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INVESTIGATIONS INTO THE NEUROCHEMICAL ABERRATIONS ASSOCIATED WITH LEAD EXPOSURE.

by

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being a thesis submitted for the degree of
Doctor of Philosophy,
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July, 1986

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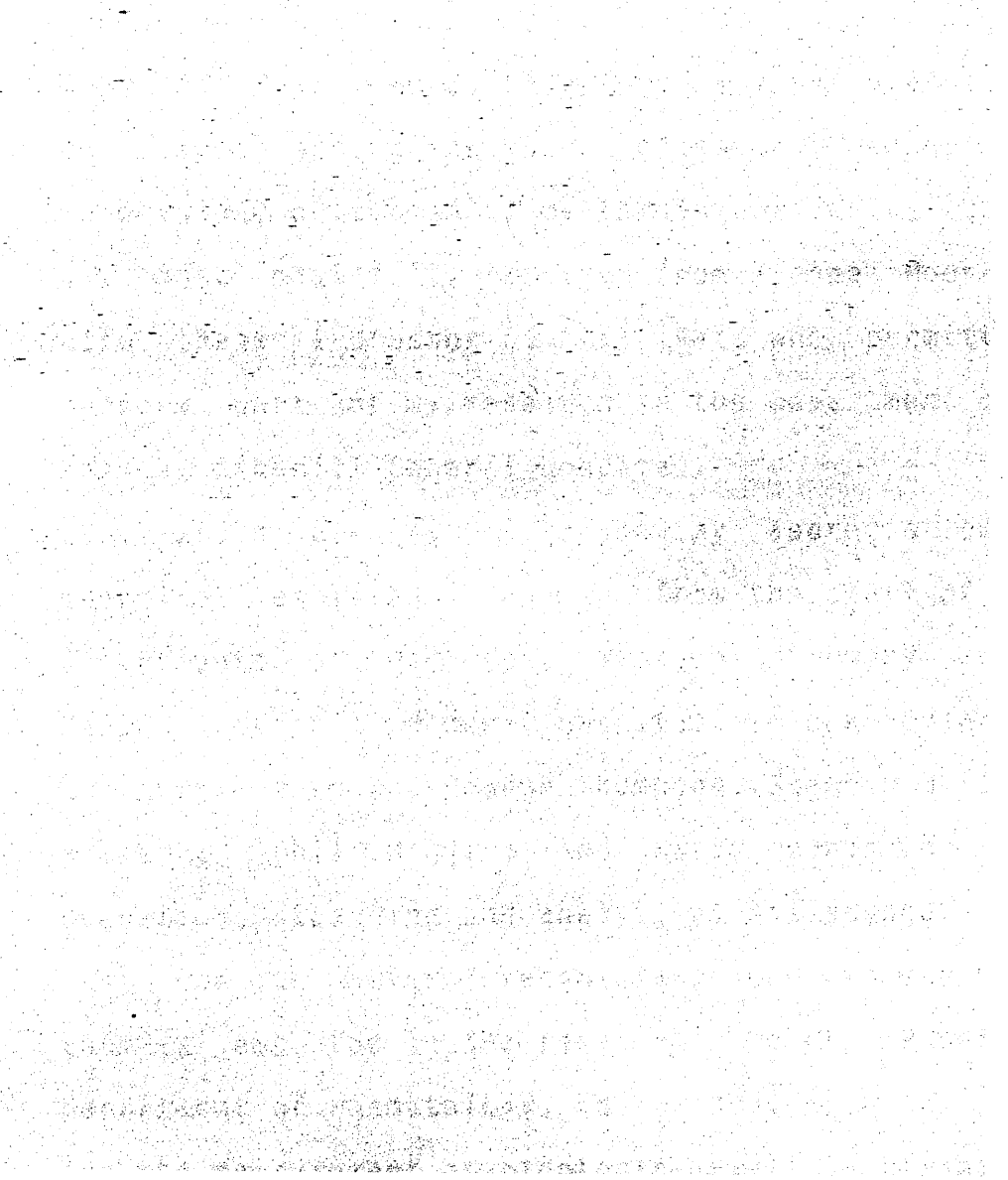


Acknowledgements

I should like to express my thanks to everyone who, in any way has assisted in the compilation of data for this thesis, and in particular I should like to thank, the following -

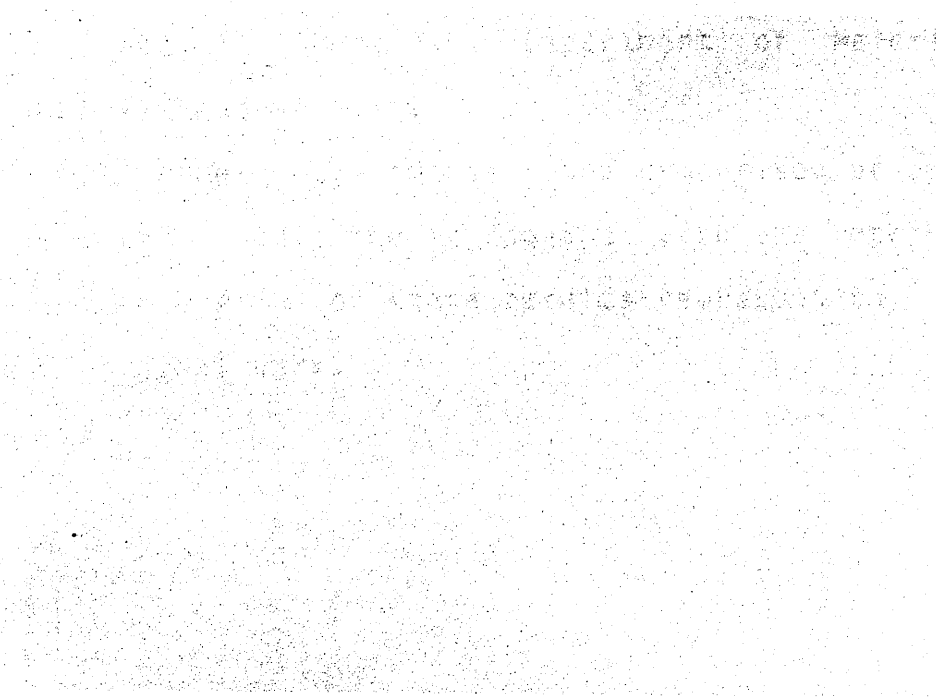
I am indebted to Professor, Sir Abraham Goldberg, who in addition to employing me to carry out the research for this project lended advice, support and supervision throughout my research period. I am also grateful to Drs. Michael Moore and Peter Meredith for their constructive discussion and-general assistance on several occasions too numerous to mention. Peter Meredith provided very useful technical assistance during the course of several experiments, and much valued criticism of my thesis during its construction was most gratefully received from Michael Moore. I must also thank Professor John L. Reid who permitted me to perform part of my research in the Department of Materia Medica, Stobhill General Hospital.

I would also like to show my appreciation for the technical assistance received from the staff of both the Departments of Medicine, Western Infirmary and Materia Medica, Stobhill General Hospital, in particular my thanks are extended to Mr. George Thompson, Stephen Miller, Daniel McSharry, Phil Higgins and Marty Hughes for their co-operation, teaching and analytical assistance. Thanks are also due to several technicians who looked after the animals employed in the research, to Dr. Robert Leeming, Department of Haematology, The General Hospital, Birmingham for the measurement of biopterin levels in plasma samples,

and to Dr. Tony Payne, Department of Anatomy, University of Glasgow for advice on brain dissection.

I should also like to take this opportunity to thank my parents for their assistence, both financial and otherwise, numerous friends and present colleagues, whose encouragement, which may at times have appeared as 'nagging' but which greatly assisted me in completing this thesis.

Finally, I am grateful to the Department of Medicine for allowing me to use their wordprocessor, without which I could not have typed this manuscript, and to Bob Kennedy, Department of Bacteriology, Stobhill General Hospital for allowing me to borrow a first class printer, and indeed last, but not least, the DHSS without whose financial support, this thesis would not have been possible.



Declaration of Collaboration

Certain aspects of the research for this thesis were performed in collaboration with workers in other research departments.

The data presented in chapter 3 collected as part of the EEC 1981 lead exposure study, was analysed in conjunction with Dr. Gordon Fell, Department of Pathological Biochemistry, Glasgow Royal Infirmary. The investigation of the effect of lead on tetrahydrobiopterin metabolism was in part performed in collaboration with Professor John A. Blair, Department of Chemistry, University of Aston and Dr. Robert J. Leeming, Department of Haematology, The General Hospital, Birmingham. These workers were involved in the early research presented in chapter 6 where analysis of biopterin status was performed by the Crithidia fasciculata bioassay. The remaining data presented in the same chapter, and indeed the results obtained in chapter 5, were obtained as a result of experiments performed in collaboration with Dr. Peter A. Meredith, Department of Materia Medica, Stobhill General Hospital.

All other experiments, the comparison of results as presented in this thesis together with the introduction to and discussion of these results represent the candidates own original work.

Summary

Lead is a toxin, of that there is no doubt. It has long been recognised that exposure to this metal can produce profound neurotoxic manifestations. Current awareness centres on the more subtle neurological deficits associated with chronic exposure to 'low-level' lead. The biochemical lesions associated with the slight cognitive derangements are uncertain. The data in this thesis is concerned with the investigation of these biochemical aberrations associated with lead exposure, using the rat as a model. The results indicate alterations in several neurochemical systems. Both the cholinergic and adrenergic nervous systems are investigated, and significantly altered levels of neurotransmitters are noted, as well as alterations in the activity of metabolic enzymes associated with these neurotransmitter systems. A chapter is also devoted to the investigation of the influence of lead on tetrahydrobiopterin, the essential cofactor for tyrosine hydroxylase as well as other enzymes. The influence of lead on the haematopoietic system is well known and associated with this effect, the influence of lead on haem biosynthesis within the rat brain is also investigated.

Aims of the project

The project, the results of which are presented in this thesis, was designed to investigate the biochemical lesion(s) responsible for the neurological sequelae associated with lead exposure, in particular, 'low-level' lead exposure.

It is well recognised that overt clinical symptoms occur as a result of both acute and chronic exposure to high doses of lead. However, current awareness has concentrated on the more subtle neurological sequelae of chronic exposure to reduced levels of the toxin, and it is the underlying pathological aberrations which will concern the bulk of the research for this thesis.

A model of 'low-level lead exposure' will be constructed in the rat which is a convenient laboratory rodent for such experiments. Various biochemical parameters will be assessed in the brains of these animals following exposure to lead by way of their drinking water.

The following publications have resulted from work carried out during the course of this project:

1. Blair, J.A., Hilburn, M.E., Leeming, R.J., McIntosh, M.J. & Moore, M.R. (1982) Lead and tetrahydrobiopterin metabolism: possible effects on IQ. Lancet, 1, 964.
2. McIntosh, M.J., Moore, M.R., Blair, J.A., Hilburn, M.E. & Leeming, R.J. (1982) Lead and tetrahydrobiopterin metabolism in man and animals. Proceedings of the Medical Research Society and Scottish Society for Experimental Medicine, Clinical Science, 63, 44-45P.

3. McIntosh, M.J., Moore, M.R., Goldberg, A., Fell, G.S., Cunningham, C. & Halls, D.J. (1982) Studies of lead and cadmium exposure in Glasgow. U.K. Ecology of Disease, 1, 177-184.

4. Moore, M.R., McIntosh, M.J., Blair, J.A., Leeming, R.J. & Hilburn, M.E. (1983) Biopterins and lead exposure. Proceedings 7th Congress, South African Biochemical Society, Stellenbosch.

5. McIntosh, M.J., Meredith, P.A., Goldberg, A. & Moore, M.R. (1984) Effect of lead on tetrahydrobiopterin levels in rat-brain. Proceedings of the Biochemical Society, Leeds 18-20 July, 1984, Biochem. Soc. Trans., 13, 204 - 205.

6. McIntosh, M.J., Meredith, P.A., Goldberg, A. & Moore, M.R. (1984) Alteration of dihydropteridine reductase activity by lead. Proceedings of the Biochemical Society, Stirling 6-7 September, 1984, Biochem. Soc. Trans., 13, 375 - 376.

7. McIntosh, M.J., Meredith, P.A., Moore, M.R. & Goldberg, A. (1985) Neurotoxic action of lead:effect on tetrahydrobiopterin metabolism in the rat. Comparative Biochemistry & Physiology, 81C, 227-231.

8. Moore, M.R., McIntosh, M.J. & Bushnell, I.W.R. (1986) The neurotoxicity of lead. In press.

Abbreviations

Ac CoA	acetyl-coenzyme A
Ach	acetylcholine
Ach E	acetylcholine esterase
ALA	5-aminolaevulinic acid
ALA D	5-aminolaevulinic acid dehydratase
ALA S	5-aminolaevulinic acid synthase
BH_4	5,6,7,8-tetrahydrobiopterin
BH_2	7,8-dihydrobiopterin
Bu Ch E	butyrylcholine esterase
Chat	choline acetyltransferase
Co A	coenzyme A
COMT	catechol-O-methyl transferase
DHPR	dihydropteridine reductase
DOMA	3,4-dihydroxymandelic acid
dopa	dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DOPEG/MHPG	3-methoxy-4-hydroxyphenylglycol
DOPET	3,4-dihydroxyphenylethanol
EDTA	disodium ethylenediaminetetraacetic acid
FAD	flavine adenine dinucleotide
GABA	γ-amino butyric acid
GTP	guanosine triphosphate
HPLC	high-pressure liquid chromatography
HVA	homovanillic acid
LCEC	HPLC with electrochemical detection
MAO	monoamine oxidase
MOPET	3-methoxy-4-hydroxy-phenylethanol
NADH	reduced nicotinamide adenine dinucleotide

NADPH	reduced nicotinamide adenine dinucleotide phosphate
ODS	octadecasilane
PBG	porphobilinogen
PNMT	phenylethanolamine N-methyl transferase
qBH_2	quinonoid dihydrobiopterin
SAM	S-adenosyl methionine
VMA	Vanillyl mandelic acid

CHAPTER 1

INTRODUCTION

1.1 Historical aspects of lead : Uses and abuses past and present

Lead, a soft, bluish-grey substance is one of the seven metals known to man since antiquity, the others being copper, gold, mercury, silver, tin and zinc. It s discovery dates back around 6,000 years. All lead is ultimately derived from the earth's crust where it exists principally as the sulphide ore, galena (Pb S) but also as cerussite, lead carbonate ($PbCO_3$); anglesite, lead sulphate ($PbSo_4$) and many other ores produced as a result of weathering of galena. The discovery of lead may well have resulted from the burning of galena in a camp fire (Waldron, 1973). On burning, the galena would have been reduced to the oxide, litharge (Pb 0) which in turn could react with unchanged galena resulting in metallic lead being recovered from the ashes (equation 1.1).

$$2Pb\,0 + Pb\,S \text{ ------> } 3Pb + SO_2 \qquad \text{Equation 1.1}$$

The metal's history dates back to the Egyptian hieroglyphics of around 1500 B.C. and both lead plate and statues were discovered in the tomb of Rameses III. There are references to lead in the Old Testament; the Israelites included lead amongst the spoil taken from the Midianites in Mosaic times (Numbers, 31.22) -

'Only the gold, and the silver, the brass, the iron, the tin, and the lead,'

Subsequently, Ezekiel described lead as a major item of trade with the Phonecians (Ezekiel, 27.12) -

'Tarshish was thy merchant by reason of the multitude of all kind of riches; with silver, iron, tin, and lead, they traded in thy fairs.'

Historically, the desire for silver was the principal stimulus for lead production in Roman and pre-Roman times. Galena, which was readily refined, provided a plentiful source of silver. The silver bearing lead ore once heated to its oxide, was absorbed onto a bulk material such as bone ash, leaving behind the precious noble silver. This process termed cupellation was alluded to by the Prophet in the Book of Jeremiah (Jeremiah, 6.29-30) -

'The bellows are burned, the lead is consumed of the fire; the founder melteth in vain: for the wicked are not plucked away. Reprobate silver shall men call them, because the lord hath rejected them.'

The importance of silver is born by the knowledge that lead mines were often termed silver mines. Mines in Asia Minor were rich in silver-lead ore, although galena mined in Britain contained considerably less silver (Tylecote, 1964).

Evidence exists suggesting the widespread use of lead by many ancient civilisations (Waldron & Stofen, 1974). Mediterranean cultures frequently employed lead in a variety of manners; for building, fishing, warfare, and many other purposes. Its use has also been implicated in India, China and Pre-Columbian Mexico.

The production of lead has steadily risen since the discovery of cupellation (Settle & Patterson, 1980) with peaks in production coinciding with the Roman era and the Industrial Revolution. Although utilized extensively throughout the ancient cultures, lead remained in the background and came to the fore only when the Romans devised elaborate projects for providing their towns and

houses with water. (Boulakia, 1972). The majority of their lead production was used to construct aquaducts and water mains. Lead pipes were constructed by soldering sheets of lead around a wooden mould whilst water reservoirs consisted of lead lined wooden tanks, a practice learned from the Greeks who collected rain water using lead lined roofs and stored olive oil in lead lined vessels (Waldron & Stofen, 1974). Lead pipes constructed 2,000 years ago have been excavated in Pompeii, Rome and Bath, and remain in working order today. The vast mining activities of ancient Rome were carried out by citizens drawn from the ranks of prisoners of war and criminals who were sent down the mines as an alternative form of capital punishment to the amphitheatre (Eisenger, 1977).

The use of lead drinking water systems certainly represented a hazard to health for Roman citizens, as indeed Vitruvius recognised in the first century B.C. (Waldron, 1973), but a far greater risk existed to the Romans. The practice of 'leading' wines can be traced with certainty to ancient Rome, where the empirical discovery was made that lead ions (Pb^{2+}) have a sweet taste and were thus added to wines in order to improve the flavour and colour and also to make sour wines more palatable and saleable. The syrup, containing lead ions, was prepared by boiling down must (unfermented grape juice). According to the degree of reduction of the must, the syrup was termed sapa, defrutum, hepsema, car(o)enum or siraeum (sireion). Many ancients have detailed the preparation of this 'sapa', such as Pliny and Columella (Eisenger, 1982). Essentially,

the important point was that the must was reduced in volume to approximately one third by heating over a slow fire in a copper vessel coated with stagnum which was a lead/silver alloy. The Romans lacked sterile conditions for fermenting and storing wines and therefore these leaded wines also had the additional property of containing a potent fungicide, namely a high concentration of lead ions, well known inhibitors of enzymatic activity. Sapa, prepared according to the detailed recipies in existence, has been prepared and observed to contain 1mg lead/ml (Eisenger, 1977; 1982). It is evident that merely one 5ml teaspoon of such a syrup would be adequate to cause chronic lead poisoning. The various recipies for adulteration of wine vary considerably but most call for a 1:48 dilution of the wine with sapa (Eisenger, 1982). The Romans and Greeks were also known to employ lead lining of their bronze pots to mask the bitter taste obtained in food cooked in such vessels. Many leaden pots have been recovered from the ruins of Pompeii, Olynthus and other cities (Nraigu, 1983). The Romans did not have access to sugar and as a substitute they employed sapa for cooking purposes. The Apician Cookbook of Roman recipies makes use of defrutum or caroenum as an ingredient in some 20% of the recipies (Nraigu, 1983). It was especially popular as an ingredient for meats and sauces (Eisenger, 1982). Lead salts have been commonly employed as colouring agents in spices, sweets, cakes and cheese (Hilburn, 1970) and the sweetness of sapa also led to its use as a vehicle for medicines.

The enthusiasm of Romans, especially the aristocracy for drinking wines is legendary, the consumption being especially collosal during the days of the Empire in which even women began consuming alcohol. Considering this prodigious consumption (Nraigu, 1983) and the fact that the practice of leading wines was widespread, a practice which Pliny complained of repeatedly (Eisenger, 1982), it is not surprising that lead poisoning was endemic in ancient Rome (Waldron, 1973). Indeed, it has been proposed that in view of the widespread sterility amongst the aristocracy of Rome (Waldron, 1973) and their reduced lifespan, lead poisoning was a contributing factor in the decline of the Roman race and the eventual fall of the Roman Empire. Certainly many emperors exhibited signs and symptoms consistent with those of lead poisoning. Claudius is an example of such an emperor (Nraigu, 1983) although the pathogenesis of his ailments is a matter of debate (Moss, 1963).

It is far from clear when the first observations were made of the potential toxic effects of lead to cause a condition which was also known under alternate names to lead poisoning such as plumbism or saturnism. However, certainly by the time of Hippocrates the clinical symptoms of the disease were accurately described (Waldron, 1973). It is generally accepted although not universally, that Hippocrates was the first of the ancients to attribute severe colic in a man who extracted metals to lead in 370 B.C. (Hunter, 1962). The first unquestioned clinical account of lead poisoning arising from the ingestion of cerusse and litharge must be accredited to the Greek

physician Nicander in the second century B.C. (Waldron, 1973; Gloag, 1980). Dioscorides in the first century B.C. gave a full but accurate description of the ill effects of consuming litharge (Hunter, 1962; Waldron, 1973). Both Dioscorides and Pliny recognised the dangers of consuming leaded wines and warned the citizens but alas to no avail. Pliny echoed the words of Dioscorides who wrote that corrected wine was 'most hurtful to the nerves' when he stated that 'from the excessive use of such wines arise dangling ... paralytic hands.' (Waldron, 1973).

Following the fall of the Roman Empire in the fourth century, the use of lead declined and remained at a low level for around 600 years (Settle & Patterson, 1980). However, the practice of sweetening wines using lead or lead salts continued until well into the eighteenth century despite a decree banning the practice by Imperial Law in 1498. As a result endemics of colic ensued frequently. The most famous incident of a colic epidemic resulting from the adulteration of wine to improve the quality was described by Francis Citois in the sixteenth century when he described what was termed the colic of Poitou or Colica Pictonum (Waldron, 1970; Eisenger, 1982). Colic termed the 'West Indian dry-gripes' was described in the early days of the American colonies (Eighteenth century), the cause of which was traced to the use of lead condensers in rum stills and to the use of pewter and glazed earthenware with a high lead content (McCord, 1953). Port, drunk liberally by the English gentry of the eighteenth and nineteenth centuries was also known to contain a considerable quantity

of lead (McCord, 1953; Ball, 1971).

As a result of the more scientific outlook of the fifteenth century onwards, which contrasted with the anti-scientific attitude of the ancient world, occupational physicians emerged. During the Renaissance of the seventeenth century, Samuel Stockhausen became concerned with the health of lead miners. However, the aetiology of the frequent colic attacks was not recognised until 1696 when Eberhard Glockel, a German physician, influenced by Stockhausen's work, associated the leadening of wines with the symptoms of colic (Eisenger, 1977).

The 'Devonshire Colic' was a disease of the cider counties of England during the eighteenth century, occurring relatively infrequently in Hereford, Gloucester, Worcester, Somerset and Cornwall, it acquired endemic proportions only in Devon itself, being first recognised by William Musgrave in 1703. There is little doubt from Musgrave's description of the illness, he is describing lead poisoning. The disease, as considered by John Huxham in 1739, was due to the consumption of cider which was incompletely fermented. However, some thirty years later in 1767, Sir George Baker diagnosed the Devonshire colic as being caused by lead poisoning (Baker, 1768). The source of this lead however became a matter of great debate. Whilst Baker considered that the vats used for preparing the cider in Devon provided the source of lead, James Hardy blamed the use of glazed earthen jugs employed for storage of such cider. Uptake of lead from such vessels had been predicted by Lind in 1754. Baker designed elaborate experiments and

demonstrated the presence of lead in the cider fermented in Devon while he could find none in the cider made in neighbouring counties (Singer & Underwood, 1962). Although Baker had many critics in his time, with opposition coming from both other physicians and the cider manufacturers who had great difficulty accepting the notion that such trivial quantities of lead could cause such harmful effects, it is now accepted that he had diagnosed the source of lead correctly (Waldron, 1970).

With the onset of the Industrial Revolution the uses to which lead was put increased and hence the demand for the metal rose dramatically. This increase in lead mining resulted in Tanquerel des Planches in 1839 publishing in Paris extensive studies describing the clinical signs and symptoms of chronic lead poisoning, a disease described in the eighteenth century in the leadhills of Scotland as the 'millreek'. Despite this work, legislation to protect workers in Britain was not introduced until the Factory Act of 1864 with further Acts in 1878 and 1883.

Factory inspectors were appointed to investigate industrial hazards especially lead, & Owing to the work carried out by Dr. Thomas Legge, the first medical inspector of factories, lead poisoning eventually became a notifiable disease in 1899. This approach has been an effective one, as despite a steady rise in lead consumption during the first half of the twentieth century, the incidence of lead poisoning has fallen.

Currently in the twentieth century, the uses of lead continue to be wide and varied, due to the diverse properties of the metal (Hilburn, 1970; Waldron & Stofen, 1974). Lead ores are mined principally in the United States of America and the Soviet Union, although mining in Australia, Canada, Mexico, Peru and many other parts of the world account for a considerable output. Metal sheets are used in roofing and as a protective screening against X-rays and radioactive emissions. The use of lead pipes, which was common practice in the first half of the twentieth century, is now declining in favour of copper or polyethylene piping to convey drinking water. Alloys of lead are employed as solder for metal food containers, whilst lead oxides and napthanates remain components of many paints for use on the outside of buildings. The incorporation of lead pigments in paint for the interior of buildings is diminishing as a result of government legislation, although much high lead-containing paint can still be observed in older housing built before the second world war. The use of lead arsenates as insecticides is also declining due to competition from organic insecticides.

The advent of the motor car brought about a new and dramatic increase in lead usage. Firstly, as a component of the lead-acid storage battery, and secondly, as a result of the discovery of the organolead 'anti-knock' additive for petrol, tetraethyl lead (TEL) by the American industrial chemist, Midgley in 1921. The incorporation of tetraethyl lead into petroleum fuels effectively increased the octane

rating, a useful property considering the comparatively primitive refining technology of the 1920's and 1930's. In 1923 the Ethyl Corporation in the United States of America commercialised this discovery. In 1940 tetraethyl lead was introduced into petrol in Great Britain while the related alkyl lead, tetramethyl lead (TML) has been employed since 1960. The modern internal combustion engine also employs lead solders and bearings in its construction. The storage battery and the lead alkyl manufacturing industries represent a large proportion of the lead consumers at the present time (Waldron & Stofen, 1974; Ratcliffe, 1981). Currently, legislation is emerging in several countries to either reduce the level of lead permissable in petroleum or remove it altogether.

The diverse properties of lead, namely its comparative ease of extraction from its ores, softness, malleability, density, resistance to corrosion, and chemical and electrical resistance, will no doubt result in a continued use of the metal long into the future.

1.2 Sources of lead exposure

1.2.1 Food

It is generally accepted that for the majority of the population alimentary exposure is the primary means by which man is exposed to lead, and it is by this route that the majority of lead is absorbed (DHSS, 1980). However, less certain are the routes by which the diet becomes exposed to and contaminated with lead, and the relative proportions of input from air, soil and water. Contamination of food with lead can occur via several routes (figure 1.1). Firstly, from the soil in which the plants are grown as a result of the natural weathering of lead minerals and ores. Over and above, lead present in the air can lead to the contamination of soil. The second route is by way of the air by direct deposition onto the crops and plants. The third vector of lead contamination of food results from positive uptake from lead -containing water during cooking, and lastly, food becomes contaminated from the vessels it is stored in. The use of old pewter and lead pottery glazes has long since been a problem. More commonly, tin cans can contain a considerable quantity of lead solder in their construction.

Plants are capable of absorbing soluble lead through their roots and studies have demonstrated a relationship between the lead content of plant roots and that of soil (MAFF, 1975). Translocation from the roots to the shoots and leaves is however very limited and hence the root regions exhibit a much higher lead concentration than the aerial regions of the plant (Motto et al, 1970). The nature

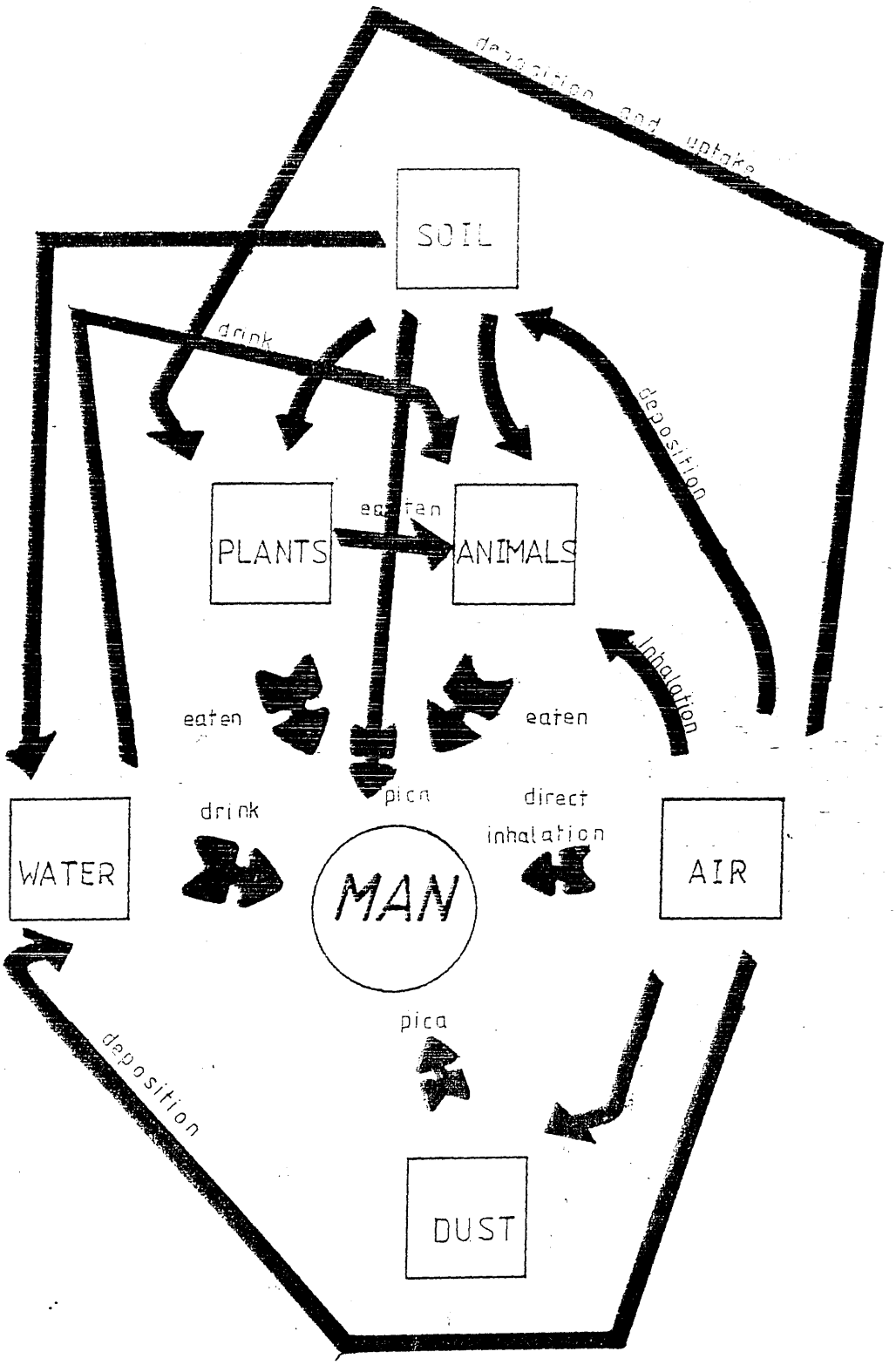


Figure 1.1 Major vectors for lead uptake by man.

Schematic representation of the major routes by which man is exposed to lead in the environment.

of the soil determines to some extent the degree of lead uptake. Acidic soils and those containing a low humus content favour lead uptake by plants (McLean, Halstead & Finn, 1969; Zimdahl & Skogerboe, 1979) since organic matter can bind the lead rendering it not bioavailable. Soil lead content varies with soil depth, with higher concentrations being found in the upper layers (Healy & Aslam, 1980) and thus poses more of a problem for those plants with shallow roots. In addition, the quantity varies with distance from the roadside, and therefore from motor exhaust fumes; the closer to the road, the greater the lead level (Warren, 1959; Healy & Aslam, 1980). Surface soils in regions close to roads and large cities exhibit raised lead levels (NAS, 1972). Areas of mining activity also have been shown to be associated with raised levels of lead in the plants (Alloway & Davies, 1971). A less common source of lead in crops today, but one which can still be a problem, is the use of lead arsenate crop sprays.

Airborne lead is an important source of lead found in the aerial parts of crops (MAFF, 1975) which is only of importance in relation to those plants whose leaves and shoots are eaten. In this context, the outer leaves of certain crops, for example cabbage, will be more contaminated with lead than those nearer the heart. In contrast, with crops whose leaves permit a free circulation of air, such as lettuce, spinach and brocolli, there will be little benefit in discarding the outermost leaves. A considerable body of evidence exists to suggest that even crops grown in farmland or mountain regions distant from

mainroads and industrial sources are contaminated with lead to a considerable degree (Hirao & Patterson, 1974; Tjell et al, 1979). Although government control is in existence to limit the lead content of commercially produced crops, many such items are produced by the consumer on his own land and thus are under no such controls. In addition, fruit growing along the roadside, commonly picked for jam making, often may contain a considerable quantity of lead which will greatly increase in concentration during jam preparation.

Although the lead content of crops can be reduced to a varying degree by thorough washing of the items before cooking, the actual process of cooking can significantly increase the lead content of food items (Moore et al, 1979b). The cooking process will denature the protein making available ligands suitable for binding of lead to sulphydryl, cysteine, methionine, carboxyl or imidazole moieties. By a similar process the lead concentration of beverages such as tea and coffee diminishes during preparation since in these cases tea leaves and coffee grounds bind significant quantities of lead from the water thus lowering the lead concentration of the beverage. It is of course evident that the importance of water as a vector for the lead content of food will depend on the level of the metal in the domestic water supply. It has been calculated that the proportion of lead in food which would have come from water will be about 15% when the water contains in excess of 0.5μM (100μg/l), whereas it would only be around 3% if the concentration were 0.1μM (20μg/l) - (DHSS,1980).

Considerable contamination of canned food can occur especially where the food is acidic in nature. Lead is still employed as solder for tin cans, and in addition, the tin plate employed also contains a limited quantity of lead although less than 0.08%. Legislation maintains the quantity of lead in canned food to a level not exceeding 2mg/kg; the equivalent value for fresh food being 1mg/kg. Today however, the use of lead soldered cans is declining in favour of welded cans. Various calculations exist as to the contribution of lead in canned food to total lead exposure (MAFF, 1983) but enormous individual variation in this figure obviously exists. The figure is generally around 15% but may well be as high as 60% if the diet contains a significant quantity of canned items. Presently lead is not employed in cans to be used for infant foods. Legislation dating from 1979 requires the lead content of such food to less than 0.2mg/kg.

It must be remembered that despite the presence of lead in the soil, crops and forage, and its incorporation into mans' food chain, either directly, or by way of grazing cattle and sheep, only a proportion of the lead taken up at each step in the chain is bioavailable. Firstly, atmospheric lead will settle directly only on the aerial parts of plants and so is important only in relation to foods where the leaves and shoots are eaten. Secondly, only a proportion of lead in the soil is available for uptake depending on factors such as the depth of the roots and type of soil. Of the lead absorbed by plants, only a small proportion is available to grazing animals, and indeed,

only a fraction of that consumed by the animals is absorbed. Finally, of the lead which does pass into the body of the animal, the great majority will be stored in the skeleton.

Despite the potential for significant contamination of foodstuffs with lead, the average lead intake in the United Kingdom is well within the recommended limits set out by the FAO/WHO joint committee on food additives of 3mg lead per week which is equivalent to 430µg/day. In a survey carried out in this country, the average diet contained 0.43µmol/kg (MAFF,1975) which was equivalent to an average weekly intake of 1mg. Duplicate diet techniques calculated the lead intake to range between 21-330 µg/day with a mean of 75 µg/day. This was comparable with total diet studies which showed the diets to contain 55-366 µg/day with a mean of 113µg/day. Regulation is in force to limit the quantity of lead in food. The lead in food regulations of 1979 restricts the amount to a maximum of 1ppm in fresh foods and 0.2ppm in food intended for infant consumption.

1.2.2 Water

In areas where limestone and galena ores are found, natural waters may contain lead in solution. Additional lead may be added to drinking water as it flows from the bulk supply which rarely contains significant quantities of lead. It has long been recognised that domestic drinking water supplies can be appreciably contaminated with lead and so represent a significant source of the metal. Numerous studies have unequivocally shown that soft, acidic water supplied to older housing which have lead plumbing

systems, not only in the form of lead piping and lead-lined storage tanks, but also where excess flux has been employed as solder for copper piping, will result in significant dissolution of lead and therefore to increased bodily uptake by the inhabitants (Bacon et al, 1967; Beattie et al, 1972a; Covell, 1975). The significance of this was suggested over a century ago by Professor Christison in 1844 and has recently been emphasised by the 'Lawther report' (DHSS, 1980). Housing built before the 1960's tends to contain lead piping to varying degrees and it is this older housing which has been associated with an increased domestic water lead content (Beattie et al, 1972b; Addis & Moore, 1974; Moore, 1977; Moore et al, 1978). Indeed the lead content of the water is related to the length of lead plumbing employed (Beattie et al, 1972b). Markedly raised blood lead levels have been found in subjects consuming water with a high lead content. Initial studies assumed a linear relationship between water lead and blood lead concentrations (Addis & Moore, 1974). Subsequent studies have demonstrated that blood lead concentration is a function of the cube root of water lead concentration (Moore et al, 1977a; 1979a; 1981a/b; Moore, 1978; Thomas et al, 1979; DHSS, 1980; Lacey et al, 1985). A raised level of lead in the domestic water supply has also been shown to be associated with a reduction in the activity of 5-amino laevulinic acid dehydrase (ALA D), a biological indicator of lead exposure (Beattie et al, 1972b; Addis & Moore, 1974) and a raised molar tooth lead content (Moore et al, 1978a). Stewart (1974) also observed an increased tooth

lead level in inhabitants of older houses with lead plumbing. Replacement of lead piping can result in a dramatic reduction in blood lead levels (Thomas et al, 1979).

The degree of plumbosolvency is related to the water pH and hardness or calcium concentration (Weston, 1920; Moore, 1973). Although a soft, acidic water supply is more plumbosolvent, hard water will also dissolve lead (DOE, 1977). However hard water does protect against lead dissolution by forming a protective layer of calcium salts over the pipe interior surface (Crawford & Morris, 1967). Plumbosolvency can be markedly decreased either by a raising of the pH or hardness (Moore, 1973), by for example addition of calcium hydroxide as lime. Data obtained by Thomas and his colleagues (Thomas et al, 1981) suggests that the availability of lead in hard water areas may be considerably less than in soft water regions. Thus the lead content of the domestic water supply is determined by the quantity of lead present in the supply, the chemical characteristics of the supply, and the residence time of the water in the conveyancing system. First flush water contains significantly greater quantities of lead than running water (Moore et al, 1979a; Thomas et al, 1979). A reduction in the blood lead concentration in a matched group of the population between 1976 and 1980 has been attributed to a fall in water plumbosolvency (Moore et al, 1981a/b). There have been a number of cases of lead poisoning resulting from the ingestion of lead contaminated water in recent years (Bacon et al, 1967; Beattie et al,

1972a). In addition, the lead content of water has been related to various disease states (Goldberg, 1974; Moore, 1977) especially mental retardation (Beattie et al, 1975b), kidney pathologies (Campbell et al, 1977a), increased incidence of renal insufficiency and gout (Campbell et al, 1978) and cardiac effects (Moore et al, 1975; Beevers et al, 1976). Perhaps the most vulnerable members of the population with respect to lead in water are babies and young children who are receiving dried milk foods reconstituted with tap water which may contain a high level of lead. Breast fed young will be protected from this source of lead to some extent as maternal milk contains only 10% of the lead concentration of the maternal blood (Moore et al, 1982). Indeed, breast fed infants have been shown to receive less lead in the diet than those fed on bottle milk (DOE, 1982; Sherlock et al, 1982).

Finally it must be remembered that exposure to lead from water comes not only as a consequence of drinking the water, but also, as was discussed earlier, from the use of such water in food preparation.

1.2.3 Air

The third major source of lead exposure for the general population is air. Lead in the environment, which has dramatically increased since ancient times (Patterson, 1983), can be derived from geological sources or individual emissions such as smelting and refining (NAS, 1980; Nraigu, 1980). However, it is generally agreed that the majority of air lead today is derived from the combustion of petroleum, with probably as much as 90% originating from this source

(NAS, 1972; Ewing & Pearson, 1974; Nraigu, 1979; DHSS, 1980). As a result of public interest in lead emitted from petroleum a wealth of data is available concerning the influence of air lead to body lead burden (Chamberlain, 1985).

Downwind pollution from local industrial sources although not the main source of air lead, can create important 'hot spots' of concern extending over a considerable area. Several regions have been identified where excessive exposure of the local community exists (Lansdown et al, 1974; Landrigan et al, 1975a). These sources of lead create three types of lead particle. Firstly, microparticulate lead aerosols with a lifetime of around 7.3 days and which can thus travel great distances from their source. Secondly, relatively large particles typically produced by industrial sources (Roberts et al, 1974) which tend to sediment out rapidly along with weathered paint as dust (Stark et al, 1982). The last group comprises the lead alkyl vapours arising from petroleum. The chemical and physical form of the lead varies widely according to the source, that emitted from petroleum varying considerably in size from 0.01μm up to several millimetres (Hirschler & Gilbert, 1964) with the vast majority being less than 0.1μm (Facchetti, 1979), and thus readily absorbed by respiration (Morrow et al, 1980).

It is not surprising considering the foregoing discussion, that the highest levels of air lead are found in the urban environment, this being a function of traffic density and flow (DHSS, 1980; NAS, 1980). The level of lead

in the blood has also been shown to be higher in the residents of an urban community compared to those people living in regions of lesser traffic flow (Johnston, Tillery & Prevost, 1975). There exists an abundance of evidence supporting the importance of petroleum combustion to air lead. Firstly, it has been demonstrated on several occasions that air lead concentration falls off rapidly with distance from the roadway (Daines, Motto & Chilko, 1970; Schuck & Locks, 1970; Smith, 1976; DHSS, 1980), the relationship being observed to be curvilinear (DHSS, 1980). In addition, soil lead content has long been shown to be related to the distance from the roadway (Smith, 1976; Healy & Aslam, 1980). Daines et al (1970) have observed a reduction in the particle size of lead as one moves away from the roadside, indicating a settling out of larger particles close to the road. Indirect evidence exists from a study carried out in the vicinity of Gravely Hill Motorway Interchange at Birmingham which is commonly known as Spagetti Junction (Waldron, 1975). This study demonstrated a significant increase in blood lead levels in the community during the first twenty months after the network was opened compared to the time prior to its opening.

In general, considering that the dissipation and precipitation of an emitted lead aerosol is dependent on particle size and meteorological factors, approximately half the lead emitted is removed by gravity within a few hundred feet of a roadway (Daines et al, 1970). With regard to petroleum lead emission, several other factors over and

above the roadway, determine the lead content of the air. Such factors include the volume of traffic (Daines et al, 1970), prevailing winds (Daines et al, 1970; Schuck & Lock, 1970; Smith, 1971), season of the year , with autumn exhibiting the greatest level (Chow & Earl, 1970; Daines et al, 1970), and diurnal rhythm where a peak occurs in the early morning and late afternoon coinciding with the times of greatest traffic flow.

In Germany where there has recently been a reduction in the lead content of petrol from 0.4 to 0.15g/l, a concurrent reduction in kerbside air lead and blood lead concentration of the inhabitants of Frankfurt has been observed (Sinn, 1980; 1981). There was a much smaller reduction in air lead in areas more distant from the roads. Although a reduction in blood lead concentrations has been demonstrated in populations in the United States of America where there has been a fall in lead in petroleum (Billick, Curran & Shiers, 1979; 1980; Annest, 1983; Billick, 1983), there has been a similar fall in blood lead levels in this country with no alteration to petrol (Oxley, 1982). Interesting data emerges from a study carried out in Turin, Italy during the 1970's and early 1980's. This experiment involved the labelling of petroleum on a regional scale, the lead isotope composition (206/207) being sufficiently different from local natural sources to allow differentiation. Lead from the Broken Hill mine in Australia with an isotope ratio of 1.04 was substituted for that found locally which was of ratio 1.18. The results indicated that as much as 95% of air lead at the kerbside

but not necessarily elsewhere is derived from petroleum (Faccetti, 1979; Faccetti et al, 1982).

Although there exists industrial emissions and ubiquitous air emissions, few people are exposed to long term air concentrations in excess of $1\mu g/m^3$. Even a congested British street typically exhibits an air lead concentration less than 24 nmoles/m^3 (Lawther et al, 1973) and the levels have been demonstrated to be much less inside than outside buildings (Daines et al, 1972). However, several local 'hot-spots' have been recognised. Examples of such industrial activity are demolition work, metal recovery in scrap yards, and the burning of old lead-acid battery cases. Lead contamination has been demonstrated over an area of 5 km^2 in El-Paso, Texas, with soil being polluted up to a distance of 10km from the source (Landrigan et al, 1975a). Following engineering work to reduce the lead emitted from this plant, there was a significant reduction in the blood lead concentration of a child living in the area (Morse et al, 1979). Similar problems have been documented in Toronto, Canada (Roberts et al, 1974; Yankel, Von Lindern & Walter, 1977). The children of workers from these plants experienced increased lead exposure via their parent's clothing becoming contaminated (Baker et al, 1976; Rice et al, 1978). Local sources often create the greatest problem where children are concerned (Duggan & Williams, 1977; Duggan, 1982; Russell-Jones & Stephens, 1982), as they tend to exhibit a lot of hand to mouth activity. In this respect both dust and soil lead are related to blood lead levels in children

(Barltrop et al, 1975; Yankel et al, 1977; Schmitt et al, 1979; Walter, Yankel & Von Lindern, 1980; Stark et al, 1982). A raised blood lead concentration has also been associated with an increased lead content on the hands (Roels et al, 1980; Charney, Sayre & Coulter, 1980) and such hand lead content can attain significant levels especially in urban children (Vostial, Taves & Sayre, 1974; Roels et al, 1980). It must also be remembered, as discussed in the previous section, that there is an inter-relationship between lead in air and in food. Sedimentation from the atmosphere results in lead contamination of vegetation either directly or via soil. Although the greatest danger of exposure to lead from the air occurs in industrialised or urban regions, more remote areas will also exhibit aerial lead. Although still a problem, the danger from petrol is on the wane. Presently the level of lead in this fuel is being reduced in several countries - U.S.A. (Annest, 1983), Japan (Ohi et al, 1981) and Europe (Sinn, 1981; DOE, 1983) but it still remains a major source in Britain.

1.2.4 Adventitious sources

Although the vast majority of the population derive their body lead burden from the food and water which they ingest, and the air in the environment, particular families or individuals may be exposed to sources of lead peculiar to a local environment or a specific product. There are several recognised sources (DHSS, 1980) which are almost invariably encountered by ingestion.

1.2.4.1 Paint

Lead compounds have long been used in the manufacture of paints, initially as one of the major ingredients, and subsequently as pigments using red and white lead, or alternatively lead napthanate and octoate which are used as driers for oil-based paints. Children living in older property with flaking paint are especially vulnerable, particularly if they are given to the habit of 'pica' (Barltrop, 1968), which is generally defined as a craving for unnatural articles of food (Ruddock, 1924; DHSS, 1980). As a result of legislation, this hazard is greatly reduced today. In accordance with an EEC directive all paints containing in excess of 0.5% lead must be appropriately labelled. In addition, a stringent level of less than 0.25% has been set for paints employed on toys, while not greater than 0.025% is permissible in paint intended for use on pencils, brushes, pens and other such items (DHSS, 1980). Children remain however, subject to exposure to leaded paints on exterior surfaces where leaded paint is generally employed because of its reputation for giving greater protection against weathering. Often old paint containing lead is not completely removed before the surface is repainted. Merely repainting over leaded paint is not adequate as children frequently indulge in chewing articles. It has been estimated that a single flake of paint of around 2-3mm in diameter can contain 1mg of lead and ingestion of such paint is common in children, accounting for one third of all cases of severe childhood lead poisoning in Britain (Barltrop, Burman & Tucker,

1976). However, data relating to rats suggests the bioavailability of such lead is low (Gage & Litchfield, 1969). In the adult population, there exists industrial hazards relating to paint. Oxy-acetylene burning of steel structures coated with many layers of lead- containing paint (Taylor, Molyneux & Blackadder, 1974; Campbell & Baird, 1977) has been shown to result in lead poisoning in some cases. Additionally, the clinical entity of lead poisoning in artists often known as 'painter's colic' has been recognised for many years. Although the artists paint 'lead white', which contains basic lead carbonate has been largely superseded by titanium white, there are still cases of lead poisoning from this source (Graham, Maxton & Twort, 1981).

1.2.4.2 Cosmetics and hair products

Many eye cosmetics from the Indian sub-continent contain lead and are frequently used on the eyes of not only adults, but also children and infants. These preparations, termed the 'khols' or 'surmas', were previously formulated from antimony sulphide but due to economic factors are now prepared from lead sulphides. Many grey and black surmas may contain up to 83% lead (Aslam, Davis & Healy, 1979). The Asian communities of Britain are particularly at risk. Although the production of such preparations is illegal in this country (EEC, 1976), the relatives of Asian families continue to bring the products into the country (Pearl, 1977).

In addition to cosmetics, some hair darkening products contain lead acetate in concentrations ranging from 0.26-

3.13% (DHSS, 1980). On contact with biological materials, dark grey to black insoluble lead-sulphur compounds form with the sulphur containing residues on amino acids of hair keratin. Such hair products must, by law, contain no more than 3% lead and must be labelled with an appropriate warning to buyers concerning their misuse.

The potential exists with these cosmetics and hair dyes, for their absorption both orally and by the percutaneous routes. However, Moore et al (1980b) have demonstrated that only around 3% of an applied dose is absorbed through the skin. The FDA (1980) however, noted that the preparations should not be used on broken skin and that they should be kept out of reach of children who are in danger of oral exposure. Within Asian and Indian communities, several cases of lead poisoning have been reported (Pearl, 1977; Ali et al, 1978; Waldron, 1979b) and it has been observed that immigrant children who use surma have significantly higher blood lead levels than children who do not (Josephs, 1977; Ali et al, 1978; Aslam et al, 1980), and indeed individuals of Asian extraction have raised blood lead concentrations over others (Waldron 1979a). This problem would appear to be a common one throughout the Moslem world (Fernando et al, 1981).

1.2.4.3 Unlicenced medicines

Lead was first introduced into the Western Pharmacopoea in the 15th century A.D. by Paraceleus (Singer & Underwood, 1962) who employed lead and opium pills to treat diarrhoea but in doing so produced several cases of lead poisoning. Recently, in Glasgow, an outbreak of lead

poisoning resulted from the intra-venous injection of such preparations by a group of drug addicts (Beattie et al, 1975). Fortunately such preparations are no longer included in the British Pharmacopoea (Singer & Underwood, 1962). However, several alternative medicines still contain lead to varying degrees. Typical of these are the aphrodisiacs and other preparations from India (Brearly & Forsyth, 1978; Aslam et al, 1979), and Chinese herbal medicines.

1.2.4.4 Domestic utensils

Domestic utensils and vessels used for the preparation of foods and beverages can, under certain circumstances, release substantial amounts of lead. Pottery with glazes imperfectly produced, are a particular hazard when coated on vessels employed for the storage of acidic fluids such as fermenting and storing beers, ciders and wines, or storage of vinegars, fruits, fruit juices, pickles and preservatives. A similar situation arises with traditional pewter mugs commonly employed for beer, although modern pewter does not represent a hazard. This problem with glazed surfaces is quite common and lead poisoning from the use of such items can occur (Klein et al, 1970; Hughes, Horan & Powles, 1976). Other domestic utensils have also been found to be a source of lead, for example kettles (Wigle & Charlebois, 1978).

1.2.4.5 Adulteration of foodstuffs

This problem has been with us since antiquity as discussed earlier, and occasional problems still occur today. Power et al (1961) reported an outbreak of lead poisoning due to the use of lead chromate to improve the

appearance of tumeric. Also, certain preparations of calcium supplement derived from bone, have been associated with lead poisoning (Crosby, 1977). Older type mills have been noted to contain lead fillings which can contaminate the flour (Eisenberg et al, 1984).

1.2.4.6 Printing inks

The lead content of newspaper may be as high as 3.6mg/g especially on pages with multicoloured print. In addition several printed bags and wrappers employed for food contain lead salts and represent a possible hazard to children who tend to chew paper (Hankin, Heichel & Botsford, 1973; 1974a/b). The burning of newsprint in the fire can also release lead (DHSS, 1980).

1.2.4.7 Alcohol

Excessive alcohol consumption has been associated with an enhanced gastro-intestinal lead absorption which is caused by two features of alcoholism, namely a metabolic acidosis and poor nutrition. Over and above, certain alcoholic beverages have been known to contain excessive quantities of lead. Illegal American 'moonshine' whiskey often contains lead, as the stills employed in its production are often made from lead piping or even car radiators containing lead solder. This, in conjunction with the high acetic acid content of the still contents, results in the formation of lead acetate in the distillate (Patterson & Jermigan, 1969). Although British beers contain little lead (DHSS, 1980), many European wines contain considerable amounts of lead (Jaulmes, Hamelle & Roques 1960; WHO, 1977).

1.3 Absorption of lead

1.3.1 Gastro-intestinal absorption

It is generally accepted that uptake and retention of lead from the gut is far less complete than from the lungs. Animal studies have indicated that a significant proportion of lead is absorbed in the duodenum, transported through the liver, before being re-excreted into the gastro-intestinal tract together with bile. In vitro experiments also suggest that jejunal absorption of lead is greater than absorption from the duodenum or colon. An inverse relationship has been reported between particle size and gastro-intestinal absorption (Barltrop & Meek, 1975). A variety of studies based either on balance techniques or using both radioactive and stable isotope tracers have been devised to estimate the percentage absorption of lead from the gastro-intestinal tract, and a figure of 10-20% has been accepted (DHSS, 1980). The balance experiments of Kehoe and his colleagues (Kehoe, 1961) estimated that 5-10% of orally ingested lead was absorbed and retained, whilst Thompson (1971) estimated the figure to be 12% and Rabinowitz et al in 1976, using the rare stable isotope of lead - ^{204}Pb, obtained a value of 8.3%. However, higher percentages such as 40% have been calculated by other authors. Other studies using the ^{212}Pb isotope have demonstrated a wide range in absorption from 1.3-16% (Hursh & Suomela, 1968). This wide variation in absorption of lead was confirmed by Blake in 1976 who observed a figure of over 60% in one subject, and by Moore, Hughes & Goldberg (1979b) who calculated that 10.4-47.7% of ^{203}Pb was

absorbed in their subjects.

Data relating to young children indicates that the proportion of ingested lead absorbed is far greater than in adults. 56% of the lead ingested has been shown to be absorbed with 18% being retained (Alexander et al, 1973). Similarly, studies in rats have evolved a picture of newborn pups absorbing a greater percentage of lead than adults (Kostial et al, 1971; Forbes & Reina, 1972). The reason for this enhanced absorption is unclear, but it has been postulated that during maturation, the gut mucosal cells develop the ability to reject a number of multivalent cations including lead, strontium and iron, which the immature gut absorbs avidly (Forbes & Reina, 1972).

The mechanism of gastro-intestinal lead absorption has not been fully elucidated, but it has been proposed to comprise of two processes which is consistent with the intra- and extracellular transport routes put forward by Blair and his colleagues (Blair et al, 1979; Hilburn et al, 1980). There may be an active carrier mediated transport process, and in addition, a passive movement of lead by diffusion across the mucosa, which, it has been suggested, is linked to the concurrent movement of water (Coleman et al, 1978; Blair et al, 1979). Hilburn et al (1980) put forward a model of lead transport in which the amount of lead transported is governed by the initial tissue uptake of uncomplexed lead ions, which then passively diffuse across the epithelium by the extracellular route. It is possible that different methods of transport predominate depending on the concentration of lead, region of

intestine, presence or absence of dietary components, nutritional status or indeed the species.

In addition to age, the gastro-intestinal absorption of lead is dependant on many factors. Where the lead exists in the gut in a readily bioavailable form, many nutritional factors influence its absorption (Moore, 1979). The mineral content of the diet in relation to the absorption of lead has been widely investigated. Several animal studies have demonstrated a decreased absorption of lead as a result of a raised level of calcium in the diet (Tompsett, 1939; Sobel et al, 1940; Shields & Mitchell, 1941; Meredith et al, 1977b) and indeed supportive data has been obtained in man (Moore et al, 1978b). A similar effect is observed with reduced dietary phosphate (Lederer & Bing, 1940; Shields & Mitchell, 1941). The converse has also been demonstrated; an increased absorption of lead in animals is observed where there is a reduced level of dietary calcium (Shields & Mitchell, 1941; Six & Goyer, 1970; Mahaffey et al, 1973; Barltrop & Khoo, 1975; Meredith et al, 1977b; Barton et al, 1978a). It has been postulated by Meredith et al (1977b) that only one of the processes of lead transport is influenced by the level of lead in the diet. This interaction between lead and calcium is believed to be of a competitive nature with both entities competing for a common mucosal binding protein (Barton et al, 1978a). This theory is supported by data obtained by Lederer & Bing in 1940., who demonstrated that intra-peritoneally administered lead is not influenced by dietary calcium content. In addition, in vitro experiments, using rat duodenal tissue,

have shown an inhibitory effect of lead on calcium transport (Gruden et al, 1974), and using everted gut sac preparations Hilburn et al (1980) demonstrated enhanced lead transport into the serosal compartment in the absence of calcium. In contrast, systemic calcium levels do not influence lead retention (Meredith et al, 1977b). Calcium, required to maintain adhesion between cells appears to play an essential role in the maintenance of the tight junction by interacting with anionic groups on adjacent cellular membranes. This, being the route utilized by ionic lead to transverse the mucosa in a similar manner to other ions (Ussing et al, 1974), may well explain the mode of action of calcium on lead absorption. It has been suggested that any process which opens these junctions will enhance lead absorption (Coleman et al, 1978; Blair et al, 1979).

It has been adequately demonstrated in rats and mice that the compensatory increase in iron absorption as a result of iron deficiency is accompanied by a raised absorption of lead (Six & Goyer, 1972; Regan, 1977; Barton et al, 1978b; Hamilton, 1978; Flanegan et al, 1979). Similarly, iron deficient human subjects absorb two to three times more lead than normals (Watson et al, 1980). Raised dietary iron leads to a fall in lead absorption (Conrad & Barton, 1978). The mode of interaction is not clear at present, although it has been postulated that lead and iron share a common high molecular weight intestinal absorptive protein in a similar manner to lead and calcium (Conrad & Barton, 1978).

Lead absorption has been demonstrated to be enhanced in

the presence of a decreased dietary protein content (Baernstein & Grand, 1942; Six & Goyer, 1972; Barltrop & Khoo, 1975; Mylroie et al, 1977) whilst a diet rich in fat promotes absorption. The influence of lipid on lead absorption has been associated with increased phospholipid and bile flow stimulation). This view is supported by the observed reduction in lead absorption produced by cannulation of the bile duct in rats (Cikrt & Tichy, 1975).

There would appear to be a relationship between lead and vitamin D. Raised dietary vitamin D levels are associated with an increased lead absorption (Sobel et al, 1940; Sobel & Burger, 1955; Smith et al, 1978). The metabolite active in stimulating gastro-intestinal lead absorption is 1,25-dihydroxy cholecalciferol, in contrast to 25-hydroxy cholecalciferol which is inactive.

The effect of dietary zinc is to reduce lead absorption (Cerkiewski & Forbes, 1976) although some authors have demonstrated lead absorption to be independent of dietary zinc content (Barltrop & Khoo, 1975). Similarly, copper and magnesium reduce lead absorption. The influence of sulphur in the diet on lead absorption is more complex. Sulphur, when administered as methionine in the diet, reduces absorption whereas absorption is enhanced when cysteine is supplied (Quarterman et al, 1976).

In essence, an entity which will interfere with the intestinal barrier, for example ethanol, will promote lead absorption (Mahaffey et al, 1974). In addition, ascorbate and citrate by increasing the solubility of lead, promote absorption. Certain drugs altering gastric function will,

by reducing gastric mobility, lead to a reduced lead absorption. The mere presence of food in the gastro-intestinal tract has been shown to cause a reduced lead absorption (Garber & Wei, 1974; Heard & Chamberlain, 1982).

1.3.2 Pulmonary absorption

By comparison with the gastro-intestinal route of absorption of lead, uptake by the lungs is more complete. It has been estimated that around 35-55% of inhaled lead is absorbed and retained (Kehoe et al, 1961; Bingham et al, 1968; Hursh & Mercer, 1970; Chamberlain et al, 1975), although the percentage depends on several factors. Generally, the uptake of an inhaled particle is a function of the particle size, its solubility and the rate of respiration of the individual. The particle size will affect its deposition, and also determine the site in the respiratory tract at which it is deposited. Many studies have demonstrated an association between the size of the lead particle and its deposition in the lung (Kehoe, 1961; 1964; Hursh & Mercer, 1970). The respiratory tract comprises of three functional areas. Firstly, the nasopharynx; secondly, the tracheobronchial tree including the terminal bronchioles; and lastly, the pulmonary compartment which includes the respiratory bronchioles, alveolar ducts, atria, alveoli, and the alveolar sacs themselves. Only a minor fraction of particles of less than 0.5μm in diameter are retained in the nasopharynx or tracheobronchial tree, the remainder being trapped by mucus and cleared by ciliary action of the respiratory epithelial cells before being swallowed. As much as 40% of such

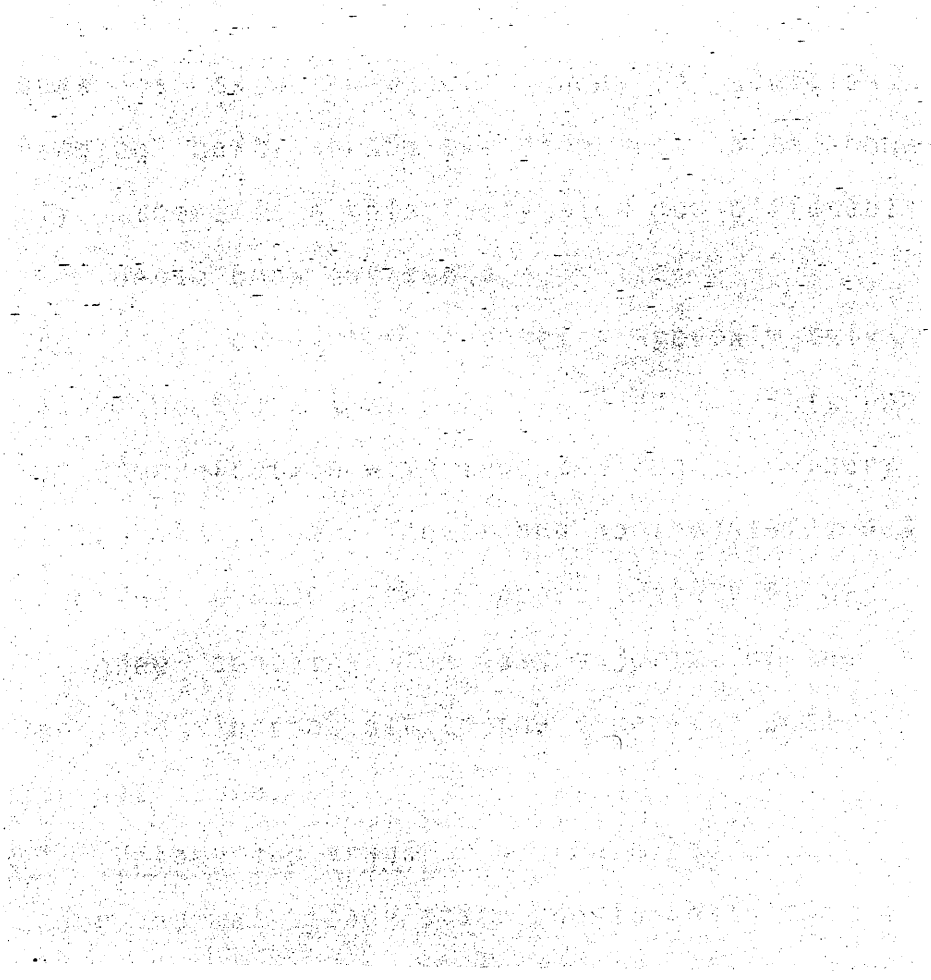
trapped large diameter particles may subsequently enter the gastro-intestinal tract in this manner (Kehoe, 1961). Alveolar cells may sequester the lead particles or they may be removed via lymphatic vessels to local lymph nodes. Dissolution of lead particles can also occur in the tissue fluids, following which they may pass into the blood stream at a rate proportional to their size and solubility. In this respect many lead compounds have a greater solubility in tissue fluids than in water (Waldron & Stofen, 1974). The rate of clearance of lead from the lungs has been calculated by several authors using radioisotopes, and has been found to be about 6-11 hours (Jacobi, 1964; Hursh & Mercer, 1970; Chamberlain et al, 1975).

1.3.3 Percutaneous absorption

The absorption of lead by percutaneous routes only attains significance in the case of organic compounds of lead, for example lead alkyls and naphthanates (Waldron & Stofen, 1974). The absorption of tetraethyl lead through the skin was adequately demonstrated not long after its discovery (Kehoe, 1927; Kehoe & Thamann, 1931) but evidence is sparse concerning the percutaneous absorption of inorganic lead compounds, and a significant proportion of the data which is available relates to animals whose skin structure is quite different from that of man. Lang & Kunze in 1948 demonstrated raised kidney lead levels following the application of various lead compounds - lead acetate, orthoarsenate, oleate and tetraethyl lead, to the bare skin of rats. However, only in the case of tetraethyl lead did the magnitude of the rise assume any significance. In 1976,

Rastogi & Clausen demonstrated the absorption of lead naphthanate through rat skin. Recently, it has been shown that the percutaneous route of absorption of inorganic lead as the acetate through human skin is infinitesimal (Moore et al, 1980b).

Considering the foregoing data, it is evident that with respect to inorganic lead, the percutaneous route of administration can be considered negligible.



1.4 Distribution of lead within the body

Following the absorption of lead via the routes previously described, it is transported via the blood stream to the various tissues and organs of the body. With regard to the distribution of lead, the total body burden can be divided into three compartments (Hilburn, 1970; Rabinowitz, Wetherill & Kopple, 1973; 1974a/b; 1976; - figure 1.2). Firstly, a lead pool which is rapidly exchangeable with other storage areas. This compartment comprises the blood, tissue fluids and most soft tissues which rapidly exchange lead with the blood. Secondly, an intermediate rate of exchange pool composing of such soft tissues as skin and muscle, and in addition, actively exchanging parts of the skeleton such as the bone marrow. Lastly, there is a relatively slow non-diffusable exchange pool of dense bone and teeth.

The first compartment contains approximately 1.7-2.0mg of lead and has a half life of 35 days. This pool is in direct communication with ingested lead, urinary lead, and pools two and three. The second pool contains 0.3-0.9mg of lead and has a half life of approximately 40 days. Lastly, pool three contains the vast majority of the body lead burden, and in contrast to the other two pools, has a much longer half life.

1.4.1 Uptake and transfer by the blood

Under normal steady state conditions, the blood will contain approximately 2-4% of the total body lead burden (Baloh, 1974; Rabinowitz et al, 1976). Within the blood, 90-95% of the lead cations are bound to the red blood cells

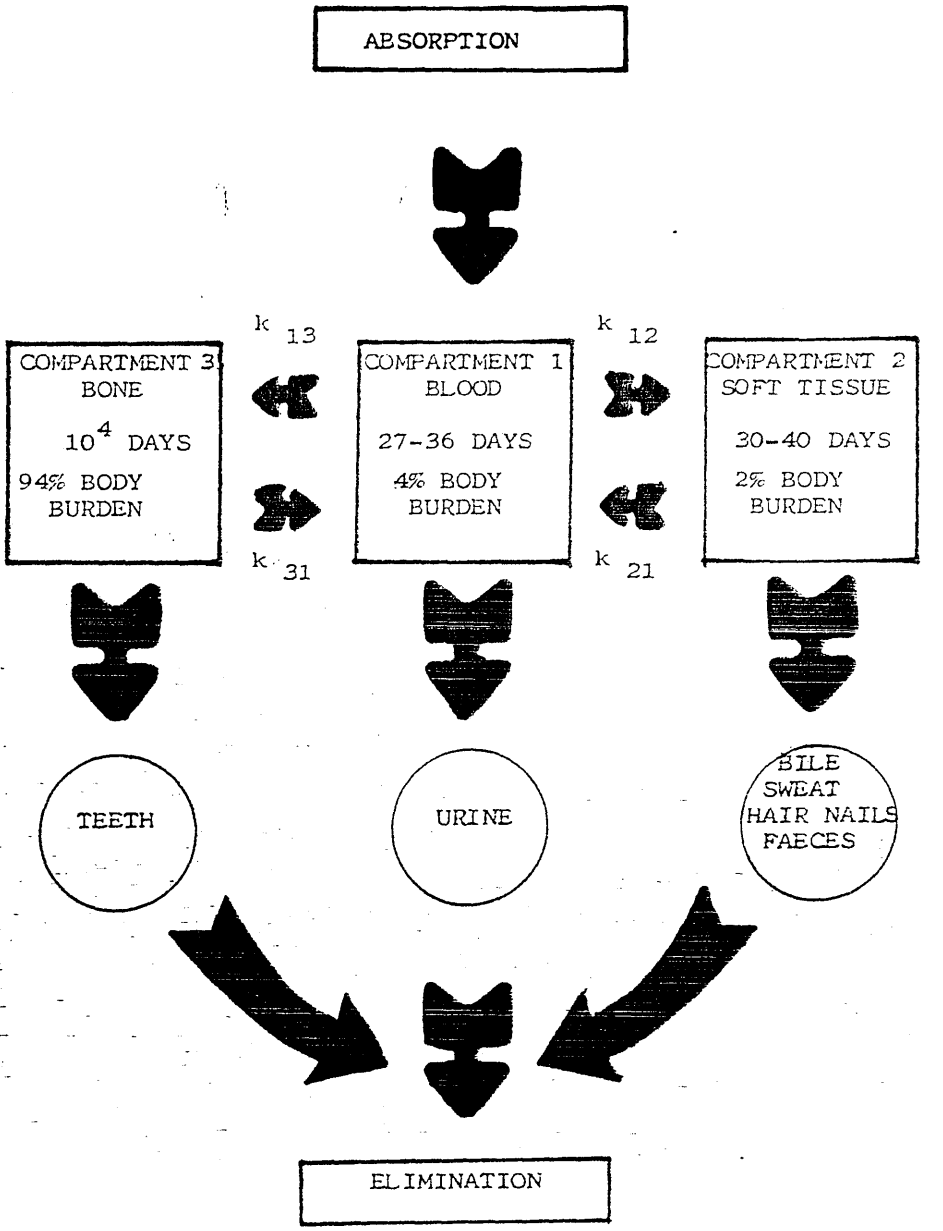


Figure 1.2 Three compartment model for the absorption, retention and elimination of lead.

Schematic representation of the absorption of lead, its relative distribution within the body, and the routes of its elimination. The diagram illustrates the half-life of lead in each compartment in addition to the main regions of the body which are involved in lead storage.

(Clarkston & Kench, 1958; Butt et al, 1964; Barltrop & Smith, 1971; Cavelleri et al, 1978), however the proportion of lead retained by the erythrocyte may be dose dependant (Waldron & Stofen, 1974). The remaining proportion, around 5%, is associated with the plasma fraction, and it is thought that it is this lead which constitutes the diffusable, metabolically active portion of the metal. Plasma lead exists principally bound to proteins with a small fraction being in a free ionic form capable of exchange with the surrounding extravascular fluid (Baloh, 1974). Binding to ligands can occur via several functional groups; OH, -COOH, $-PO_3H_2$, -SH and imidazole.

The uptake of lead by the erythrocyte is a rapid process (Morteson & Kellog, 1944; Clarkston & Kench, 1958). Early studies suggested the cell membrane as the principal lead binding site (Aub et al, 1925; Watanabe & Yana, 1953) with lead probably being associated with the lipids or lipoproteins (Teisinger, Zumanova & Zezula, 1958), but more recently, lead has been found to be bound primarily to haemoglobin (Barltrop & Smith, 1971) probably as an insoluble lead-haemoglobin complex (Clarkston & Kench, 1958, Ong & Lee, 1980). Barltrop & Smith (1975) have advanced the theory that there are two compartments for lead binding in the red blood cell, a weak binding compartment in addition to the firm binding to haemoglobin. Plasma lead may be in dynamic equilibrium with the loosely bound erythroctye lead (Morteson & Kellog, 1944) with lead being surrendered from the cells as plasma levels fall, and, under certain conditions, the lead-haemoglobin complex

dissociating. Such theories of dynamic equilibrium between firstly, erythrocyte and plasma lead, and secondly, intracellular and extracellular lead, are however not consistent with the data of Hursh & Suomela (1968), who suggest that lead may be released from the red blood cell slowly, and only on its death.

1.4.2 Uptake by soft tissue

Lead which is absorbed into the blood may be transported to virtually all the organs and tissues of the body, the concentration at any site and time being determined by many complex dynamic interchanges amongst various tissues and fluids of the body. Indeed, this dynamic equilibrium between blood and tissues predicts that lead will diffuse from plasma into the cell via the extracellular fluid. Removal of lead from the soft tissues also occurs, and the kinetics of this movement closely resemble those for the removal of lead from blood (Castellino & Aloj, 1964), although the exchange rate for certain tissues, notably muscle and skin, are slower. Exposure of the tissues to lead can occur under two sets of circumstances. Firstly, after absorption of lead from the environment via transport in the blood; and secondly, as a result of mobilization of lead from storage regions of the skeleton.

Around 2% of the body burden of lead is present in the soft tissues (NAS, 1980). Variations in relative tissue lead content occur with geographical location (Schroeder & Tipton, 1968). However in general the highest concentrations are found in the aorta, liver and kidney with much lower levels being found in the muscle, brain,

spleen, heart, skin and testis. There is a wide variation in the actual levels of lead in tissues between individuals. Observed concentrations depend on many factors, not least the level of lead exposure to which the individual is exposed. The lead concentraton in soft tissues tends to reach equilibrium during the second decade in life after which no rise in the metals' concentration is observed throughout adult life (Barry & Mossman, 1970; Barry, 1975). Schroeder & Balassa (1961), however observed a rise in lead content in certain tissues up to around the fifth decade, after which the levels once again fell. This pattern was evident only in tissues of individuals from certain geographical regions. The concentrations in tissues from infants and young children tend to be lower than in the adult (Barry & Mossman, 1970; Barry, 1981). At moderate levels of exposure, there is no significant difference in soft tissue lead content between males and females (Barry & Mossman, 1970; Barry, 1975) although males may tend to have about 30% more lead in their tissues if they are especially exposed to the metal (Barry, 1975).

Uneven distribution of lead within an organ is often present. The kidney cortex contains more lead on a weight basis in comparison with the medulla, both in adults (Barry & Mossman, 1970; Barry, 1975) and children (Barry, 1981). Grandjean (1978) has observed an uneven distribution of lead within the brain. The hippocampus and amygdala contained a greater lead concentration than the mean in the half brain or the cerebellum, in non lead-exposed individuals. Relatively high concentrations were also found

in the medulla, while the corpus callosum and optic tract were lower in lead. This distribution in lead content of the brain complies with data obtained from individuals especially exposed to lead. In general it also coincides with the distribution of certain essential trace elements (Harrison, Netsky & Brown, 1968), being localised mainly in densely cellulated regions.

The organs with the greatest uptake of lead form intracellular inclusion bodies which are composed of a lead-protein complex. When the lead is bound in this non-diffusable complex, cellular function can continue even in the presence of large quantities of intracellular lead. Intracellular lead is also associated with organelles. Binding is thought to occur principally via sulphydryl groups. Intracellular distribution varies with organ, an example being the liver, in which the lead is bound in a stable manner to the mitochondrion (Castellino & Aloj, 1969) with little being attached to lysosomes. Ultrastructural changes in the myocardial mitochondrion have been observed in animal studies following lead exposure (Asokan, 1974; Moore, 1975).

1.2.3 Storage of lead in the skeleton.

The skeleton contains by far the greatest proportion of the body burden of lead, with a figure of over 90% of the body lead content often being calculated (Schroeder & Tipton, 1968; Barry & Mossman, 1970; Barry, 1975). In particular, lead seeks out areas of active bone formation. Storage of lead in bone occurs as the relatively insoluble triphosphate salt, and for this reason less than 1% of the

skeletal load is exchangeable (Rabinowitz et al, 1973). The bone mineral contains two calcium phosphate pools. Firstly, a crystalline phase of apatite crystals similar to hydroxyapatite, and secondly, an amorphous non-crystalline phase predominately of calcium and phosphate (Posner, 1969). In a similar manner to strontium, lead can replace calcium in the crystal. Under normal conditions, a constant, slow diffusion of lead from bone occurs (Castellino & Aloj, 1964). However, during certain disease states in which there is a disturbance in the acid-base status of the body, a more rapid mobilization of skeletal lead can occur (Waldron & Stofen, 1974) in a similar manner to the movement of calcium (DHSS, 1980). The biological half-life of lead in the skeleton has been calculated to be around 10^4 days (Rabinowitz et al, 1973; 1976) although other workers have calculated a figure in the region of 670-840 days (Black, 1962). The lead content of bone steadily rises throughout life (DHSS, 1980). The bones of children contain around 1mg lead/kg., while the level in a 50 year old adult is some forty times greater (Barry, 1975). In contrast to the observations of Schroeder & Balassa (1961) who observed a fall in bone lead after the seventh decade, the post-mortem data of Barry and his colleague (Barry & Mossman, 1970) observed no fall in bone lead in extreme old age. Variations exist in bone lead concentrations. Rib and vertebrae concentrations tend to reach a maximum between the ages of sixty to seventy, while the long bones continue to accumulate lead till death. Lead poisoning can be confirmed in children by radiography which

shows the presence of a line of increased radio-density in the metaphysis of the growing end of bone. Although formerly thought to be due to deposited lead it is now believed to be caused by calcium deposits.

The level of lead in bone represents the cumulative exposure of the individual to the toxin since birth. Similarly, the lead content of exfoliated teeth represents a measure of both past and present exposure. The highest concentration of lead occurs in the circumpulpal dentine zone (Shapiro et al, 1973; 1975) although enamel concentrations are higher than dentine. Even across the enamel layer concentrations vary, and the root portion of the tooth contains a higher lead content than the crown. Similar to bone, the lead content of tooth dentine has been observed to rise with age (Al-Naimi, Edmonds & Fremlin, 1980), but in contrast, the enamel lead content does not follow such a pattern.

1.5 Excretion of lead

The principal routes of elimination of lead from the body are via urine and faeces, although small amounts are excreted in sweat and milk or via tissues which are shed such as deciduous teeth, hair and nails. The vast majority of faecal lead originates from that which was ingested orally but not absorbed. Absorbed lead which is excreted into the gastro-intestinal tract, is eliminated by way of biliary, pancreatic, salivary and gastric secretions (Blaxter & Cavie, 1946; Castellino et al, 1966; Cikrt, 1972; Rabinowitz et al, 1976). Cikrt (1972) also demonstrated that the cells of the gut wall which contain

lead, may also contribute to intestinal elimination to a minor degree.

Although faecal elimination is quantitatively more important than renal (Kehoe, 1925; 1964), the renal route of lead excretion accounts for the elimination of 95% of absorbed lead (NAS, 1980). For the most part, the kidneys eliminate lead by glomerular filtration, especially in the modestly exposed individual (Vostal, 1963). However, following sudden increases in blood lead concentration, as caused by intra-venous injection of lead, tubular resorption of filtered lead can occur (Vostal, 1963). This process is pH dependant with the amount of resorbed lead being greater at low pH values. Active secretion of lead by the renal tubule has also been demonstrated (Vostal & Heller, 1968).

Excretion in other fluids such as breast milk does not attain any great significance, since the concentration of lead in this fluid is similar to that in plasma, being only 10% of the level in blood.

Pathological effects of lead

Pathological effects of lead have been noted on many body systems. There exists a spectrum of 'threshold' blood lead concentrations for the various toxic effects (WHO, 1977). A summary of these effects will be presented.

In addition to the most important sites of action of lead; the central nervous system and the haematopoietic system, numerous other target organs exist, including the kidney, skeleton, gastro-intestinal tract, cardio-vascular system, endocrine, immune, reproductive and peripheral neuro-muscular systems. Additionally, one should consider the influence of lead on the ageing process, chromosome abnormalities and mutations, and cancer. Lead has diverse biological effects both in humans and animals. There exists a wide dose-response curve for the collection of biological effects with some biochemical changes occurring at very low levels of exposure while other aberrations are only noted at much higher exposure levels.

Haematopoietic system The observed effects of lead can be attributed to the combined results of an inhibition of haemoglobin synthesis, due to diminished haem levels, and a shortened life span of the erythrocyte, which results in anaemia. The multiple actions of lead on the haem biosynthetic pathway, which will be discussed in further detail in chapter 7, result in an excess in specific porphyrins in the red blood cell and a vast increase in urinary excretion of certain porphyrins and 5-aminolaevulinic acid. An increase in free erythrocyte protoporphyrin is an early indication of the detrimental

effect of lead on the haem biosynthetic pathway. These effects which can result in a quite pronounced anaemia are common in lead poisoning.

Kidney The association between lead poisoning and kidney disease has been noted for many years, with both adults and children being susceptible. Structural changes can be readily observed following lead exposure within the kidney and in this respect the proximal convoluted tubule is most affected. The characteristic renal response to these lesions is a Fanconi syndrome of aminoaciduria, glycosuria and phosphaturia. The resultant clinical manifestations vary dramatically depending on the level and duration of exposure. At one end of the spectrum, sub-clinical effects on various aspects of renal function have been observed in lead workers, while more severe levels of exposure can result in chronic nephropathy and gout, which results as a consequence of impaired tubular secretion of urate. Both short-term apparently reversible damage to renal tubules associated with acute lead poisoning and a progressive loss of renal function characteristic of chronic elevated exposures have been demonstrated. Depending on the degree of lead exposure, reversible or irreversible changes in kidney function may ensue. It has not been established whether acute lead poisoning in childhood may subsequently result in pathologies of renal function later in life.

Cardio-vascular system Several studies in animal species have demonstrated effects ranging from vasoconstriction to abnormal heartbeats, structural damage to heart tissue, and arteriosclerosis following exposure to lead. Evidence in

man is inconclusive. Some epidemiological studies have shown both an elevated prevalence of hypertension and excess mortality rate from cerebrovascular diseases among lead workers, while other studies have shown no effects of this sort in similar populations (NAS, 1980). It should be noted that it is not a simple matter to distinguish the primary action of lead associated with hypertension, since lead acting on the kidney may by causing kidney disease thus indirectly producing an elevation in blood pressure.

Immune system Evidence suggests that lead renders animals more susceptible to endotoxins and infectious agents. However, although available data indicates that lead affects immunity, it is not at all clear the mechanism by which it exerts its immunosuppressive action and indeed data indicating immunosuppression in humans is lacking.

Skeleton The possibility cannot be ignored that lead can cause a disturbance in bone metabolism since a great percentage of lead is stored in the skeleton. Little evidence exists to support this hypothesis. One must consider however, the possibility that cumulative long-term lead exposure is partly or even wholly responsible for the osteopenia and/or osteoporosis found in the elderly members of the population.

1.7 Effect of lead on the nervous system

1.7.1 General considerations

When one is considering lead exposure, there are really two distinct situations. No doubt exists that lead is a neurotoxin and general poison. There is firstly the case of overt or clinical acute lead poisoning which was commonplace in the ancient world and is characterised by severe abdominal pains, constipation or diarrhoea, anaemia and peripheral neuropathy with encephalopathy, heart failure, coma and even death ensuing (Waldron, 1973). Knowledge of such an illness of the nervous system caused by lead extends historically to antiquity. Indeed, Dioscorides in the first century A.D. foreshadowed us -

'the mind gives way; the limbs are paralysed'.

Secondly, chronic lead poisoning is more of a problem today. It occurs as a result of industrial exposure to relatively high concentrations of lead over a lengthy period, and results in small but discernable health effects such as kidney disease or cardiovascular effects. Until relatively recently however, it was thought that lead poisoning was almost exclusively a disease of industrial exposure. Presently, great interest is being shown in the possibility of insidious effects resulting from long-term exposure to environmental lead. The profound effects of overt lead poisoning are not in dispute but great controversy exists in the literature as to the effects of lesser exposure and indeed at what level of exposure they begin to occur.

Recognition of a neurotoxin usually begins with the

identification of often dramatically high dose effects. As knowledge of the biochemical toxicity of the toxin broadens, more sensitive measures of toxicity are developed and the threshold for effect is revised accordingly downwards. Vulnerable segments of the population are then identified and the question of whether ordinary community exposure is toxic is raised. Epidemiological and experimental studies are then conducted to define the extent of the problem and to elucidate the potential toxic mechanism(s). This is exactly the course of events that have occurred with lead.

The most prone tissue to lead is the brain and central nervous system (CNS), the tissue least capable of repair. The effect on the nervous system can be observed both biochemically and by histopathological techniques. The toxic effects are most probably biphasic. Firstly, an effect on nervous system development and growth (Louis-Ferdinand et al, 1978), an effect which is almost certainly irreversible and will result in permanent neurological changes especially where exposure occurs in early life; and secondly, an action on various biochemical systems to alter the concentrations of neuro-active metabolites. This action is in contrast to the first one, probably reversible.

