylglutamic acid. Moreover, experiments in which there was concurrent administration of folic acid plus phenytoin have been inconclusive (Kernis et al., 1973), suggesting the absence of a direct relationship between folic acid and phenytoin in relation to teratogenicity. Possibly phenytoin teratogenicity is mediated by effects on polyamine levels (via ornithine decarboxylase), and the folic acid-antagonist effect is secondary (Buehler and Smith, 1979).

Some findings have implicated the epoxide metabolite of phenytoin in the mechanism of teratogenicity (Blake and Fallinger, 1976). It has been suggested that the reactive epoxide binds covalently to gestational tissue, thereby disrupting macromolecular functioning during critical periods of embryonic development (Spielberg et al., 1981). It is not possible to test this hypothesis directly, since the metabolite has yet to be isolated in sufficient quantities to be available for **in vivo** studies. The fact that some infants escape all teratogenic manifestations, despite prolonged **in utero** phenytoin exposure, may be due to a genetically determined ability of some fetuses to cope with harmful metabolites (Millicovsky and Johnson, 1981), either by the low production of the suspected harmful epoxide, or by its rapid excretion.

3.2 Results

3.2.1 Drug Administration

Phenytoin sodium (B.P.) (Lennon Ltd) was suspended in distilled water and the pH adjusted to 7,6 using phosphoric acid (Patsalos and Wiggins, 1982). The drug was administered by gastric intubation (see Section 2.1.4). The suspension was made up freshly on the day of administration. Controls received distilled water (pH adjusted to 7,6)

It was essential that the drug was administered during the critical period of teratogenic susceptibility. In the mouse, the development of the brain as an organ begins at approximately day $8^1/_2$ post conception (p.c.), with the development of somites and neuromeres (Rugh, 1968). However, it is between day $8^1/_2$ and day $9^1/_2$ p.c. that organogenesis is greatly accelerated, and according to Robert Rugh, "the most devastating congenital anomalies can be produced in embryos subjected at this time (day $8^1/_2$ to day 9 p.c.) to various types of trauma" (Rugh, 1968). On day 9 p.c. the

neural tube has largely closed, and the entire nervous system is undergoing cellular differentiation. It was therefore decided to administer phenytoin as single dosages on days $8^{1}/_{2}$ and $9^{1}/_{2}$ p.c. Both days fell within the period of greatest teratogenic susceptibility, and it was hoped that phenytoin administration on day $8^{1}/_{2}$ and day $9^{1}/_{2}$ p.c. would produce markedly different teratogenic outcomes, due to the profound and rapid developmental changes taking place within the fetal brain during this period.

It was also essential to select the dosage of phenytoin very carefully. On the one hand, too high a dosage causes maternal toxicity; on the other hand, enough drug had to be administered to show up any potential teratogenicity. The literature is not helpful because of the wide variety of experimental protocols that are described. Not only have several different strains of mice been used in experimental teratology, but a number of different routes of administration as well. Some protocols call for the daily administration of the teratogen over the entire course of the pregnancy (for example, Finnell 1981), and others for two or three (or more) administrations on consecutive days during the pregnancy (for example, Hanson and Hodes 1983).

No single published protocol was found to be suitable, and an empirical approach was adopted to determine the optimal dosage. A toxicity study was done to establish the threshold of maternal toxicity. Daily administrations of 300 mg phenytoin per kg. body weight to non-pregnant females produced restless, aggressive behaviour, and because the animals were clearly under stress this regimen was not continued. When the daily dosage was reduced to 200 mg/kg phenytoin, no overt signs of toxicity resulted apart from a small decrease in weight (Table 3.1). The weight of control animals remained unchanged. The experiment was not continued beyond the sixth day due to increased resistance from the animals to intubation.

Table 3.1

Adult mouse body weight after 6 consecutive daily administrations of 200 mg/kg phenytoin

WEIGHT IN GRAMS		
Day 0	Day 6	Change(g)
22.69	20.92	-1.77
20.34	19.71	-0.63
21.17	20.15	-1.02
20.08	19.15	-0.93
21.92	20.39	-1.53
21.36	21.34	-0.02
21.80	20.43	-1.37
21.15	19.57	-1.58
23.49	22.18	-1.31
21.02	19.88	-1.14
22.67	21.75	-0.92
20.73	19.61	-1.12

It was judged that a dosage of 200 mg/kg, administered on one day rather than on six consecutive days, was likely to fall below the threshold of maternal toxicity and yet be high enough to produce a teratogenic outcome.

3.2.2 Effects of Acute *in vivo* Phenytoin Exposure on Fetal Weight and Fetal Brain Weight

Mean fetal weight of $18^{1}/_{2}$ day fetuses that had been exposed to phenytoin (200 mg/kg) on gestational day $8^{1}/_{2}$ was significantly less ($P < 0,01$) than unexposed fetuses. There was also a significant decrease in fetal brain weight ($P < 0,001$). These findings are summarized in Table 3.2.

There was likewise a significant reduction in the mean weight of $18^{1}/_{2}$ day fetuses that had been exposed to phenytoin (200 mg/kg) on day $9^{1}/_{2}$ ($P < 0,02$). Fetal brain weight, however, was not significantly reduced (see Table 3.3).

These results are in accordance with the findings of earlier studies that dealt with the effects of phenytoin on the mouse fetus. For example, Harbison and Becker (1969) reported a statistically significant dose-related reduction in fetal body weight after single treatments of phenytoin on gestational days 9 to 15. Unexpectedly, Harbison and Becker found that on day 8 only the lowest of four dosages of phenytoin gave a significant result, while one day later, on day 9, the highest dosage resulted in the greatest weight reduction.

Hanson and Hodes (1983) reported a significant reduction in the mean weight of mouse fetuses from the ICR, A/J, (B6A)F_2 and C_3H/He strains, after exposure to 75 mg/kg of phenytoin on gestational days 10, 11 and 12, as compared to controls. Finnell (1981) also analysed the effects of phenytoin on the fetuses from a number of

different mouse strains, and found that fetal weights decreased significantly in all the strains as the dosage of phenytoin increased.

Fritz and colleagues (1976), found a significant decrease in the mean fetal weight of MNRI fetuses after treatment with 100 mg/kg and 170 mg/kg phenytoin on gestational days 6 to 15. Gibson and Becker (1968) observed a significant reduction in fetal weight of Swiss-Webster and A/J mice after phenytoin exposure (50 mg/kg) on gestational days 11,12 and 13, but not on gestational days 7,8 and 9. It appears from the data supplied by Gibson and Becker that phenytoin is not teratogenic to either Swiss-Webster or A/J mice when administered during early embryogenesis (days 7, 8 and 9). Only Sullivan and McElhatton (1977) found no significant difference in fetal weight between the phenytoin treated mice and untreated controls.

These findings cannot be directly compared to the data on fetal weights presented Tables 3.2 and 3.3, due to both the differences in strain of mice used and also differences in methodolgy. Clearly, phenytoin induces fetal weight reduction under a diversity of experimental conditions. In terms of mechanism, the action of phenytoin in causing a decrease in fetal weight may be non-specific, and the decrease in weight may reflect an embryotoxic rather than a teratogenic manifestation.

The statistically significant reduction in fetal brain weight after phenytoin exposure on day $8^{1}/_{2}$ is to be expected in view of the corresponding reduction in fetal body weight. The absence of a statistically significant reduction in brain weight of fetuses exposed to phenytoin on day $9^{1}/_{2}$ is difficult to explain in the light of a significant reduction in body weight, and there are no available literature reports on fetal brain weights after phenytoin exposure to put this finding into suitable context.