It is long since known that lead has abortifacient properties. In this respect it is evident that lead is well capable of acting in utero, the very time that the human body is probably at its most vulnerable with respect to development of its organs including the nervous system. The blood-brain barrier which is permeable to lead, is not as

well developed in the young as in later life. Bryce-Smith et al (1977) have demonstrated that stillbirths contained between five and ten times the lead that was observed in live newborn. Wibberly et al (1977) have also demostrated a significant increase in placental lead in those who failed to survive both birth and the neonatal period. An interesting study with possible detrimental implications with regard to the foetus was carried out by Alexander & Delves (1981). These workers noted that blood lead fell during pregnancy, the magnitude of the fall being greater than would be expected by the dilutional effect of the increased plasma volume normally observed in pregnancy. The authors put forward several possible explanations for the fall -

1. A displacement of the equilibrium which exists between the maternal blood and soft tissues or skeletal tissue
2. Transfer of lead from the mother to the placenta or foetal tissue
3. Enhanced maternal excretion by way of the urine
4. Altered lead exposure, for example as a result of moving from the work to the home environment or a change in smoking or dietary habits.

Obviously if the second case were true then the foetus may well be exposed in utero to relatively high concentrations of lead presumably if the mother has herself a significant lead burden.

1.7.2 Studies in man

Research began at the turn of the century when the risks to children especially became apparent. Gibson in Australia described a lead induced oculo neuritis in a child and identified lead paint as the source (Gibson, 1917). This observation was rapidly followed by a case noted by Ruddock in 1924 when he described lead poisoning in a child the cause of which was pica. For many years it was generally assumed that upon recovery from the acute phase of intoxication, children were left without significant residual deficit in central brain function. The classic paper of Byers & Lord (1943) put an end to these beliefs. Randolph Byers demonstrated that children who were assumed to be asymptomatic following an episode of lead intoxication were, in fact, profoundly impaired. Following through the early school years of twenty children who had previously sustained episodes of clinical lead poisoning as evidenced by clinical symptoms such as encephalopathy and peripheral neuritis and also lead lines on X-ray, nineteen subsequently exhibited impaired cognitive function and altered behaviour; shortened attention span, antisocial behaviour, impaired visual-motor co-ordination. Considering this data, it is reasonable therefore to look for lesser impairments after a less extreme exposure to lead.

Spurred on by Byer's classic paper, a great wealth of data on the postnatal neurobehavioural and developmental effects of lead exposure have appeared during the 1970's and 1980's. During the 1960's screening programmes were set up to determine how widespread the problem of 'elevated'

lead exposure amongst children was, if such a definition of elevated could be decided on. In 1972, an article in the New England Journal of Medicine summed up the dilemma that confronted health workers and researchers of that decade, and indeed this one, as follows -

'a critical question...is whether lead can damage the central nervous system of young children in the absence of overt signs and symptoms referable to that system'

- Lin-Fu 1972

These studies can be separated into two types; those investigating the neurotoxic effect of lead on the peripheral nervous system and those studying the central effect. Several lengthy reviews of available data prior to 1980 have been published; Bornschein, Pearson & Reiter (1980), DHSS (1980), Rutter (1980), each attempting to draw conclusions from the wealth of confusing data published. Effects of lead on the central and peripheral nervous systems will be considered separately.

1.7.2.1 Effects on the central nervous system

Multiple approaches have been taken to investigate the central neurotoxic effects of lead. Two basic approaches have been undertaken-

1. The identification of a population with minimally increased lead levels and search for a central nervous system dysfunction

or,

2. The converse, that is the investigation of a population with central dysfunction in which to establish lead levels.

Within the second approach, many studies have shown significantly increased lead in the blood of mentally

retarded children (Moncrieff et al, 1964; Beattie et al, 1975; David et al, 1976; 1982; Moore et al, 1977b; Youroukos et al, 1978). Many studies have selected 3 groups

1. Normal subjects
2. Mildly retarded subjects whose retardation is of unknown aetiology
3. Retarded subjects whose abnormality origin is known.

The incorporation of this third group is vital since these studies are subject to a cause or effect problem. The presence of increased blood lead in retarded compared to normal children does not prove an aetiological relationship between mental retardation and raised but non-encephalopathic blood lead levels. The arguement critics put forward runs along the lines that the mentally retarded children may well ingest lead more readily than their normal peers and are hence predisposed to an increased lead burden. However, it is noted in these studies that there is no significant difference in blood lead in the mentally subnormal children of known aetiology by comparison with control normal children (David, Clark & Voeller, 1972; David et al, 1982). A supportive observation to the hypothesis that lead is involved in the aetiology of the retardation in these children, is that these subjects exhibited hyperactivity, a feature said to occur as a result of lead exposure. The data presented by David et al (1982) has however been critised (Ernhart, 1982) for several reasons. Firstly, the authors failed to consider any confounding variables such as parental factors and social class, which may be related to the retardation.

Additionally, it should be noted that the blood lead levels observed in this group of 'unknown aetiology' mentally retarded children were not extremely high with the mean being 1.2μM (25μg/100ml) and the highest value being in the region of 2.6μM (54μg/100ml). A slightly different approach has been taken in the retrospective study of Moore et al (1977b). This group obtained phenylkenonuria cards containing blood spots obtained a few days after birth from mentally retarded children, again of unknown aetiology. A clear correlation between mental subnormality and blood lead was observed. Beattie et al (1975b) reported a strong association between high levels of lead in household water supplies used during pregnancy and mental retardation in children born to mothers using such water. Blood samples obtained a few days after birth showed mean lead levels of 1.2μM (25.4μg/100ml) in the retarded group compared with 0.9μM (17.8μg/100ml) in the control group. The case that lead is a causal factor in this impaired intellectual development is more strongly supported in this study since the children's lead exposure cannot be explained by their behaviour. The doubt does remain however, as to the fine distinction that exists between unknown and known aetiology with regard to subnormal mentality. Other studies have obtained similar data. Youroukos et al (1978) also observed that the mean blood lead of mentally retarded children exceeded that observed in either a retarded group but of known aetiology or a control normal group. A group in Wales however, have noted no difference in water lead content of educationally subnormal children compared to normals

(Elwood et al, 1976) and have sewn doubts on the influence of lead on mental retardation. These doubts however only stand if a significant proportion of the lead these children are exposed to is derived from water.

On the other side of the equation, children with known lead exposure have been identified and resultant deficits in brain function sought. De la Burde & Choate (1972; 1975) performed a longitudinal study on children at the ages of four and seven, who as toddlers, had blood lead concentrations in excess of 1.9μM (40μg/100ml) or lead lines in the long bones on radiography. Psychological testing at school age indicated deficits in behavioural tests, I.Q., visual and fine motor co-ordination. Not surprisingly, there was no significant difference in blood lead concentrations between the previously lead exposed children and a lesser exposed group, since blood lead reflects the recent exposure of the individual. Mean tooth lead contents, a more cumulative and long-term exposure index, were however divergent. In a study of black pre-school age children in New York, who were divided into a low lead group whose blood lead was less than 1.4μM (30μg/100ml) or a high group if the blood lead exceeded 1.9μM (40μg/100ml), psychological testing indicated impaired cognitive and perceptual performance in the high lead group. This study controlled for covarients such as parental intelligence, age and birth weight (Perino & Ernhart, 1974). Other studies have investigated children with known lead exposure. Albert et al (1974) observed New York children in which, not surprisingly, those with

encephalopathy and lead exposure symptoms had a deficit in I.Q. However, those who were asymptomatic but whose blood lead exceeded 2.9μM (60μg/100 ml) did not differ significantly from controls with respect to I.Q. although they had more attention and concentration difficulties. No lead related deficiency in cognitive tests were observed in children whose blood lead exceeded 2.4μM (50μg/100ml) compared to those with a lead level less than 1.9μM (40μg/100ml) - Baloh et al (1975). A greater percentage of the 'high' lead group however were considered hyperactive by either the parents or teachers.

A great many studies have selected a group of children residing close to a lead source such as a smelter, and compared them to a similar group living more distant, and therefore presumably less exposed. Whereas some studies have demonstrated detrimental effects of lead, others have not. One much quoted study performed in the east end of London and which failed to demonstrate a detrimental effect of lead, is that of Lansdown et al (1974). After measuring I.Q. and classroom behaviour in two groups of children, living either close or more distant from a lead smelter, no relationship was found between I.Q. and either distance from the smelter or blood lead. However, grave doubt is placed on the results of this study as selection of the 'control' group was performed rather inappropriately. The group of children residing further from the lead source exhibited an increased proportion of disturbed children, and as a result lower levels of intelligence and higher rates of disturbance were more related to social factors

than lead exposure. Using a lead smelter in El-Paso, Texas Landrigan et al (1975a/b) divided a batch of asymptomatic children into two matched groups exhibiting a blood lead of either less than 1.9μM (40μg/100ml) or greater (observed range 1.9-3.9μM; 40-80μg/100ml). The groups were matched for socioeconomic status, sex, length of residence and proximity to the smelter and the language spoken at home. Using both a parental questionnaire and psychological testing performed by a physician, subtle but significant impairments in non-verbal cognitive and perceptual-motor skills using the Wechsler Intelligence scale, were noted. Performance I.Q. was impaired by 8 points but there were no significant differences in verbal I.Q., behaviour or hyperactivity between the groups. In the same location, an independent study conducted by McNeil and his colleagues (McNeil, Ptasnik & Croft, 1975) dividing asymptomatic children on the grounds of proximity to the smelter, failed to detect any lead related defects by means of physical, neurological, psychometric or school performance measures. The Werry-Weiss-Peters scale for hyperactivity also did not detect any difference as a result of lead exposure. The control group in this study lived in another area of El-Paso. These results may well not be inconsistent with the data obtained by Landrigan and co-workers since they employed a different research strategy. Following the publication of the Lawther report (DHSS, 1980) two members of the committee carried out a study near a leadworks in London (Lansdown et al, 1981; Yule et al, 1981), the results of which greatly contradicted the conclusions of

their earlier study (Lansdown et al, 1974). This later study reported a 7 point I.Q. difference related to body lead, a deficit which remained after subtraction of the component of I.Q. related to a varying degree of social class. The range of blood lead values observed was in the range 0.3-1.6μM (3-33μg/100ml) with mean 0.7μM (14μg/100ml). Blood lead was significantly associated with attainment scores on tests of reading, spelling and intelligence but not mathematics, even after social class effects had been partialled out. One criticism of the paper however, was that the blood lead measurements reflecting recent exposure, were taken 9 to 12 months prior to psychological testing. Teachers were asked to complete three behavioural rating scales, including the eleven item forced-choice scale employed by Needleman et al (1979) on each child. The pattern of results obtained in this British study was similar to that observed by Needleman's group. A battery factory in Birmingham provided the lead source for a study performed by Hebel, Kinch & Armstrong (1976). The data suggested a tendency, though not statistically, for children living closer to the factory to score 1-2 points lower on verbal reasoning. In another study a non-significant reduction in I.Q. of 108 in a 'moderate' lead group (mean 1.4μM, range 0.9-1.7μM; 28μg/100ml, 18-35μg/100ml) compared to 102 in a 'high' group (mean 2.1μM, range 1.7-3.1μM; 44μg/100ml, 36-64μg/100ml) was observed in children again residing close to a battery works in Manchester (Ratcliffe, 1977). The lead estimations were however performed at the age of two whereas psychological

testing did not take place until school age.

Much of the remaining data available relating to the central neurotoxic action of lead is obtained from general population studies, none of which have been performed in the United Kingdom. The possibility therefore exists that these studies have been performed in areas where social and educational conditions differ from Britain.

Undoubtedly the largest population study was carried out in two primarily white working class towns adjacent to Boston, Massachusetts (Needleman et al, 1979; Needleman, 1983). This was a very well designed study which attempted as best as is possible to take into consideration confounding non-lead related variables. Considering other methodological problems the first design advantage over many other previous studies was the use of an alternate means to blood lead in order to assess lead status of the children. In this study in excess of 2,000 asymptomatic children were recruited and body lead burden assessed by way of the lead content of shed decidious tooth dentine, a reflection of cumulative past exposure to the metal (Needleman et al, 1974). From these levels the extremes were selected, the highest and lowest tenth percentiles. The high lead group had a tooth dentine lead level of 20μg/g or more, while in the low group the lead content did not exceed 10μg/g. For the purpose of comparison with other studies, blood lead levels in these groups were measured, and observed to be 0.9-2.6μM, with mean 1.7μM (18-54μg/100ml; 35.5μg/100ml) in the high group and 0.6-1.8μM with mean 1.2μM (12-37μg/100ml; 23.8μg/100ml) in the low

group. These figures indicate quite clearly that blood lead cannot be interchanged with tooth lead levels for the purposes of selecting 'high' and 'low' lead groups. Each child included in the study of these extreme groups with regard to body lead burden, underwent a 4 hour neuropsychological examination. In addition, the mother of the child completed a lengthy questionnaire designed to test 39 non-lead confounding co-variates. Comparison was considered for physical, medical, socioeconomic, family variables and also parental attitude, namely aspirations for the child, home learning environment, attitudes to school and the child, and restrictiveness. Teachers provided crude rating measures of behaviour by way of an 11 question forced choice questionnaire covering distractability, not persistent, disorganised, hyperactive, impulsive, easily frustrated, day dreamer, does not follow a sequence of directions and low overall functioning. Needleman paid great attention to the problem of confounding non-lead variables. The groups were broadly similar on background variables, but the high lead group were slightly older at the time of testing, slightly more socially disadvantaged and had parents with a slightly lower I.Q. As a result, 5 co-variates were included in the analysis. The battery of tests of neuropsychological function were analysed by analysis of covariance, and the outcome demonstrated that the two groups differed significantly on several tests including verbal intelligence, verbal and auditory processing, attention span and full-scale I.Q., the deficit being 4.5 points as measured by the Weschler Intelligence

Scale for Children (WISC). The high lead group had a corrected mean full-scale WISC-R I.Q. of 102.1 compared to 106.6 in the low lead group, a difference which was highly significant. The high lead group also scored significantly lower on 9 of the 11 indices of classroom performance rated by teachers. Although the teachers' reports showed increased distractability and prevalence of daydreaming, and lack of persistence and an inability to follow a series of directions, all features said to constitute 'hyperactivity', this feature was not specifically reported to be elevated. This increase in frequency of detrimental classroom behaviour was reported to be dose related. Statistical analysis demonstrated that none of the non-lead confounding variables could account for the observed difference in I.Q. Although the difference in I.Q. of 4.5 points is statistically significant one should consider the difference in another light. It must be pondered over as to whether this difference loses significance when compared with the conventional standard deviation of 15 points employed on the WISC-R scale. Another point to note is that the occurrence of pica was three times as common in the high lead group as in the low group and yet was not included as a covariate; 30% of the high group exhibited pica in comparison to 11% in the lower group. Is then, pica not a marker for pre-existent behavioural deviation, and hence if this were the case, the increased lead burden would be an effect rather than a cause of the deficit? Data however indicates there is no relation between the pica and the teachers ratings and therefore this is an unlikely

proposition (Needleman, 1983). It would have been interesting to know whether the intermediate group were also intermediate psychologically since if it were not, then it would weaken the argument that the association represented a causal influence of lead.

Subsequent to this study, supportive data of altered central nervous system functioning as a result of chronic low lead exposure in these children was obtained by Birchfiel et al (1980). A random subset of the same group of children was selected for quantitative electroencephalography (EEG) pattern analysis. The data in this study shows an overall altered pattern of alpha and delta waves. Measurement of brainwave energy over four wavebands, delta (0.5-3.5 Hz), theta (4-7.5 Hz), alpha (8-12 Hz) and beta (12.5-31.5 Hz) indicated a significant increase in low frequency delta waves over the central parietal and occipital cortices and a reduction of alpha waves over the parietal and occipital regions.

Re-analysis of the same data obtained by Needleman et al (1979) to investigate a possible deficit in the child's I.Q. from the expected I.Q. as calculated from maternal I.Q. was performed by Bellinger & Needleman (1983). A regression line for the maternal I.Q. and child's I.Q. was calculated for the sample as a whole, that is high and low lead groups together, and also for each group separately. Then, if the complete sample regression line is used to obtain an estimate of the child's expected I.Q., the difference between the child's observed I.Q. and this expected I.Q. can be calculated. This value differed

significantly between the two groups. The observed I.Q. was on average 3.94 points (±12.90) below the expected in the high lead group, while in the low group it was 1.97 (±11.57) greater than expected. The next question to be asked in this study was if the differences varied with the child's dentine lead. Within the low lead group the correlation was essentially zero. In the high group however, the difference was significantly correlated with dentine lead. In summary, for every part per million rise in dentine lead over 20, the observed I.Q. fell 0.42 below the expected on the basis of maternal I.Q.

If it could be shown that a reduction in body lead was followed by intellectual gains and/or behavioural improvement, powerful evidence would be provided for a causal link between lead and behaviour. Chelation studies theoretically provide a suitable model but such studies to date suffer from methodological drawbacks. Pueschel, Kopito & Schwachman (1972) reported a rise in I.Q. of 8 points amongst children who previously had raised blood lead and many of whom had symptoms of exposure, and who subsequently had chelation therapy. Hyperkinetic children with blood lead in excess of 1.2μM (25μg/100ml) were studied following chelation therapy (David et al, 1976). The authors concluded that those children whose hyperactivity was of unknown cause showed improved behaviour following treatment while those whose abnormal behaviour was of a known origin, failed to improve.

Although the vast majority of data relating to the central neurotoxic action of lead has been obtained in

studies of young children since these are the most vulnerable members of the population, a handful of studies have relatively recently been performed in adults.

In a Danish battery plant, a study of males exhibiting blood lead in the range 2.8-4.0μM (58-82μg/100ml) having neuropsychological testing, showed that they lacked concentration and memory; I.Q. however was normal or above average (Arnvig, Grandjean & Beckmann, 1980). At a slightly lower level of exposure, in a foundry, a study by Baker et al (1983) demonstrated an increased rate of non-specific symptoms such as depression, confusion, anger, fatigue and tension. Those workers who had a blood lead between 1.9-2.9μM (40-60μg/100ml), also exhibited impairment of other aspects of neurobehavioural functioning including verbal concept formation, memory and visual-motor performance. Zimmerman et al (1983) divided workers at an electric storage battery plant into a 'low' group who had a blood lead of less than 1.7μM (35μg/100ml) or a 'high' group whose blood lead fell within the range 2.2-2.9μM (45-60μg/100ml). An additional group of 'non-exposed' persons who were locally employed male nurses were also included in the study. Each volunteer was asked to complete a questionnaire of psychological and physical symptoms. The 'high' lead group reported a significantly greater number of symptoms especially neurological than either the 'low' lead group or the control group which did not differ from each other. This study attempted to control the bias which may exist when asking lead workers who are aware of the dangers of lead exposure to self-report symptoms. The

inclusion of an additonal 'low' lead group alleviated these problems. Additionally, a battery of performance tests indicated an impairment of general functioning in cognitive, visual and psychomotor areas (Campara et al, 1984).

Further electrophysiological evidence of central nervous system dysfunction as a result of lead exposure comes from Otto and co-workers (Benignus et al, 1981; Otto et al, 1981; 1983). These workers studied children aged 13-75 months from low income black families exposed to lead from a variety of sources. Lead status was performed by blood lead concentration before electroencephalographic analysis was performed. The data obtained showed an alteration of EEG potentials which changed in a linear fashion with blood lead over the observed range 0.3-2.7μM (6-55μg/100ml). Thus there may well be a continuum of effects of lead on the central nervous system either with no, or a very low threshold level of exposure. These changes in brain function appeared to remain over the two year interval between the studies despite a fall in blood lead.

As will be evident from the foregoing discussion, the literature is rife with confusion and controversy concerning minimally elevated blood lead levels and their meaning. Indeed the first controversy must be the definition of elevated lead exposure. In the United States of America, an upper limit of acceptability and action level of 1.5μM (30μg/100ml) has been set by the Centre for Disease Control (CDC, 1978) for the prevention of lead poisoning in children. Additionally, biological variability

must be considered; that is at similar levels of exposure, some subjects may manifest central nervous system symptoms, while others may not. The confidence with which performance deficits, if observed, can be attributed to past lead exposure, depends on whether the study has successfully addressed methodologic issues. There are four well recognised basic methodologic problems which have been identified and considered to varying degrees in various studies (Rutter, 1980). -

1. Inadequate markers of exposure.

'Low level lead' is a vague and ambiguous phrase and if exposure is not assessed adequately then it is most unlikely that an existing effect will be identified. Blood lead measurements reflect recent lead exposure. When investigating children, they may well have acquired a considerable lead burden as a toddler which will not be reflected in a blood lead measurement. Similarly, vice versa; single blood lead estimations can reflect a temporary and perhaps unimportant rise. It is a very serious drawback that blood lead measurements are not able to distinguish between short-term and long-term lead intoxication, since it may well be that only chronic lead exposure is damaging. More appropriate is the use of serial blood measurements or alternatively, tooth lead estimations. Primary dentine of deciduous teeth is a useful index of the six months in utero until the time at which the tooth becomes functional, whereas the lead exposure from the time the tooth becomes functional until it is shed is reflected in circumpulpal

dentine (Shapiro et al, 1973; 1975). Thus tooth lead content reflects cumulative exposure to the metal. There is a limitation on using deciduous tooth lead as an index in that one is confined to those children who are at the age of shedding such teeth.

2. Insensitive measures of performance.

The importance of a study lies in the battery of tests applied. At present only gross changes in central nervous system functioning can be reliably detected. Attributes which have long been regarded as associated with central dysfunction such as attention span, distractability and poor learning cannot be readily measured and certainly not by rating scales. Where tests of cognitive development and function are available, many are standardised for children older than three to five years and are therefore not entirely suitable for this age group. It is of course the younger child and toddler who is at greatest risk to the adverse health effects of lead.

3. Biased ascertainment of subjects.

Selection of individuals from a relatively restricted source may lead to a reduction in the population to which findings may be generalised. In addition restriction to a population most at risk for the target condition, in this case lead exposure, increases the possibility of establishing an effect given that it exists. Subject selection may be biased if factors leading to non-participation are related both to lead level and to outcome measures, such as lack of parental interest or unwillingness to co-operate within the structures of the

study protocol; subjects who enter a study may differ in a systematic fashion from those who reject participation. The study must recruit a large enough sample size in order to detect subtle defects if present.

4. Inadequate identification and consideration of non-lead related confounding variables affecting development.

Poor educational attainment is clearly related to a wide range of indicies of social disadvantage. Since such measures also appear to be related to higher lead exposure, it is necessary to demonstrate statistically that lead and attainment are still related after the influence of mediating social factors are taken into account. Most hard to control are the genetic factors as estimated from parental I.Q., socioeconomic status, and early parent-child interactions which all influence greatly the child's cognitive development. Deficiences in the general caregiving environment in the home during the vital first two years of life can increase the risk of increased lead absorption, when over-exposure to environmental lead occurs. Studies have shown that children of lower socioeconomic status tend to have higher blood lead concentrations than those from a more priviliged background. There are a great many medical and social conditions which can cause or contribute to the deficit of measured intelligence. A carefully controlled study must incorporate appropriate methods of statistical analysis to assess the relative contributions made by the various factors. It must also be considered that there are medical conditions which may have a depressive effect even on a transitory basis on a child's response to an I.Q.

test. Although most studies exclude permanently brain damaged children, a minor degree of illness such as infection, anaemia or poor nutrition can cause a temporary lack of responsiveness and most psychological tests are performed on a single occasion.

Although it is impossible for an epidemiological study to achieve complete control of an infinite number of variables that contribute to outcome, it is vital however to take into account as many variables as possible, since the direction of bias in evaluating a possible detrimental agent associated with disadvantage will be towards falsely ascribing a deficit to the agent. Where differences are small as they are in studies reporting positive effects of low level lead exposure, biases are critical. An additional problem is that even where confounding variables are identified and measured as reliably as possible, there are no fully satisfactory statistical methods for their control. Neither analysis of covariance, multiple regression or matched pairing on all known confounding variables completely corrects. It has also been pointed out that there is a subtle but distinct difference between backwardness and underachievement which must be observed. An additional point may also be considered.

5. Multiple comparisons.

By far the most important factors are the issues of confounding variables and assessment of lead exposure. However, it should be noted that although it is desirable to employ several measures of behaviour and intelligence, in any large number of independent statistical comparisons,

a specified proportion will reach statistical significance in the absence of a true effect. A significance level of 5% implies that 1 in 20 of the differences will be a chance finding.

As with all studies, there is a need for independent replication. Hrdina & Winneke (1978) performed a similar population study to that of Needleman et al (1979) but on a smaller scale. 458 school age children (aged 7-10 years) in Duisburg, Germany were selected and segregated into 'low' and 'elevated' lead exposure groups by way of incisor tooth lead content. The low group had a whole incisor tooth lead content less that 3 parts per million (mean 2.4 ppm), while if the lead content exceeded 7 parts per million the child was classified as 'elevated exposure' (mean of group being 9.2 ppm). The elevated group represented approximately the upper 15% of the distribution which covered the range 1.4-12.7µg/g. Non-lead variables were accounted for by pair matching the children for age, sex and parental occupation. Several psychological tests were performed and the data indicated that two of the tests of perceptual motor integrity were significantly different between the groups. A deficit of 5-7 points in I.Q. was however not significant. These results supported the hypothesis of an association between increased lead exposure and disturbances of neuropysiological development. However, they were not unequivocal and pronounced enough to prove a relationship between the lead burden and observed neuropsychological deficit. A second study was performed by the same group (Winneke, Hrdina & Brockhaus, 1982; Winneke,

1982) near a smelter at Aachen. Tooth lead contents were significantly higher than those observed in Duisberg. Three groups were identified, 'low' (<4 ppm), 'moderate' (>4-10 ppm) and 'high' (>10 ppm). The study revealed no detrimental effects of lead on verbal I.Q., performance I.Q. or full scale I.Q. and the same was the case for several other neuropsychological parameters. However, there was a significant association between tooth lead content and perceptual-motor integration.

Again using tooth lead as an index of cumulative exposure, Maracek et al (1983) studied a group of black children in urban Philadelphia. The two groups selected exhibited median tooth leads of 5 and 59.8 µg/g with the groups being selected from a skewed distribution. A battery of neuropsychological tests was applied and overall, exposure to lead was associated with a decrement in performance. Statistical significance was reached most often on tests of visual-motor functioning and perceptual integration. Motor functioning was however not affected. With regard to non-lead variables, the authors concluded that socioeconomic factors were not related to lead exposure.

One further population study, again performed in the United States (Ernhart, Landa & Schell, 1981) selected urban black children living in New York. Here multiple markers of exposure were employed, namely pre-school blood lead, school age blood lead and free erythrocyte protoporphyrin, and deciduous tooth dentine lead. A battery of tests was performed- McCarthy scales, reading tests,

teachers assessments, and the results suggested impairment being associated with lead exposure. With no control of parental I.Q. one could conclude from the data that lead may be associated with deficiences in general cognition, verbal skills, motor performance and reading test performance. However, when a brief measure of parental I.Q. was considered, the statistical deficits were nulled out, and the authors concluded that lead had if any very minimal effects of central nervous system dysfunction. However, Rutter for one, suggests that the authors are over emphasising their non-significant results. There was a significant association between blood lead and I.Q. even after controlling for confounding variables, even though not significant. A significant proportion of the association was due to confounding variables rather than lead per se.

1.7.2.2 Effects on the peripheral nervous system

In contrast to the infant and young child, the principal neurotoxic effects of lead in the adult, appear in the peripheral nervous system. Radial palsy in the past was a classical sign of lead neuropathy. Within the peripheral nervous system, a spectrum of lead related deficits can appear, from paresis to slight functional defects only capable of detection by sensitive electrophysiological techniques. Although peripheral paresis is well known, the occurrence of lead palsy in severe lead poisoning is now rare. Many studies have selected a group of men exposed to lead in the working environment, but who are neurologically asymptomatic and have demonstrated impaired motor

conduction nerve velocities (Catton et al, 1970; Seppalainen & Hernberg, 1972; Seppalainen et al, 1975; Araki & Honma, 1976; Feldman et al, 1977; Ashby, 1980). Seppalainen, Hernberg & Kock (1979) measured a battery of functions of the peripheral nervous system; maximum motor conduction velocity of the median and tibial nerves, motor conduction velocity of the slow fibres of the ulnar nerve, sensory conduction velocity of the forearm region of the median and tibial nerves, distal sensory conduction velocity of the median nerve from the finger to wrist and the- motor distal latency of the median nerve. All these parameters correlated with the maximum blood lead during the entire period of occupational lead exposure, with a time weighted average blood lead, and with the actual blood lead at the time of testing. It would appear that the most sensitive indices of the peripheral neurotoxic action of lead are the slowing of the conduction velocity of the slower motor fibres of the ulnar nerve and the motor latency of the median nerve (Seppalainen & Hernberg, 1972; Seppalainen et al, 1975). In order to investigate the time span between the start of lead exposure and the onset of these manifestations, the same laboratory (Seppalainen & Hernberg, 1982) selected a group of newly exposed lead workers. After one year exposure to the metal, these workers exhibited significantly longer motor distal sensory conduction velocities of the median nerve. The data in this study suggested that lead has detrimental effects on the peripheral nervous system in these lead workers at a blood lead level of less than 1.9μM (40μg/100ml). Electromyo-

graphic analysis has demonstrated abnormalities such as fibrillation and a diminished number of motor units in maximal contraction (Seppalainen et al, 1975).

In their study of children exposed to chronic low level lead from a nearby smelter, Landrigan et al (1975b) noted a lead related slowing of wrist-tapping which may be an indication of low grade motor neuropathy. The slowing of nerve conduction velocities described above will precede the development of signs and symptoms of peripheral neuropathy. The asymptomatic slowing of sensory nerve conduction velocities precedes the development of motor nerve effects (Singer, Valciukas & Lilas, 1983).

1.7.3 Animal studies

Similarly to the data available in man, the studies performed in animals, mainly mice and rats, have provided data which is inconsistent and contradictory. Early behavioural studies in animals tended to study locomotor activity in response to the observations of David et al (1972) indicating a relationship between hyperkinesis and lead exposure in children. Although research in the early 1970's suggested that lead produced hyperactivity (Silbergeld & Goldberg, 1973; Sauerhoff & Michaelson, 1973; Michaelson & Sauerhoff, 1974a; Silbergeld & Goldberg, 1974; Golter & Michaelson, 1975), an observation subsequently supported by other laboratories (Overmann, 1977; Dolinsky et al, 1981), this observation is not universally supported. Other groups have noted either no effect of lead on activity (Sobotka & Cook, 1974; Kostas, McFarland & Drew, 1976) or even hypoactivity (Reiter et al, 1975;

Driscoll & Stegner, 1976; Hastings et al, 1977). Such discrepancies may be due to methodological issues such as different experimental protocols, time period of lead administration, age of testing or perhaps the specific test(s) employed. A study measuring activity levels in mice has demonstrated that the effects of lead are not invariant and are influenced by factors such as the time of day the testing is performed (Dolinsky et al, 1981). Several investigators have examined a number of behavioural paradigms including mazes, discrimination tasks and shock-avoidance situations. The available data demonstrates a lead associated impaired performance in certain types of learning tasks (Winneke et al, 1977; Lanthorn & Isaacson, 1978; Laporte & Talbott, 1978), although it has been suggested by one group that learning may well be improved by lead (Driscoll & Stegner, 1976); altered social behaviour including changes in aggressiveness, reduced social interaction and stereotyped behaviour (Silbergeld & Goldberg, 1973; Allen, McWey & Suomi, 1974; Cutler, 1977; Drew et al, 1979). It has been generally consistently noted that where lead affects learning tasks, the deficiency is greatest where the tasks are of a more complex nature. Studies involving the use of learning tasks of different complexity, such as orientation, which is relatively easy, and size discrimination, a more demanding task, have demonstrated no deficits on easy task learning (Winneke et al, 1977). However since performance in a learning task is complex and based on a number of functional processes such as motor functions, motivation, sensory functions,

cognitive functions namely memory process, it is not easy to pin point which function is being affected, although cognitive function is thought not to be the deficit (Winneke, Lilienthal & Werner, 1982; Kishi et al, 1983). It must be realised however that neurobehavioural studies on learning and memory in animals are relatively poor models of human cognitive function. It has been suggested that the deficit is associated with hippocampal damage (Jason & Kellog, 1980). The influence of lead on classical (Pavlovian) conditioning has been investigated. Adult rats exposed to lead in their chow showed enhanced conditioned emotional response; that is they showed an increased frequency of lever pressing in response to a cue representing the occurrance of an electric shock. However, these same animals were slower to learn this task than non-exposed animals (Nation et al, 1981). Data obtained by Alfano & Petit (1985) suggests the cholinergic system is involved in lead induced behavioural disorders.

The question arises when investigating the behavioural and learning effects of lead in animals as to the age of animals which should be employed in the study. The main problem of lead exposure in man peaks at age 1-3 years (NAS, 1972), a stage of neural and metabolic maturation more advanced than that of infant rodents. The effects of lead on adult rodents by comparison to younger animals are uncertain, with some authors claiming older rodents are insensitive to lead effects (Bornschein et al, 1980; Jason & Kellog, 1980; Michaelson, 1980) while other studies have

demonstrated quite clear effects in older animals (Donald et al, 1981; Angell & Weiss, 1982). However, it is not easy to separate the effects of lead pre- and post- weaning, since the feeding dam will impart lead to the offspring via the milk if she is exposed to lead while carrying her young, even if not exposed after parturition. A study by Dolinsky, Burright & Donovick (1983) suggests that the effects of lead administered postnatally is greater than those effects resulting from in utero exposure. Cross-fostering experiments however would suggest a largely prenatal effect of lead in causing a delay in development of exploratory and locomotor activity (Croften et al, 1981).

The level of exposure of the rodents is important since at high levels of exposure the observed effects are confounded by undernutrition. Nursing dams will comsume significantly reduced quantities of food and water when exposed to lead.

The question arises as to the reversibility of lead related behavioural changes. Krass et al (1980) studied the degree of recovery from lead induced neurobehavioural deficits in rats after the blood lead concentration had declined back to non-exposed levels. Following an exposure-free period of four months, during which time the blood lead had fallen, persistence of neurobehavioural symptoms occurred and so the question of irreversibiltiy arises. If indeed the neurotoxic effects of lead are irreversible, then young children whose nervous system is still developing, are at great risk.

1.7.4 Discussion and aims of this thesis

There is no doubt that lead is a toxic substance which in sufficient quantities can cause encephalopathy which may result in permanent brain damage to babies and children. The response of a particular child exposed from any source depends upon so intricate a network of interrelated factors that a simple relationship between dosage and degree of injury which would allow the risks to be calculated has not, and possibly cannot be established. As with all biological phenomena, the sensitivity of any individual child to lead exposure will vary; nevertheless it is recognised that once a child shows signs of lead encephalopathy, there is a danger that permanent damage may result.

None of the types of study discussed here provides definitive proof that lead causes, or is a primary contributing cause of impaired intellectual development. Significant methodological issues discussed previously limit the inferences that can be drawn from any one approach.

The Lawther Committee (DHSS, 1980) were convinced that at two ranges of blood lead, there is no doubt of the effect; that is if the blood lead exceeded 3.9μM (80μg/100ml), symptoms of exposure occur. At the other end of the spectrum, they were equally convinced that there was no convincing evidence of deleterious effects at a blood lead of less than 1.7μM (35μg/100ml). However, the committee were unable to come to any conclusions regarding effects within the range 1.7 - 3.9μM (35 - 80μg/100ml)

although they did recommend action be taken if a child's blood lead was of this order of magnitude. The inferences drawn from published work differ between reviews. At the other extreme of views, the Conservation Society concluded that there was most probably no threshold for toxicity and that neurotoxicity was liable if blood lead exceeded 0.24μM (5μg/100ml) (Conservation Society, 1980). Rutter (1980) takes a middle line with respect to the Lawther Committee and the Conservation Society. He concludes that although the research findings are somewhat contradictory, there is good evidence, especially from clinic type studies, that a blood lead persistently exceeding 2.9μM (60μg/100ml) is probably associated with an average reduction of 3 - 4 points even in asymptomatic children. Adverse cognitive sequelae are also possible within the range 1.9 - 2.9μM (40 -60μg/100ml) but this conclusion rests on less firm foundations. The cognitive deficit, when it occurs, is not of any specific type. He is unable to draw conclusions where the blood lead does not exceed 1.9μM (40μg/100 ml). However, it is noted in this report that in the studies using dentine lead as an index of exposure, the 'highest' lead group were selected from the general population and represented the top 10% of such, and not a rare, extreme group. Therefore the data observed refers to levels of lead exposure which had previously been regarded as 'safe' or 'acceptable'. The negative findings of Needleman et al (1979) and Lansdown et al (1974) are not discordant with the hyperactivity observed by David et al (1972; 1976) since the latter authors were dealing with a deviant group.

Uncertainties arise in comparing Needleman's study with those of other investigators. Findings are strongest with respect to behaviour whereas in other investigations the association between raised lead levels and behavioural disturbance have been both weaker and less consistent than those with impaired intelligence. No obvious explanation for this disparity is evident. A 4 point I.Q. difference with respect to dentine lead levels within the normal range suggests very much greater I.Q. differences should be found with blood lead in the range 1.9-3.9μM (40-80μg/100ml) and yet this has not been observed. Nevertheless this represents an important study and the results arising from it should not be dismissed lightly.

Studies of children living nearby or far from an extrinsic source of lead contamination should constitute a good test of the hypothesis that raised lead levels lead to intellectual deficits or below deviance. In fact this has not proved the case partly because very large samples are needed to test for small differences and also because of incomplete reporting and inadequate analysis in some studies. However, certain studies are consistent with a small intellectual deficit associated with raised lead levels in the range 1.9-3.9μM (40-80μg/100ml). Even if an average deficit in I.Q. is only a few points in any group of children, this implies more damaging impairment in some, and that a given dose of lead may possibly do more harm to the socially disadvantaged children. However, doubt still remains that even if it can be shown that children who have been exposed to lead are consistently less intelligent or

CHAPTER 2

GENERAL METHODOLOGY

2.1 Animals

2.1.1 Experimental model of lead neurotoxicity

The nature of the research for this thesis necessitates the selection of an animal model to study the neurological effects of lead exposure which would as closely as possible mimic those seen in man, and more importantly in young children, the most susceptible members of the population, to the neurotoxic effects of lead. The problems of using animal models of lead exposure and extrapolating data obtained from animal models to man has been adequately reviewed (Silbergeld & Goldberg, 1980). With any animal model comes an inherent problem, namely the existence of fundamental differences among species. The sensitivity of specific organs and systems to lead varies among species.

The mode of exposing the animals, which in the experiments for this thesis, will be rats, is an important consideration. From the following possibilities: intra-peritoneal, sub-cutaneous, intravenous, gastro-intestinal gavage, addition to food or water, or by inhalation of suspended particles, several can be eliminated as the chosen technique for various reasons. Following administration of lead acetate by intravenous, sub-cutaneous or intra-peritoneal routes, there is a rapid deposition of insoluble lead compounds especially carbonates from which lead is only slowly leached, and which can cause non-specific trauma (Bischoff & Bryson, 1977). Taking into consideration the views that man is exposed to lead primarily via the oral route (DHSS, 1980), the chosen method of administration for these experiments

will be orally, by addition of lead to drinking water. Lead may be administered as the chloride, carbonate, nitrate or acetate. The nitrate salt is not a common choice in neurochemical studies due to its inherent cardiac effects. In accordance with many other studies, the acetate salt will be employed.

One must also decide at which age and for what duration of time the rats should be exposed to lead. Even within a given species, the toxic effects of lead vary with age, sex and specific organ systems. The duration of exposure must account for the difference in life span between man and the rat. There is a compounding factor concerning lead, in that there is a complex relationship between dose and duration (Browder, Joselow & Louria, 1973). Acute exposure to high concentrations of lead is associated with effects such as encephalopathy, seizures, coma and even death, effects not seen even after very prolonged exposure to low lead levels.

Animals can be exposed to lead orally either directly or by indirect methods. The well quoted method of exposing rodents to lead, that of Pentshew & Garro (1966), involves exposing suckling rodents to the metal by administering 4% lead carbonate to the dams. This technique delivers significant doses of lead via the maternal milk (Bornstein, Michaelson & Fox, 1975). The regime preserves two fundamental aspects of lead exposure in children; firstly, the oral route of intake, and secondly, the pre-weaning period in the rat exhibits analagous brain growth patterns to the first two years in man. The data in this thesis will be derived from exposing rats to lead both directly and

indirectly. Mature animals are generally considered more resistant to the central neurotoxic effects and thus the majority of studies involve exposure of neonates and developing young to the metal. These experiments will involve either exposing the rats to a lead drinking solution from weaning, or alternatively the indirect technique will be employed. Following exposure of pups in utero and then via maternal milk, the young will then be exposed once again directly to the metal.

Finally the level of lead exposure must be considered. One must remember the level of exposure via the diet to which man is exposed, and the fact that the rat absorbs and retains only one tenth that of man, in order to select a level of exposure in the model. There are however compounding factors. For example, it is known that absorption of lead in rodents is not dose dependant until the lead concentration is relatively high (Gerber & Wei, 1974). In addition, the gastro-intestinal absorption of lead in the rat has been calculated using standard rat chow, and it has been observed that these absorption figures tend to rise towards the values obtained in man, when the animals are fed on human diets. Therefore, from the foregoing discussion, it is evident that in experimental toxicity it is not easy to determine an equivalence in exposure between the rodent and man and thus a compromise must be attained.

2.1.2 Housing and feeding considerations

Male Sprague-Dawley rats were supplied by Bantin & kingman Ltd., Grimston, Hull, England. The animals were kept in either wire mesh stainless steel base or plastic base cages and were supplied with food and liquid ad. libitum. Stainless steel base cages were employed as other metals had previously been demonstrated to be a potential source of lead. The rats on arrival were divided randomly into groups and supplied with either distilled water or a solution of lead acetate of a selected concentration as a drinking solution, in addition to laboratory chow 41B. The cages containing those animals receiving distilled water were invariably positioned on a rack above those animals supplied with lead acetate. This ensured that no lead could be spilled into cages of animals in the distilled water control group. Similarly, the rats receiving the highest dose of lead were placed nearest the bottom of the rack. The lead acetate solution was prepared as a concentrated stock solution (120 mM) stored at 4 ^{o}C in a plastic container and diluted to the desired concentration as required. Lighting and heating conditions were maintained as constant as possible throughout the period of each experiment and animals were sacrificed in the morning. Prior to each animal being sacrificed, the weight was noted.

2.1.3 Breeding experiments

The majority of experiments in this thesis employed rats which were supplied with distilled water or lead acetate from weaning, in which case the animals were obtained from

the suppliers as weaners weighing approximately 50g. However a group of rats were bred in the department. The procedure employed in this study was as follows:- female Sprague-Dawley rats weighing in the region of 150g and male rats (180 - 200g) were obtained from Bantin & Kingman Ltd. The females were supplied with distilled water or a lead solution for four weeks after arrival. During this period, the heating and lighting were maintained constant with a twelve hour light period to ensure that the female rats came into oestrous regularly every four days. At the end of this period, the females were mated on the Harem system. Groups of one male and four females were allowed to run together in one cage, mating taking place when each female came into oestrous. After seven days the male rats were removed from the cages. Just prior to parturition, (the gestation period of the rat being 21-23 days), the pregnant females were segregated into individual cages and supplied with paper shavings as bedding to cover their offspring. When born, the young remained with the dam until four weeks old at which time the mothers were removed and the offspring segregated according to sex. The adult female rats continued to receive distilled water or the lead acetate solution throughout pregnancy and when rearing their young. After the young were weaned the male offspring were then supplied with the appropriate solution as drinking fluid until being sacrificed at one month post-weaning.

All animals were sacrificed by decapitation. A sample of blood and femur were removed for lead analysis.

2.1.4 Brain dissection

The skulls of the rats were opened and the brains removed intact. Freehand dissection was then carried out as rapidly as possible. The areas of rat brain which were studied in all experiments were the cerebellum, midbrain, diencephalon and telencephalon (cerebrum). Plate 1 shows these regions in the intact brain and the procedure employed to obtain them is described diagramatically in figure 2.1.

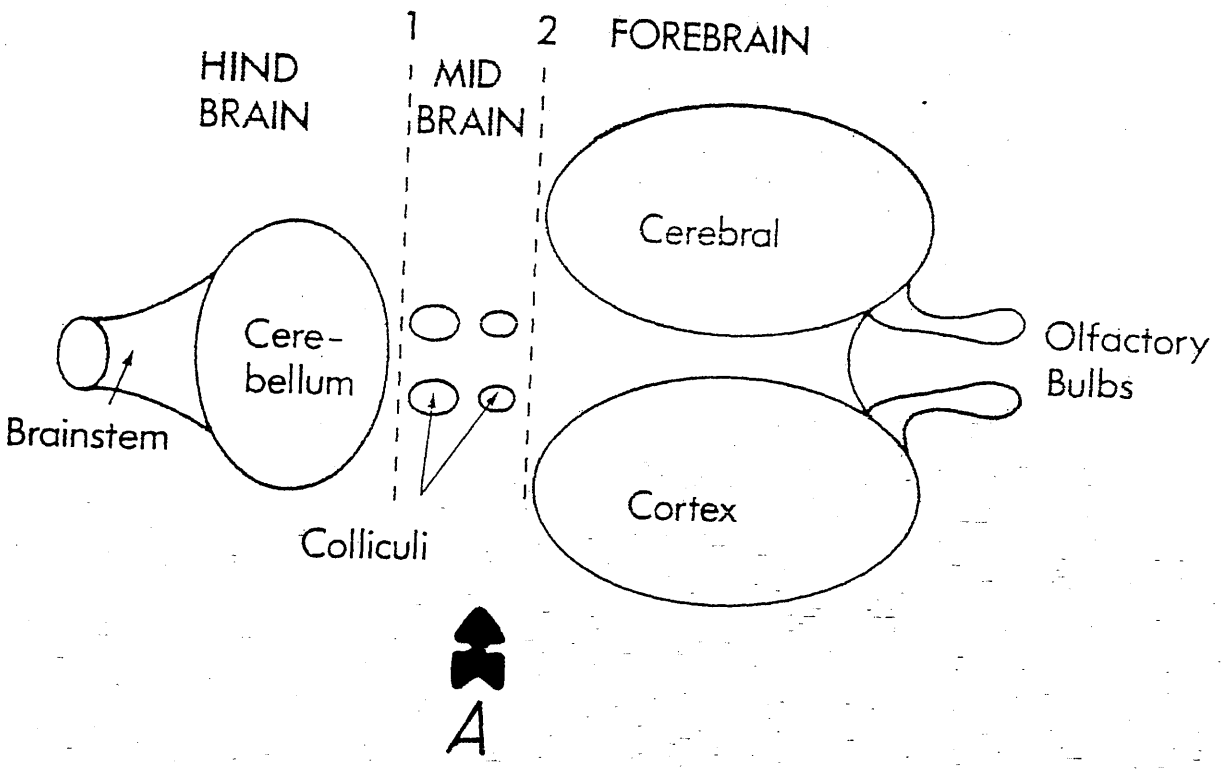
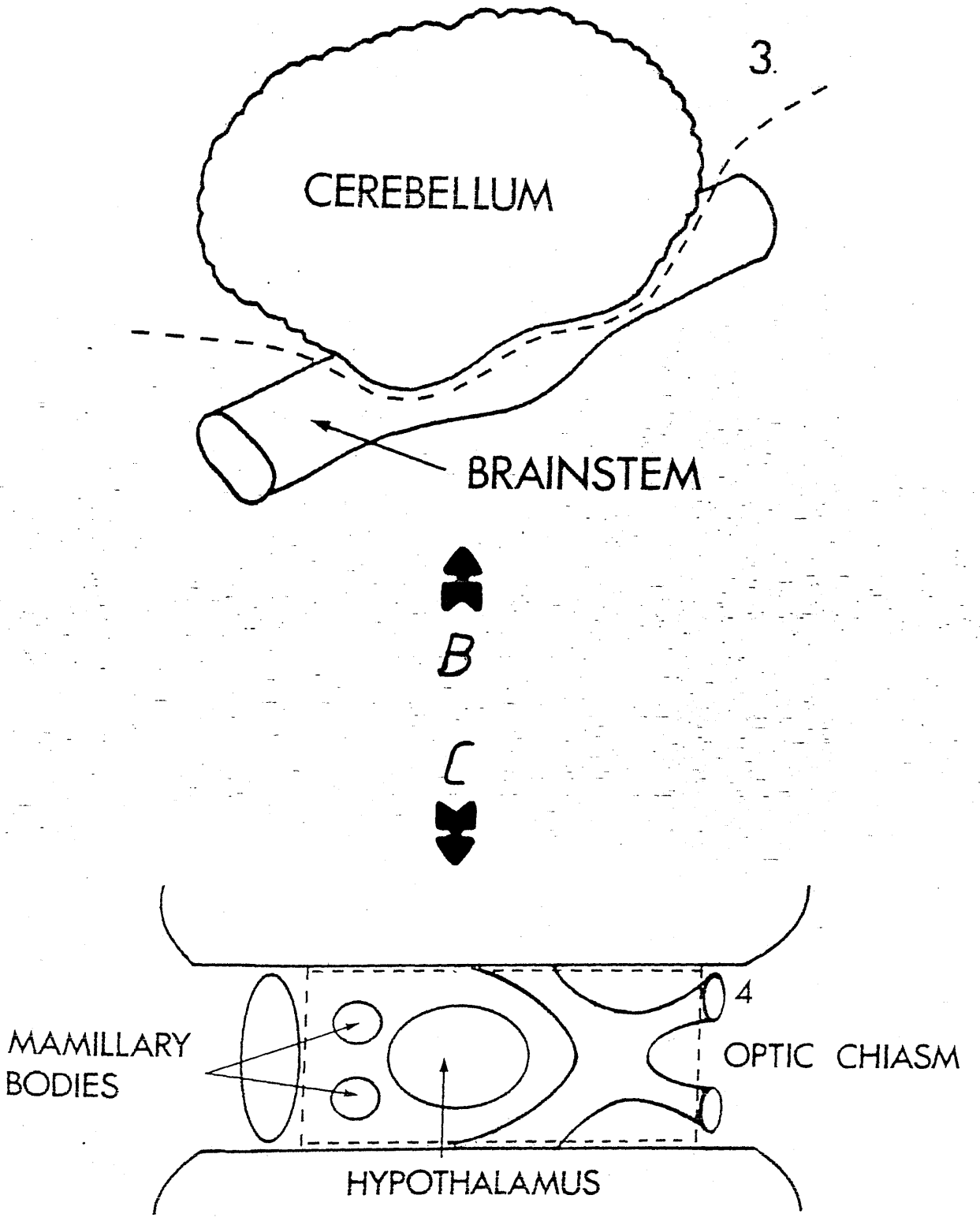


Figure 2.1 Schematic representation of the procedure employed to dissect the rat brain

(a) The rat brain was placed on the bench with the hemispheres facing uppermost. Using a scalpel, the hemispheres were carefully dissected away from each other and from the cerebellum to expose the 4 colliculi of the midbrain. Cuts labelled 1 and 2 in the diagram were then made, thus separating the hindbrain, midbrain and forebrain.

(b) Using a scalpel the cerebellum is readily dissected from the brainstem (cut 3).

(c) With the underside of the forebrain uppermost a block is dissected out of the tissue as shown by cut 4.


3.
CEREBELLUM
BRAINSTEM
B
C
4
MAMILLARY BODIES
OPTIC CHIASM
HYPOTHALAMUS

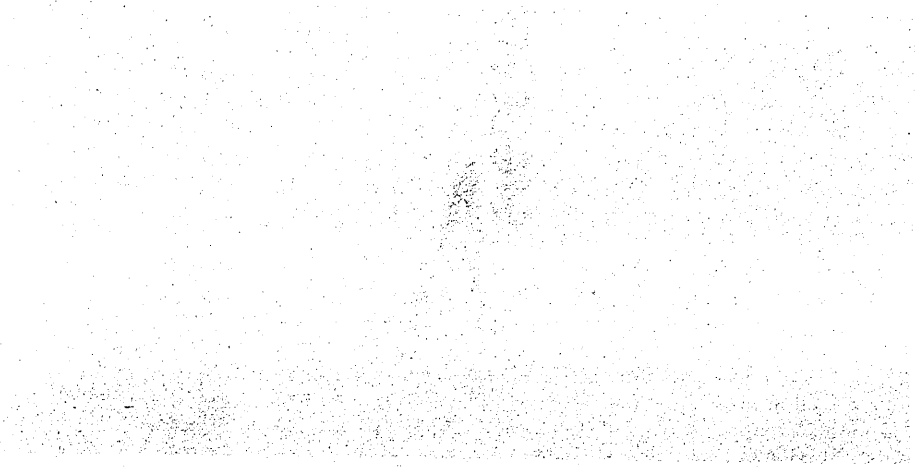
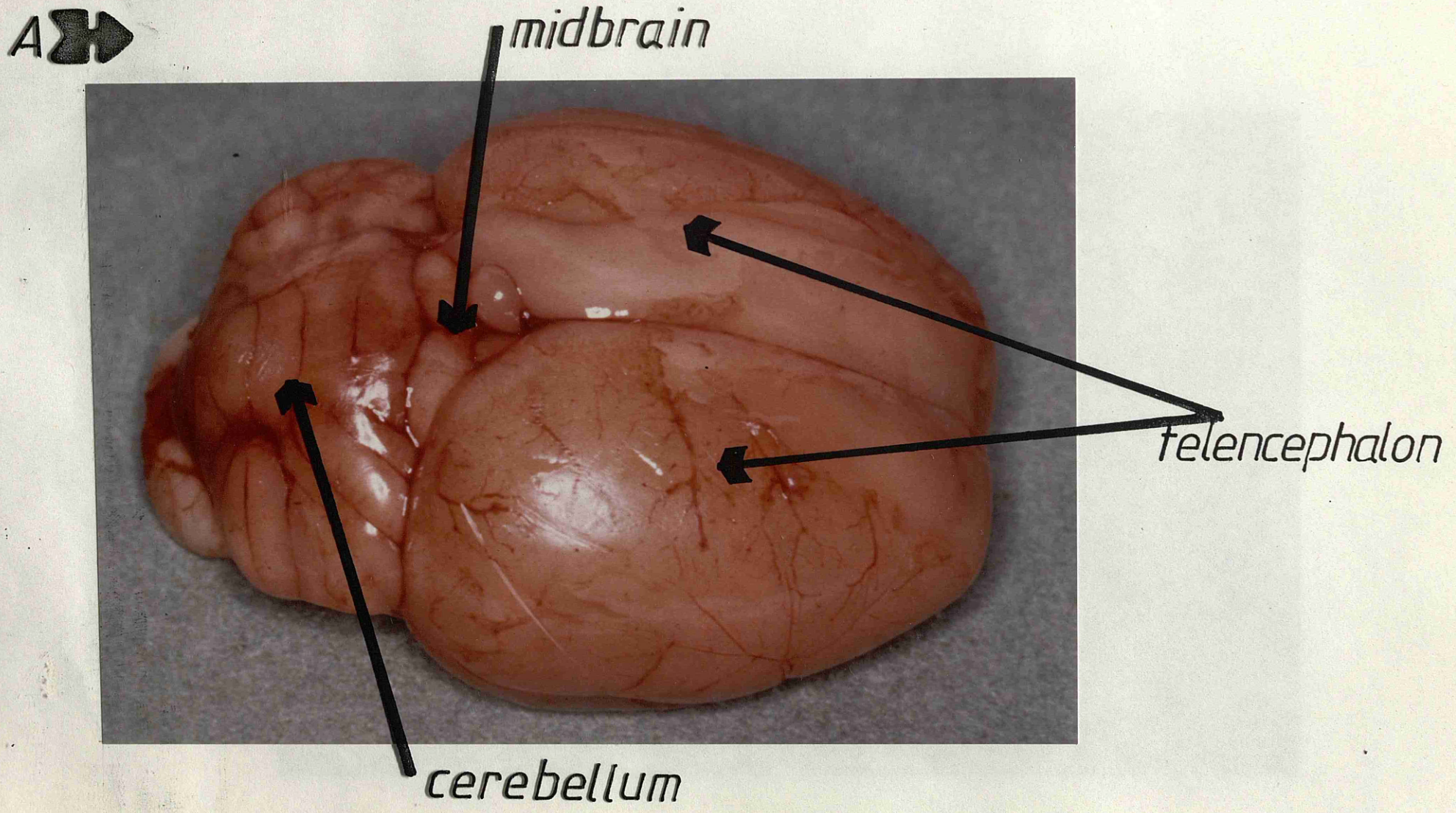
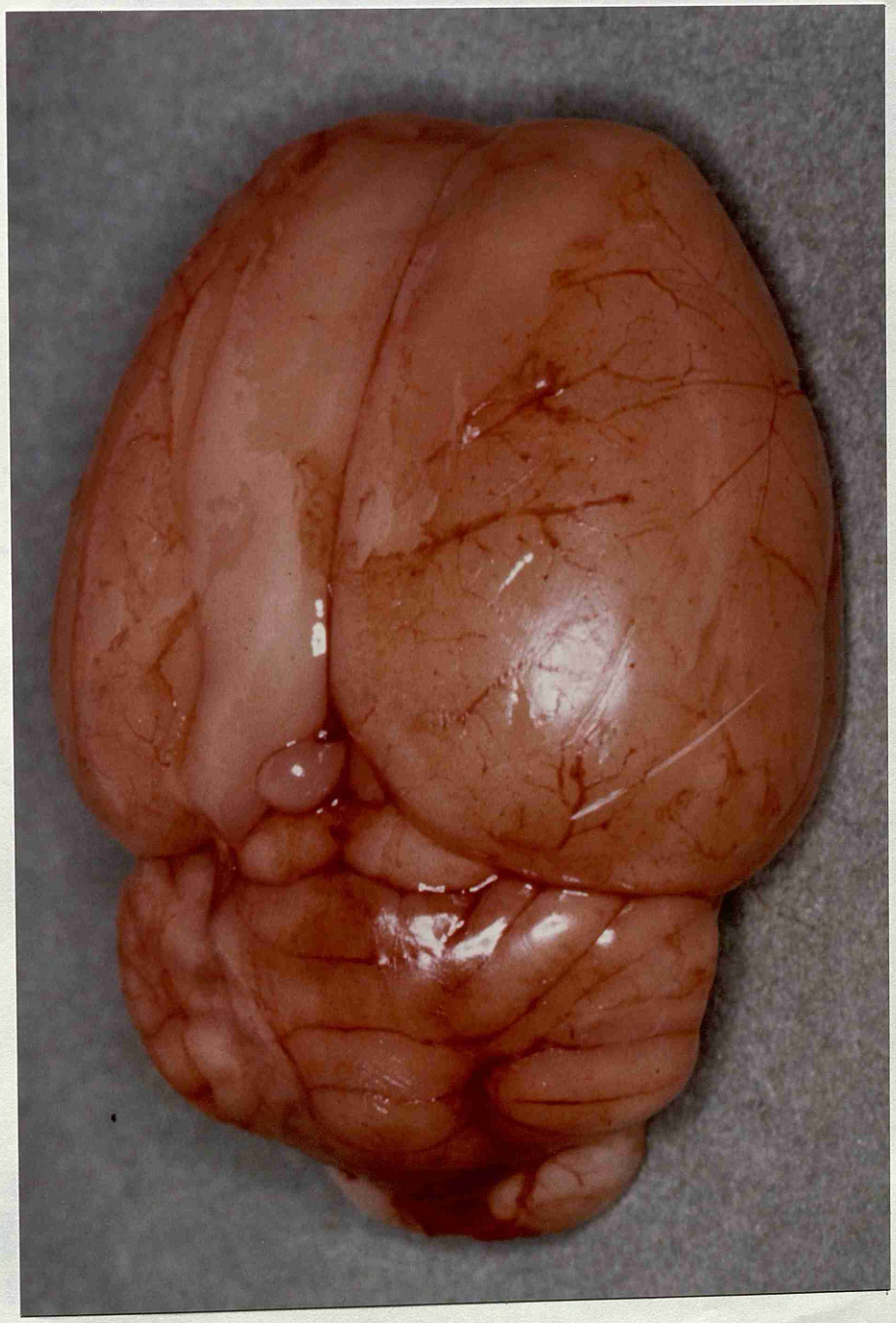


Plate 1. Photographs of rat brain showing the regions to be studied.

The photographs show the upper side of the rat whole brain (a) illustrating the cerebellum, midbrain and telencephalon regions. (b) shows the underside depicting the diencephalon.


A
midbrain
telencephalon
cerebellum



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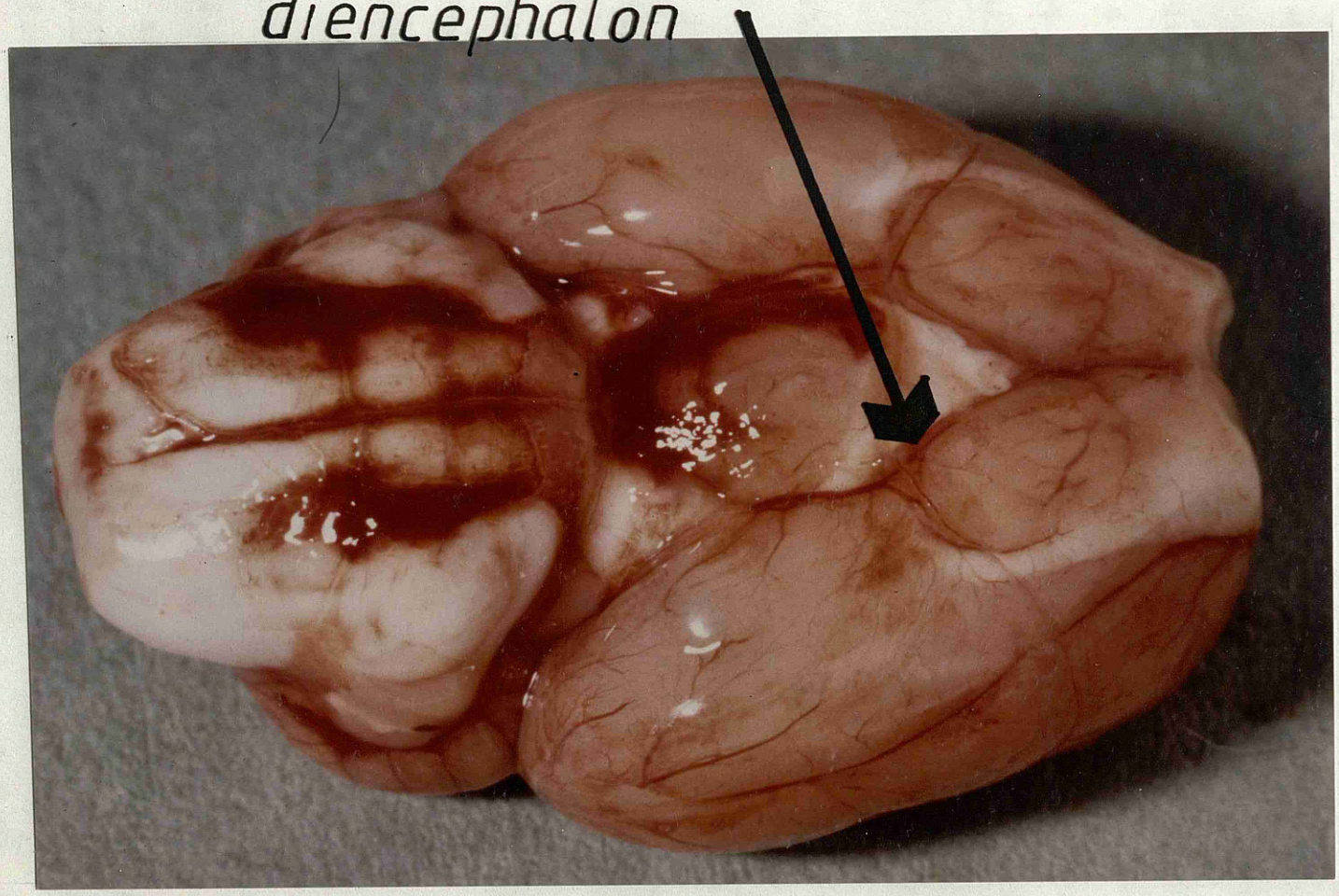
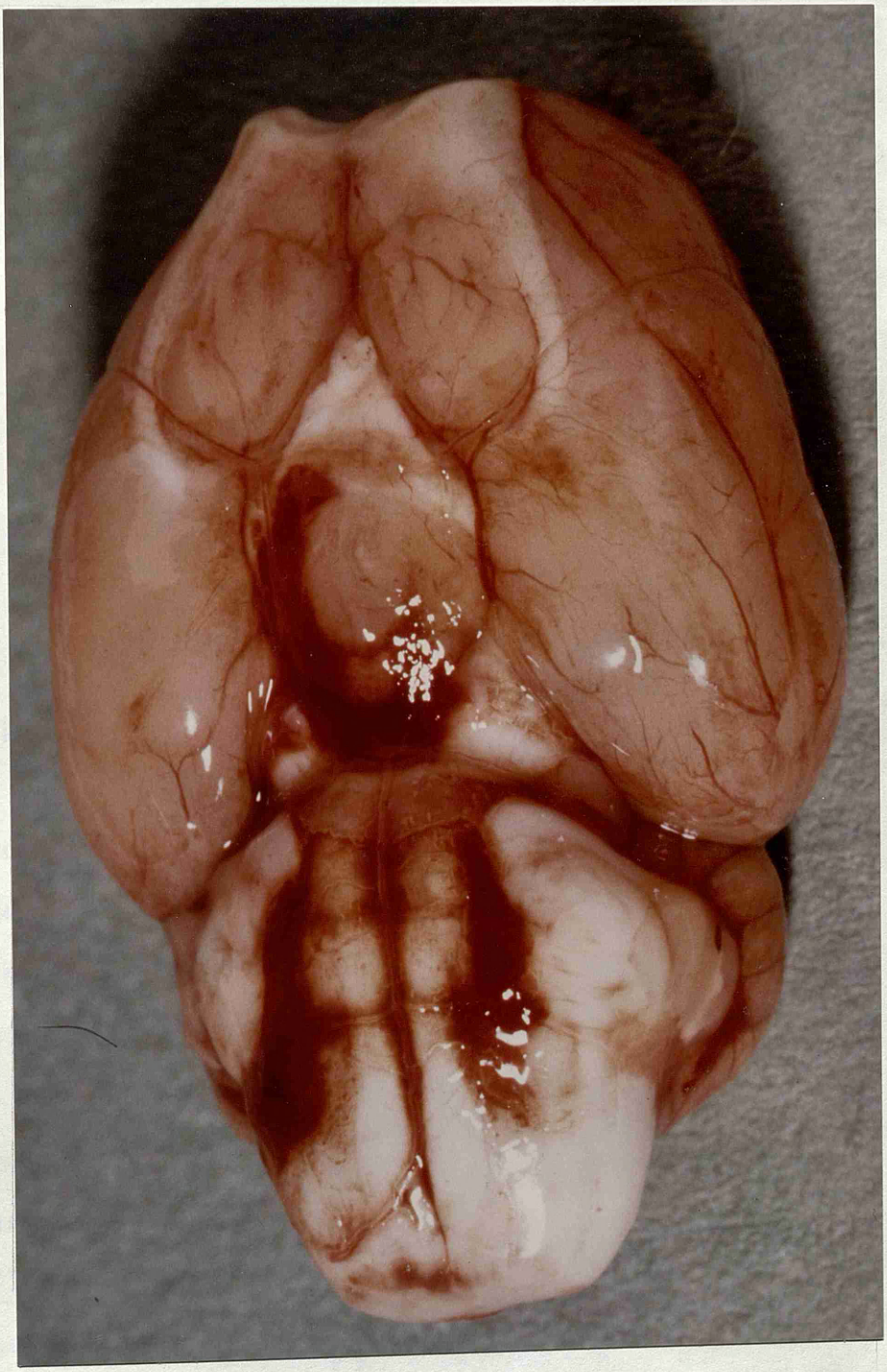




2.2 Blood lead analysis

Whole blood lead concentrations were measured by flameless atomic absorption spectrophotometry (Perkin-Elmer 703 with HGA 500 and AS-1 autosampler or Perkin-Elmer 306 with an HGA 72 furnace). Blood, collected in lead-free polystyrene or polypropylene tubes containing either potassium ethylenediaminetetraacetic acid (EDTA) or sodium heparin as anticoagulant was stored at 4 °C until analysis. It has previously been demonstrated that no appreciable absorption of lead onto the container materials occurs during storage under these conditions. (Moore & Meredith, 1977). At the time of analysis, the samples were mixed for at least 45 minutes to make certain that the lead was evenly distributed throughout the sample. After mixing, a 1 in 10 dilution of each sample was prepared in triton X-100 (in duplicate). Assay calibration was by the technique of matrix matching. A commercially available lead nitrate standard (4.8mM; 1mg/ml) was added to whole human blood and the standards aliquoted before being stored at -20 °C. A set of standards was then carried through the assay (in duplicate) with each batch of samples.

Each tube containing either sample or standard was capped and mixed on a whirlimixer for a few seconds before being analysed for lead content by atomic absorption.

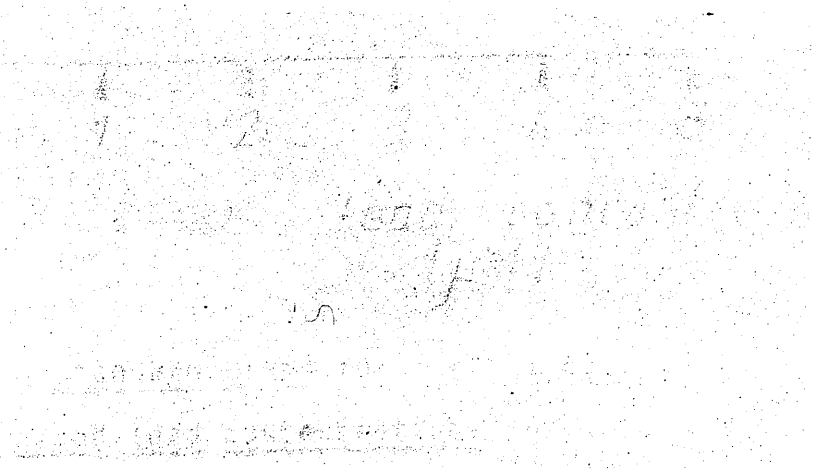
P-E 703 The autosampler was set to inject a volume of 20µl into the furnace, and to sample each cup twice. The furnace was programmed as in table 2.1. The absorbance due to lead atomisation was recorded on a Perkin-Elmer 706 single pen recorder from which the peak heights were manually

measured. The average absorbance of the two readings from each cup was used in calculating the lead concentration except where they differed greatly in which case the cup was re-analysed.

P-E 306 Sample volumes of 20µl were injected manually into the furnace. The conditions employed are shown in table 2.1 and again absorbances were manually measured from a Perkin-Elmer 706 recorder.

A standard graph was constructed by plotting blood lead concentration versus atomic absorption absorbance for each standard, absorbances being corrected for blank values (figure 2.2).

All glassware, pipette tips, autosampler cups and tubes used in the analysis were soaked in 50% nitric acid ('analar grade') overnight before being rinsed with distilled water and dried in an oven prior to use.



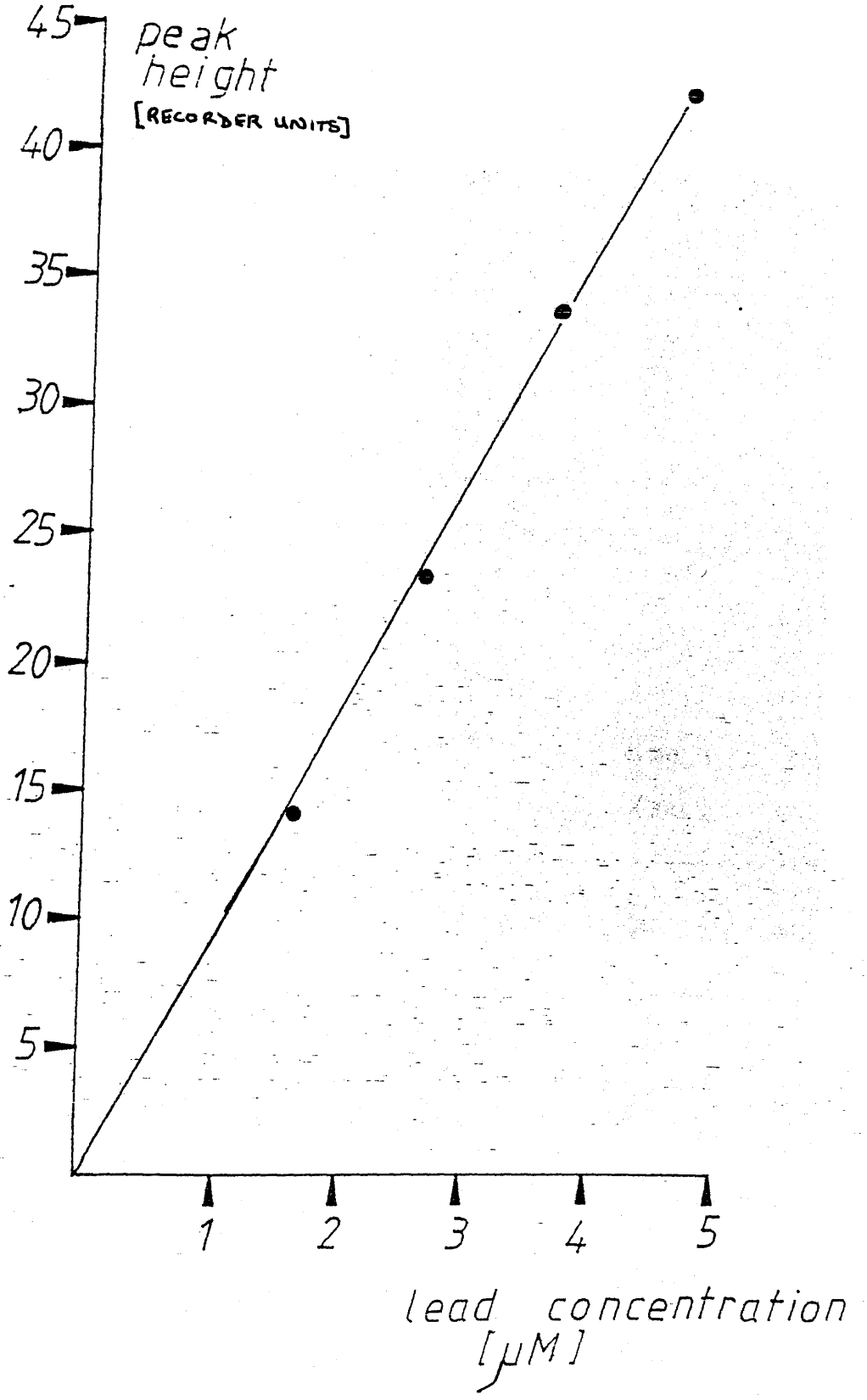


Figure 2.2 Standard graph for the calculation of blood lead concentration.

Example of a calibration graph for the calculation of blood lead estimation. Each point represents the average of 2 estimations.

Table 2.1 Programmer settings for blood lead

HGA 500

Stage	Temperature (^{o}C)	Ramp time (sec)	Hold time (sec)	Gas flow
drying	100	20	10	
ashing	540	25	15	
atomisation	2300	4	5	10
cleaning	2500	3	2	

3 seconds prior to atomisation the recorder is initiated and the baseline set.

HGA 72

Stage	Temperature (^{o}C)	Time (sec)
drying	100	20
ashing	450	20
atomisation	2040	4

2.3 Bone Lead Analysis

Rat femur lead content was measured by the method of Wittmers, Alich & Aufderheide (1981). Bone samples were dry-ashed, dissolved and analysed directly for lead content by electrothermal atomic absorption spectrophotometry. This technique is prone to severe negative bias owing to interferences from the complex sample matrix of bone. These effects can be minimised by employing the method of serial additions, but analysis time is greatly increased. Several reports have been published expressing the usefulness of lanthanum in overcoming this interference problem (Bertenshaw, Gelsthorpe & Wheatstone, 1982). The technique employed in this thesis involves the addition of lanthanum ions to both the standard solutions and bone ash in order to measure the lead content without extraction.

The bone samples were scraped to remove any adhering tissue, weighed and placed in a crucible, before being dried at 100 ^{o}C to constant weight in a muffle furnace. After recording the dry weight of each bone, they were ashed in a muffle furnace at 450 ^{o}C again to constant weight. When ashed the bones had turned completely white. The temperature was selected to avoid loss of lead by volatisation of the sulphide or chloride salt. (Middleton & Stuckley, 1953; Gorsuch, 1962; Kellow, kostial & Harrison, 1975). The ash weight was obtained and the samples were ground to a fine powder in an agar mortar. 20-25mg of each bone powder (in duplicate) was dissolved in 0.5ml of 9.4M nitric acid ('aristar grade') and diluted with 1.5ml of distilled water. A 0.2ml portion of this

dissolved bone solution was transferred to a polystyrene autosampler cup and diluted with 1ml of distilled water containing 853µg/ml lanthanum ion (3.1mM). The lanthanum ion was prepared as a stock solution consisting of 2g lanthanum oxide dissolved in 160ml of nitric acid and diluted to 1 litre with distilled water. This yielded a stock solution containing 1,705µg/ml lanthanum ion (6.2mM) which was diluted 1 in 2 with distilled water to produce the working solution containing 853µg/ml La^{3+}. The purpose of the lanthanum ion was to suppress matrix interferences caused by other ions present in the bone.

A calibration graph was constructed using lead standard solutions:- 0, 2, 4, 8 µg lead/ml standards were freshly prepared for analysis by pipetting 0, 0.2, 0.4 & 0.8 ml of a commercially available lead standard solution (4.8mM; 1mg/ml) into 100ml volumetric flasks, adding 1ml concentrated nitric acid ('aristar' grade) and diluting to volume with distilled water. 20µl of each standard (in duplicate) was then transferred to an autosampler cup containing 1ml of working lanthanum solution (853µg/ml). The resulting standards contained 0.0, 39.2, 78.4 & 156.9 ng lead/ml, which are equivalent to 0.000, 0.189, 0.377 & 0.756 µM. A typical calibration curve can be seen in figure 2.3.

The bone samples and standard solutions were analysed by flameless atomic absorption spectrophotometry (Perkin-Elmer 703 with HGA 500 and AS-1 autosampler). The furnace was programmed as shown in table 2.2. The AS-1 autosampler was set to inject a volume of 20µl into the furnace, and to sample each cup twice. The average absorbance of the two readings was used in subsequent calculations except where the two absorbance readings differed by greater than 4mm on the chart recorder, in which case the sampling of the cup was repeated. Absorbance readings were recorded using a Perkin-Elmer 706 single pen chart recorder. Calcium deposits which built up on the walls of the furnace tube and cones and interfered with the lead signal (Okuno, Whitehead & White, 1978), were regularly removed by abrasion.

All glassware, crucibles, sample cups and pipette tips used in the analysis were made lead-free by soaking in 7.8M nitric acid ('analar grade') overnight, rinsing with distilled water before drying in an oven.

In preliminary experiments, the validity of utilizing lanthanum ion to minimise matrix interferences was investigated. The lead content of several bone samples was determined by the method of standard additions:- once again, 20-25mg of bone ash was dissolved in 0.5ml nitric acid (9.4M) and diluted as before with 1.5ml of distilled water. 0.2ml of the dissolved bone ash was pipetted into each of 8 autosampler cups . To 6 sample cups 20, 40 & 60µl of the 2µg/ml lead standard was added (in duplicate) and all cups were diluted to 1ml with the 853µg/ml working

solution. Figure 2.4 shows a typical calibration line obtained by this procedure and for comparison the line obtained using aqueous standards. There is no significant difference between the slopes of these two lines at the 1% significance level.

Table 2.2 HGA 500 programmer settings for bone lead.

Stage	Temperature (^{0}C)	Ramp time (sec)	Hold time (sec)	gas flow
drying	100	7	20	
ashing	500	7	20	
atomisation	2500	7	5	10
cleaning	2500	2	1	

2 seconds prior to atomisation, the recorder is initiated and the baseline set.

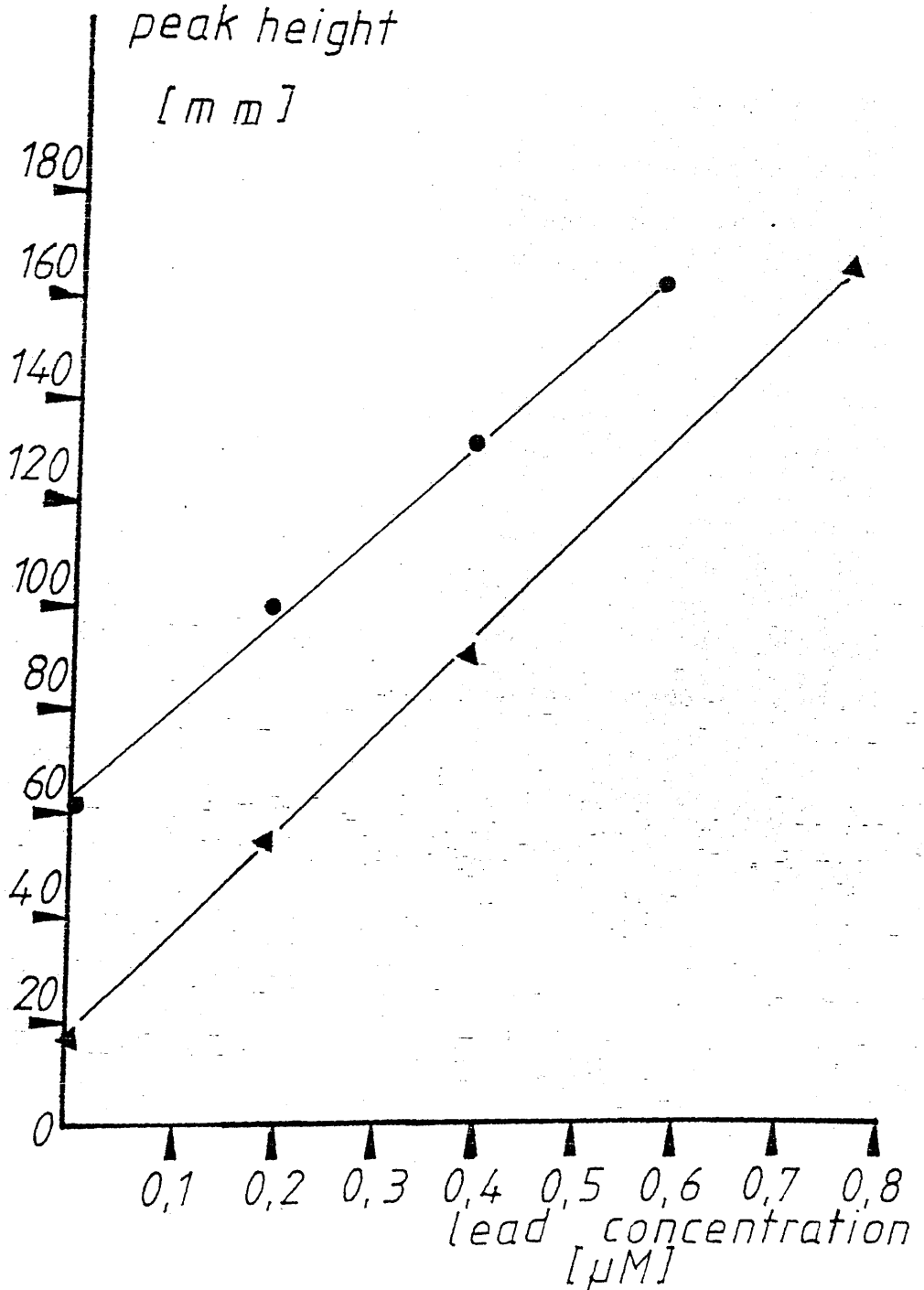


Figure 2.4 Comparison of an aqueous calibration curve with serial addition.

Comparison of an aqueous calibration graph represented by the triangles with a curve constructed by serial addition techniques -circles. Statistical analysis demonstrates the slopes of the 2 lines are not statistically different. Each point represents the mean of 2 observations.

2.4 Tissue lead analysis.

A 1 in 5 dilution of each tissue homogenate was prepared in 0.025% (w/v) trition X-100. The lead content of each sample was then quantitated by direct injection of 20µl into an HGA 72 flameless atomisation furnace which was employed in conjunction with a Perkin-Elmer 306 atomic absorption spectrophotometer. The furnace conditions employed were as detailed for blood lead estimation (table 2.1).

A standard curve was prepared based on peak height, by the method of standard additions.

2.5 Water lead measurements

Analysis of domestic water samples for lead content was performed by the Strathclyde Regional Council laboratory, the method employed being described below.

100ml portions of each acidified domestic water sample were concentrated by evaporation to a volume of 10±5ml in covered, glass beakers, before being diluted to 25ml with distilled water in calibrated 'lead-free' flasks. Each batch of water samples included blank(s) and lead solutions of known concentration, which were treated in an identical manner to the unknown samples. Lead quantitation was performed by flame atomic absorption spectrophotometry by direct aspiration of each treated sample, blank or lead solution into the air-acetylene flame. Standard lead solutions were also aspirated into the atomic absorption spectrophotometer and from these a standard calibration curve was constructed. Preliminary experiments had demonstrated that the process of evaporation and re-

dilution did not affect the calibration curve. The concentration of lead in each unknown sample was then read from the graph and corrected for the absorbance of the blank. All glassware and other equipment employed throughout the assay was prepared 'lead-free' in a similar manner to that described in previous assays involving lead quantitation. Samples of lead concentration less than 0.05μM were not able to be quantitated by this method as described.

2.6 Blood cadmium analysis

Blood cadmium concentration was determined by carbon furnace atomic absorption spectrophotometry. Whole blood was collected in polystyrene tubes containing potassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant and stored at 4°C until cadmium analysis could be carried out. The analysis was carried out by the Department of Pathological Biochemistry, Glasgow Royal Infirmary using the procedure similar to that described by Stoeppler & Brandt, 1978; 1980).