Table 3.2 Summary of results of Phenytoin Exposure at 200 mg/kg on Gestational Day $8^{1}/_{2}$

	Test (n=27)	Control (n=13)	P
Total conceptions	38	18	ns
Resorptions	5	3	ns
Intra-uterine deaths	3	1	ns
Abnormalities	3 2 anencephaly 1 exomphalos	1 1 exomphalos	ns
Mean fetal weight (g)	1,0472 (1,0044-1,0900)	1,1347 (1,0879-1,1815)	p<0,001
Mean fetal brain weight (mg)	70,01 (68,14; 71,88)	76,85 (72,69; 81,01)	p<0.001

(Figures in brackets are the 95% confidence interval for the population mean).
ns = no significance

Table 3.3 Summary of results of Phenytoin Exposure at 200 mg/kg on Gestational Day $9^{1}/_{2}$

	Test (n=22)	Control (n=14)	P
Total conceptions	31	17	ns
Resorptions	2	2	ns
Intra-uterine deaths	5	1	ns
Abnormalities	2 1 anencephaly 1 exomphalos	-	ns
Mean Fetal weight (g)	1,0350 (0,9866;1,0834)	1,1031 (1,0510; 1,1552)	p <0,02
Mean fetal brain weight (mg)	71,86 (68,90; 74,82)	74,85 (71,85; 77,85)	ns

(Figures in brackets are the 95% confidence intervals for the population mean)
ns = no significance

3.2.3 Morphologic Abnormalities to the Fetus after *in vivo* Phenytoin Exposure

In this study very few external anomalies were found (see Tables 3.2 and 3.3). There was no significant difference in the numbers of abnormalities between test and control animals. Anencephaly and exomphalos were the only abnormalities recorded in both treated and untreated mice. In literature reports the incidence of these abnormalities, and any other potentially lethal abnormalities, are extremely low. For example, Fritz and coworkers (1976), in an analysis of a total of 879 fetuses that had been exposed to phenytoin during gestation, recorded only one major malformation (spina bifida). Hanson and Hodes (1983) mentioned anencephaly, but did not give any incidence of frequency. Harbison and Becker (1969) obtained a 4% incidence of exencephaly in fetuses that had been exposed to a single dose of phenytoin (150 mg/kg) on gestational day 9.

In the present study no examples of cleft lip, cleft palate, open eyes or abnormal head shape were found. This is an unexpected result in view of the extensive reports of orofacial abnormalities in the literature. An explanation for this apparent discrepancy may be found in the protocols for drug administration used to obtain orofacial abnormalities. Harbison and Becker (1969) obtained orofacial abnormalities in fetuses that were exposed to phenytoin on days 11, 12, 13 and 14, which is considerably later than the time of phenytoin exposure in the present study. Harbison and Becker (1974) obtained an extremely high rate (85%) of orofacial anomalies after phenytoin exposure (at 87,5 mg/kg) on days 11, 12 and 13. On day 10, at almost double the dosage of phenytoin, the rate of orofacial anomalies was much less, and although the rate was still very high (42%), the corresponding resorption rate (67%) suggests that the dosage was excessive. Sullivan and McElhatton (1977) obtained a 5,1% frequency of full-length cleft palate; their schedule of drug administration called for daily dosages of phenytoin on days 6 to 16, and conceivably the abnormal palates could have arisen

as a result of susceptibility to phenytoin teratogenicity during the latter half of the dosing period. Susceptibility to orofacial abnormalities appears to be strain related. For example, Hanson and Hodes (1983) administered phenytoin on each of gestational days 10, 11 and 12, but obtained only one orofacial abnormality out of 131 C_3H/He fetuses; the A/J and AB6F strains produced a high percentage of orofacial abnormalities. These findings were to some extent confirmed by Gibson and Becker (1968), who obtained a 15,2% incidence of cleft palate in Swiss-Webster mice, and a 30,8% incidence of cleft palate in A/J mice, when phenytoin was administered during late embryogenesis (gestational days 11, 12 and 13).

Visual examination of the fetuses revealed no limb abnormalities. Abnormal digits are frequently the result of **in vivo** phenytoin exposure, and abnormalities include ectrodactyly, syndactyly (Harbison and Becker, 1969), digital hypoplasia (Finnell, 1981), and retarded ossification of the digits (Sullivan and McElhatton, 1977). The mouse fetus appears to be susceptible to digit abnormalities when phenytoin is administered in the mid-gestational period, since Harbison and Becker (1969) obtained a 9% frequency of ectrodactyly in the offspring of Swiss-Webster mice that were exposed to phenytoin on the ninth day of gestation. The absence of digital anomalies in the present study may be accounted for by the C_3H strain being relatively resistant to this kind of injury.

No morphologic abnormalities of the fetal brain were noticed. Harbison and Becker (1969) reported the symmetrical enlargement of the ventricles of the fetal mouse brain after a single administration of phenytoin (150 mg/kg) on the tenth day of gestation. This kind of abnormality, however, can only be detected by the preparation of ultrathin sections of brain tissue and their examination under a microscope. Similar techniques were presumably available to Finnell (1981), who reported immaturely developed cerebral ventricles in fetuses exposed to phenytoin, and also to Sullivan and

McElhatton (1977), who found enlarged cerebral ventricles after **in vivo** administration of phenytoin to fetal mice.

3.2.4 Effect of *in vivo* Phenytoin Exposure on Resorption Rate and Rate of Intra-uterine Death

The rate of fetal mortality as a result of phenytoin exposure was not significantly different from the mortality rate among unexposed fetuses (see Tables 3.2 and 3.3). The implication is that phenytoin at 200 mg/kg is not potently embryotoxic, although the reduced fetal weight among exposed fetuses suggests that there is some degree of embryotoxicity.

In most of the reports on the effects of teratogenic dosages of phenytoin on fetal mice, there is no increase in fetal mortality among treated fetuses, as compared to untreated fetuses. Gibson and Becker (1968) found that phenytoin at 50 mg/kg did not significantly increase the resorption rate in Swiss-Webster mice, although in a later study using a similar methodology, but a higher dosage of phenytoin (150 mg/kg), a significant difference in resorption rates between treated and untreated mice was obtained (Harbison and Becker, 1969). Sullivan and McElhatton (1977), using CDI mice, found no significant difference in resorption rates in any of the phenytoin treated groups compared with controls.

Some strains of mice, including the C_3H/He strain used in this study, have a high resorption rate among the untreated animals. In this study the spontaneous resorption rate varied between 12% and 17%, while Hanson and Hodes (1983) report a rate of 17% for C_3H/He mice in this study. Sullivan and McElhatton (1977) gave a 11,8% rate of dead and resorbed fetuses among the untreated animals in the strain they used (CDI). The A/J strain also has a significant spontaneous resorption rate, although in

this strain phenytoin does have a significant effect on the resorption rate, with phenytoin administration on days 11, 12 and 13 producing a resorption rate of 35%, compared with 14,3% for untreated animals (Gibson and Becker, 1968).

The resorption rate appears to be dependent on the route of phenytoin administration. Harbison and Becker (1969) found that a single intra-peritoneal injection of 150mg/kg phenytoin on the tenth gestational day resulted in approximately 80% resorptions, while oral and subcutaneous administrations resulted in significantly fewer resorptions (no data given).

It is difficult to review the frequency of intrauterine deaths (as distinct from resorptions) due to ambiguities in the literature. Many studies do not distinguish between resorptions and intrauterine deaths. Fritz and coworkers (1975) usefully distinguished between embryonic resorptions (those deaths which they estimate to have occurred before day 14), and fetal resorptions (deaths after day 14). Gibson and Becker (1968) distinguished between resorptions occurring in early embryogenesis (drug administration on days 7, 8, 9) and resorptions occurring in late embryogenesis (drug administration on days 11, 12 and 13). Harbison and Becker (1969) administered phenytoin up to day 15, and included all deaths at laporotomy (day 19) under "resorptions", while in the present study fetal deaths that occurred as early as day 12, were considered to be intrauterine deaths.

3.2.5 Changes to the Protein Complement of Fetal Brain

Although phenytoin-associated fetal weight reduction, resorptions, and morphological abnormalities, have been intensively investigated in mice, comparatively little is known about the molecular events that accompany these changes. Part of the aim of this study, therefore, was to analyse some of the molecular events that occur in fetal brain

after phenytoin exposure, with the view to obtaining some insight into the mechanism of action of phenytoin.

Two-dimensional gel electrophoresis was chosen as a potentially suitable technique for approaching this task. The technique has the advantage of displaying a mixture of a large number of different proteins; drug effects on a particular tissue may produce alterations in the pattern of proteins, and these alterations may then become the starting point of an investigation into the nature and cause of the drug effect. Such an experimental approach was thought to be particularly advantageous to use on the brain, in view of the great difficulty otherwise of finding a starting point in so complex an organ.

A representative two-dimensional polyacrylamide gel that was generated from fetal mouse brain and electrophoresed under the conditions described in Chapter Two is shown in Figure 3.1. The ordinate and the abscissa are marked for molecular weight and isoelectric point respectively. Only proteins in a molecular weight range approximately of 14 to 120 kd, and a limited pH range of 4,8 to 7,0, were separated. Up to 1250 proteins were discernible on a freshly stained gel. The actual gels measured approximately 20x24 cm; the photographs therefore represent a size reduction of about 15%.