All glassware, tubes and autosampler cups employed in the assay were soaked in 20% nitric acid ('analar grade') for one hour, then rinsed in deionised water prior to use. Blood samples were mixed for at least fifteen minutes before analysis (Denley Spiramix). 300μl whole blood was treated with an equal volume of 10% nitric acid ('aristar grade') in LP3 tubes. After thorough mixing, the tubes were capped and centrifuged at 2,500rpm for fifteen minutes. The deproteinised supernatant was then transferred to an autosampler cup to be loaded onto the autosampler tray.

20µl of each sample was injected into the carbon furnace. A reagent blank was prepared by replacing the blood with distilled water in the above procedure. Calibration was achieved by the technique of matrix matching- addition of cadmium nitrate standard to whole human blood. The standards were prepared as shown in table 2.3, and stored at -20 °C for up to six months in 0.5ml aliqots. A set of standards was then analysed with every batch of samples.

The instrumentation employed to measure the cadmium content was a Perkin-Elmer 370 atomic absorption spectrophotometer with deuterium arc background correction and HGA 76 carbon furnace with AS-1 autosampler. The instrument conditions for analysis can be seen in tables 2.4 & 2.5. Absorbance signals were recorded as peak height on a pen recorder set at a full scale deflection of 10 millivolts. A standard graph was constructed of peak height against cadmium concentration of standard, from which the cadmium concentration in each sample was read. The method was linear over the range 0-90 nanomoles cadmium per litre. Samples exhibiting a higher concentration than this were diluted in distilled water prior to analysis, and re-analysed. The within batch precision (C.V.) at low cadmium levels was 9.1% (mean ± S.D. of 21±2 nmol/l, n=20) and 1.9% at high blood cadmium levels (mean ± S.D. of 213±4 nmol/l, n=20). Between batch precision was 22.9% at low cadmium levels (mean ± S.D. of 23±5 nmol/l, n=20) and 10.6% at high cadmium levels. (mean ± S.D. of 222±23 nmol/l, n=20).

The recovery of added cadmium was satisfactory and comparison of results using an alternative fluorescence

technique (Michel et al, 1979) was acceptable. In addition, participation in two external quality control schemes -a National quality control scheme (SAS) and a European scheme (IUPAC) indicated that the method was of a satisfactory level.

Table 2.3 Preparation of standards for blood cadmium quantitation.

Standard	0	1	2	3	4	5	6
ml Cd(NO3) - 0.5mg Cd/l	0	0.1	0.2	0.4	0.6	0.8	1.0
ml blood	50.0	49.9	49.8	49.6	49.4	49.2	49.0
cadmium concn. (nmol/l)	0	8.9	17.8	35.6	53.4	71.2	89.0

- stock cadmium nitrate prepared from a commercial standard (BDH - 1 mg/l), diluted 1 : 10 followed by 1 : 200 to produce a cadmium concentration of 0.5mg/l.
Standards were prepared in acid-washed polypropylene flasks and stored in 0.5ml aliquots at -20 ^{0}C for up to 6 months.

Table 2.4 HGA 76 programmer settings for cadmium analysis

Stage	Temperature ^{o}C	Rate	Time (sec)	Additional conditions
drying	100	2	20	
ashing	350	1	10	auto zero
atomisation	1900	0	5	purge gas stop
cleaning	2400	0	5	

Table 2.5 Spectrophotometer conditions for cadmium analysis

wavelength	228.8 nm
lamp current	6 mA
mode	absorbance
slit setting	0.7 nm
purge gas	argon
background correction	on
sample volume	20.0 µl

2.7 Instrumentation:- high-pressure liquid chromatography (HPLC)

2.7.1 General considerations

The analytical technique of high-pressure liquid chromatography (HPLC) will be employed in order to measure several parameters in the research for this thesis. The terms high-performance and high-speed are synonymous with high-pressure. The basic components of an HPLC system are illustrated diagramatically in figure 2.5.

HPLC, as a separation technique is rapidly gaining popularity over thin-layer (TLC) and gas chromatography (GC) and is extremely useful when combined with a selective detector exhibiting low detection limits. The solvent or mobile phase travels through the column at a constant rate. The sample is introduced onto the column via the injector and its components are separated according to differences in their molecular groups. Such components differing in their distribution between the stationary phase (column packing) and mobile phase, migrate through the column at differing rates. Each separated component elutes from the column and passes through the detector as a 'band'. The detector then sends an electrical signal to the recording instrument where it is measured as a deviation from a baseline to produce a chromatogram.

2.7.2 Column

All experiments in this thesis will employ a stainless steel column of dimensions 250 X 4.6mm I.D. which will be slurry packed at a pressure of 7.5kpsi using a Shandon column packing pump (Shandon Southern Products Ltd.,

Cheshire, England) with Spherisorb 5μm ODS packing material. Column packing was performed according to the recommendations of Shandon Southern Products Ltd; that is 3.8g packing material will be utilized per column and propan-2-ol employed as both slurry and packing solvent. Conditioning of each column will be achieved using methanol and water in the ratio 1:1 (v/v) prior to use. Ultimately, since the main concern is the column's ability to perform a desired separation, it is useful if each column packed is compared only briefly with its predecessor. To this end, a mixture of amino acids -cytosine, adenine and guanine will be separated on each column under standard conditions. -

mobile phase:-

0.01 M potassium dihydrogen orthophosphate

0.01 M heptane sulphonic acid

- pH 2.9

95% of above buffer : 5% acetonitrile (v/v)

flow rate 2 ml/minute

From this chromatogram, a typical example of which is shown in figure 2.6, the number of theoretical plates can be calculated from equation 2.1.

$$\text{Theoretical plates, } N = 16 \left(\frac{t_r}{W}\right)^2$$

-Equation 2.1

where t_r is the retention time of the peak in seconds and W the peak width also in seconds. Figure 2.7 illustrates these parameters used in the equation.

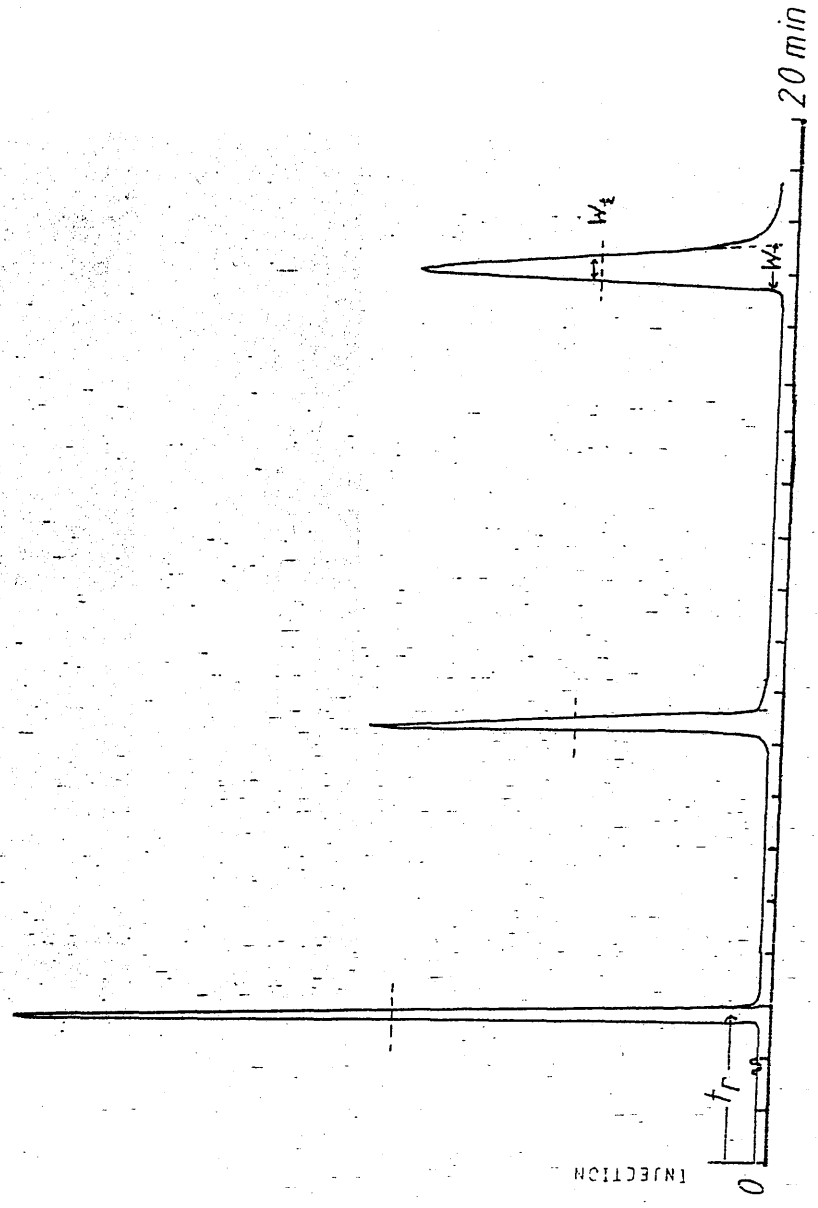


Figure 2.6 Typical example of a test chromatogram.

Test chromatogram employed to evaluate the quality of a column. Cytosine, adenine and guanine were injected onto the column using conditions described in the text, and monitoring at 340nm. Illustrated are the parameters used to calculate the number of theoretical plates-

w - peak width in seconds

$w_{1/2}$ - width of the peak at half maximum height in seconds (alternative parameter to w)

t_r - retention time of the peak also in seconds.

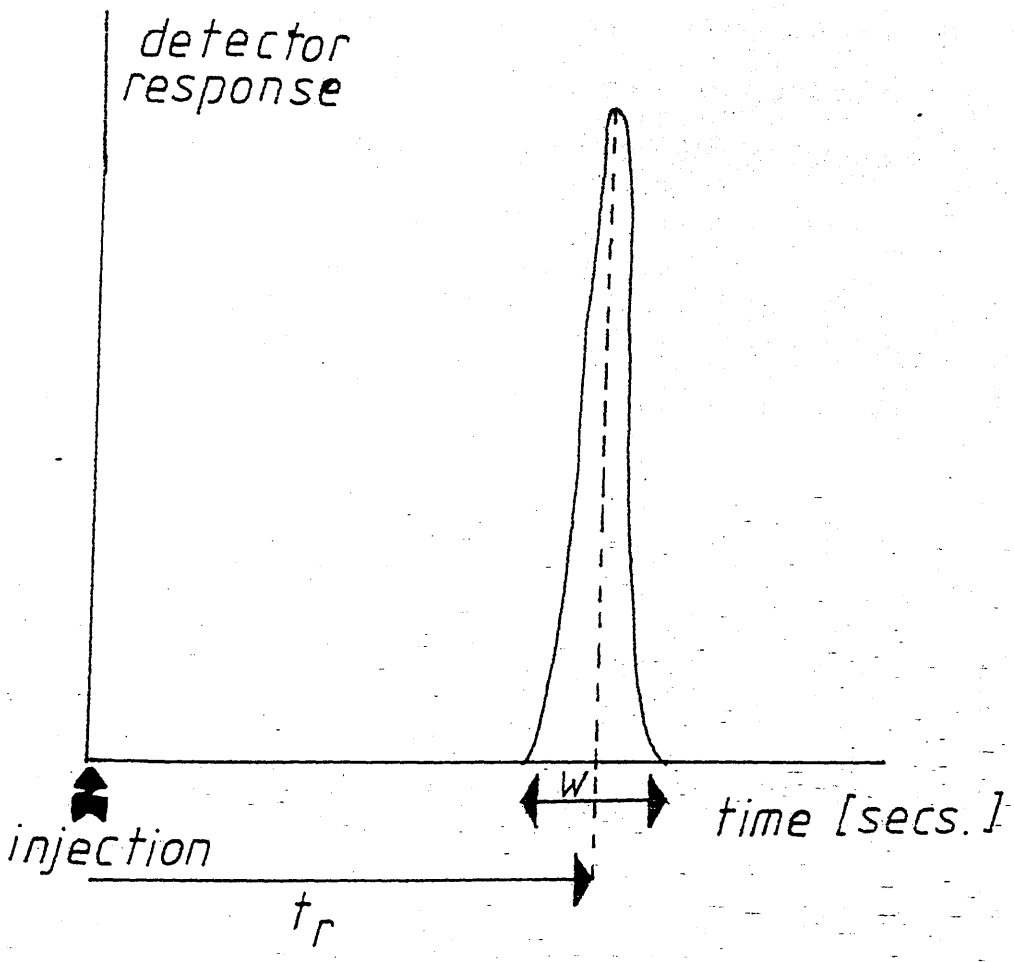


Figure 2.7 Parameters used to calculate the number of theoretical plates in a column.

The peak shown in the diagram is expanded in width for clarity.

W represents the peak width inseconds

tr the retention time also in seconds.

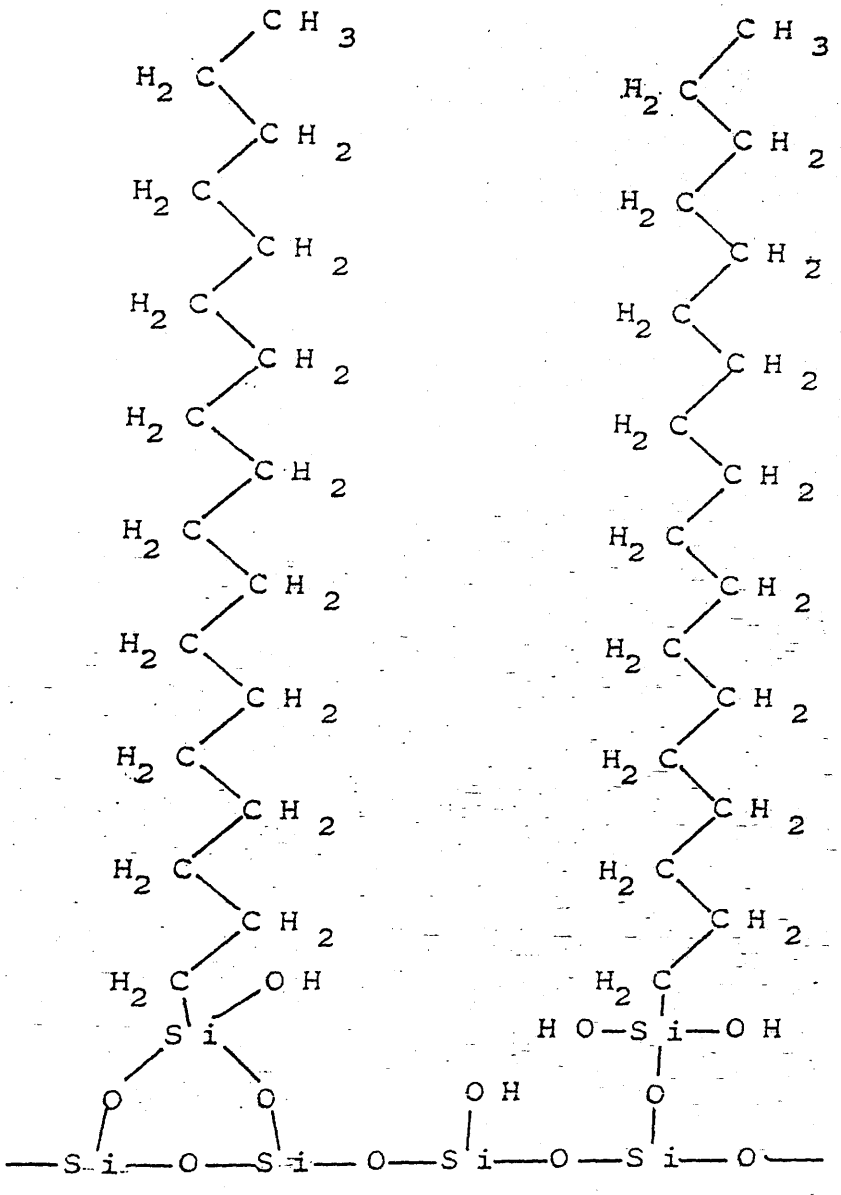


Figure 2.8 Structure of the stationary phase of a reverse-phase column.

Schematic representation of the octadecylsilane groups (ODS) constituting the stationary phase of the HPLC column.

stationary phase (the degree of retention being dependant on the species). This technique termed ionic suppression is explained in equation 2.2 -.

$$R\text{-}COOH \underset{<------}{------>} R\text{-}COO^{-} + H^{+}$$

<---------- buffer drives equilibrium to left

- Equation 2.2

However, over the pH range employed in reverse-phase chromatography - pH 2.5-9.0 (outside this pH range the silica support is altered), strong acids and bases remain in their ionic form and are therefore poorly retained or elute as skewed peaks. A second technique is therefore required if these are to be separated. Paired-ion chromatography involves inclusion of a large organic counter-ion in the mobile phase, the nature of this counter-ion depending on the solutes to be separated. Basic solutes can be separated by employing alkyl sulphonates; while for acidic solutes, a quaternary amine may be used as the counter-ion. Sodium octyl sulphate will be used as an ion-pairing agent in the chromatographic system. Thus paired-ion chromatography, whose development can be attributed to Schill and his co-workers (Eksborg et al, 1973), is a means to separate ionic species on a reverse-phase system by forcing the ionic solutes to behave as non-ionic moieties with some lipophilic characteristics. This technique is known under a variety of synonymous terms -

'soap chromatography' - Knox & Laird (1976)

'ion-pair chromatography' - Fransson et al (1976)

'solvent-generated (dynamic) ion-exchange

- Kraak, Jonker & Huber (1977); Terweij-Groen, Heemstra & Kraak (1978)

'Paired-ion' - Waters associates

'detergent-based cation-exchange' - Kraak et al (1977)

'solvophobic-ion' - Hoffman & Liao (1977)

'surfactant' - Tomlinson, Jefferies & Riley (1978)

'hetaeric chromatography' - Horváth et al (1977)

Such a variety of nomenclature indicates the uncertainty which exists by which retention of an ionised solute is affected by ion pairing materials on the chromatographic column. There are basically two hypotheses-

1. The eluate, present in its ionic form, combines with the counter-ion in the mobile phase -equation 2.3.

$$\text{sample}^{+} + \text{counter-ion}^{-} \underset{\text{----->}}{\text{<-----}} [\text{sample}^{+}\ \text{counter-ion}^{-}]^{0}$$

- equation 2.3

this complex then behaves as an electrically neutral and lipophilic compound. The extent to which the ionised sample and counter-ion form a complex affects the degree to which the complex partitions into the bonded, hydrophobic stationary phase. The more lipophilic the ion-pair complex, the greater will be its attraction for and retention on the non-polar stationary phase.

Or alternatively,

2. the lipophilic counter-ion is adsorbed onto the reverse-

phase surface with its ionic group orientated towards the surface. The column then behaves in a manner akin to an ion-exchanger, with the charged sample ions partitioning between the mobile phase and the stationary phase.

The main equilibria that are generally recognised to be important in separation are as follows -

(a) ion-pairing in the mobile phase

$$\text{sample}^{+}_{m} + \text{counter-ion}^{-}_{m} \rightleftharpoons [\text{sample}^{+}\ \text{counter-ion}^{-}]^{0}_{m\ \text{pair}}$$

followed by reversible binding to the non-polar surface

$$[\text{sample}^{+}\ \text{counter-ion}^{-}]_{m} \rightleftharpoons [\text{sample}^{+}\ \text{counter-ion}^{-}]_{s}$$

(b) dynamic ion exchange - the solute forms a complex with the counter-ion already adsorbed onto the non-polar surface

$$\text{sample}^{+}_{m} + \text{counter-ion}^{-}_{s} \rightleftharpoons [\text{sample}^{+}\ \text{counter-ion}^{-}]_{s}$$

(c) dynamic complex exchange - metathesis exchange of solute between the ion-pairing agent bound to the column and the ion-pair formed in the mobile phase

$$[\text{sample}^{+}\ \text{counter-ion}^{-}]_{s} + \text{counter-ion}^{-}_{s} \rightleftharpoons [\text{sample}^{+}\ \text{counter-ion}^{-}]_{s} + \text{counter-ion}^{-}_{m}$$

-where the subscripts 'm' and 's' represent mobile and stationary phase respectively.

It is not known which of the above mechanisms represents the method of solute retention and perhaps Melander & Horvath (1980) are correct in concluding that the above mechanisms represent limiting cases and the retention process is not expected to follow any one or other mechanism over a wide range of chromatographic conditions.

2.7.3 Pump

The pump in the HPLC system must deliver a continuous, uniform, pulse-free flow of solvent through the chromatographic system. For this purpose an Altex 110 reciprocating piston pump will be employed. In addition, a pulse dampener will be incorporated to minimise fluctuations in flow rate and thus stabilize the detector baseline.

2.7.4 Injector

The sample will be introduced onto the column by means of a Rheodyne 7010 or 7125 syringe loading sample injection valve (Rheodyne Inc., Berkeley, USA). Typically, sample loops employed will be either 50µl or 100µl.

2.7.5 Solvent

The mobile phase composition will vary with the assay and condition of column at the time of analysis. However, the general procedure employed to make up all solvents for HPLC use follow a similar pattern -

Buffer is prepared by dissolving the salt in distilled water which has been redistilled from alkaline potassium permanganate in order to oxidise any impurities in the water. This procedure helps to stabilize the baseline (minimise the detector noise) and hence increase

sensitivity. After adjusting the hydrogen ion concentration to the desired pH, the buffer is filtered through a 0.45μm membrane using millipore vaccuum apparatus (Millipore S.A., Molshein, France). The organic modifier, which in these experiments will be methanol, is filtered through a 1um membrane before being combined with the filtered buffer in the desired ratio. After addition of the ion-pairing agent, the solvent will be degassed by helium displacement, before being pumped through the column. The relative proportions of buffer and methanol will vary with application and column conditions. Basically, retention times of solutes will decrease with increasing methanol content. In the same manner, increasing concentrations of sodium octyl sulphate cause solutes to be retained by the column to a greater extent, but only up to a certain concentration of ion-pairing agent, above which the retention of solutes is not affected.

2.7.6 Detector

The recent advances in column technology (Majors, Barth & Lochmuller, 1982) have meant that the major limitation of liquid chromatography is the sensitivity of the detector system. Combining the very small extra column dead volumes with electrochemical detectors which have dead volumes in the order of microlitres, the technique of HPLC:ECD (LCEC) is very attractive. The possibility of using electrochemistry to monitor the effluent from a chromatographic column has been recognised since the 1950's (Kemula, 1952). The main characteristics of a good detector are selectivity, sensitivity, noise and linear range. Each

characteristic will be defined and discussed briefly with regard to the system to be employed in these experiments -

Selectivity: A non-selective detector, for example a refractive index detector, responds to sample components of all types whereas a selective detector responds only to a certain type of compound. An electrochemical detector is selective since the compounds must be electroactive to be detected. This feature is especially important in situations where the samples to be analysed are of a complex nature containing not only the components of interest but, in addition, other compounds of potential interference, as is the case with many biological samples.

Sensitivity: Defined as the slope of a plot of detector response versus sample concentration, and will depend on the type of sample being analysed.

Noise: Random or periodic pattern superimposed on the steady-state background signal and represents the summation of spurious contributions from pump pulsations, flow hydrodynamics, cell-surface reactions, static electricity, power line noise, electronic amplification effects etcetera.

Noise and sensitivity lead to a quantity termed minimum detectable quantity (MDQ) or detection limit which is defined as the amount of sample that produces a baseline shift of twice the standard deviation of the noise.

Linear range: Range in which the detector signal is directly proportional to the sample concentration (figure 2.9).

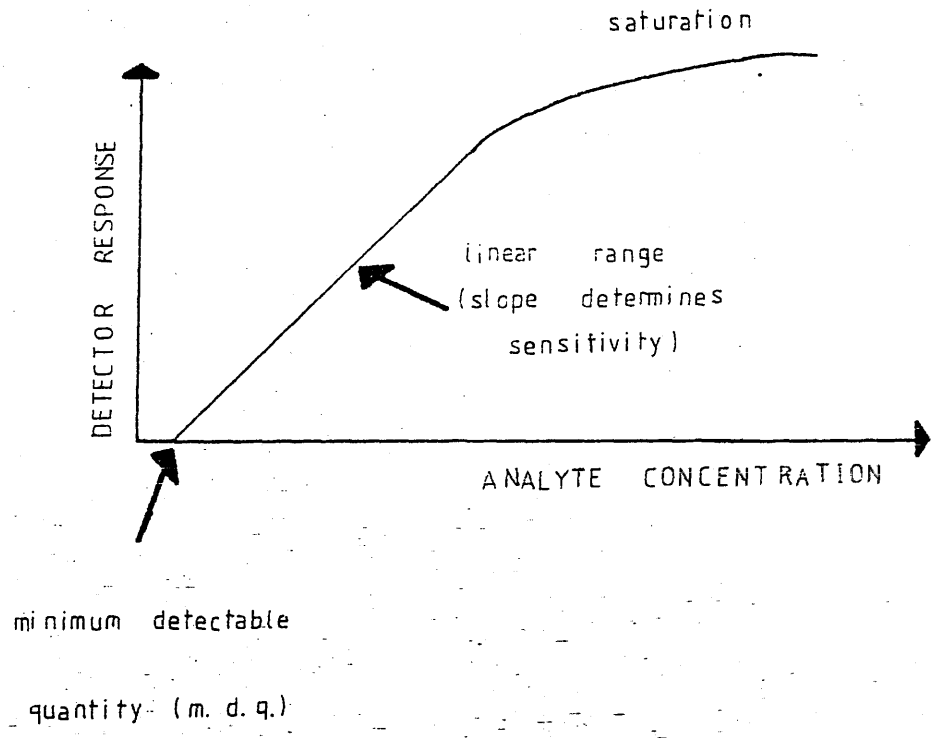


Figure 2.9 Linear range of electrochemical detector.

From a plot of analyte concentration against detector response 2 clear regions of the graph are identifiable. Firstly, a linear range in which the analyte concentration is directly proportionable to detector response; and secondly, at a specific analyte concentration detector response does not increase further with increases in analyte injected.

The first report on the successful use of an electrochemical flow cell as a detector component for liquid chromatography appeared in the early 1970's when Kissinger and his colleagues reported the detector as having a detection limit in the picogram range (Kissinger, 1973). A schematic representation of the electrochemical cell employed can be seen in figure 2.10 (Bioanalytical Systems Inc., West Lafayette, USA). The detector working electrode is located in a block consisting of two parts separated by a 50μm teflon gasket. The eluent from the column passes over the electrode surface via a slit in the gasket. The outstanding features of this type of thin-layer cell are its low volume and high sensitivity. The outlet from the cell is connected to a silver/silver chloride reference electrode from which the eluent passes through a stainless steel tube serving as an auxiliary electrode, and a waste tube leading back to the solvent reservoir; that is the mobile phase is recycled. The purpose of the reference electrode is to provide a stable, reproducable voltage to which the working electrode potential can be referenced. The auxiliary electrode merely serves to complete the current loop. The function of the potentiostat amplifier is to apply a fixed potential difference between the working cell and the solvent with respect to the reference electrode. In addition, it also serves to amplify the oxidation current produced at the electrode surface, as this is often in the nanoampere range, and to convert the current into a voltage in a proportional manner.

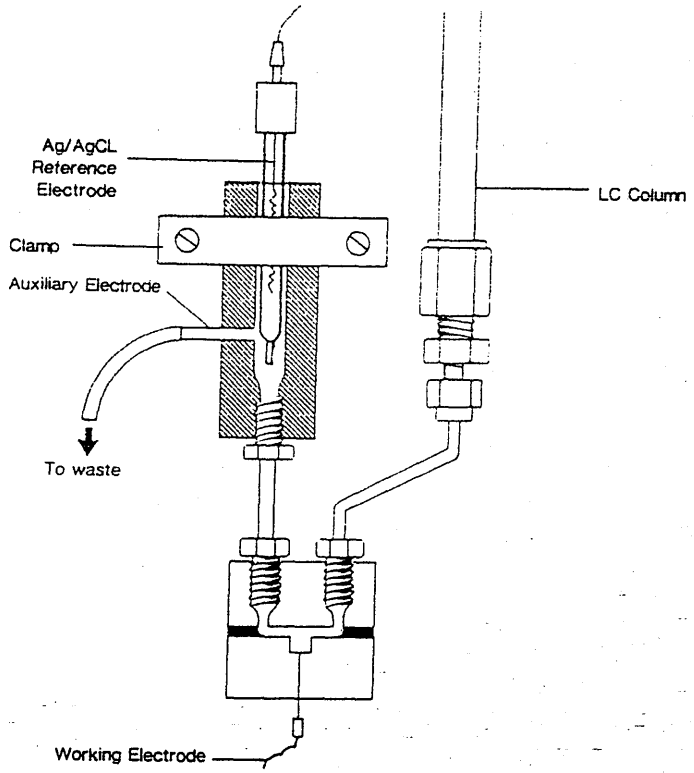


Figure 2.10 Electrochemical flowcell.

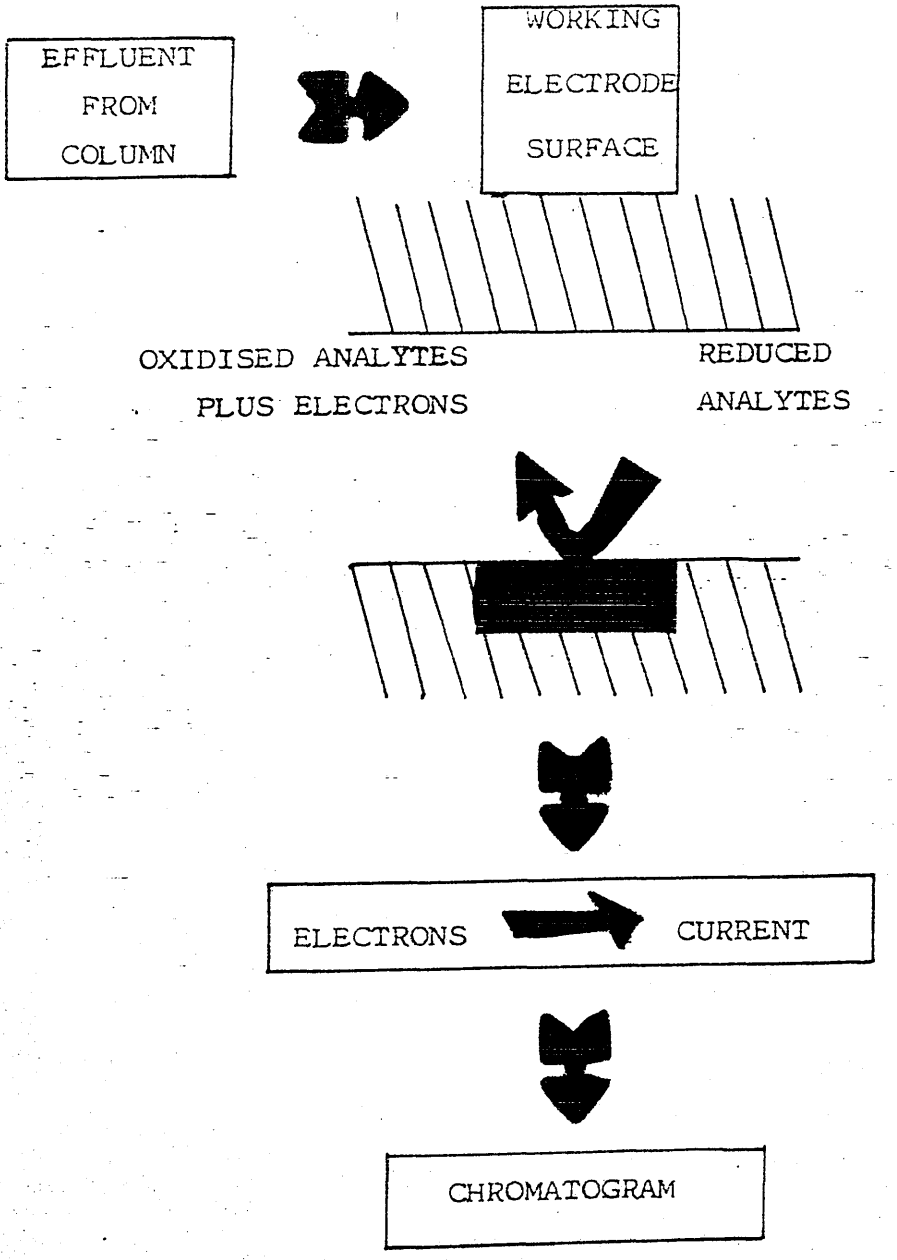
Schematic representation of the electrochemical flowcell comprising a carbon paste working electrode, silver/silver chloride reference electrode, and the auxiliary electrode completing the circuit.

The process of LCEC may be considered in terms of electrolysis at a fixed point along a flowing stream, the stream or column eluate being composed of a sequence of solute zones separated with varying degrees of resolution. The zones of analytes pass through the thin-layer cell over the working electrode held at a fixed potential. If the potential exceeds that required for electrolysis of the analyte, electrons pass from the analyte to the electrode. The resulting current, proportional to the solute concentration passing over the electrode, is amplified by the controller and observed as a function of time on the recorder (Figure 2.11). The detector is selective since only species electroactive at the applied potential will be detected.

Although the original development of the detector employed a carbon paste working electrode, there are now several types of surfaces available -glassy (vitreous) carbon, 3 types of carbon paste, mercury/amalgamated gold, and platinum. Carbon paste is a very fine graphite with an inert binder mixed to a semi-dry paste. The carbon with its sheets of delocalised electrons acts as an excellent conductor to convey charge. The binder employed may be paraffin oil, ceresin wax or silicone grease. Carbon paste and glassy carbon are the most commonly employed cell types. Glassy carbon is a hard, brittle, solvent impervious glass-like material which is highly conductive and capable of being polished to a mirror-like finish. The decision on which type of electrode to employ in an LCEC system depends on the chromatographic conditions and sample type to be

Figure 2.11 Schematic representation of the principle of electrochemical detection.

Diagramatic representation of the basis of the electrochemical detector.



analysed. Glassy carbon when compared to carbon paste exhibits a similar sensitivity but the noise on a good carbon paste electrode is generally less. The reduced noise leads to a reduced detection limit. The time required for a new carbon paste electrode to achieve a steady baseline, termed the conditioning time, is shorter than that for glassy carbon - when the working electrode is turned on a large current signal termed the charging and transient background current is observed. This gradually decays over a period of minutes or hours depending on the cell type and conditions (figure 2.12). The steady state background current which arises from oxidation of electroactive impurites in the mobile phase is nulled out using offset controls on the controller. A good detector electrode will exhibit fast electron transport. It has been demonstrated that carbon paste with a paraffin oil binder is a favourable surface in this respect.

It would appear from the above considerations that carbon paste is superior to glassy carbon as an electrode surface. However glassy carbon does have its advantages. The lifetime of a carbon paste electrode can vary considerably from a second to several months whereas glassy carbon tends to be more stable. Both surfaces are subject to electrode passivation whereby components of the mobile phase or products of the electrode reaction adsorb onto the surface. When this occurs the surface of the glassy carbon cell must be repolished, whilst the carbon paste cell must be repacked. The glassy carbon cell is capable of withstanding high concentrations of organic solvents

whereas the limit of organic solvents is around 25% (v/v) methanol or 5% acetonitrile if carbon paste is employed. Since the mobile phases to be employed in these studies will not contain in excess of 10% methanol the electrochemical cell employed will have a carbon paste with paraffin oil binder working electrode surface.

Electron transfer at the electrode is essential for detection and such transfer will only occur if the electrode is polarised at or greater than a certain potential. The potential is selected for each analyte by constructing a plot of applied potential to the cell against electrode current produced. This is termed a hydrodynamic voltammogram and exhibits the features shown in figure 2.13. In the zero region the potential is not sufficient to force oxidation to occur. The current then rises with applied potential until in the plateau region current is independent of potential. In most situations the potential applied to the cell is selected from this plateau region.

2.7.7 Recording device

The chromatograms obtained will be recorded by means of a single channel pen recorder set at full scale deflection of 1 volt.

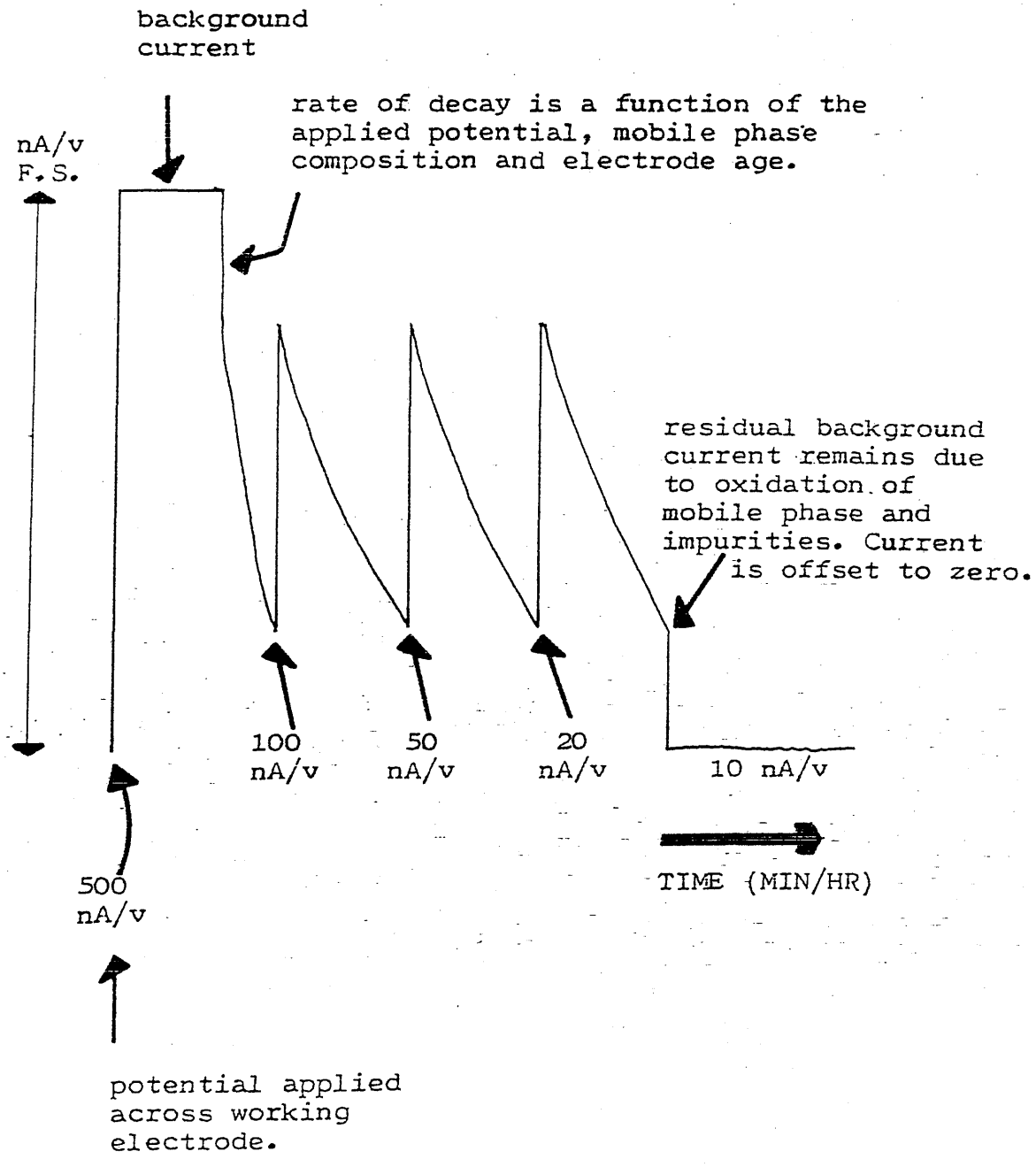


Figure 2.12 Electrode response on applying a potential.

A large transient current is produced on application of a potential across the working electrode surface. This circuit gradually decays with time until a small residual remains which can be offset.

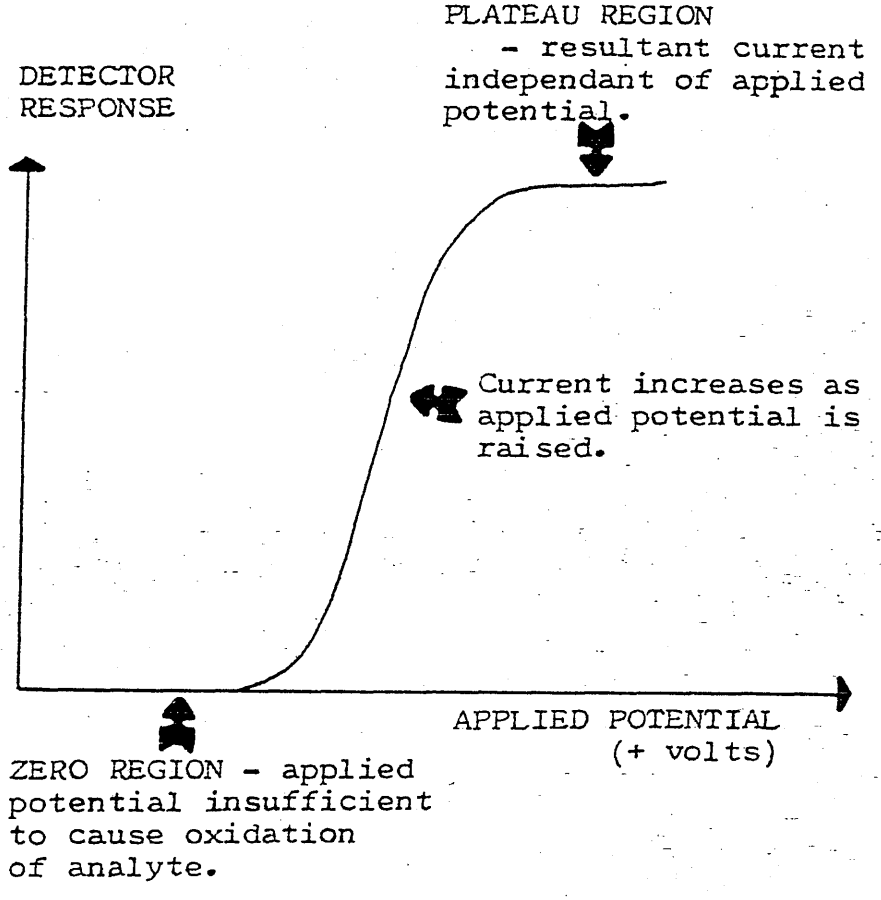


Figure 2.13 Hydrodynamic voltammogram

Plot of applied potential versus detector response showing the various regions of the voltammogram; zero region, linear region and plateau region.

2.8 Purification of aluminium oxide (alumina)

The alumina required for the analysis of tissue catecholamines and tyrosine hydroxylase activity was purified according to the method of Anton & Sayre (1962).

500g high purity alumina (Woelm neutral activity, grade 1) was slurried with 2M hydrochloric acid. The slurry was heated to 100 ^{0}C for approximately 30 minutes with stirring. After aspiration of the acid, the procedure was repeated until the acid no longer turned yellow. A distilled water wash was then performed repeatedly until the pH rose to 3.4 and the wash water was no longer cloudy. Finally after drying by heating to 100 ^{0}C, the alumina was stored in an air-tight container till required. The alumina will be employed in a purification step in the analysis of tisuue catecholamines and the activity of the enzyme tyrosine hydroxylase. At alkaline pH the catechol group is adsorbed onto the alumina by ionic interactions. Following the adsorption of the basic catechols, the alumina is washed to remove neutral and acidic compounds, before desorption of the catechols is achieved by formation of an acidic environment. Figure 2.14 explains this mechanism of absorption and elution.

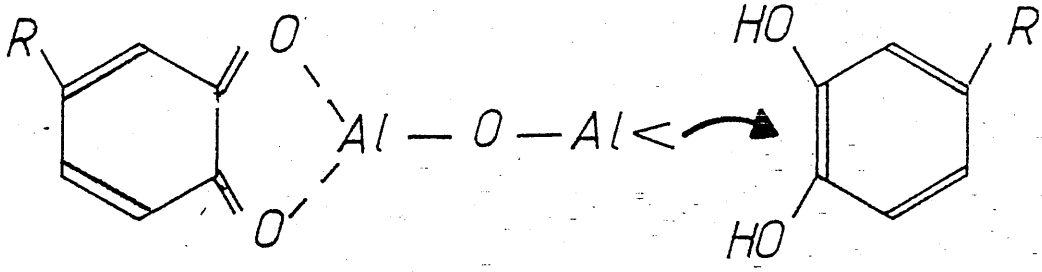


Figure 2.14 Mechanism of adsorption and elution of catechols onto alumina.

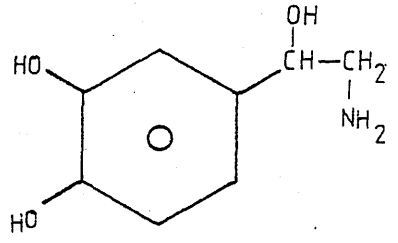
Schematic representation of the mechanism of extraction of catecholamines by use of alumina adsorption and subsequent elution.

2.9 Analysis of tissue catecholamines

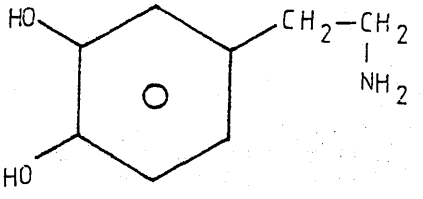
The technique which will be employed to measure the levels of catecholamines in the rat brain samples is high pressure liquid chromatography coupled to electrochemical detection (LCEC). Traditionally radioenzymatic techniques have been employed to measure tissue catecholamine levels, methodology which can detect catecholamines in the picogram range but whose running costs are exceedingly high. Such assays have been based on the use of enzymes of catecholamine metabolism and catabolism, catechol-O-methyl transferase (COMT) and phenylethanolamine-N-methyl transferase (PNMT; Henry et al, 1975; Petty & Reid, 1979). Recently however, LCEC has been shown to be a relatively inexpensive and sensitive method which is adequately suited to such tissue analysis. It has been demonstrated that this technique is no less precise than the phenylethanolamine N-methyl transferase or the catechol-O-methyl transferase radioenzymatic assays when employed to measure rat brain noradrenaline levels (Miller, 1981). Although a variety of methods to quantitate catecholamines in tissue samples have been published (for example Refshauge et al, 1974; Felice, Felice & Kissinger, 1978; Davis et al, 1978; Wagner, Palfreyman & Zraika, 1979; LCEC application note, 1980; Hegstrand & Eichelman; 1981), the method selected to analyse samples in these experiments will be based on that described by Miller (1981). Methodology which will be used in this thesis is described below.

The rat brain samples were homogenised in 5 volumes 0.4M perchloric acid and the acid extracts centrifuged for 10

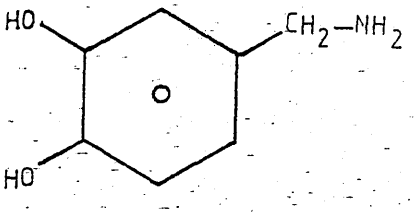
minutes at 2,000 rpm in 3ml tubes. 400µl of the supernatant was pipetted into polypropylene LP3 tubes containing 200µl 1µM 3,4 -dihydroxybenzylamine hydrobromide as internal standard, and the samples stored at -70°C. The internal standard was chosen since it was structurally related to the catecholamines (figure 2.15). Initially it was envisaged to analyse the samples by direct injection onto the HPLC-ECD system with no extraction as in the Method of Miller (1981). However, this proved impractical since although the chromatograms obtained exhibited satisfactory resolution, the nature of thesamples rapidly created an intolerable rise in pressure through the column. In order to overcome this problem an extraction step to clean up the sample was introduced. To each sample was added 50mg acid washed alumina and 0.5ml 3M tris/EDTA buffer (30.6g tris base dissolved in 100 ml distilled water; add 5g disodium EDTA and adjust the pH to 8.6 with 6M hydrochloric acid). The samples were shaken for 10 minutes and when the alumina had settled, the supernatant was aspirated. The alumina was then washed twice with distilled water with aspiration in between. After the final wash, 200µl 0.35M hydrochloric acid was added and the samples were vortexed before centrifugation at 2,000 rpm for 5 minutes. The supernatant was transferred into a fresh LP3 tube and stored at -70°C until chromatography could be performed.



Noradrenaline



Dopamine



3,4-Dihydroxybenzylamine

Figure 2.15 Structure of catecholamines and internal standard, 3,4-dihydroxybenzylamine.

The diagram illustrates the chemical similarity in structure of the catecholamines and the compound used as an internal standard in their quantitation, 3,4-dihydroxybenzylamine.

2.10 Tyrosine hydroxylase (TH) activity

The activity of tyrosine hydroxylase in the rat brain samples was measured by reverse-phase high-pressure liquid chromatography with subsequent electrochemical detection (LCEC) using the system previously described (section 2.7). The assay procedure employed was a modification of the methods of Nagatsu, Oka & Kata (1979) and Blank & Pike (1976). The basic approach involves incubating the system with the appropriate substrate and cofactor, followed by isolation and quantitation of the enzymatic product, dopa.

The brain samples were weighed and a 1 in 6 homogenate prepared in 0.25M sucrose. 30µl portions (in duplicate) were pipetted into polypropylene tubes on ice; the remaining homogenate being retained for protein estimation using the technique of Lowry et al (1951) which will be detailed in section 2.17 of this chapter.

To each sample was then added:-

10 µl	1 M	sodium acetate buffer pH 6.0
20 µl	1 mM	L-tyrosine in 0.01 M HCl
10 µl	10 mM	6-methyl-5,6,7,8-tetrahydropterin in 1M mercaptoethanol
10 µl	1mg/ml	catalase
10 µl	10 mM	ferrous ammonium sulphate
10 µl	625 uM	benzyloxyamine

The 6-methyl-5,6,7,8-tetrahydropterin acts as a cofactor for tyrosine hydroxylase and being readily oxidisable, it is protected from oxidation, by mercaptoethanol.

The samples were incubated for 30 minutes at 37 ^{0}C after which the reaction was stopped by the addition of 0.6ml

0.5M hydrochloric acid containing 150ng/ml of the internal standard, 3,4-dihydroxybenzylamine hydrobromide. 0.1ml 1M sodium hydrogen sulphite and 0.1ml 0.1M disodium ethylenediaminetetraacetic acid (EDTA) were then added, and the samples vortexed before being stored at -70°C until analysis by LCEC could be carried out.

At the time of analysis, 0.5ml of each sample was placed in 13ml polypropylene tubes containing 60mg acid-washed alumina (as prepared by the method of Anton & Sayre (1962) - section 2.8) and 0.5ml of 0.5M tris(tri-hydroxymethyl-aminomethane) buffer, pH 8.6. After shaking at room temperature for fifteen minutes, the alumina was allowed to settle and the supernatant aspirated. The alumina was then washed three times with 2ml portions of a solution containing:-

100 µl 0.1 M EDTA

100 µl 1 M disodium sulphite

1 ml tris buffer (0.5 M, pH 8.6)

-per 100 ml distilled water

After the final washing and aspiration, the catecholamines were eluted from the alumina by the addition of 200µl 0.35M HCl. The samples were vortexed for 20 seconds and centrifuged at 3,000rpm for 3 minutes. The acid, which was to be injected onto the column, was pipetted into fresh tubes to prevent decomposition of the catecholamines and stored at -70°C if not injected immediately. A 100µl aliquot of the hydrochloric acid extract was injected onto the LCEC system which was set at a potential of +0.6 volts versus a silver/silver chloride

reference electrode. The working electrode surface was composed of carbon paste (oil based), and the following mobile phase was employed:-

0.1 M potassium dihydrogen orthophosphate

0.1 mM disodium EDTA

0.2 mM sodium octylsulphate

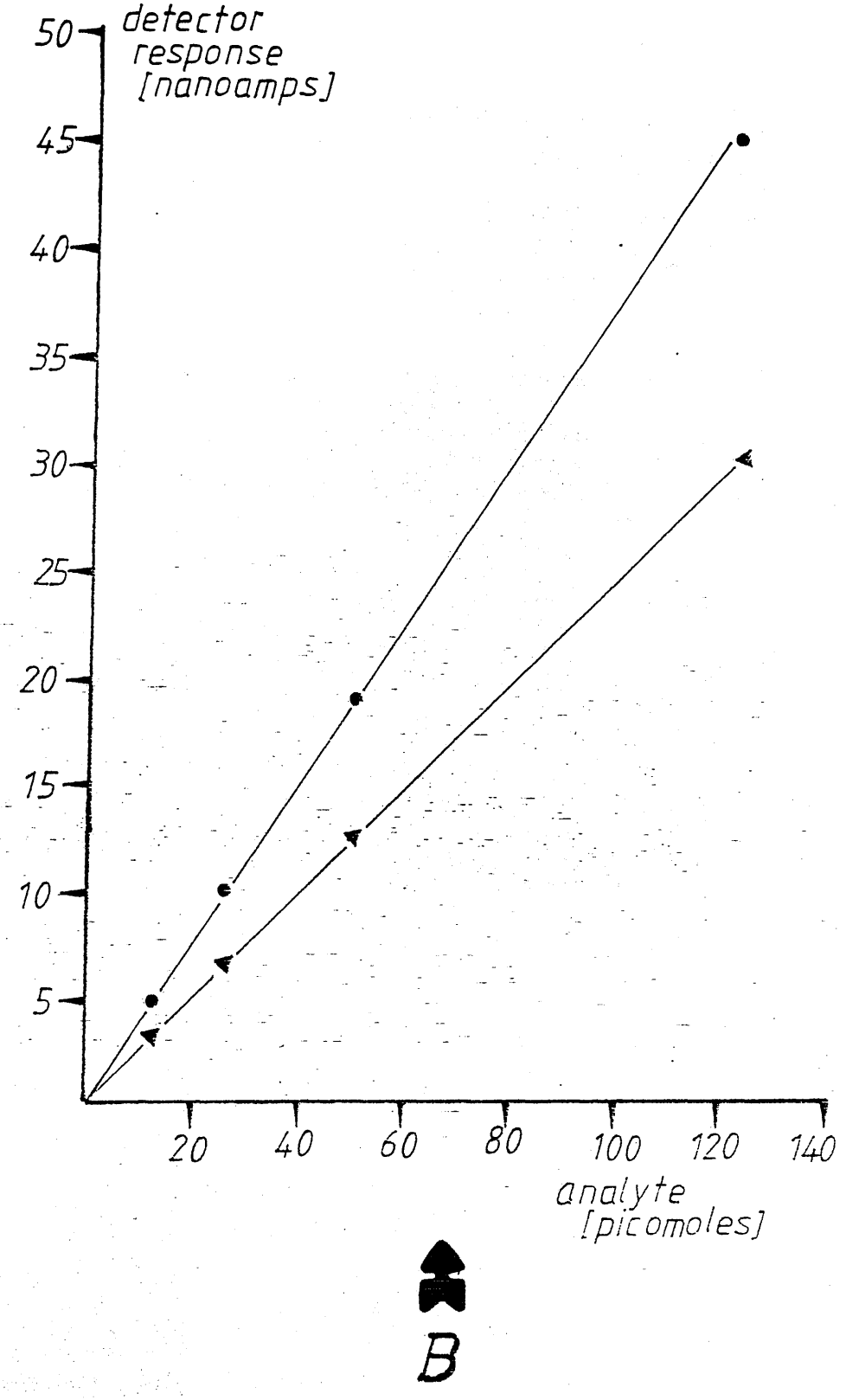
-pH to 4.5 with orthophosphoric acid

5 - 10 % methanol (v/v)

Flow rates employed were in the region of 1ml/minute. The column employed contained Spherisorb 5µm ODS stationary phase (250 X 4.6 mm).

The oxidation potential was selected by constructing a voltammogram to dopa (figure 2.16). A potential of +0.6 volts was sufficiently positive to cause oxidation of dopa at the working electrode surface, whereas higher potentials of around +0.8 volts can oxidise impurities present in the sample resulting in the presence of interfering peaks in the chromatogram.

The voltammetric detector system is highly sensitive for catecholamine compounds. Therefore dopa, the product of the tyrosine hydroxylase reaction, can be assayed with extremely high sensitivity. A calibration graph showing the linear response of the peak height of the detector for the amounts of dopa and internal standard injected (1-125 picomoles) can be seen in figure 2.17).


detector
response
[nanoamps]
50
45
40
35
30
25
20
15
10
5
20
40
60
80
100
120
140
analyte
[picomoles]

B

The activity of tyrosine hydroxylase in the brain samples was calculated by including in each assay, standard samples which contained a known amount of dopa in place of the enzyme homogenate. The standards employed were of concentrations 5, 10 and 15 micromolar, producing 150, 300 and 450 picomoles dopa per incubation.

A standard graph was constructed (figure 2.18) from which the amount of dopa formed in each sample during the thirty minute incubation period could be calculated.

Since the assay includes a step in which dopa is adsorbed onto alumina with subsequent recovery into acid, the recovery of dopa from alumina was investigated. Figure 2.19 shows that the recovery was 58.3%. However, the use of an internal standard (3,4-dihydroxybenzylamine) makes it unnecessary to correct for recovery of dopa from the alumina in this assay.

Considering the high sensitivity of the detector for dopa (the minimum detectable quantity defined as the quantity of solute producing a baseline shift twice the standard deviation of the noise, was 1 picomole), the sensitivity of the assay is determined by the blank value. Blank values were obtained by replacing L-tyrosine with D-tyrosine which is not a substrate for tyrosine hydroxylase. The dopa in the blank sample is derived from either dopa formed by non-enzymatic routes from both L- and D-tyrosine or from endogenous dopa present in the crude brain tissue. In addition, tyrosine solutions contain dopa as an impurity. It is likely that only a small percentage of the dopa in the blank is attributable to endogenous dopa since

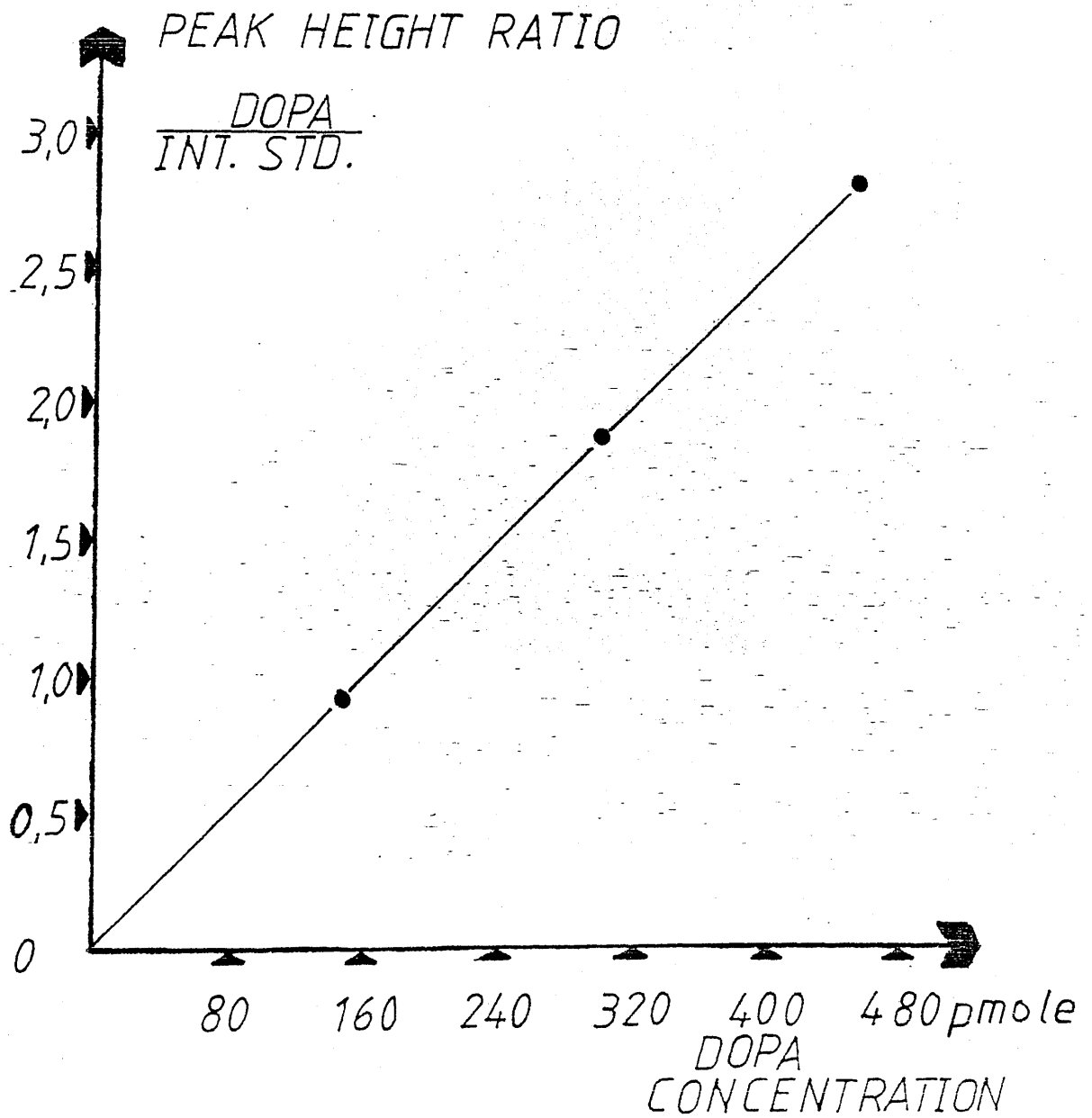


Figure 2.18 Calibration graph for assay of tyrosine hydroxylase.

Example of a graph employed to calculate the activity of tyrosine hydroxylase present in the rat brains. Each point represents the mean of 2 observations.

it is present in low concentrations (Anton & Sayre, 1964).

A typical chromatogram obtained from a rat brain homogenate sample is shown in figure 2.20 (a). The retention times being:-

'solvent front'	1.5 minutes
dopa	4 minutes
internal standard	8 minutes
unidentified endogenous peak	12 minutes

The identity of the peak at 12 minutes is uncertain, although it may be endogenous dopamine in the crude enzyme preparations as the retention time coincides with that of a pure dopamine standard. Iron ions can be detected under the conditions employed but they are eluted in the void volume. This chromatogram can be compared to one obtained from a blank sample (figure 2.20b), and for a standard sample containing 150 picomoles dopa (figure 2.20c).

Preliminary experiments demonstrated that the amount of tissue assayed was linearly related to tyrosine hydroxylase activity and that the rate of dopa formation was linear up to thirty minutes incubation (figures 2.21 & 2.22). The effect of varying the concentration of cofactor, DL-6-methyl-5,6,7,8-tetrahydropterin and the substrate, tyrosine on dopa formation was investigated. (figures 2.23 & 2.24). These figures demonstrate that the concentration of each used in the assay was saturating with respect to tyrosine hydroxylase. Several other assay parameters were investigated- The presence of Fe^{3+} are known to stimulate purified preparations of tyrosine hydroxylase (Nagatsu, Levitt & Udenfriend, 1964) and therefore the effect of

their removal from the incubation medium was investigated. The absence of Fe^{2+} resulted in a significant ($p < 0.01$ by Mann-Whitney U significance test) fall in tyrosine hydroxylase activity, and hence Fe^{2+} were included in all incubation media. Nagatsu et al (1979a) included catalase in their incubation media to produce oxygen via equation 2.4.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

- equation 2.4

Using rat brain homogenate, it was observed that the removal of catalase resulted in a significant ($p<0.02$) fall in enzymatic activity. An inter-assay coefficient of variation of 4.7% was calculated for the assay system as described above.

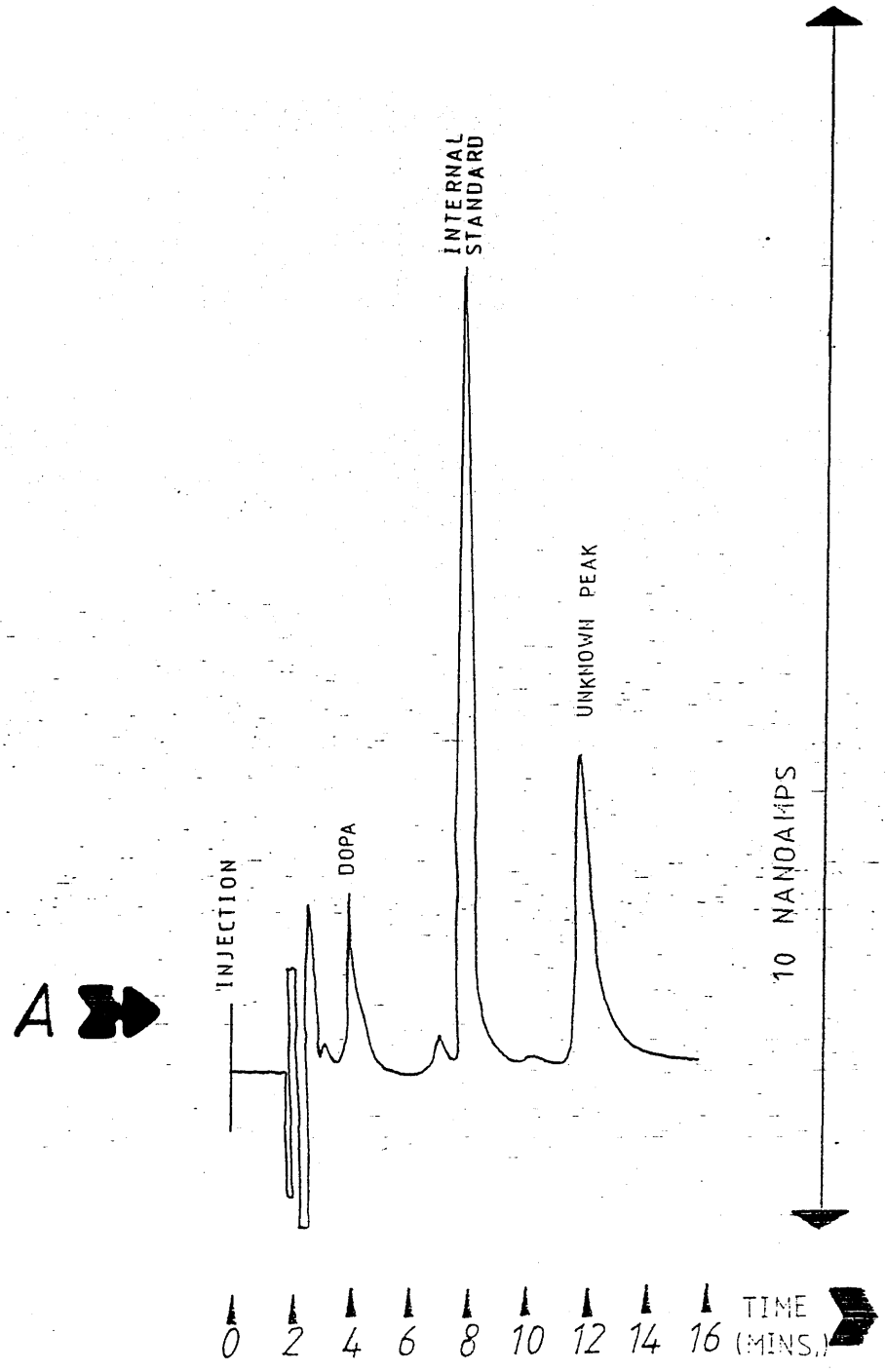


Figure 2.20 Typical examples of chromatograms.

Typical chromatograms -

(a) shows a chromatogram from rat brain showing the dopa and internal standard peaks in addition to an unidentified endogenous peak.

(b) standard sample containing 150 picomoles dopa.

(c) blank sample illustrating the absence of a dopa peak.

INTERNAL STANDARD
UNKNOWN PEAK
injection
DOPA
INTERNAL STANDARD
UNKNOWN PEAK
injection
DOPA
0 2 4 6 8 10 12 14
0 2 4 6 8 10 12 14
time [mins.]
B
C

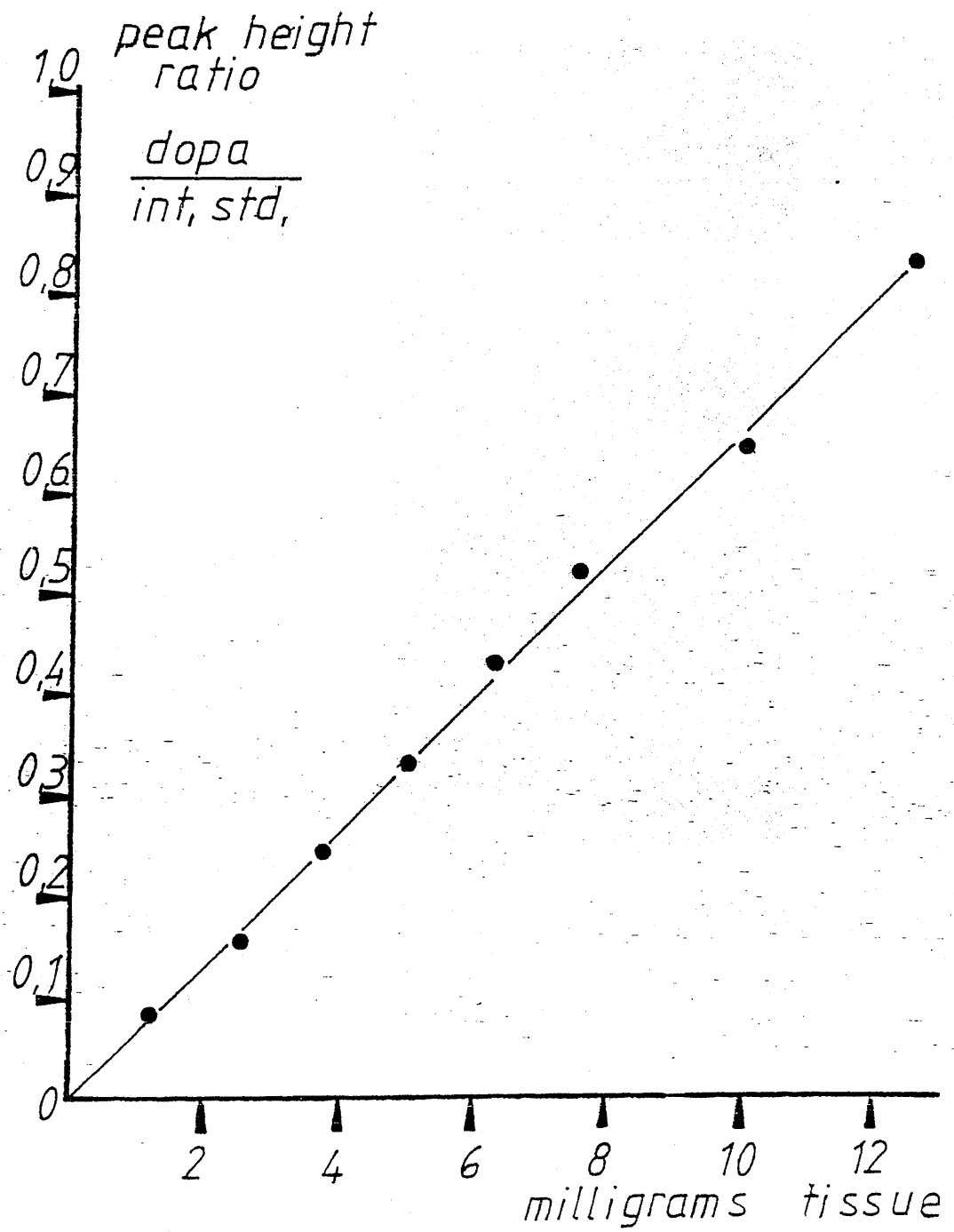


Figure 2.21 Linearity of tyrosine hydroxylase assay with amount of tissue assayed.

The graph illustrates that the assay is linear up to at least 12mg rat brain tissue. Each point represents the mean of 3 observations.

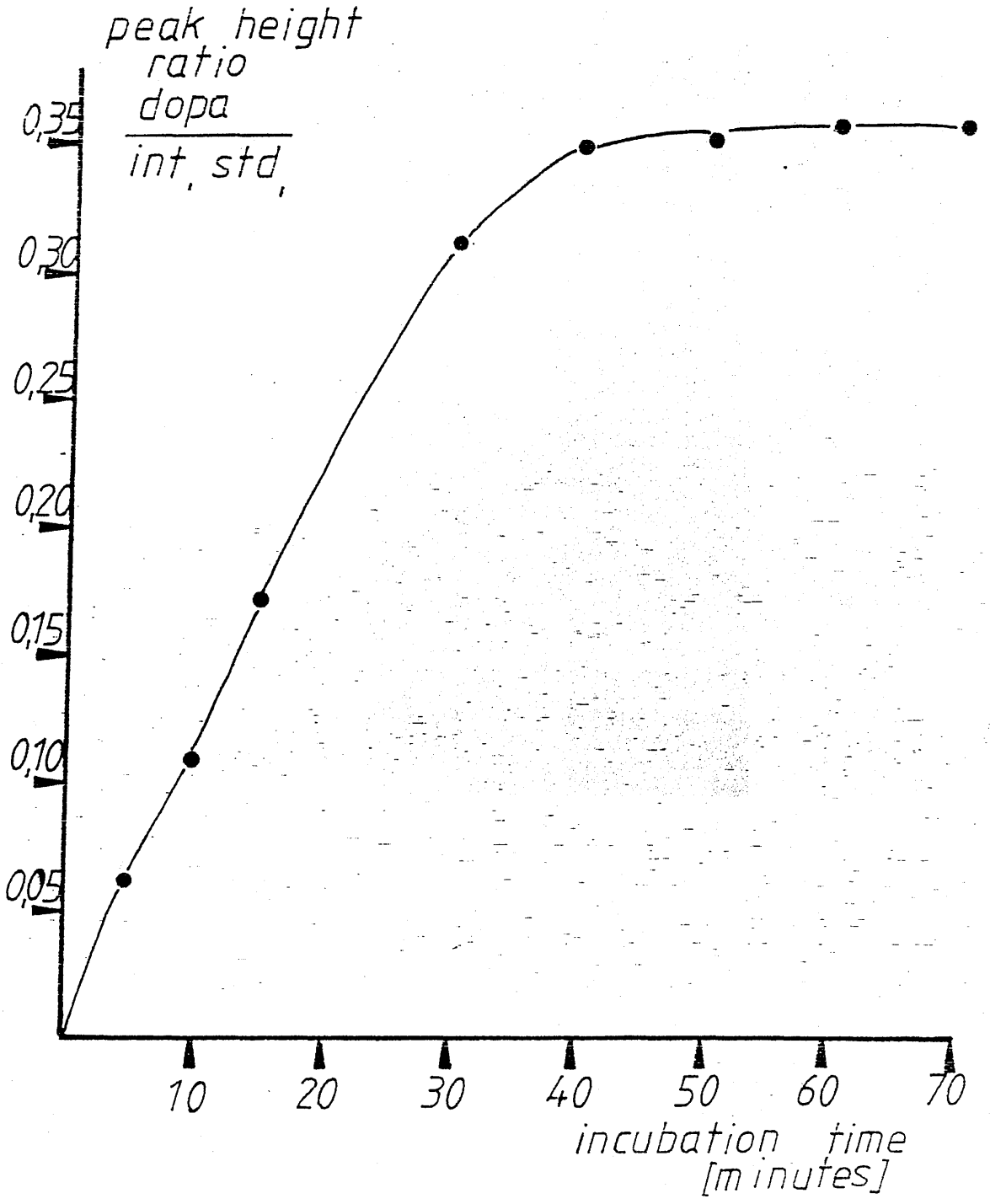


Figure 2.22 Linearity of tyrosine hydroxylase assay with incubation time.

The graph illustrates that the assay is linear up to 30 minutes incubation time. Each point represents the mean of 3 observations.

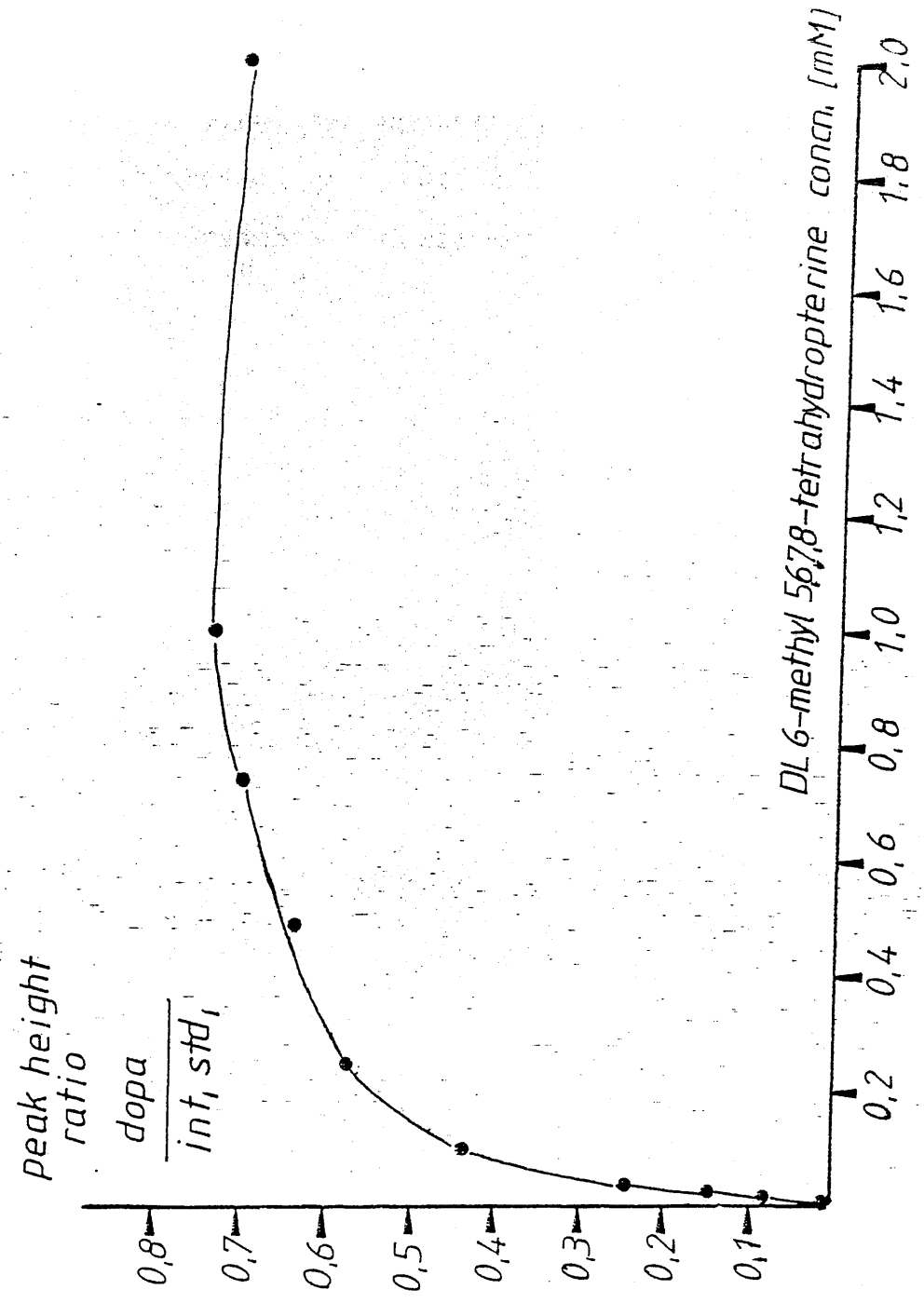
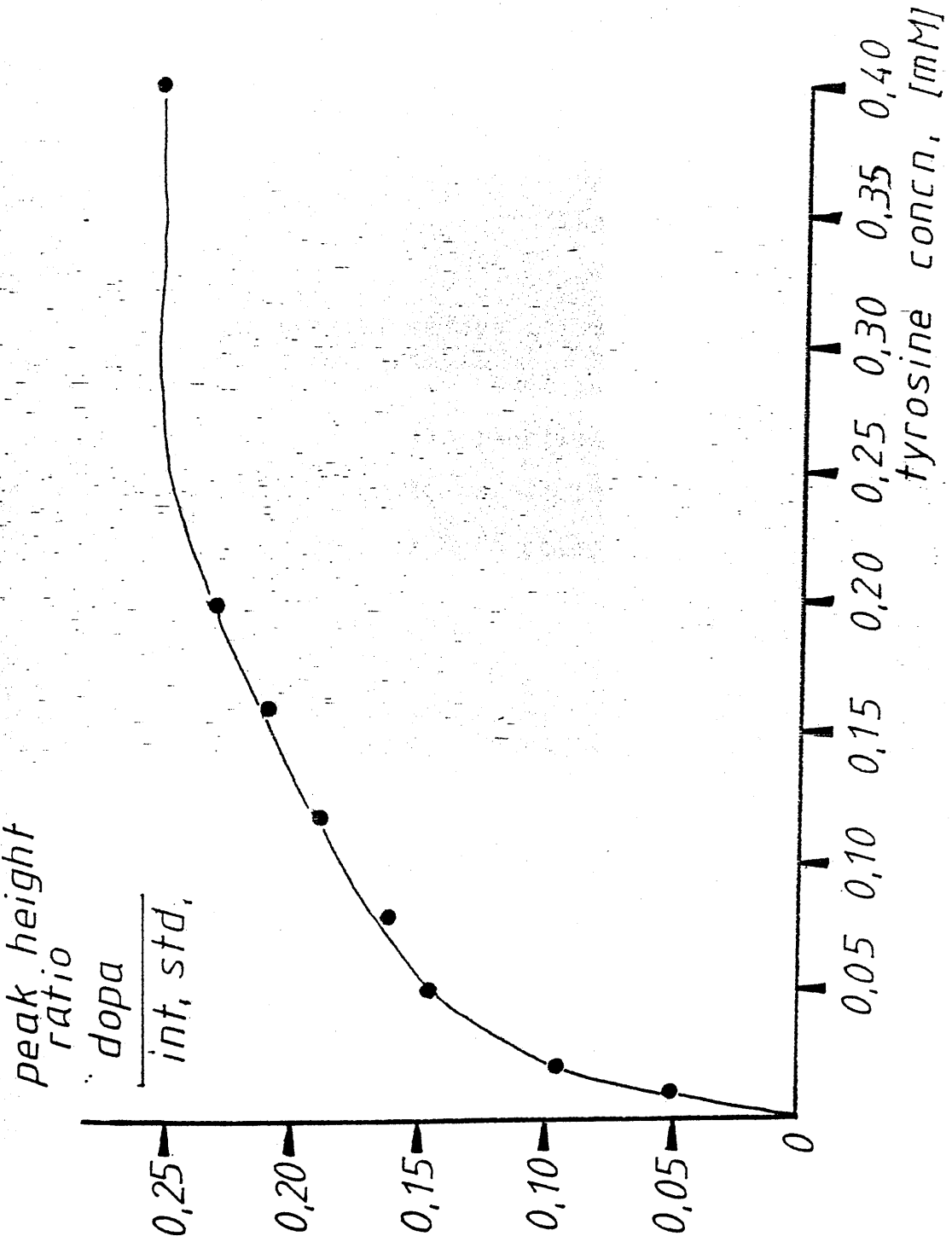


Figure 2.23 Activity of tyrosine hydroxylase as a function of pterin concentration.

Effect of varying the concentration of 6-methyl-5,6,7,8-tetrahydropterin on tyrosine hydroxylase activity. Each point represents the mean of 3 observations.

Figure 2.24 Activity of tyrosine hydroxylase as a function of tyrosine concentration.

Effect of varying the concentration of tyrosine in the incubation medium on tyrosine hydroxylase activity. Each point represents the mean of 3 observations.



2.11 Phenylethanolamine-N-methyl transferase (PNMT) activity

2.11.1 General considerations

At present there exist three techniques available to measure the activity of the enzyme, phenylethanolamine-N-methyl transferase (PNMT) which catalyses the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to noradrenaline, thus producing adrenaline (figure 5.1). These methods involve fluorometric, radiochemical or chromatographic techniques (Axelrod, 1962; Fuller & Hunt, 1966; Saavedra et al, 1974; Borchardt, Vincek & Grunewald, 1977; Petty, 1980).

The radiochemical assays utilize either (^{14}C) or (^{3}H) methyl-labelled SAM; the other substrate for the enzyme being noradrenaline. The labelled product, adrenaline is then isolated either by extraction procedures (Axelrod, 1962; Petty, 1980) or by precipitation of the unreacted methyl donor (Fuller & Hunt, 1966). The radiochemical methods have traditionally been the most frequently employed techniques. These assays have been developed to a level exhibiting great sensitivity. However reagent costs are high, and extraction procedures tedious and time consuming. The assays which necessitate fluorometric detection of the reaction products often involve a conversion of the products to more intensely fluorescent species which adds complexity to the assay procedure.

2.11.2 HPLC assay for PNMT activity

In order to reduce assay costs and simplify the procedure, the method of Borchardt *et al* (1977) was selected as the technique of choice to measure the activity of PNMT in the rat brain samples. These authors employed HPLC with electrochemical detection (LCEC) in order to measure the product of the reaction, adrenaline. The general scheme of the assay can be seen in figure 2.25. The substrate noradrenaline was separated from the N-methylated product, adrenaline by HPLC on a Zipax SCX cation exchange stationary phase (500 X 2mm). The mobile phase employed by these authors was that of Keller *et al* (1976) - citrate/acetate buffer, pH 5.2 with a flow rate in the region of 0.4 ml/minute. Although adequate chromatographic separation was achieved using the conditions described above, a reverse-phase system was selected to separate noradrenaline, adrenaline and the internal standard, dihydroxybenzylamine hydrobromide (DHBR). This alteration to the method was made for two reasons. Firstly, ion-exchange columns are less reproducable and less stable than reverse-phase systems. There is a variability in the separation characteristics of the resin from batch to batch which could necessitate the need to re-optimise chromatographic conditions if the cation exchange column currently being employed had to be renewed. Secondly, a reverse-phase column could be more efficient and hence sensitivity may well be improved. The reverse-phase paired-ion chromatographic system employed a 5µm Spherisorb ODS column (250 X 4.6mm) with the following mobile phase:-

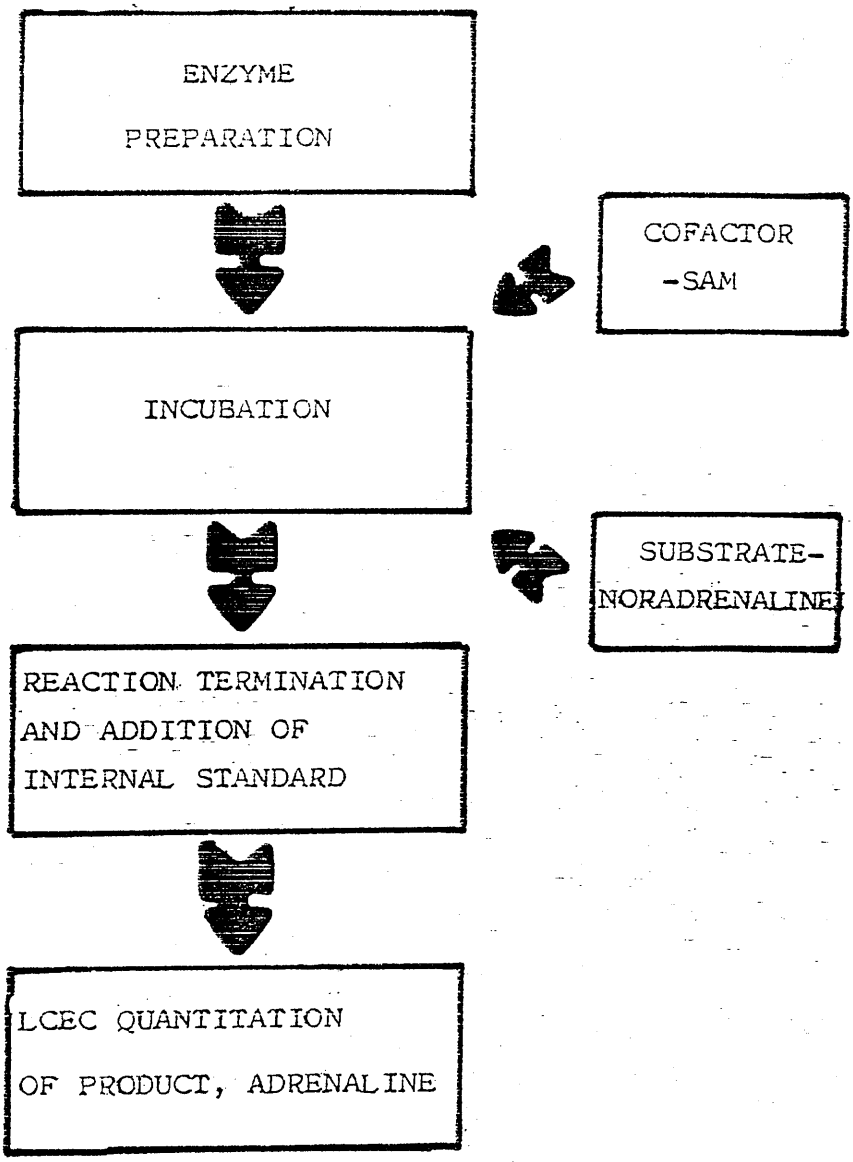


Figure 2.25 Schematic representation of PNMT HPLC assay.

Diagramatic representation of the principles of the HPLC assay for PNMT activity.

0.1 M potassiium dihydrogen orthophosphate

0.1 mM disodium EDTA

0.2 mM sodium octylsulphate

-pH adjusted to 3.5 with orthophosphoric acid

5 - 15 % methanol (v/v)

The separation achieved can be seen in figure 2.26 from which it is evident that the system is capable of separating the product adrenaline, from the internal standard, DHBA and the large amount of noradrenaline substrate present. Typical retention times were as follows:-

noradrenaline	8 minutes
adrenaline	12 minutes
DHBA	17 minutes

The enzyme preparation and incubation conditions described by Borchardt et al (1977) were as follows:- The rat brain samples were homogenised in five volumes isotonic potassium chloride (0.15M). After centrifugation for 10 minutes at 100,000g, portions of supernatant were assayed for PNMT activity using the incubation conditions:-

tissue homogenate supernatant		175 µl
phosphate buffer	0.5 M,pH 8.0	50 µl
noradrenaline	0.6 mM	15 µl
SAM	1.8 mM	10 µl

This regime resulted in an incubation medium containing 0.1M phosphate buffer, 36µM noradrenaline and 72µM SAM. After incubation at 37 °C for 60 minutes, the reaction was stopped by the addition of 250µl 0.1M perchloric acid containing the internal standard, DHBA. The authors

employed blank samples which omitted noradrenaline from the incubation medium, in order to correct for any endogenous adrenaline present in the enzyme preparation. A sample of the quenched reaction mixture was then simply injected onto the chromatographic system.

When the assay as described above was performed in our laboratory using the rat brain as source of enzyme, the results shown in table 2.6 were obtained. These results demonstrate that there is PNMT activity present in the rat brain greater than the blank when the blank sample employed is one lacking noradrenaline in the incubation medium. However, if a blank sample is employed in which noradrenaline is incubated with SAM, phosphate buffer and potassium chloride but no brain homogenate is present, a much higher blank value is obtained. In an attempt to elucidate the reason for the above results the assay was repeated as described above except that the homogenate was prepared in 0.2M tris buffer, pH 8.6 containing 0.2% (v/v) triton X-100, a homogisation buffer employed in a radioenzymatic assay procedure for PNMT activity (Petty, 1980). The results of such an experiment are tabulated in table 2.7. A possible explanation for these observations is that the noradrenaline present in the incubation medium is breaking down into one or more substances, one of which includes adrenaline. This breakdown at 37 ^{0}C is much greater in the presence of potassium chloride than tris buffer.

Various parameters of the assay were investigated using the rat brain as a source of PNMT; the homogenate being prepared in 0.2M tris buffer, pH 8.6/0.2% triton X-100.

After carrying out these preliminary experiments, the decision was made that this LCEC assay for PNMT activity was not sensitive enough to measure the enzyme activity in the relatively small brain samples in which we intend to have available in the experiments which will be performed for this thesis. In addition, practical experience in the laboratory suggested that the assay would not be robust enough for the purpose which it was intended.

Table 2.6 Adrenaline production in blank samples using a potassium chloride homogenisation buffer

Sample type	Ratio Adrenaline / Internal standard
rat brain homogenate	1.21
blank - no noradrenaline added to incubation	0.07
blank - no enzyme added to incubation	1.11

Table 2.7 Adrenaline production in blank samples using a tris homogenisation buffer

Sample type	Ratio Adrenaline / Internal standard
rat brain homogenate	1.30
blank - no noradrenaline added to incubation	0.03
blank - no enzyme added to incubation	0.19

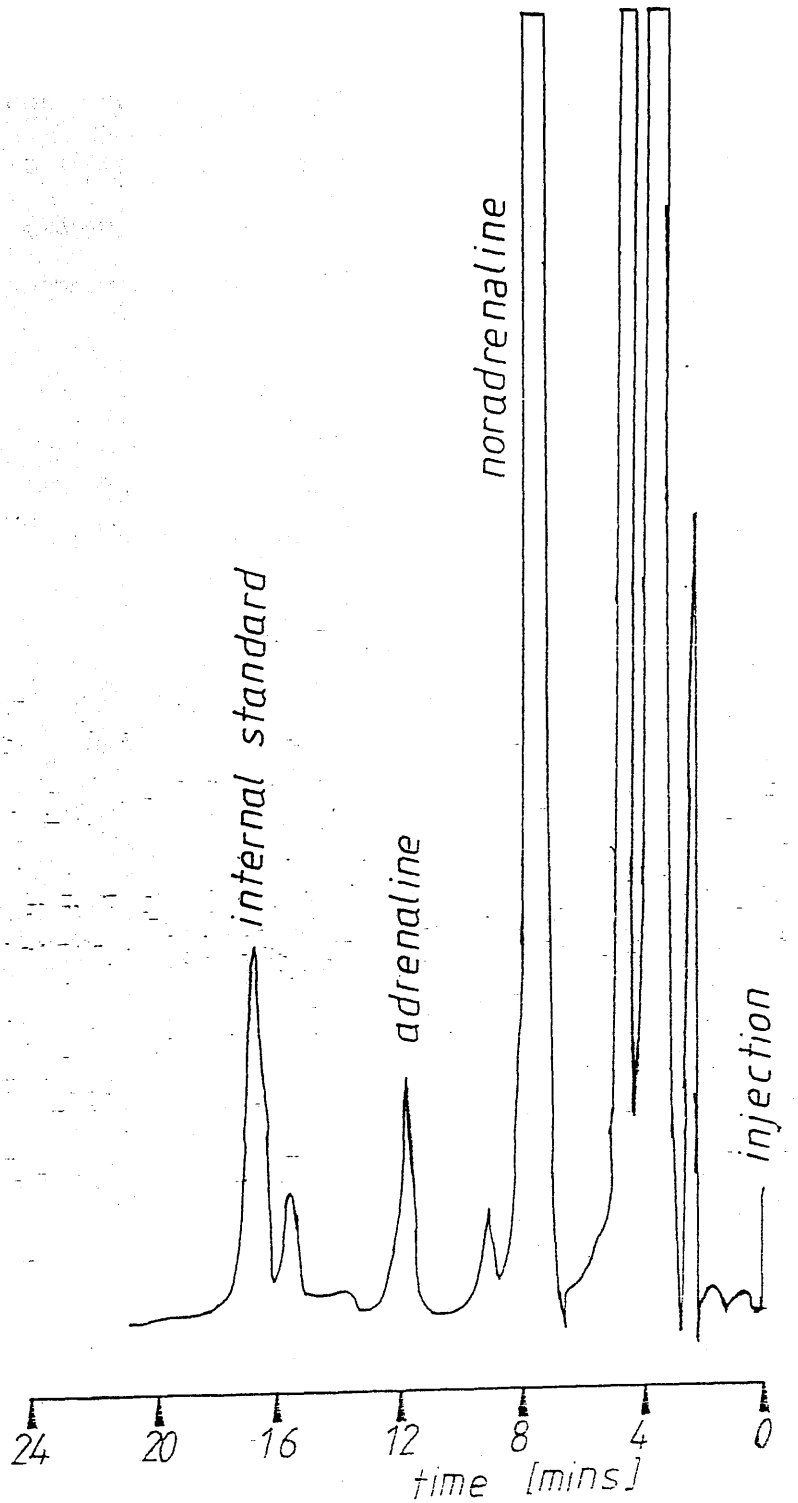


Figure 2.26 Typical example of a chromatogram from PNMT assay

The chromatogramshows the separation between the large noradrenaline substrate peak and the adrenaline and internal standard peaks which can be quantitated.

2.11.3 Radioenzymatic assay for PNMT activity

The activity of the enzyme phenylethanolamine N-methyl transferase in the rat brain samples was measured by a modification of the method of Saavedra et al (1974) as detailed by Petty (1980). The brain tissue was homogenised in 20 volumes 0.2M Tris hydrochloride buffer pH 8.6 (w/v) containing 0.2% triton X-100 (v/v) and kept on ice. After the removal of a sample of homogenate for protein estimation, the samples were centrifuged at 2,000 rpm for 15 minutes at 4 ^{0}C. Duplicate portions (50µl) of supernatant were pipetted into ice-cold 12ml polypropylene conical tubes . Blanks were prepared (four per assay) by incubating 50µl of the homogenisation buffer containing no brain tissue. These blanks consistently produced counts per minute of 200 - 300. Higher counts were obtained when the blanks consisted of either the absence of phenylethanolamine substrate or the substitution of phenylethanolamine by B-phenyl-ethylamine or tryptamine which are not substrates for this reaction (typically 1,300cpm). These observations confirm that the enzyme under study is PNMT and not a non-specific N-methyltransferase (Saavedra, Coyle & Axelrod, 1973). The observation that these blanks are higher than if no enzyme is incubated can be attributed to the methylation of endogenous noradrenaline present in the enzyme homogenate.

To the samples and blanks was added 100µl of the following mixture:-

1 ml	1 mg/ml phenylethanolamine
0.5 ml	S-adenosyl-L-(methyl-^{3}H)methionine - specific activity 15 curies/mmol
per 18.5 ml	distilled water

After an incubation at 37^{0}C for 30 minutes, the reaction was terminated by the addition of 0.5ml 0.5M borate pH 10. 6ml of 3% amyl alcohol in toluene (v/v) was added and the tubes vortexed for 15 seconds (multitube vortexer, Scientific Manufacturing Industries, U.S.A.) before being centrifuged for 5 minutes at 1,000rpm. This step served to separate the radioactive product from the labelled substrate, S-adenosyl-L-(methyl-^{3}H)methionine. After freezing the borate layer in an acetone-dry ice bath, the organic layer containing labelled product was poured into fresh tubes containing 0.5ml 0.1M hydrochloric acid. The samples were then vortexed and centrifuged. After freezing, the toluene layer was discarded and 2ml 1M borate pH 12.5 plus 6ml 3% amyl alcohol in toluene (v/v) was added to the acid layer. The samples were vortexed, centrifuged and frozen once more, and the toluene layer was poured into polyethylene scintillation vials containing 10ml Fisofluor 'mpc'. Finally, the activity was counted in a Packard Tricarb Liquid Scintillation Spectrometer automatically pre-programmed to count tritium.

The assay was repeated using partially purified PNMT which had a known specific activity - 1 unit was capable of converting 1 nanomole of normetanephrine to metanephrine in

1 hour at 37°C and pH 8.5. 3.33×10^{-3} and 6.67×10^{-3} units of this PNMT were carried through the assay as described above (in triplicate) in order to calculate the activity of the enzyme in each of the samples. The samples, standards and blanks were corrected for inter-sample variations in quenching by constructing a quench calibration curve using tritium labelled toluene - figure 2.27. PNMT activity was expressed as fmoles or pmoles ^{3}H-methyl-phenylethanolamine formed/mg tissue or protein/hour.

Preliminary experiments demonstrated that the rate of ^{3}H-methyl-phenylethanolamine formation was linear up to 60 minutes incubation and that the amount of tissue assayed was linearly related to PNMT activity. Figures 2.28 & 2.29). In addition, the inter-assay coefficient of variation of the assay as described above was calculated to be 8.6% (N=12).

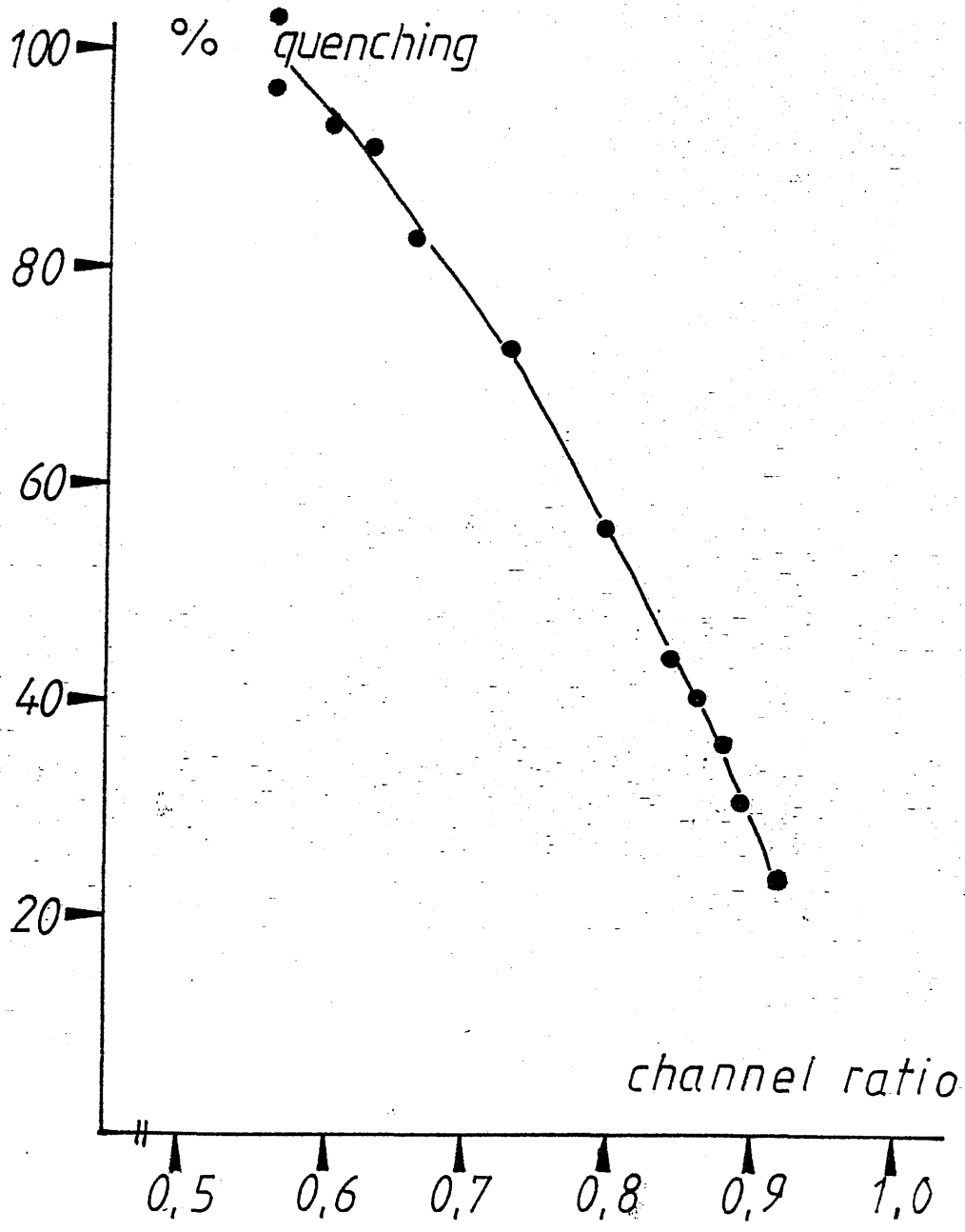


Figure 2.27 Quench correction curve.

Inter-sample variations in quenching were corrected by the construction of a quench correction curve, an example of which is shown in the diagram. The curve was constructed using standardised tritium labelled toluene supplied by Amersham International plc.

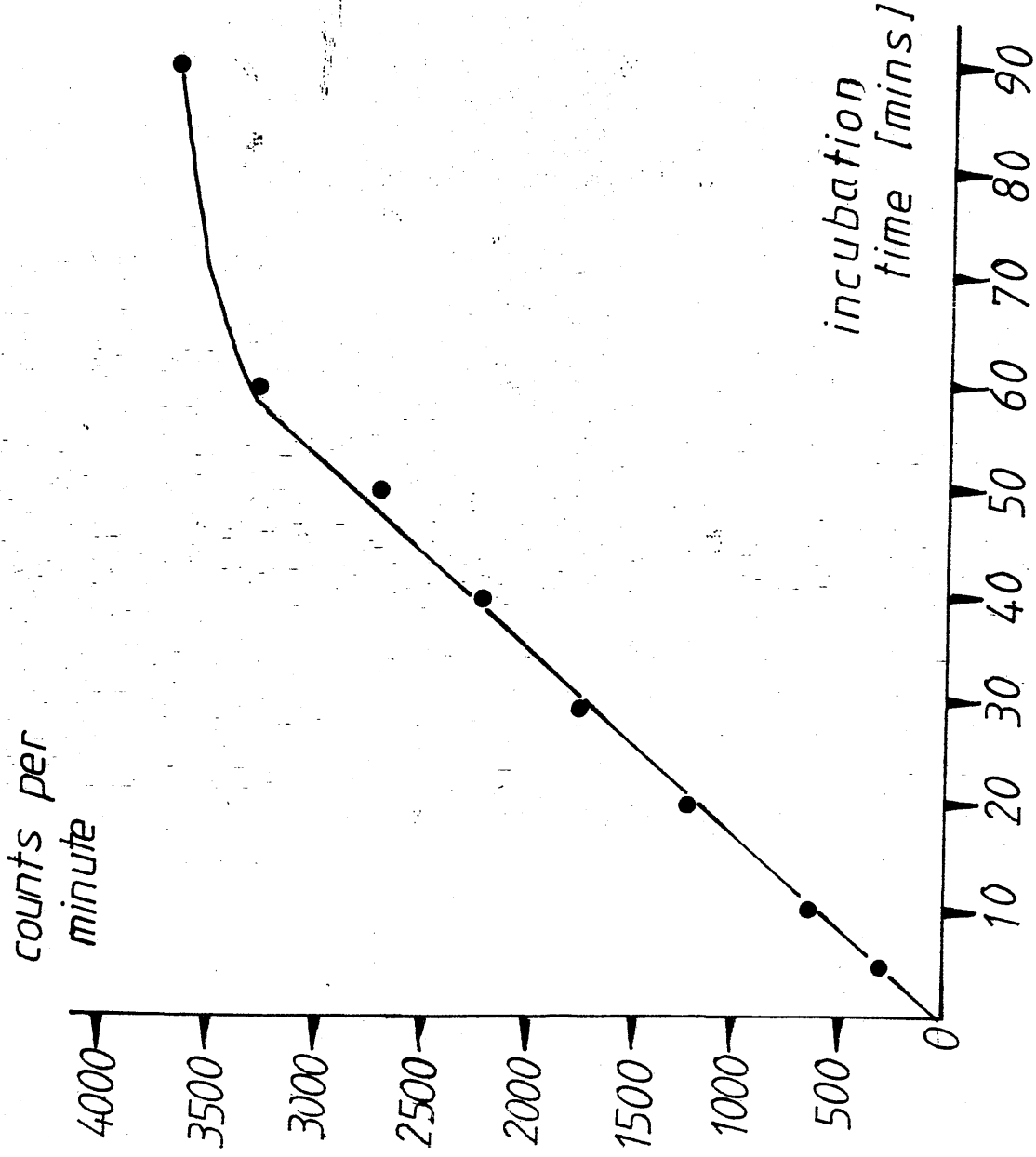


Figure 2.28 Linearity of PNMT assay with incubation time.

The figure illustrates that the activity of PNMT was linearly related to time until 60 minutes.

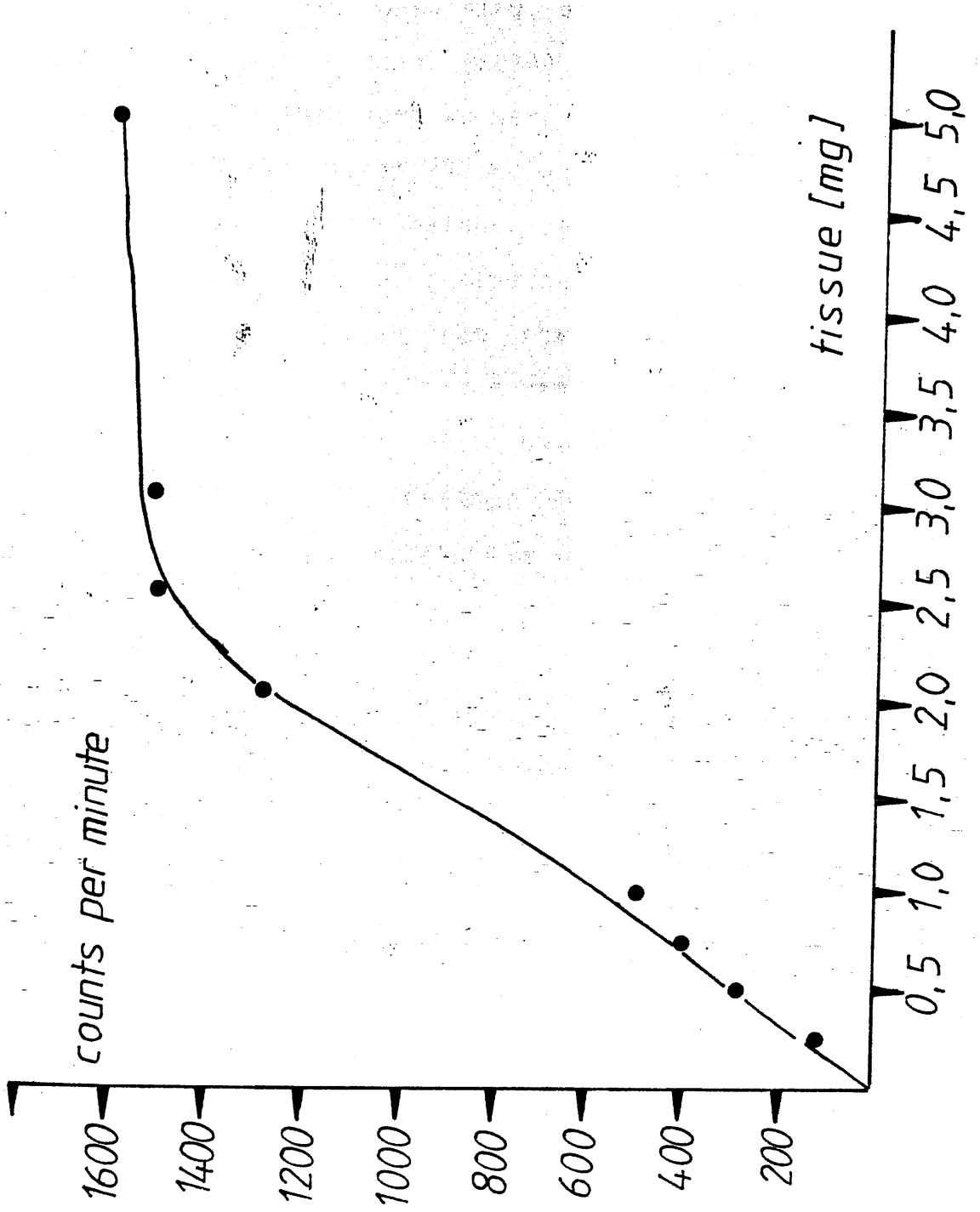


Figure 2.29 Linearity of PNMT assay with amount of tissue assayed.

The figure shows that the assay for PNMT activity was linearly related to the amount of brain tissue in the incubation medium.

2.12 Plasma Biopterins Assay

Blood samples were collected in lead-free polystyrene or polypropylene tubes containing either potassium ethylene-diaminetetraacetic acid (EDTA) or sodium heparin as anticoagulant. The samples were mixed thoroughly for 30 minutes (Denley Spiramix) and an sample of blood removed into LP3 tubes; the remainder being stored at 4°C until lead analysis could be carried out. The samples were then centrifuged at 2,000 rpm for ten minutes. After centrifugation, the plasma was removed from each sample and stored at -20°C in fresh LP3 tubes. Plasma biopterins were measured by Dr. R.J. Leeming, Department of Haematology, The General Hospital, Birmingham who employed the Crithidia fasciculata protozoological bioassay (Leeming et al, 1976b).

Members of the genus 'Crithidia' were first described by Leger in 1902 and are trypanosomatid insect parasites ranging from 4-10μm in length. The first chemically defined growth medium for Crithidia fasciculata was described by Cowperthwaite et al, (1953) who reported an extremely high folic acid requirement. In addition, they demonstrated that this ostensible requirement for folate was greatly spared by a virtually folate-free liver fraction. Subsequently, it was demonstrated that Crithidia fasciculata required considerably less folate than previously anticipated but in addition it also required the presence of a second factor, termed 'Crithidia factor' probably related to folic acid. (Nathan & Cowperthwaite, 1955). Patterson et al in 1955 demonstrated that the 'non-physiological' folate

requirement could be spared by an acid-stable, new, unconjugated pteridine, named biopterin. (figure 2.30).

The name 'pterin' was suggested by Pfeiderer in 1964 for derivatives of 2-amino-4-hydroxy-pteridine. There are now known to be several unconjugated 2-amino-4-hydroxy-pteridines which are capable of reducing the folic acid requirement of Crithidia fasciculata from 1.0µg/ml to 0.001µg/ml. Each is characterised by possessing an aliphatic substituent in the 6-position at least 2 carbons long which bears at least 2 hydroxyl groups. The group of active unconjugated pteridines are termed 'crithidia factor'. (Note that the nomenclature used throughout this thesis for folic acid and related folates is that given by the IUPAC-IUB Commision on Biochemical Nomenclature (1966)).

The presence of folic acid gives enhanced growth in medium containing biopterin, and crithidia factor is produced in conditions where folate is the sole source of pteridine (Guttman & Wallace, 1964). In addition, Crithidia fasciculata can synthesise crithidia factor in a medium containing a carbohydrate substrate such as sorbitol and 2,4,5-triamino-6-hydroxy-pyrimidine together with one of several sugars- L-rhamnose, D-mannose, D-galactose, gluconolactose, sucrose or glucose. (Nathan & Funk, 1959). Hence it can be stated from these observations that crithidia factor is required for growth of Crithidia fasciculata and it can be supplied (a) as such (b) as a conjugated pteridine, part of which the organism can convert to a crithidia factor such as folate, and (c) as a non-pteridine

precursor from which the factor can be synthesised (de-novo synthesis).

There are several potent, naturally occurring 6-substituted pterins capable of supplying the pteridine requirements of Crithidia fasciculata. The bioassay is specific for tetrahydrobiopterin, dihydrobiopterin, biopterin and L-neopterin, the first three of which show equal activity. (Leeming & Blair, 1974; Leeming, 1975). L-neopterin has a reduced activity of 56% by comparison with biopterin, but using thin layer chromatography it has been demonstrated that this pteridine is absent from human plasma (Leeming, 1975). Pteroic acid will also spare Crithidia's need for biopterin but again it is not present in plasma (Leeming, 1975). Several other pterins can support Crithidia but they, like folate require to be present in large quantities, examples being pteroyl-L-aspartate, pteroyl-glycine, pteroyl-alanine (Broquist & Albrecht, 1955), and D-neopterin (Leeming, personal communication). Thus the Crithidia assay is very selective in its response to pteridines with folic acid exhibiting an activity of 0.07% relative to biopterin, while the figure for the folate analogues 5-methyltetrahydrofolate, 10-formyltetrahydrofolate is less than 0.01% (Leeming, 1975). Therefore the assay described in this chapter measures the aggregate of the pterins biopterin, dihydrobiopterin and tetrahydrobiopterin present in the plasma samples. The assay exhibits a coefficient of variation (C.V.) of 7.9% (Leeming, 1975) and the sensitivity is less than 0.2µg/ml (Leeming & Blair, 1980).

The culture medium for Crithidia fasciculata is that described by Guttman & Wallace in 1964 with the addition of 1% casamino acids as suggested by Iwai, Kobashi & Fugisawa (1970). The complete culture medium which does not differ substantially from the original medium of Cowperthwaite et al, (1953) with more closely defined nutritional requirements, is shown in table 2.8. The inclusion of casamino acids produces a considerable increase in maximum growth (Leeming, 1975). The ingredients, with the exception of vitamins, haemin, triethanolamine and casamino acids were made to a 10-fold concentration, steamed for 20 minutes to promote solution and for partial sterilisation before being stored in sterile screwcapped bottles at 4°C. The vitamins, apart from folic acid were prepared as a dry mix and again stored at 4°C. Folic acid was kept as a stock solution at a concentration of 100ng/ml and 0.5ml added to every 100ml of double-strength medium. The haemin was dissolved at a concentraion of 5mg/ml in 50% triethanolamine. When preparing the medium, half the triethanolamine was added bringing the base above pH 7.0 to prevent precipitation on subsequent addition of the haemin; with the addition of the haemin the other half of the triethanolamine was added. Crithidia fasciculata will grow over a wide range of pH values (3.8 - 6.3) in a defined medium with the precipitation of haemin not appearing to hinder growth (Tamburro & Hutner, 1971). However it is difficult to measure growth using turbimetric techniques when there is a background of precipitated haemin. Since the addition of carbohydrate to the medium permits growth

up to pH 8.2 (Nathan & Cowperthwaite, 1955), the complete double-strength medium was adjusted to a more basic pH of 7.5. The addition of the buffered sample or standard altered the pH to 6.5 which agrees with the optimum pH observed by Baker et al (1974).

The plasma samples were diluted in buffer to produce a biopterin concentration in the growth range for Crithidia fasciculata. Each sample was diluted 1 in 20 with 0.2M phosphate buffer pH 5.0 and autoclaved for three minutes at 115°C to cause deproteinisation, after which 0.5ml of diluted sample was added (in triplicate) to tubes containing 1.5ml of distilled water and 2ml of the double-strength medium cocktail. Standards (in triplicate) were prepared by adding 0, 10, 20, 30, ... 100 picograms of biopterin per tube in 0.5ml quantities of 0.2M phosphate buffer pH 5.0. The volume was adjusted to 4ml by the addition of 1.5ml of distilled water and 2ml of double-strength medium. Each tube was then autoclaved at a temperature of 115°C for five minutes before being inoculated with the Crithidia fasciculata culture. The stock culture was subcultured in maintenance medium (Leeming & Blair, 1974)- table 2.9. 1mg/ml ampicillin was added, to prevent bacterial contamination without affecting the growth of Crithidia fasciculata, and the culture was incubated for 2 days at 29°C in the dark before being stored at -20°C. The inoculum was prepared 4 days prior to inoculation of the samples by adding 1 drop of the 2 day culture asceptically to 15ml of single-strength medium and incubating at 29°C for 4 days to exhaust the pteridines in

the Crithidia fasciculata culture. 0.2ml of the resultant growth was added to 20ml of single-strength medium and this constituted the inoculum. After the addition of ampicillin at a concentration of 25mg/ml, to the inoculum, 1 drop was added to each of the samples and standards using a sterile pasteur pipette. The inoculated tubes were incubated at 29 °C in the dark for 4 days, after which the growth was read at 590nm against uninoculated medium containing the diluted serum sample, on a Gilford micro-sample spectrophotometer 300 using a semi-automated system (Leeming & Graham, 1973). The system consisted of the spectrophotometer, a chart recorder and dispensers operated by a sample changer (Hook & Tucker). The sample changer had 40 stations each with 3 (75 x 15 mm) tubes, sited radially. It had 2 programmes; the first for reading moved a probe inwards sampling from each of the 3 tubes whilst simultaneously mixing the adjacent tubes before moving to the next station, and the second programme operated the 3 dispensers which delivered aliquots simultaneously to the three tubes at each station and could thus be used for setting up assays.

Table 2.8 Culture medium for Crithidia fasciculata

Part 1

Component	Quantity (g)
L-arginine hydrochloride	5.0
L-glutamic acid	10.0
L-histidine hydrochloride	3.0
DL-isoleucine	1.0
DL-leucine	1.0
L-lysine hydrochloride	4.0
DL-methionine	1.0
DL-phenylalanine	0.6
DL-tryptophan	0.8
L-tyrosine	0.6
DL-valine	0.5
Ethylenediaminetetraacetic acid	6.0
Boric acid	0.005
Calcium chloride	0.005
Cobalt sulphate	0.025
Copper sulphate	0.025
Ferric ammonium sulphate	0.010
Manganese sulphate	1.4
Magnesium sulphate	6.5
Tri-potassium phosphate	1.5
Zinc sulphate	0.5
Sucrose	150.0

- make up to a volume of 1 litre and steam for 20 minutes at 100 ^{0}C and store in sterile bottles in the dark at 4 ^{0}C for up to 3 months.

Part 2

Component	Quantity (g)
Adenine	1.0
Biotin	0.001
Calcium pantothenate	0.3
Nicotinic acid	0.3
Pyridoxamine dihydrochloride	0.1
Riboflavin	0.06
Thiamine hydrochloride	0.6

- grind together and store dry in the dark at 4 ^{0}C.

Part 3

Haemin - 5mg/ml in 50% triethanolamine; freshly prepared.

part 4

Folic acid - 100ng/ml; freshly prepared.

Double strength medium - 100 ml

Distilled water	78 ml
Part 1	20 ml
Part 2	4.8 mg
Vitamin free casamino acids	2.0 mg
Triethanolamine (added after part 3)	0.5 ml
Part 3	1.0 ml
Part 4	0.5 ml

- adjust pH to 7.5 with sulphuric acid.

Table 2.9 Maintenance medium for Crithidia fasciculata

Component	Quantity
Yeast extract	0.3 g
Trypticase	0.3 g
Sucrose	0.25 g
Liver fraction L	0.01 g
Haemin	5 mg/ml in 50% triethanolamine - 0.5 ml

- the volume is brought to 100 ml with distilled water and the pH adjusted to 7.5. 8% glycerol is added to allow cultures to be frozen at -20 ^{0}C for up to 3 months.

2.13 Tetrahydrobiopterin analysis

The concentration of tetrahydrobiopterin, the cofactor for the enzyme tyrosine hydroxylase in the rat brain samples was estimated by high pressure liquid chromatography with subsequent electrochemical detection (LCEC). The chromatographic system employed has been described previously in section 2.7 of this chapter. Separation was achieved using a 4.6 X 250 mm column containing Spherisorb 5 µm ODS reverse-phase packing material. Initially, the citrate-phosphate isocratic (elution without change in solvent composition) solvent system of Brautigam, Dreesen & Herken (1982) was employed:-

13.3 mM citric acid

6.6 mM disodium hydrogen orthophosphate

60 µM disodium ethylenediaminetetraacetic acid

1.4 mM 1-octanesulphonic acid, sodium salt

-adjusted to pH 3.3 with sodium hydroxide

methanol - 5 - 10% (v/v)

The reduced biopterin standard, 7,8-dihydro-L-biopterin (BH_2) was obtained as the free base whilst 5,6,7,8-tetrahydrobiopterin (BH_4) was supplied as the dihydrochloride. The BH_4 standard employed consisted of a mixture of the two 6-diastereoisomers as this was the only form of the pteridine available when supplied. The natural isomer is the 6-R form and is the sole isomer observed in biological tissue.

Storage of BH_2 was in sodium phosphate buffer pH 6.8 at -20^0 C while BH_4 was stored in 0.1 M hydrochloric acid again at -20^0 C.

Although the citrate-phosphate mobile phase produced separation of the reduced biopterins as was observed by Brautigam et al (1982), excessive baseline noise was experienced using both glassy carbon and carbon paste (oil based) working electrode surfaces. Other workers have previously reported adverse experiences using citrate buffers. Crombeen, Kraak & Poppe in 1978 stated that metal ions in the eluent from the column (as a result of corrosion of the stainless steel column by the citrate buffer) may cause difficulties when using an electrochemical detector. This criticism was later supported by Behner & Hubbard in 1979. The presence of excessive baseline noise will limit the sensitivity of the electrochemical detector and therefore an improved isocratic solvent system was sought. A phosphate based mobile phase previously employed to separate catecholamines (Millar, 1981) was found to be as effective as the citrate-phosphate buffer in separating the reduced biopterins, and also produced a more stable baseline. EDTA was added as a precautionary measure to complex any metal ions and to improve the stability of the baseline. In addition, the mobile phase was prepared using distilled water which had been redistilled from potassium permanganate. The final choice of solvent employed in the assay was as follows:-

0.1 M potassium dihydrogen orthophosphate

0.1 mM disodium EDTA

0.2 mM sodium octylsulphate

-pH adjusted to 3.5 with orthophosphoric acid

2 - 8% methanol (v/v)

The 5 μm ODS column was capable of resolving the two diastereoisomers of BH_4 (figure 2.31). Under typical conditions in which the mobile phase contained 3% methanol and the flow rate was 1ml/minute, the capacity factors were 2.87 for the natural diastereoisomer and 4.00 for the other S-isomer. (The capacity factor is a measure of the capacity of the column to retain a given sample, and is defined as follows:-

$$\text{Capacity factor} = \frac{t_s - t_o}{t_o}$$

where t_s is the retention time of the solute, and t_o is the retention time of a non-retarded material - that is the void volume).

By calculating the area of each of the two peaks from the chromatogram, it is evident that the two compounds are present in the ratio 3:1, thus confirming that the two peaks observed are the two stereoisomers of BH_4 since this corresponds to the proportion of the two isomers present in the standard. (Schircks, personal communication). In addition, an identical chromatogram was obtained on injection of the BH_4 standard onto a high pressure liquid chromatograph employing a Pye Unicam variable wavelength ultraviolet detector set at 267 nm (figure 2.32), the peak wavelength at which tetrahydrobioperin absorbs. This adds weight to the argument that the second peak is an isomer of BH_4 and not an impurity in the sample.

Henceforth, all weights of BH_4 will be expressed as the actual weight of the natural stereoisomer, that is the

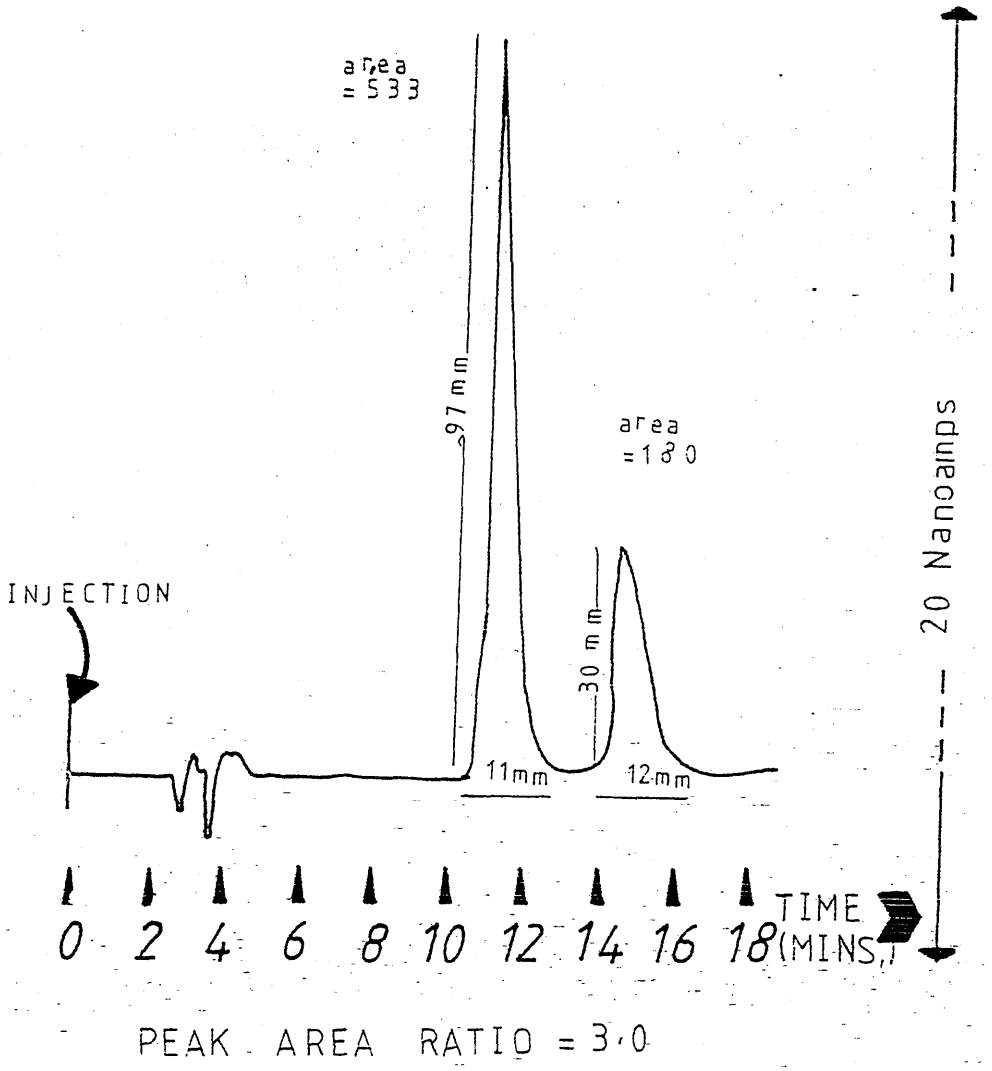
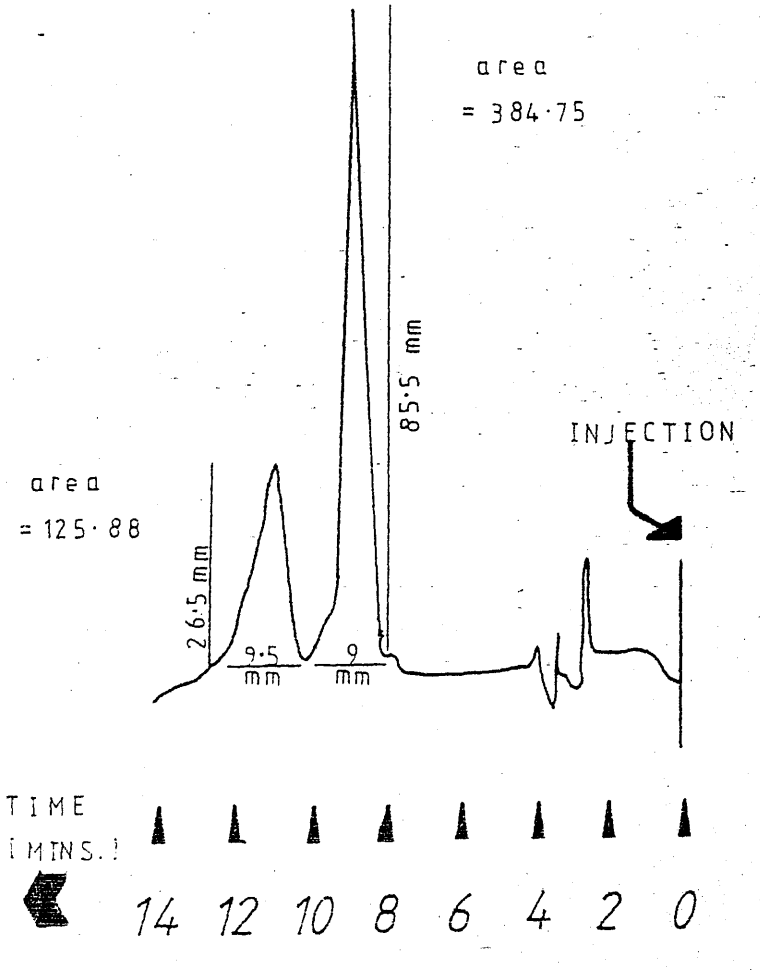


Figure 2.31 Chromatographic separation of tetrahydrobiopterin isomers as detected electrochemically.

Separation of the 2 isomers of tetrahydrobiopterin (R and S) by HPLC with electrochemical detection. Mobile phase conditions are as described in the text; 3% methanol (v/v) with a flow rate of 1ml/min. A potential of +0.25 volts was employed and 56ng of a mixture of isomers of tetrahydrobiopterin was injected in a volume of 100µl.

Figure 2.32 Chromatographic separation of tetrahydrobiopterin isomers as detected at 267 nm.

Separation of the 2 isomers of tetrahydrobiopterin at 267nm (Pye-Unicam LC3). Mobile phase conditions are as described in the text; 3% methanol (v/v) with a flow rate of 1ml/min. 333ng of a mixture of the dihydrochloride of both isomers was injected in a volume of 20µl.



PEAK AREA RATIO = 3·06

amount of standard injected onto the column will be corrected to account for the unnatural isomer and for the presence of the dihydrochloride in the standard.

To determine the relationship between the applied oxidation potential and the output from the detector, voltammograms were constructed for BH_4 and BH_2 (figures 2.33 & 2.34). It is evident that an oxidation potential of only +0.25 volts is sufficiently positive for the analysis of BH_4 whereas +0.8 volts is required to measure BH_2. It was not possible to detect biopterin under these conditions.

The linear response of the detector over the range of BH_4 standards injected (15 pg - 2.85 ng) is shown in figure 2.35, with the minimal detectable quantity being 15pg.

The concentration of tetrahydrobiopterin in rat brain was measured by the reverse-phase LCEC technique using the extraction procedure of Brautigam & Dreesen (1982). The rat brain samples were homogenised in 4 volumes of freshly prepared 0.2M perchloric acid containing 10mM ascorbic acid which was precooled to 0-4 ^{0}C and degassed. The ascorbate was present to prevent oxidation of the unstable BH_4. The samples were then centrifuged for 20 minutes at 15,000g (4 ^{0}C) with storage on ice before and after. Following centrifugation, the BH_4 content of the supernatant was determined by direct injection onto the column. The high sensitivity and selectivity of the electrochemical detector and the low oxidation potential (+0.25V) employed to detect BH_4 allows direct injection of the supernatant with no preceeding purification step being required.

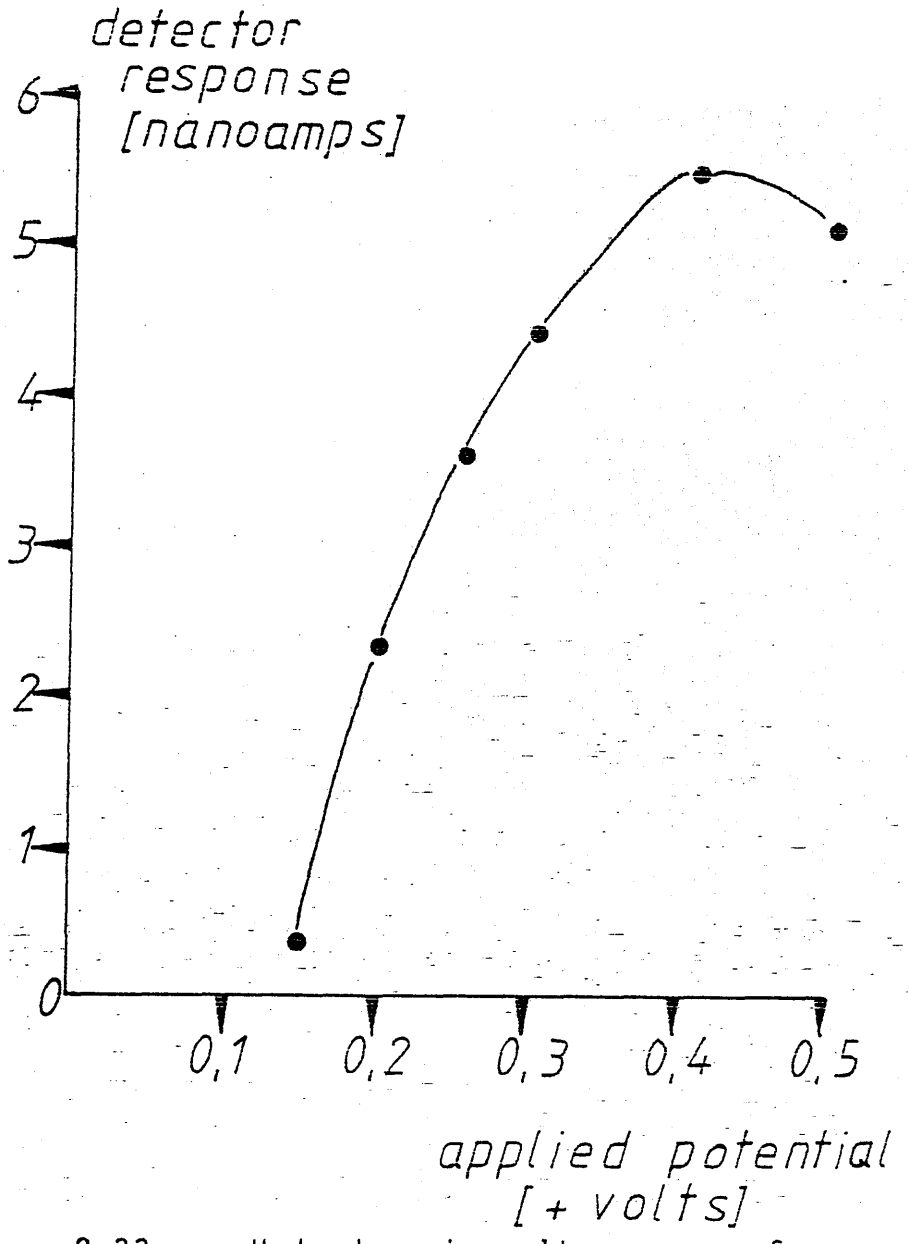


Figure 2.33 Hydrodynamic voltammogram of tetrahydrobiopterin

The response of the detector to constant quantities of analyte injected onto the column will vary with the potential applied to the working electrode. A characteristic sigmoid curve is produced when the applied potential is plotted against the detector output. The mobile phase is as described in the text; 5% methanol (v/v) with a flow rate of 1.5ml/min. 6.3ng tetrahydrobiopterin is injected onto the column in a volume of 100µl +0·25V is sufficiently positive to detect BH4 while minimising oxidation of sample impurities.

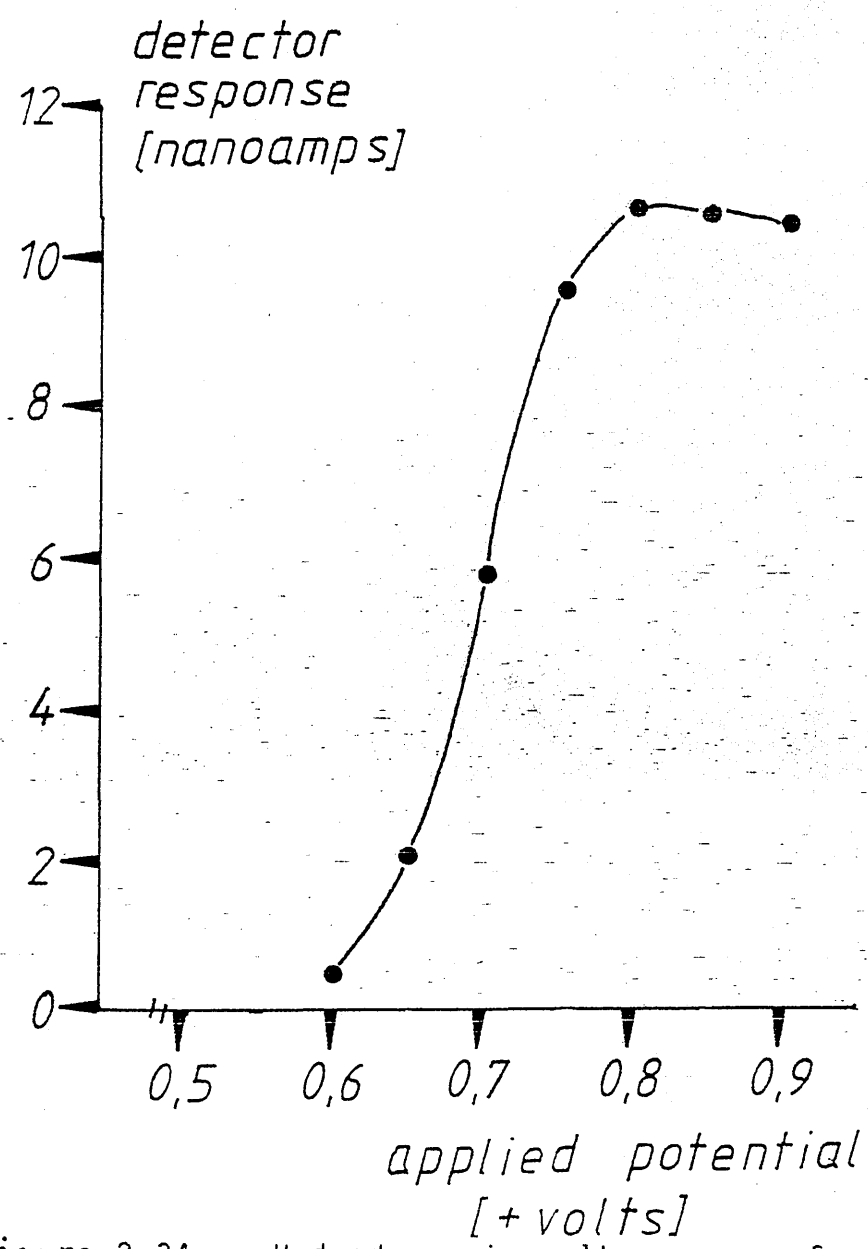


Figure 2.34 Hydrodynammic voltammogram of dihydrobiopterin.

Detector response to a constant amount of dihydrobiopterin injected onto the column at differing potentials applied to the working electrode. The mobile phase is as described in the text; 7% methanol (v/v), flow rate 2ml/min. 30ng dihydrobiopterin is injected onto the column in a volume of 100µl.

Figure 2.35 Detector linearity to tetrahydrobiopterin.

The graph illustrates the linearity of the electrochemical detector to tetrahydrobiopterin. Each point represents the mean of 2 injections. The minimal detectable quantity was calculated to be 15 picograms.

The chromatographic separation of dihydrobiopterin and the two stereoisomers of BH_4 in the reverse-phase HPLC system is shown in figure 2.36 which can be compared to a chromatogram obtained from rat cortex. BH_2 cannot be detected in tissue samples using the method described above due to a number of disturbing peaks being present in the chromatogram as a result of the much higher oxidation potential (+0.8 V) which must be applied.

The recovery of BH_4 from tissue homogenates was estimated by adding increasing quantities of BH_4 standard to 400µl 0.2M perchloric acid (containing 10mM ascorbic acid) and 50mg rat brain cortex. Each sample was then subjected to the extraction procedure described above and the BH_4 content measured. The results are shown in figure 2.37 which demonstrates that the extraction of BH_4 was satisfactory.

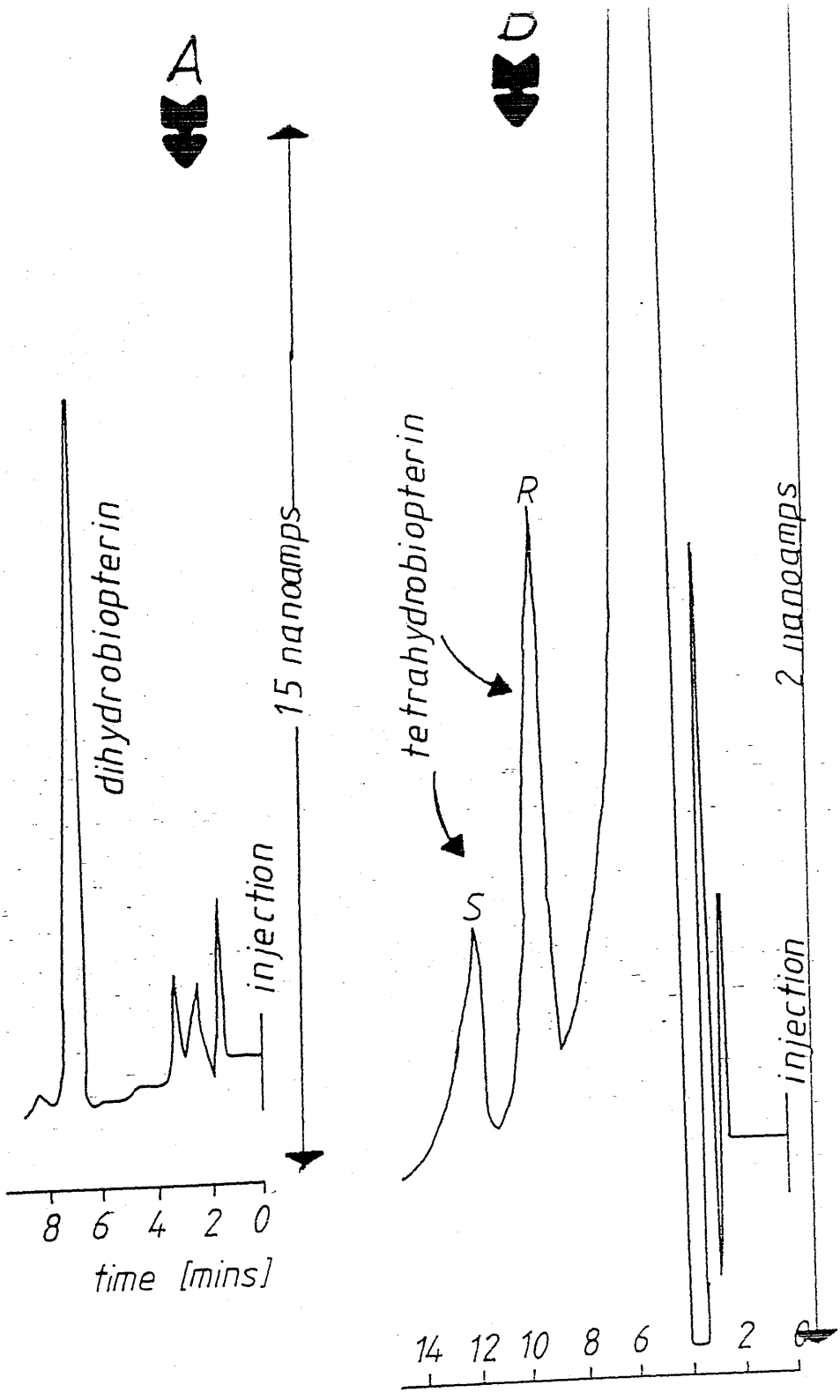


Figure 2.36 Typical examples of chromatograms.

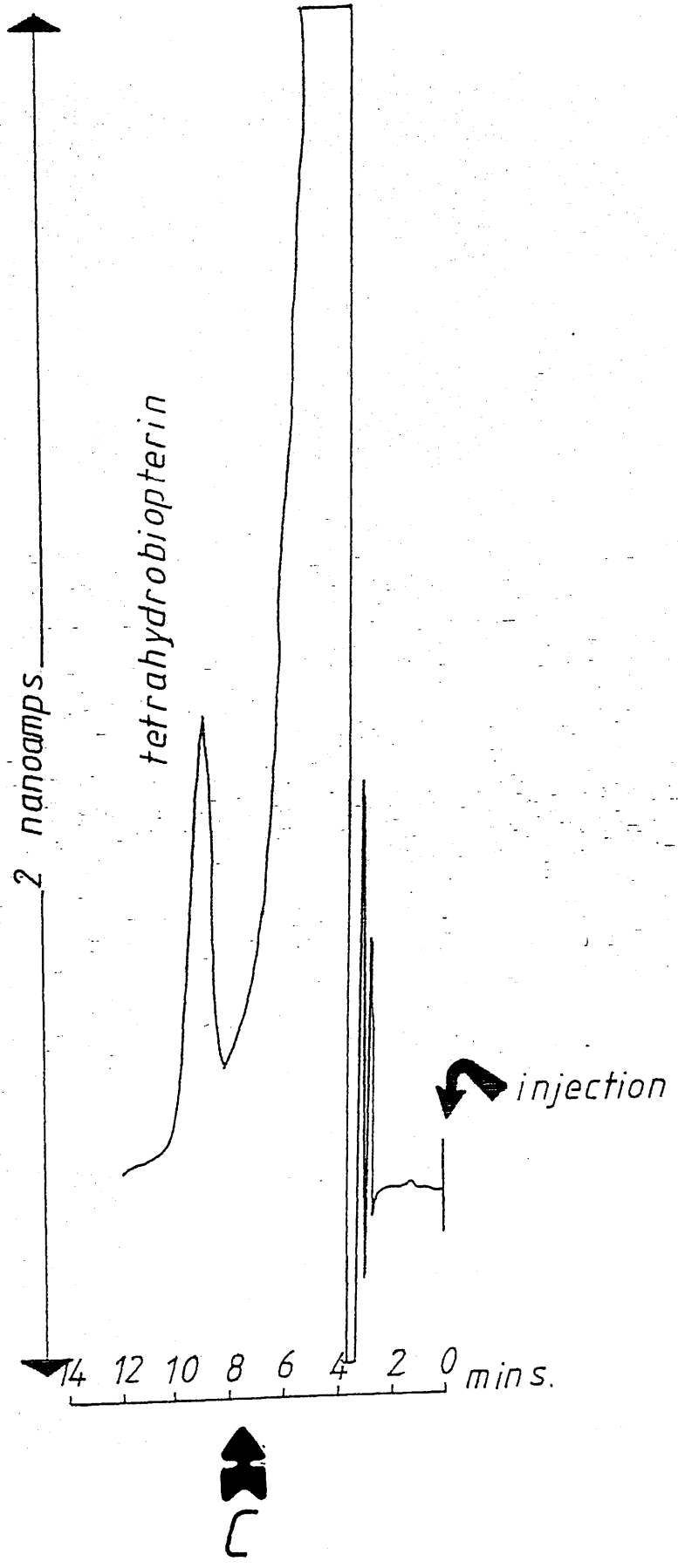
Typical separation of the pteridines on the reverse-phase column

(a) dihydrobiopterin standard

(b) standard solution consisting of a mixture of the R and S isomers of tetrahydrobiopterin

(c) rat brain homogenate sample showing the presence of only the R isomer of tetrahydrobiopterin

chromatographic conditions are as described in the text.


2 nanoamps
tetrahydrobiopterin
injection
14 12 10 8 6 4 2 0 mins.

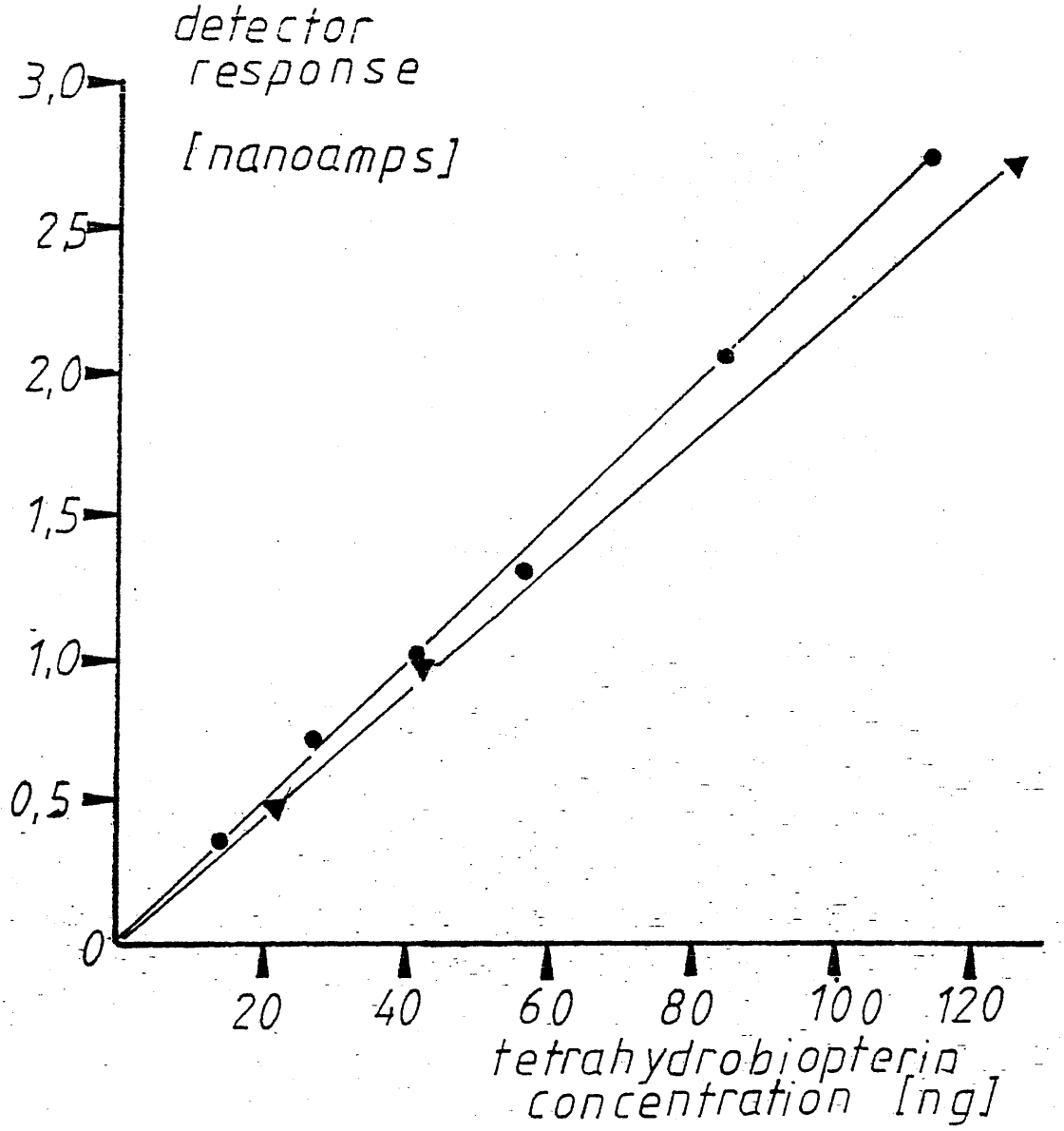


Figure 2.37 Recovery of tetrahydrobiopterin.

The graph shows that the recovery of tetrahydrobiopterin from rat brain homogenates was constant over the concentration range shown. The triangles represent the unextracted standards while the extracted samples are represented by the circles. Each point represents the mean of 2 observations. Statistical analysis demonstrates no significant difference between the slopes of the two lines.

2.14 Dihydropteridine Reductase (DHPR) Activity

Dihydropteridine reductase activity (DHPR) in rat brain was measured by the method of Purdy et al (1981) which was similar to the techniques employed by Craine, Hall & Kaufman (1972) and Nielsen, Simonsen & Lind (1969) with the addition of sodium azide as a catalase inhibitor. A 1 in 4 homogenate of the rat brain samples was prepared in 0.1M Tris, 0.04M potassium chloride buffer pH 8.0 and centrifuged at 19,000g for 1 hour at 4 ^{0}C. Dihydropteridine reductase activity was assayed spectrophotometrically by following the disappearance of reduced nicotinamide adenine dinucleotide (NADH) at 340nm at 25 ^{0}C in 20μl of the brain supernatant diluted to 1ml. The assay relies on the ability of peroxidase to generate quinonoid dihydropterin, one of the substrates for dihydropteridine reductase, from the corresponding tetrahydropterin (Storm & Kaufman, 1968) - equation 2.5.

$$\text{Tetrahydropterin} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{Quinonoid dihydropterin} + 2H_2O$$

- Equation 2.5

Figure 2.38 shows the scheme for this assay system. The reaction catalysed by peroxidase thus replaces the hydroxylase catalysed reactions normally employing tetrahydrobiopterin as a cofactor in vivo. The assay utilizes sodium azide as a catalase inhibitor in order to prevent the reduction of hydrogen peroxide. Peroxidase and hydrogen peroxide continually reoxidise the reduced

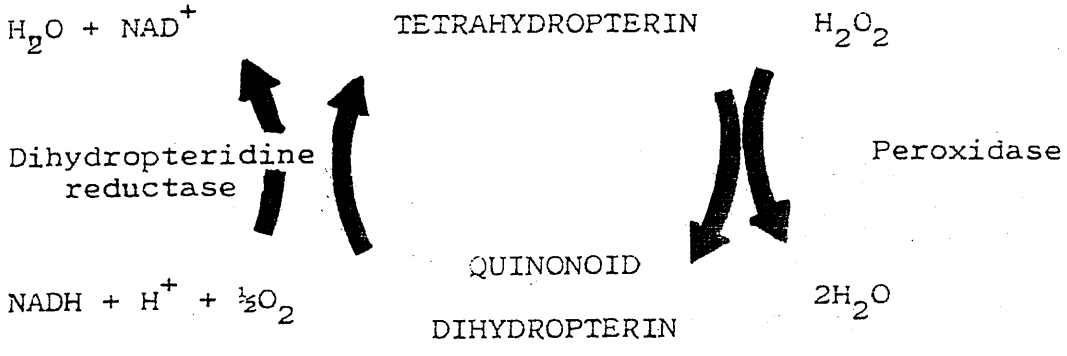


Figure 2.38 Schematic representation of dihydropteridine reductase assay.

The diagram illustrates the principle of the assay for DHPR activity in which the disappearance of NADH is measured spectrophotometrically.

tetrahydropterin to the quinonoid dihydropterin and thus maintain the concentration of the latter virtually constant; that is, the concentration of the quinonoid form in the reaction mixture is equal to the concentration of the tetrahydropterin present initially (Craine et al, 1972).

The incubation mixture consists of the following components:-

1) Tris-(hydroxymethyl)aminomethane	0.1 M, pH 7.2	480 µl
2) Sodium azide	2.5×10^{-3} M	100 µl
3) Horse raddish peroxidase	8 mg/100 ml	100 µl
4) Hydrogen peroxide	10^{-2} M	100 µl
5) Rat brain supernatant		20 µl
6) 6-Methyl-5,6,7,8-tetrahydropterin	10^{-4} M	100 µl
7) NADH	10^{-3} M	100 µl

After the addition of NADH which was invariably the final component added, the mixture was briefly vortexed. The addition of each component resulted in assay conditions as follows:- 0.05M Tris/HCl buffer pH 7.2, 2.5×10^{-4} M sodium azide, 10^{-3} M hydrogen peroxide, 10^{-5} M 6-methyl-5,6,7,8-tetrahydropterin, 8µg peroxidase, 10^{-4} M NADH and enzyme in a final volume of 1ml.

It has previously been demonstrated that NADH is a better substrate than NADPH (Nielsen et al, 1969) for dihydropteridine reductase from several species of mammalian liver. Hence the use of NADH in the assay system described above in preference to NADPH.

The reduction of quinonoid dihydropterin to the corresponding tetrahydropterin can occur both spontaneously

or by the enzyme dihydropteridine reductase (Kaufman, 1961). Blank rates, that is the nonenzymatic reduction of quinonoid dihydropterin by pyridine nucleotide were determined by omitting brain supernatant from the assay system.

The rate of reaction was measured by the decrease in absorbance at 340nm in a Pye-Unicam SP500 spectrophotometer from 1 minute of the addition of NADH to ensure that mixing was complete and the reaction established. The optical density changes were recorded for a period of 7 minutes (samples and blanks being read against water). Enzyme activities were calculated as the rate of oxidation of NADH and expressed as nanomoles NADH/minute/milligram tissue or protein. The reaction was linear with time (figure 2.39) indicating that the formation of 7,8-dihydropterin by spontaneous isomerisation of the unstable quinonoid dihydropterin was insignificant during the course of the assay. Figure 2.40 demonstrates the effect of varying the concentration of the substrate, 6-methyl-tetrahydropterin on enzymatic activity. Preliminary experiments demonstrated that this compound was as appropriate a substrate as 6,7-dimethyltetrahydropterin for dihydropteridine reductase from the brains of the species of rats employed in our experiments (Sprague-Dawley). Using 6-methyl-tetrahydropterin as a substrate, the activity of dihydropteridine reductase was 10.3 nmoles NADH/minute/mg protein (n=8) while it was 8.9 nmoles NADH/minute/mg protein (n=6) when the dimethyl compound was the substrate (not significant at the 5% level). the effect of altering the NADH

concentration in the incubation medium on dihydropteridine reductase activity can be seen in figure 2.41. In addition, the activity of the enzyme was linearly related to tissue concentration (figure 2.42). An inter-assay coefficient of variation of 7.2% was calculated for the assay as described above.

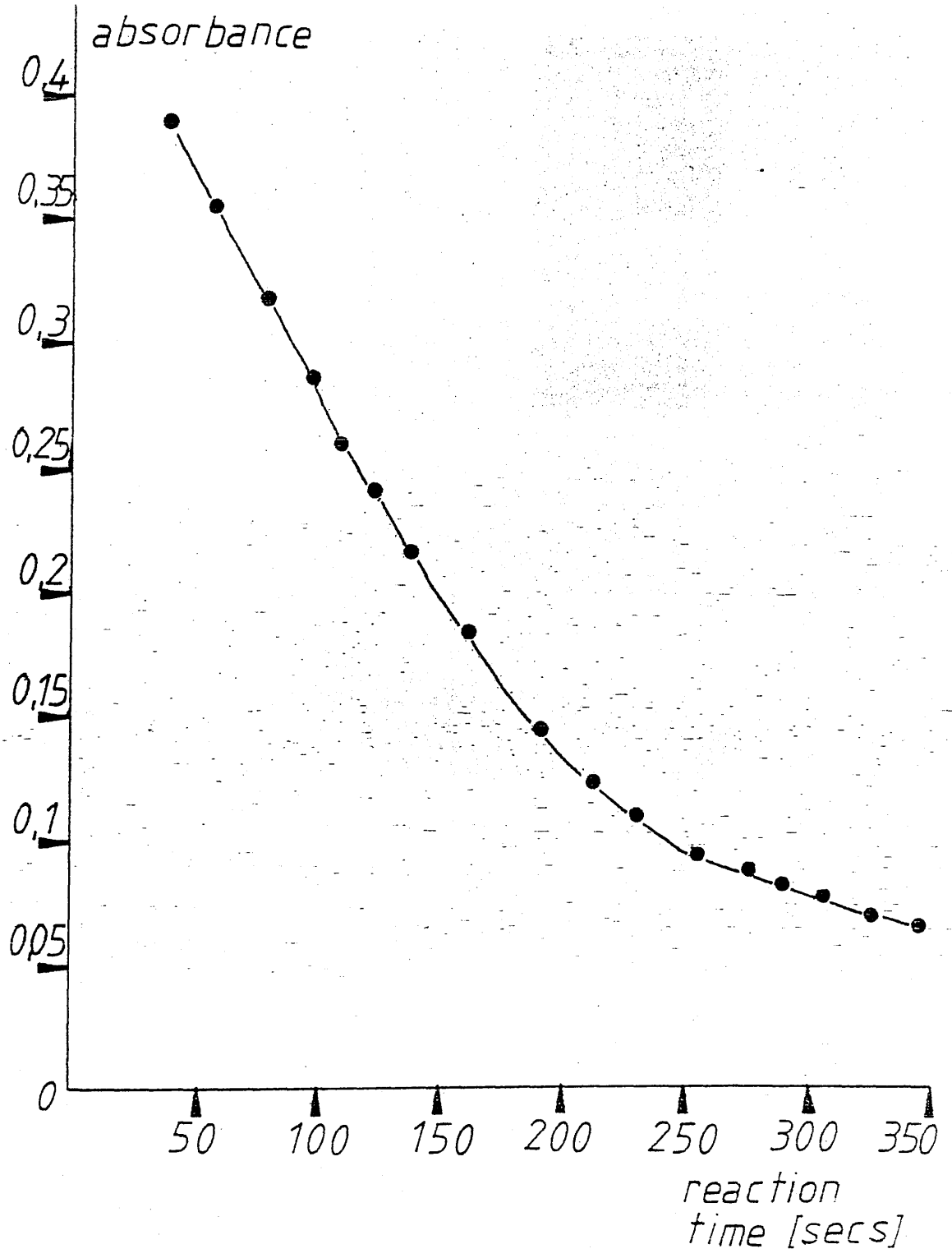


Figure 2.39 Activity of dihydropteridine reductase with time.

The graph illustrates that the assay is linear up to 3 minutes.

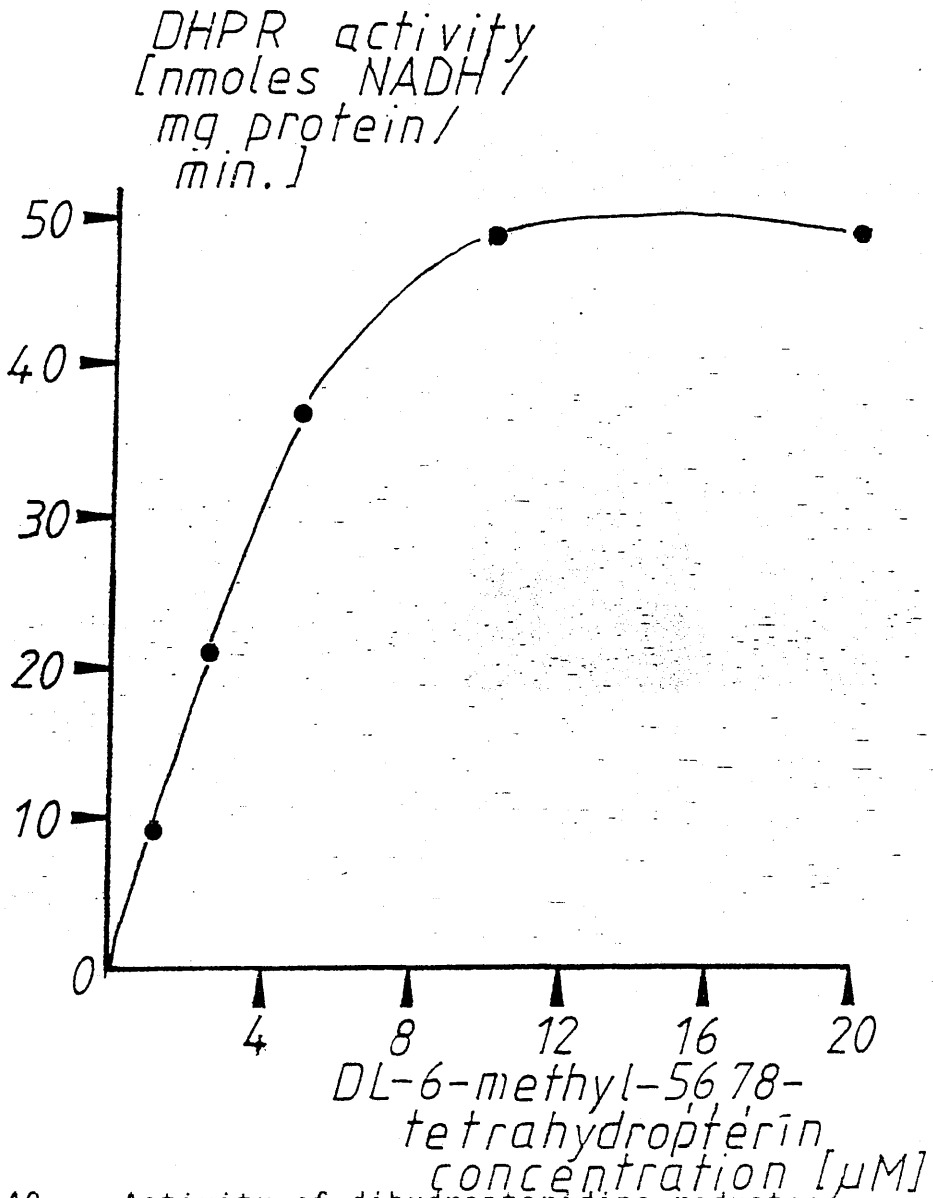


Figure 2.40 Activity of dihydropteridine reductase as a function of pterin concentration.

The graph illustrates the kinetics of DHPR with respect to the substrate, 6-methyl-5,6,7,8-tetrahydropterin concentration. Each point represents the mean of 3 observations.

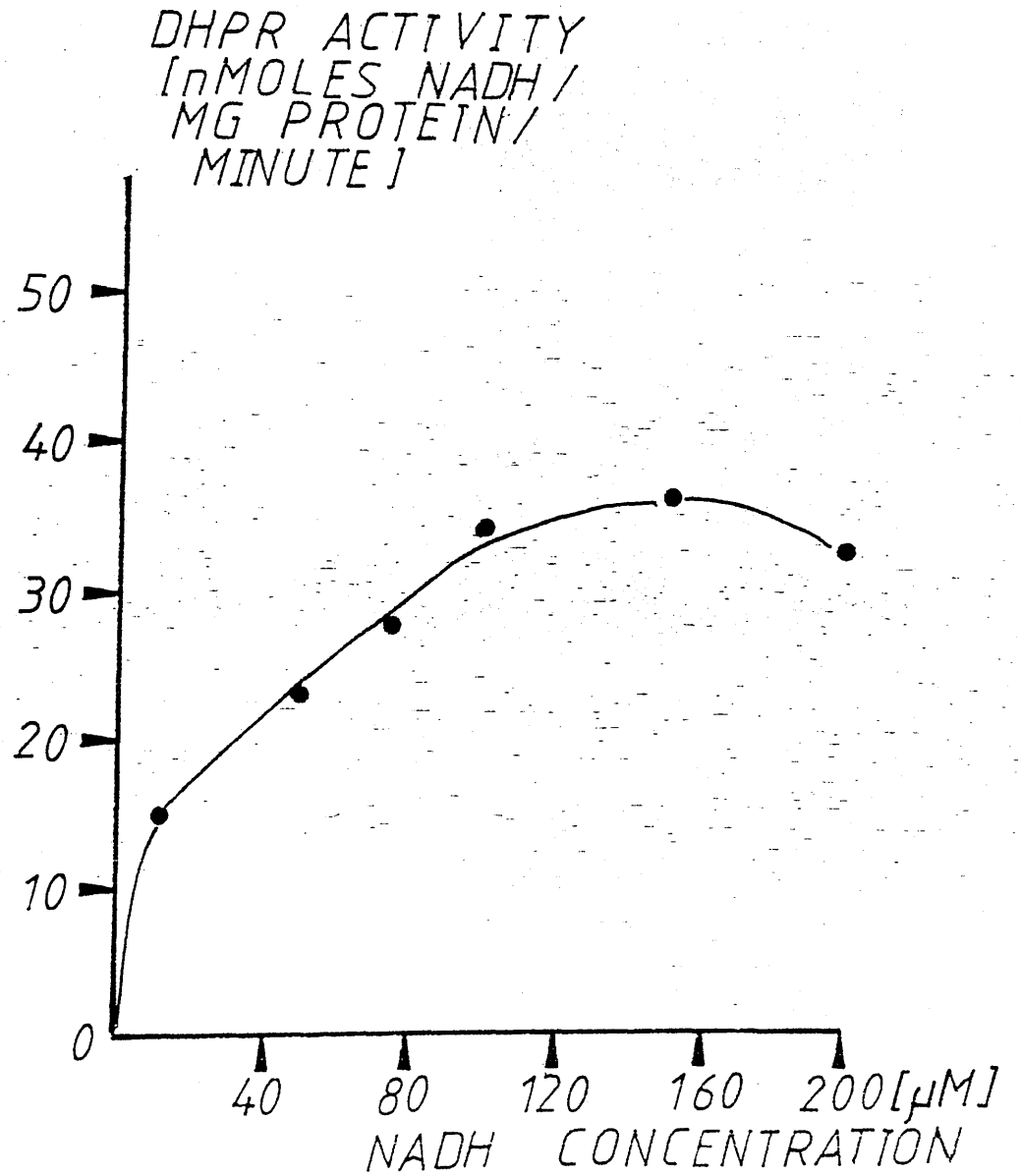


Figure 2.41 Activity of dihydropteridine reductase as a function of NADH concentration.

The kinetics of DHPR with respect to substrate, NADH concentration. Each point represents the mean of 3 observations.