A number of changes were found in a comparison of brain protein patterns obtained from fetuses that had been exposed to phenytoin (200 mg/kg) on day $8^{1}/_{2}$ and day $9^{1}/_{2}$, and untreated controls. Five proteins in all underwent pronounced modifications, three of them in concentration, and two in position. These changes are significant because they appear to represent an explicit response by the fetal brain tissue to phenytoin exposure, and indirectly provide the means for an eventual analysis of this response.

Figure 3.1 Silver stained gel electrophoretic protein pattern of $18^{1}/_{2}$ day fetal mouse brain (control)

Figure 3.2 Silver stained gel electrophoretic protein pattern of $18^{1}/_{2}$ day fetal mouse brain (phenytoin at 200 mg/kg administered on day $8^{1}/_{2}$ p.c.)

Figure 3.3 Silver stained gel electrophoretic protein pattern of $18^{1}/_{2}$ day fetal mouse brain (phenytoin at 200 mg/kg administered on day $9^{1}/_{2}$ p.c.)

The unequivocal changes are indicated and numbered on Figure 3.1:

Protein 1 (approximate molecular weight 80 kd, pI 4.9). This protein is apparently absent from fetal brain tissue that had been exposed to phenytoin (see Figures 3.2 and 3.3). In fact, protein 1 was present in about 28% (7 out of 24) of gels that were generated from brain tissue of fetuses exposed to phenytoin at gestational day $8^{1}/_{2}$; these gels displayed protein 1 at intermediate intensities. Fetuses exposed at day $9^{1}/_{2}$ did not express this protein at all.

Protein 2 (molecular weight 25 kd, pI 5.1) is absent from the control gels, but is present on all the gels derived from fetal brain tissue that had been exposed to phenytoin (see Figures 3.2 and 3.3). The spot of comparable intensity directly above protein 2 may be used as a yardstick to assess this unambiguous change. In Figure 3.3 a vertical smear (representing an area of protein overloading) lies across protein 2, raising the possibility that protein 2 is an artifact of protein overloading. However, in Figure 3.2 protein 2 is clearly present without being bisected by the band of overloading.

Protein 3 (molecular weight 55 kd, pI 5.3) was too faint to be detected by means of Coomassie staining. The absence of this protein spot from Figures 3.2 and 3.3 is not due to unsatisfactory staining, since the protein patterns on the gels represented by Figures 3.2 and 3.3 are in fact more intensely stained than in the control gel.

Proteins 4 and 5 (molecular weight 35 kd, pI 6.0 and 6.1) show no change in concentration between gels derived from phenytoin treated animals, and gels derived from control animals. However, phenytoin administration on day $9^{1}/_{2}$ had the effect of shifting the pI of both these proteins towards a higher pH - a shift of approximately 0,1 pH units. The molecular weight of proteins 4 and 5 remained unchanged on all the

gels. The fuzziness of these two spots in Figure 3.3 was not present on other equivalent gels. The possibility was considered that protein 5 did not undergo a shift in position, but that protein 4 had a shift of 0,2 pH units, taking it to the other side of protein 5. However, it is thought more likely that both proteins had a positional shift, because protein 5 has a slightly smaller molecular weight than protein 4, and the pattern of the left spot being slightly further down (in Figure 3.2) is preserved in Figure 3.3.

All other differences noticeable between Figures 3.1, 3.2 and 3.3 were not consistently present in the other equivalent gels, and the presence of these other, variable, differences was attributed to non-uniformities in the staining process, and also to non-uniformities in the casting and running of the gels. The difficulties in achieving reproducibility hampered any investigation of subtle quantitative differences between proteins.

None of the five proteins of interest has been identified. It is impossible at this stage to say to which classes of proteins they belong (enzymes, glycoproteins, structural proteins etc), or to assign them to subcellular fractions. Knowing this, and knowing their relationship to one another, is likely to go a long way towards establishing their intracellular function, and their role in the modulation by phenytoin of protein expression.

The mechanism responsible for the elevation or reduction in the concentration of these proteins (or the shift in position) cannot be deduced without knowing more about them. The complete absence of protein 1 from fetal brain tissue after phenytoin exposure on day $9^{1}/_{2}$ is presumably due to the inhibition of its expression. That inhibition could have occurred at the level of the gene, during the translation of RNA into protein, or during post-translational processing. The agent responsible for the inhibition may have been phenytoin, a metabolite of phenytoin, or it may have followed

on from another, more primary event induced by phenytoin. The apparent inhibition of protein 3 may have occurred by a similar mechanism.

The presence of protein 1 in 7 out of 24 samples obtained from fetuses exposed to phenytoin on day $8^{1}/_{2}$ presents additional questions. The total absence of this protein one day later in gestation suggests that this inhibition may be developmentally regulated, a suggestion that can be verified by exposing the fetus to phenytoin on day $7^{1}/_{2}$, and also on days following day $9^{1}/_{2}$.

That there is also an apparent induction of a brain protein (protein 2) as a result of exposing the fetus to phenytoin, suggests that the intracellular actions of phenytoin are diverse and complex. The types of cells in which these deletions and deductions occurred are not necessarily the same, and in view of the great cellular variety in the brain, it is unlikely that they are the same. Protein 2 may conceivably be one of the drug metabolizing enzymes, although not cytochrome P-450, which, although it is present in the mouse brain and is susceptible to induction by phenytoin (Volk et al., 1988), has a moderately basic pI (Vlasuk and Walz, 1980), which is incompatible with the pI of 5,1 of protein 2. The induction of protein 2 is unlikely to be due to gene amplifications, since gene amplification is normally the result of chronic drug treatment. Nor is protein 2 likely to be one of the so-called "heat shock" proteins, since they are synthesized in minute quantities. Establishing the functional characteristics of this protein is likely to cast new light on the way in which phenytoin modulates cellular function. However, it may be that these protein changes are merely the more conspicuous features of widespread cellular perturbations, and that the primary actions of phenytoin may continue to remain occluded by the intricacies of cellular biology.

Phenytoin administration on gestational day $9^{1}/_{2}$ (but not day $8^{1}/_{2}$) produced a shift in the isoelectric points of proteins 4 and 5. Both proteins underwent the same shift in

pI, suggesting that phenytoin induced the same quantitative modification to both proteins. The fact that proteins 4 and 5 have similar molecular weights, and have pIs so close together, plus the observation that phenytoin appears to produce a near identical shift in the pI of both proteins, suggests that these proteins may be structurally closely related. If this is so then any phenytoin-induced change would affect both proteins equally. It is possible that proteins 4 and 5 are so similar because they were originally the same protein, and that during the sample solubilization process they were subjected to modifications. If roughly 50% of the protein molecules were modified (for instance, by the removal of a charged side-chain), then the molecular weight would remain virtually unchanged, but there would be a significant difference in pI. Another possibility is that proteins 4 and 5 are the products of two genes that diverged very recently in evolutionary terms.

Phenytoin is recognized as having widespread protein modulatory effects in the brain and in other organs. Hicks and coworkers (1983) reported that in the primary palates of phenytoin-treated embryos, DNA synthesis was decreased and protein synthesis was increased by 2,2 times, compared with control primary palates. A general effect such as this may presumably account for the reduction in fetal body weight and brain weight found in this study. Numerous reports have dealt with the effects of phenytoin on protein synthesis in mature animals. Yanagihara and Hamberger (1971) found that phenytoin inhibited the incorporation of tritiated leucine into proteins in the cerebral cortex of the rat. A more recent report dealt with the phenytoin-associated decrease in the phosphorylation level of (Na^+, K^+)- ATPase in mouse brain (Guillaume et al., 1983). In rats exposed to high dosages of phenytoin, histologically detectable changes and degenerative changes in the cerebellum have been reported (Kokenge et al., 1965).

Because of these diverse and widespread effects of phenytoin, it is important to ask if the protein changes reported in this study are necessarily teratogenic manifestations. Teratogenic effects, by definition, result in gross or subtle abnormalities close to or at birth. While the present protein changes are evidently not part of the developmental programme of the fetal mouse brain, they should not for that reason be labelled abnormal, and there is no evidence that the fetuses are at any disadvantage for having a different protein complement. The protein changes induced by phenytoin in this study appear to be uniform and general, in that they were experienced by all the fetuses to the same extent (except for protein 1). Phenytoin-related teratogenic effects, however, seem randomly to affect some fetuses in a litter, but not others (at dosages which give a moderate resorption rate). It seems, therefore, that it cannot be taken for granted that we have a teratogenic effect, and that alternative hypotheses must be considered:

(a) A developmental toxic manifestation. The modified protein expression is possibly the result of a toxic insult by phenytoin on certain target cells within the developing brain, without concomitant necrosis or a grossly modified developmental programme. This hypothesis is supported, in the present study, by a significant reduction in fetal weight, without a significant increase in the resorption or malformation rate. The induction and deletion of specific proteins may be due to a cellular "stress response", a hypothetical homeostatic mechanism whereby the tissue produces new proteins and eliminates others in order to minimize the toxic effects of the drug.