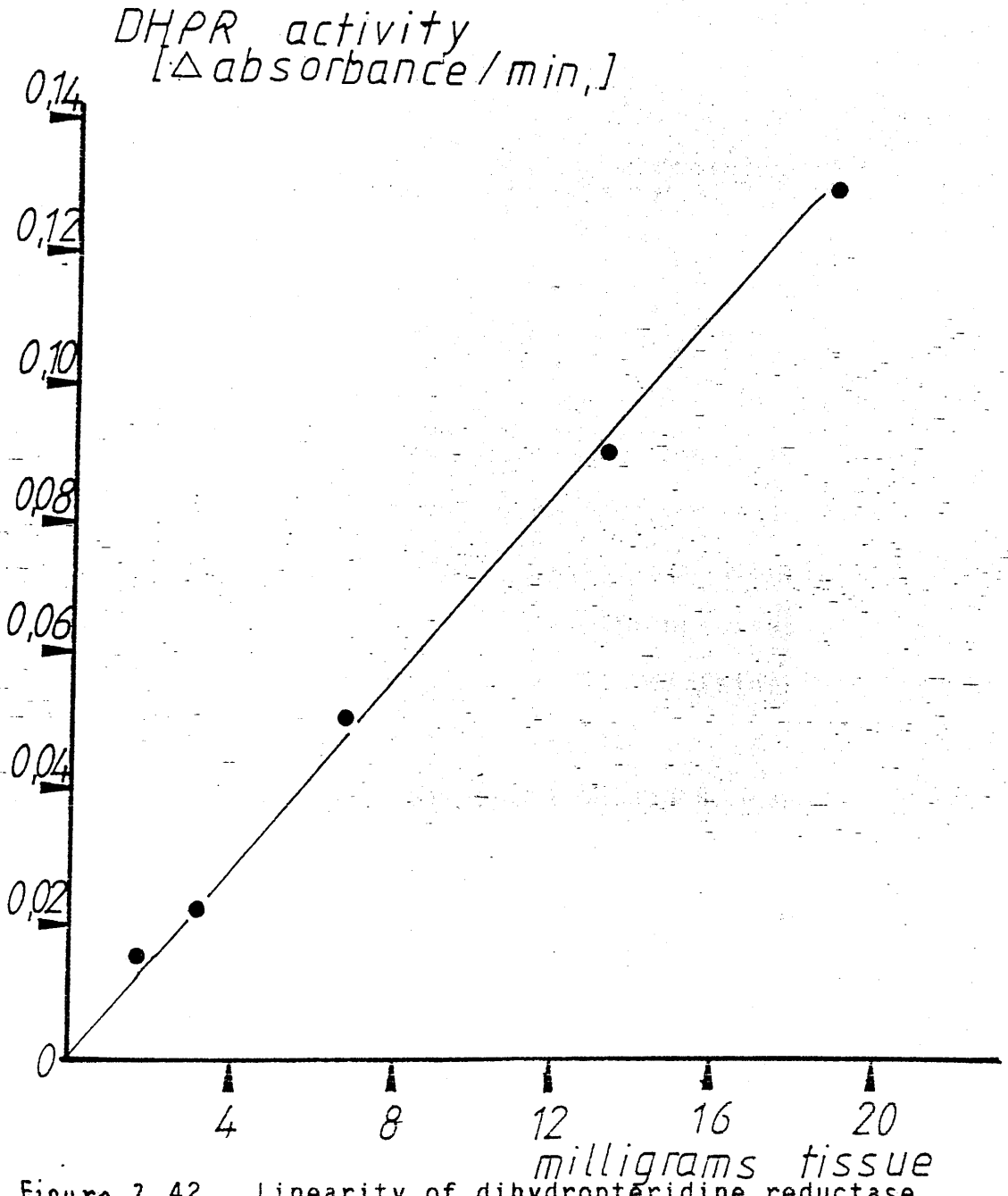


Figure 2.42 Linearity of dihydropteridine reductase assay with amount of tissue assayed

The graph illustrates that the assay was linear up to at least 20mg rat brain tissue. Each point represents the mean of 3 observations.

2.15 Choline acetyltransferase (Chat) activity

The dissected regions of rat brain were stored at -20 °C until cholineacetyltransferase activity could be estimated. This was possible since this enzyme is stable in frozen tissue for relatively long periods (Fonnum, 1979c). At the time of analysis, the tissues were removed from the freezer and allowed to thaw on ice. Each sample was then homogenised in 5 volumes 10mM disodium ethylenediamine-tetraacetic acid (EDTA), pH 7.4 containing 0.5% (v/v) triton X-100. The enzyme is mainly localised in the synaptosomal fraction of the cell and hence generally found in an occluded form in the homogenate. The purpose of the detergent is to release full enzymatic activity from the tissues. The tissue homogenates were maintained on ice before being incubated with the appropriate substrates. Several radiochemical assays exist for choline acetyl-transferase (McCamen & Hunt, 1965; Fonnum, 1966; Goldberg, Kaita & McCamen, 1969; Bull & Oderfeld-Nowak, 1971; Tsuchida et al, 1982) but in this study, the activity of this enzyme was estimated by the method of Fonnum (1979c). Using this method, acetylcholine is formed from ^{14}C labelled acetyl-CoA and unlabelled choline during an incubation step. The product is then separated from excess substrate by a liquid cation exchange resin, and the labelled acetylcholine counted in a scintillation counter. The incubation medium employed in the assay is shown in table 2.10. The inclusion of EDTA in the incubation medium provided protection and stabilization of choline acetyl-transferase. The enzyme from different sources can be

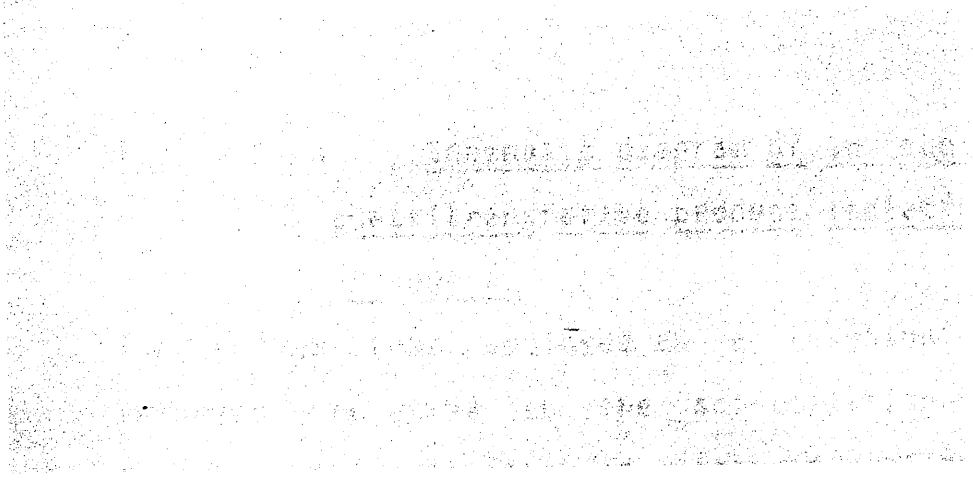
activated by high concentrations of salts; either sodium or potassium chloride can be employed. Buffering of the incubation medium can be accomplished by either phosphate or tris H Cl as pH is not a critical factor due to the enzyme exhibiting a broad pH optimum. In order to prevent hydrolysis of acetylcholine formed by cholinesterases present in the homogenate, a cholinesterase inhibitor, physostigmine was employed.

The most critical factor in cholineacetyltransferase activity assays is the purity and concentration of acetyl-CoA. This must be of high quality otherwise erratic results are obtained. In all assays carried out for this thesis, ^{14}C acetyl-CoA was obtained from Amersham International plc and the unlabelled compound was supplied by Sigma Chemical Company and was 90-95% pure. The actual assay procedure was as follows:-

2µl of each brain homogenate (in triplicate) was pipetted into small glass durham tubes, on ice. After the addition of 5µl of incubation mixture, the samples were incubated for 15 minutes at 37°C. At the end of this period the tubes were transferred to scintillation vials into which had been dispensed 10ml toluene containing 0.5% 2,5-diphenyloxazole (PPO) and 0.02% 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (POPOP) and 2ml acetonitrile containing 10mg kalignost (sodium tetraphenylboron). The contents of the tube were then washed out with 5ml of 10mM sodium phosphate buffer, pH 7.4. Sodium tetraphenylboron acts as a liquid cation exchanger (Fonnum, 1969a); that is, it is able to form ionic complexes which are highly soluble

in an organic phase of acetonitrile relative to an aqueous phase, buffer (Fonnum, 1969b) Moreover, the acetylcholine-Kalignost complex is very soluble in acetonitrile but almost insoluble in water.

After shaking the vials lightly for two minutes, they were then left to stand until the two layers separated out under gravity. Once the layers had separated, the activity was counted in the biphasic system. The labelled acetylcholine formed by cholineacetyltransferase was present in the toluene-acetonitrile solvent and unreacted labelled acetyl-CoA remained in the aqueous layer. Since no scintillation arises in the aqueous phase, the labelled acetyl-CoA was not counted and only the labelled acetylcholine in the organic layer could be counted (figure 2.43). Counting was carried out in a Packard Tricarb Scintillation Counter.



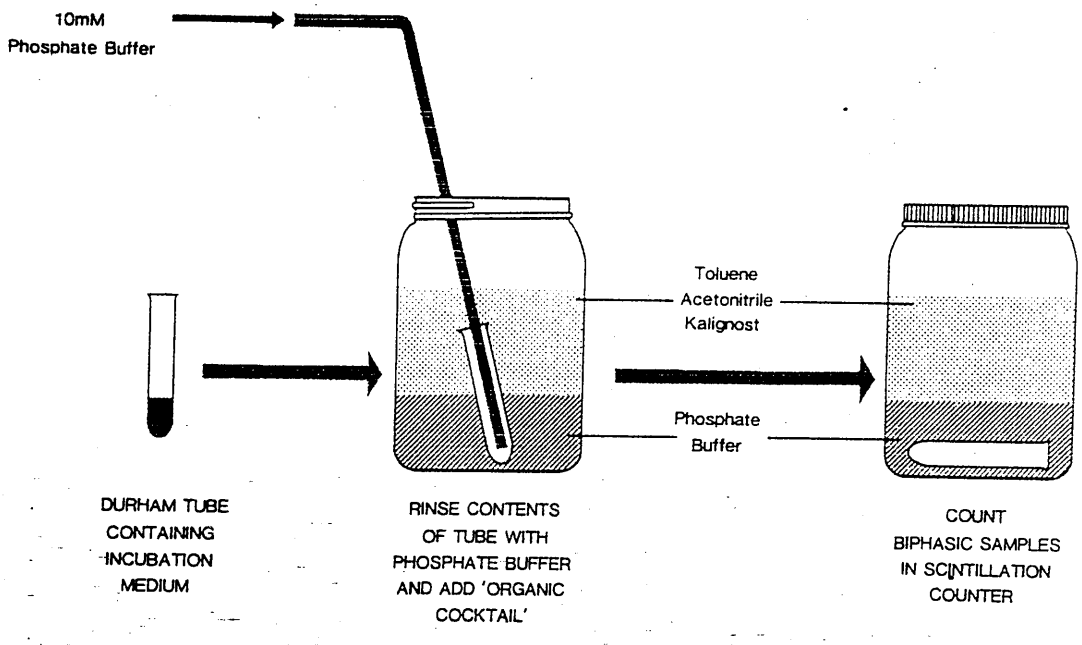


Figure 2.43 Schematic diagram of the choline acetyltransferase product isolation procedure.

The Durham tube employed as a reaction vessel is enlarged relative to the scintillation vial for clarity. Scintillation counting is confined to the toluene-acetonitrile organic layer containing the product, acetylcholine, while excess labelled acetyl-CoA remains in the buffer layer and is not counted.

Table 2.10 Assay incubation conditions for choline acetyltransferase activity

Component concentration	Volume added	Final concentration
3.6M NaCl (21g/100ml)	25 µl	300 mM
0.5M NaH_2PO_4, pH 7.4 (7.8g/100ml)	25 µl	41 mM
0.43mM physostigmine salicylate (0.26g/ml) dissolved in EDTA		0.1 mM
0.066M EDTA (adjusted to pH 7.4 with Na OH)	50 µl	10 mM
0.2M choline bromide	15 µl	10 mM
0.6mM acetyl-CoA	100 µl	0.2 mM
10% triton-X-100	1 µl	0.05%
total volume	216 µl	

2.16 Delta-aminolaevulinic acid synthase (ALA S) activity

The activity of the enzyme delta-aminolaevulinic acid synthase (E.C. 2.3.1.37) which is responsible for the conversion of glycine and succinyl Co A into delta-aminolaevulinic acid was measured in the brains of rats by a modification of the isotopic method of Freshney & Paul (1970).

Following removal of the brains of the rats the tissue was homogenised in a solution comprising equal volumes of 0.9% sodium chloride and a buffer prepared as follows -

a 0.12 M solution of dipotassium hydrogen orthophosphate (20.902g/l) and of potassium dihydrogen orthophosphate (16.32g/l) are prepared. To approximately 700ml of dipotassium hydrogen orthophosphate is added 171.15g of sucrose, 8.53g magnesium chloride, 7.44g ethylenediaminetetraacetic acid, sodium salt (EDTA Na_2), 0.075g glycine and mercaptoethanol. The resultant solution is then adjusted to pH 7.0 with the potassium dihydrogen orthophosphate. The remaining 300ml of the dipotassium hydrogen orthophosphate is also titrated with the potassium dihydrogen orthophosphate to pH 7.0 and used to adjust the buffer volume to 1 litre. The buffer prepared as above contains 0.5M sucrose, 90mM $MgCl_2$, 20mM EDTA, 1mM glycine and mercaptoethanol.

The actual assay is performed in a small 5ml polypropylene tube in duplicate. 200µl of the tissue homogenate is pipetted into the tube along with 200µl of reaction mixture prepared as shown below, and the tubes are

incubated at 37°C for 1 hour in a shaking water bath.

Reaction buffer - A 0.05M solution of potassium dihydrogen orthophosphate (3.4g/500ml) and of dipotassium hydrogen orthophosphate (4.335g/500ml) are prepared. To 100ml of dipotassium hydrogen orthophosphate is added

9.5 mg glycine (0.13M)

22.25 mg magnesium sodium maleate (0.11M)

108 mg magnesium chloride (1.13M)

66 mg pyridoxal phosphate (0.25M)

the pH of the resultant solution is then adjusted to 6.8 with potassium dihydrogen orthophosphate. The remaining 400ml of dipotassium hydrogen orthophosphate is titrated to pH 6.8 with the potassium dihydrogen orthophosphate and used to adjust the volume of the reaction mixture to 250ml. The reaction mixture employed in the assay is prepared by taking 20ml of the above buffer and spiking it with 100µl of 2 (^{14}C) labelled substrate, glycine (53.5mCi/mmol.).

The reaction is terminated by the addition of 40µl of a 51µM solution of delta-aminolaevulinic acid in 2M trichloroacetic acid to 200ul of the incubation mixture. The tube is then mixed, sonicated and stored at -20°C until high voltage electrophoresis can be performed. Separation of the labelled substrate, glycine from the product, delta-aminolaevulinic acid is achieved by high voltage electrophoresis (Lecarte). The precipitate from each sample is removed by centrifugation and the clear supernatant spotted onto Whatman 3MM chromatography paper for electrophoresis. Separation is performed at 2000V at 4°C in 0.05M potassium phthalate buffer. After

electrophoresis and drying the two spots are identified by spraying with ninhydrin, cut out and burned using a oxidiser (Packard), before being counted (Packard tricarb liquid scintillation counter) for a period of 100 minutes in order to obtain sufficient counts due to the low activity present.

2.17 Tissue protein concentration

Tissue protein concentrations were measured by the method of Lowry et al (1951). The samples were diluted using water to give a protein concentration in the range 0-200µg/ml. To 1ml of each diluted sample (in duplicate) was added 5ml of a solution containing:-

0.5ml 2% sodium tartrate

0.5ml 1% copper sulphate.5H_2O

per 50ml 2% sodium carbonate in 0.1M sodium hydroxide.

Standards were prepared using bovine serum albumin. Protein solutions in water containing 25, 50, 100 & 200µg/ml protein were prepared and to 1ml of each (in duplicate) was added 5ml of the above solution.

The samples and standards were vortexed and allowed to stand for 10 minutes. The purpose of this step was to allow the initial reaction of Cu^{2+} and protein in alkali. After standing for this period of time, 0.5ml of a 1:1 dilution of Folin & Ciocalteu's reagent in water was added and the samples vortexed once more. This step involved the reduction of phosphotungstic and phosphomolybolic acids to molybenum blue and tungsten blue, both by a copper-protein complex and by tyrosine and tryptophan of the protein.

Although both tyrosine and tryptophan give a colour reaction without Cu^{2+}, the majority of the colour is due to protein.

After a period of 30 minutes the samples and standards were read on a spectrophotometer set at a wavelength of 750nm against a reagent blank - 1ml distilled water instead of the sample or standard. (Pye Unicam SP500 series 2). The concentration of protein in each of the diluted samples calculated from the standard graph, an example of which is shown in figure 2.44 was multiplied by an appropriate factor to correct for the dilution of the sample, thus producing a tissue protein concentration expressed in mg/ml.

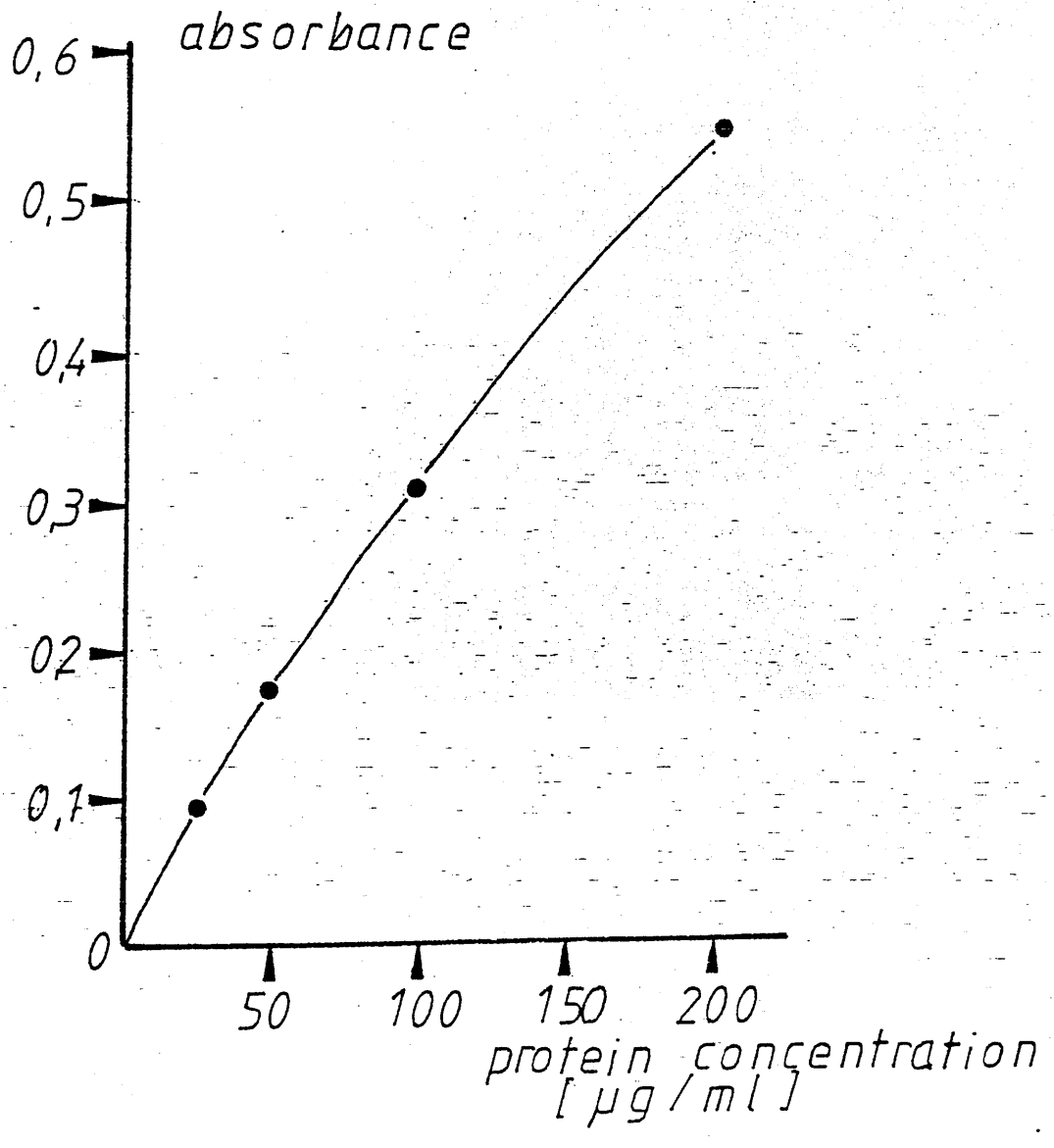


Figure 2.44 Standard curve for protein estimation.

A typical calibration curve employed in the calculation of protein concentrations. Each point represents the mean of 2 observations.

2.18 Statistical techniques

The data in this thesis will be calculated as the mean plus or minus one standard deviation ($\bar{x} \pm$ S.D.), the mean being calculated as -

$$\bar{x} = \frac{\sum x_i}{N}$$

and the standard deviation being derived from the formula -

$$S.D. = \sqrt{\frac{\sum (x_i - \bar{x})^2}{N - 1}}$$

where x_i is the i'th value, $\bar{x}$ the mean of the data and N the number of observations being considered. For each set of data points, 3 parameters termed the mean (average), median (central point) and mode (most frequent observation) can be calculated. These parameters are shown graphically for a moderately skewed distribution in figure 2.45. In the case of a perfectly symmetrical distribution the variables all coincide. In situations where the data obtained are found to distribute in a skewed fashion, in which case there will be a large difference in the values of the mean, mode and the median, then the geometric mean (Figure 2.45) will be calculated along with an appropriate scatter variable. Calculation of the geometric mean will be according to the following formula-

$$\text{Geometric mean} = ([x1] X [x2] X [x3] \ldots\ldots x_n)^{1/N}$$

where x is the first, ...n'th variable, and N is the number of observations under consideration.

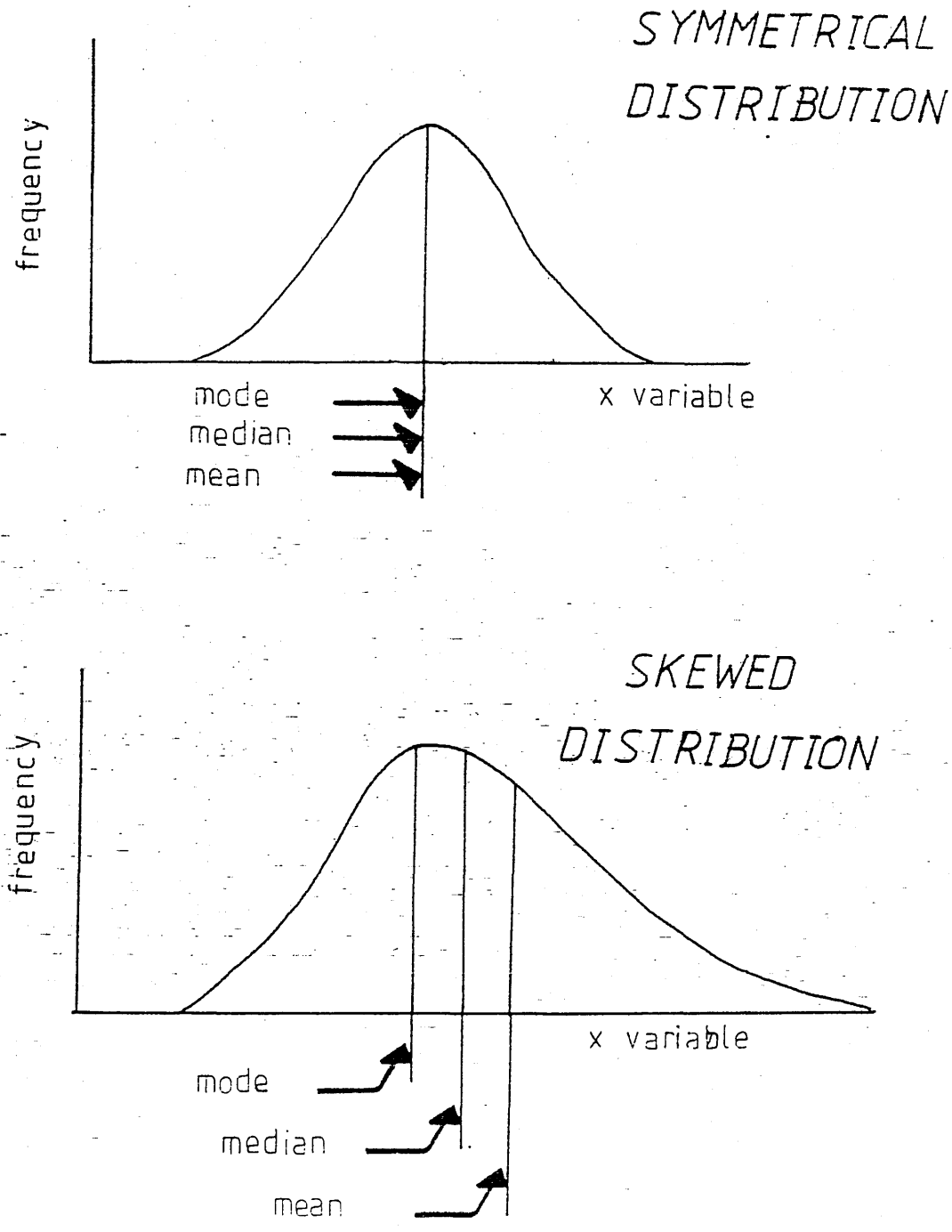


Figure 2.45 Mean, median and mode of a symmetrical and skewed distribution.

Diagram illustrating the divergence of the mean, median and mode of a skewed distribution in contrast to a symmetrical distribution in which they coincide.

The geometric mean, a useful parameter for skewed distributions falls to the left of the arithmetic mean in the skewed distribution shown above.

Statistical testing techniques employed will depend on the nature of the data. Parametric tests, which can be applied to normally distributed data, require that certain conditions be met -

1. The observations must be independent from each other
2. They must be drawn from normally distributed populations
3. The populations must have equal variances within certain limits.

If these conditions are met then a parametric test can and should be applied since this type of test will be the most powerful for rejecting the null hypothesis (Ho - the hypothesis of no difference) when it should be rejected. Selection of the appropriate test for each set of data was performed with the aid of several references (Siegel, 1956; Snedecor & Cochran, 1969; Bradford Hill, 1977; Green & Margerison, 1978; Bailey, 1981).

Comparison of two groups of data by non-parametric techniques was performed using the Mann-Whitney U test. The data are first of all combined and ranked in ascending order. The values U_1 and U_2 are then calculated according to the following formulae -

$$U_1 = N1\ N2 + \frac{N1(N1+1)}{2} - R1$$

$$U_2 = N1\ N2 + \frac{N2(N2+1)}{2} - R2$$

Where R1 is the sum of the ranks assigned to the group

whose sample size is N1, and R2 is the sum of the ranks assigned to the group whose sample size is N2. These two values, U_1 and U_2 are related by the formula -

$$U_1 = N1\ N2 - U_2$$

and it is the smaller value of U which is employed to test the null hypothesis. When tied scores occur, each of the tied observations is given the average score they would have had if no ties had occurred.

The parametric least squares regression analysis of Pearson was employed to analyse linear relationships. A regression line of y on x for N pairs of data points (x,y) of the form:

$y = a + bx$ is formed where,

$$b = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\Sigma(x-\bar{x})^2}$$

and $a = \bar{y} - b\bar{x}$

The correlation coefficient, r can be calculated:

$$r = \frac{\Sigma (x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2\ \Sigma(y-\bar{y})^2}}$$

The degree of association can be examined statistically.

Non-parametric correlation analysis was carried out using the ranking test of Spearman which calculates a coefficient termed rho, r_s. Each value of X and Y is listed and ranked independently. The difference between the X and

the Y rank for each data point is then calculated (d_i), squared and summated (Σd_i^2). This value for the sum of the squares of the differences, along with the number of data points, N is entered into the following formula -

$$r_s = 1 - \frac{6 \Sigma d_i^2}{N3 - N}$$

Once again each tied score receives the average rank which would have been assigned had no ties occurred. Having computed a value for r_s, the null hypothesis that the two variables under study are not associated in the population, and that the observed value of r_s differs from zero only by chance is tested. When the value of N is less than 10, the probability is calculated from appropriate tables published in Siegel (1956). However, where more data points are available the following formula was applied to produce a t value with N - 1 degrees of freedom. -

$$t = r_s \sqrt{\frac{N - 2}{1 - r_s^2}}$$

Where appropriate the chi-squared test was employed to compare several groups. In addition analysis of variance was also performed to compare more than two groups of data.

CHAPTER 3

GLASGOW LEAD EXPOSURE SURVEY 1981

3.1 Introduction

Studies have demonstrated that soft, plumbosolvent water supplies in older houses with lead-soldered water pipes and lead-lined storage tanks will result in significant uptake of lead by the population living in these areas (Moore, 1977). The West of Scotland, with special reference to Glasgow, represents a region where such factors exist.

The history of the water supply to the residents of Glasgow has recently been reviewed (Goldberg, 1984) and dates back to the middle of the Nineteenth Century when a Mr. John Bateman provided a report on the suitability of Loch Katrine as a water supply for the city. Even at this time the presence of lead in such water was noted and as a result of opposition from residents living in the north side of the city, an enquiry was set up. Many eminent people of the time gave evidence, including Professor Frederick Penny, a professor of Chemistry in Glasgow. Although he initially stated that he had, on analysis of the water, found lead 'abundantly present" and that the water being 'highly charged with lead' was 'decidedly poisonous', he then proceeded to play down these findings and subsequently stated that the water was 'unexceptionable in every respect.' Additional statements were added by eminent engineers of the time such as Robert Stephenson and Isambard Kingdom Brunel -

'water of precisely similar quality and having the same power has been hitherto so used in contact with lead without any evil consequences.'

- Burnett, 1869

Despite elegant experiments performed by Professor Sir Robert Christison of Edinburgh (1844) which clearly demonstrated that all pure waters can act on lead to dissolve it and that the presence of neutral salts would prevent such an action, this scientist was not consulted, and the water works at Loch Katrine on the north side of the city with its holding reservior at Mugdock was opened in 1859. A few years later an extension reservoir was constructed at Craigmaddie (Burnett, 1869).

Before long, however, cases of lead poisoning from water became evident both in Glasgow and indeed throughout the United Kingdom. Despite such cases being noted at the turn of the century, which undoubtedly resulted at least partly from water (Oliver, 1914), lead pipes and lead-lined storage tanks continued to be employed as standard practice until 1968, and legislation to limit the quantity of lead permissible in drinking water was not in force until 1985. Even then, the European Economic Community guidelines are exceedingly vague:- Running drinking water may not contain lead in excess of 0.24μM (50μg/l), but where there exist lead pipes and the lead content exceeds 0.48μM (100μg/l) either 'frequently or to an appreciable extent' then measures must be taken to reduce the consumers' exposure. (Official Journal of the European Community, 1980).

Not until the 1970's was the full significance of the conclusions of Christison realised -

1. Lead pipes ought not to be used for the purpose of conducting water, at least where the distance is

considerable, without a careful examination of the water to be transmitted

2. The risk of dangerous impregnation with lead is greatest in the instance of the purest waters,

and

3. Water with less than about one in 8000th of salts in solution cannot be safely conducted in lead pipes without certain precautions.

A relatively recent study carried out by the Department of the Environment (DOE, 1977) revealed that some 9% of the households in the United Kingdom had random water samples containing in excess of 0.48μM lead, and this figure for Scotland was 29%. This study agrees well with the data obtained in Glasgow by Moore et al, (1977a) which showed that 56% of first flush water samples and 31% of running samples exceeded 0.48μM.

Clear relationships have been demonstrated between water hardness or calcium concentration, temperature and pH with plumbosolvency (Moore, 1973). A positive relationship has been demonstrated between temperature and plumbosolvency while the relationship with calcium concentration is of an inverse exponential nature. The influence of pH on plumbosolvency is of a more complex form. Plumbosolvency falls sharply as the pH is raised from 4 to around 7. During the period when these studies cited above were being carried out, the pH of the water supply to Glasgow was 6.3, a hydrogen ion concentration which would result in significant lead dissolution (Richards et al, 1980; Richards & Moore, 1982). Finally, in the late 1970's the

conclusions of Christison were put into practice. Initial studies in the east end of the city suggested that the addition of 3ppm lime (calcium hydroxide) would adequately reduce plumbosolvency (Richards et al, 1980). Following this pilot study, a fully automated closed loop lime dosing plant was built at Mugdock and commenced operation in April 1978. However, the addition of 4mg/l lime proved insufficient to achieve minimum plumbosolvency (Richards et al, 1980). Although the pH of the water supply leaving Milngavie (close to the source) was 7.8, values of less than 7 were common at the distal ends of the distribution system. Subsequently in April 1980, the pH was raised to 9 by increasing the lime to 5mg/l which maintained a pH greater than 8 at the tap, a value previously demonstrated to be required to reduce plumbosolvency (Britton & Richards, 1981). A study performed prior to the increase in lime (Richards et al, 1980) illustrated that a significant number of water samples with elevated lead content continued to be found in houses with certain features -

1. Older housing, pre 1919
2. Housing with lead-lined storage tanks
3. Housing with runs of copper to lead pipes
4. Housing occupied by a single inhabitant, who would tend to run water less frequently.

This chapter reports the results of a survey carried out in Glasgow early in 1981 at a time when the water was being treated with 5mg/l lime. The water lead levels and the concentrations of heavy metals, lead and cadmium in the blood of inhabitants, will be compared to similar data of 2

years prior, in 1979 (DOE, 1981). During this period no other source of lead was identified to have altered.

3.2 Methods

Random selection of two hundred adult volunteers (82 males and 118 females) residing in the Glasgow area was performed by including in the study every twelve hundredth name appearing in the current electoral register. If, however, the selected subject declined to participate in the survey then another adult of the same sex, living in the same household, or if this was not possible in an adjacent household, was chosen. A venous blood sample was obtained from each volunteer for lead and cadmium estimation, and a random water sample from the domestic water supply was estimated for lead content. In addition, each volunteer was invited to complete a short questionnaire designed to obtain background information on each subject. The questionnaire, reproduced in appendix 2, in addition to providing information about each subjects' age, sex, ethnic origin, smoking and drinking habits, established if there was a potential for the volunteer to be exposed to lead either in the home or at their place of employment.

Analytical techniques employed are as described in the general methodology chapter.

3.3 Results

3.3.1 Water lead

The distribution of domestic water lead values obtained in the survey are shown in table 3.1 and figure 3.1. 9.4% of the samples are in excess of 0.48μM (100μg/l) and 13.4%

exceed 0.24μM (50μg/l). Differentiation of these values into those obtained from houses built before 1945 and those built later indicates that the domestic water supply from the older buildings contains significantly greater quantities of lead than those from newer buildings (table 3.2 & figure 3.2). Whilst 17.3% of the samples from the older house group were in excess of 0.24μM and 12.7% in excess of 0.48μM, the figures for the newer house group were 7.4% and 3.7% respectively.

Table 3.1 Distribution of water lead values in Glasgow during 1981.

Distribution of lead in domestic water samples in a random selection of houses in Glasgow in 1981.

Water lead concentration (μmol/l)	Percentage of samples (No. = 197)	Cumulative percentage
<0.05	43.7	43.7
0.05	16.8	60.5
0.10	11.2	71.7
0.14	6.6	78.3
0.19	5.1	83.4
0.24	3.0	86.4
0.29 - 0.48	4.0	90.4
0.53 - 0.72	3.5	93.9
0.77 - 0.96	2.5	96.4
>1.01	3.5	100

Table 3.2 Distribution of domestic water lead values with respect to house age.

Distribution of domestic water lead contents in a random selection of houses in Glasgow in 1981. The houses are segregated into 2 groups by way of age of the building.

Water lead (μM)	Pre 1945 % of samples (N=110)	Pre 1945 Cumulative percentage	Post 1945 % of samples (N=81)	Post 1945 Cumulative percentage
<0.05	39.1	39.1	50.6	50.6
0.05	15.6	53.6	21.0	71.6
0.10	10.9	64.6	12.4	84.0
0.14	8.2	72.7	3.7	87.7
0.19	6.4	79.1	3.7	91.4
0.24	3.6	82.7	1.2	92.6
0.29-0.48	4.5	87.3	3.7	96.3
0.53-0.72	2.7	90.0	2.5	98.8
0.77-0.96	3.6	93.6	1.2	100
>1.01	6.4	100	0.0	

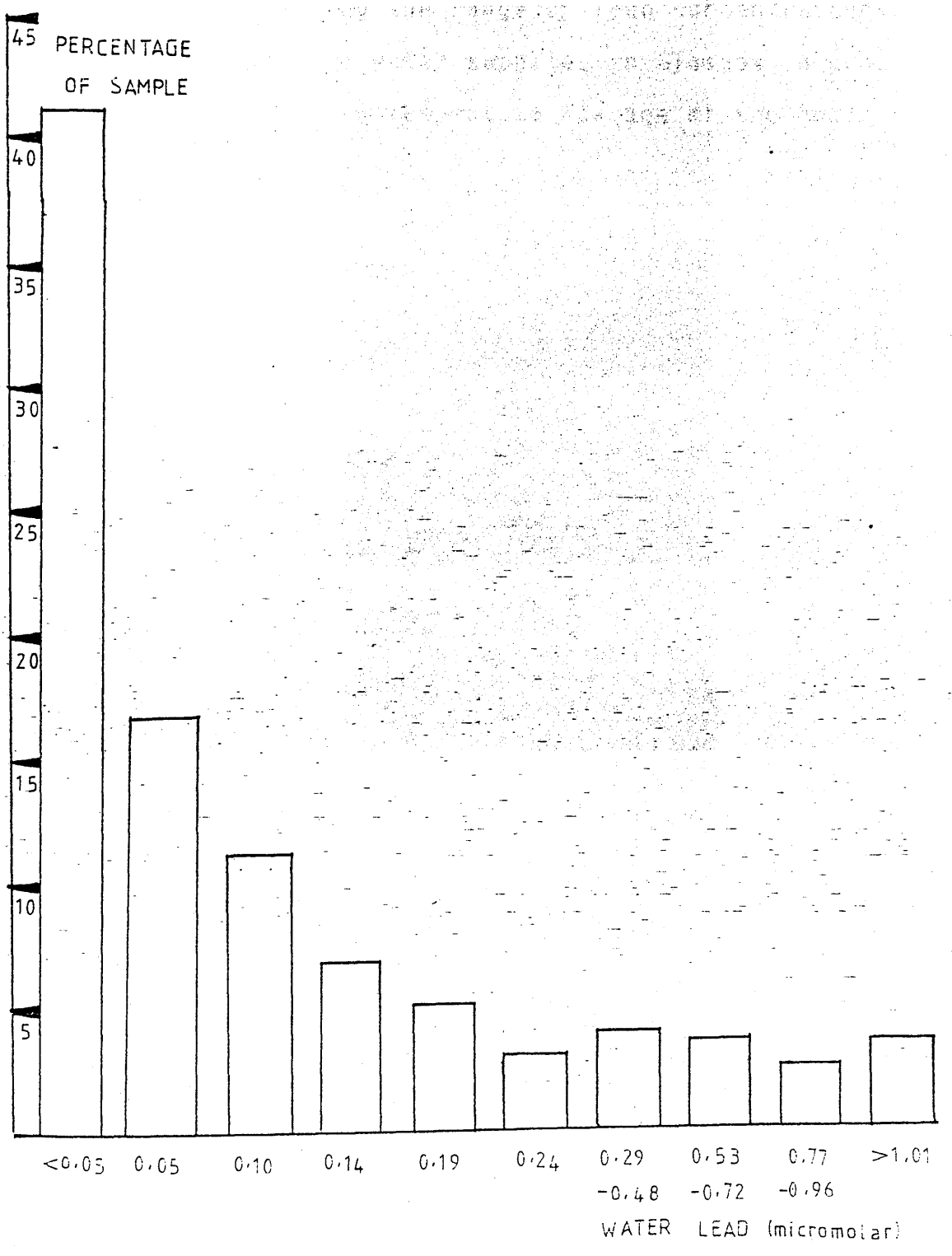
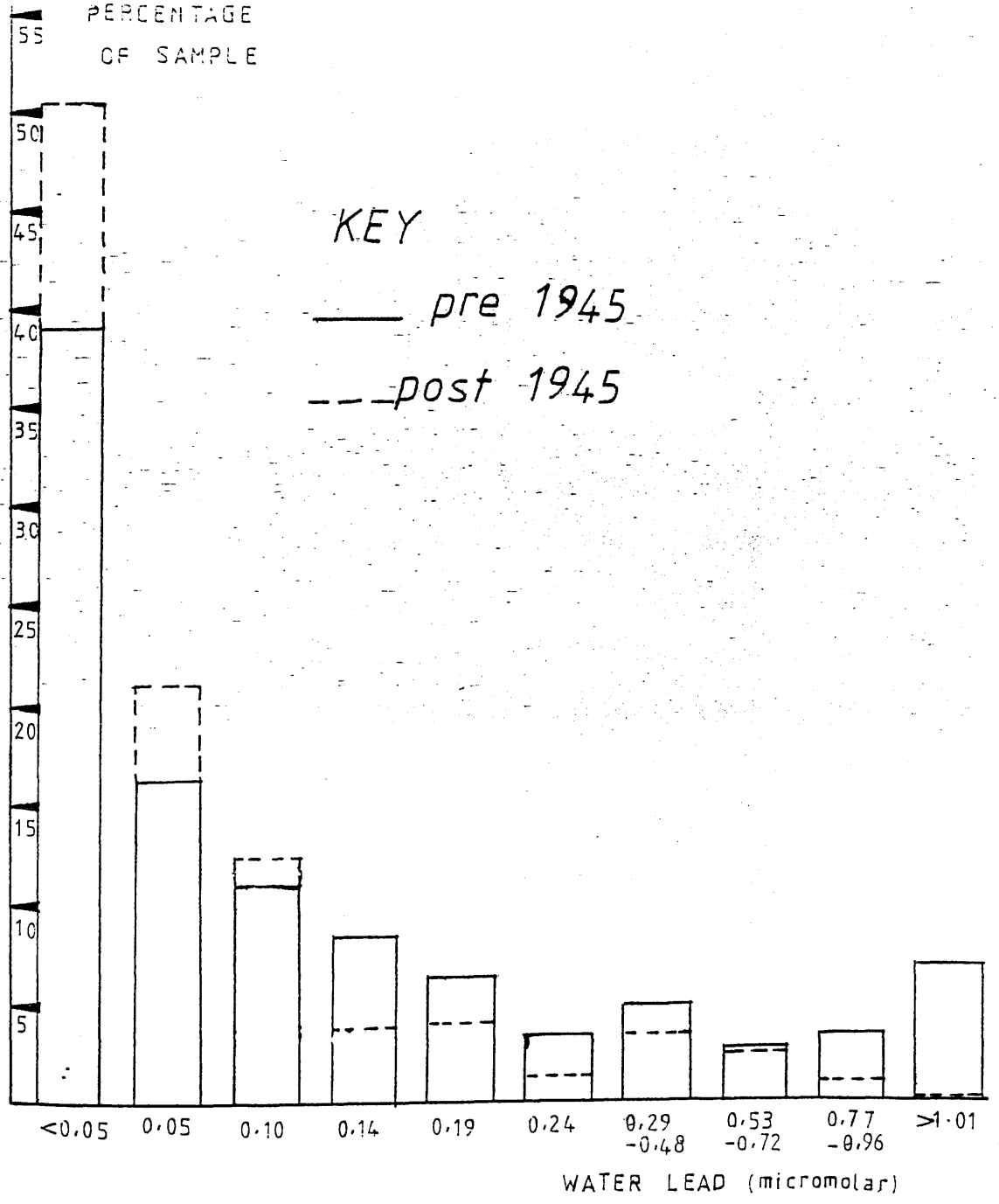


Figure 3.1 Distribution of water lead values -1981

Histogram presenting the range of lead concentrations observed in the domestic water supply of Glasgow houses during 1981.

Figure 3.2 Differential distribution of water lead values; pre- and post- 1945.

Histogram representing the range of lead concentrations observed in domestic water supplies in Glasgow houses in 1981 separated with respect to the age of the house.



3.3.2 Blood lead

The distribution of blood lead concentrations obtained in this survey in Glasgow is shown in figure 3.4. From this histogram it is evident that the distribution is skewed, deviating markedly from normality. Comparison of this data with results obtained in a previous study carried out 2 years prior (DOE,1981) demonstrates a marked alteration in blood lead concentrations of the Glasgow population over this period (figure 3.3). In addition, the histograms reveal that there is a difference in blood lead levels between the sexes, with females exhibiting lower levels than males. This observation is supported by the data in table 3.3 which tabulates the geometric mean blood lead concentrations for both years (the use of geometric means being required by the skewed distribution). Comparing figures 3.3 and 3.4, it is clear that there has been a reduction in blood lead levels in the Glasgow population during the two year interval.

Using a two-way analysis of variance, it was demonstrated that there has been a significant reduction in blood lead levels between the two years ($p<0.001$) and also between the two sexes ($p<0.001$) - table 3.3.

Table 3.4 compares the distribution of volunteers in the two surveys with respect to age. Analysis of the data using a chi-squared test demonstrates that the scatter of adults over the age ranges is not significantly different in 1981 from that in 1979 ($p=0.5$).

The possibility of a relationship existing between blood lead concentration and several social factors and habits

was also investigated. Table 3.5 clearly demonstrates that smoking does not have any influence on blood lead concentration. Similarly, no significant relationships were found between blood lead concentration and either the age of the house or the length of time the volunteer had been resident in the building (table 3.6). As with smoking, the drinking habits of the volunteer did not have any influence on the blood lead concentration (table 3.7). Likewise there was no relationship with the occupation or social class of the individual (table 3.8).

Table 3.3 Blood lead concentrations in Glasgow during 1979 and 1981.

Blood lead concentration in a random selection of the Glasgow population in 1979 and 1981. Males and females are segregated into separate groups.

Year	Sex	No. in group	Geometric mean ± geom. dev. (μmol/l)
1979	M	97	0.96 ± 1.49
	F	99	0.74 ± 1.62
1981	M	82	0.78 ± 1.45
	F	118	0.57 ± 1.61

Analysis of variance was performed on the data after log transformation to form a normal distribution.

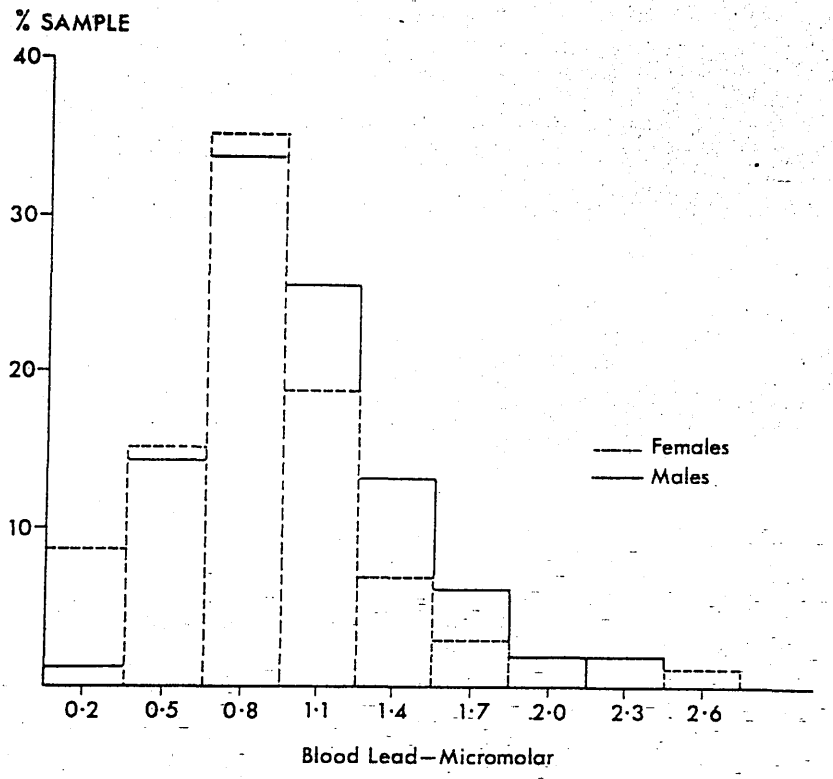


Figure 3.3 Distribution of blood lead concentrations in Glasgow during 1979.

The histogram illustrates the skewed distribution of blood lead values. The solid lines represent the male population while females are shown by a broken line.

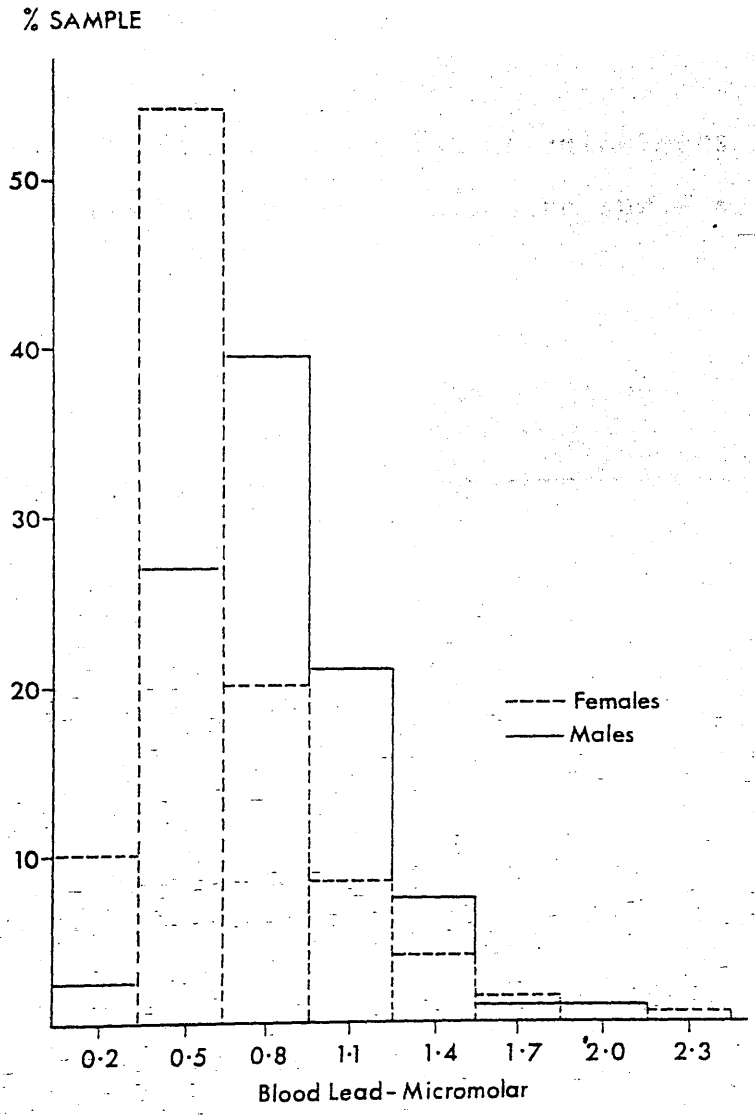


Figure 3.4 Distribution of blood lead concentrations in Glasgow during 1981.

Similarly to 1979, the distribution of blood lead values in 1981 is also skewed. Solid lines represent males and broken lines females.

Table 3.4 Distribution of volunteers with respect to age -a comparison of 1979 with 1981.

Distribution of the random selection of volunteers included in the 1979 and 1981 Glasgow lead exposure surveys.

		No. of subjects in age group				
Year	Sex	15-29	30-44	45-59	60-74	75+
1979	M	28	15	28	23	3
	F	26	25	27	19	2
1981	M	16	24	24	15	3
	F	35	22	27	27	7

Table 3.5 Blood lead concentration with respect to smoking habits.

Blood lead concentration in the random selection of the Glasgow population in 1981, the group being divided according to smoking habits and by sex.

No. cigarettes smoked per day	Sex	No. in group	Geometric mean ± geom. dev.(μmol/l)
Non-smoker	M	35	0.75 ± 1.51
	F	66	0.56 ± 1.65
< 5	M	4	0.85 ± 1.47
	F	9	0.58 ± 1.70
5 -20	M	25	0.75 ± 1.37
	F	30	0.57 ± 1.59
> 20	M	18	0.88 ± 1.43
	F	13	0.66 ± 1.45
Non-smokers	M/F	101	0.62 ± 1.63
smokers	M/F	99	0.68 ± 1.53

Table 3.6 Blood lead concentration with respect to house age and length of residence.

Blood lead concentration in the random selection of the Glasgow population in 1981, the sample being grouped according to the age of their house and by sex.

	Sex	No. in group	Geometric mean ± geom. dev. (μmol/l)
Age of building			
Pre 1945	M	45	0.81 ± 1.49
	F	69	0.61 ± 1.67
Post 1945	M	34	0.73 ± 1.38
	F	49	0.53 ± 1.52
Length of residence (yrs)			
1 - 5	M	33	0.80 ± 1.43
	F	46	0.54 ± 1.55
6 - 10	M	15	0.70 ± 1.33
	F	19	0.55 ± 1.66
11 - 15	M	14	0.71 ± 1.36
	F	17	0.52 ± 1.64
16 - 20	M	6	0.89 ± 1.49
	F	18	0.65 ± 1.68
> 20	M	13	0.80 ± 1.68
	F	19	0.77 ± 1.64

Table 3.7 Blood lead concentration with respect to drinking habits.

Blood lead concentration of the random group of Glasgow volunteers divided according to the type of liquid they drink in significant quantities.

Drinking habit	sex	number in group	geo. mean. ± geom. dev. (μM)
tap water	M	78	0.76±1.46
	F	115	0.58±1.61
milk	M	22	0.75±1.31
	F	34	0.54±1.51
juice	M	6	0.80±1.54
	F	8	0.69±1.64
beer	M	35	0.79±1.37
	F	7	0.58±1.57
wine	M	8	0.72±1.63
	F	13	0.55±1.34
spirits	M	19	0.83±1.43
	F	15	0.55±1.28
bottled water	M	2	*
	F	2	*

* This group comprised only 4 individuals with blood lead values as follows - males: 0.63 & 0.30 μM

females: 0.16 & 1.59 μM

Table 3.8 Blood lead concentration with respect to social class.

Blood lead concentration of the random group of Glasgow volunteers, the group being divided according to social class, with males and females being considered separately.

Social class	Sex	Number in group	Geometric mean ± geom. dev. (μM)
1	M	3	0.75 ± 1.47
	F	0	
2	M	13	0.69 ± 1.51
	F	15	0.54 ± 1.62
3	M	26	0.77 ± 1.49
	F	48	0.63 ± 1.66
4	M	22	0.85 ± 1.49
	F	26	0.59 ± 1.56
5	M	17	0.80 ± 1.31
	F	29	0.50 ± 1.56

3.3.3 Blood cadmium

The markedly skewed distribution of blood cadmium levels found in 1981 (figure 3.5) is similar to that noted in the 1979 survey. In contrast to the data observed for blood lead values, the geometric mean, being taken as representative of the values, is not significantly different between the sexes or over the two year period (table 3.9). A marked increase in blood cadmium is observed in smokers (table 3.10), the increase being related to the degree of smoking.

Table 3.9 Blood cadmium levels in Glasgow during 1979 and 1981.

Blood cadmium levels in the random selection of volunteers in Glasgow in 1979 and 1981. Within each year, males and females are considered separately.

Year	Sex	No. in group	Geometric mean ± geom. dev. (nM)	Range
1979	M	97	12 ± 3	<3 - 54
	F	97	11 ± 3	<3 - 70
1981	M	82	14 ± 2	<3 - 65
	F	118	12 ± 2	<3 - 61

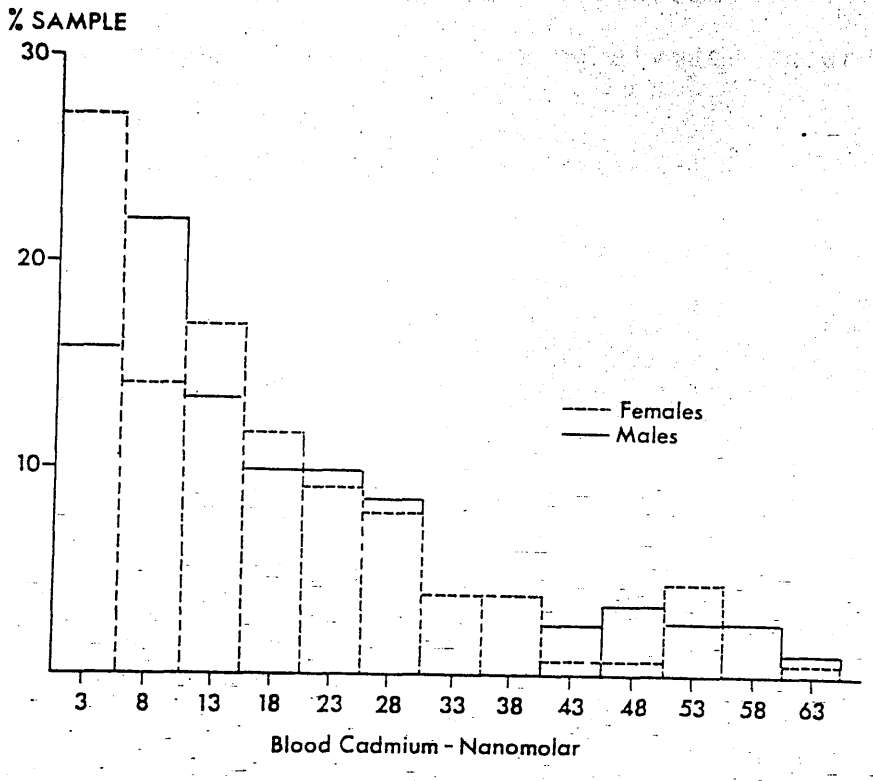


Figure 3.5 Distribution of blood cadmium concentrations in Glasgow during 1981.

The distribution of blood cadmium values observed during 1981 is divided into males and females represented by solid and broken lines respectively.

Table 3.10 Relationship between cigarette smoking and blood cadmium levels.

Blood cadmium levels in the random selection of volunteers in Glasgow in 1981, the group being divided according to smoking habits.

	No. cigarettes smoked per day			
	0	<5	5-20	>20
No. in group	101	13	55	31
Geometric mean ± geom. dev. (nM)	8 ± 2	12 ± 2 *	18 ± 2 **	25 ± 2 **
Range	<3-65	<3-33	<3-52	<3-59

* $p = 0.06437$ compared to group of non-smokers

** $p < 0.0004$ by Mann-Whitney U test.

3.4 Discussion

The present study confirms the views that the acknowledged soft, acidic water supply to the West of Scotland, and Glasgow in particular, can be made less plumbosolvent by specific treatment. Raising the pH has indeed ameliorated the situation, with a reduction in the number of dwelling houses exhibiting water supplies with lead concentrations in excess of the World Health Organisation (WHO) limit of 0.48μM and the European Economic Community (EEC) maximum of 0.24μM. Unfortunately, water lead concentrations were not measured in the previous EEC study in 1979 (DOE, 1981). However, the present study carried out in 1981, at a time when the Glasgow area had been supplied with a water supply of pH 9 for approximately 1 year, demonstrates that 9.5% of the domestic water samples were in excess of 0.48μM and a further 4% exceeded 0.24μM. These figures compare with those obtained before treatment when more than 50% of random daytime samples exceeded 0.48μM (Richards & Moore, 1982). Additionally, they also demonstrated that greater benefit in terms of a reduction in plumbosolvency could be achieved by further increasing the water pH from 7.8 to 9 since 20% of samples contained in excess of 0.48μM lead when the pH as measured at Milngavie was 7.8. The figures observed in the present study are exceedingly close to those predicted by Britton & Richards (1981) who proposed that more than 90% of water samples would contain less lead than the WHO limit if a pH of greater than 8 were to be maintained at the tap.

Considering the curvilinear relationship known to exist between blood lead and water lead (Moore et al, 1977a; 1979a; Moore, 1983) and the reduced lead content of the Glasgow water supply, then a reduction in blood lead levels would be anticipated. Indeed, a downward shift in the distribution of blood lead levels of the Glasgow population between 1979 and the present study was observed (figures 3.3 & 3.4, table 3.3). The age distributions of the 1979 and the 1981 groups were similar, and so there was therefore a real effect on blood lead by chemical modification of the water. No other factors with regard to lead exposure sources could be identified as altering during this time span. This reduction in the exposure of the local environment to lead is supported by data obtained by Moore et al (1981a/b) who measured the blood lead concentration in a group of pregnant women living in the north side of the city in 1980-81. The geometric mean blood lead concentration was 0.39μM with the median being 0.32μM. A similar group of women observed prior to water treatment in 1976/77 exhibited a geometric mean blood lead of 0.7μM and median of 0.8μM. Whereas before water pH adjustment, 6% of these women had a blood lead in excess of 0.7μM (35μg%), none exceeded this value in 1980-81. Although blood lead levels obtained in 1981 were within the EEC reference levels defined as the distribution:

< 2% of samples with blood lead concentrations in excess of 1.69 μmol/l (35 μg%)

our figure, 1981 - 1.5%

< 10% of samples with blood lead concentration in excess of

1.45 μmol/l (30 μg%)
our figure, 1981 - 4%

< 50% of samples with blood lead concentration in excess of 0.97 μmol/l (20 μg%)
our figure, 1981 - 21%

(Commission of European Communities, 1977; DOE, 1981). 4% of the 1981 group still had increased blood lead levels (greater than 1.45μM), indicating the need for continued surveillance of susceptible groups such as pregnant women and young children (Moore, 1980). However, it must be noted that the blood lead concentrations in the 1981 group as a whole showed a marked improvement over the 1979 group, which was not within the EEC reference levels. Similar studies performed once more in Glasgow support the view that there has been a general reduction in the level of blood lead observed in the population of this city over the last few years (Moore et al, 1985).

In contrast to the blood lead results, the values for cadmium did not alter appreciably from 1979 to 1981. The dietary intake of cadmium is around 10-30μg per day and unpolluted drinking water at 1μg cd/l adds only a negligible amount (Friberg et al, 1979). Thus the study confirms the view that water borne lead is important in soft water regions where the housing stock has lead piping and lead storage tanks. Such problems exist especially in houses built several years ago when lead was popularly employed for water conveyancing. The higher water lead content in these houses compared to more recently built buildings is confirmed in this study (figure 3.2 & table

3.2). These problems, which exist in other parts of the West of Scotland, such as Renfrew (Forteath, 1981) and Ayr (Sherlock et al, 1982), can be greatly alleviated by alkalinisation of the water supply. The lead content of the water supply as it exists in Loch Katrine is extremely low as the water authorities have a statutory requirement to supply water without contaminants. The problem emerges as it travels through the conveyancing system, both from piping and tanks which not only allow a large surface area of lead to be in contact with the water but also allow the water to absorb carbon dioxide from the atmosphere and thus become more acidic. However, the problem has been greatly reduced in Glasgow and is in fact still being actively tackled today. Following a pilot study in the south side of the city which demonstrated that water lead concentration could be further reduced by 75% (Richards & Moore, 1982) by buffering with orthophosphate, as was proposed by Christison in 1844, the water supply is now supplemented with 2mg phosphate per litre in an attempt to alleviate the problem further. These techniques represent relatively inexpensive methods of reducing plumbosolvency in a soft, acidic water region, and as such are convenient solutions to the problem if lead plumbing replacement is not feasible.

Several social related factors are known to perhaps influence blood lead concentration, one of which is cigarette smoking. The results of this study demonstrate that smoking does not contribute to the blood lead concentration (table 3.5), which is in agreement with

several other studies (Lehnert et al, 1967; Lehnert, 1968; Daines et al, 1972; Jones, Cummins & Cernik, 1972; McLaughlin & Stopps, 1973; DOE,1981). There are however, several other authors who have reported that smokers exhibit a raised blood lead level compared to non-smokers (Tepper, 1975; Zielhuis et al, 1977; Grandjean, Olsen & Hollnagel, 1981; Shaper et al, 1982). There would therefore appear to be debate as to the influence of cigarette smoking on blood lead burden. This study certainly showed no contribution from cigarettes in this local population. Contrasting with this data, smoking contributed significantly to blood cadmium levels even at modest levels of exposure (table 3.10), an observation consistent with other studies (Zielhuis et al, 1977; Stoeppler & Brandt, 1978; Ward, Fisher & Tellez-Yudilevich, 1978). It would thus appear from this data that the subjects in this survey were certainly smoking to a significant degree since this habit had a substantial effect on their cadmium exposure, and indeed one would expect that the level of smoking was underestimated since it is perhaps considered socially unacceptable to smoke in this country today. Despite this degree of exposure, no significant contribution to blood lead content was derived from cigarettes.

Although alcohol has been implicated to be an important factor with regard to lead absorption, no relationship was observed in this study between alcohol intake and blood lead concentration. Similarly, several studies have emphasised the importance of social class with regard to lead exposure and psychological effects of the metal, but

no relationship was observed in this study. The age of the house has been selected as an important variable in determining the lead content of the domestic water supply (Richards et al, 1980) and hence to lead exposure of the inhabitants as exemplified by blood lead concentration. Although houses built prior to 1945 did indeed have a higher water lead concentration than those built more recently (table 3.2), this difference was not reflected in the blood lead levels of the inhabitants (Table 3.6), possibly because there are more sources of lead exposure to the individual than water.

This study clearly demonstrates that water lead is an important vector of lead exposure in the population of Glasgow, and social determinants appear to have little if any influence. There are obviously however, other source(s) of lead playing a part in contributing to the body lead burden of this population, and consideration of the 1981 data with that obtained in 1979 would indicate that perhaps water lead is now playing a less significant part in the exposure of the inhabitants of the West of Scotland than was previously the case. But even today, at a time when active measures have been taken to reduce this source of lead, it should not be ignored.

CHAPTER 4

EFFECT OF LEAD ON CHOLINERGIC NERVOUS SYSTEM FUNCTION

4.1 Introduction

Acetylcholine (Ach) is the neurotransmitter released both from the nerve terminals of the parasympathetic nervous system and also from the pre-ganglionic neurones of the sympathetic nervous system. Synthesis of the transmitter from the substrates, choline and acetyl-coenzyme A (Ac Co A) is catalysed by the enzyme choline acetyltransferase, sometimes termed choline acetylase (Chat; acetyl-CoA:choline-O-acetyltransferase; E.C. 2.3.1.6) according to equation 4.1, and occurs mainly in the axon terminals.

$$\text{Choline} + \text{Ac Co A} \xrightarrow[\text{Chat}]{} \text{Ach} + \text{Co A}$$

- Equation 4.1

Choline acetyltransferase, which is synthesised in the ribosomes of the nerve cell body and transported to the axon terminals by axoplasmic flow, is almost exclusively localised in the nervous system of both vertebrates and invertebrates, the only exception being that a high concentration of enzyme is found in the placenta where its functional role is uncertain. Within the nervous system, its presence can be regarded as a specific marker for cholinergic neurones. The close correlation between choline acetyltransferase activity and acetylcholine concentration indicates that the enzymatic activity is a good marker of the level of neurotransmitter (Hebb, 1963). There are conflicting views on whether a low level of choline acetyltransferase occurs in presumably non-cholinergic tissues such as dorsal spinal roots, optic nerves and red blood cells (Hebb, 1963). This may indeed be the case since

various pieces of superficial evidence exist to suggest that acetylcholine is involved in the release of noradrenaline at adrenergic nerve endings. Histochemical staining of many adrenergic neurones demonstrates the presence of acetylcholine esterase. Subcellular fractionation of tissues shows a bimodal distribution of choline acetyltransferase; most occurring in the synaptosomal fraction and representing that present in the cholinergic nerve terminals of the tissue, and a smaller fraction being isolated in the soluble fraction derived from cholinergic cell bodies, axons and dendrites. The substrate, acetyl-coenzyme-A, is produced in the mitochondrion during the oxidation of glucose, fatty acids and amino acids. However, it is unable to pass through the mitochondrial membrane and so must undergo conversion to citrate in order to leave the mitochondria (figure 4.1). Choline however is derived from the extracellar fluid, plasma therefore being the ultimate source which obtains its supply mainly from hepatic synthesis but also from dietary sources. Being a cation, choline is not lipid soluble and hence must be taken up into the nerve terminal by specific mechanisms of which there are two -

1. Low affinity transport into cells which is of an uncertain nature, and
2. High affinity transport present in the neural axons and is of a carrier mediated nature. The carrier existing in the membrane shuttles across taking choline with it and the process is of an active nature.

This second process enables choline transport to be

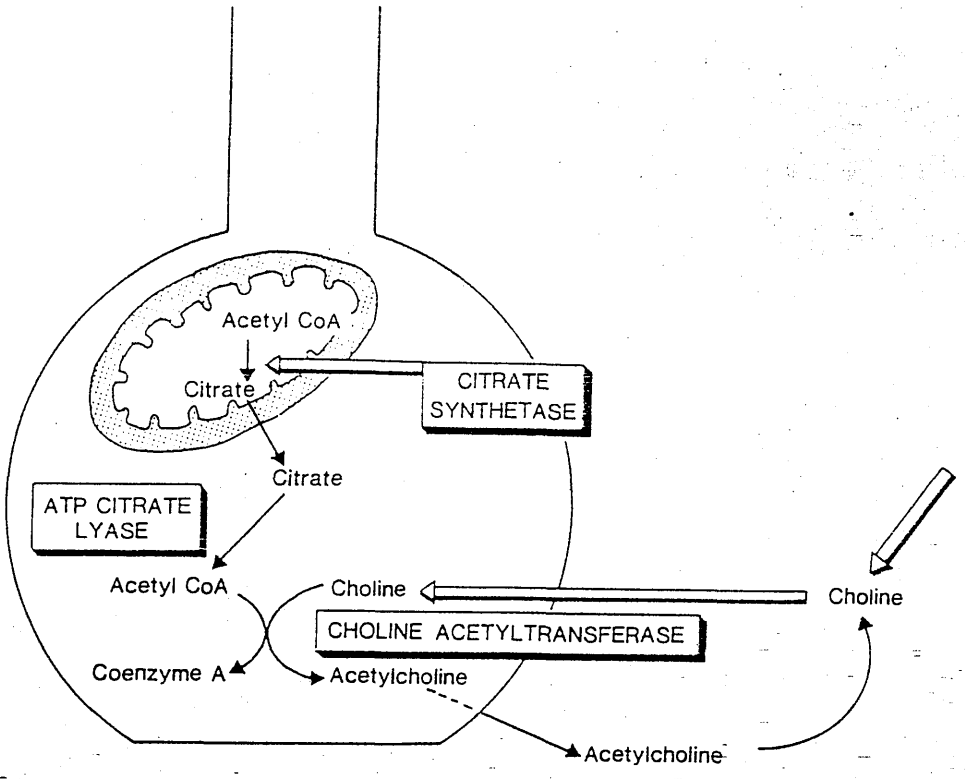


Figure 4.1 Synthesis of acetylcholine.

Citrate, synthesised within the mitochondrion, is transported out of the organelle and utilized to synthesize substrate, acetyl coenzyme A. Choline, derived mainly from the systemic circulation but also from the breakdown of released acetylcholine, is actively taken up into the nerve terminal and forms the second substrate for choline acetyltransferase, the enzyme responsible for the formation of acetylcholine.

maintained at a level sufficient for acetylcholine release even when nerve impulses are occurring at a rapid rate. Much of the choline employed for acetylcholine synthesis is derived from acetylcholine which has been released from the neurone, broken down into its components, and the choline actively reclaimed via this transport process which may well play a regulatory role over adrenergic neurotransmition (Simon, Atweh & Kumar, 1976). The process is however not entirely specific for choline, and in fact some acetylcholine can be transported under conditions where its hydrolysis is prevented, for example by drugs.

A certain degree of understanding of the release mechanism for acetylcholine is necessary to comprehend the proposed action(s) of lead on the cholinergic system.

Certain conditions are necessary for optimal acetylcholine synthesis and release, such as the presence of glucose, oxygen, sodium and calcium. In the absence of a nerve action potential there is continuous spontaneous release of acetylcholine from the terminal in a quantal fashion; that is, each spurt of transmitter release is of approximately equal size and occurs at a frequency of around 1-4 Hz. The post-junctional depolarisation, termed the miniature endplate potential (mEPP) which ensues is however below the threshold to initiate an action potential in the post-junctional cell membrane. Upon arrival of an action potential at the axon terminal the release of quanta of acetylcholine is enormously but transiently accelerated. Calcium ions play an essential role in the coupling of the nerve impulse to the release mechanism (excitation-release

coupling). Within limits the quantity of acetylcholine released is proportional to the intracellular calcium concentration. The arrival of the action potential at the terminal results in the entry of calcium into the terminal through voltage sensitive channels resulting in a transient rise in intracellular calcium and subsequent release of acetylcholine (Llines & Heuser, 1977). Spontaneous release is also dependant on intracellular calcium, such that an inhibition of the calcium buffering system within the nerve terminal raises the calcium ion concentration and an increase in the spontaneous transmitter release follows (Rahamimoff et al, 1980). There are a variety of buffering systems affecting the level of intracellular calcium. -

1. Influx of calcium through voltage sensitive channels (Llinas, Steinberg & Walton, 1981).
2. Efflux of calcium by active transport (Baker & Crawford, 1975).
3. Sequestering of calcium by intracellular organelles
   - a. Mitochondrion (Alnaes & Rahamimoff, 1975)
   - b. Synaptic vesicles (Rahamimoff & Abramovitz, 1978)
   - c. Smooth endoplasmic reticulum (McGraw, Somlyo & Blaustein, 1980)

   and d. Possibly intracellullar proteins such as calmodulin.

Of the above organelles, the mitochondrion is very important. Agents capable of blocking energy-requiring processes of the mitochondria or inhibiting directly calcium binding to the mitochondria, result in an increase

in miniature endplate frequency. An understanding of the involvement of calcium in the excitation-release coupling mechanism is necessary in order to postulate the mode of action of lead in altering cholinergic function. Following release of the acetylcholine from the nerve terminal, which occurs as a result of the synaptic vesicles containing the neurotransmitter fusing with the axonal membrane and releasing their contents, the transmitter must then be inactivated so that future nerve action potentials can produce a post-junctional effect. Degradation of the acetylcholine is affected by the enzyme, acetylcholine esterase (acetylcholine acylhydrolase; AchE - E.C. 3.1.1.7) which reforms choline which is then available for uptake back into the nerve terminal along with acetate as in equation 4.2.

$$\text{Acetylcholine} + HOH \xrightleftharpoons{\text{AchE}} \text{acetate} + \text{choline}$$

Equation 4.2

Cholinesterases can be broadly classified into true or specific and pseudo or non-specific, and it is the true cholinesterases which are responsible for destruction of the transmitter. Pseudocholinesterases however, occur in plasma, intestine, skin and many other tissues, and it is proposed they play a role in the control of acetylcholine release, acting as a local hormone in the intestine. In mammals, butyrylcholine esterase is the main cholinesterase (acetylcholine acylhydrolase; BuChE - E.C. 3.1.1.8), although a propionyl cholinesterase has also been found in

rat brain.

Acetylcholine esterase is present in many animal tissues together with other cholinesterases and simple esterases. In general, the distribution of acetylcholine esterase parallels that of acetylcholine in nervous tissue with some striking exceptions such as cerebellum, substantia nigra and globus pallidus which contain an abnormally high activity of enzyme. The enzyme therefore has a wider distibution than acetylcholine and cannot be used as a true marker for cholinergic neurones. In addition, outside the nervous system, a high level is found in erythrocytes, placenta and muscle membranes especially myotendanous junctions.

Various aspects of cholinergic function have been investigated with regard to the effects of lead, and the data is complex and varied (Shih & Hanin, 1978a; Hrdina, Hanin & Dubas, 1980; Silbergeld & Hruska, 1980; Skorka & Bien, 1983; Winder & Kitchen, 1984).

The effects of lead on cholinergic neurotransmitters have been investigated because of the long history of clinical data describing peripheral neuropathies of lead poisoning. The peripheral neuromuscular pathology of lead, clinically described as lead palsy, may result at least in part from the action of lead on prejunctional sites of the peripheral cholinergic nervous system. Increased exposure to lead has been shown to result in a reduction in mean nerve conduction velocity (Feldman et al, 1973) and an impairment in fine motor co-ordination (De la Burde & Choate, 1972) in children. It must be remembered however,

that these peripheral pathologies may well not ensue as a result of a specific effect of lead on cholinergic function but as a consequence of the segmental demyelination and axonal degeneration of peripheral nerves known to occur in lead exposure (Fullerton, 1966; Lampert & Schochet, 1968). Studies to date have concentrated on the investigation of the action of lead both in vitro and in vivo in the rat and mouse. A variety of approaches have been taken to investigate the effect of lead in vivo. -

1. Measurement of steady state levels of the neurotransmitter itself and its precursor choline in animals exposed to lead by various methods.

2. Measurement of the activity of metabolic enzymes involved in acetylcholine synthesis and degradation

   a. Choline acetyltransferase -responsible for the in vivo synthesis.

   b. Acetylcholine esterase -responsible for the degradation and inactivation of released acetylcholine back into choline

   c. Butyrylcholine esterase/pseudocholine esterase -found endogenously but as yet has an undefined functional role

   d. Choline phosphokinase -catalyses the in vivo conversion of choline into phosphoryl choline.

4. Measurement of the turnover rate of acetylcholine.

5. Studies on the spontaneous and induced acetylcholine release and the extent of a subsequent modification of transmitter release as a result of a physiological response caused for example by drugs.

And,

6. Analysis of the dynamics of availability of choline for acetylcholine synthesis as measured by high- and low-affinity transport of choline.

The observations recorded on cholinergic function by various groups as a result of lead exposure, are not consistent. Such variability is probably due to differences in approaches used in the various laboratories to expose the animals to lead, as well as methodological techniques used to kill the animals, and to analyse the tissue for acetylcholine and choline content. In particular, the method of sacrifice is extremely significant because there are rapid post mortem changes in the levels of both substances. Ideally one should be able to inactivate instantaneously in situ all post mortem degradative changes in order to obtain a reflection of the levels of choline and acetylcholine in the brain at the time of death.

The effects of lead on the cholinergic nervous system have been studied both peripherally and centrally. The data relating to the peripheral effects are less equivocal. Although early work in frogs attempted to locate the site of action to a lesion within the muscle itself, showing changes in inorganic phosphorus and creatine phosphate (Reznikoff & Aub, 1927; Steiman, 1939), it soon became evident that the lesion was of a synaptic nature (Kostial & Vouk, 1957). These workers demonstrated that lead in vitro produced a pre-synaptic block of acetylcholine release in response to nerve stimulation in the superior cervical ganglion of the cat, an observation later confirmed at the neuromuscular junction of the frog sartorius-sciatic nerve

preparation (Manalis & Cooper, 1973; 1975) and in the rat hemidiaphragm-phrenic nerve preparation (Silbergeld, Fales & Goldberg, 1974 a/b). On the other hand lead has no effect on muscle contraction in response to direct stimulation or exogenous application of acetylcholine or acetyl B-methylcholine (Silbergeld, Fales & Goldberg, 1974 a/b). In contrast to the effect of lead on the response to the evoked action potential, in vitro exposure to the metal results in an increase in spontaneous acetylcholine release as evidenced by an increase in frequency of miniature endplate potentials (Manalis & Cooper, 1975). It would thus appear that the blockage by lead is of a synaptic nature and very little action is of a curariform nature, that is it does not interact significantly with motor endplate receptors (Manalis & Cooper, 1975; Manalis, Cooper & Pomeroy, 1984), a hypothesis which was earlier proposed (Savay & Csillik, 1959). There is strong evidence to suggest that the action of lead on the peripheral cholinergic nervous system may in part be modulated through calcium. Similarly to the presence of lead, reducing the calcium concentration in the bathing medium around an isolated muscle preparation leads to a fall in acetylcholine released in response to nervous stimulation (Silbergeld et al, 1974b; Kober & Cooper, 1976). In addition, increasing the calcium concentration can partly overcome the block caused by lead (Silbergeld et al, 1974b; Kober & Cooper, 1976). Using radiolabelled ^{45}Ca, Kober & Cooper (1976) have demonstrated a lead induced reduction in calcium uptake by preganglionic nerve terminals in the

frog; in other words lead is preventing acetylcholine release in response to nerve stimulation by inhibiting calcium entry. Manalis et al (1984) have put forward a hypothesis based on calcium interactions to explain the reason why the blockage of evoked release caused by lead preceeds the increase in spontaneous release. Lead, acting on external calcium receptors on the nerve terminal causes an inhibition of evoked release, while there is a delay as the metal enters the axon to disrupt one or more of the calcium buffering systems described earlier and thereby raise the intracellular calcium concentration and hence miniature endplate potential frequency. Calcium and lead can bind the same protein and probably interact with carboxyl groups (Ong & Lee, 1980). Indirect evidence suggests lead can enter the nerve terminal via calcium channels. Since the rise in miniature endplate potential frequency is prevented by the presence of cadmium (Manalis & Cooper, 1982), which can specifically block inward calcium currents (Kostuyk & Kishtal, 1977; Llinas et al, 1981). Lead has been demonstrated to be associated with the mitochodrion (Silbergeld, Adler & Costa, 1977) and to reduce calcium uptake by these organelles (Goldstein, 1977). If this hypothesis were true, lead would not be alone amongst the heavy metals in reducing evoked acetylcholine release via a competitive action with calcium (Kober & Cooper, 1976; Picket & Bornstein, 1978; Manalis et al, 1984), as cobalt (Balnave & Gage, 1973), manganese (Nilson & Volle, 1976) and cadmium (Weakly, 1973) have a similar action. This hypothesis is not however supported

universally. Silbergeld & Adler (1978) for example, proposed that lead does not enter the peripheral synapse and its actions are mediated by competition with calcium at the receptor site situated externally on the neuronal axon.

Less well clearly defined are the neurotoxic effects of lead on the central cholinergic system. In vivo studies involving exposure of rodents to lead at an early age either via their drinking water or via exposure of the nursing dams have shown in general no alteration or an increase in acetylcholine concentration in specific brain regions. The levels of the precursor choline are unaltered except in one study where they were reduced by 30% in rat midbrain (Shih & Hanin, 1977; 1978b). Both choline and acetylcholine were unaltered by lead exposure in mouse forebrain (Silbergeld & Goldberg, 1975; Carroll, Silbergeld & Goldberg, 1977), rat cerebellum, hippocampus, midbrain, pons-medulla, cortex and striatum (Modak, Weintraub & Stavinoha, 1975a/b; Shih & Hanin, 1977; 1978b). The rat diencephalon exhibited a small but significant rise of 20% in acetylcholine content (Modak et al, 1975a/b; Hrdina, Peters & Singhal, 1976). However this study by Modak and his colleagues employed a high dose of lead (10,900 ppm - 1%) and as a result, the animals were significantly stunted in growth with respect to non-exposed control rats. Hrdina et al (1976) also observed a significant increase of 32-48% in rat cortex as a result of lead exposure.

More recently the use of microwave irradiation to sacrifice the animals has led to the observed transmitter levels being closer to the in vivo situation, and a

regional diminution of brain acetylcholine as a result of lead exposure has since been noted; reductions being observed in the cerebellum, medulla, diencephalon, cerebrum, striatum, midbrain and indeed whole brain following 30 days lead exposure (Modak, Purdy & Stavinoha, 1978). However, exposure for a longer period resulted in the levels returning to normal in whole brain (Modak et al, 1978), midbrain, hippocampus, striatum and cortex (Shih & Hanin, 1977; 1978a).

Less contradictory is the data existing concerning the effect of lead on the central cholinergic nervous system involving turnover studies which indicate a downgrading of cholinergic metabolism as a result of lead exposure (Shih & Hanin, 1977; 1978a). These studies in rats have demonstrated a fall in acetylcholine turnover rate in the cortex, hippocampus, midbrain and striatum of 35, 54, 51 and 33% respectively. Similar to the data observed in the periphery, in vivo lead treatment results in a reduction in potassium induced acetylcholine release (16-30%) and also of choline (34-57%) in mouse cortical minces (Carroll et al, 1977). Spontaneous release of acetylcholine was also significantly increased by 40% but spontaneous release of choline was unaltered.

Using synaptosomes, the high affinity transport of choline has been shown to be inhibited by lead. A reduction of 50% has been demonstrated by Silbergeld & Goldberg (1975) using synaptosomes prepared from mouse forebrain tissue; low affinity transport being unaffected. Other groups of workers have however failed to demonstrate any

alteration in choline transport in mouse cortical minces (Carroll et al, 1977; Ramsay, Krigman & Morrell, 1980).