(b) A "pre-teratogenic" manifestation. The altered protein pattern may represent one of the inceptive steps in the complex process that eventually results in a full-blown teratogenic manifestation. This postulate arises out of the apparent contradiction of having phenytoin-induced molecular changes that are universal (occurs in every

fetus), while the reported pattern of phenytoin-induced morphological abnormalities is random (affects some fetuses in the litter, but not others). In this scenario, the early step is not sufficient to produce a teratogenic outcome, and must be succeeded by one or more additional steps before the fetus suffers a developmental aberration. Increasing the dosage of phenytoin (in this case, beyond 200 mg/kg) may increase the likelihood of subsequent steps taking place. Genetic factors in individual fetuses may determine why, at a given dosage of phenytoin, some fetuses pass through the hypothetical multistep process leading to morphological defects, while others do not.

(c) The altered proteins have no developmental role. This hypothesis proposes that changes to the protein complement may occur in a variety of fetal and adult tissues after exposure to phenytoin. This may be a general response to a particular environmental insult, and the mechanism of this hypothetical response can be expected to be quite different from the mechanism responsible for phenytoin teratogenicity. The phenytoin-induced dephosphorylation of (Na^+, K^+)-ATPase in adult mouse brain (Guillaume et al., 1986) may, for example, also take place in fetal mouse brain without producing a developmental outcome. This hypothesis can easily be tested by examining the protein patterns obtained from various tissues, both fetal and adult, of phenytoin-exposed animals. The fact that these protein changes are present 10 days after the insult argues against this hypothesis, because there was likely to have been adequate time for repair to occur.

3.3 Conclusions

In this study phenytoin at 200 mg/kg produced a significant reduction in fetal weight and brain weight when exposure occurred on gestational day $8^{1}/_{2}$, and a significant reduction in fetal weight only when exposure took place on day $9^{1}/_{2}$.

No significant increase in the rate of resorptions or of abnormalities was found.

Two-dimensional gel electrophoresis of total brain proteins revealed a phenytoin-associated change in the protein pattern; alterations included two deletions, an induction and two changes in pI. The proteins that underwent modulation could not be identified, and their function could not be determined.

To my knowledge, this is the first teratological study in which two-dimensional gel electrophoresis was used to show protein changes induced in the fetal mouse brain by phenytoin. However, the protein changes are not necessarily teratogenic manifestations, because it cannot be assumed that they are associated with injury to the fetal brain. The changes can only be characterized as teratogenic once they have been correlated with histological, morphological or behavioural abnormalities.

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CHAPTER 4

EFFECTS OF *IN VIVO* DISULFIRAM EXPOSURE ON FETAL MOUSE BRAIN WEIGHT AND BRAIN PROTEIN COMPLEMENT

4.1 Introduction

4.1.1 Pharmacological Properties of Disulfiram

Disulfiram has been used for years in the treatment of chronic alcoholism. The drug is thought to act through the inhibition of aldehyde dehydrogenase and alcohol dehydrogenase (Carper et al., 1987). The failure by a person to abstain from ethanol intake while under treatment with disulfiram results in a rise in serum acetaldehyde, leading to a wide variety of unpleasant systemic effects, including nausea, vomiting, headache, sweating and thirst. The enhancement of the adverse effects of ethanol is therefore the basis of disulfiram aversion therapy. However, the use of disulfiram as a therapeutic agent has been curtailed in recent years because the concomitant ingestion of disulfiram and ethanol by sensitive individuals may result in severe cardiac effects, convulsions, and death (Goodman and Gilman, 1985).

Disulfiram, when taken by itself, is relatively nontoxic. Minor side-effects include drowsiness, headache, memory impairment, decreased libido, gastro-intestinal symptoms, halitosis, and skin rashes (Bradley and Hewer, 1966). Kump and colleagues (1979) reported a disulfiram-induced acute organic brain syndrome that was reversed after discontinuance of the drug. Cases of peripheral neuropathy in persons on long-term disulfiram therapy have been reported by Bradley and Hewer (1966), and Watson (1980). Histological investigations on material obtained from nerve biopsies by Watson (1980), revealed dissolution of axoplasmic organelles as well as collapsed and distorted myelin sheaths. All of the patients examined by

Watson had stopped taking alcohol before the onset of disulfiram-associated symptoms, making the possibility of an alcohol-associated neuropathy improbable.

4.1.2 Is Disulfiram a Central Nervous System Teratogen?

Case reports suggest that disulfiram has some teratogenic potential. For example, Nora and co-workers (1977) reported on two infants with severe limb reduction and other skeletal anomalies, whose mothers had been maintained on a disulfiram sobriety regimen during the first trimester of pregnancy. A prospective cohort of five human pregnancies in which there was maternal exposure to disulfiram revealed one spontaneous abortion, two infants with clubfeet and two normal infants (see Nora et al., 1977). Gardner and Clarkson (1981) gave a single case report of a severely malformed and retarded child whose previously alcoholic mother had taken disulfiram early during pregnancy.

Animal experimentation suggests that disulfiram has embryotoxic potential. Salgo and Oster (1974) were able to obtain a fetal resorption rate of 83% in rats on the twelfth day of pregnancy; reproductive failure was attributed to the copper-chelating activity of disulfiram. Thompson and Folb (1985) found that the **in vivo** administration of high dosages of disulfiram to mice resulted in a significant increase in early resorptions. An **in vitro** mouse embryo culture system revealed that disulfiram has widespread morphological and histological effects on 8-day mouse embryos; among the organs affected was the central nervous system (Thompson and Folb, 1985).

The possibility therefore exists that disulfiram does have teratogenic potential, and the aim of this study was partly to seek preliminary data in support of this possibility, using the methodology described in Chapter 2.

4.1.3 Effects of Disulfiram on Protein Expression: Literature Reports.

The ability of the major metabolite of disulfiram to chelate heavy metals has widespread enzyme effects. Several metalloenzymes, including alcohol dehydrogenase, aldehyde dehydrogenase, and dopamine beta-hydroxylase, are inhibited as a result of the chelating of copper and other heavy metals by the principal metabolite of disulfiram, diethyldithiolcarbamate (Levinson et al., 1978). The malaria parasite, Plasmodium is particularly rich in cytochrome oxidase, a metalloenzyme, and disulfiram has been effectively used as an **in vitro** anti-malarial agent (Scheibel et al., 1979). Disulfiram has also been shown to have anti-viral activity **in vitro**, by the inhibition of RNA-dependent DNA polymerase of Rous sarcoma virus, which is a metalloenzyme (Levinson et al., 1978).

Disulfiram is also an ionophore of copper. Hannan and McAuslan (1982) have postulated that treatment with disulfiram may achieve more efficient localization of metal ions at the target, than treatment with metal salts, at a given concentration.

The drug has also a marked general effect on the synthesis and expression of both cellular and secretory proteins. For example, Hannan and co-workers (1982) found that disulfiram caused a 50 to 70% inhibition of the total incorporation of ^{35}S-methionine into clonal cell lines of vascular endothelial cells. Over and above this general suppression of protein synthesis, there was also the total inhibition of specific secretory proteins, for example fibronectin and collagen (Hannan et al., 1982). This inhibition was both rapid (within 30 minutes) and irreversible (still effective after 24 hours). Two hybridoma cell lines treated in a similar way failed to produce IgM and IgG chains (Hannan et al., 1982).

The mechanism responsible for the general inhibition of protein synthesis by disulfiram is unknown. A possible explanation is that disulfiram has this effect on protein expression because the drug inhibits the copper-dependent enzyme responsible for

peptide amidation (Mains et al., 1986). Peptide amidation, which involves the conversion of peptides terminating in -x-gly to amidated peptides terminating in -x-NH_2 is an essential process during protein biosynthesis, and any alterations in the levels of amidated peptides may be expected to have wide-ranging effects. The effect of disulfiram on specific secretory proteins is postulated by Hannan and co-workers (1982) to result from its role as an ionophore; other ionophores also act as selective inhibitors of the expression of extracellular proteins.

Diethyldithiolcarbamate, the major metabolite of disulfiram, has the ability to act as a mercaptan, and therefore may inhibit enzymes with sulfhydryl groups (Mains et al., 1986; Goodman and Gilman, 1985). It appears, therefore, that there are a number of different ways by which disulfiram may induce changes to protein expression. The inhibition of peptide amidation occurs in the rat at dosages of disulfiram similar to the human therapeutic dosages (Mains et al., 1986), and the disulfiram-associated protein modulatory effects may well account for some of the side-effects that result from long-term disulfiram use. It seems reasonable to suppose that some or all of these protein-modulatory effects may also occur in fetal tissues, and if so, two-dimensional gel electrophoresis may be an appropriate technique for detecting such manifestations in fetal mouse brain.

4.1.4 Disulfiram and the Heat-Shock Proteins

In addition to the abovementioned protein modulatory effects apparently mediated by disulfiram, the drug also appears to be capable of inducing the synthesis of the so-called heat-shock proteins. These proteins first became the subject of study when it was noticed that transient hyperthermia induced the same set of proteins (or a very similar set) in a wide variety of animal and plant tissues. Subsequently it was discovered that the heat-shock response may be evoked by a diversity of

environmental agents, including toxic substances, a number of therapeutic agents, and some teratogens (see Schlesinger et al., 1982).

Although the evidence is still indirect, disulfiram appears to be capable of inducing the synthesis of the heat-shock proteins. Levinson and co-workers (1978) reported that disulfiram induced the synthesis of four proteins, of approximately 100 kd, 70 kd, 35 kd and 25 kd, in chick embryo and human foreskin cells; Hannan and McAuslan (1982), using bovine endothelial cells, found that disulfiram induced a similar set of proteins, of 83 kd, 73 kd, 32 kd and 28 kd. The predominant heat-shock protein found in other tissues is the 70 kd species, while heat-shock proteins of 22-27 kd and 80-90 kd are found in virtually every species (Schlesinger et al., 1982). Significantly, Johnston and colleagues (1980) reported that four proteins induced by disulfiram in chick fibroblasts were the same as those induced by sodium arsenite, and that the latter co-migrated with heat-shock proteins expressed in the same tissue.

The heat-shock response has been reported to occur in a great variety of tissues, including brain, thymus, heart, lung, spleen, liver, and kidney (Currie and White, 1981). Tissues of the developing organism also appear to be susceptible to the heat-shock response; this has been shown in Drosophila and Xenopus, and in cultured mammalian cells (mouse embryonal carcinoma stem cells from brain and nervous tissue), as well as the rat fetus at mid-gestation (Walsh et al., 1987). German (1984) demonstrated that heat-shock manifestations occurred in the 12-day-old mouse embryo. It was therefore decided to determine whether disulfiram induces the synthesis of heat-shock proteins in the fetal mouse brain, since such a finding could conceivably be relevant to an investigation of the potential teratogenic manifestations of disulfiram.

4.2 Drug administration

A suitable dosage of disulfiram was difficult to establish because so few teratogenic investigations have been reported. Salgo and Oster (1974) administered disulfiram to rats at 100 mg per animal continuously over a number of days during pregnancy. This dosage protocol was not considered to be suitable for the present study because of the extremely high dosages used (approximately 2 g/kg), and also because an acute exposure was required to coincide with a specific stage in the development of the mouse brain. The protocol of Thompson and Folb (1985) could also not be used, because the animals were exposed to disulfiram for the duration of pregnancy

It was therefore attempted to estimate an adequate teratogenic dosage by using the toxic dosage (in the adult animal) as a guideline. Salgo and Oster (1974) described the toxic manifestations in adult rats after chronic exposure to high dosages of disulfiram. Similar manifestations were not observed in the present study after the acute exposure of C_3H mice to disulfiram, and single dosages of up to 1,5g/kg appeared to have no untoward effects. Additional criteria were therefore considered to establish guidelines for the selection of a teratogenic dosage. In view of the widespread disulfiram-associated protein effects, sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on homogenized adult mouse brain at various intervals after drug administration. An unknown brain protein of approximately 25 kd was induced, and first became discernible following the ingestion by mice of 450 mg disulfiram per kg; the effect appeared to be dose related and 600 mg/kg had a marked effect. Since this effect was possibly a toxic manifestation, the higher dosages were not considered for teratogenic investigations. Pettersson and Tottmar (1982) had found that single dosages of 300 mg disulfiram/kg resulted in enzyme inhibition in rat brain and liver. In view of the above findings it was decided that disulfiram at 450 mg/kg was potentially an adequate teratogenic dosage.

Drug administration was by means of gastric intubation. Disulfiram is insoluble in water and only sparingly soluble in alcohol; Pettersson and Tottmar (1982) administered the drug as a suspension in 5% gum arabicum. In the present study disulfiram was administered as a partial suspension in arachis oil, because arachis oil had been used previously and found to be acceptable to mice (Pillans et al., 1988). A maximum volume of 100 µl was administered and none of the mice showed any ill-effects. Controls received arachis oil only.

In order to expose the fetus to disulfiram during critical stages of central nervous system development, the drug was administered as single dosages on either gestational day $8^{1}/_{2}$ or $9^{1}/_{2}$, as in the phenytoin study.

4.3 Effects of in vivo disulfiram exposure on fetal weight and fetal brain weight

Disulfiram (450 mg/kg), when administrated to pregnant mice on day $8^{1}/_{2}$ post conception, had no significantly effect on fetal weight at day $18^{1}/_{2}$; there was, however a significant increase in fetal brain weight ($p<0,05$). There were no significant differences in the occurrence of resorptions, intra-uterine deaths, or macroscopic abnormalities between test and control fetuses.

When the same dosage of disulfiram was administered on gestational day $9^{1}/_{2}$, there was a significant increase in fetal body weight ($p<0,05$), but not in fetal brain weight of test animals. This result was unexpected in view of the findings by Salgo and Oster (1974), namely, that disulfiram caused a significant reduction in fetal weight. (See Tables 4.1 and 4.2).

The results of Salgo and Oster (1974), however, were obtained with dosages that were toxic to the dams. At much lower dosages of disulfiram, administered for the duration of pregnancy, Thompson and Folb (1985) found no significant reduction in fetal weight. No explanation can be offered for the **increase** in body weight of fetuses exposed to

Table 4.1 Summary of the effects on fetal C_3H mice after *in vivo* exposure to 450 mg/kg disulfiram on gestational day $8^1/_2$

	Test (n=14)	Control (n=10)	P
Total conceptions	18	13	-
Resorptions	1	1	ns
Intra-uterine deaths	2	1	ns
Abnormalities	1 exomphalos	1 exomphalos	ns
Mean fetal weight (g)	1,1761 (1,1338-1,2184)	1,1313 (1,0458-1,2168)	ns
Mean fetal brain weight (mg)	78,76 (76,08-81,44)	74,60 (72,07-77,13)	$p<0,05$

(Figures in brackets are the 95% confidence intervals for the population mean)
ns = no significance

Table 4.2 Summary of the effects on fetal C_3H mice after *in vivo* exposure to 450 mg/kg disulfiram on gestational day $9^{1}/_{2}$

	Test (n=20)	Control (n=12)	P
Total conceptions	26	15	-
Resorptions	2	1	ns
Intra-uterine deaths	2	1	ns
Abnormalities	2	1	ns
	exomphalos	monster	
Mean fetal weight (g)	1,1048 (1,0733-1,1363)	1,0336 (0,9757-1,0915)	p<0,05
Mean fetal brain weight (mg)	75,55 (74,46-76,64)	72,83 (69,31-76,35)	ns

(Figures in brackets are the 95% confidence intervals for the population mean).

ns = no significance

disulfiram on day $9^{1}/_{2}$ of gestation or for the increase in fetal brain weight of fetuses exposed on day $8^{1}/_{2}$, and this kind of finding is not generally associated with fetal drug exposure.

There were no significant differences in the incidence of resorptions, intra-uterine deaths, or macroscopic abnormalities among fetuses exposed to disulfiram on gestational day $9^{1}/_{2}$, compared with controls (see Table 4.2). Both Salgo and Oster (1974), and Thompson and Folb (1985) found a significant increase in fetal resorptions after continuous disulfiram administration during gestation. It appears from the findings of this study that the mouse fetus is not susceptible to acute exposure to disulfiram at days $8^{1}/_{2}$ and $9^{1}/_{2}$, at the dosage used. Thompson and Folb (1985) proposed that disulfiram does in fact have teratogenic potential, and further research may be required to establish at what stage of gestation, and at what dosages, the fetal mouse central nervous system is susceptible to disulfiram teratogenesis.