Various groups have measured the activities of enzymes involved in cholinergic metabolism, namely, choline acetyltransferase, acetylcholine esterase, choline phosphokinase and butyrylcholine esterase, and where changes in activity were observed, there were differences when measured in different brain regions. Such an observation would point to a selectivity of action of lead in discrete brain regions of the rat and mouse in favour of an overall uniform effect on the entire brain. Modak and his colleagues (1975a/b) have demonstrated a significant reduction in the activity of acetylcholine esterase (9-12%) in the rat diencephalon, pons-medulla and midbrain while there was an increased activity of choline acetyltransferase (14-18%) in the cerebral cortex, hippocampus, and medulla-pons. The reduction in the diencephalon correlated with an increased concentration of acetylcholine also observed in this study, although this was true of only this one brain region. Choline acetyltransferase activity was not altered in the cerebellum, diencephalon or striatum, while the same was true of acetylcholine esterase in the cerebellum, cortex, hippocampus, pineal and striatum. The drawback of this study however, was that the rats were exposed to a relatively high dose of lead (1% lead acetate) post partum and subsequently post weaning for 60 days, and as a result these animals exhibited a significantly reduced weight gain. Although there was no alteration in the activity of

acetylcholine esterase in the cortex or cerebellum in this study, a study by Sobotka, Brodie & Cook (1975) has demonstrated a reduction in the activity in these brain regions with no significant change in the brainstem as was also observed by Anca (1983). In support, Anca has additionally observed a fall in acetylcholine esterase in the cerebellum, diencephalon and whole rat brain. However, similarly to the data of Modak and co-workers, Hrdina et al (1976) observed no alteration in acetylcholine esterase activity in rat cortex following lead exposure. Analysis of forebrain following lead exposure has demonstrated no alteration in the activities of acetylcholine esterase, choline acetyltransferase or choline phosphokinase in the mouse (Carroll et al, 1977). These findings, observed mainly in rats but also in mice, are in accord with data obtained in man (De Bruin, 1971). In this study, serum acetylcholine esterase activity was significantly reduced in man exposed to high concentrations of lead in the environment and in patients with lead poisoning. Acute exposure of rats to lead (7.5mg/kg lead acetate intra-peritoneally from birth for 10 days) has shown a significantly decreased activity of acetylcholine esterase in the hippocampus (40%) and the medulla-oblongata (17%) - Louis-Ferdinand et al (1978). No changes were however seen in other brain regions -cerebellum, cerebrum, midbrain and striatum.

The activity of butylrylcholine esterase, an enzyme involved in acetylcholine metabolism, although of uncertain physiological significance, has been investigated following

lead exposure. Sobotka et al (1975) have observed a significant reduction in the enzyme in the cortex and brainstem with no change or a slight increase in the cerebellum following exposure of rats via gavage which resulted in a slight state of undernutrition in the animals. Similarly, acute intravenous exposure of rats to lead for 15-20 days after birth resulted in a significant reduction in activity in selected brain regions, namely cerebrum, hippocampus and midbrain.

In addition to these effects on specific aspects of the cholinergic system, lead has been observed to have a general inhibitory effect on energy metabolism (Holtzman, Hsu & Desautel, 1981; Bull et al, 1979), an extremely important factor where the brain is concerned due to its specific requirements for glucose as an energy source. Bull and his colleagues in 1975 induced rat cerebral cortex to perform osmotic work in response to a change in potassium concentration in the medium. The data they obtained strongly suggested an inhibition of the oxidation of reducing equivalents produced in the cytosol by the mitochondria. Thus lead upon binding to the mitochondrion, can cause a reduction in the activity of the Krebs or citric acid cycle and hence a reduction in energy production. This data is supported by studies of Goldstein (1977), Holtzman, Hsu & Mortell (1978) and Gmerek et al (1981). Again using rat cerebral cortex slices from animals exposed to 600 ppm lead in water for 20 days, Sterling et al (1980) have demonstrated a reduced incorporation of ^{14}C and ^{3}H labelled glucose into its metabolic products such as

lactate, citrate and also acetylcholine. A reduction of 13-17% of glucose incorporation into intermediates of the Embden-Meyerhof glycolytic pathway: glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, ∝-glycerophosphate and pyruvate, and also a reduced conversion of ketone bodies such as β-hydoxybutyric acid into citrate and acetylcholine. Such an effect of lead would appear to be a general one on glucose metabolism as against being directed at a specific site. Such observations are of importance since glucose and pyruvate are major precursors to the acetyl moiety of acetylcholine in adult rat brain (Quastel, Tennenbaum & Wheatley, 1936; Browning & Shulman, 1968; Tucek & Cheng, 1974). Although several mechanisms for the transport of acetyl groups across the mitochondrial membrane into the cytosol have been proposed (Jope, 1979; Dolezal & Tucek, 1981; Tucek et al, 1981), the mechanism in the rat is probably that which occurs in man, involving the conversion of acetyl-CoA into citrate intramitochondrially, and its subsequent reconversion into acetyl CoA within the cytosol by ATP-citrate lyase (Sterling & O'Neill, 1978; Sterling, McCafferty & O'Neill, 1981) -figure 4.1.

Data supporting a reduction in availability of acetyl moieties for acetylcholine synthesis has been obtained in rats exposed to lead via their dams which were supplied with 1% lead acetate in their water for 45 days post partum. The offspring exposed to lead exhibited an increased activity of hexokinase, the enzyme responsible for the conversion of glucose-6-phosphate into glucose-1,6-diphosphate, part of the glycolytic path, and an increased

pyruvate concentration in the brain of non-exposed animals. These observations are suggestive of a marked glucose consumption and incomplete glucose degradation via the krebs cycle - figure 4.2.

Acetylcholine synthesis can be reduced as a result of -

1. A reduction in choline acetyltransferase activity
2. Reduced concentration of one or other of the precursors, choline or acetyl CoA

or

3. Indirectly by a decrease in acetylcholine release.

Debate exists as to the mechanism(s) controlling acetylcholine synthesis and release and which factor is rate limiting. The action of lead on central and peripheral acetylcholine levels may well result by competitive interaction of lead ions with calcium at presynaptic cholinergic nerve terminals (Silbergeld & Goldberg, 1975; Carroll et al, 1977; Silbergeld, 1977; Silbergeld & Adler, 1975). Lead and calcium ions are similar in being divalent cations although of greatly different mass:charge ratio. However, perhaps equally important, inhibition of glucose metabolism has been shown to decrease the availability of acetyl CoA essential for acetylcholine synthesis (Gibson, Jope & Blass, 1975; Gibson & Blass, 1976), an action also observed as a result of lead exposure as discussed above. The effect of lead on cholinergic metabolism will be investigated in this chapter by measuring the activity of Chat in the brains of rats exposed to lead via their drinking water.

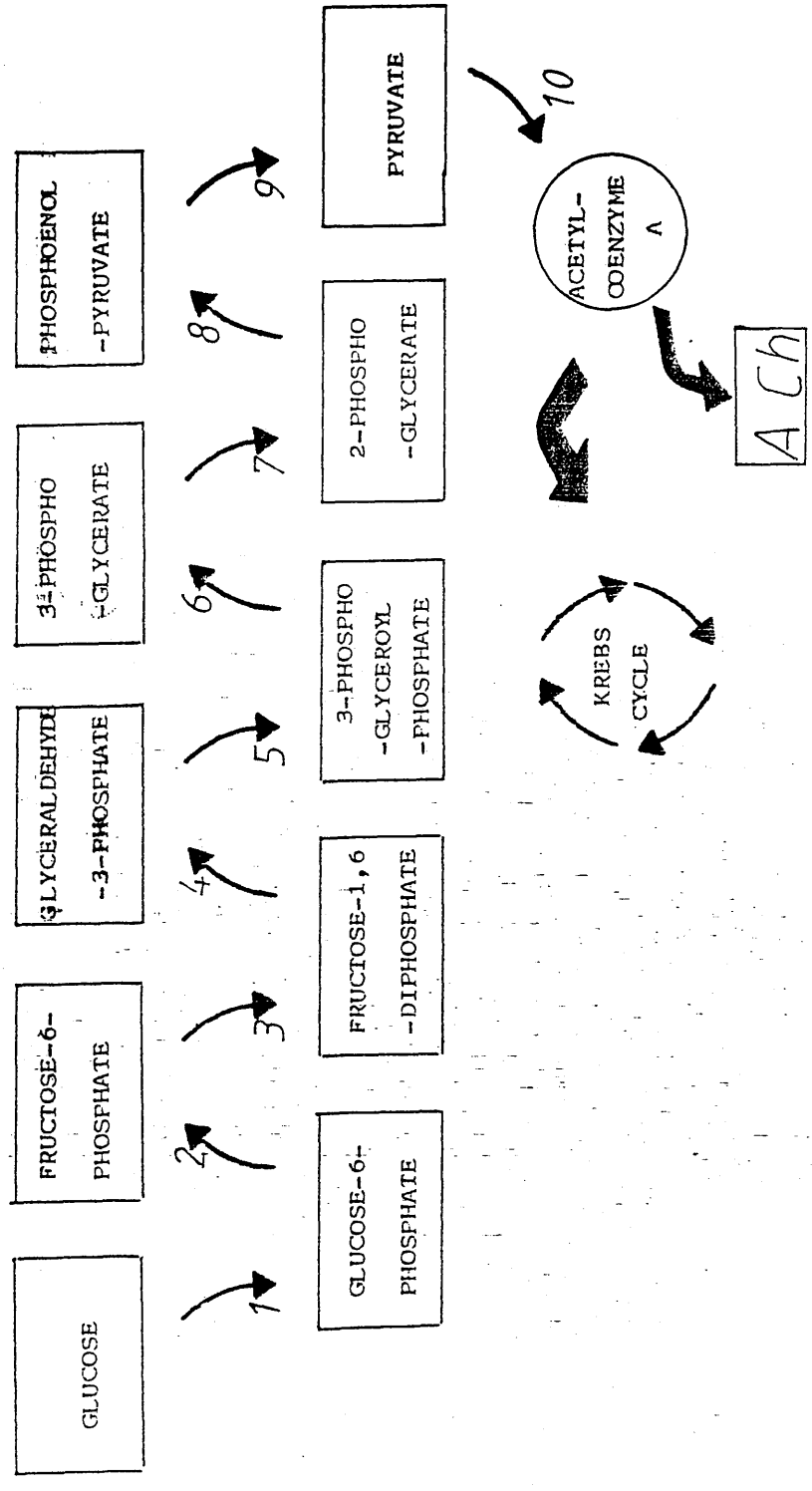


Figure 4.2 Metabolic pathway of glycolysis and the Krebs cycle.

The figure illustrates the relationship of the glycolytic and Krebs cycles with the availability of acetyl-coenzyme A the precursor of acetylcholine.

4.2 Methods

Male Sprague-Dawley rats were exposed to lead (as the acetate) at a level of 24μM (5mg lead/l) in their drinking water from weaning with a control group receiving distilled water. The animals were allowed access to food and water ad. libitum. Following a period of exposure of either 3 or 7 months, the rats were sacrificed by decapitation. The brains were removed, dissection as described in the methods section carried out, and choline acetyltransferase activity subsequently measured in each region. A sample of heparinised blood was obtained for lead analysis, as was the femur. The analytical techniques to measure choline acetyltransferase activity, tissue protein concentration, blood and bone lead concentrations are as detailed in the general methodology section.

4.3 Results

Lead exposure in these two groups of animals was assessed by means of blood and bone lead content (table 4.1). While blood lead concentration was significantly raised in the exposed group compared to the non-exposed controls ($p<0.002$ - by Mann-Whitney U test) following an exposure period of 3 months, there was no significant difference between the two groups when the length of exposure was extended to 7 months. However, the animals were still exposed to, and accumulating lead as demonstrated by the significantly elevated level of lead in the femur of exposed compared to non-exposed animals after both periods of exposure. The degree of lead accumulation was not directly related to length of exposure. A large variation in blood lead levels was observed between the 3 and 7 month control groups which may be related to a variation in lead from rat chow known to occur during the year.

Tables 4.2 and 4.3 present the activity of choline acetyltransferase in the four discrete brain regions analysed in the non-exposed and exposed rats following exposure periods of 3 and 7 months. Of the regions studied, cerebellum, midbrain, diencephalon and telencephalon, alterations in the activity of this enzyme were noted in only two, namely, midbrain and diencephalon. Following exposure to 24μM lead for 3 months there was a significant reduction in choline acetyltransferase activity in the midbrain ($p<0.02$ by Mann-Whitney U test) as a result of lead exposure. This alteration only reached significance however, when the results were expressed per unit of tissue, and although the trends remained when expressed per unit of protein, the alterations failed to reach statistical significance at the 5% level. In the diencephalon, lead exposure was associated with a rise in choline acetyltransferase activity both when expressed per unit of tissue and protein, although the rise was only significant when the data were expressed with respect to protein content of the tissue ($p<0.05$), perhaps due to the large distribution in enzymatic activity present within each group. Extending the period of exposure to 7 months did not result in any significant alterations in choline acetyltransferase activity, although the patterns of change observed at 3 months were again evident albeit non-significant.

In an attempt to explain this feature that the data demonstrated a strong dependence on the method of presentation of results, the protein concentration of each

brain region was calculated and a comparison between lead exposed and non-exposed groups performed (table 4.4). Although no statistically significant effect of lead on brain protein concentration was observed, lead exposure was in general associated with a reduction in protein content of the tissue.

Table 4.1 Blood and bone lead concentrations

Blood lead and bone lead concentrations in rats exposed to 24μM lead or distilled water for a period of 3 or 7 months. 10 rats are included in each group in the 3 month group while each 7 month group contains 6 rats.

Treatment group	Blood lead ($\bar{x}$±S.D.) - μmoles/l		Bone lead ($\bar{x}$±S.D.) -μmoles/kg dry wt.	
	Control	24μM Pb	Control	24μM Pb
3 month	0.57±0.06	0.74±0.08*	7.0±2.8	26.8±5.0*
7 month	0.28±0.03	0.3±0.05	4.2±0.5	23.8±3.0*

* $p < 0.002$ compared to control non-exposed group by Mann-Whitney U test.

Table 4.2 Choline acetyltransferase activity - 3 months exposure

Choline acetyltransferase activity in rat brain following exposure to 24μM lead acetate for 3 months since weaning. Results expressed as the mean ± S.D. of 10 observations.

Brain Region	Choline acetyltransferase activity - μmoles/g tissue/hr.		- μmoles/g protein/hr.	
	control	lead	control	lead
Cerebellum	0.67±0.23	0.72±0.26 *	5.05±1.17	6.28±2.33
Midbrain	6.06±1.04	4.78±1.00	50.3±6.9	46.9±8.2
Diencephalon	6.33±1.77	8.08±2.20	77.9±47.5	127.9±56.6 **
Telencephalon	6.59±1.67	5.72±1.49	67.3±12.8	59.1±15.1

* $p < 0.02$
** $p < 0.05$

compared to control non-exposed group by Mann-Whitney U test.

Table 4.3 Choline acetyltransferase activity - 7 months exposure

Choline acetyltransferase activity in rat brain following exposure to 24μM lead acetate for 7 months since weaning. Results expressed as the mean ± S.D. of 6 observations.

Brain region	Choline acetyltransferase activity -μmoles/g tissue/hr.		-μmoles/g protein/hr.	
	control	lead	control	lead
Cerebellum	0.53±0.10	0.54±0.11	4.94±0.64	6.97±2.29
Midbrain	6.22±2.51	5.44±2.03	59.8±20.5	49.8±20.2
Diencephalon	5.42±1.82	7.56±1.90	51.0±20.8	69.2±21.5
Telencephalon	5.58±1.35	6.17±1.29	49.1±13.6	49.6±12.3

Table 4.4 Tissue protein concentration

Protein concentration of brain tissue from rats exposed to 24μM lead acetate from weaning.

Brain region	3 month exposure ($\bar{x}$±S.D. 10 observations - mg/ml)		7 month exposure ($\bar{x}$±S.D. 6 observations - mg/ml)	
	control	lead	control	lead
Cerebellum	6.53±1.03	5.91±0.68	5.40±1.56	4.08±0.95
Midbrain	5.86±1.13	5.05±0.97	5.48±0.94	5.42±0.72
Diencephalon	4.56±1.06	3.72±1.21	5.35±0.86	5.48±0.64
Telencephalon	5.28±1.11	4.96±0.75	5.84±0.27	6.58±1.03

4.4 Discussion

The first neurotransmitter pathway to be examined with regard to identifying the neurochemical disturbance in lead toxicity was the cholinergic system. This was a logical direction in which to proceed since parallelism was assumed to occur between the actions of lead on the peripheral and central cholinergic synapses and such actions of lead aimed at peripheral cholinergic neurons have indeed been demonstrated as discussed in the introduction to this chapter. However, it soon became evident that the central cholinergic effects of lead were not as simple and clear cut as the observed peripheral actions.

It is unfortunate that the concentration of acetylcholine and the precursor choline were not able to be quantitated in this study due to the unavailability of expensive microwave irradiation equipment necessary to sacrifice the animals. Rapid post mortem changes occur within the cholinergic system and therefore the animals must be killed extremely quickly in order to obtain transmitter levels close to the in vivo situation. The alternative choice was therefore to measure the activity of the enzyme responsible for transmitter synthesis.

The regulation of acetylcholine synthesis is complex and not entirely understood, but it is thought that the availability of the precursors, choline and acetyl coenzyme-A, is an important factor. Since acetylcholine is derived from the metabolism of glucose and other carbohydrates, any interferences with metabolism may result in a reduction in acetyl coenzyme A and ultimately

acetylcholine. The activity of choline acetyltransferase present in nerve endings appears to be in excess of that required to support the observed rates of transmitter synthesis (Jope, 1979). The reaction is reversible and since it is normally close to equilibrium, the net rate of the reaction will depend on the relative concentrations of substrate and product. If indeed the activity of choline acetyltransferase is not of primary importance in determining the concentration of the neurotransmitter acetylcholine, then the measurement of the enzymatic activity may not be a useful tool in the elucidation of a possible cholinergic effect of lead.

The data presented in the results section of this chapter however suggests an effect of lead on cholinergic transmission which results in an alteration in choline acetyltransferase activity. There is no evidence from this study to point to the site of such an action. Whether lead is having a specific inhibitory effect on the enzyme itself in the midbrain or the reduced activity is a secondary factor resulting from an alternative action of the metal remains to be discovered. We can however see that the neurotoxic action is of a selective nature; that is lead does not have a general effect on the brain as a whole. The action would appear to be localised to specific brain regions since in this study although four anatomical brain regions were studied, namely the cerebellum, midbrain, diencephalon and the telencephalon, significant changes in enzymatic activity were confined to the midbrain and the diencephalon. Indeed, the observed direction of change in

enzymatic activity differed in each region with a rise being observed in the diencephalon whilst the activity was depressed in the midbrain. A better insight into the cholinergic effects of lead may have been possible had the concentrations of acetylcholine and choline been measured.

The discrepancies between the data presented in two different ways is difficult to interpret and may partly be explained by the large variation in activities observed in the animals. In the midbrain, there was a significant decrease in activity after three months exposure when the results are expressed per unit of tissue, which is lost when expressed per unit of protein. The two groups, control and lead treated, did not exhibit significantly different brain protein concentrations (table 4.4). Similarly, three month lead exposure resulted in a significant rise in choline acetyltransferase activity in the diencephalon when the data was expressed per unit of protein; there was an increase in activity expressing per unit of protein but it failed to reach statistical significance at the 5% confidence level. Once again there was no significant difference in protein content of the diencephalon between the two groups. One suspects that the rise fails to reach significance because of the large variation in observed activities but this cannot be proven.

Where significant alterations in choline acetyltransferase activity were noted, they were only observed in the group of rats exposed to lead for three months; no significant alterations were observed in the group exposed for a longer period of seven months, although

similar trends of a reduced activity in the midbrain and enhanced activity within the diencephalon were apparent. These alterations were however not statistically significant. The longer exposure study comprised of only 6 animals in each group in comparison with 10 in the 3 month study. Thus one can speculate that the changes may well have reached significance had the groups been larger.

Previous studies quantitating the activity of choline acetyltransferase have in general failed to note significant changes as a result of lead exposure (Carroll et al, 1977), although a rise has been observed in one study (Modak et al, 1975a/b) in the cerebrum, hippocampus and pons-medulla. The increase in activity in our study may well be due to a direct effect of lead on the enzyme but it is more likely to be a compensatory rise resulting from another action of lead. If there were a shortage of precursors for transmitter synthesis, the activity of choline acetyltransferase may increase in an attempt to meet transmitter requirements. The introduction to this chapter discussed the fact that the main source of the acetyl moiety for acetylcholine synthesis is from glucose and pyruvate by way of glycolysis. It is well recognised that lead has profound effects on intermediary metabolism and the mitochondrion in general, although its specific actions are rather hazy (Bull, 1980). Thus it is not unrealistic to consider a link between the actions of lead on energy production and alterations in central neurotransmitters such as glutamate, GABA and indeed acetylcholine. Whether or not such an action of lead on

central cholinergic metabolism is mediated through an effect of calcium as proposed by Silbergeld's group, remains to be seen although it does present an attractive possibility.

It is evident that if lead does indeed have a neurotoxic action associated with the central cholinergic nervous system, then a great deal of work is still required in order to elucidate the mechanism. The evidence presented in this chapter would certainly not refute such an effect of lead on central cholinergic metabolism.

CHAPTER 5

LEAD RELATED DERANGEMENTS OF CATECHOLAMINE METABOLISM

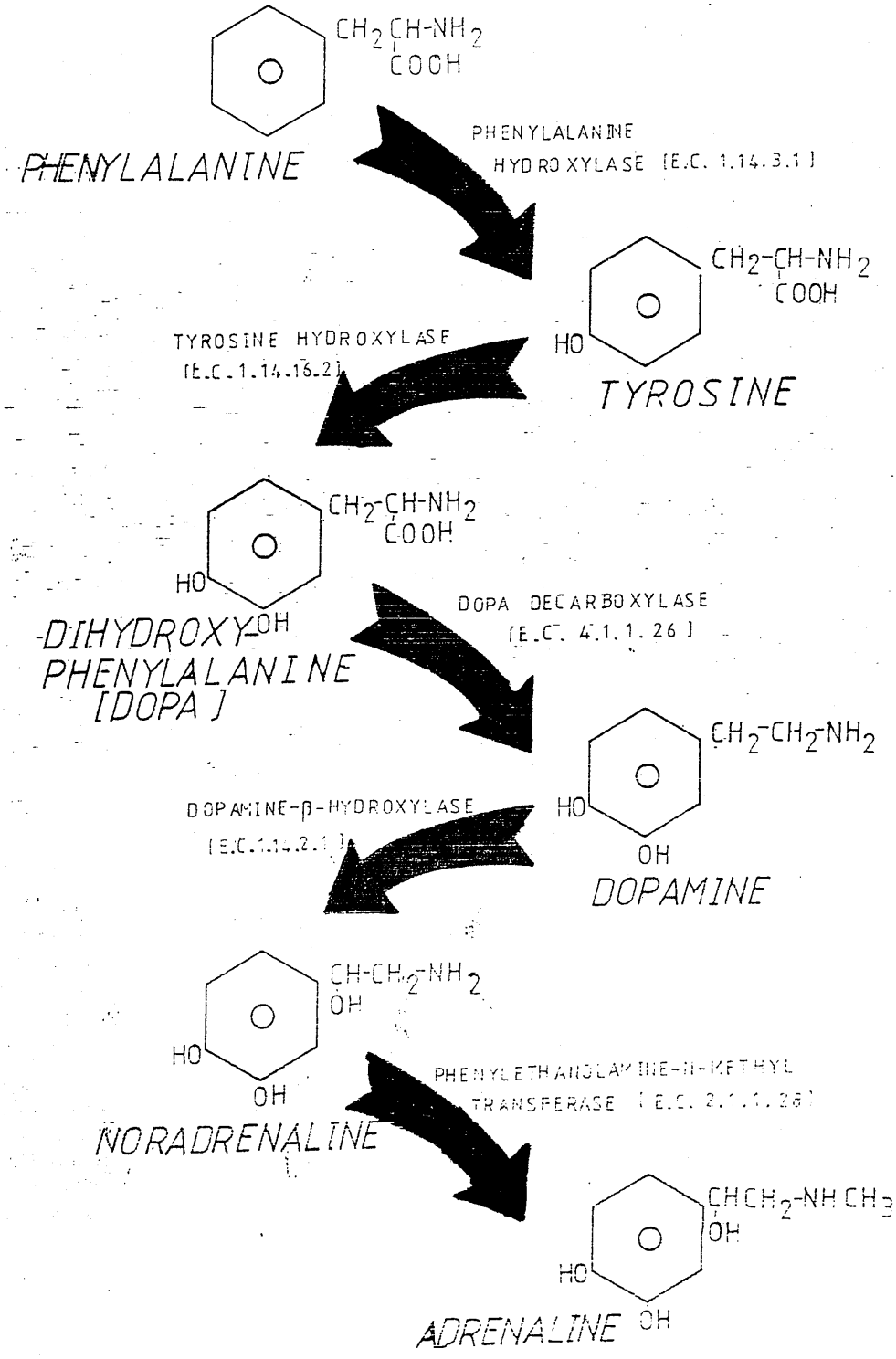
5.1 Introduction

The catecholamines are widely distributed throughout both the peripheral and the central nervous systems, and in addition, a high concentration can be found in the adrenal medulla. Within the nervous system the essential enzymes for biosynthesis are synthesised in the cell body and transported by axoplasmic flow to the terminal axons. The synthetic path, shown in figure 5.1, relies on a supply of dietary amino acids in the form of phenylalanine and tyrosine, two amino acids which, in the western world at least there is an adequate supply of in the diet.

The first step in the pathway involves the conversion of phenylalanine into tyrosine, a reaction catalysed by the enzyme phenylalanine hydroxylase (E.C. 1.14.3.1). The further addition of a 3-hydroxy moiety to tyrosine thus converting a phenol group into the corresponding catechol amino acid and producing dihydroxyphenylalanine (dopa), is catalysed by the rate-controlling enzyme of the path, tyrosine hydroxylase (E.C. 1.14.3a), which is loosely associated with the endoplasmic reticulum. Both tyrosine hydroxylase and the preceeding enzyme, phenylalanine hydroxylase, require the presence of cofactors for maximal activity. In this case both iron and tetrahydrobiopterin are essential. The level of neuronal activity is an important factor in the control of tyrosine hydroxylase and hence of catecholamine synthesis. If the neuronal activity is raised, the synthesis rate of tyrosine hydroxylase is increased, and the converse is true when neuronal activity is low. In this way the rate of synthesis of catecholamines

Figure 5.1 Major route of catecholamine synthesis.

The diagram shows the major route of synthesis which occurs in all catecholamine synthesising tissues. The international enzyme classification code for each enzyme is included in parenthesis.



is adjusted to meet the requirements of transmission. Such alterations in synthesis of tyrosine hydroxylase are inhibited by inhibitors of purine synthesis such as puromycin, cyclohexamide or actinomycin D. The activity of tyrosine hydroxylase is also under direct end-product inhibitory control from noradrenaline, and the K_M of the enzyme is approximately equivalent to the concentration of tyrosine in blood, that is around 0.1mM.

Dopa decarboxylase (E.C. 4.1.1.26) or aromatic amino acid decarboxylase as it is often termed, reflecting the non-specific nature of its substrate specificity, is the next enzyme in the path, and is responsible for the decarboxylation of dopa to form dopamine, the first of the catecholamines. The combination of the slow rate of formation of dopa with the rapid conversion into dopamine, results in very little dopa being found in adrenergic tissues. Dopa decarboxylase is found free in the cytoplasm and not associated with subcellular structures. Similarly, to the other decarboxylases, pyridoxal phosphate is required as cofactor. Within sympathetic adrenergic neurones, dopamine represents around 10% of the total catecholamines present and it is thought to constitute a reserve of precursor for the synthesis of noradrenaline.

Dopamine, formed in the cytoplasm of the axon, is taken up and bound by storage vesicles within the neuron which can be looked upon as the equivalent of storage granules in the chromaffin cells. Uptake serves to protect the amine from degrative enzymes present within the neurone and which will be discussed later.

Noradrenaline is formed from dopamine via the enzyme dopamine-B-hydroxylase (E.C. 1.14.2.1), an enzyme which contains copper as a prosthetic group and during the hydroxylation reaction, the cofactor ascorbic acid is converted to dehydroascorbate. The enzyme is incorporated within the catecholamine storage organelles, a proportion being bound to membranes and the remainder releasable.

Finally, in chromaffin cells of the adrenal medulla (Axelrod, 1962), adrenaline is the catecholamine product of the biosynthetic pathway. Phenylethanolamine N-methyl transferase (S-adenosyl-L-methionine:phenylethanolamine-methyltransferase; PNMT -E.C. 2.1.1.28) is responsible for this conversion from noradrenaline, the methyl group being derived from adenosylmethionine, and it is thought that this enzyme may be involved in the control of adrenergic synthesis. The synthesis of the enzyme is under positive control from adrenocortical hormones. Similarly to dopa decarboxylase, phenylethanolamine N-methyl transferase is not substrate specific for noradrenaline and will also form epinine from dopamine & convert adrenaline into dimethyl-noradrenaline. Since these enzymes of catecholamine synthesis are not specific for substrate requirements, a minor path of catecholamine synthesis exists in adjunct to the major path and is shown in figure 5.2. As a result, a number of related species, tyramine, octopamine, synephrine, epinine and dimethylnoradrenaline are normally present in small amounts in catecholamine synthesising tissues and in urine.

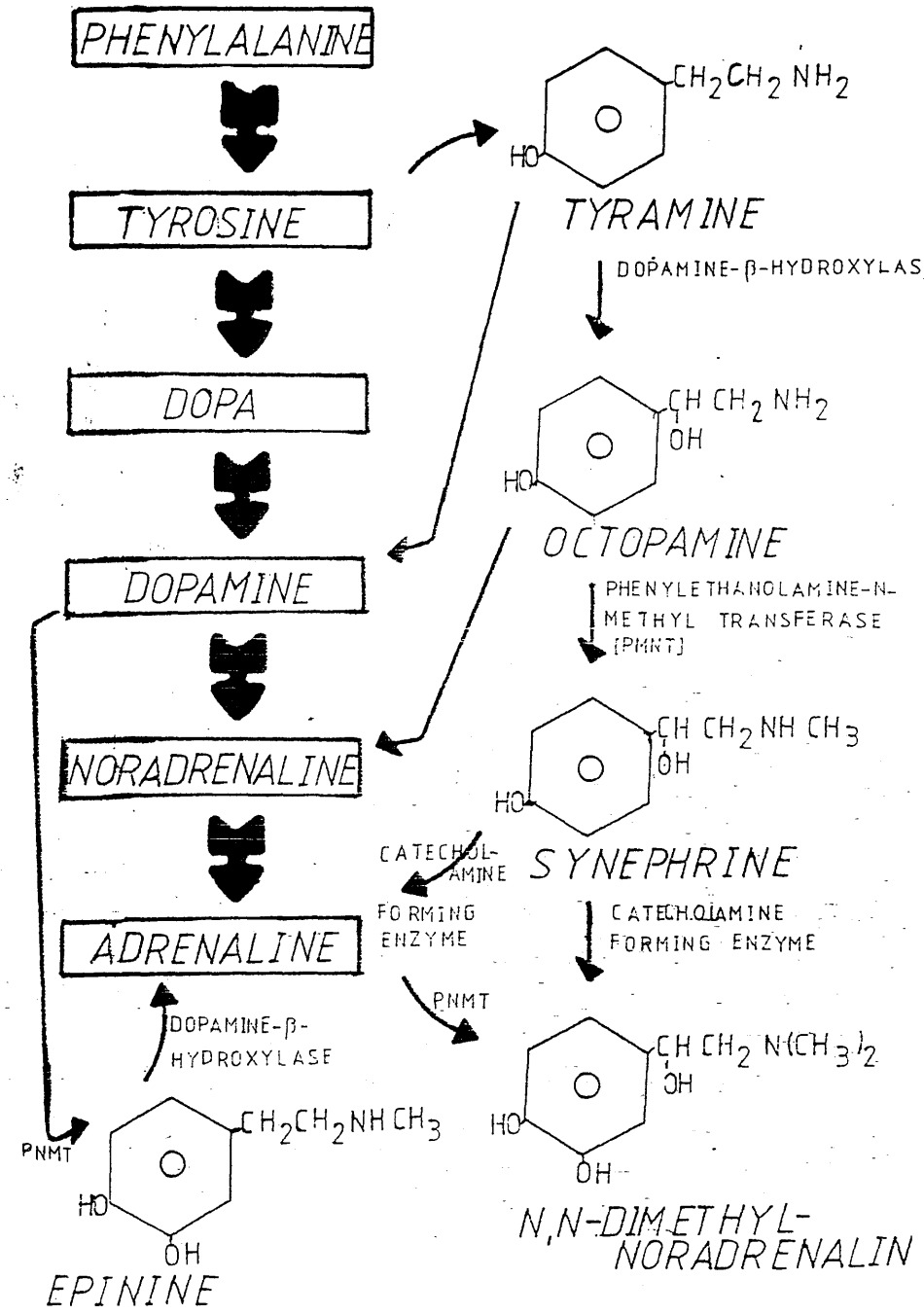


Figure 5.2 Minor metabolic pathways concerned with catecholamine biosynthesis.

The pathways shown down the right hand side of the diagram show the minor route leading to catecholamine formation. The relationship between this route and the major route is also shown by the inclusion of the major pathway down the centre of the illustration.

Catabolism of catecholamines is effected by two enzymes acting in concert, namely catechol-O-methyl transferase (COMT; E.C. 2.1.1.6), and monoamine oxidase (MAO: E.C. 1.4.3.4). In contrast to the inactivation of acetylcholine at cholinergic junctions where the neurotransmitter is inactivated largely by re-uptake, COMT, a divalent cation requiring enzyme, catalyses transfer of a methyl group from adenosylmethionine to the meta (3-) hydroxy group of the catecholamines. Thus dopamine is converted to 3-methoxytyrosine, noradrenaline to normetanephrine, and adrenaline to metanephrine (figure 5.3). The enzyme is widely distributed in tissues being located intracellularly as a cytoplasmic enzyme. Although enzymatic activity is high in liver and kidneys, it is also present in smooth and cardiac muscle, other tissues innervated by adrenergic nerves, in certain adrenergic neurones and in the brain.

MAO catalyses the oxidative deamination of catecholamines to form the corresponding aldehyde derivative with the aid of FAD as a prosthetic group. The resultant aldehyde is rapidly oxidised or reduced to the acid or alcohol via the appropriate aldehyde dehydrogenase or alcohol dehydrogenase. This reduction of aldehyde to alcohol occurs especially in the brain. Thus dopamine will be converted into 3,4-dihydroxyphenylacetic acid (DOPAC) or 3,4-dihydroxyphenylethanol (DOPET) and adrenaline or noradrenaline into 3,4-dihydroxymandelic acid (DOMA) or 3,4-dihydroxyphenylethylglycol (DOPEG; DHPG). These reactions of MAO are shown in figure 5.4. MAO is located within the mitochondrion, probably between the inner and

outer membranes. The substrates for the enzyme, can readily pass through the outer membrane. Enzymatic activity is found in almost all tissues including adrenergic nerves and chromaffin cells and in central neurones utilizing other monoamine transmitters, that is 5-hydroxytryptamine and dopamine. In practice MAO and COMT act in conjunction with each other physiologically. The 3-methoxy compounds formed from catecholamines by COMT are substrates for MAO, and the catechols with alcoholic or acidic side chains formed from catecholamines after MAO has acted are substrates for COMT. The complex series of catabolic paths of catecholamines are summarised in figure 5.5.

Since early reports of the possible involvement of brain catecholamines in the lead-induced hyperactivity commonly observed in animals (Sauerhoff & Michaelson, 1973; Golter & Michaelson, 1975), attempts have been made to correlate the observed hyperactivity with changes in the content of established and some putative neurotransmitters. Catecholamines, especially dopamine, appear to be involved in the central regulation of motor activity (Creese & Iverson, 1975); Jones, Mogenson & Wu, 1981). The nucleus accumbens would appear to be the primary locus of this dopamine-mediated locomotor activity. Evidence supporting these observations comes from studies demonstrating increased locomotor activity following apomorphine, a dopamine agonist (Kelly, Seviour & Iverson, 1975) and following amphetamine administration, which induces catecholamine release (Azzaro & Rutledge, 1973).

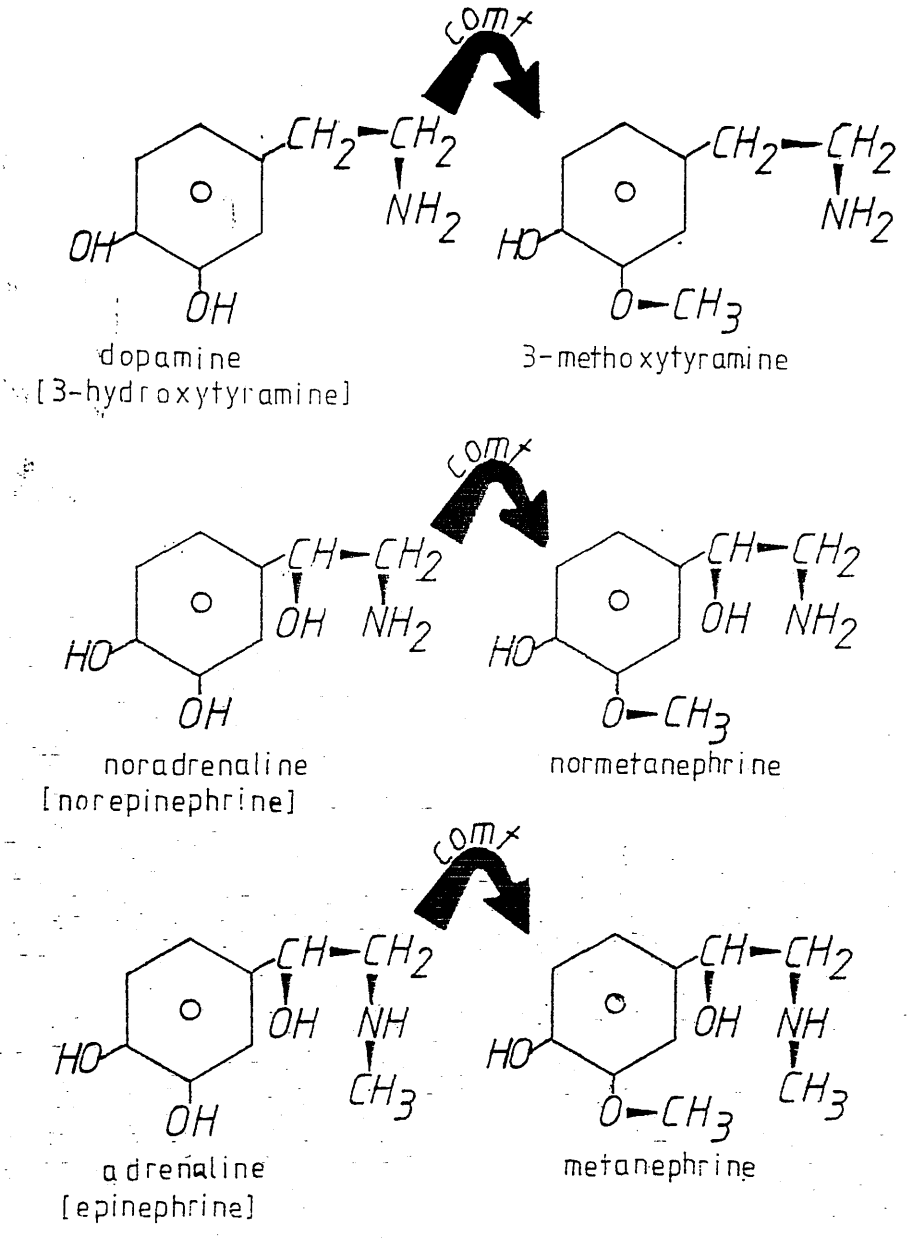


Figure 5.3 Reactions catalysed by COMT.

Products formed by the action of catechol-O-methyltransferase (COMT) on noradrenaline, adrenaline and dopamine. Alternative names for the catecholamines are shown in parenthesis to indicate the derivation of the names of the meta-O-methylated products.

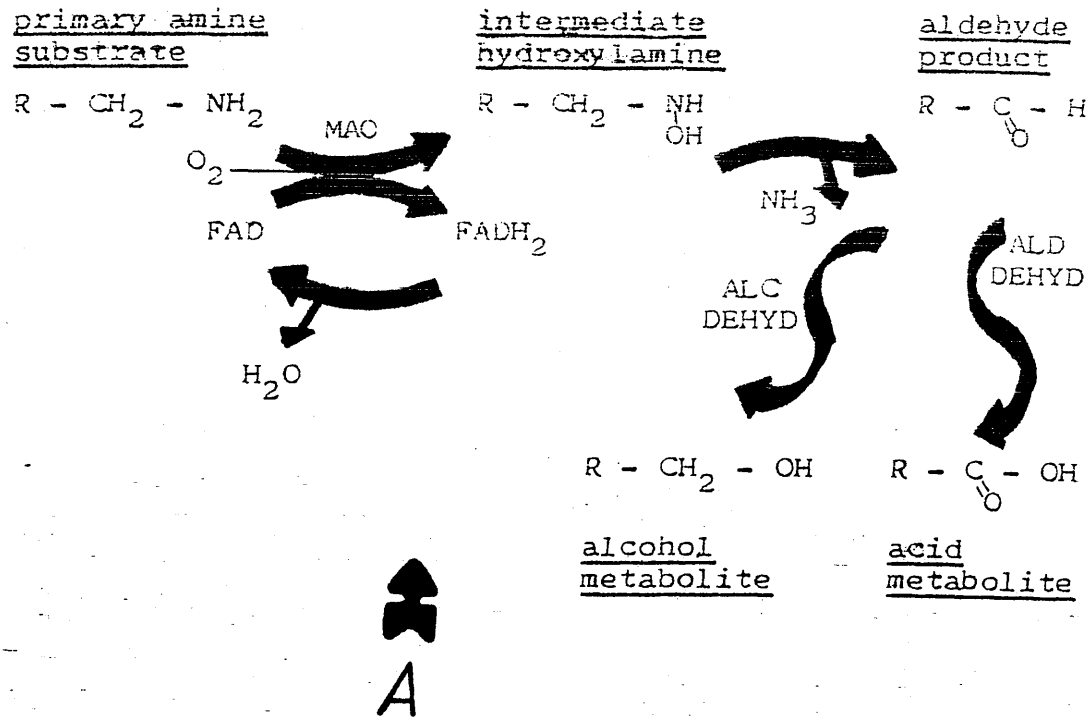


Figure 5.4 Reactions catalysed by MAO.

Products formed by the action of monoamine oxidase (MAO) on noradrenaline, adrenaline and dopamine.

(a) shows the general reaction which this enzyme is responsible for

(b) specific products formed from the catecholamines

DOPAC - 3,4-dihydroxyphenylacetic acid

DOPET - 3,4-dihydroxyphenylethanol

DOMA - 3,4-dihydroxymandelic acid

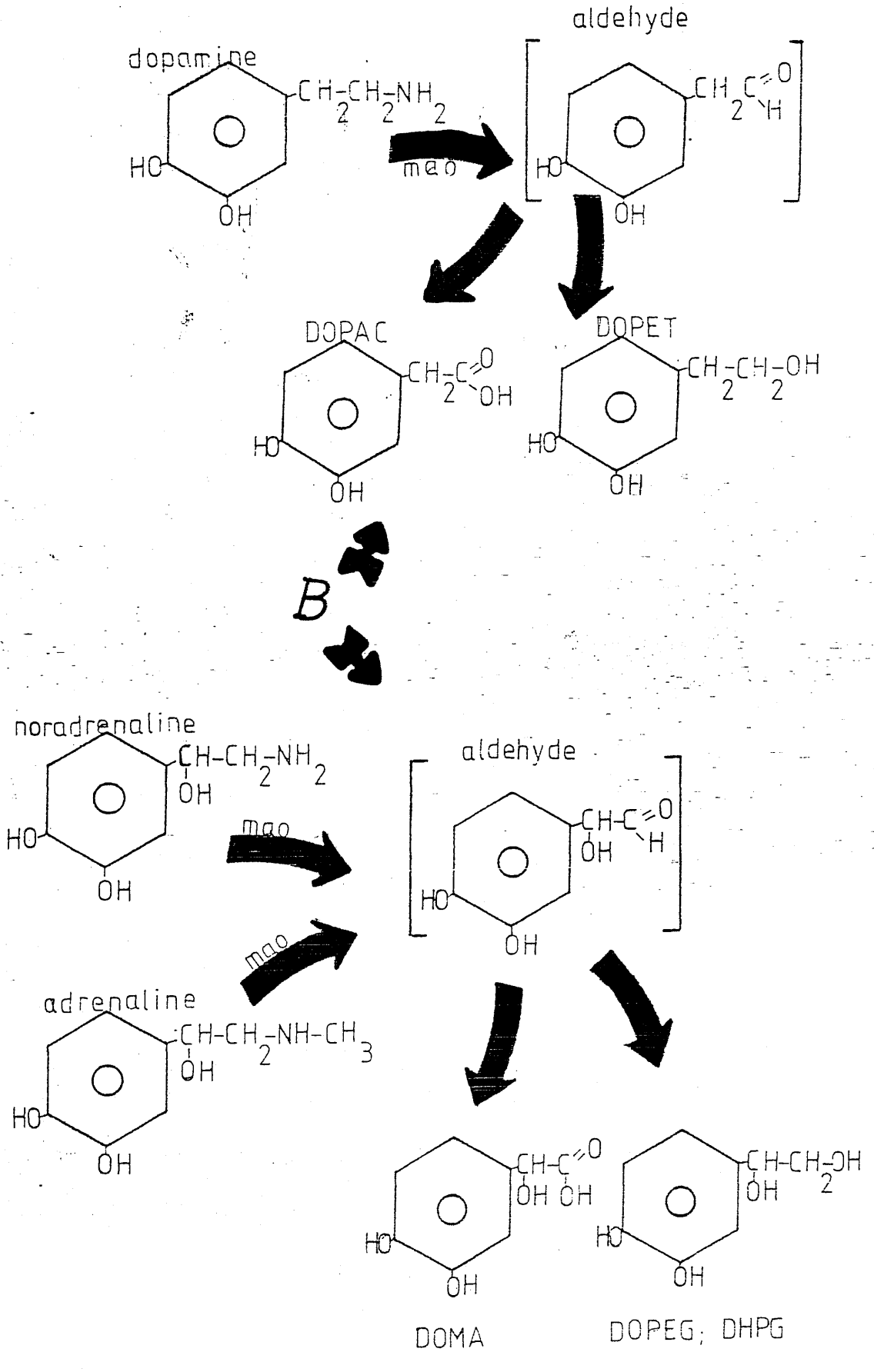
DOPEG/DHPG -3,4-dihydroxyphenylethylglycol

ALC DEHYD - alcohol dehydrogenase

ALD DEHYD - aldehyde dehydrogenase

FAD - flavin adenine dinucleotide

FADH2 - reduced flavin adenine dinucleotide


dopamine
aldehyde
mao
DOPAC
DOPET
B
noradrenaline
aldehyde
mao
mao
adrenaline
DOMA
DOPEG; DHPG

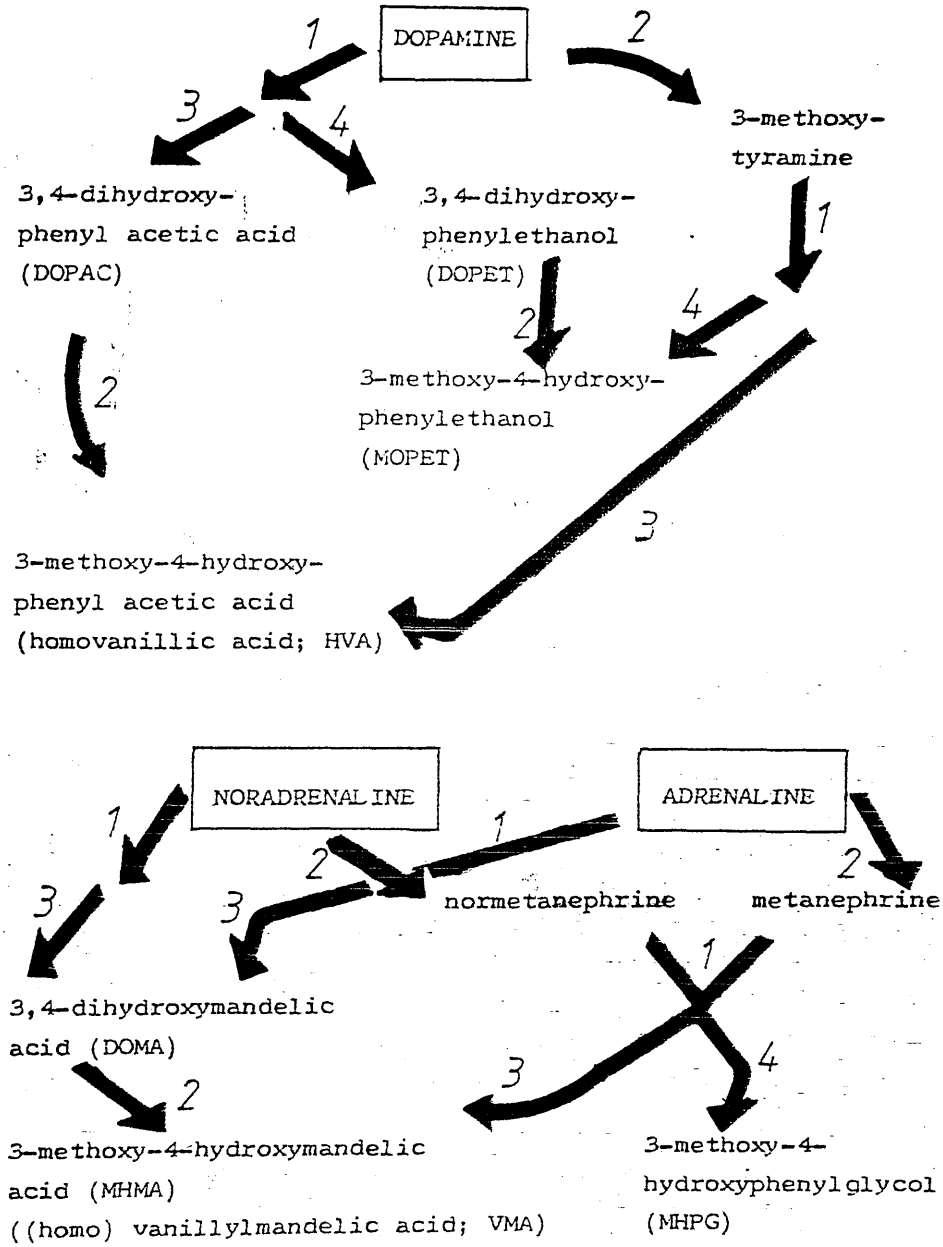


Figure 5.5 Catabolic actions of COMT and MAO.

The catabolic enzymes perform physiological roles in conjunction with each other. Products of one enzyme subsequently act as substrates for the other enzyme and vice versa. The diagram illustrates these inter-related roles.

Early research concentrated on the effects of lead on endogenous levels of the neurotransmitters, mainly noradrenaline and dopamine but also tyrosine to a limited degree. Studies have investigated these parameters in either rats or mice exposed to lead during the neonatal period or as young adults. Several studies have investigated the levels of catecholamines in the brain as a whole. These studies initially indicated no change in noradrenaline concentrations in the rat (Sauerhoff & Michaelson ,1973; Michaelson & Sauerhoff, 1974) and mouse (Schumann, 1977; Schumann et al, 1977), but subsequent data obtained by the same group of workers showed increased noradrenaline levels in the brain (Michaelson, Greenland & Roth, 1974; Golter & Michaelson, 1975), an effect also confirmed by Silbergeld & Goldberg (1975). These same studies initially indicated that dopamine levels were reduced by 20% (Sauerhoff & Michaelson ,1973; Michaelson & Sauerhoff, 1974), and later they reported no alteration to the level of this catecholamine (Michaelson et al, 1974; Golter & Michaelson, 1975), an observation in agreement with that of Silbergeld and Goldberg (1975) in the mouse. Regional analysis of the brain however, demonstrates a slightly clearer picture. Rat forebrain, midbrain and brainstem exhibited an increased concentration of noradrenaline as a result of lead exposure (Silbergeld & Goldberg, 1975; Jason & Kellog, 1977; Dubas & Hrdina, 1978; Dubas et al, 1978). Other studies however, failed to demonstrate any effect in any region. No change in noradrenaline was observed in the cortex, brainstem,

cerebellum, hypothalamus, striatum or diencephalon (Sobotka & Cook, 1974; Sobotka et al, 1975; Grant et al, 1976a/b; Jason & Kellog, 1977). Even more data shows a fall in noradrenaline in the hypothalamus, striatum and brainstem (Hrdina et al, 1976; Dubas et al, 1978; Jason & Kellog, 1981). There would appear therefore to be no consistency in the data obtained concerning the effect of lead on brain noradrenaline concentrations even within the same brain region.

Turning now to dopamine levels in discrete regions of brain, although significant reductions in this catecholamine were observed in the straitum (Jason & Kellog, 1977) and cortex, midbrain, hypothalamus (Dubas & Hrdina, 1978), other studies have shown no change in various regions; cortex, brainstem, hypothalamus, striatum and forebrain (Sobotka & Cook, 1974; Sobotaka et al, 1975; Grant et al, 1976a/b; Schumann, 1977; Schumann et al, 1977; Jason & Kellog, 1981). Indirect measurements of dopamine levels were obtained in a study which quantitated the level of prolactin. Lead exposure caused an increase in prolactin levels (Govani et al, 1978). Since prolactin secretion is controlled mainly under hypothalamic dopaminergic inhibitory control this data implies a reduction in dopaminergic activity in this brain region. The study of Dubas & Hrdina (1978) cited above showing a reduced concentration of dopamine in the midbrain and hypothalamus is interesting since this same laboratory in another paper quoted an increase in dopamine in these same regions (Dubas et al, 1978).

There exist only two papers from the same group of researchers which have quantitated endogenous levels of tyrosine. Data in these papers indicates that lead does not affect the level of this amino acid at least in whole brain (Schumann, 1977; Schumann et al, 1977).

A series of studies by Meredith and his coworkers have investigated the effects of lead on brain noradrenaline, dopamine and adrenaline in certain very small discrete regions, following both acute and chronic exposure to the metal. Acute exposure consisted of the administration of either 5 or 20μmol/kg intraperitoneally in rats for 14 days while chronic exposure was achieved by supplying a 2mM lead acetate drinking solution for a period of 8 or 26 weeks. Following acute exposure, noradrenaline was increased in both the anterior and posterior hypothalamus and adrenaline was increased in the anterior pituitary but only in the 20μmol/kg group. Additionally, there was a slight rise in dopamine in this same group of animals, again in the anterior hypothalamus. No significant changes in either catecholamine were observed in other brain regions examined - cortex, pons-medulla or hippocampus. In the chronic study, however, there were significant reductions in both noradrenaline and dopamine after 26 weeks of exposure. After the shorter exposure period of 8 weeks, the level of catecholamines was increased but this failed to reach statistical significance (Meredith, Petty & Reid, 1979; 1981; Meredith et al, 1981b). These studies suggest that there may be a biphasic response to lead depending on the length of exposure and /or exposure level.

The effect of lead on both noradrenaline and dopamine catabolism has been investigated. Turnover rates have been studied by looking at levels of metabolites present in the tissues (figure 5.5).

Concerning noradrenaline catabolism, Silbergeld & Chisholm (1976) demonstrated a significant increase in the noradrenaline metabolite, vanillylmandelic acid (VMA), both in the brain (48%) and urine (216%) of mice exposed to 5g lead/l from birth. Urinary metabolites most probably are derived from, and hence reflect peripheral catecholamine metabolism, and therefore both a central and peripheral action of lead is implicated from this data. Silbergeld & Goldberg (1978) also observed a 15% increase in brain VMA which was associated with an enhanced MAO activity (20%), a feature which could account for the increased metabolite levels. Taking another approach, Michaelson et al (1974) observed an increased rate of decline of noradrenaline in rat wholebrain followng α-methyltyrosine. However, although this data would indicate an enhanced utilization of noradrenaline as a result of lead exposure, Schumann et al (1977) have failed to demonstrate an alteration in the rate of noradrenaline synthesis from tritiated tyrosine in the mouse brain.

More data exists concerning dopamine catabolism. In mouse forebrain, a significant increase of 33% in homovanyllic acid (HVA) was demonstrated following lead exposure indicating enhanced dopamine turnover. Concordant with this tissue rise, there was also a 265% increase in the urine (Silbergeld & Chisholm, 1976). However, following

labelled tyrosine administration, no change in dopamine synthesis was observed in the mouse brain (Schumann et al, 1977) or rat forebrain (Wince, Donovan & Azzaro, 1980). On the other hand, other studies of dopamine metabolism indicate that the metabolism is altered with the direction of change being regionally specific. 3,4-dihydroxyphenyl-acetic acid (DOPAC), considered a reliable indirect indicator of the functional ability of dopaminergic neurones, was first demonstrated to be significantly reduced in the striatum (20%) of rats exposed to 2.5g lead/l throughout life (Govani et al, 1978). Confirming this observation and extending their data, the same laboratory demonstrated a significant increase in DOPAC in the rat nucleus accumbens (20%, 33%) and frontal cortex (35%, 45%), with no change in the substantia nigra. Once again, there was a reduced DOPAC level in the striatum (24%) - Govani et al (1979; 1980). In both these studies the animals exhibited an increased locomotor activity. Supportive data was also obtained in a study by Memo et al (1981). In the rat DOPAC was diminished by 15% in the striatum and enhanced by 17% in the nucleus accumbens, effects which appeared to be reversible since they had returned to normal 30 days after cessation of lead treatment.

Using a different approach of following the decline in DOPAC after α-methyltyrosine administration, data is obtained which is consistent with the previously discussed results and with an increased dopamine turnover. A reduction in DOPAC decline following α-methyltyrosine

administration was observed in the striatum (Govani et al, 1978; 1979; 1980), while an enhancement was seen in the nucleus accumbens and frontal cortex (Govani et al, 1979; Memo et al, 1981). Again in the striatum, Jason & Kellog (1977) observed a reduction in dopamine turnover (35%) following lead exposure which was based on a decline in dopamine levels after α-methyltyrosine treatment.

Although the above cited studies were performed in either the mouse or rat, a study of children provides data which is generally in agreement with these animal studies. Similarly to the data in the mouse, where Silbergeld & Chisholm (1976) observed an increase in VMA and HVA in the brain and urine, the same authors noted these metabolites to be also increased in the urine of children who were significantly exposed to lead; that is they had blood lead levels in the range 2.8-3.3μM (59-68μg/100ml). The data would thus implicate increased peripheral catabolism of catecholamines in these exposed children. Since the regional alterations being probably opposite in nature would possibly be cancelling each other out, caution must be taken when interpreting turnover studies in which an enzyme of the catecholamine synthetic pathway, often tyrosine hydroxylase, is inhibited since the accumulation of intermediates is measured, as if lead were to alter the activity of a synthetic enzyme then a change may not be identified by these studies.

Most studies investigating the uptake and release of catecholamines centre on the high affinity uptake of dopamine by synaptosomes. A single paper has reported data

on noradrenaline uptake. Silbergeld & Goldberg (1975) observed no alteration in high affinity noradrenaline uptake by mouse forebrain synaptosomes. The same authors also observed a significant reduction on dopamine uptake (19%) following in vivo exposure and, in addition, a 15% enhancement in uptake of tyrosine. Other groups performing similar uptake studies following in vivo lead exposure, failed to exhibit alterations in dopamine uptake in rat forebrain slices (Wince et al, 1980), striatal synaptosomes (Ramsay et al, 1980) or striatal minces (Jason & Kellog, 1982). One group even observed enhanced dopamine uptake into rat synaptosomes (Wince & Azarro, 1978). The report of enhanced tyrosine uptake quoted above (Silbergeld & Goldberg, 1975) is consistent with an increase in dopamine turnover in the same brain regions.

Similarly to dopamine uptake studies, data relating to dopamine release is also conflicting. Following a load of tritiated dopamine, Wince and co-workers (1980) observed a 12-30% fall in potassium stimulated release from brain slices, data in conflict with results from Jason & Kellog (1981) who observed no effects on potassium stimulated release from striatal minces. Kent et al (1984) measured the noradrenaline release from the cortex, hippocampus, the release of dopamine from the striatum, substantia nigra, and the release of both transmitters from the nucleus-o-tubercle and hypothalamus. No difference was observed between the spontaneous, potassium-stimulated or amphetamine-stimulated release of catecholamines from tissues of lead treated compared to control rats. The

addition of lead in vitro resulted in a concentration dependant diminished high affinity dopamine uptake in rat striatal synaptosomes (Silbergeld, 1977), an observation not confirmed by either Bondy et al (1979) in mouse wholebrain synaptosomes or by Ramsay et al (1980) using striatal synaptosomes. The same picture is seen when looking at dopamine release following in vitro exposure to lead. While Silbergeld (1977) and Komulainin & Tuomisto (1981) reported no effect, two papers reported stimulated release (Bondy et al, 1979a/b; Ramsay et al, 1980).

Data relating to the activity of individual enzymes of the biosynthetic pathway is relatively sparse. Although evidence of Wince & Azarro (1978) and Wince, Donovan & Azzaro (1976) suggests that tyrosine hydoxylase activity is stimulated as a result of lead exposure, a study by Deskin, Bursian & Edens (1980) suggests this enzyme is unaltered in two specific brain regions at least - striatum and hypothalamus. Yet another series of studies by Meredith and colleagues (Meredith et al, 1979; 1981a/b) have demonstrated a lead-induced fall in tyrosine hydroxylase activity both following acute exposure of rats (5 or 20μmol/kg i.p. for 14 days) and chronic exposure (2mM for 8 or 26 weeks) in the hypothalamus. No alterations were noted in other regions; cortex, pons-medulla or hippocampus.

In vitro experiments have shown a progressive irreversible inhibition of phenylethanolamine-N-methyl transferase (PNMT) by lead (Caspers, 1982), an effect which the author suggests is due to an action of lead on sulphydryl groups on the enzyme. Studies involving in vivo

lead exposure have not however demonstrated an alteration to the activity of this enzyme (Meredith et al, 1979; 1981).

Finally, the possibility of an alteration in receptor populations must be considered, and in this respect only the dopamine receptor has been studied extensively. The dopamine D_1 receptor which is linked to adenylate cyclase can be assessed for functional ability using dopamine stimulation. Reports indicate either no change in dopamine stimulated activity in rat striatum and accumbens (Govani et al, 1979; Lucci et al, 1981), or alternatively, Wince et al (1980) reported a significant lead-induced reduction in cyclase activity and a marked inhibition of apomorphine-induced stimulation. These workers employed rat forebrain synaptosomes as tissue. Concerning the dopamine D_2 receptor, conflicting results emerge depending on the ligand selected for binding. While ^{3}H-spiroperidol binding is not altered (Govani et al, 1979; Lucci et al, 1981) in rat straitum and accumbens following neonatal lead exposure, increased striatal binding and decreased binding in the nucleus accumbens was reported when ^{3}H-sulpiride, which is reputed to be more specific for the D_2 receptor, was employed (Lucci et al, 1981).

These findings concerning the dopamine D_2 receptor are in the opposite direction to alterations of DOPAC levels (Govani et al, 1978; 1979) and are thus in agreement; that is increased dopamine synthesis as a result of a decrease in receptor sensitivity and vice versa.

Many reports have demonstrated a lead-induced block in post-synaptic adenylate cyclase activity associated with both the noradrenaline and adrenaline receptor, at relatively low lead levels (Nathanson & Bloom, 1975; Wince et al, 1976; Taylor et al, 1978), although another group have failed to confirm this effect (Williams et al, 1977).

However, it is difficult to relate these in vitro effects on enzymatic activity to the effects of lead on the intact nervous system. The data in this chapter of my thesis will be of an in vivo nature whereby various parameters of catecholamine metabolism will be investigated in rat brain following exposure of the animals to lead in drinking water.

5.2 Methodology

Within this chapter the data obtained in several experiments will be reported. Principally the experimental procedures can be segregated into those studies in which the male Sprague-Dawley rats were exposed to lead for a variable period of time after they were weaned, and secondly, experiments where exposure was in utero, pre-weaning and post-weaning.

5.2.1 Methods - exposure post-weaning

Male Sprague-Dawley rats were exposed to a lead acetate drinking fluid for a period of 1,3 or 7 months post-weaning; a control group receiving distilled water. Laboratory rat chow and fluid were supplied ad. libitum. Those rat groups exposed to lead for either 1 or 3 months were supplied with the metal at a concentration of 120μM (25 mg/l) or 480μM (100 mg/l), whilst the level of exposure

was 48μM (10 mg/l) or 480μM (100 mg/l) in the longer exposure group of duration 7 months. At the end of the appropriate period of exposure, the animals were killed by decapitation. A sample of blood was obtained for lead estimation, as was the femur. The brains were removed and dissected as described under the general methodology section in preparation for the estimation of catecholamines, tyrosine hydroxylase activity and phenylethanolamine-N-methyl transferase (PNMT) activity in each distinct brain region. The assays employed to measure these parameters and tissue protein concentration are also detailed in the methodology chapter 2.

5.2.2 Exposure in utero, pre- and post-weaning

Female Sprague-Dawley rats were supplied with lead acetate drinking fluid or distilled water for a period of 4 weeks prior to being mated with non-lead exposed male rats. When born, the young were exposed to lead via the dams' milk, and when weaned, exposure continued again via the drinking fluid for a further period of 1 month. At the end of this period, the animals were sacrificed and treated as described above. The level of lead exposure employed in these experiments was 480μM (100 mg/l) and 1930μM (400 mg/l).

5.3 Results

5.3.1 Influence of lead exposure on rat weight

As an index of whether exposure of the rats to lead is resulting in a reduced growth rate, the animals were weighed before being sacrificed. Table 5.1 shows the weights of the animals exposed to lead for 1, 3 and 7

months post-weaning and the group of animals exposed both pre- and post- weaning. At the 5% significance level only 2 lead groups exhibited a significant reduction in weight gain by comparison with non-exposed animals. The animals exposed to 120μM lead for 1 month post-weaning were significantly lighter than their non-exposed controls. Although the mean weight of the rats exposed to the higher lead dose (480μM) was less than the control group, this difference was not significant. Exposure to a relatively high dose of lead, 1930μM both in utero and pre-and post-weaning, resulted in a significant reduction in weight gain of these animals ($p<0.02$). A lower dose of 480μM did not however affect weight gain.

5.3.2 Lead concentrations in blood, brain and bone.

The resultant blood lead concentrations as a consequence of exposure to lead acetate drinking fluid for varying lengths of exposure and dose regimes are shown in table 5.2. The animals exposed to lead exhibited a significantly elevated level of blood lead than their non-exposed control group after all lengths of exposure. In addition it should be noted that the elevation in blood lead concentration was dose related although the relationship was not of a linear fashion. This data confirms that the animals are indeed absorbing significant quantities of lead.

The levels of the metal which had accumulated in the various brain regions under study was also measured and the data is presented in tables 5.3 to 5.5. Exposure to lead at a dose of either 120 or 480μM for a relatively short period of 1 month post-weaning was sufficient to result in an

accumulation of the metal within all brain regions studied, cerebellum, midbrain, diencephalon and telencephalon. Only within the cerebellum at the lower dose level was the metal not significantly elevated above control levels. In addition, increasing the level of exposure resulted in a further accumulation in each brain region. The data also indicates that there does not appear to be a selective uptake of lead by a specific brain region. However, extending the period of post-weaning lead exposure does not cause an enhanced tissue accumulation of the metal.

Neither dose of lead (48 or 480μM) in the 7 month exposure group caused an increase of lead to occur in the brain. Those animals exposed to 1930μM lead in utero showed significantly elevated levels of lead in the cerebellum, midbrain or diencephalon but not in the telencephalon. Levels of lead in all brain regions were higher in the lower lead group than in controls but this difference was not significant at the 5% level.

The concentration of lead in the femur of the rats was measured as an estimation of the quantity of lead the animals accumulated over the various exposure periods. The results are tabulated in table 5.6. All groups of lead exposed animals irrespective of exposure time or degree of exposure exhibited a significantly elevated concentration of lead in the femur by comparison with non-exposed rats, although the degree of elevation was not linearly related to water lead intake. **It is noteworthy that there is a fall in brain lead between 3 & 7 months exposure (tables 5.3 & 5.4). No explanation can be put forward for this observation except that it may be related to a variable lead burden resulting from rat chow or be related to the complex movement of lead between body compartments.**

5.3.3 Brain catecholamine concentrations.

The catecholamines, noradrenaline and dopamine were measured in 4 brain regions, cerebellum, midbrain, diencephalon and telencephalon in the rats exposed to lead acetate or distilled water for 1, 3 or 7 months post weaning or 1 month post weaning after additional exposure pre-weaning and in utero. The concentrations of noradrenaline are shown in tables 5.7 to 5.10 and tables 5.11 to 5.14 presents similar data for dopamine.

Firstly, considering the noradrenaline data. Tables 5.7 to 5.10 illustrate the complex results noted with regard to the concentration of this catecholamine following lead exposure for the varying periods of time. There is however, a concensus from the various experiments that the telencephalon does not exhibit changes in noradrenaline concentration under any condition employed in these experiments. Alterations in noradrenaline levels are manifest most frequently in the midbrain and diencephalon. Following 3 months lead exposure to both 120 and 480μM lead, there is a significant elevation in noradrenaline concentration in the diencephalon, whilst the same parameter was decreased in the midbrain but only in the higher exposure group. Extending the period of exposure to 7 months once more results in significant changes being noted in the same two brain regions, but in this case there is a significant reduction in noradrenaline in the diencephalon following exposure to 480μM lead when the results are expressed with respect to protein. Although the much lower dose of 48μM resulted in a reduction in

noradrenaline this failed to reach statistical significance at the 5% level. It is unclear as to the influence of lead on noradrenaline in the midbrain with a suggestion of an enhanced concentration in the lower dose and a diminution in the higher dose group. In contrast to the longer exposure groups of 3 and 7 months, the group exposed for 1 month in addition to in utero, showed no alteration in noradrenaline in these 2 brain regions. However, a significantly reduced noradrenaline concentration was noted in the cerebellum but only in the low dose group.

Turning now to dopamine concentrations (tables 5.11 to 5.14). The data obtained from those animals exposed to lead for 3 or 7 months indicates a reduction in dopamine concentration in the midbrain. Indeed, observing the results as a whole, it is evident that where dopamine concentrations are altered the direction of change is always a lead associated reduction. The lower dose exposure level of 48μM for 7 months resulted in significantly decreased dopamine in the diencephalon and cerebellum. Reductions were also noted in this same brain region in those animals exposed in utero. Similarly to the data obtained for noradrenaline, there was no significant alteration in dopamine in the telencephalon.

5.3.4 Effect of lead on tyrosine hydroxylase activity

Tyrosine hydroxylase activity was measured following lead exposure in the 4 regions of brain and the results can be seen in tables 5.15 to 5.18. Exposure in the experiments was for a period of 1 month (table 5.15), 3 months (table 5.16) or 7 months post weaning (table 5.17), or both pre-

and post- weaning (table 5.18). Similarly to the other 3 parameters, the most lead related changes in tyrosine hydroxylase activity are in the midbrain and diencephalon. No significant alterations were observed in the telencephalon. Where the cerebellum is concerned, there is a suggestion of a lead related increase in activity under certain conditions of exposure. The group exposed to 480μM lead both in utero and post-weaning showed an elevated TH activity in the cerebellum compared to non-exposed animals but only when the data was expressed per unit of protein did the rise reach significance. Within the diencepnalon, lead would appear to cause a reduction in TH activity; however not all groups of exposed animals exhibited this inhibition. Exposure for a longer period of 7 months resulted in a significant diminution in enzymatic activity in the midbrain when the animals were exposed to 48μM lead. No other region showed significantly altered activity.

5.3.5 Effect of lead on PNMT activity.

The influence of lead exposure on the activity of PNMT in the cerebellum, midbrain, diencephalon and telencephalon is shown in tables 5.19 to 5.22. The exposure in these experiments was for a period of 1 month (table 5.19), 3 months (table 5.20) or 7 months (table 5.21) post weaning or both pre- and post- weaning (table 5.22). Similarly to the data obtained in the previous two sections, the effect of lead on the activity of PNMT in this series of experiments is dependant on the dose and duration of exposure and on the specific brain region under examination. Following all levels of exposure for a period

between 1 and 7 months, there is no significant alteration in PNMT activity in the telencephalon. Where the other three regions are concerned however, a complex picture emerges. After 3 months lead exposure, significant elevations in activity are noted in the high dose group in the midbrain by comparison with control animals but this rise is only of significance when the data are expressed per unit of tissue. There is also a suggestion that there is a reduction in enzymatic activity in the cerebellum, but this is only in the lower dose group. In contrast to this observed enhanced activity in the midbrain, following exposure to 480μM lead, extending the time to 7 months results in a significant depression in PNMT activity when the data is expressed per unit of tissue and protein. A lead related reduction in activity is also noted in the diencephalon.

Exposing the rats to lead for a relatively brief period of only 1 month post-weaning or indeed additional in utero exposure, did not result in any significant alterations, either enhancement or reduction in activity, in any of the 4 regions studied. However, there were non-significant trends noted which were similar to the significant changes just described. 1 month exposure post-weaning resulted in a tendency for PNMT activity to be reduced in the cerebellum, and enhanced in both the midbrain and the diencephalon; once more no differences between the groups were noted in the telencephalon. No obvious pattern of change was clear from the study where the rats were exposed both pre- and post-weaning.

5.3.6 Effect of lead on rat development

In an attempt to assess whether the lead exposure levels employed in these experiments was resulting in a reduced gross brain development, the weight of each brain region was noted. Table 5.23 shows this information for each brain region following 3 months exposure to 120μM and 480μM lead and compares these brain weights to those noted in non-exposed animals. The data indicates no reduction in brain growth as a result of lead exposure for 3 months. Similar data extending the exposure period to 7 months is shown in table 5.24. Again no reduction in brain weight following both 48μM and a higher dose of 480μM was observed.

Protein content relative to tissue weight was also calculated for each region and exposure group and the results can be seen in tables 5.25 - 5.27. The data relating to 1 & 3 months post-weaning exposure is shown in table 5.25. Exposure to 120μM and 480μM lead acetate did not result in a reduced protein content of the brain in any region studied. Similarly, no significant differences were noted when the exposure period was extended to 7 months (table 5.26) or when the rats were exposed to lead in utero in addition to 1 month post-weaning (table 5.27).

Table 5.1 Rat weights

- Weight of rats prior to being sacrificed in the various exposure groups. The results are expressed as the mean ± S.D. with the number in each group being tabulated.

group	weight of rat (g)					
	control		120 μM Pb		480 μM Pb	
	No.	Wt.	No.	Wt.	No.	Wt.
1 Month post-weaning	24	150±39	21	122±24 +	22	136±25
3 Months post-weaning	23	298±27	19	308±34	20	298±26

	Control		48 μM Pb		480 μM Pb	
	No.	Wt.	No.	Wt.	No.	Wt.
7 Months post-weaning	37	416±62	18	419±64	20	443±27

	Control		480 μM Pb		1930 μM Pb	
	No.	Wt.	No.	Wt.	No.	Wt.
1 Month post-weaning + exposure in utero & pre-weaning	12	145±14	18	142±33	18	118±28 ++

\+ $p < 0.01$ compared to the

++ $p < 0.02$ control group

Table 5.2 Blood lead concentrations in rats

Concentration of lead in the blood of rats exposed to lead compared to non-exposed animals. The results are expressed as the mean ± S.D. of at least 6 animals in each group.

group	blood lead (μM) control	120 μM Pb	480 μM Pb
1 month post-weaning	0.19±0.17	0.30±0.09	0.55±0.25 +*
3 months post-weaning	0.12±0.04	0.44±0.09 ++	0.76±0.15 ++**

	control	48 μM Pb	control	480 μM Pb
7 month post-weaning	0.19±0.04	0.46±0.15 ++	0.20±0.07	0.96±0.38 ++

	control	480 μM Pb	1930 μM Pb
pre- & post-weaning	0.22±0.06	0.80±0.18 @	0.95±0.34 ++

\+ $p<0.02$ ++ $p<o.002$ @ $p<0.005$ compared to the control group

* $p<0.05$ ** $p<0.002$ compared to the 120μM lead group

by Mann-Whitney U test

Table 5.3 Tissue lead concentrations in rats - 1 and 3 months lead exposure

Concentration of lead in brain regions of rats exposed to lead compared to non-exposed rats. Results are expressed as the mean ± S.D. of 6 observations.

group	tissue lead (nmoles/ml homogenate) control	120 µM Pb	480 µM Pb
1 month post-weaning			
cerebellum	0.21±0.15	0.25±0.05	0.58±0.12 +*
midbrain	0.10±0.02	0.24±0.07 +	0.50±0.08 +*
diencephalon	0.14±0.03	0.26±0.06 +	0.58±0.09 +*
telencephalon	0.15±0.03	0.25±0.12 ++	0.55±0.10 +**
3 months post-weaning			
cerebellum	0.27±0.10	0.37±0.11	0.54±0.10
midbrain	0.18±0.08	0.24±0.11	0.26±0.13
diencephalon	0.21±0.13	0.26±0.08	0.54±0.35
telencephalon	0.30±0.07	0.34±0.06	0.88±0.27

\+ $p < 0.005$ compared to
++ $p < 0.01$ control group

* $p < 0.005$ compared to
** $p < 0.01$ 120 µM group

Table 5.4 Tissue lead concentrations in rats - 7 months lead exposure

Concentration of lead in brain regions of rats exposed to lead compared to non-exposed controls rats. Results are expressed as mean ± S.D. of 6 animals in each group.

group	tissue lead (nmoles/ml homogenate) control	48 μM Pb
7 month post-weaning		
cerebellum	0.11±0.04	0.14±0.10
midbrain	0.11±0.09	0.21±0.12
diencephalon	0.12±0.08	0.18±0.06
telencephalon	0.15±0.11	0.14±0.06
	control	480 μM Pb
7 month post-weaning		
cerebellum	0.28±0.15	0.22±0.06
midbrain	0.24±0.10	0.25±0.07
diencephalon	0.23±0.13	0.24±0.09
telencephalon	0.19±0.09	0.17±0.08

Table 5.5 Tissue lead concentrations in rats - pre-weaning exposure

Concentration of lead in brain regions of rats exposed to lead in utero, during the pre-weaning phase and 1 month post-weaning. Results are expressed as mean ± S.D. of 6 observations.

group	tissue lead (nmoles/ml homogenate) control	480 µM Pb	1930 µM Pb
pre- & post-weaning exposure			
cerebellum	0.70±0.15	0.77±0.13	0.88±0.06 +
midbrain	0.75±0.08	0.81±0.12	0.94±0.07 ++
diencephalon	0.62±0.08	0.68±0.05	0.85±0.06 ++*
telencephalon	0.66±0.04	0.70±0.12	0.74±0.11

+ $p < 0.01$ compared to
++ $p < 0.005$ control group

* $p < 0.01$ compared to 480µM group

Table 5.6 Bone lead concentrations in rats

Concentration of lead in the femur of rats exposed to lead compared to non-exposed animals. The results are expressed as the mean ± S.D. of 6 observations.

group	bone lead (μmol/kg dry bone wt.) control	120 μM Pb	480 μM Pb
1 month post-weaning	24±12	69±17^{+}	187±36^{++*}
3 months post-weaning	12±6	92±21^{++}	216±38^{++*}

	control	48 μM Pb	control	480 μM Pb
7 month post-weaning	8±4	56±14^{+}	9±4	229±36^{++}

	control	480 μM Pb	1930 μM Pb
pre- & post-weaning	10±5	196±35^{++}	480±60^{++**}

+ $p < 0.05$ compared to control
++ $p < 0.001$ group

* $p < 0.01$ compared with lower dose
** $p < 0.001$ lead group

Table 5.7 Tissue noradrenaline levels in rat brain - 1 month post-weaning

Rats exposed to 120μM and 480μM lead for 1 month post-weaning.
The results are expressed as the mean ± S.D. of 6 observations.

Brain region	noradrenaline concentration (nmoles/g tissue)			(pmoles/mg protein)		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	2.68±0.62	2.25±0.28	2.33±0.22	29±7	26±3	24±2
midbrain	4.05±0.52	3.34±0.13 ++	4.19±0.66	48±6	44±2	47±8
diencephalon	6.51±0.76	6.15±0.85	4.96±1.05 +	73±13	69±10	59±12
telencephalon	2.97±0.64	2.92±0.56	3.84±0.95	39±8	34±6	44+11

\+ $p < 0.02$ compared with
++ $p < 0.05$ control group

Table 5.8 Tissue noradrenaline levels in rat brain - 3 months lead exposure

Rats exposed to 120 μM and 480 μM lead for 3 months post-weaning. The results are expressed as the mean ± S.D. of 8 observations in the control group and 6 in each lead group.

Brain region	noradrenaline concentration (nmoles/g tissue)			noradrenaline concentration (pmoles/mg protein)		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	2.50±0.48	2.65±0.49	2.52±0.32	27±5	26±5	31±4
midbrain	7.33±1.10	6.47±1.14	4.65±1.13 +	82±13	72±13	54±13 +
diencephalon	8.12±1.66	11.18±1.37 +	10.90±1.58	79±16	122±15 ++	110±16 @
telencephalon	5.03±1.02	4.29±0.56	5.24±0.58	52±11	49±7	56±6

\+ $p < 0.01$ compared with
++ $p < 0.005$ control group
@ $p < 0.05$

Table 5.9 Tissue noradrenaline in rat brain - 7 month lead exposure post-weaning

Rats exposed to 48 μM and 480 μM lead for 7 months post-weaning. The results are expressed as the mean ± S.D. of 6 observations.

Brain region	noradrenaline concentration (nmoles/g tissue)			
	control	48 μM Pb	control	480 μM Pb
cerebellum	4.17±0.58	3.86±0.50	2.96±0.41	2.72±0.20
midbrain	4.32±0.50	6.09±1.55	6.92±1.08	5.00±1.18 ++
diencephalon	7.87±2.01	6.26±1.12	9.92±1.62	8.62±1.31
telencephalon	5.18±2.22	4.79±1.57	4.66±0.50	3.93±0.32

	(pmoles/mg protein)			
	control	48 μM Pb	control	480 μM Pb
cerebellum	41±6	35±3	45±6	33±4
midbrain	73±11	88±21	63±7	64±16
diencephalon	177±29	144±22	106±27	54±10 +
telencephalon	76±8	67±5	57±23	54±18

\+ $p < 0.05$ compared to
++ $p < 0.02$ control group

Table 5.10 Tissue noradrenaline levels in rat brain - pre- and post- weaning lead exposure

Rats exposed to 480 μM and 1930 μM lead for 1 month post-weaning in addition to exposure in utero and pre-weaning via the dam. The results are expressed as the mean ± S.D. of 6 observations.

Brain region	noradrenaline concentration (nmoles/g tissue)			(pmoles/mg protein)		
	control	480 μM Pb	1930 μM Pb	control	480 μM Pb	1930 μM Pb
cerebellum	0.92±0.07	0.73±0.10+	0.79±0.15	8.52±0.66	6.47±0.87++	7.72±1.46
midbrain	1.53±0.42	1.49±0.27	1.73±0.34	15.0±4.1	15.3±2.8	15.8±3.1
diencephalon	2.55±0.45	1.78±0.28	2.75±0.48	26±5	18±3	28±5
telencephalon	0.96±0.14	1.00±0.17	0.85±0.17	9.25±1.37	8.74±1.51	7.72±1.54

+ $p < 0.05$ compared to
++ $p < 0.01$ control group

Table 5.11 Tissue dopamine levels in rat brain - 1 month post-weaning

Rats exposed to 120μM and 480μM lead for 1 month post-weaning.
The results are expressed as the mean ± S.D. of 6 observations.

Brain region	dopamine concentration (nmoles/g tissue)			(pmoles/mg protein)		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	1.45±0.27	1.60±0.33	1.47±0.43	15.8±2.9	18.4±3.9	15.2±4.5
midbrain	1.43±0.23	1.09±0.35	1.61±0.25	17.0±2.6	14.0±4.6	18.2±2.8
diencephalon	10.87±1.72	10.31±2.02	11.16±1.28	130±20	117±23	133±15
telencephalon	4.91±0.63	5.12±0.88	6.40±0.71 +	64±9	60±10	72±7

+ $p < 0.005$ compared to control group

Table 5.12 Tissue dopamine levels in rat brain - 3 months lead exposure

Rats exposed to 120 μM and 480 μM lead for 3 months post-weaning. The results are expressed as the mean ± S.D. of 8 observations in the control group and 6 in each lead group.

Brain region	dopamine concentration (nmoles/g tissue)			(pmoles/mg protein)		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	0.88±0.26	1.06±0.36	1.11±0.36	8.86±1.92	9.13±1.82	13.7±4.1
midbrain	1.92±0.41	2.07±0.40	1.27±0.13	21.5±4.7	23.0±4.4	14.7±1.5+
diencephalon	15.5±4.7	16.7±3.5	17.6±5.8	151±46	182±38	177±59
telencephalon	10.9±3.3	10.7±1.4	11.1±1.1	113±36	102±52	118±11

+ $p < 0.02$ compared to control group

Table 5.13 Tissue dopamine levels in rat brain - 7 month lead exposure post-weaning

Rats exposed to 48 μM and 480 μM lead for 7 months post-weaning. The results are expressed as the mean ± S.D. of 6 observations.

Brain region	dopamine concentration (nmoles/g tissue)			
	control	48 μM Pb	control	480 μM Pb
cerebellum	2.65±0.20	1.68±0.43 +	1.47±0.79	1.99±0.85 @
midbrain	1.57±0.45	1.53±0.13	1.96±0.26	1.30±0.44
diencephalon	8.44±1.77	6.45±2.19	13.6±5.5	12.5±3.4
telencephalon	5.89±0.68	5.87±0.53	9.52±1.03	8.17±1.10

	(pmoles/mg protein)			
	control	48 μM Pb	control	480 μM Pb
cerebellum	28±2	14±4 +	20±1	26±11
midbrain	23±6	16±2	21±3	23±8
diencephalon	113±24	55±19 ++	243±98	208±56
telencephalon	66±8	65±6	156±17	139±19

+ $p < 0.01$ compared to control group
++ $p < 0.02$
@ $p < 0.05$

Table 5.14 Tissue dopamine levels in rat brain

- pre- and post- weaning lead exposure

Rats exposed to 480 μM and 1930 μM lead for 1 month post-weaning in addition to exposure in utero and pre-weaning via the dam. The results are expressed as the mean ± S.D. of 6 observations.