4.4 Effects of disulfiram on the protein complement of fetal mouse brain

No consistent disulfiram-associated changes were found in the fetal mouse brain protein complement. An analysis of the two-dimensional gel electrophoretic patterns obtained from disulfiram-exposed fetuses (gestational day $9^{1}/_{2}$) revealed an apparent protein induction (MW 90 kd; pI 5.2). However, this apparent change was present in only four out of 16 gels and was therefore regarded with scepticism, particularly in view of the fact that it occurred in an area of local protein overloading.

The apparent absence of any disulfiram-induced changes is unexpected in view of the wide-ranging protein changes with which disulfiram is associated in adult tissues. Changes may in fact have occurred in the fetus immediately after drug administration, and these changes may then have been reversed by an as yet unknown fetal repair mechanism.

Another possibility is that the dosage of disulfiram used was in fact not adequate to give rise to a teratogenic (or other) effect. It is noteworthy that the protein modulation induced by disulfiram in the adult mouse brain (see section 4.2) was absent from the two-dimensional gels of fetal brain proteins.

Among the protein changes that were anticipated but not found, were those associated with the heat-shock response. These proteins have acidic pIs (between 5 and 6) and molecular weights between 24 kd and approximately 100 kd, and they may therefore be expected to resolve within the parameters of the electrophoretic system that was used. Although the heat-shock proteins are minor components (even after induction) of the tissue protein complement, the higher molecular weight species (particularly the 70 kd protein) may readily be visualized on silver-stained two-dimensional gels. Even careful scrutiny, however, revealed no protein modifications in those regions of the gels where the heat-shock proteins were most likely to be found.

This inability to detect the heat-shock proteins cannot be taken as conclusive evidence that disulfiram does not induce the heat-shock response, and indeed, it would be highly significant if it was found that the heat-shock proteins are **not** inducible in fetal mouse brain. It is known that the expression of the heat-shock proteins is transitory, and adequate allowance should be made for the kinetics of heat-shock protein induction. Since the low-molecular weight heat-shock proteins are expressed in low copy number, labelling by means of the metabolic incorporation of radioactive amino acids may greatly increase sensitivity, as well as enhancing visualisation by limiting the number of protein spots displayed to only the newly synthesized proteins.

4.5 Disulfiram and the embryonic stress hypothesis of teratogenesis

James German (1984) proposed that the induction of the heat-shock response in the mammalian embryo provides a common pathway by which diverse environmental agents may lead to developmental abnormalities. This hypothesis has profound implications for teratogenesis, and it will therefore be examined here, within the context of a discussion of the effects of disulfiram on the fetus.

German proposed that the induction of the heat-shock proteins pre-empts the normal schedule of protein synthesis during development. The established program of activation and inactivation of genetic loci, essential for normal intra-uterine development, is therefore altered, leading to developmental abnormalities. German suggested that the precise period during gestation when the response is induced may determine the nature of the abnormalities.

German's hypothesis flows from the long-established idea that fever during pregnancy may interfere with normal human embryonic development. Previously it was assumed that transient hyperthermia resulted either in outright cell death or a disruption of mitosis or cellular migration (see German, 1984). Transient hyperthermia was also found, however, to stimulate the heat-shock response. Other environmental agents, some of them teratogens, were likewise observed to stimulate the heat-shock response. The possibility therefore exists that the heat-shock response itself is responsible for the disruption to embryonic development, and indeed, it may be the common pathway whereby a variety of environmental agents induce fetal injury.

German unfortunately presents no evidence to back up his hypothesis, and he also ignores some contradictions. For example, he fails to explain why sodium salicylate, which is not generally considered to be teratogenic, induces the expression of the heat-shock proteins. Although he notes that thalidomide does not elicit the heat-

shock response, German does not explain why this powerful human teratogen should not share the common pathway with other known or suspected teratogens.

German also fails satisfactorily to refute an alternative hypothesis for the biological role of the heat-shock response. Ashburner and Bonner (1979) formulated the idea that the expression of the heat-shock proteins may serve a **protective** role. Evidence from many studies has suggested that the threshold to injury, when a tissue is exposed to harmful environmental agents, is increased once the heat-shock response has been evoked, enabling the tissue to tolerate concentrations of toxins that may otherwise be lethal (see Schlesinger et al., 1984). This putative homeostatic role of the heat-shock response has received widespread support, and recent studies have revealed some of the molecular details of how these proteins may exercise a protective function (see, for example, Deshaies et al., 1988).

The second hypothesis is particularly attractive because it is open to being tested. Little research has been done on the protective heat-shock response to drugs in the fetus. Disulfiram may be especially suitable in this role, since it appears to have teratogenic potential, and it is also suspected of being an inducer of the heat-shock response. A low dosage of disulfiram may well stimulate the protective response in fetal tissues, which may then prevent manifestations associated with toxic or teratogenic dosages of disulfiram.

As for German's "common pathway" hypothesis, it seems more likely that there are diverse mechanisms whereby drugs, and xenobiotics, may cause injury to the fetus. Rather than seeking a single all-embracing mechanism, it may ultimately be more productive to concentrate on the mode of action of individual teratogens, and in this way establish how teratogens resemble one another, or differ from one another, in their mechanisms of action.

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CHAPTER 5

EVALUATION AND FUTURE PROPOSALS

5.1 Two-dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis is a powerful analytical technique that has been used by a number of researchers to evaluate the effects of xenobiotics on the protein complement of several different cells and tissues. In teratological research the technique has not been extensively used, and part of the aim of the present study was to explore the potential of two-dimensional gel electrophoresis for uncovering some of the molecular events that accompany the disruption of development by drugs.

Approximately 1250 different proteins were detected by the electrophoretic system and staining procedure used in the present study. Using much larger, so-called "giant" gels, Young (1984) was able to detect approximately 3000 different proteins from rat thymus cells; an additional 2000 proteins were resolved from the same tissue when non-equilibrium gels were also used. Klose and Zeindl (1984) resolved concentrated subcellular fractions on "giant" two-dimensional gels, and after multiple autoradiographic exposures (to detect proteins of extremely low copy number) they succeeded in detecting approximately 10 000 different proteins from a single cell type.

Although the development of "giant" gels represents a significant advance in the capability of the technique, these enormous gels are difficult to prepare and manipulate (Young, 1984). The gels used in the present study were approximately twice the area of the "standard" O'Farrell gel (O'Farrell, 1975), and produced a level of detection intermediate between the "standard" and the "giant" gels, without introducing the daunting technical difficulties associated with the latter. In the present study some attention was given to the statistical evaluation of differences between test and control

fetuses, and the task of running a complex "giant" gel on each specimen, and the examination of thousands of minor spots would therefore have been overwhelming. Nevertheless, the decision not to use the extremely large gels may have resulted in some drug-induced modulations of minor components remaining undetected.

The formulation of the iso-electric focussing dimension dictated which sub-set of the total proteins was incorporated for electrophoretic analysis. For instance, the pH range employed in this study (pH 4.8 - 7.0) was chosen for the inclusion of the largest number of proteins combined with high-quality resolution, and ease of operation (see O'Farrell, 1975). This range does not extend above pH 7 because the basic proteins resolve poorly on equilibrium (or steady-state) gels, and separate non-equilibrium electrophoresis is required for their resolution (see Young, 1984). The recent development of "immobilized" pH gradients has made iso-electric separation possible over an extended range (Hanash and Strahler, 1989).

Streaking can result in a large increase in spot size, which makes the evaluation of quantitative changes difficult or impossible (O'Farrell, 1975). Some streaks may in fact consist of multiple spots that are poorly resolved, and these may in turn mask other, more indistinct, spots. In the present study (see Figure 3.1) streaking at the basic end of the gels could not be eliminated, and the analysis of the gels must therefore have been biased towards the detection of protein changes among the more acidic proteins.

5.2 The Nature of the Protein Modifications

The phenytoin-associated protein modulations reported in Chapter 3 appear to be largely unambiguous, with only one protein spot (spot 1 in Figure 3.1) present

intermediate concentrations. The apparent induction, deletions and changes in pI represent the full spectrum of protein modulatory manifestations detectable on two-dimensional gels, and suggests that phenytoin may have wide-ranging biological effects in the fetal mouse brain.