Brain region	dopamine concentration (nmoles/g tissue)			(pmoles/mg protein)		
	control	480 μM Pb	1930 μM Pb	control	480 μM Pb	1930 μM Pb
cerebellum	0.86±0.25	0.77±0.14	0.89±0.23	8.03±2.37	6.85±1.21	8.71±2.27
midbrain	0.77±0.09	0.83±0.23	0.81±0.18	7.04±1.41	8.47±2.35	7.36±1.62
diencephalon	8.24±1.19	5.69±1.16	8.80±0.91	83±12	59±12+	89±9
telencephalon	3.95±0.45	4.36±0.74	4.17±0.51	38±5	38±7	30±13

+ $p < 0.05$ compared to control group

Table 5.15 Activity of tyrosine hydroxylase in rat brain - 1 month post-weaning

Activities expressed as the mean ± S.D. of 6 observations after exposure to 120 μM or 480 μM lead for 1 month post-weaning.

Brain region	Tyrosine hydroxylase activity (pmoles/mg tissue/hr.)			(nmoles/mg protein/hr.)		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	10.3±2.1	11.6±4.6	10.4±3.8	109±25	124±50	93±3
midbrain	33.7±1.7	25.8±6.5	33.2±6.6	325±30	297±79	319±44
diencephalon	32.1±5.4	31.3±7.7	32.5±6.1	349±52	286±56	327±77
telencephalon	15.5±1.0	14.5±3.1	16.8±2.4	159±19	172±38	180±24

Table 5.16 Activity of tyrosine hydroxylase in rat brain - 3 month lead exposure post-weaning

Activities expressed as the mean ± S.D. after exposure to 120 μM or 480 μM lead for 3 months post-weaning. The control group comprises 8 observations and each lead group, 6 observations.

Brain region	tyrosine hydroxylase activity (pmoles/mg tissue/hr.)			(nmoles/mg protein		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	31±8	22±2	28±9	0.335±0.13	0.26±0.08	0.32+0.13
midbrain	102±33	118±12	97±16	1.20±0.35	1.44±0.11	1.02+0.30
diencephlon	117±13	92±9 +	92±18 ++	1.00±0.19	1.28±0.37	0.95±0.40
telencephalon	64±8	61±9	63±13	0.79±0.19	0.83±0.21	0.71±0.12

\+ $p < 0.02$ compared with control group
++ $p < 0.05$

Table 5.17 Activity of tyrosine hydroxylase in rat brain - 7 months lead exposure post weaning

Activites expressed as the mean ± S.D. of 6 observations after exposure to 48 μM or 480 μM lead for 7 months post-weaning.

Brain region	tyrosine hydroxylase activity (pmoles/mg tissue/hr.)	
	control	48 μM Pb
cerebellum	20.8±5.5	20.7±1.7
midbrain	86±20	76±20
diencephalon	101±25	117±30
Telencephalon	76±20	93±13

	(nmoles/mg protein/hr.)	
	control	48 μM Pb
cerebellum	0.21±0.03	0.21±0.09
midbrain	1.49±0.23	0.85±0.20+
diencephalon	1.29±0.38	1.03±0.39
telencephalon	0.93±0.35	1.00±0.23

+ $p < 0.01$ compared to control group

Table 5.18 Activity of tyrosine hydroxylase in rat brain - pre- and post- weaning lead exposure

Activities expressed as the mean ± S.D. of 6 observations after exposure to 480 μM or 1930 μM for 1 month post weaning in addition to exposure in utero and pre-weaning via the dam.

Brain region	tyrosine hydroxylase activity (pmoles/mg tissue/hr.)			(nmoles/mg protein/hr.)		
	control	480 μM Pb	1930 μM Pb	control	480 μM Pb	1930 μM Pb
cerebellum	25±9	35±5	32±10	0.24±0.06	0.36±0.08 +	0.27±0.13
midbrain	73±8	68±7	72±5	0.93±0.17	0.97±0.18	0.77±0.14
diencephalon	96±13	86±9	111±12	1.12±0.08	0.82±0.22 ++	1.04±0.07
telencephalon	50±9	47±5	47±4	0.51±0.10	0.47±0.03	0.46±0.06

+ $p < 0.05$ compared to
++ $p < 0.01$ control group

Table 5.19 Activity of phenylethanolamine N-methyltransferase (PNMT) in rat brain - 1 month lead exposure.

Activities expressed as mean ± S.D. after exposure to 120 μM or 480 μM lead for 1 month. Each group comprises 6 observations.

Brain region	PNMT activity (fmoles/mg. tissue/hr.)			PNMT activity (pmoles/mg. protein/hr.)		
	Control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	63±25	61±14	57±16	0.66±0.33	0.56±0.08	0.44±0.12
midbrain	160±40	167±16	192±40	1.45±0.24	1.74±0.33	1.79±0.38
diencephalon	164±25	193±24	193±25	1.46±0.18	1.63±0.1	1.75±0.41
telencephalon	81±8	98±3	78±18	0.82±0.16	0.86±0.04	0.74±0.18

Table 5.20 Activity of phenylethanolamine N-methyltransferase (PNMT) in rat brain - 3 months lead exposure.

Activities expressed as mean ± S.D. after exposure to 120 μM or 480 μM lead for 3 months. Each group consists of 8 animals in the control group and 6 in each lead treated group.

Brain region	PNMT activity (fmoles / mg tissue / hr.)			PNMT activity (pmoles / mg protein / hr)		
	Control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	64±15	57±10	72±17	0.50±0.14	0.28±0.06	0.43±0.17
midbrain	189±23	199±9	234±25 ++	1.13±0.25	1.09±0.20	1.24±0.15
diencephalon	210±37	216±84	243±60	1.13±0.28	1.20±0.38	1.31±0.29
telencephalon	87±22	84±15	89±14	0.40±0.08	0.42±0.08	0.42±0.11

\+ $p < 0.005$ compared to
++ $p < 0.02$ control group

Table 5.21 Activity of phenylethanolamine N-methyl-transferase (PNMT) in rat brain -7 months lead exposure.

Activities expressed as mean ± S.D. after exposure to 48 μM or 480 μM lead for 7 months. Each group comprises 6 observations.

Brain region	PNMT activity (fmoles/mg tissue/hr) Control	48 μM Pb	PNMT activity (pmoles/mg protein/hr) Control	48 μM Pb
cerebellum	66±14	68±18	0.52±0.12	0.54±0.10
midbrain	168±26	173±38	1.56±0.45	1.39±0.17
diencephalon	246±48	254±44	2.66±0.74	2.08±0.55
telencephalon	88±17	99±17	0.74±0.22	0.86±0.17

	control	480 μM Pb	control	480 μM Pb
cerebellum	157±30	176±56	1.30±0.39	1.39±0.40
midbrain	472±44	269±54 + @	3.59±0.41	2.64±0.43 ++ +++
diencephalon	414±97	250±24	3.74±0.86	2.23±0.32
telencephalon	222±43	180±28	1.80±0.78	1.70±0.40

+ $p < 0.005$
++ $p < 0.02$
+++ $p < 0.01$
@ $p < 0.002$

compared to control group

Table 5.22 Activity of phenylethanolamine N-methyltransferase (PNMT) in rat brain - pre- and post- weaning lead exposure.

Activities expressed as the mean ± S.D. after exposure to lead during the pre-weaning phase and for 1 month post-weaning. Each group comprises 6 observations.

Brain region	PNMT activity (fmoles / mg tissue / hr.)			PNMT activity (pmiles / mg protein / hr.)		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
cerebellum	48±10	38±4	46±3	0.35±0.08	0.31±0.07	0.39±0.08
midbrain	134±20	135±30	156±14	1.21±0.31	1.13±0.34	1.29±0.19
diencephalon	159±27	132±25	159±50	1.48±0.52	1.30±0.35	1.27±0.64
telencephalon	50±10	50±8	56±7	0.47±0.16	0.41±0.05	0.49±0.08

Table 5.23 Brain weights - 3 month lead exposure

Weight of each brain region of rats exposed to lead for 3 months compared to non-exposed control animals. The results are expressed as mean ± S.D. of 8 animals in each control group and 6 in each lead group.

brain region	brain weight (g)		
	control	120 μM Pb	480 μM Pb
cerebellum	0.27±0.04	0.29±0.04	0.29±0.07
midbrain	0.20±0.04	0.21±0.03	0.15±0.02
diencaphalon	0.24±0.08	0.27±0.09	0.22±0.05
telencephalon	0.92±0.13	0.90±0.12	0.90±0.09

Table 5.24 Brain weights -7 month lead exposure

Weight of each brain region of rats exposed to lead for 7 months compared to non-exposed control animals. The results are expressed as mean ± S.D. of 6 observations.

brain region	brain weight (g) control	48 μM Pb
cerebellum	0.28±0.03	0.28±0.04
midbrain	0.17±0.02	0.15±0.03
diencephalon	0.21±0.02	0.19±0.03
telencephalon	0.96±0.11	0.96±0.07

	control	480 μM Pb
cerebellum	0.26±0.04	0.30±0.01
midbrain	0.13±0.04	0.13±0.02
diencephalon	0.18±0.04	0.17±0.03
telencephalon	0.96±0.12	0.97±0.10

Table 5.25 Brain protein concentration - 1 & 3 months lead exposure post weaning

Protein concentration of brain regions of rats exposed to lead compared to non-exposed control animals. The results are expressed as the mean ± S.D. of 8 animals in each control group and 6 in the lead groups.

Group	Brain protein (mg/ml homogenate) control	120 µM Pb	480 µM Pb
1 month post-weaning			
cerebellum	12.2±2.2	11.6±1.9	12.9±0.6
midbrain	11.2±2.1	10.0±1.9	11.8±1.6
diencephalon	11.2±0.8	11.8±0.6	11.2±1.1
telencephalon	10.2±1.3	11.4±0.9	11.6±0.9
3 month post-weaning			
cerebellum	19.4±3.0	20.6±0.9	17.4±2.4
midbrain	16.9±3.9	17.0±0.8	18.3±1.0
diencephalon	20.1±3.7	19.5±2.4	18.7±1.0
telencephalon	20.1±1.5	19.9±1.4	19.7±3.3

Table 5.26 Brain protein concentration - 7 month lead exposure

Protein concentration of brain regions of rats exposed to lead for 7 months compared to non-exposed control animals. The results are expressed as the mean ± S.D. of 6 observations.

group	brain protein (mg/ml homogenate) control	48 μM Pb
7 month post-weaning		
cerebellum	18.6±3.9	21.4±5.0
midbrain	15.3±5.0	18.9±2.7
diencephalon	18.8±2.6	19.8±2.3
telencephalon	19.8±4.6	17.9±2.8

	control	480 μM Pb
7 month post-weaning		
cerebellum	15.7±3.0	18.5±3.8
midbrain	12.0±2.8	12.2±3.1
diencephalon	11.7±2.6	13.3±2.9
telencephalon	14.0±2.9	11.5±1.8

Table 5.27 Brain protein concentration -pre- & post-weaning

Protein concentrations of brain regions of rats exposed to lead in utero, pre- and post- weaning compared to non-exposed control animals. Results are expressed as mean ± S.D. of 6 observations.

group	brain protein		
	control	480 μM Pb	1930 μM Pb
pre- & post-weaning			
cerebellum	12.1±2.1	13.2±1.8	11.8±2.7
midbrain	11.7±1.2	12.3±1.8	12.2±1.8
diencephalon	11.1±2.2	10.5±1.2	10.7±2.5
telencephalon	10.8±1.9	12.0±1.5	11.8±2.2

5.4 Discussion

The data presented throughout this chapter would support the studies in the literature observing a complex effect or effects of lead on the catecholaminergic nervous system which is manifest in several parameters. Over and above this, the results also indicate that even under the same conditions, such as exposure route, method of animal sacrifice, and assay conditions, the effect of lead is not uniform and is observed differently depending on the degree and duration of exposure.

The observed differences in all the parameters between the various experiments presented in this chapter may either be the result of a multisite mode of action of lead, or alternatively, they may be the result of secondary effects of lead occurring as a result of a primary action of lead. In other words, one or more of the observed 'lead-related' effects may be compensatory effect(s) coming into play as a result of one or more primary actions. Looking at all the experiments reported in this chapter, there is a wealth of evidence indicating that there are differences in lead related effects, either primary or secondary, depending on the degree of exposure. Homeostasis may well play a vital role in attempting to correct the aberrations caused be lead. The data presented in this chapter certainly indicates an effect of lead on catecholaminergic transmission. However exactly where the primary biochemical sites of action are remains rather hazy. The neurotoxic action was manifest in several parameters studied in these experiments; the levels of neurotransmitters, noradrenaline

and dopamine, in addition to alterations in the activity of the rate limiting enzyme tyrosine hydroxylase and in PNMT. Taken as a whole, these findings provide strong evidence of an effect of lead on catecholaminergic transmission. However the data provides further information as to the effect of lead on this system. What is certain, is that the effect of lead is regionally specific within the brain. When one investigates the cerebellum, midbrain, diencephalon and telencephalon, it is the midbrain and diencephalon which exhibit the most changes in the various parameters. It is noteworthy that these are the same two regions which appeared to be the target regions with regard to the cholinergic system (chapter 4).

It is not surprising, considering the data presented in this chapter, that there exists such a lack of agreement in the literature with regard to the catecholaminergic nervous system and lead. There would appear to be a difference in the manifestation of the lead effect, depending on the duration and degree of exposure. Such observations strongly suggest that homeostatic mechanisms are coming into play to attempt to correct the pathologies. This of course complicates the situation and makes it even more difficult to identify the primary site of action of lead.

With regard to the activity of the rate limiting enzyme, tyrosine hydroxylase, significant lead related alterations in activity were indeed noted in this study. However both enhancement and suppression of activity were observed. These changes may well be related to alterations in tetrahydrobiopterin metabolism. However, not enough is as

yet known about the factors, including BH_4, which are involved in controlling tyrosine hydroxylase activity in order to speculate about this association.

Whether or not these observed effects on catecholaminergic function are related or can be attributed to the influence of the metal on tetrahydrobiopterin metabolism (chapter 6) remains to be determined. It is most certainly a possible mechanism of action which should be considered seriously since this cofactor plays an important role in the biosynthesis of these essential neurotransmitters. The data presented in this chapter cannot be directly compared with other studies unless they employed similar dosage regimes. Our data indicates a varying effect of lead depending on the dosage and duration of exposure, suggesting a complex effect or effects of lead on this system.

Many studies in the literature are confounded even more by so called non-specific effects such as under-nutrition. We have to a large extent eliminated these effects. There were no lead related reductions in brain weight between the various lead groups (tables 5.23, 5.24). In addition brain protein concentration was not altered in any group of lead treated animals by comparison with their respective non-exposed controls (tables 5.25 - 5.27). However, those animals exposed to 1930μM lead both pre- and post-weaning and in utero did not gain weight to the same degree as the non-exposed rats or indeed the animals exposed to the lower lead dose (480μM) -table 5.1. This reduction in total body weight would appear to be a feature of the group of rats

exposed to lead for a short period of 1 month post-weaning. Lengthening the exposure period allowed the lead exposed animals to gain weight since those groups exposed for longer periods of time were not significantly reduced in weight.

Although all lead exposed groups of animals absorbed lead, as shown by its accumulation in bone, there is evidently a complex movement of lead occurring between the brain and other body compartments since not all groups of exposed animals exhibited significantly elevated brain lead concentration compared to their respective controls. The animals exposed for the relatively brief period of 1 month either with or without prior exposure in utero, exhibited significantly elevated brain lead levels compared to controls. In addition, it should be noted that this uptake was non-selective; that is all brain regions studied showed uptake of the metal. However, extending the period of exposure resulted in no enhancement in lead uptake in any brain region. Short term lead absorption as manifest by blood lead concentration showed that all lead exposed groups were absorbing the metal into the bloodstream although, as was the case with bone lead, the relationship between exposure and concentration of lead was not linear.

From the foregoing data and discussion one can conclude that although there are certainly lead related effects on catecholamine function in specific brain regions, the primary sites of action remain undetermined. Such effects are evident at even low levels of lead exposure and after only a brief period of exposure. Much further studies will

have to be performed and a better understanding of the control of catecholamine synthesis will be required before one can pin point the biochemical lesion.

CHAPTER 6

EFFECT OF LEAD ON TETRAHYDROBIOPTERIN (BH_4) METABOLISM

6.1 General Introduction

L-Erythro-tetrahydrobiopterin (2-amino-4-hydroxy-6-L-erythro-dihydroxypropyl-5,6,7,8-tetrahydropteridine; BH_4) functions as the cofactor for several mixed function oxygenase enzymes in mammalian systems. To date the only well defined roles of this pteridine are: Firstly, as a cofactor for tyrosine hydroxylase (tyrosine-3-monooxygenase, E.C. 1.14.16.2) which converts the amino acid tyrosine into dihydroxyphenylalanine (DOPA; Nagatsu et al, 1972; Levitt et al, 1965): Secondly, tetrahydrobiopterin also serves as a cofactor for another enzyme of catecholamine synthesis, namely, phenylalanine hydroxylase (phenylalanine-4-monooxygenase, E.C. 1.14.16.1) which functions to form tyrosine from its precursor, phenylalanine (Kaufman, 1958; 1959; 1963). A fuller discussion of these two enzymes in catecholamine synthesis can be found earlier in this thesis (chapter 5). And, thirdly, the role of tetrahydrobiopterin as a cofactor is in the conversion of tryptophan into 5-hydroxytryptophan catalysed by the enzyme tryptophan hydroxylase (tryptophan-5-monooxygenase, E.C. 1.14.16.4), the role being described in the 1960's (Hosoda & Glick, 1966; Lovenberg, Jequier & Sjoerdsma, 1967). It is evident from the foregoing that tetrahydrobiopterin plays an important part in the formation of catecholamines, since tyrosine hydroxylase is the rate controlling enzyme in this pathway (Levitt et al, 1965). There are two types of mechanism by which the activity of tyrosine hydroxylase can be regulated in vivo, namely, by synthesis of new enzyme molecules, and by

alteration of the activity of existing molecules. Although the synthesis of new molecules is effective at increasing tissue enzyme levels, the process is relatively slow since the new molecules once produced in the cell body must then be transported down the axon to the nerve terminal. A more rapid technique involves alteration of the kinetic parameters of the enzyme. In these terms there are several factors which may alter the rate of hydroxylation of tyrosine-

1. The concentration of apoenzyme - as discussed this can only be altered relatively slowly.

2. Availability of molecular oxygen (Nagatsu et al, 1964a)

3. Concentration of the substrate, tyrosine

4. Concentration of the cofactor, tetrahydrobiopterin

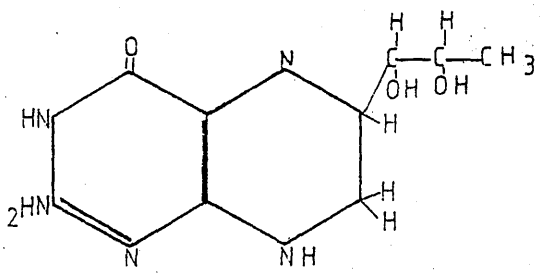
5. Activity of dihydropteridine reductase, an enzyme involved in tetrahydrobiopterin metabolism.

The tissue concentration of tyrosine is close to or greater than the K_m for the enzyme, and hence the concentration of this substrate is probably not normally rate limiting for the enzyme in vivo (Lovenberg & Victor, 1974). However, in contrast to the concentration of cofactors for dopamine-B-hydroxylase and phenylethanolamine-N-methyl transferase which appear to be above saturation, the concentration of tetrahydrobiopterin is below the K_m for both the high affinity tyrosine hydroxylase (24μm) and the low affinity enzyme (135μm) - (Abou-Donia & Viveros, 1981). Considering this data, it would appear that the concentration of tetrahydrobiopterin

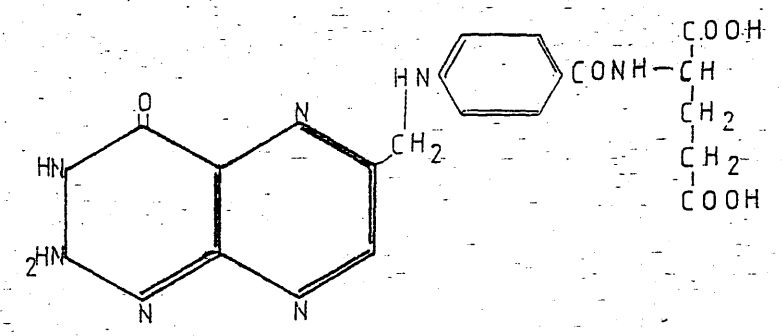
is the rate limiting factor for tyrosine hydroxylase activity. Evidence to support this hypothesis exists both in vitro (Musacchio, D'Angelo & McQueen, 1971), and in vivo (Kettler, Bartholini & Pletscher, 1974). Thus changes in tyrosine hydroxylase activity may arise as a result of modifications in the availability of reduced pteridine cofactor at the enzyme site (Musacchio et al, 1971).

The structure of tetrahydrobiopterin is shown in figure 6.1 which also details folic acid for comparison since the two differ only at carbon number 6. Despite this structural similarity between the two compounds, both containing a 2-amino-4-hydroxy pteridine moiety, and the fact that they are both derived from a common precursor, guanosine triphosphate (GTP), this is where the connection ends with folate and tetrahydrobiopterin having quite different roles in mammalian cells. In addition, mammalian cells have lost the ability to synthesis folates and consequently it is required as an essential vitamin. In contrast, the capability to synthesis tetrahydrobiopterin has been retained by the brain, adrenals and other tissues where metabolism depends on its presence.

The function of tetrahydrobiopterin is shown diagramatically in figure 6.2. During the hydroxylation reactions, the cofactor donates two hydrogen atoms to form water together with an atom of oxygen derived from molecular oxygen; the other oxygen atom being transferred to the substrate, phenylalanine, tyrosine or tryptophan; that is the tetrahydrobiopterin serves as an electron donor in the reaction. The actual mechanism of the reaction is a matter



5,6,7,8-TETRAHYDROBIOPTERIN



FOLIC ACID

Figure 6.1 Chemical structure of tetrahydrobiopterin and folic acid.

The diagram serves to illustrate the structural similarity between folic acid and tetrahydrobiopterin.

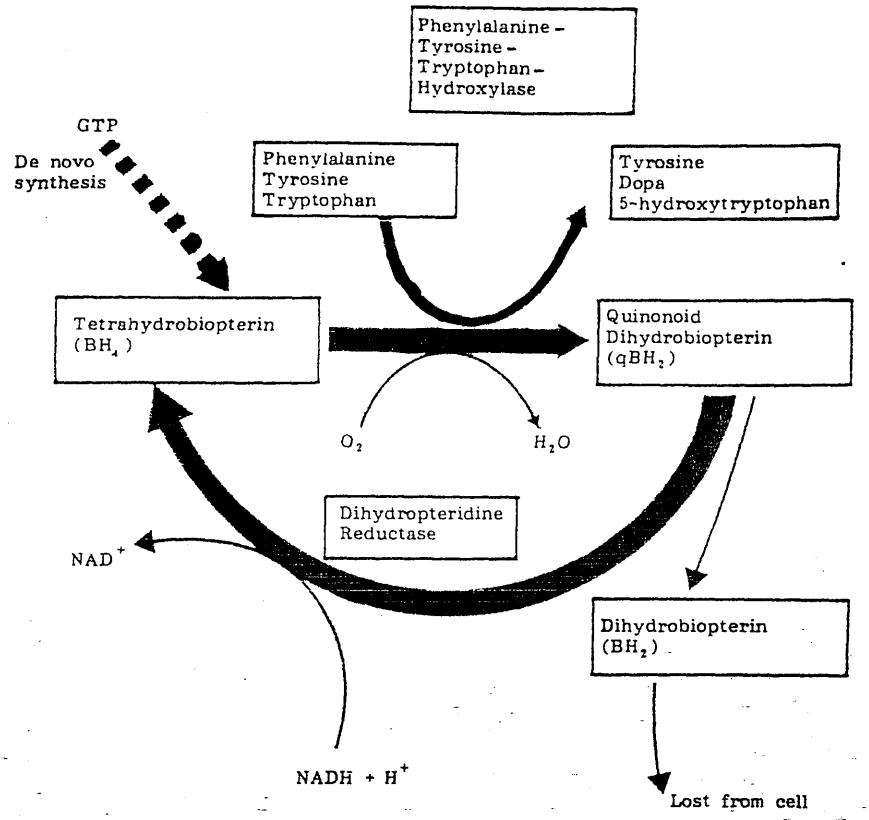


Figure 6.2 Tetrahydrobiopterin metabolism.

Tetrahydrobiopterin acts as essential cofactor in the hydroxylation of phenylalanine, tyrosine and tryptophan. Synthesis of the cofactor is by a de novo pathway from guanosine triphosphate and also via a salvage route catalysed by dihydropteridine reductase.

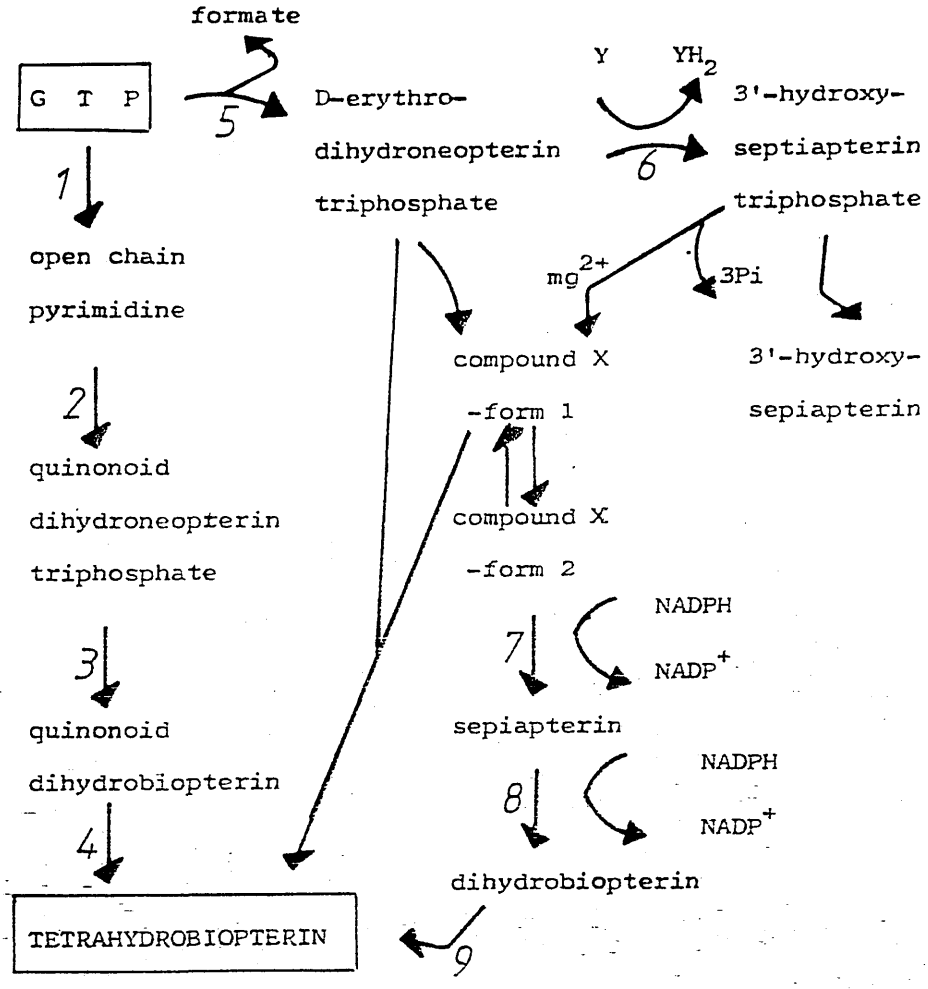
of some debate. However, during the reaction, the cofactor is converted via one or more unstable intermediates into quinonoid dihydrobiopterin (qBH_2) which can then rearrange into the more stable compound dihydrobiopterin (BH_2). However, a salvage pathway exists in order to maintain cellular tetrahydrobiopterin levels. Quinonoid dihydrobiopterin can be reduced once more to tetrahydrobiopterin, a reaction catalysed by dihydropteridine reductase (DHPR; E.C. 1.6.99.7; Craine et al 1972;) - figure 6.2. This reaction also necessitates the presence of reduced nicotinamide adenine dinucleotide (NADH). Dihydropteridine reductase is a common enzyme in mammalian cells, and tissues generally contain sufficient enzyme to maintain the cofactor in the fully reduced state (Craine et al, 1972). Polyacrylamide gel electrophoresis has demonstrated that dihydropteridine reductase is a dimer of molecular weight 41-42,800 which agrees well with the data of Scrimgeour and his colleagues who observed each identical subunit to have a molecular weight of 27,000. Quinonoid dihydrobiopterin is a relatively unstable species and will spontaneously rearrange into the more stable dihydrobiopterin, a reaction which will occur in both acidic and basic buffers. (Kaufman, 1967). The resultant compound, dihydrobiopterin will be mainly lost from the cell but a small proportion can be re-converted back into the active cofactor, a reaction catalysed by dihydrofolate reductase (figure 6.2).

Tetrahydrobiopterin is widely distributed in mammalian tissues (Baker et al, 1974; Leeming et al, 1976a; Fukushima & Nixon, 1980; Duch & Nichol, 1983) being found in many tissues where its function is far from clear. Analysis of several rat tissues has demonstrated high levels to be present in brain, pineal gland, pituitary gland, adrenal glands, ovary, thyroid gland and spleen. Analysis of brain regions demonstrates a differential distribution of the cofactor throughout the organ (Leeming et al, 1976b; Bullard et al, 1978; Levine, Kuhn & Lovenberg, 1979), a distribution consistent with the variation observed in neuroblastoma lines (Albrecht et al, 1978). As mentioned previously, mammalian cells have retained the ability to synthesize tetrahydrobiopterin. This is in fact essential since although biopterins are present in normal diets, the fully hydrogenated biopterin is poorly absorbed by man (Blair, Ratanasthien & Leeming, 1974; Leeming, 1975; 1979), although when administered parentally the cells are capable of retention. However, tetrahydrobiopterin will rapidly dehydrogenate into dihydrobiopterin and subsequently into biopterin (Blair & Pearson, 1974). Kettler and his colleagues (1974) have demonstrated that when administered intravenously, tetrahydrobiopterin is not capable of entering the brain and thus this tissue at least, having an essential requirement for the cofactor, must synthesize it within the cell. Further evidence for the synthesis of tetrahydrobiopterin is derived from the observation that rats fed for several generations on a biopterin-free diet develop normally and continue to excrete 33µg/day

biopterins in their urine (Leeming, 1975). In spite of a common pterin ring between folates and biopterins, there is no evidence for interconversion between the two groups of compounds. Urine and serum biopterin levels are not affected by folate status nor altered by the administration of excess folates (Fleming & Broquist, 1967; Fukushima & Shiota, 1972; Leeming et al, 1976b).

From the foregoing discussion and the data presented in figure 6.2, it is evident that the cellular concentration of tetrahydrobiopterin is maintained by two mechanisms. Firstly, de-novo synthesis from guanosine triphosphate, and secondly in addition by way of either the dihydropteridine reductase catalysed reduction of quinonoid dihydrobiopterin or via the minor contribution from dihydrofolate reductase catalysed reduction of the more stable dihydrobiopterin. Although there is not direct interconversion between folate and biopterin, this represents a curious relationship between their metabolism. In addition, the enzyme methylenetetrahydrofolate reductase can reduce quinonoid dihydrobiopterin although not as rapidly as dihydropteridine reductase (Kaufman, 1967; Mathews & Kaufman, 1980). Since the relatively recent awareness that tetrahydrobiopterin is derived from guanosine triphosphate in mammalian cells (Fukushima & Shiota, 1974) much interest in the biosynthetic path has been shown. The original sketchy route proposed by Gal and his co-workers (Gal et al, 1978) - figure 6.3 was initally accepted (Leeming, 1979; Leeming, Pheasant & Blair, 1981) but has since been challenged on numerous occasions, although an agreed alternative pathway

has not been proposed as yet. Curtuis et al (1983a) have reviewed the proposed pathways suggested by various authors. The work of Gal suggested the crucial step in the biosynthesis involved the conversion of guanosine triphosphate into D-erythro-7,8-dihydroneopterin triphosphate via an open chain pyrimidine intermediate (2-amino-6(5-triphosphoryl-benzyl)-amino-5-formamide-6-hydroxy-pyrimidine), the first reaction being catalysed by guanosine triphosphate cyclohydrolases A1 and A2, and D-erythro-7,8-dihydroneopterin triphosphate synthetase being responsible for the cyclisation. L-erythro-7,8-dihydro-biopterin is subsequently formed by the appropriate synthetase enzyme. Finally, the active pteridine, tetra-hydrobiopterin is produced by a dihydropteridine reductase catalysed reaction (figure 6.3). This scheme of reaction steps was formulated using rat brain as the tissue. No further evidence exists to corroborate the formation of an open chain pyrimidine intermediate. It is now proposed that there may well exist two pathways being studied by various research groups as the 'de-novo' biosynthetic path, although various pieces of evidence suggest that either some of the proposed intermediates are artifacts of the isolation procecures employed, or, alternatively one of the paths is not per se the 'de-novo' route of synthesis and as such has been termed a 'salvage path' (Nichel et al, 1983). The scheme proposed by Tanaka et al (1981) is shown in figure 6.3. In contrast to the proposals of Gal and his colleagues, the first reaction of this pathway, catalysed by guanosine triphosphate cyclohydrolase forms D-erythro-



KEY TO ENZYMES -

1	cyclohydrolase
2	dihydroneopterin triphosphate synthetase
3	dihydrobiopterin synthetase
4	dihydropteridine reductase
5	GTP cyclohydrolase
6	fraction A2
7	fraction A1
8	sepiapterin reductase
9	dihydrofolate reductase

Figure 6.3 Proposed pathways from guanosine triphosphate to tetrahydrobiopterin.

The diagram illustrates the proposed pathways for de novo synthesis of tetrahydrobiopterin. The pathway down the left hand side of the figure represents the proposals of Gal et al (1978). Tanaka et al (1981) put forward the pathway shown on the right hand side of the figure, and finally Nichol et al (1983) proposed the rather sketchy route shown down the middle of the diagram.

7,8-dihydroneopterin triphosphate directly. This work was carried out in chicken liver but supporting data has been obtained by other workers using rat liver (Dhont et al, 1983; Blau & Neiderweiser, 1983) and chicken liver (Fukushima, Richter & Shiota, 1977). Debate exists as to whether the product of guanosine cyclohydrolase exists as the quinonoid form (Gal et al, 1978) or not (Yim & Brown, 1976; Fukushima et al, 1977). However it is generally accepted that in these tissues at least, no intermediate exists. It has been suggested that this may be an important regulatory step of the pathway (Viveros et al, 1981). The pituitary gland may well exert a regulatory role on tetrahydrobiopterin synthesis. Treatment with reserpine or insulin-induced hypoglycaemia results in stimulation of adrenocorticotrophic hormone (ACTH) production (Gaunt, Chart & Renzi, 1963) with a resultant rise in guanosine triphosphate cyclohydrolase activity and tetrahydrobiopterin levels in rat adrenal cortex (Abou-Donia & Viveros, 1981; Viveros et al, 1981). This effect is by way of enzyme induction since it can be blocked by the protein synthesis inhibitor, cyclohexamide. Tanaka et al (1981) fractionated their chicken liver into three fractions designated A1, A2 and B. Fraction A2, a magnesium dependant fraction, catalysed the conversion of D-erythro-dihydroneopterin triphosphate into a compound designated X. This reaction occurred via several intermediates. Firstly, 3'-hydroxysepiapterin triphosphate was formed, a step which required an unknown prosthetic group which was simultaneously reduced ($Y \rightarrow YH_2$ - figure 6.3). Subsequently this

species was converted into X, the keto and enol forms of which are probably in equilibrium with each other. The second step was the magnesium dependant stage of the reaction. The incorporation of 3'-hydroxy-sepiapterin triphosphate as an intermediate is supported by the work of Niederwieser et al (1980). Although the exact nature of the labile species X is uncertain, it is capable of degradation into pyruvate and pterin and is proposed to be 6-(1,2-dioxopropyl)-dihydropterin (Tanaka et al, 1981). According to this scheme, compound X is reduced to sepiapterin (6-lactyl-7,8-dihydroxypterin), a reaction catalysed by fraction A1 and requiring the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The nature of X is uncertain, and indeed the work of Dorsett, Flanagan & Jacobson (1982) suggests the existence of more than one intermediate 'X'. Subsequently, sepiapterin reductase (E.C. 1.1.1.153), another NADPH requiring enzyme present in fraction B, is responsible for the conversion of sepiapterin into dihydrobiopterin. The terminal step, the conversion of dihydrobiopterin into the active cofactor is then mediated by dihydrofolate reductase (Spector et al, 1975). Evidence exists however challenging the involvement of 7,8-dihydrobiopterin and sepiapterin in the de-novo biosynthetic pathway of tetrahydrobiopterin. Although methotrexate can completely inhibit dihydrofolate reductase in mammalian cells in culture, tetrahydrobiopterin levels are not reduced (Duch et al, 1982a; Nichol et al, 1983). Additionally, the distribution of sepiapterin reductase in mammalian cell lines does not parallel that of tetrahydro-

biopterin or guanosine triphosphate cyclohydrolase (Duch & Nichol, 1983) and no correlation is observed between the ability of cells to convert 7,8-dihydrobiopterin into tetrahydrobiopterin with the levels of the cofactor, or the activity of guanosine triphosphate cyclohydrolase. In contrast, the activity of the two enzymes dihydrofolate reductase and 7,8-dihydrobiopterin reductase parallel each other (Duch & Nichol, 1983). Although not probably the sole path of tetrahydrobiopterin biosynthesis, this route termed the 'pterin salvage path' (Nichol et al, 1983), may well exist in certain cells or situations. An alternative true 'de-novo' path independent of dihydrofolate reductase and methotrexate insensitive has been proposed (Nichol et al, 1983). This scheme (figure 6.3) has not been elucidated but proposes the conversion of 7,8-dihydroneopterin triphosphate, formed directly from guanosine triphosphate by the same cyclohydrolase, into tetrahydrobiopterin via several labile intermediates of which one may well be the compound labelled X. The important feature of this path however is its insensitivity to inhibition by dihydrofolate reductase inhibitors.

From the foregoing discussion it may well be that the biosynthetic pathway proceeds by different routes depending on the tissue concerned. If this is the case the properties of rat brain enzymes as observed by Gal and co-workers, may be quite distinct from those of chicken and rat liver or kidney. First of all in the rat cerebral system, the conversion of guanosine triphosphate to D-erythro-dihydroneopterin triphosphate is a two step process

requiring either A1 or A2 and enzyme B. In the chicken liver, this process is catalysed by a single enzyme, does not require magnesium and shows no free intermediates. In addition, the cerebral system catalyses the conversion of D-erythro-dihydroneopterin triphosphate to dihydrobiopterin by a single enzyme, and furthermore does not require any cofactors. In contrast, in chicken kidney this conversion requires at least three enzymes, each needing a cofactor. At present it is not possible to exclude one or more of the proposed schemes and an open mind concerning the details of tetrahydrobiopterin biosynthesis will require to be kept.

Consideration of the behavioural and cognitive effects of lead both at relatively high levels (Rutter, 1980), and the known consequences of deranged tetrahydrobiopterin metabolism, led to the proposal to investigate the effect of lead on the levels of such an important cofactor and its metabolism. There are numerous clinical states documented in the literature in which tetrahydrobiopterin synthesis or degradation is altered (Leeming et al, 1981). Any defect in the metabolism of tetrahydrobiopterin which leads to a decreased concentration of the cofactor in the brain will impair neurotransmitter biosynthesis and may thus have neuropathological consequences. One of the most serious derangements in this category is malignant hyperphenylalanaemia which unlike the common form of the disease classical phenylketonuria in which the patient has a gross deficiency of phenylalanine hydroxylase, the subject has a reduced synthesis of tetrahydrobiopterin. The prognosis for these patients is poor, and progressive neurological

disease with lowered neurotransmitter production ensues unless treated with daily supplements of tetrahydrobiopterin. This reduction in the cofactor can arise from a failure of synthesis, or a failure to salvage quinonoid dihydrobiopterin by dihydropteridine reductase. Examples of both of these types of inherited disorder have been reported (Kaufman et al, 1975; Leeming et al,1976c). These two types of malignant hyperphenylalaninia exhibit differing levels of biopterin derivatives. Reduction in the de-novo synthesis of the cofactor leads to a lowering of plasma biopterin derivatives, whilst a reduced activity of dihydropteridine reductase has the effect of raising the level of plasma biopterins although in both types the tissue levels of the active cofactor are reduced. Reports suggest however, that the neurological sequelae of the form resulting from a reduced salvage pathway are more profound (Brewster et al, 1979; Rey et al, 1980). Although classical phenylketonuria is due to a deficiency of phenylalanine hydroxylase leading to a massive increase in the tissue concentration of phenylalanine and of phenylpyruvate in the plasma, it has been suggested that there may be an inhibition of dihydropteridine reductase by phenylpyruvate in vivo (Leeming et al, 1981), although alternative suggestions have been proposed (Kaufman, 1976; Youdim, 1979). The hypothesis that phenylpyruvate inhibits this enzyme is supported by data showing raised levels of biopterin derivatives in patients on a normal diet compared to those on a phenylalanine restricted diet (Leeming et al, 1981). These patients present with a picture of increased

plasma biopterins (Leeming et al, 1976a; Dhondt et al, 1981a).

Biopterin metabolism appears to be disturbed in a number of other disease states. Active coeliac disease results in a lowering of serum biopterins (Leeming et al, 1976b; Leeming & Blair, 1980; Leeming et al, 1981), an observation also evident in malignant disease (Leeming & Blair, 1980; Rokos et al, 1980) and senile dementia (Leeming, Blair & Melikian, 1979). Patients with carcinoid disease have been shown to exhibit an increased neopterin concentration in relation to biopterin both in urine and serum (Dhont et al, 1981b) along with 6-formyl-pterin and 6-hydroxypterin appearing in the urine (Stea et al, 1978). Senile dementia is associated with an alteration in tetrahydrobiopterin metabolism. Serum dihydrobiopterin levels are reduced (Leeming et al, 1979) and brain tetrahydrobiopterin levels are lower. This lowering of cofactor levels in the brain may then result in a depression of catecholamine synthesis.

Reduced levels of tetrahydrobiopterin in cerebrospinal fluid (CSF) have been reported in a variety of neurological diseases such as Parkinson's disease (Lovenberg et al, 1979; Williams et al, 1980), Alzheimer's disease, Steel-Richardson syndrome, Huntington's chorea (Williams et al, 1980), and dystonia (Williams et al, 1979). In the case of Parkinson's, a reduced level of active cofactor has been demonstrated specifically in the caudate nucleus (Nagatsu et al, 1981). It must be noted that CSF levels of active cofactor are more representative of the levels likely to be found in the brain than the serum values.

Raised biopterin derivatives have also been observed in the blood of patients with gout, uraemia and alcoholic liver disease (Baker et al, 1974) and general kidney dysfunction (Leeming, 1975; Leeming et al, 1976b). A reduction in the biopterin content of the urine of rheumatoid arthritic patients has been observed (Leeming et al, 1976b). Whilst there is an increase in the urine from psychiatric diagnosed depressed patients (Duch et al, 1982b), schizophrenics show no alteration in either urinary biopterin or neopterin, or CSF biopterin content (Garbutt et al, 1982). Other studies however have suggested that depression is characterised by an underproduction of tetrahydrobiopterin (Curtuis et al, 1982; 1983b). Finally, drugs used to treat various conditions can affect biopterin metabolism. Methotrexate used to treat acute lymphoblastic leukemia can produce neurological damage (Meadows & Evans, 1976; Eiser,1978) and such patients have markedly raised serum biopterin levels (Leeming & Blair, 1980), an effect which may be the result of an inhibition of dihydropteridine reductase by methotrexate, an action previously observed in vitro (Craine et al, 1972). Amphetamine is also capable of causing a reduction in biopterin levels in rat straitum, possibly due to the uncoupling of tyrosine hydroxylase and dihydropteridine reductase (Mandell, 1978). Other psychotropic drugs however are not capable of a similar effect. Aluminium ions are well documented as a causal agent of dialysis dementia (Alfrey, Le Gendre & Kachny, 1976, McDermott et al, 1976) and this metal is also known to inhibit quinonoid dihydrobiopterin salvage in

vitro (Leeming & Blair, 1979) at concentrations close to those found in brains of such patients.

Similarly, altered activity of dihydropteridine reductase has been observed in pathological disease states. Dhont et al (1981b) observed a positive correlation between dihydropteridine reductase activity and oestrogen receptors in human breast tumours, and the enzyme activity was significantly increased over normal in this neoplastic breast tissue. No significant increase was observed however in neoplastic tissue from the large intestine (Eggar et al, 1983).

The essential role of tetrahydrobiopterin in production of neurotransmitters must be clear from the dire consequences of its absence in these rare and sometimes not so rare conditions just described, either when tetrahydrobiopterin synthesis is defective, or dihydropteridine reductase activity reduced or non existent.

Lead has been implicated as an environmental agent that can act at more than one site of tetrahydrobiopterin metabolism, both in the de-novo pathway of biosynthesis, and the salvage path. Purdy et al (1981) demonstrated inhibition of tetrahydrobiopterin synthesis in a rat brain homogenate system 'in vitro', and dihydropteridine reductase activity from both rat brain and liver was irreversibly inhibited. Lead, at a concentration of 1nM, produced a 25% reduction in the synthesis of tetrahydrobiopterin, and at higher concentrations of 10μM the synthesis was only 55% of the control value. The effect on dihydropteridine reductase occurred at slightly higher

lead concentrations. The enzymatic activity was significantly ($p<0.05$) inhibited by about 14% in the presence of 1μM lead, and much higher concentrations were capable of a 50% inhibition. These results must be considered in terms of tissue lead levels encountered in man and consideration that the data was obtained in vitro. Additionally, a significantly decreased level of serum biopterins has been observed in subjects with clinically expressed lead poisoning (Leeming & Blair, 1980). Acute exposure to lead in rats (0.3mg/kg body weight injected intra-peritoneally every 48hr) has been shown to produce a significant fall in serum biopterins after 2,4 and 6 days (Hilburn ,1970).

These studies would suggest that lead has indeed an effect on tetrahydrobiopterin metabolism. This chapter reports data obtained from a series of experiments both in man and the rat to try to elucidate the mode of action of lead on tetrahydrobiopterin metabolism.

6.2 Studies with Crithidia fasciculata

6.2.1 Introduction

The range of serum biopterin derivatives observed under normal physiological conditions is narrow (Baker et al, 1974; Leeming, 1975; Leeming et al, 1976b), an observation suggesting the existence of mechanisms operating to maintain the levels within a close range. Various pathological states result in the manifestation of altered serum biopterin levels as discussed in the general introduction. The term 'biopterin derivatives' or 'serum biopterins' will be used in this thesis to signify the

conglomerate of biopterin and its biologically active reduced derivatives, dihydrobiopterin and tetrahydrobiopterin.

Serum biopterins can be measured by several techniques, such as radio-immuno assay (Nagatsu et al, 1979b), by an enzymatic assay using the phenylalanine hydroxylase system (Guroff, Rhoads & Abramovitz, 1967; Kaufman et al, 1978), by the bioassay using Crithidia fasciculata (Danks, Cotton & Schlesinger, 1975; Leeming et al, 1976b) or by chromatographic determination using high pressure liquid chromatography (Fukushima et al, 1978; Stea et al, 1980). Crithidia fasciculata has an absolute requirement for exogenous pteridines, different pteridines exhibiting a varying degree of stimulation of growth. Relative activities of various pteridines are as follows:-

Biopterin	100%
7,8-dihydrobiopterin	100%
tetrahydrobiopterin	80%
sepiapterin	100%
L-neopterin	56%
D-neopterin	0.2%

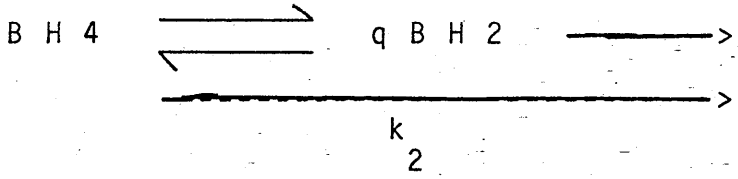
-values being quoted relative to biopterin (Leeming, 1975; Blair et al, 1983).

Thin layer chromatography has demonstrated that quantitatively the most important biopterin derivative in human serum is dihydrobiopterin (Leeming et al, 1976b). Bioautoradiography shows the absence of sepiapterin (Blair et al, 1983), and although neopterin is present it mainly exists as the D-erythro form. Thus in essence the Crithidia

fasciculata assay will measure the aggregate of biopterin, dihydrobiopterin and tetrahydrobiopterin present in the serum or plasma, with the majority being present as the dihydro form.

If one considers the kinetics of tetrahydrobiopterin metabolism, two reactions can be described as in equations 6.1 & 6.2, which have rate constants K_1 and K_2 respectively (Leeming, 1979).

Equation 6.1



Equation 6.2

Since $K_2 << K_1$ and dihydropteridine reductase is present in excess (Craine et al, 1972), K_2 represents the rate determining factor for the appearance of dihydrobiopterin in serum, and only exceptionally will K_1 be rate limiting. Assuming only one reaction process alters at a time, changes in the levels of dihydrobiopterin in blood and tetrahydrobiopterin in the cell may be grouped under four headings:-

1. Dihydropteridine reductase inhibition

The resultant manifestation will be an increase in dihydrobiopterin formed and therefore as the rate limiting step, an increase in dihydrobiopterin in the serum. Cell levels of active cofactor will simultaneously fall.

2. Enhanced activity of dihydropteridine reductase

This will result in a reduction in dihydrobiopterin in serum and an increase in cellular tetrahydrobiopterin.

3. Reduction in cofactor synthesis via de-novo routes

The level of intracellular cofactor will fall. In order to effect the rate of dihydrobiopterin appearance, the rate of synthesis must be very low; that is K_1 must become rate limiting. At this point the rate of cofactor production will be slow and hence serum biopterins will be low.

4. Enhanced tetrahydrobiopterin synthesis

This will not affect the rate of dihydrobiopterin appearance in serum since this is not the rate limiting step. However intracellular cofactor levels will rise.

Consequently, alteration in serum biopterins may be interpreted to elucidate alterations in tetrahydrobiopterin metabolism. It must be remembered that the main biopterin derivative in serum is dihydrobiopterin since many of the arguements presented in this chapter depend on this observation.

Two series of experimental data are reported in this section. Firstly, a study carried out in man exposed to lead in the environmental range, or at industrial levels, and secondly, experiments carried out in the rat.

6.2.2 Human studies

6.2.2.1 Methods

Blood samples received in the laboratory for lead analysis were collected and plasma biopterins measured in the same blood sample; blood lead and Crithidia active biopterins being analysed as detailed in the general methodology section. Details concerning each patient such as sex, age and possible exposure to lead at work, if applicable, were also noted.

6.2.2.2 Results

The distribution of blood lead concentrations observed in the blood samples obtained from the 146 subjects in the study (44 adults males, 47 adult females, 55 children of which 22 were female and 33 male; all children being under the age of 10 and the oldest adult being 76) is shown in figure 6.4. This markedly skewed distribution has a geometric mean of 1.02μM with deviation 1.86μM. There is a greater concentration of lead in the blood of the adult male subjects (geometric mean ± deviation of 1.42 ± 1.67μM) than in the adult females (0.78 ± 1.68μM), this difference being significant at the 5% level by Mann-Whitney U test, the group of children as a whole having a geometric mean of 0.97 ± 1.96μM. This differential sex distribution is shown graphically in figure 6.5. In contrast, the distribution of serum biopterins as measured by Crithidia fasciculata does not exhibit such a skewed distribution (figure 6.6), and the difference between the sexes is not nearly as marked, although females do tend to show slightly reduced plasma biopterin levels compared to males (figure 6.7).

The arithmetic mean ± standard deviation for adult males and adult females being 1.8 ± 0.5ng/ml and 1.4 ± 0.3ng/ml respectively. The level of serum biopterins observed in the children being 1.6 ± 0.5ng/ml.

Figures 6.8 - 6.11 illustrate the plot of blood lead concentration against plasma biopterin concentration for the group of subjects as a whole, adult males, adult females and children alone respectively. The Spearman Rank correlation coefficient was calculated in each case and the values are tabulated in table 6.1 which also shows the level of significance for each coefficient.

Figure 6.8 illustrates that there is a positive correlation between these two variables. When the sample as a whole was divided into four groups according to blood lead concentration, the mean plasma biopterins concentration significantly increased with blood lead concentration (table 6.2), supporting the relationship between the two variables. However, the relationship becomes less clear when the group is divided into groups of smaller numbers by way of sex and age (table 6.1). The reason for this observation may well be the small number of subjects in each group.

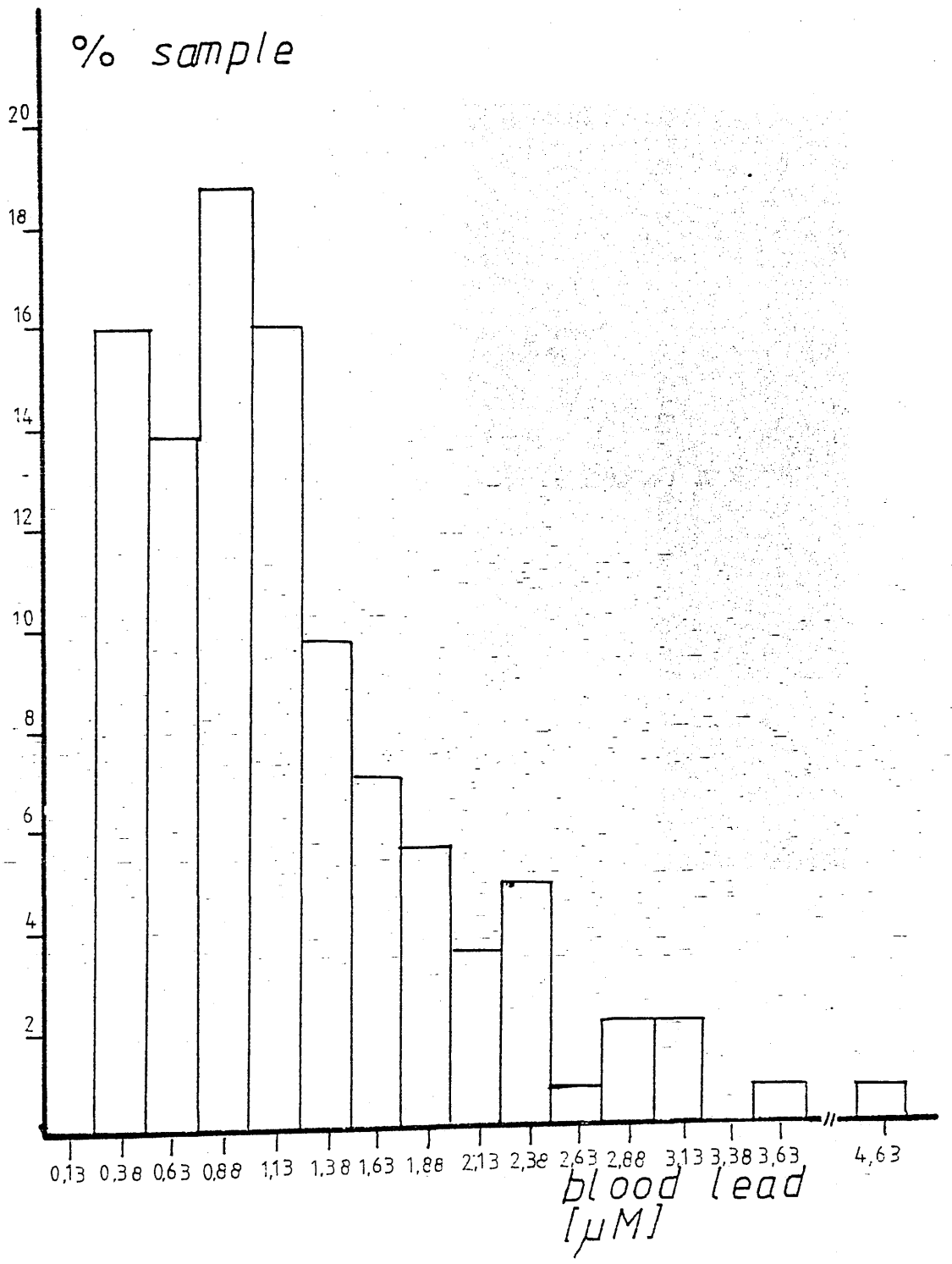


Figure 6.4 Distribution of blood lead values.

The histogram illustrates the skewed distribution of blood lead values observed in the subjects under study.

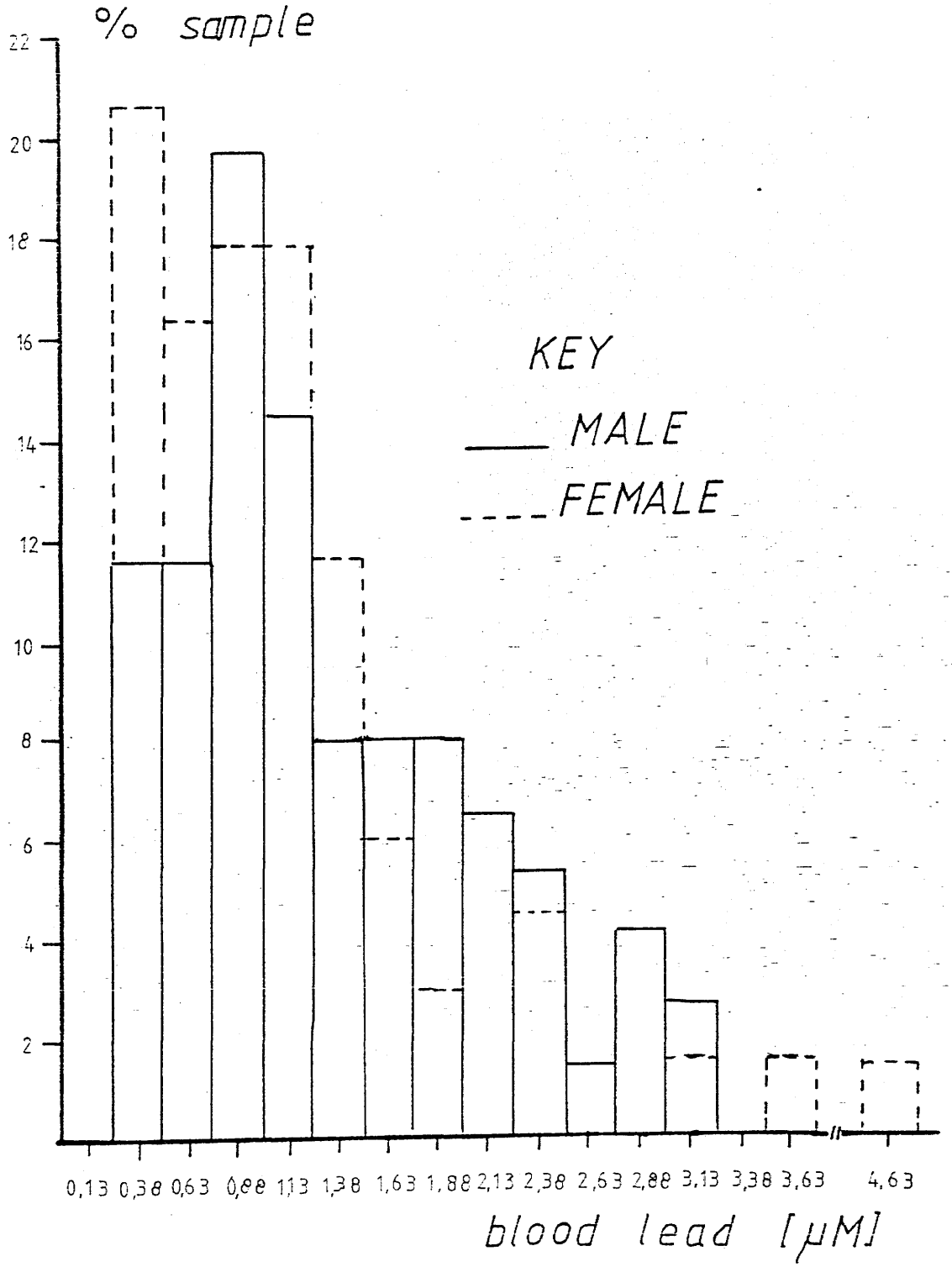


Figure 6.5 Differential distribution of blood lead values; males and females.

The histogram illustrates the differing distribution of blood lead values noted in the female compared to male subjects employed in the study.

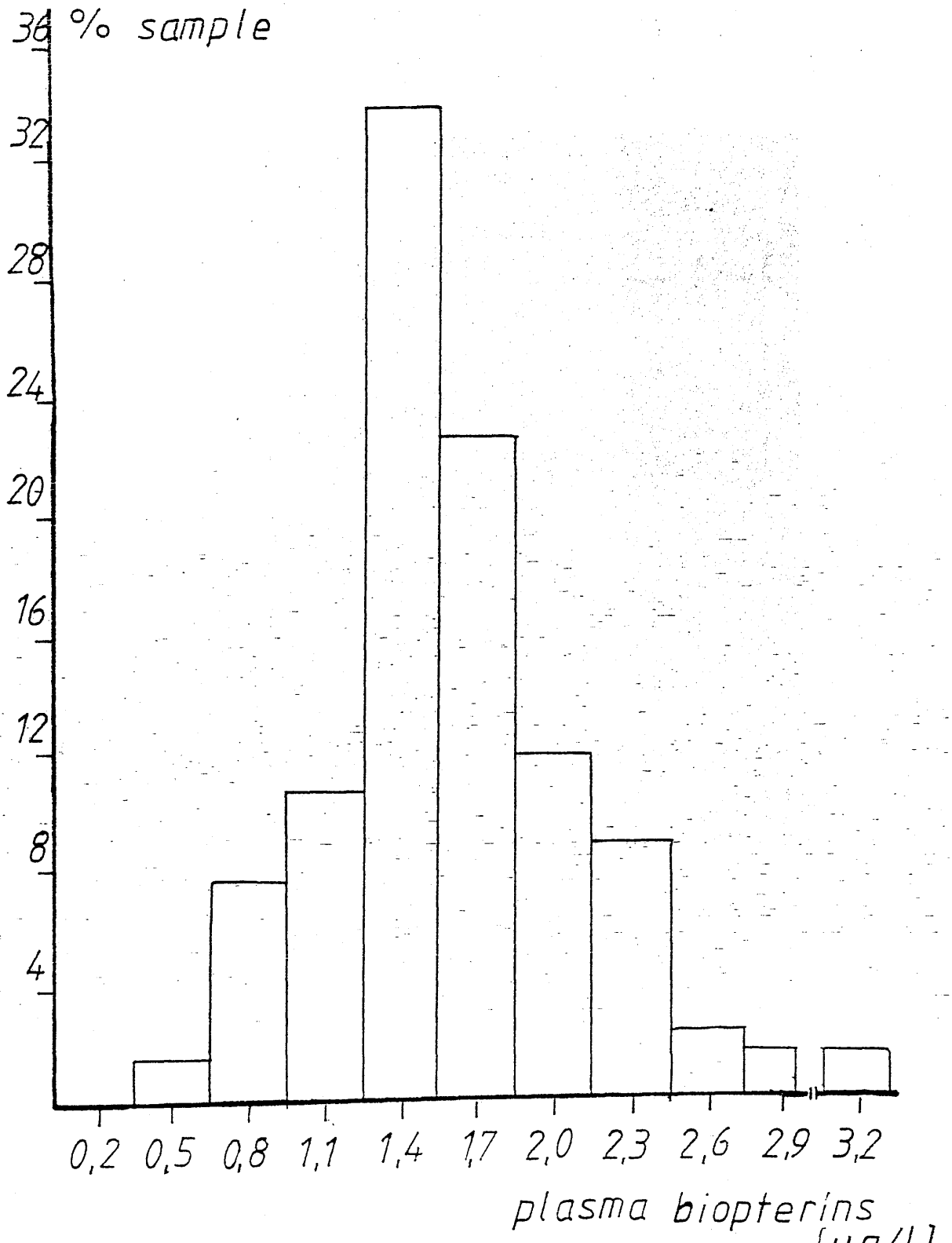


Figure 6.6 Distribution of plasma biopterin values

In contrast to the distribution of blood lead values, the range of plasma biopterins is symmetrically distributed.

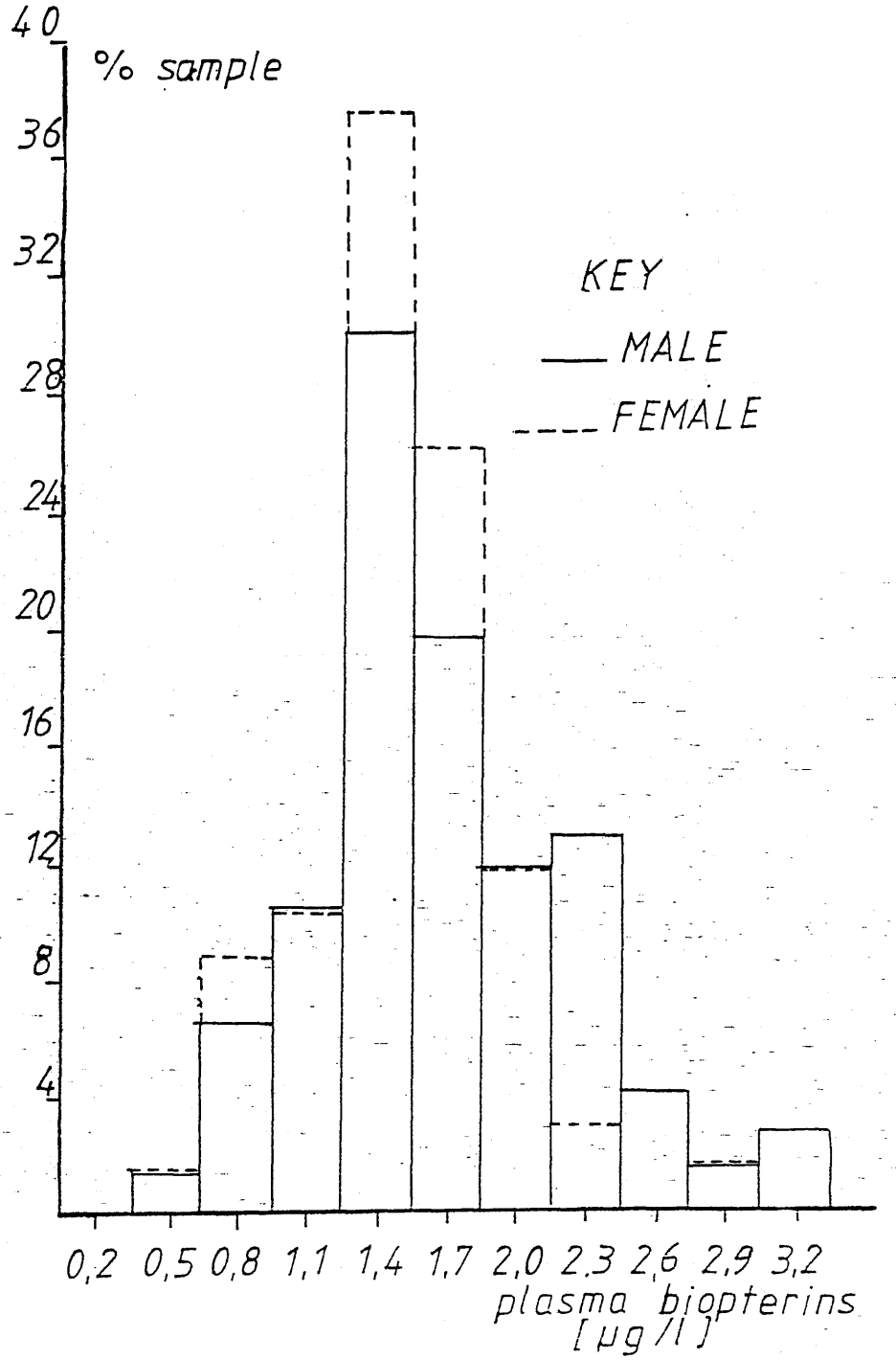
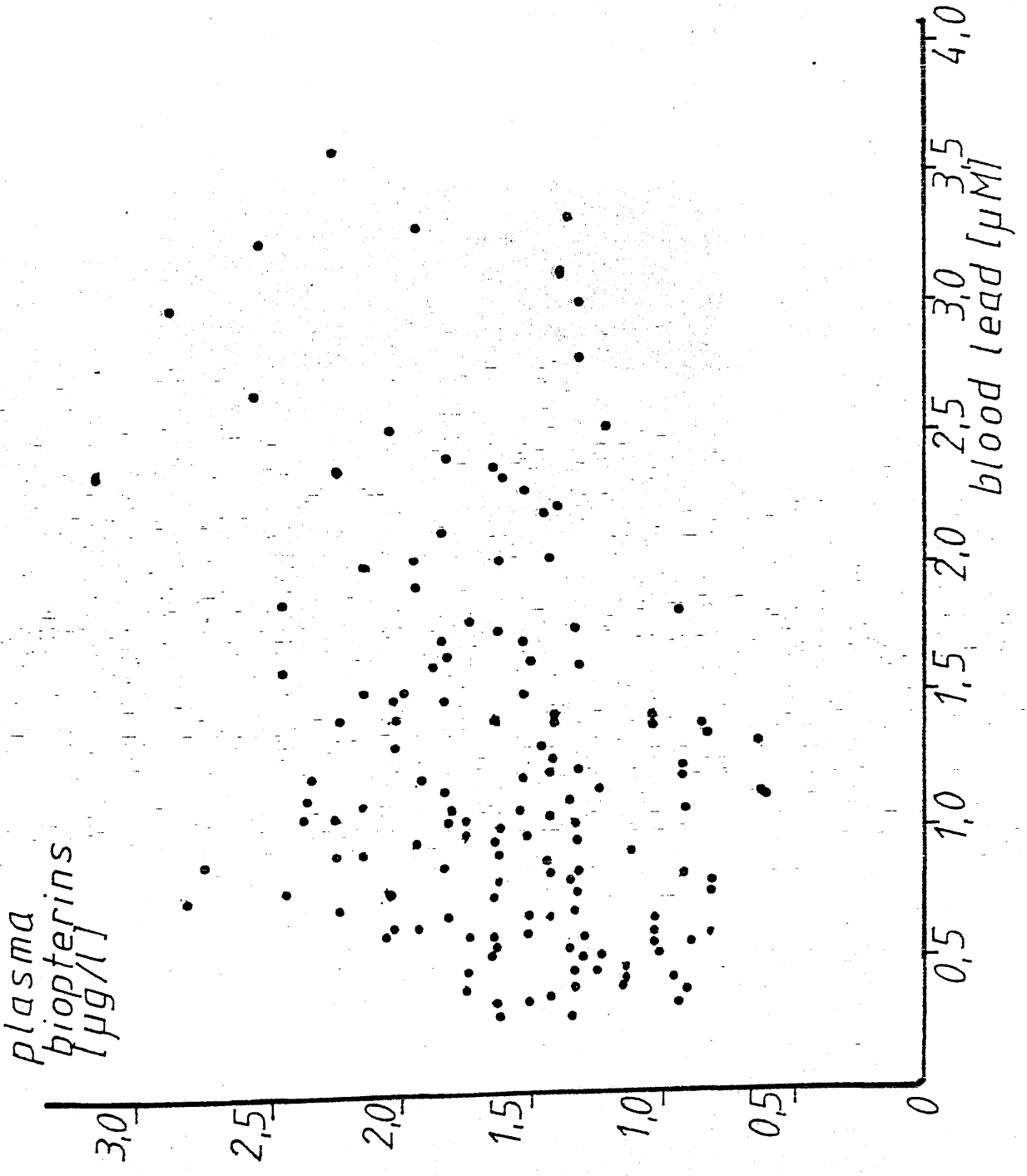


Figure 6.7 Differential distribution of plasma biopterins values; males and females.

The histogram depicts the range of plasma biopterins in the males employed in the study and compares this distribution to that observed in the female subjects.

Figure 6.8 Plot of blood lead versus plasma biopterins; all subjects.



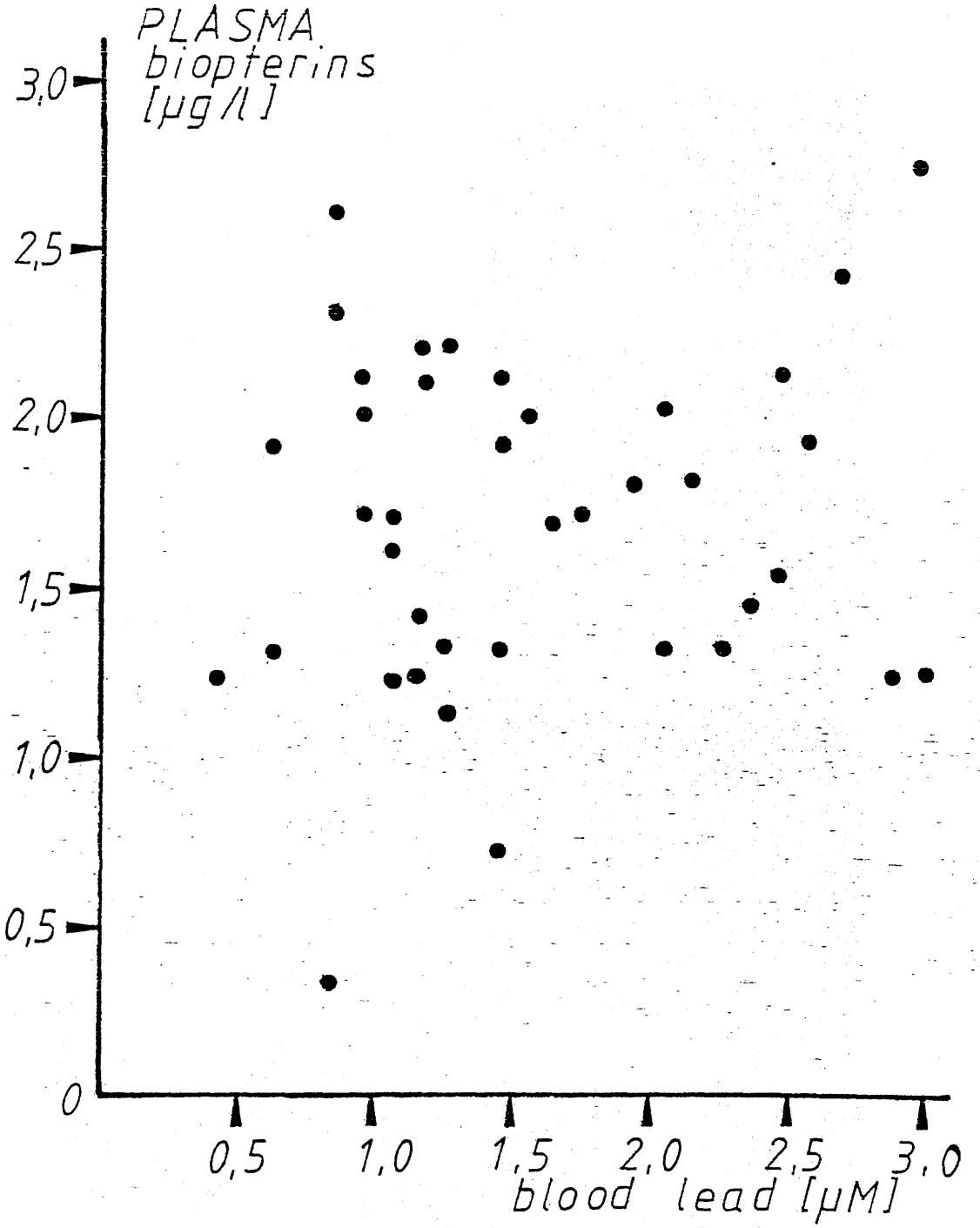


Figure 6.9 Plot of blood lead versus plasma biopterins; adult males.

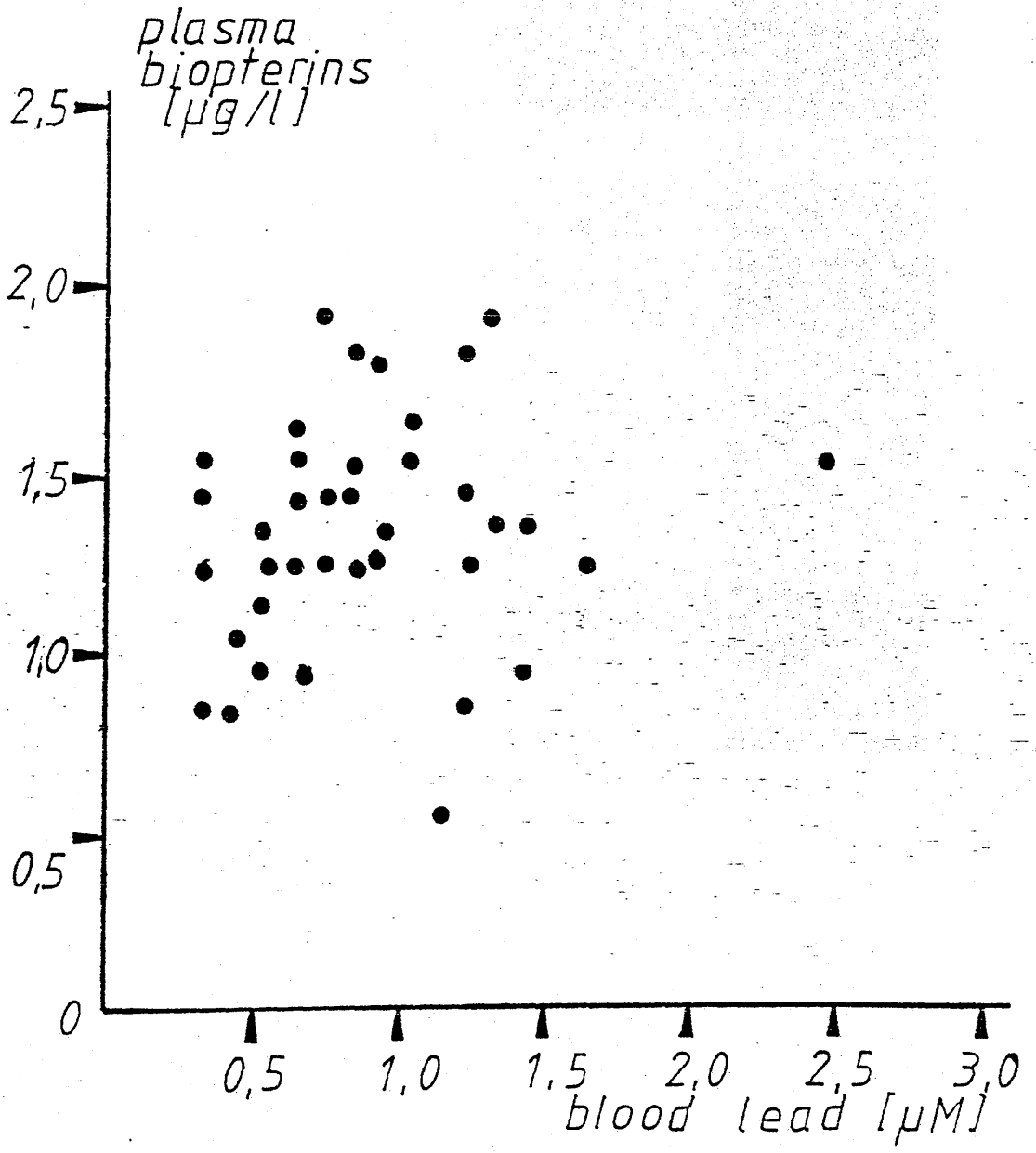


Figure 6.10 Plot of blood lead versus plasma biopterins; adult females.

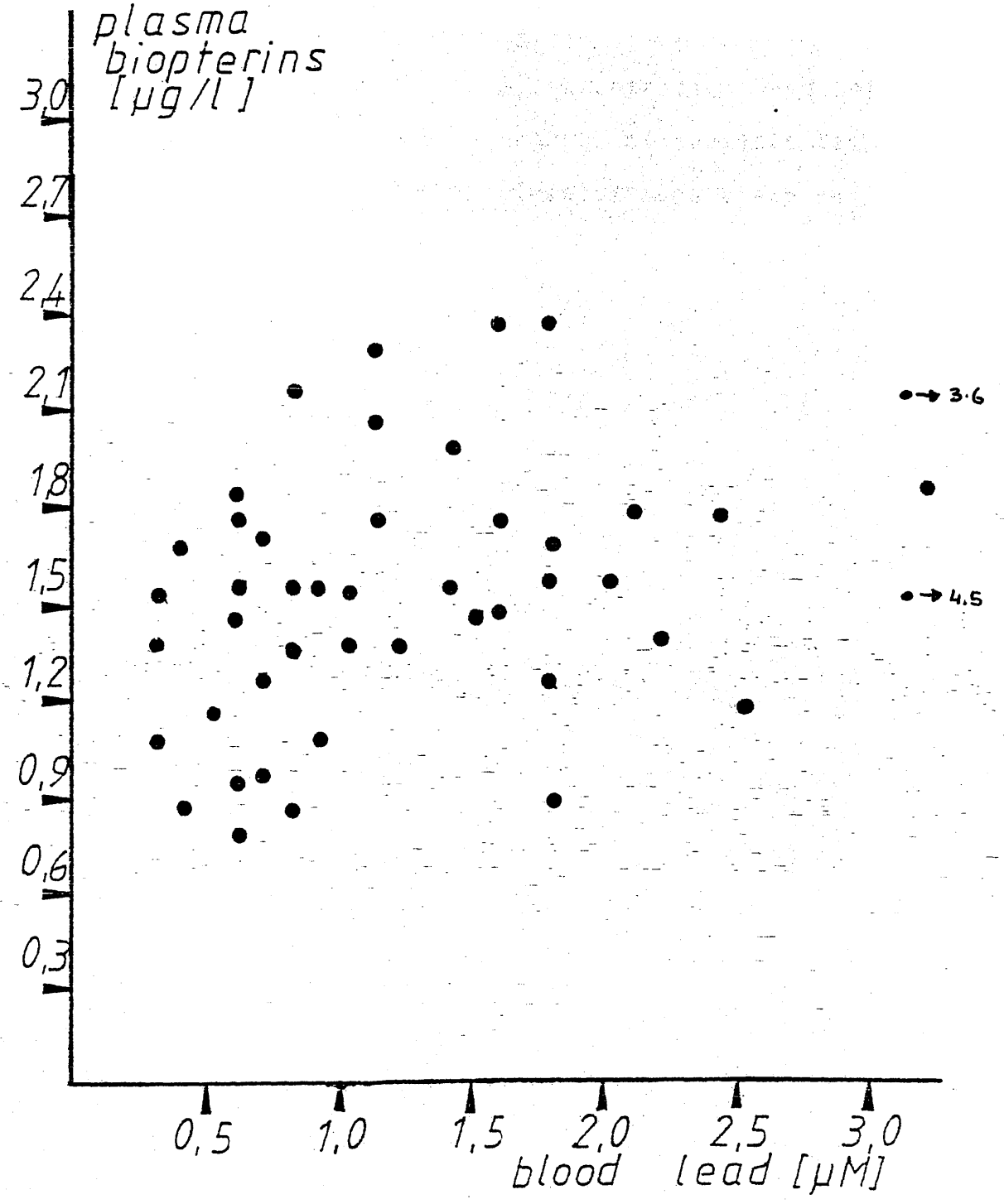


Figure 6.11 Plot of blood lead versus plasma biopterins; children.

Table 6.1 Correlation coefficients and significance levels for the relationship between blood lead and plasma biopterin concentrations.

Correlation between blood lead concentration and plasma Crithidia active biopterins in a group of subjects taken as a whole, for adult males and females separately and for children alone.

Figure	Group	Number in group	Spearman Rank coefficient	Probability
6.8	all	146	0.36	< 0.001
6.9	adult ♂	44	0.146	
6.10	adult ♀	47	0.112	
6.11	children	55	-0.042	

Table 6.2 Effect of blood lead concentration on plasma biopterins in a group of subjects

Group	No. in group	Blood lead concentration Range -μM (ug/dl)	g.mean ± dev. (μM)	Plasma biopterins mean ± S.D. (μg/l)
A	46	<0.72 (<15)	0.48±1.31	1.4±0.4
B	81	0.72-1.93 (15-40)	1.14±1.32	1.6±0.5
C	18	1.93-2.89 (40-60)	2.28±1.10	1.8±0.5
D	7	>2.89 (>60)	3.32±1.16	2.0±0.6

6.2.2.3 Discussion

The data presented in this section suggests an effect of lead on tetrahydrobiopterin metabolism observed as an increased concentration of plasma biopterins which, as measured by Crithidia fasciculata bioassay, is probably mainly dihydrobiopterin (Leeming et al, 1976b) although there is doubt surrounding this observation (Leeming, personal communication). Increased concentrations of plasma biopterins would indicate an inhibition of dihydropteridine reductase (Leeming, 1979) and hence the data would suggest that the predominant effect of lead ions at the blood concentrations observed in this group (0.28-4.51μM, although the modal value is closer to the lower end of the range due to the skewed distribution) is the inhibition of dihydropteridine reductase. At the cellular level, one would expect that there would be a reduced level of neurotransmitter synthesis and consequently neurological sequelae. Indeed such neurological disease resulting from an inhibition of dihydropteridine reductase is a feature of malignant hyperphenylalanaemia (Leeming et al, 1981). An increase in plasma biopterins from 1.8 to 4.9μg/l is associated with a drop in I.Q. score from 104 to 96 (Leeming et al, 1976c; Berry et al, 1979). Similar detrimental effects may then be anticipated from the inhibition of dihydropteridine reductase and subsequent increase in plasma biopterins caused by lead ions.