The fetal brains that were analysed electrophoretically were all macroscopically normal, and there is no evidence that the phenytoin-induced protein changes reported here are teratogenic manifestations. A correlation will have to be made between morphological or histological abnormalities and protein changes before the latter may be described as teratogenic. It is not obvious how the present study may be expanded to establish such a correlation, since the brain samples were solubilized immediately after removal from the fetuses. If only a portion of the brain is solubilized for electrophoresis, then the remainder may be used for other investigations, but the division of the sample will have to be made with a high degree of consistency if the protein patterns between samples are to be reproducible. Gel electrophoresis may be used to determine to what extent the two hemispheres of the fetal brain are homologous. If there is a high degree of homology then it may be possible to do an electrophoretic analysis on the one hemisphere, and morphological and histological examinations on the other; the assumption would be that drug-induced lesions in one hemisphere may be mirrored in the other. Behavioural studies may be conducted to clarify whether the phenytoin-induced brain protein changes are evidence of teratogenic impairment. Mice exposed to phenytoin at 200 mg/kg may well have learning or developmental deficiencies after birth, but it would still be necessary to establish a direct link between these deficits and anomalies in brain protein complement. The teratogenicity or otherwise of the phenytoin-induced protein changes is an important question and to solve it an experimental approach altogether different from the present one may be required.

It is possible that teratological damage, as evinced in histological or morphological anomalies, may not be conclusively reflected in correspondingly disrupted protein patterns. Klose and Putz (1983) found that gross chromosomal abnormalities (trisomies) in embryonic mice were accompanied by unexpectedly few protein changes. The implication of their work is that cells have powerful protein synthesis regulatory mechanisms, and if so, there is every reason to suppose that analogous homeostatic mechanisms may come into play during drug-induced injury to the fetus.

The phenytoin-induced changes could conceivably be a generalised response by fetal tissue to exposure to high levels of xenobiotics, although the apparent absence of similar disulfiram-induced changes argues against this suggestion. The phenytoin-associated protein changes need not even be fetus-specific, and all or some of the changes may also occur in the adult mouse brain after phenytoin administration. If such a phenytoin-associated response does occur in adult brain tissue, it may be related to the drug's anticonvulsant properties, or to one of the diverse and complex biological effects that phenytoin is known to have (see Chapter 3).

These difficulties in establishing the significance of protein changes are found to a greater or lesser extent in other studies that are heavily reliant on two-dimensional gel electrophoresis. For example, Anderson and co-workers (1986) found that no less than 31 proteins from mouse liver showed quantitative differences following exposure to chlorinated hydrocarbons. The aim of their study was to establish the mechanism of toxicity of chlorinated hydrocarbons, but the realization of this aim will entail the determination of the identity and function of the majority of the modulated proteins, and even after the completion of this formidable task the mechanism may still remain elusive. Marshall and co-workers (1985) found that dimethylformamide induced minor but reproducible protein changes in rat serum. The aim of the study was largely technical (a comparison of two-dimensional electrophoretic methods) and the authors

included little discussion on the nature of the dimethylformamide-induced changes. Heydorn and colleagues (1984) found desmethylimipramine- and reserpine-induced modulations after the electrophoretic separation of the proteins of discrete regions the rat central nervous system. The authors attempted to integrate these findings into what is known about noradrenergic neurotransmission, but the study appears to be preliminary and the conclusions speculative. It seems, therefore, that there is some difficulty in attaching biological significance to data generated by means of two-dimensional gel electrophoresis, and without the careful establishment of goals the generation of such data may become an end in itself.

Some other studies have utilised two-dimensional gel electrophoresis not in order to generate primary data but as a tool to analyze narrowly focussed biological entities. For example, Heikkila and co-workers (1981) studied the transient disaggregation of polysomes to monosomes in rabbit brain. Lysergic acid diethylamide mediates this process in both free and membrane-bound polysomes, and the use of two-dimensional gel electrophoresis made possible the detection of a 74 kd protein involved in the disaggregation process. Vlasuk and Walz (1980), in an electrophoretic study of liver microsomes, found that phenobarbitone and beta-methylcholanthrene induced differences in the synthesis of putative cytochrome P-450 polypeptides. Pipkin and co-workers (1985) found that isoproterenol and sodium phenobarbitone enhanced the metabolism associated with regeneration in proliferating liver, and two-dimensional gel electrophoresis was used to study protein synthesis during specific phases of the liver cell cycle.

5.3 Strategies for the Identification of Specific Proteins

The information on individual proteins derived from the gel viz. molecular weight, iso-electric point, and relative copy number, is usually insufficient for protein identification. Additional identification techniques have to be utilized.

The most direct way of establishing the identity of a specific protein involves first isolating it from the other brain proteins and then obtaining the amino-acid sequence. Protein isolation may be achieved by excising the spot from the gel, although the percentage recovery is likely to be low, and there is also likely to be a problem with contamination (particularly sodium dodecyl sulphate). Nevertheless, interest in obtaining a specific polypeptide directly from the gel has recently been revived and refinements have been introduced (Kennedy et al, 1988; Eckerskorn et al., 1988). An alternative strategy for the isolation of a specific protein involves subjecting the source material (homogenized fetal brain) to column chromatography. Two-dimensional gels may then be run at various stages during the isolation procedure to confirm that the correct protein fraction is being enriched (Torres et al., 1985). Since there is no restriction on the amount of starting material, a protein present even in low concentration may be isolated. Liquid chromatography is being increasingly used in the separation and isolation of proteins, and is an alternative to column chromatography for the isolation procedure mentioned above (see Hunkapiller et al., 1984). Whatever the technology used, the isolation of a protein is a time-consuming task that represents a major research commitment.

The determination of the amino-acid sequence of the purified protein usually requires access to specialist protein chemistry facilities, and the use of an automated amino-acid analyzer, although an alternative is a liquid chromatograph that has been set-up specially for the task (Hunkapiller et al., 1984).

Once the amino-acid sequence is known, then the identity of the protein may be obtainable from a protein "library" (see Eckerskorn et al., 1988). The identification of a murine polypeptide does not necessarily require a murine protein database; there appears to be sufficient homology between the proteins of mammalian species, and also different cell types, for this not to be essential. For example, McConkey (1982) was able to show, using two-dimensional gel electrophoresis, that at least half of the 370 denatured polypeptides from hamster cells and human cells are indistinguishable in terms of isoelectric point and molecular weight.

All the phenytoin-modulated proteins are represented by minor spots, and this makes the determination of their amino-acid sequence an essential route to identification. Some prominent proteins (eg. actin) may be identified simply from their positions on the gels, since they occur in the same positions on gels derived from other tissues. Proteins available commercially in pure form may be added to the sample containing the protein of interest to test whether or not co-migration occurs. Any information, even of a general nature, that is available about the target protein may be utilized to speed up the purification and identification process. If it is known, for instance, that the unknown protein is a protease, then substrate affinity chromatography may be performed for rapid purification.

Once enough of the unknown protein has been isolated it may be used as an antigen to raise antibodies. Radioactive markers conjugated to these antibodies should reveal the distribution within the brain of the drug-modulated protein. The intracellular locality of the protein may also be determined, and this information may well give important hints as to the function of the protein.

Until the drug-modulated proteins are identified, the data available from the two-dimensional gels must necessarily be seen as preliminary. However, it cannot simply be assumed that the identification of drug-modulated proteins will reveal the

fundamental biological events that occur when a teratogen stimulates the modulation of those proteins. The observed modulations may be secondary, or even tertiary, manifestations, in which case the available data may well be insufficient to allow a meaningful assessment to be made of biological responses that occur after drug exposure. If a strong relationship can be found between the various drug-modulated proteins, either in terms of function, structure, or intracellular position, then it may be much easier to hypothesize on the sequence of events that follows from drug exposure.

5.4 The Conceptual Difficulties of an Holistic Approach

The interpretational difficulties mentioned in the previous section are more or less inherent to an holistic approach. In such an experimental procedure the investigator subjects the intact organism to an intervention, and then records the morphological, histological or molecular outcome. Although this is a long-established and valuable scientific method, it is not without limitations. For one thing, the achievement of positive results is more or less fortuitous, and negative results represent a cul-de-sac, leaving no alternative approach and giving no hint as to how the problem may be reformulated. Moreover, it is often difficult or impossible to grade the various items of data in terms of their importance, and unless the findings can be placed in some or other context, the investigator may not be able to assign to them value or significance. These comments certainly apply to the data on the gels, but they may also apply after the drug-modulated proteins have been identified, in which case the task of identification will have been an end in itself.

The alternative is to adopt a reductionistic approach (see Nagel (1971) for general comments). Experimentally this involves the selection of a specific biological process or entity, which is then studied independently of the organism as a biological

approximation of what actually happens. Ideally, the material under study should be highly defined and in some situations it is feasible that most, if not all, of the components of the entity may be known. Simplicity is the key, since simplicity allows the investigator to design experiments in such a way as to obtain unequivocal answers to specific questions. A series of carefully designed experiments may reveal a great deal about the biological entity under investigation, and this knowledge may then be "extrapolated" to more complex levels. As Karl Popper (1981) puts it: "...whenever we can explain entities and events on a higher level by those of a lower level... we can say that we have added much to our understanding of the higher level. As a **research programme,** reductionism is not only important, but it is part of the programme of science whose aim is to explain and to understand."

Clearly the formulation of an experimental protocol that will fulfill some or all of the criteria of such a reductionistic approach is crucial. In fact, such a formulation must presuppose detailed knowledge of, and extensive familiarity with, the biological entity of choice, and the selection of a suitable area of research is therefore in itself a significant step to the eventual solution of the problem. It is not clear, however, how the present study may be reformulated in terms of a reductionistic approach. In fact two-dimensional gel electrophoresis may be used more productively in an analytical capacity, rather than as a means of generating primary data. In the following section some tentative comments will be offered on how reductionistic principles may be utilised to investigate protein modulations in the fetal brain, within the context of teratology.

In teratological research an holistic approach has long been favoured. This is partly due to the clinical contribution expected of teratological research e.g. strategies for the prediction of the teratogenic potential of new drugs, and screening procedures for assigning relative teratogenic risk to drugs currently in use (see for example Schmid,

1987). While such endeavours are essential, the strategies used to address these issues are not wholly appropriate to answer mechanistic questions, or to solve complex biological problems. Many of the advances in modern biochemistry and molecular biology have come as a result of the acceptance that vital processes may be removed from their normal contexts and reassembled in vitro in a form where they retain a specific and limited function, and where simple manipulations may provide YES/NO answers. Even if initially the information obtained may appear fragmentary and inconsequential, the data will be precise enough to lead to the formulation of other, more wide-ranging, objectives. Such a strategy may provide insight into fundamental and important teratological processes, and at the same time greatly assist in the search for effective screening procedures.

5.5 Research ideas for the study of drug-modulated fetal proteins

An examination of the heat-shock response within the framework of a teratological investigation may be extremely rewarding. The induction of these proteins may in fact occur in fetal mouse brain during mid- and late-gestation, and if so, then it would be interesting to know how different drugs affect the level and the kinetics of expression. It would also be interesting to confirm whether the fetal mouse brain expresses the heat-shock proteins constitutively (see for example Barnier et al., 1987), and whether this expression differs between fetal and adult brain. If the fetal heat-shock proteins are inducible, then the effects of disulfiram and phenytoin may be analysed and compared. The level of expression of the heat-shock proteins may conceivably be used as a measure of the stress that drugs exert on the fetus, and this may constitute the basis of a teratogen screening procedure.

The protective function that has been attributed to the heat-shock response is potentially of the highest relevance to the study of drug-induced injury to the fetus.

Only one literature report has, to my knowledge, mentioned this protective capacity of the heat-shock response within the context of teratology. Buzin and Bournias-Vardiabasis (1982) were able to show that mild heat pretreatment of Drosophila embryonic cells protects them from exposure to teratogens (coumarin and phenytoin). It may therefore be fruitful to explore the protective role of heat-shock inducers in the fetal mammalian brain. For example, will the prior administration of a non-teratogenic dosage of disulfiram, followed by a teratogenic dosage of phenytoin, prevent the manifestations of phenytoin teratogenicity? Will the protein modulations that follow phenytoin administration (as detected in the present study) be suppressed by the prior administration of disulfiram? Answers to these questions may reveal a great deal about the kinetics of unscheduled protein synthesis in response to drug exposure. The ability to elicit a protective response in the fetus (if it exists) may be useful therapeutically, for example, in cases of high fever during critical stages of pregnancy. The administration of a sub-teratogenic dosage of a drug, that induces the heat-shock response, immediately after the accidental or deliberate ingestion of large amounts of phenytoin (or any other teratogen), may provide the fetus with a measure of protection.

As in the case of the heat-shock proteins, ornithine decarboxylase expression appears to be sensitive to diverse environmental factors. Pegg and McCann (1982) have reported that ornithine decarboxylase activity may be increased many-fold within a few hours of exposure to environmental stimuli, including drugs. Dienel and Cruz (1984) were able to show that ornithine decarboxylase activity in the rat brain increased two- to sixfold after different modes of injury, including mechanical and thermal injury, and also in response to exposure to neurotoxins.

Ornithine decarboxylase does not appear to belong to the family of heat-shock proteins, although it too may be useful as an indicator of stressful or noxious stimuli

(Slotkin et al., 1985). The likelihood exists that it is inducible by toxic or potentially teratogenic dosages of disulfiram and phenytoin. However, it is unlikely that ornithine decarboxylase will be detectable on gels, because Dienel and Cruz (1984) reported that the enzyme represents only about 10^{-6} per cent of the protein in a 17 000g brain supernatant. Whether or not the enzyme is in fact modulated by phenytoin and disulfiram, and the extent of the modulation, may be determined from an enzyme activity assay.

The importance of ornithine decarboxylase in a teratological context lies in the fact that it is the rate-limiting enzyme in the biosynthesis of the polyamines spermine and spermidine. These are percusors of macromolecular biosynthesis, and are known to play an essential role in many aspects of normal cellular growth and differentiation (Pegg and McCann, 1982). Ornithine decarboxylase is very active in the fetus and its expression in the mouse fetus fluctuates during gestation, reaching maximum activity on gestational day 8 (O'Toole et al., 1989). The enzyme, however, appears to participate only in discrete developmental events, since inhibition studies have shown that not all growth processes are highly dependent on elevated ornithine decarboxylase activity (Slotkin et al., 1984).

Pharmacologically active substances therefore appear potentially to have a wide spectrum of effects on fetal tissues. Some of these substances have been shown to induce the unscheduled synthesis of proteins, although as yet little is known about what effects drug-induced protein synthesis may have on the highly ordered and sensitive processes of development. From a reductionistic viewpoint, it seems possible to utilize selected drugs as tools in order to manipulate specific molecular processes within the fetus, and thereby to improve our understanding of fetal vulnerability to xenobiotics, and to shed light on the developmental process itself.

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APPENDIX

A. FORMULATIONS

Chromic Acid 100g potassium dichromate, 250 ml H_2SO_4 made up to 1 L.

Methanolic Alkaline Solution KOH dissolved in methanol (saturated).

Lysis Buffer 9,5M urea, 2% (w/v) Triton X-100, 2% Ampholines (comprised of 1,6% pH range 5-7, and 0,4% pH range 3-10), and 5% 2-mercaptoethanol.

Molecular Weight Standards Lyzozyme (14,4 kd), trypsin inhibitor 28 kd), bovine serum albumin (68 kd), phosphorylase b (rabbit muscle) (97,4 kd), and alcohol dehydrogenase (150 kd).

B. SUPPLIERS

C_3H Mice supplied by Animal Unit, University of Cape Town Medical School.

Mice cubes Atlas Feed (Pty) Ltd.,
392 Main Road,
Wynberg, 7800

Phenytoin sodium Lennon Ltd.,
7 Fairclough Road,
Port Elizabeth, 6001

Disulfiram MPS Laboratories (Pty) Ltd,
PO Box 260778
Johannesburg, 2000

Glass tubing Pyrex capillary tubing, 2,5 mm internal diameter.
Scientific Glassblowers
332 Victoria Road,
Salt River, 7925

Glass plates P.G. Glass
88 Buitengracht Street,
Cape Town, 8001

Palladium electrodes Johnson Matthey (Pty) Ltd.,
PO Box 14078,
Wadeville, 1422
Obtained with S.A. Police permit: Cape Town 42/2/8/1232

Hoefer Power Supply PS1200 Hoefer Scientific Instruments, San Francisco.
Local agents:
Scientific Associates,
PO Box 262,
Tokai, 7945

Urea, TEMED, Sulfosalicylic Acid, Tris and Triton X-100 were supplied by Merck; Agarose, Ampholines by LKB; Lyzozyme, Trypsin Inhibitor, Alcohol Dehydrogenase by Boehringer Mannheim; Coomassie blue, Phosphorylase b by Sigma; Bovine serum albumin by Miles Laboratories; Glycine, Mercaptoethanol, Silver nitrate by BDH.