6.2.3 Studies with Crithidia in rats

6.2.3.1 Methods

Male Sprague-Dawley rats were exposed to lead (as the

acetate salt) at a level of either 24μM (5mg lead/l) or 120μM (25mg lead/l) in their drinking water since weaning; control rats receiving distilled water or tap water depending on the experiment. Food and water were supplied ad. libitum. After a variable period of such exposure the animals were sacrificed by decapitation, a heparinised sample of blood obtained and the femur removed for lead analysis. Blood and bone lead analyses were performed as described in the general methodology section, as was analysis of the plasma for Crithidia active biopterins.

6.2.3.2 Results

In order to establish the concentration of plasma biopterins observed in the species of rat to be used in the studies, a group of male Sprague-Dawley rats being fed on normal laboratory chow and tap water ad. libitum. were selected. These animals were eight months old and had been in the same environment since weaning. The range of plasma biopterins was relatively large ranging from 9.9 to 32.4μg/l with an arithmetic mean ± standard deviation of 18.0 ± 6.2μg/l (10 rats).

The first group of experiments involved exposing a group of rats to either distilled water or 24μM lead as drinking fluid for a period of 3 months after weaning. The results obtained from this experiment can be seen in table 6.3 and figure 6.12, which show that lead exposure resulted in a significant elevation in the level of lead in the blood of these rats compared to distilled water controls. Accumulation of lead in the body was also demonstrated by the significantly increased bone lead levels which these

animals exhibited. Exposure to lead resulted in an increase in the concentration of plasma biopterins (Table 6.3; $p<0.02$). Shown graphically, there is a significant positive correlation between the blood lead concentration in these animals and the level of plasma biopterins, with a Spearman Rank correlation coefficient of 0.52 being calculated (figure 6.12 - $p<0.02$).

Data relating to an increased length of exposure and an increased level of exposure are shown in table 6.4. The rats were exposed to either 24μM or 120μM lead for a period of 7 months from weaning. Table 6.4 shows that exposure to lead resulted in an increased level of lead both in the blood and the bones of these animals. However, the data obtained on plasma biopterins does not parallel the results obtained in the previous study in rats or the data obtained in man. Exposure to the 24μM dose of lead resulted in a small statistically non-significant fall in plasma biopterins, whilst those rats supplied with 120μM lead showed no alteration in biopterins from the control animals. The level of blood lead did not correlate with plasma biopterins (figure 6.13). It is noteworthy that one animal exposed to 120μM lead exhibited a very high blood lead concentration of 1.56μM and it was this same animal which had an exceedingly high concentration of plasma biopterins, in excess of 135μg/l, a level beyond the linearity range of the Crithidia assay.

Since the exposure of the rats to 24μM lead for a relatively long period of exposure of 7 months resulted in a slight fall in plasma biopterins, an observation not

consistent with previous data, the study was repeated with a further group of animals and the results are shown in table 6.5. These rats exposed to lead had accumulated the metal as shown by the significantly elevated bone lead concentrations compared to control animals, although there was no difference in the level of blood lead between the two groups. Similar to the previous group of animals, exposure to 24μM lead for a period of 7 months resulted in a fall in plasma biopterins compared to control animals and this fall was significant at the 5% significance level.

Figure 6.14 shows a plot of blood lead concentration against plasma biopterins for all the rat groups studied. Statistical analysis of the data calculated that the relationship was not significant.

Table 6.3 Blood lead, bone lead & plasma biopterins in male rats

-Lead exposure for 3 months from weaning in water

Group	No. in group	Blood lead (g.mean±dev. - μM)	Bone lead x±S.D. μmol/kg dry bone wt.	plasma biopterin (mean±S.D -μg/L)
Distilled water	10	0.57±1.11	8±3	11.0±1.1
Lead acetate-24μM	10	0.73±1.12	22±5 +	14.7±2.7 ++

+ $p < 0.002$ compared to distilled water group
++ $p < 0.02$ by Mann-Whitney U test.

Table 6.4 Blood lead, bone lead & plasma biopterins in male rats

- Lead exposure for 7 months from weaning in water.

Group	No. in group	Blood lead (g.mean±dev - μM)	Bone lead x±S.D. umol/ kg dry wt	plasma biopt (x±S.D. - μg/L)
Distilled water	13	0.56±1.21	7±2	16.9±5.6
Lead acetate-24μM	15	0.70±1.22 ++	30±6 ++	13.4±7.6
Lead acetate-120μM	6	0.82±1.47 +	180±20 +++	16.8±4.6

+ $p < 0.05$ compared to distilled water
++ $p < 0.02$ group by Mann-Whitney
+++ $p < 0.001$ U test.

Table 6.5 Blood lead, bone lead & plasma biopterins in male rats

- Lead exposure for 7 months from weaning in water.

Group	No. in group	Blood lead (g.mean+dev - μM)	Bone lead (g.mean+dev -μmol/kg dry wt)	Plasma biopt (x+S.D. - μg/l)
Distilled water	6	0.30+1.12	4.2+1.13	24.1+1.9
Lead acetate-24μM	6	0.30+1.17	23.5+1.14 ++	12.7+0.8 +

+ $p < 0.05$ compared to distilled water group
++ $p < 0.002$ by Mann-Whitney U test

Figure 6.12 Plot of blood lead versus plasma biopterins following 3 month lead exposure in rats.

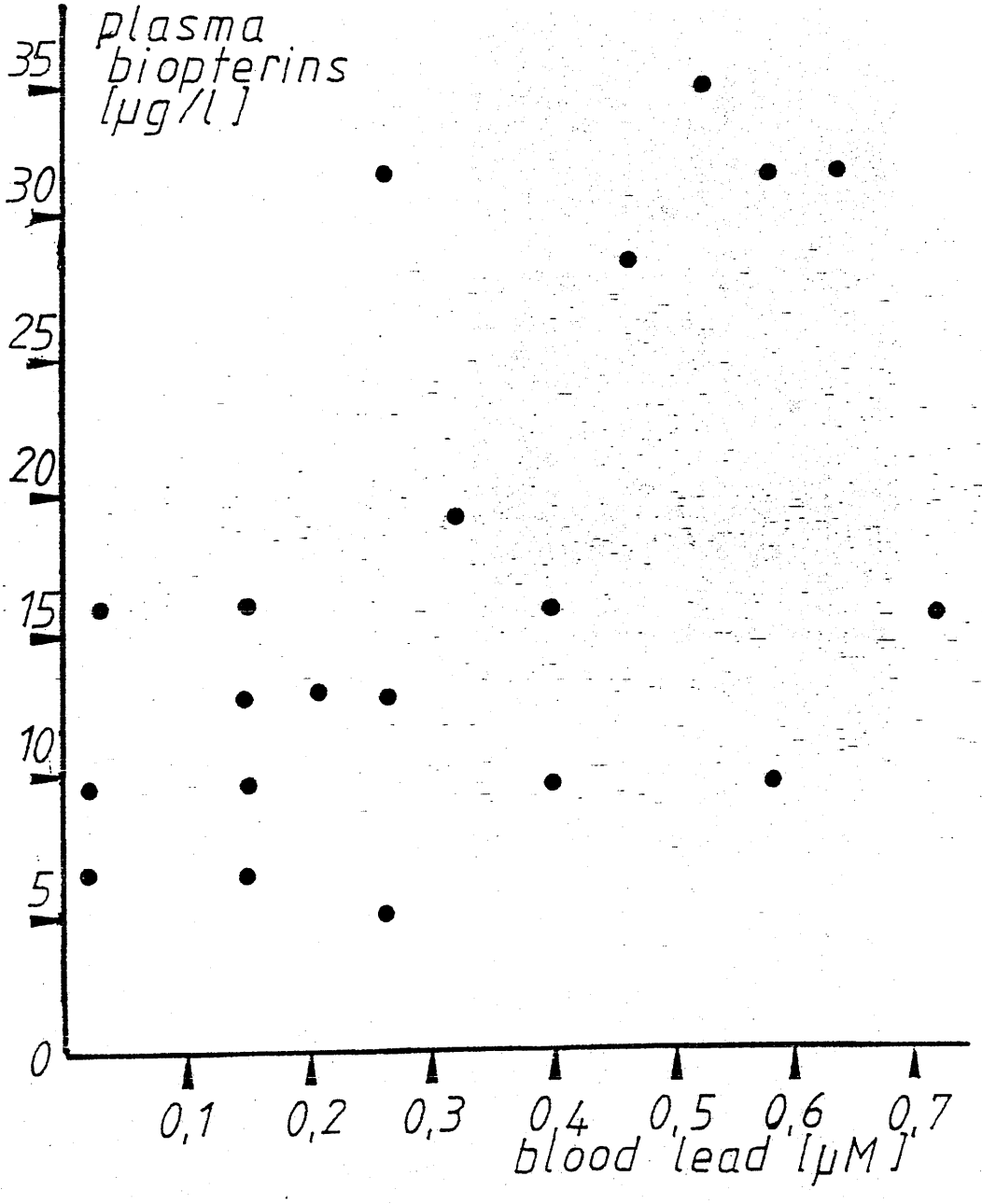


Figure 6.13 Plot of blood lead versus plasma biopterins following 7 month lead exposure in rats.

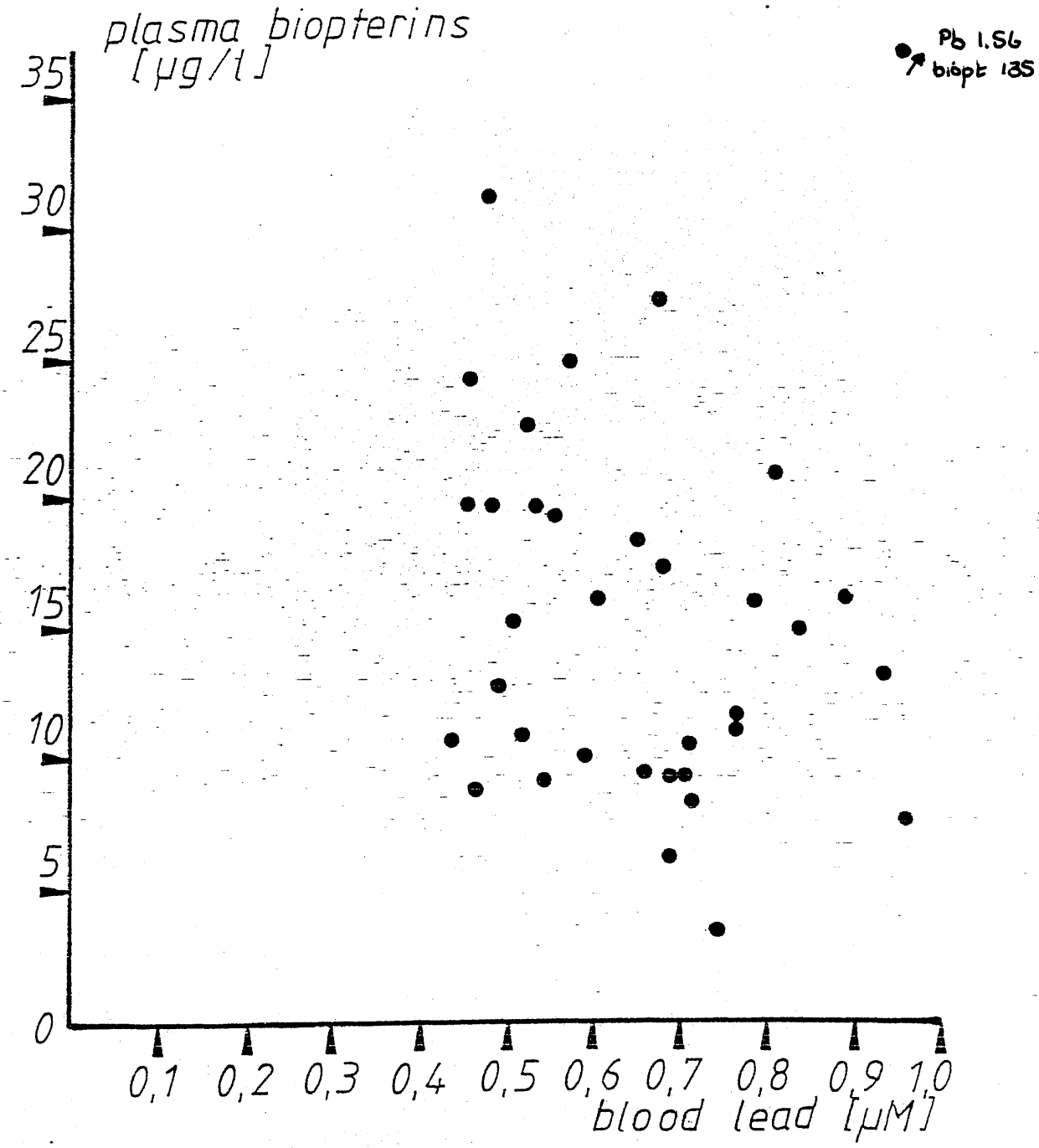
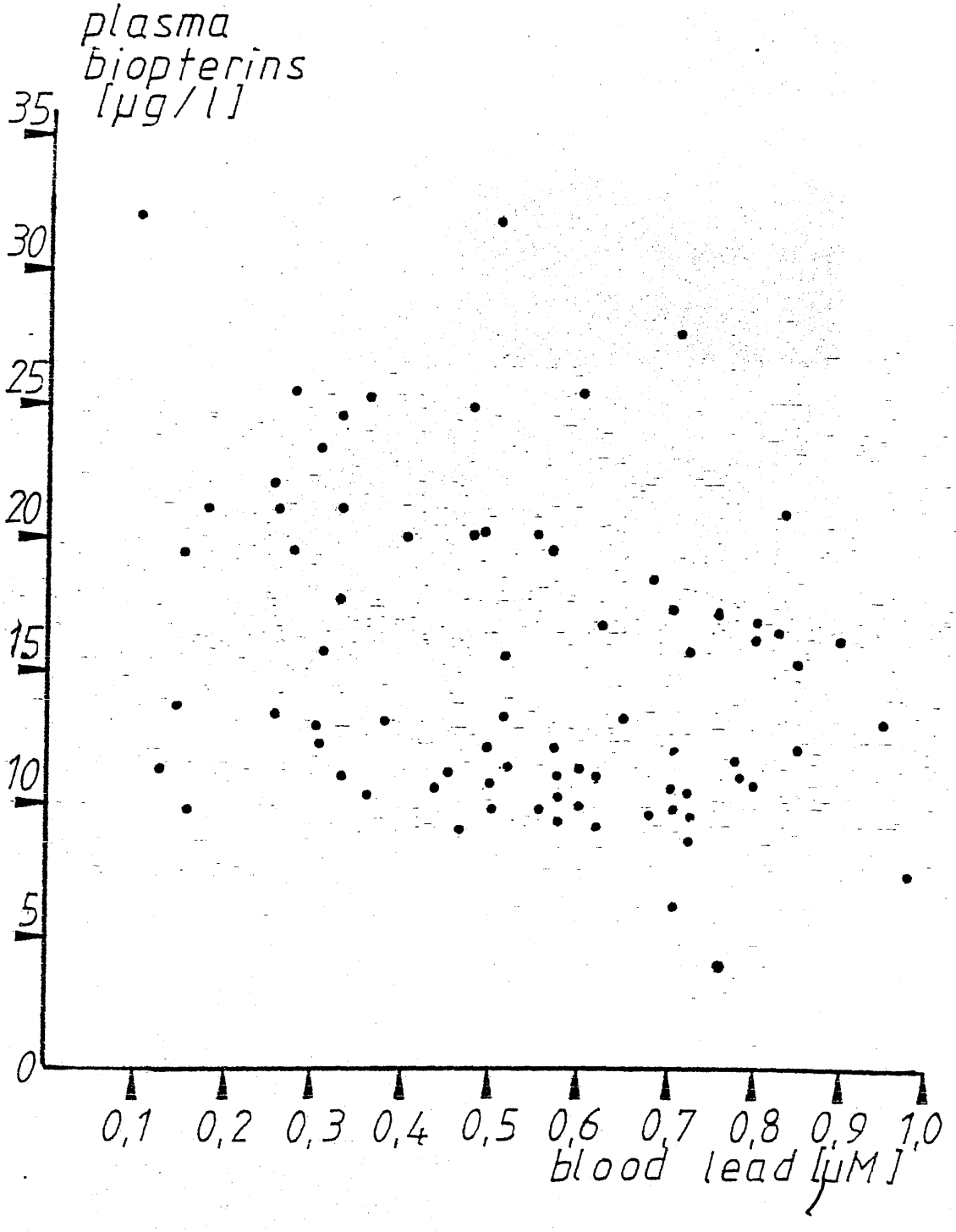


Figure 6.14 Plot of blood lead versus plasma biopterins in rats.



6.2.3.3 Discussion

The data obtained in the first experiment involving a relatively short exposure period of 3 months to a low dose of lead (24μM) showing a significantly enhanced level of plasma biopterins in the lead exposed group compared to controls is consistent with the data obtained in the previous study in man and suggests an inhibition of dihydropteridine reductase activity in these animals. The potential thus exists for a reduction in cellular tetrahydrobiopterin concentration as a result of this inhibition. The data obtained from the studies in which the dose is raised to 120μM lead and/or the period of exposure is lengthened are divergent and raise several questions. Exposing rats to the same low dose of lead (24μM) for a period of 7 months would appear to lower the level of plasma biopterins, data which would indicate that the activity of dihydropteridine reductase may be enhanced in these rats, in which case there will be a rise in intracellular tetrahydrobiopterin concentration. Alternatively, the de-novo synthesis of the active cofactor may be reduced, although this inhibition would require to be substantial to result in a reduced appearance of dihydrobiopterin in the serum (Leeming, 1979). No evidence is available from this study to elucidate which of these two possibilites is the mode of action of lead in these animals. The enhancement of dihydropteridine reductase activity may not be the primary effect of lead but may merely be a secondary compensatory effect occurring as a

result of the primary action. Such a primary effect may well be inhibition of de-novo synthesis at one or more sites. Exposure of the rats to 120μM lead for a period of 7 months resulted in no alteration in the level of plasma biopterins. This does not signify that lead is not having an effect on tetrahydrobiopterin metabolism in this group of animals. The relatively narrow variation in plasma biopterins observed in man (1.81 ± 0.06ng/ml - Leeming et al, 1976b; 1.75 ± 0.03ng/ml in males and 1.53 ± 0.04ng/ml in females - Leeming & Blair, 1980; Leeming et al, 1981) suggests homeostatic mechanisms coming into play to maintain these values within such a range. Thus in these animals compensatory mechanisms may be operating to maintain the level of tetrahydrobiopterin in the cell at the appropriate concentration as a result of an action of lead.

6.2.4 Conclusions on studies with Crithidia fasciculata

The data presented from these studies both in man and the rat suggest an action of lead to inhibit the dihydropteridine reductase catalysed path of tetrahydrobiopterin production. Additionally, there appears to be another mode of action of this environmental neurotoxin and it is suggested that this may be either an inhibition of de-novo synthesis of the cofactor or an enhancement of salvage pathway activity, but this action is not clear from these studies. Furthermore, compensatory mechanisms appear to come into play to combat the effect of lead in the rat.

6.3 Effect of lead on brain tetrahydrobiopterin concentration and dihydropteridine reductase activity in rats.

6.3.1 Introduction

The data presented in the previous sections and in the introduction to this chapter suggest a pathological effect of lead on tetrahydrobiopterin metabolism. However, all studies to date have involved measurement of plasma biopterins by the Crithidia fasciculata protozoological bioassay and thus measure the aggregate of biopterin, dihydrobiopterin and tetrahydrobiopterin. The active cofactor for the mixed function oxygenases is however tetrahydrobiopterin, and it is this compound that is vital for normal neurotransmitter function, both centrally and peripherally. More information may be gained as to the mode of action of lead on tetrahydrobiopterin metabolism if the effect of lead exposure on the concentration of the cofactor alone could be measured. To this end, a high-pressure liquid chromatography (HPLC) technique has been developed which is capable of measuring tissue tetrahydrobiopterin concentration. The data in this section will investigate the effect of lead on brain tetrahydrobiopterin concentration in rats exposed to lead in their drinking water. Additionally, the activity of dihydropteridine reductase (DHPR), the enzyme principally responsible for the salvage of oxidised forms of tetrahydrobiopterin back into the active cofactor, will be simultaneously measured in the same brain regions of these animals.

6.3.2 Methods

Male Sprague-Dawley rats were exposed to lead (as the acetate salt) at a level of either 120μM (25mg lead/l) or 480μM (100mg lead/l) in their drinking fluid from weaning; a control group received distilled water. The animals were allowed access to food and water ad. libitum. At the end of a period of exposure of either 4 or 12 weeks, the rats were weighed prior to being sacrificed by decapitation. The brain was removed and dissected as described in the General Methodology Chapter. Tetrahydrobiopterin and dihydropteridine reductase activity were subsequently measured in these brain regions. A sample of heparinised blood was obtained for lead analysis as was the femur. The methods employed to measure brain tetrahydrobiopterin concentration, dihydropteridine reductase activity, blood and bone lead concentrations and tissue protein concentration are also as detailed in the methods.

6.3.3 Results

There was no significant difference in the weight of the rats exposed to either the low dose of lead (120μM) or the high dose (480μM) - table 6.6.

The concentrations of lead in the blood and femur of the rats are shown in tables 6.7 & 6.8. The levels of lead in the blood were significantly elevated in the animals exposed to lead at both doses compared to the non-exposed control rats. In addition, the group exposed to the higher dose exhibited a greater blood lead concentration than the lower dose group. Bone lead levels paralleled the blood lead data.

Tables 6.9 & 6.11 show the effect on brain tetrahydrobiopterin concentration and dihydropteridine reductase activity after four weeks lead exposure post-weaning. The data presented in these tables can be compared to that obtained after a further eight weeks of exposure (tables 6.10 & 6.12). The concentration of tetrahydrobiopterin in the diencephalon was significantly increased after four weeks exposure to both levels of lead ($p<0.05$), and after a further eight weeks, exposure to 480μM lead but not to 120μM ($p<0.05$); in all groups the absolute concentration in this region had risen between the fourth and twelfth weeks of exposure. No significant alterations in cofactor concentration were noted in any other brain region analysed, either in the low dose group or the higher exposure group, after either period of exposure (Tables 6.9 & 6.10). Considering now the activity of dihydropteridine reductase (Tables 6.11 & 6.12); similarly to tetrahydrobiopterin levels, the only region of the brain analysed which showed any alteration in dihydropteridine reductase activity was the diencephalon. Enzymatic activity was significantly raised in the low dose 120μM lead group compared to the control group after twelve weeks lead exposure ($p<0.01$). Enhanced activity was also observed in the higher dose group but this increase failed to reach statistical significance at the five percent level, possibly due to the relatively large scatter of activities in this group. The effect of lead on this enzyme is however unclear as the enhanced activity is no longer apparent when the activities are expressed per unit of protein. No

alteration in the activity of dihydropteridine reductase was observed in either group of exposure after the shorter period of exposure of four weeks.

Table 6.6 Rat weights

Comparison of the weights of rats exposed to lead for a period of 4 or 12 weeks compared to non-exposed controls. Results expressed as mean ± S.D. with the number in each group in parenthesis.

Treatment	Weight (g) control	120 μM Pb	480 μM Pb
BH group			
4 weeks	205 ± 13 (7)	224 ± 12 (7)	207 ± 37 (7)
12 weeks	316 ± 63 (6)	363 ± 27 (6)	401 ± 40 (6)
DHPR group			
4 weeks	160 ± 14 (7)	157 ± 21 (7)	166 ± 32 (7)
12 weeks	386 ± 35 (6)	356 ± 38 (6)	417 ± 45 (6)

Table 6.7 Blood lead concentrations

Blood lead concentrations in rats exposed to lead for a period of 4 or 12 weeks. Results expressed as mean ± S.D. with the number in each group in parenthesis.

Treatment	Blood lead (μM)		
	control	120 μM Pb	480 μM Pb
BH group			
4 weeks	0.29 ± 0.12 (5)	0.53 ± 0.21 (6)$^{+}$	0.98 ± 0.36 (6)$^{++*}$
12 weeks	0.27 ± 0.10 (6)	0.66 ± 0.23 (6)$^{+}$	1.12 ± 0.25 (6)$^{++**}$
DHPR group			
4 weeks	0.18 ± 0.06 (6)	0.35 ± 0.18 (6)$^{+++}$	0.60 ± 0.23 (6)$^{++***}$
12 weeks	0.22 ± 0.08 (6)	0.53 ± 0.16 (6)$^{@}$	0.78 ± 0.23 (6)$^{+}$

+ $p < 0.005$
++ $p < 0.001$ compared with
+++ $p < 0.05$ control group
@ $p < 0.01$

* $p < 0.005$
** $p < 0.01$ compared with
*** $p < 0.05$ 120 μM group

Table 6.8 Bone lead concentrations

Bone lead concentrations in rats exposed to lead for a period of 4 or 12 weeks. Results expressed as mean ± S.D. of 6 observations.

Treatment	Bone lead (μmol/kg dry bone weight)		
	Control	120 μM Pb	480 μM Pb
BH group			
4 weeks	28 ± 14	67 ± 19 +	190 ± 34 ++*
12 weeks	10 ± 5	108 ± 14 ++	210 ± 35 ++*
DHPR group			
4 weeks	8 ± 10	86 ± 18 ++	195 ± 86 ++**
12 weeks	1 ± 2	80 ± 25 ++	212 ± 38 ++**

\+ $p < 0.05$ compared with
++ $p < 0.001$ control group

* $p < 0.001$ compared with
** $p < 0.01$ 120 μM group

By Mann-Whitney U test

Table 6.9 Tetrahydrobiopterin concentrationS - 4 weeks exposure

Tetrahydrobiopterin concentrations in the brains of rats exposed to lead for a period of 4 weeks. Results expressed as mean ± S.D. with the number in each group in parenthesis.

Brain Region	Tetrahydrobiopterin (pg/mg tissue)		
	Control	120 μM Pb	480 μM Pb
Cerebellum	102 ± 19 (6)	105 ± 19 (6)	94 ± 15 (6)
Midbrain	137 ± 23 (6)	146 ± 32 (6)	155 ± 20 (5)
Diencephalon	197 ± 18 (5)	243 ± 33 (6)+	230 ± 24 (6)+
Telencephalon	106 ± 18 (6)	118 ± 3 (6)	104 ± 19 (6)

+ $p < 0.05$ compared with control group

Table 6.10 Tetrahydrobiopterin concentrations - 12 weeks exposure

Tetrahydrobiopterin concentrations in the brains of rats exposed to lead for a period of 12 weeks. Results expressed as mean ± S.D. with the number in each group in parenthesis.

Brain region	Tetrahydrobiopterin (pg/mg tissue)		
	control	120 µM	480 µM
Cerebellum	121 ± 55 (6)	94 ± 28 (5)	104 ± 35 (6)
Midbrain	134 ± 48 (6)	142 ± 27 (5)	139 ± 25 (6)
Diencephalon	156 ± 29 (6)	145 ± 29 (6)	205 ± 22 (6) +*
Telencephalon	172 ± 56 (6)	135 ± 28 (6)	164 ± 29 (6)

+ $p < 0.05$ compared with control group

* $p < 0.05$ compared with 120 µM group

Table 6.11 Dihydropteridine reductase activity - 4 weeks exposure

Dihydropteridine reductase activity in the brains of rats exposed to lead for a period of 4 weeks. Results expressed as mean ± S.D. of six animals in each group.

Brain region	DHPR activity (nmole NADH/mg tissue/min)			DHPR activity (nmole NADH/mg protein/min)		
	control	120 μM Pb	480 μM Pb	control	120 μM Pb	480 μM Pb
Cerebellum	0.73±0.15	0.90±0.47	0.68±0.18	20.4±6.3	29.7±11.3	17·2±3·5
Midbrain	0.46±0.11	0.40±0.16	0.57±0.15	18.1±4.3	16.5±5.2	21.1±4.1
Diencephalon	0.79±0.36	0.61±0.13	0.51±0.14	20.3±11.6	13.8±4.4	11.8±2.3
Telencephalon	0.80±0.26	1.09±0.41	0.84±0.17	9.4±3.2	11.9±4.5	10.6±2.7

Table 6.12 Dihydropteridine reductase activity - 12 weeks exposure

Dihydropteridine reductase activity in the brains of rats exposed to lead for a period of 12 weeks. Results expressed as mean ± S.D. of six animals in each group.

Brain region	DHPR activity (nmole NADH/mg tissue/min)			DHPR activity (nmole NADH/mg protein/min)		
	control	120 µM	480 µM	control	120 µM	480 µM
Cerebellum	1.21±0.33	1.28±0.49	1.68±0.68	19.7±1.8	18.8±6.3	24·7±8·3
Midbrain	2.39±0.86	2.02±0.77	2.17±0.65	49.6±23.6	34.3±10.3	33.4±4.4
Diencephalon	1.14±0.21	1.53±0.13 +	1.54±0.43	19.1±5.3	24.7±4.3	22·6±3·1
Telencephalon	1.59±0.43	1.79±0.36	1.92±0.57	22.2±5.5	26.8±8.8	27.4±7.8

+ $p < 0.01$ compared to control group

6.3.4 Discussion

The foregoing data indicate an effect of lead on tetrahydrobiopterin metabolism selectively in the diencephalon of the rat brain with no apparent effect in the cerebellum, midbrain or telencephalon in this model. In the diencephalon, lead caused an elevation in the levels of tetrahydrobiopterin after 4 weeks exposure (post weaning). There were, however, no significant alterations in the activity of dihydropteridine reductase, the enzyme re-converting the inactive quinoniod dihydrobiopterin back into the active enzyme cofactor. After a further 8 weeks exposure to lead, the increase in tetrahydrobiopterin concentration observed in the group exposed to the higher dose of lead (480μM) was less than previously found and the lower dose group had no change in the concentration of tetrahydrobiopterin. Indeed dihydropteridine reductase activity was significantly elevated in the low dose group when the results were expressed in relation to tissue weight. This rise failed to reach significance when protein concentration was taken into consideration.

Increased levels of tetrahydrobiopterin could be caused by:

(a) Increased 'de-novo' synthesis (figure 6.2)

(b) Increased activity of dihydropteridine reductase

(c) Decreased activity of hydroxylation enzymes.

The third possibility is unlikely to explain the rise in the level of cofactor since the cofactor concentration is thought to determine the activity of tyrosine hydroxylase activity (Kettler et al, 1974) rather than the converse. An

increase in the activity of the salvage pathway enzyme is a possibility supported by the data obtained in this study. However it may still be the case that lead is having an action on the 'de-novo' synthetic pathway of tetrahydrobiopterin metabolism. This possibility must, however, be judged in the knowledge that lead inhibits pyrimidine 5'-nucleotidase (E.C. 3.1.3.5; Valentine et al, 1976; Rocco et al, 1981; Angle et al, 1982), the enzyme responsible for the dephosphorylation of pyrimidine nucleoside monophosphates. This will clearly alter the balance between pyrimidine and purine bases within the cell and consequently the availability of GTP for pteridine synthesis.

It is not possible to determine from the present data the action or actions of lead on tetrahydrobiopterin metabolism. The possibility exists that an observed effect is a compensatory mechanism as a result of another effect of lead. An action of lead on an enzyme in the pathway of 'de-novo' synthesis may lead to compensatory alterations in other enzyme(s) in this same path.

The data presented in this chapter would support the hypothesis that lead acts to elevate cellular tetrahydrobiopterin concentrations by an as yet undetermined mechanism. After a relatively short exposure and also after a chronic exposure period, dihydropteridine reductase activity is altered either as a result of a direct action of lead or by a homeostatic mechanism.

6.4 Conclusions

The series of experiments presented in this chapter demonstrate alteration to the metabolism of tetrahydrobiopterin, the essential cofactor required for the synthesis of catecholamines and 5-hydroxytryptamine. However the exact site or sites of action of lead remain elusive. There would appear to be an inhibition of dihydropteridine reductase in vivo which supports the data obtained in vitro (Purdy et al, 1981). However the inhibition of tetrahydrobiopterin synthesis observed in vitro (Purdy et al, 1981) remains to be confirmed in vivo. Further research into possible effects of lead on the individual enzymes of the 'de-novo' pathway of tetrahydrobiopterin synthesis, when indeed it has been elucidated, is required. The interplay between the various reactions in which tetrahydrobiopterin and related species are involved, would appear to be complex and alteration in the activity of one or more enzymes compensates for altered activity of others in the same or another pathway.

One cannot neglect however, the other neurochemical effects of lead such as alterations in catecholamine synthesis which may in part relate to changes in cofactor concentration (Meredith et al, 1981) and other biochemical events such as alteration in haem synthesis and over-production of 5-aminolaevulinic acid as mediators of the neurochemical action of lead (Moore & Goldberg, 1984). The influence of lead at a site of catecholamine synthesis distant from tyrosine hydroxylase or phenylalanine hydroxylase may well elicit changes in the activities of these

enzymes perhaps by way of changes in tetrahydrobiopterin metabolism.

Whatever the reason, the influence that lead has upon the particular pathway means that there is more than sufficient reason to include this mechanism as one which is part of the constellation of biochemical events which may contribute to the proven psychological, neurological and behavioural changes observed during lead exposure.

CHAPTER 7

EFFECT OF LEAD ON 5-AMINOLAEVULINIC ACID SYNTHASE ACTIVITY IN RAT BRAIN

7.1 Introduction

The anaemia of lead poisoning is long since known and numerous studies have adequately demonstrated altered haem metabolism in lead exposed subjects. The relatively complex pathway leading to haem formation is shown in figure 7.1. The first step, the most important reaction in the path, is the formation of 5-aminolaevulinic acid (ALA) by the condensation of succinyl-coA and glycine with the aid of cofactor pyridixal phosphate. δ-Aminolaevulinic acid synthase (ALA S; E.C. 2.3.1.37); the enzyme responsible for this rate-controlling step is exclusively located within the mitochondrion being associated mainly with the inner membrane of the organelle. Control of haem biosynthesis through ALA synthase is achieved by feedback inhibition; the enzyme is repressed by haem or a haem-like compound and hence if the rate of haem production is reduced, ALA synthase becomes de-repressed and increased amounts of ALA are formed. ALA enters the cytoplasm of the cell where it is metabolised to the monopyrrole, porphobilinogen (PBG) by δ-aminolaevulinic acid dehydratase (ALA D; E.C. 4.2.1.24). 4 moles of PBG are involved in condensation, cyclization and finally isomerisation to form the first porphyrin, uroporphyrinogen I, a reaction catalysed by uroporphyrinogen I synthetase (PBG deaminase; E.C. 4.3.1.8). Uroporphyrinogen I is unstable and readily converts to the more stable species, uroporphyrinogen III, a reaction catalysed by the enzyme uropophyrinogen III cosynthetase. Uroporphyrinogen decarboxylase (E.C. 4.1.1.37) is then responsible for the formation of

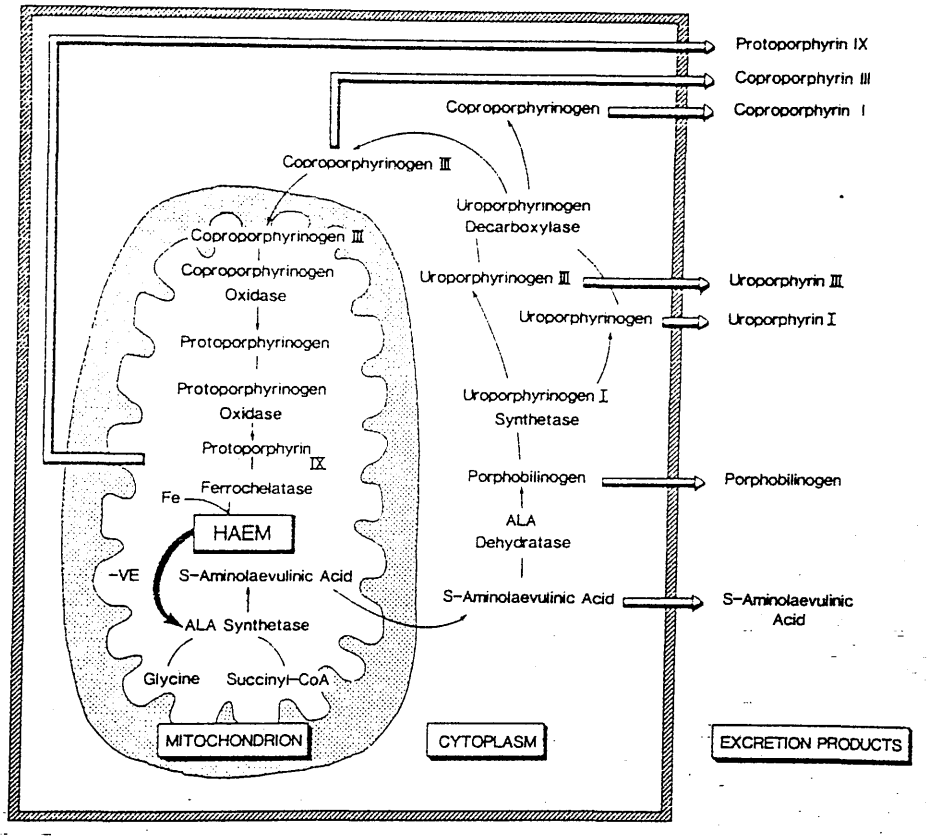


Figure 7.1 The haem biosynthetic pathway.

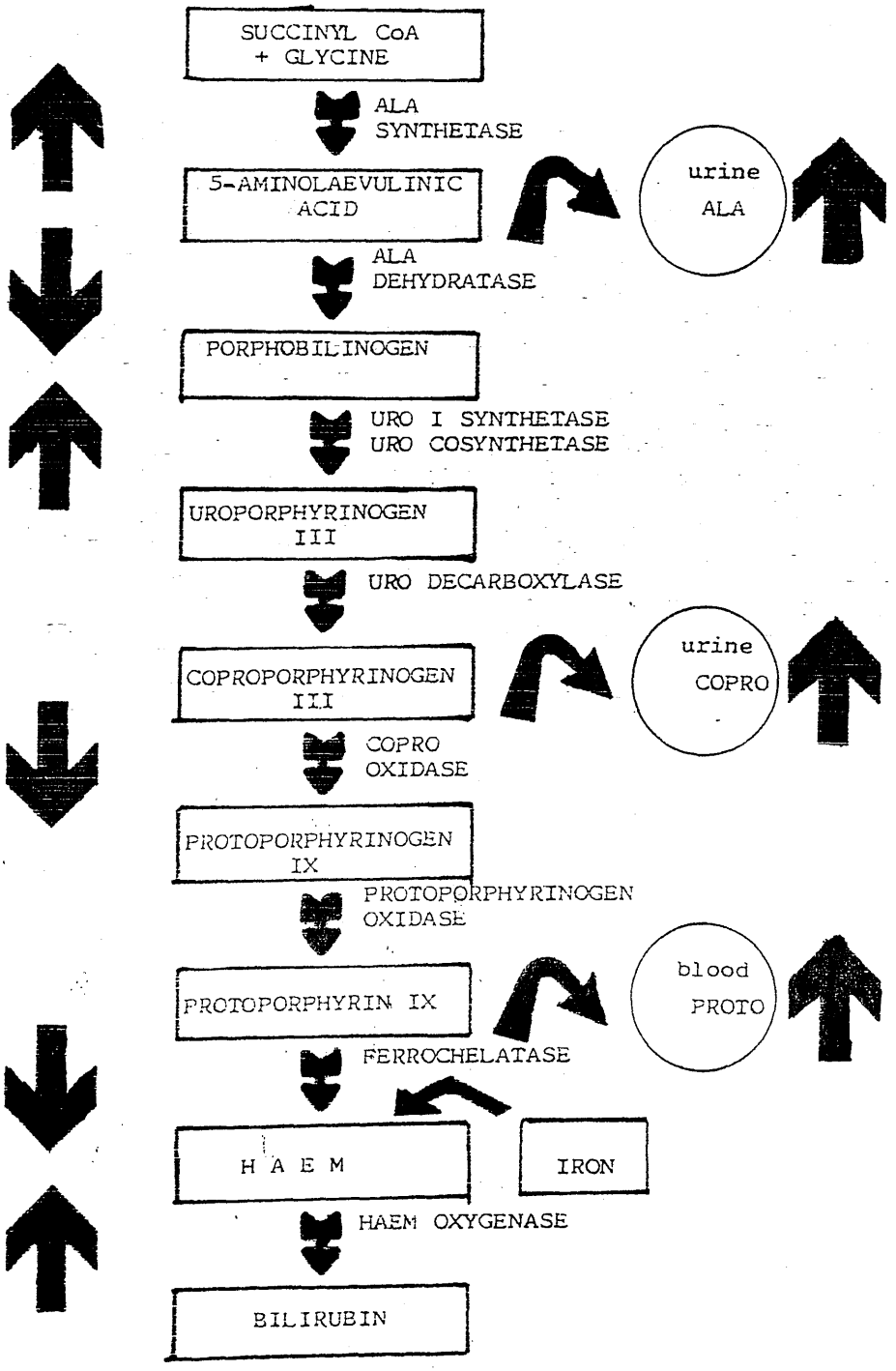
Haem biosynthesis commences within the mitochondrion of the cell with the rate-limiting enzyme of the pathway, 5-aminolaevulinic acid synthase. It then proceeds down the path in the cytoplasm before re-entering the same organelle for the final 3 stages leading ultimately to haem. Control is by way of negative feedback by haem upon ALA synthase. The series I porphyrinogens, uroporphyrinogen I and coproporphyrinogen I cannot be utilized and are therefore obligatory excreted.

coproporphyrinogen which transfers the haem biosynthetic pathway back into the mitochondrion. Coproporphyrinogen III is decarboxylated to protoporphyrinogen IX by coproporphyrinogen oxidase (E.C. 1.3.3.3) and oxidation of this porphyrin then occurs by the enzyme protoporphyrinogen oxidase forming protoporphyrin IX. The final step is the insertion of ferrous iron into protoporphyrin IX by ferrochelatase (E.C. 4.99.1.1) to form haem, which is ultimately degradaded to bilirubin via haem oxygenase (E.C. 1.14.99.3).

This pathway is well controlled via ALA synthase, producing only sufficient amounts of intermediates and ultimately haem to service the requirements for haemoglobin and other haemoproteins such as cytochrome P450. However under certain pathological conditions this coordination falls down. Lead has been demonstrated to upset haem metabolism significantly (figure 7.2). In lead poisoning there are early rises in urinary ALA and coproporphyrin with rises in PBG in severe cases. In the blood, erythrocyte protoporphyrin is increased. Studies performed both in the rat and man on the individual enzymes of the path indicate two factors are responsible for the accumulation of ALA by lead, namely a reduction in the activity of ALA D and an enhancement in the activity of ALA S (Miller et al, 1972; Campbell et al, 1977b; Meredith, Moore & Goldberg, 1977a; 1979; Goldberg et al, 1978; Meredith et al, 1978), although an in vitro study by Morrow and colleagues (Morrow, Urata & Goldberg, 1969) suggested an inhibition of ALA S by lead. Additionally lead is

Figure 7.2 Effect of lead on haem biosynthesis.

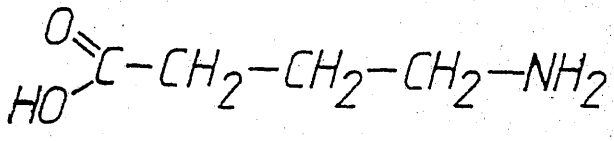
Pathological effects of lead on the haem biosynthetic pathway showing the overproduction of ALA, coproporphyrinogen III and protoporphyrin IX.



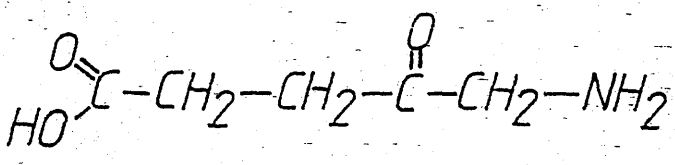
responsible for the depression in activity of copropophyrinogen oxidase and ferrochelatase (Campbell et al, 1977b) in human leucocytes. The inhibition of these enzymes combined with enhanced haem degradation also caused by lead exposure, leads to a diminished concentration of free haem and hence a feedback induced increased activity of the rate limiting enzyme, ALA S (Goldberg, 1972; Moore, Meredith & Goldberg, 1980).

From the foregoing description of the haem biosynthetic pathway and the effects of lead thereon, it is evident that there is an overproduction of ALA during lead exposure. It has relatively recently been suggested that this haem precursor may be responsible for at least some of the neurological features of lead exposure. Although PBG and the porphyrins are pharmacologically inactive, ALA has potent activity. ALA can penetrate into many tissues such as heart, liver, kidney, spleen, gut and fat in which it appears to be bound or maintained in tissues by active mechanisms (McGillion, Thomson & Goldberg, 1975), and it is capable of crossing the blood-brain barrier (Becker, Kramer & Viljoen, 1974; McGillion et al, 1974), the relationship between blood ALA concentration and that in brain being of the familiar curvilinear nature (McGillion et al, 1974).

ALA and the putative amino acid neurotransmitters, glutamic acid and γ-amino butyric acid (GABA) are structural analogues (figure 7.3) with ALA and GABA differing by only one carbon. Although ALA does not affect GABA synthesis or metabolism as demonstrated in rabbits (Becker, Cayanis & Kramer, 1980), evidence suggests that



GABA



ALA

Figure 7.3 Chemical structure of GABA and ALA.

The diagram illustrates the structural similarity between GABA and ALA.

ALA is capable of acting as a false transmitter at the GABA receptor (Nicholl, 1976; Dichter et al, 1977). Data obtained in synaptosomes prepared from rabbit cerebral cortex suggests that in vitro at least, ALA is capable of causing inappropriate release of GABA (Becker, Viljoen & Kramer, 1976).

Both in vitro and in vivo experiments have demonstrated the pharmacological potency of ALA in a variety of tissue preparations and species. In 1968 Feldman and his colleagues (Feldman, Levere & Lieberman, 1968) demonstrated the presynaptic neuromuscular inhibition of potassium augmented miniature endplate potential (mepps) frequency. The response of the frog sciatic-gastrocnemius preparation to electrical or nervous stimulation was also inhibited by ALA (Cutler, Dick & Moore, 1978), as was evolked release of acetylcholine in a low calcium- high magnesium bathing fluid (Bornstein, Picket & Diamond, 1979). Again in the frog, ALA caused depolarisation of the sartorius muscle (Becker, Goldstuck & Kramer, 1975) and caused inhibition of both the spinal cord ventral and dorsal root responses (Loots et al, 1975; Nicholl, 1976). Both spontaneous and acetylcholine induced contractions of rabbit duodenum were inhibited by ALA in a dose dependant manner, causing decreasing muscle tone, contraction rate and amplitude of contractions (Cutler, Moore & Dick, 1980).

Studies in small rodents suggest ALA is capable of altering activity. Intra-peritoneal injection of ALA into mice resulted in increased spontaneous activity (McGillion, Moore & Goldberg, 1973).The same group of workers later

demonstrated a differential effect of ALA depending on the mode of exposure. Acute exposure in mice resulted in an initial depression of spontaneous locomotor activity followed by a period of hyperactivity, whilst mice exposed chronically exhibited a reduction in spontaneous activity and reduced excitability (Moore, McGillion & Goldberg, 1975a). Cutler, Moore & Ewart (1979) studied the social behaviour of male mice 30-40 minutes following an injection of ALA. These rodents explored the cage and scanned less frequently than saline injected controls and also showed longer periods of immobilisation. A hypotensive action of ALA has been demonstrated in the anaesthetised and pithed rat (McGillion, Moore & Goldberg, 1975). The mode of action of ALA which is responsible for the actions on nerve-muscle function is uncertain but possibly the action of ALA to inhibit the sodium-potassium dependant ATPase, and thus the associated increase in capilliary permeability is important. The significant reduction in resting membrane potential observed in the frog sartorius muscle preparation by Becker et al (1975) is consistent with an action of ALA on the ATPase. The data of Cutler et al (1979) also indicates an effect of ALA on ion transport when they demonstrated the presence of polyphasic action potentials again in the frog neuro-muscular preparation following application of ALA.

It would therefore seem possible that certain neurological manifestations of lead exposure may be mediated through its proven haemopoietic effects. However, although ALA is capable of crossing the blood-brain

barrier, and hence causing effects within the brain, lead may, in addition, enter the brain and alter haem biosynthesis in situ. To investigate this possibility, the effect on haem biosynthesis in rat brain following lead exposure will be investigated in this chapter. To this end, the activity of the rate controlling enzyme, ALA synthase will be measured, since although it is not necessarily directly affected by lead, its activity will be elevated as a result of diminished haem production and the co-ordinated control of haem synthesis which occurs in mammals if lead is indeed having an effect on haem metabolism within the brain.

7.2 Methods

A group of male Sprague-Dawley rat weaners were divided into one of five groups destined to receive either distilled water or a lead acetate solution of concentration 24μM, 48μM, 140μM or 240μM lead which are equivalent to 5, 10, 30 and 50mg lead/litre as drinking solution. Each lead group consisted of 6 animals except the unexposed control group which comprised 8 rats. At the end of an 8 week period, the animals were sacrificed by decapitation and the brains removed for analysis of ALA synthase activity. Lead was also measured in a sample of heparinised blood and the femur. Analytical techniques for the measurement of lead concentrations, tissue protein and enzymatic activity are as described in the General Methodology section.

7.3 Results

In all lead exposed groups, the level of exposure selected resulted in a significant lead burden developing in these male rats, as demonstrated in this experiment by the elevated blood lead concentrations in the exposed animals by comparison with the control group (table 7.1; significance being obtained at the 2% level or less), and by the quantity of lead being stored in the skeleton; femur lead content being used as an index of skeletal lead accumulation (table 7.1). Although the level of lead in the drinking fluid of these rats was not high by comparison with many other studies performed by other groups investigating the neurotoxic action of lead, the possibility still remained that the brain growth of the exposed animals was retarded by comparison with the unexposed rats. Analysis of variance demonstrated that there was no significant difference in the protein content of the brain of the animals in each group (table 7.2) and thus any observed differences in 5-aminolaevulinic acid synthase activity if noted cannot be attributed to an indirect effect of lead on brain growth and development. Table 7.3 tabulates the activity of 5-aminolaevulinic acid synthase in each of the 4 lead exposed groups and the control unexposed group. From this data, it can be seen that there is a wide range in the activiy of this enzyme seen in these rats.

Table 7.1 Blood and bone lead concentration in male rats

Blood and bone lead concentration in rats exposed to 24, 48, 140 and 240 μM lead acetate solution compared to the concentration in the tissues of rats not exposed to lead. Results are expressed as the mean ± S.D. of 6 observations in each lead group and 8 in the control group.

Group	Blood lead (mean ± S.D. - μM)	Bone lead (μmol/kg dry bone wt.)
Control	0.41 ± 0.13	10±4
24 μM Pb.	0.58 ± 0.08 +	49±12 ++
48 μM Pb.	0.66 ± 0.06 ++	60±9 +++
140 μM Pb.	0.66 ± 0.03 ++	81±15 +++
240 μM Pb.	0.84 ± 0.20 ++	145±31 +++

\+ $p < 0.02$
++ $p < 0.002$
+++ $p < 0.001$

by comparison with the control group by Mann-Whitney U test

Table 7.2 Brain protein concentration in male rats exposed to lead acetate

Brain protein concentration of rats exposed to 24, 48, 140 and 240μM lead acetate solution compared to that of control unexposed animals. Results expressed as the mean ± standard deviation of 6 observations in each lead group and 8 in the control group.

Group	Brain protein concentration (mg/ml)
Control	27.2 ± 3.9
24 μM Pb.	22.8 ± 2.8
48 μM Pb.	25.0 ± 2.3
140 μM Pb.	27.4 ± 2.9
240 μM Pb.	25.9 ± 3.3

Table 7.3 Activity of 5-aminolaevulinic acid synthase in rat brain following exposure to lead

Activity of 5-aminolaevulinic acid synthase in rat brain following exposure to 24, 48, 140 and 240μM lead acetate solution compared to control non-exposed animals. The results are expressed as the mean ± standard deviation of 6 observations in each lead group and 8 in the control group.

Group	5-Aminolaevulinic acid synthase activity (mmoles 14C ala formed/g protein/hr.)
Control	1.45 ± 0.61
24 μM Pb.	1.32 ± 0.39
48 μM Pb.	0.90 ± 0.28
140 μM Pb.	1.13 ± 0.44
240 μM Pb.	1.77 ± 0.72

7.4 Discussion

Recently obtained evidence is quite strong suggesting that it may not be lead itself, but perhaps a product of deranged haem biosynthesis, which is adversely affecting the nervous system during lead exposure. Such an association between two systems, the haematological system and neural changes in lead poisoning would not be a unique bond as similar parallels can be observed with other pathologies such as vitamin B_{12} deficiency. Several possible modes of interaction can be hypothesised. Firstly, the neurological manifestations of lead neuropathy and the observed haematological changes may result from a common underlying disturbance of haem biosynthesis; Secondly, they may be due to a possible toxic action of an intermediate produced in excessive quantities; and thirdly the final proposal is that thay are the result of a deficiency state resulting from depletion of an essential cofactor. Although the biochemical lesion of lead poisoning is observed essentially in the bone marrow, it has not been ruled out that a similar lesion occurs in other tissues including neurons.

One of the characteristic features of lead poisoning, excessive production of ALA, is also a feature of the genetically transmitted inborn error of metabolism, acute intermittent porphyria (AIP). Following an extensive review of cases of AIP and lead poisoning, Dagg and his colleagues (Dagg *et, al*, 1975) noted similarities between the two pathologies. Both ailments are variably accompanied by abdominal pain, constipation, vomiting, paralysis,

demyelination and psychiatric disturbances. Voluminous data exists to indicate a significant increase in ALA both in blood and urine of lead exposed animals and man. Increases in ALA in tissues of experimental animals exposed to lead have also been documented. In a study by Silbergeld and colleagues (Silbergeld et al, 1982) there was a rise in the ALA of the spleen and kidney following acute administration of lead in a high dose, while chronic exposure resulted in an increase of ALA in brain, kidney and spleen in rats compared to control animals. In view of the observation that there is a common neurological feature of AIP and lead poisoning, the idea of a common aetiological agent such as ALA should at least be considered seriously. A difference however between the two pathologies does exist in that in AIP there is an endogenous biochemical abnormality resulting in excessive production of ALA, whilst in lead poisoning, an exogenous biochemical abnormality is responsible for the ALA production. If such a link exists between the excessive production of ALA and the neurological manifestations of lead exposure, one must then consider the mode of action of ALA.

It has been suggested that ALA may act as a GABA agonist on presynaptic receptors and by feedback inhibition may reduce the amount of GABA available at the synapse. Interference of GABAergic function is compatible with such clinical and experimental signs of lead neurotoxicity such as excitability, hyperactivity, and in severe cases convulsions (Silbergeld & Lamon, 1980). As noted in the introduction to this chapter, the two compounds have very

similar chemical structures (figure 7.3) with ALA possessing **an additional C = O group not present on the GABA neurotransmitter molecule.** One of the leading authors in this field, Silbergeld has suggested (Silbergeld et al, 1979) that the convulsions noted in severe cases of lead poisoning are indeed due to a decreased GABAergic neurotransmission. Studies in rats have demonstrated altered levels of GABA in the cerebellum following chronic lead exposure (Silbergeld & Lamon, 1980). Uptake and release of the neurotransmitter was also inhibited. Considering the observation that lead in vitro has no effect on GABA uptake or release from synaptosomes, then some other factor other than lead may be responsible for the inhibition of GABAergic function in vivo. One such possibility is the involvement of ALA which may reduce GABA release by a presynaptic action. An alternative proposal is that an intact multi-pathway system is necessary for this action of lead. In vitro studies (Nicoll, 1976; Silbergeld et al, 1980) have demonstrated that ALA is capable of displacement of GABA from synaptosomal membranes but that it is considerably less potent than GABA. This data would suggest that levels of ALA likely to be observed during lead exposure probably fall short of those required to be competitive. More plausibly, the role for ALA in initiating the neurotoxicity of lead exposure may well be through its action to reduce GABA release via the presynaptic negative feedback mechanism which operates on GABAergic nerve terminals.

Another possibility exists in that the key factor

involved in the neurotoxic manifestations of lead exposure, are related to a reduction in haem, rather than an effect of ALA. Lead is associated with a reduction in hepatic haem-requiring enzyme system, tryptophan pyrrolase via a reduction in the hepatic free haem pool. This results in a siginificant rise in brain tryptophan, 5-hydroxytryptophan and 5-hydroxyindoleacetic acid (LItman & Correia, 1983) which are all correctable by initiating haem infusions.

Whether or not the observed stimulation of ALA synthase activity is due to a direct action of lead is under debate. This induction of ALA synthase observed during lead exposure, may result from an increased demand for haem which is necessary for haemoglobin and other haemoproteins, or it may be due to a primary action of lead on the enzyme. Whatever the mechanism the consequence is a production of ALA. Patients with iron-deficient and megaloblastic anaemia have been observed to exhibit an increased concentration of ALA in blood although the activity of ALA dehydratase was noted to be decreased (Campbell et al, 1977b). Noting these observations it is most possible that the rise in ALA was due to the enhanced activity of ALA synthase stimulated by the lack of haem. Thus perhaps the same mechanism is in operation in lead exposure.

Consumptive depletion of pyridoxal phosphate due to induction and overactivity of ALA synthase might also account for lead neuropathy, but as yet, no evidence is available to confirm or refute this hypothesis. However, if confirmed, lead neuropathy would not be alone in a neurological pathology involving pyridoxal phosphate

deficiency, other proposed examples being AIP, isoniazid therapy and alcoholism.

It must be noted however that a fundamental difference exists between the two pathologies, lead poisoning and AIP. Whereas in AIP the increase in ALA synthase marks the beginning of an episode of attack, this same lesion is probably the resulting compensatory end stage of lead poisoning.

Although studies have noted an enhanced activity of ALA S in blood of lead exposed subjects, there is no available data relating to levels of activity of this enzyme in tissue. Our study, the results of which are reported in this chapter, measured activity of ALA S in whole rat brain. Table 7.3 demonstrates that no significant alterations were noted in this parameter at 4 levels of lead exposure compared to unexposed animals. This observation does not however imply that levels of ALA were not elevated in these rat brains. The standard deviations presented in table 7.3 indicate the large range of activities of ALA S measured in the brains. Most probably a very large number of animals would require to be included in each study group in order to identify any significant differences in ALA S activity if it were to be found. The data does not preclude an increase in ALA occurring in the brain as a result of lead exposure. Additionally, if the activity of ALA D, a sensitive enzyme to the action of lead, were depressed then haem availability may be reduced but to a degree insufficient to cause a feedback enhancement of ALA S activity.

Although it is well established that lead exposure results in a reduction in ALA D activity at least in the blood of exposed subjects (Meredith et al, 1979), it is almost certain that ALA D has the capacity to be reduced to a considerable degree before alterations in haem production begin to occur. If this were indeed the case then it is quite possible that no compensatory increase in ALA S would be noted, either in the blood of exposed animals or humans, or in the brain of experimental rats if lead is having an effect on haem biosynthesis in situ within the brain of such animals. One must also consider the data presented in this chapter in the light of the results obtained in previous chapters in the cholinergic, catecholaminergic and biopterin systems. The results noted in these previous sections indicate a specific effect of lead within only 2 of the 4 brain regions under study. This chapter investigates the activity of ALA S following lead exposure in the rat brain as a whole and therefore the possibility must be considered that lead may cause changes in ALA S activity either directly or indirectly, but that they are cancelling each other out when the brain as a whole is investigated.

In conclusion, the data presented in this chapter would not provide any evidence in support of a role of ALA in relation to the neurotoxic action of lead, but as discussed above the non-significant observations do not rule out such an association.

CHAPTER 8

GENERAL DISCUSSION

The studies performed in order to compile this thesis have been diverse and covered a range of aspects of the possible neurotoxic effects of lead. Although there has been a general reduction in the level of lead exposure within the population as a whole, there still remain certain individuals who are exposed to significant quantities of lead via one source or another. With particular regard to the neurotoxic effects, the younger members of the population are especially at risk. Locally in Glasgow, traditionally a region of high lead exposure, the main source being the drinking water supplied from Loch Katrine in the Trossachs, there has been a great improvement with regard to the quantity of lead to which the population is exposed. The data presented in chapter 3 indicates the significant reduction in water lead which can be achieved by the relatively inexpensive measures of raising the pH of the water and thus reducing plumbosolvency. By these means, the lead exposure of the population of a city can dramatically be reduced. Of course, this is a technique which will only be of value in a population in whom a major percentage of the lead exposure is derived from water and to a certain extent food cooked in such water. Glasgow conforms to this picture very well as do other areas in the West of Scotland, and indeed Wales where a significant reduction in lead exposure levels can be achieved by similar water treatment. However, it must be remembered that there still remains a proportion of the population who will still be at risk of lead exposure even after such treatment, and it is this group

which one should concentrate on to reduce their exposure by alternative means.

Amongst the numerous actions of lead upon the human body the most poorly understood is that affecting the nervous system. Turning to the specific neurotoxic effects at the molecular level, despite many years of modern sophisticated techniques the old saying of Tanquerel de Planches still holds true -

'.... but we can see this damage only through its symptoms and nil through its anatomy'

and today many years on, his words are being repeated -

'the neurotoxic effects of lead can occur before histopathological alterations are evident'
- Silbergeld & Hruska, 1980

Although pathologists and electrophysiologists report damaged neuronal axons and myelin sheaths as a result of lead poisoning, it is undoubtedly the biochemist who will provide the answer to the primary cause of lead neuropathy. Great effort must be expended to identify biochemical lesions which express themselves clinically prior to pathological alterations. It is generally accepted that the severe neural damage due to lead poisoning may be irreversible. It is precisely this irreversibility which, if it also applies to the more subtle neural effects of lead, has given rise to the concern about the exposure of young children, who are especially susceptible to lead in the environment due to their greater intake of lead both from the diet and atmosphere per unit of body weight, and their enhanced absorption by comparison with adults.

A fundamental assumption to the discussions concerning the mechanisms of lead neurotoxicity is that lead does not

play a useful role in normal cell biochemistry or physiology; to date no function has been described. Bearing this assumption in mind, the notion of a 'no-threshold level' for the neurotoxic effects of lead is not hard to comprehend. This is in contrast to other metals such as copper which can be classified either as a trace metal or a neurotoxin depending on its concentration. Although an ancient problem, the interest in lead toxicity remains to the fore today for many reasons. Where lead is concerned, there is less than an order of magnitude separating those levels commonly proposed to be toxic and the concentrations observed in the general population. There remains a scientific desire to elucidate the specific site(s) and mode(s) of action of the neurotoxic action of lead. On the other hand, a more recent interest has emerged; that of a threshold of effect and an interest in so called 'low, subclinical' environmental levels of lead previously considered subtoxic. Progression through the twentieth century has seen changes in the history of lead neuropathy. Increasing concern over the deleterious neurotoxic effects of lead have resulted in the near eradication of chronic occupational and non-occupational exposure to the metal. Today, overt lead poisoning is a rarity by comparison with the past. Lead at a relatively high body burden no doubt causes encephalopathy but susceptibility between individuals is extremely variable (Winder & Kitchen, 1984). The presence of lead within the human body has been adequately demonstrated with the sensitive detection methodology available today. What is questionable is the

degree of lead burden responsible for pathological sequelae.

Silbergeld (1982) proposed 3 basic principles with regard to lead neurotoxicity. Firstly, the alterations in neural functioning are observed prior to neuropathological damage, as was noted by Tanquerel de Planches; Secondly, the various neurotoxic effects, which are still uncertain, are specific for different neurotransmitter pathways within the central and peripheral nervous systems; and thirdly, these specific transmitter related effects result from highly specific effects of lead on ionic mechanisms of neurotransmission, such as enzyme regulation, transport processes, phosphorylation or transmitter release. Once the metal enters the body compartments it is difficult to remove since there is no biochemical mechanism present for its clearance.

Although it is perfectly justifiable to propose that elevated levels of environmental lead resulting in exposure to the metal for a lengthy period of time may result in mental retardation, intelligence and/or psychological impairment, hyperactivity and other behavioural changes, this hypothesis has not been inconclusively proven. Neither have the neurochemical alterations caused by lead been elucidated and matched with the associated neurological impairment. It is however, most probably the case that the toxic action(s) of lead on brain biochemistry are specific to each particular neurochemical pathway and vary between each anatomical brain region. Current investigations into the neurotoxic action of lead can be divided into two

spheres; Firstly, there are the numerous epidemiological studies on children being performed all over the world, and Secondly, the laboratory animal studies attempting to identify a biochemical lesion. It is this second type of study with which this thesis is concerned. Although the epidemiological research studies generally employ I.Q. tests which are reliable, well standardised tests used in both the educational and clinical fields, there is an inherent problem in this type of research. No definitive means is available to decide whether an observed statistical association between an environmental factor, such as lead exposure, and a measure of health outcome, for example I.Q. deficit is due to a cause and effect relationship. The involvement of a third factor linking the two cannot be definitively ruled out. The majority of studies involve the measurement of cognitive or mental deficiences or sensory-motor, that is co-ordination deficiencies at a degree of lead exposure termed 'low-level' or alternatively 'sub-clinical', a phrase which is inadequate and indeed self-contradictory. Considerable variation exists between individual children for any neuropsychological measurement and consequently any proposed study must recruit a large number of children in order to overcome the natural scatter of behaviour or ability and detect any small, but nevertheless important association with lead. A complex inter-relation exists in determining a child's behaviour and intelligence involving hereditary factors and a wide range of environmental influences. Over and above this variation in a child's

ability there is a variation in susceptibility to lead existing within the population, and indeed even within a group of children of similar age. A variety of physiological factors come into play in determining the detrimental effect of a specific dose of lead on a particular child, a feature well described by Dickens in the last century -

'Some of them gets lead-pisoned soon, and some of them gets lead-pisoned later, and some, but not many, niver; and 'tis all according to the constitooshun, sur; and some constitooshuns is strong and some is weak'

- The Uncommercial Traveller
Charles Dickens, 1868.

The data obtained from such population studies performed prior to 1980 has been extensively reviewed by a DHSS working party of 12 scientists, set up in 1978 under the leadership of Professor Patrick Lawther (DHSS, 1980). Its terms of reference were to review the overall effects on health of environmental lead from all sources, and in particular its effects on the health and development of children, and to assess the contribution lead in petrol makes to the body burden. No definite conclusions were recognised. Subsequent data was then considered by the MRC who were asked by the DHSS in 1983 for advice on the validity and interpretation of the results on research on neuropsychological effects of lead in children (MRC, 1983). The MRC lead advisory group noted significant lead related effects obtained outside the United Kingdom but dismissed the data on the grounds that the studies exhibited methodological deficits (Ernhart, Landa & Schell, 1981; McBride, Blank & English, 1981; Thatcher et al, 1982) but

tended to come down on the side of a 'no detrimental lead related effects', a conclusion derived from studies performed in the U.K. (Smith et al, 1983; Harvey et al, 1984; Yule et al, 1984).

A major drawback in the elucidation of the neurotoxic action of lead in man is that we must depend on animal experimentation in order to investigate the site and mode of action at the molecular level. Various enzyme systems are sensitive to the action of lead; notably, the enzymes of the haem biosynthetic pathway, particularly ALA dehydratase; and those involved in tetrahydrobiopterin metabolism. There is a vast wealth of data in the literature investigating the biochemical aberrations caused by lead within the brain. Various studies have been performed mostly in rodents, concentrating on the cholinergic, catecholaminergic, GABAergic, opioid and tryptaminergic neurotransmitter systems. In addition, more recently levels of various amino acids, including those now considered putative neurotransmitters have been studied in lead dosed rodents. Data to date has centred on glutamate, aspartate, glutamine, tyrosine, proline, glycine, phenylalanine and leucine. However, the vast majority of data is concerned with the cholinergic, catecholaminergic and GABAergic systems. The literature is contradictory with regard to all of these systems and the reason for this must be sought if any progress is to be made in elucidating the neurotoxic effects of lead at the biochemical level. Most certainly, the use of different exposure levels, time of exposure, age, sex and species of animals, must all

contribute to the disparity between data in the literature. Particularly important with regard to cholinergic effects of lead is the method of sacrifice at the end of the animals exposure period. Consideration of the data as a whole reveals a general downgrading of the cholinergic system while catecholaminergic activity is enhanced by lead. The data obtained in chapter 4 would certainly substantiate an effect of lead on cholinergic nervous function but the specific effect remains elusive. The action is almost certainly regionally specific within the brain and not a generalised action. The enhanced activity of choline acetyltransferase when related to protein content noted in the diencephalon may well be a compensatory effect as a result of a primary action of lead; it is unlikely to be a direct stimulatory effect of lead on the enzyme itself although this possibility must not be ignored. On the other hand, the depression in enzymatic activity observed in the midbrain may either be a direct effect of the metal or secondary to another action, which is as yet unknown. The possible influence of lead on cholinergic metabolism will require to remain uncertain at present and most certainly a great deal of work will be necessary in order to identify the specific biochemical effect of lead on central cholinergic metabolism. Included in these possible cholinergic effects of lead must be an influence on glucose metabolism resulting in altered availability of precursors for acetylcholine synthesis, notably acetylcoenzyme A which is derived by way of the glycolytic metabolic pathway. Evidence is accumulating to suggest a link between energy production of some sort and a

cholinergic effect of lead.

There is even more data available concerning the catecholaminergic nervous system. It is not possible to determine from the experiments performed in chapter 5 as to whether the observed lead related alterations in catecholaminergic system are related to the known effects on tetrahydrobiopterin metabolism. The effects of lead on both systems appear to be complex and vary depending on the dose and duration of exposure. Certainly the data collected in this thesis is not in dispute with the studies in the literature which show great disparity in results between the various research groups. It is thus evident that with regard to the catecholaminergic system only a true comparison can be made between studies employing similar conditions of exposure. Certainly an effect of lead even at relatively low levels of exposure on catecholaminergic function is confirmed by the data presented in chapter 5. It is important however to disregard those studies in the literature in which the data obtained may be complicated by the presence of non-lead variables influencing the measured parameters. Most importantly, the presence of under-nutrition in the animals must be noted if it occurs as a result of lead exposure. Studies where the animals are under-nourished should be interpreted with great caution since those effects may influence catecholaminergic function and subsequently be attributed to the effect of lead.

Whether there are several primary sites of action of the metal remains to be elucidated. The data presented in

chapter 5 shows lead related alterations in catecholaminergic function being manifest in several parameters such as levels of the catecholamines themselves, noradrenaline and dopamine, activity of the rate limiting enzyme of the synthetic pathway tyrosine hydroxylase, or in PNMT activity the enzyme responsible for formation of adrenaline. There may be only one primary site of action for lead and all these noted alterations are observations resulting from this one action, or alternatively, multiple target sites for lead on this system may exist. Over and above, alterations to this relatively complex pathway at one site may lead to compensatory changes in order to maintain the levels of neurotransmitters within optimum range. It is important to maintain the concentration of noradrenaline and other catecholamines at an optimal level since they play an important role in brain neurotransmission.

The observed alterations in catecholaminergic metabolism noted in this thesis or by other workers, may be related to the availability of the pterdine cofactor for tyrosine and phenylalanine hydroxylases, tetrahydrobiopterin. The data presented in chapter 6 clearly demonstrates that lead does have a profound effect on tetrahydrobiopterin metabolism, and indeed these influences are evident at relatively low levels of exposure since the data obtained in man was observed in subjects not suffering from clinical lead poisoning. The animal studies reveal that the effects of lead are not simple and there are most probably multiple sites of action which may depend on the dose or duration of

lead exposure. What is certain however is that lead is capable of interfering with normal tetrahydrobiopterin availability which may well influence the activity of phenylalanine hydroxylase and more importantly tyrosine hydroxylase, the rate limiting enzyme of catecholamine synthesis. More knowledge concerning the factors controlling the activity of tyrosine hydroxylase are necessary in order to gain an insight into the relative importance of tetrahydrobiopterin concentrations with regard to catecholaminergic neurotransmission. In addition it should not be forgotten that tetrahydrobiopterin plays an essential role in determining the activity of another hydroxylation enzyme, tryptophan hydroxylase. To date, only limited research into the possible effects of lead on tryptaminergic neurotransmission has taken place, and the importance of the role of the cofactor tetrahydrobiopterin in monoamine transmitter systems remains unclear. Tetrahydrobiopterin may well provide a link regarding the neurotoxic action of lead on two central neurotransmitter pathways, the catecholaminergic and the tryptaminergic systems. With regard to all the neurotransmitter systems studied throughout this thesis, significant changes in the various biochemical parameters may not have been noted because any alterations noted were too minor to reach significance. The use of higher exposure levels may or may not have resulted in alterations reaching significance. However, dose selection must be performed cautiously since at higher levels of exposure non-specific effects begin to dominate, a principal example being undernutrition. The

data presented throughout this thesis indicates that such effects have been largely eliminated.

Studies have clearly demonstrated that in immature animals especially, chronic lead exposure alters cholinergic and catecholaminergic functioning (Shih & Hanin, 1978a). Silbergeld has proposed (Silbergeld & Adler, 1978) that these effects are produced at the subcellular level through some kind of interaction between lead, calcium and sodium, perhaps with the mitochondrion being a crucial organelle for the observed effects (Silbergeld et al, 1978). In this thesis the data obtained with regard to the neurotransmitter systems was limited to the cholinergic and catecholaminergic nervous systems as time was a limiting factor in this research. However, although there are lead related changes occurring within these two systems, these are most probably only a contributing part of the vast conglomerate of effects of the neurotoxin. The restriction of investigation which most of the data in the literature is concerned with to catecholaminergic, cholinergic and GABAergic systems, should not be interpreted as implying that these systems are either the exclusive or most sensitive for the toxic action. This development picture is merely the result of historical developments. Indeed, data obtained by my own group, recently published and included in this thesis, indicates that the tetrahydrobiopterin metabolic pathway is extremely sensitive to alteration by very low levels of lead exposure. Only recently has this system been investigated with regard to the neurotoxic mechanism of lead.

Increasing data is being accumulated concerning the effect of lead on GABAergic transmission. Studies have either investigated an effect of lead on GABAergic function, or alternatively, a role for GABA has been proposed in association with ALA. The haematopoietic system is most certainly altered by lead. ALA is greatly increased in lead exposure and it is proposed that it is this compound that may well act as a false transmitter at the GABA receptor due to the structural similarities between the two compounds (Nicell, 1976; Dichter et al, 1977; Silbergeld & Lamon, 1980). Similarly to the other neurotransmitter systems, available data on the GABAergic system is not consistent. Although some workers have observed no alterations in forebrain GABA levels (Sauerhoff & Michaelson, 1973), other groups have noted altered transmitter levels in addition to changes in several other aspects of GABAergic function including various enzymes (Silbergeld et al, 1979). These alterations in enzymatic activity have not however been confirmed by other workers (Govoni et al, 1980) although this group employed a lower dose of lead. It should be noted that where such alterations are observed they are regionally specific, a feature in accord with effects on other neurotransmitter systems. If there were a reduction in GABA in certain brain regions this could provide some explanation for the reported increased central nervous system excitability since GABA is a major inhibitory neurotransmitter. In consequence, any effect of lead on GABAergic neurotransmission would be expected to produce an increase

in central excitability or alternatively, inhibition of neurochemical paths which mediate inhibition of the central nervous system. However, these effects remain to be proven.

More recently, the opioid neurotransmitters have been studied. One group have noted an increase in striatal enkephalons following lead exposure (Govani et al, 1980; Memo et al, 1980) whilst other studies have demonstrated a depression in this same brain region (Clayton et al, 1983, Winder et al, 1984) although these studies employed a lesser degree of lead exposure.

Sparse data is available concerning the tryptaminergic system. The data from one group (Dubas & Hrdina, 1978; Dubas et al, 1978) reported reduced levels of 5-hydroxytrypamine and in two brain regions, the cortex and hypothalamus, whilst the level of metabolite, 5-hydroxyindoacetic acid was also depressed in these two same brain regions in addition to the midbrain.

Lastly, concerning other putative neurotransmitters such as amino acids, Patel and his colleagues (Patel et al, 1974) measured the developmental increase in glutamate, aspartate and glutamine and noted it to be reduced in lead treated compared to control animals. Uptake of other amino acids into certain brain regions, such as glycine, phenylalanine, leucine, proline and tyrosine has been observed to be either unchanged (Silbergeld & Goldberg, 1975) or increased (Gerber et al, 1978). The most important nonspecific effect of lead is undernutrition. The conclusions drawn from studies where a degree of undernutrition is present must be considered carefully.

Although the haemopoietic system has long since been recognised as a target system for the toxic action of lead, and it was briefly investigated with regard to the neurotoxic actions of lead (chapter 7), it remains far from clear as to the involvement of ALA in brain neurotransmission. Alterations in the concentration of this compound may well occur as a result of lead exposure even at relatively low levels, but we must prove that ALA is neuroactive at concentrations noted within the brain during lead exposure before we can plausibly consider a role for ALA in lead neurochemistry.

It would therefore appear that there are a great many proposed biochemical effects of lead, and whether or not all do indeed occur remains to be proven, but it would seem more probable that several observed alterations are secondary to alternative primary effects of lead. Preistell sums up the situation accurately -

'with the passage of time the only measurement of agreement reached among various experts was that the problem was complex, and with the advent of modern laboratory methods it seems to have become even more complicated.'
- Preistell, 1958

In addition to these specific observed alterations on the neurotransmitter systems, there are known interferences in intermediary metabolism. Amongst these the one most likely to be of supreme importance, especially within the nervous system, are those biochemical effects upon terminal oxidative metabolism. In vivo and in vitro studies have shown changes in mitochondrial function. Changes in the morphology of the organelle have been noted in several organs including Kidney (Goyer & Phyre, 1973), heart

(Malpasse et al, 1971; Moore et al, 1975), liver (Hoffman et al, 1972) and in the central and peripheral nervous systems (Press, 1977; Brashear et al, 1978). Such effects on the mitochondrion will result in changes in terminal oxidative metabolism. Uncoupling of energy and metabolism occurs and cell respiration is inhibited (Bull, 1980). Such alterations in mitochondrial functioning may well be more important in their own right, but more significantly they may result in reduced availability of precursors for neurotransmitter synthesis, such as acetylcholine, as discussed earlier. The reported neurochemical effects of lead are enormous. Broadly speaking, the cholinergic system is associated with changes at relatively high levels of exposure and indeed many changes are of a non-specific nature. The data presented in this thesis would however support specific changes, although minor, occurring in cholinergic function at low levels of exposure. Effects on the catecholaminergic system are noted more frequently at lower levels of exposure.

In conclusion therefore, it is not unexpected that lead will be neurotoxic in view of the diverse biochemical effects it is observed to have, both in vivo and in vitro. As a result of the vast confusion which exists in the literature, the mode and sites of action of lead upon the nervous system are not understood but it is clear that there are several components to this action involving both neurochemical and cytological features. Most probably all relate to the neurochemical features which result from over exposure to the metal. The relative importance of these

various neurochemical events is difficult to apportion. There are obviously changes in catecholaminergic function which may be linked to the availability of pteridine cofactor. Other systems such as the cholinergic, tryptaminergic or GABAergic system are less markedly affected but undoubtedly especially at higher levels of exposure, contribute to the observed changes associated with lead exposure and the same applies to the plethora of other neurotransmitters. The scientific world must move on from the simple identification of such changes in the various neurotransmitter systems to proceed towards a goal of apportionment of the importance of these various effects.

Most probably lead does not have a widespread action over the whole nervous system where gross anatomy is concerned, but a more realistic view when investigating biochemical effects is that the metal is exerting specific actions on discrete anatomical areas of the central nervous system and thus the biochemical aberration will be quite specific to the brain region under consideration. The data presented throughout this thesis would certainly conform to this hypothesis. If this is indeed the case, the observation will go some way to explaining some of the discordant observations in the literature.

There remain several questions still unanswered which were first asked many years ago. Firstly, the concept of a threshold level of lead at which effects begin to occur, and secondly tied in with this notion, the idea of a 'normal' blood lead level. Today, we still remain in the

dark as to the effects observed both in pathology and in biochemical pathways. Over and above this, if such effects do indeed occur, do they have a clinical significance? The answers to these questions remain to be elucidated.

It is most probably correct to say that lead neuropathy is an example of a concept proposed by Sir Rudolph Peters; it was suggested that there was a necessity to identify a metabolic defect as the biochemical basis for the neurotoxic action of chemical substances. Identification of a primary biochemical lesion is not easy, since there may be a time lag between the period of exposure and the effect. Lead neuropathy ensues as a result of a chronic, perhaps long term exposure to lead. Thus it is difficult to correlate observed biochemical lesions with electrophysiology and neurochemical data since it cannot be assumed that peak lead concentrations coincide with the time of measurement, and indeed peak biochemical changes may well not occur simultaneously with maximum physiological change. Alterations in neurochemistry may precede and be the basis for the altered behaviour and pathologies previously attributed to lead exposure. Alternatively such noted neurochemical changes although present, may not result in any clinical manifestations and therefore their relevance is questionable. Due to the complexity of the situation it has proven difficult to relate neurochemical and neuropathological effects of lead. Many pathological changes reported in the literature were noted at exceedingly elevated lead levels, levels which have no relevance to the currently employed lower

concentrations now used in animal experimentation. Although certain chemical changes within the central nervous system may not be manifest clinically, I think it naive to accept these changes as of no detrimental significance. The levels of lead which the population as a whole, and young children in particular, are exposed to must be reduced to a level at which no detectable changes of any sort can be observed within the human body. If this is not possible, then every effort should be made to reduce lead exposure to a minimum from every source known to man today.

A great wealth of data is emerging daily concerning the neurochemical aberrations associated with lead toxicity, but as yet no consensus of opinion has been attained in the identification of a single critical biochemical lesion responsible for the damage to the neurone. More probably ultimately one specific alteration will not be found, but a group of effects will be identified. I believe it remains essential that the quandary which has troubled scientists and physicians alike for so many years be solved, despite the views of other eminent people -

'as long as everyone is occupied in the search after truth, it matters little if all arrive at different conclusions.'
- J. Priestly, 1733 - 1804

The one conclusion must be reached even if this means arriving at different lead related effects within each neurotransmitter pathway, but the biochemical lesion within each pathway must be agreed upon.

APPENDICES

Appendix 1

Materials and methods

Animals The rats employed in all experiments were supplied by Bantin & Kingman, Grimston, Hull, England.

Lead & cadmium estimation Lead and cadmium nitrate solutions were of atomic absorption spectrophotometry grade supplied by BDH Biochemicals, Poole, England. Nitric acid used in bone lead estimation was 'aristar' grade from BDH; glassware and disposible polypropylene equipment was presoaked in 'analar' nitric acid (BDH).

HPLC HPLC columns were packed with Spherisorb 5μm ODS packing material obtained from Phase-Separations Ltd., Queensferry, Clwyd. Methanol employed in the mobile phase was of 'HPLC' grade obtainable from several commercial suppliers; sodium octyl sulphate was from Eastman Kodak; potassium dihydrogen orthophosphate was HPLC grade from Fisons. Distilled water used to prepare mobile phase was redistilled from alkaline potassium permanganate.

Alumina Woelm neutral activity grade 1 alumina was employed for extraction of catecholamines.

Tyrosine hydroxylase activity L-tyrosine, benzyloxyamine and 3,4-dihydroxybenzylamine hydrobromide were from Sigma; 6-methyl-5,6,7,8-tetrahydropterin and catalase from Calbiochem, Cambridge.

Phenylethanolamine N-methyltransferase Noradrenaline, tryptamine, B-phenyl-ethylamine, phenylethanolamine and PNMT were supplied by Sigma; Fisofluor 'mpc' was from Fisons; labelled S-adenosyl-L-(methyl-^{3}H)methionine was obtained from Amersham International plc., Buckinghamshire.

Crithidia fasciculata plasma biopterins all components for part 1 of the culture medium were obtained from BDH as was haemin and sucrose; triethanolamine and all components of part 2 were supplied by Sigma; vitamin free casamino acids were from Difco; yeast extract from Oxoid; Trypticase from BAL; liver fraction L from Nutritional Biochemical Co.; Folic acid from Kodak.

Tetrahydrobiopterin 7,8-dihydro-L-biopterin and 5,6,7,8-tetrahydrobiopterin dihydrochloride were obtained from Dr. B. Schircks Laboratory, Buechstr. 17, CH-8645, Jona, Switzerland.

Dihydropteridine reductase horseraddish peroxidase and NADH were obtained from Sigma; 6-methyl-5,6,7,8-tetrahydropterin and 6,7-dimethyl-5,6,7,8-tetrahydropterin were from Calbiochem, Cambridge.

Protein estimation bovine serum albumin was obtained from Armour Pharmaceutical Co., Eastbourne, England; Folin & Ciocalteau's reagent from BDH.

Choline acetyltransferase unlabelled acetylcoenzyme A and physostigmine were supplied by Sigma whilst the labelled compound was from Amersham International plc.

5-Aminolaevulinic acid synthase labelled 2(14C)-glycine was from Amersham International plc.

All other reagents employed in the assays used in this thesis were of 'analar' grade and obtained from commercial suppliers.

Appendix 2 Questionnaire - Glasgow lead study 1981

Identification number

LA Code Personal number letter

PART 1: PERSONAL DETAILS

1.1 Surname _____

1.2 First name(s) _____

1.3 Home address _____

_____

Post code _____

1.4 Telephone Home _____

Work _____

1.5 Result to be reported (i) To subject Yes

No

only if elevated PbB

(ii) To general practitioner Yes

No

only if elevated PbB

1.6 General Practitioner

Name _____

Address _____

_____

PART 2: BACKGROUND INFORMATION

2.1 Member State 0 9

2.2 Area Code 8 7 5 0

2.3 Type of survey 0 0 8

2.4 Personal number

2.5 Date questionnaire completed

day month year

2.6 Ethnic origin

White	0
African/W.Indian	1
Indian/Pakistani/Bangladeshi	2
Other	3

2.7 Sex

Male	Female
0	1

2.8 Date of birth

month year

2.9 Years of residence at present address

2.10 House built

Before 1945	After 1945	Not known
0	1	2

2.11 Current occupation

2.11.1 Working / Not working

Working	Not working
0	1

if NOT Working, go to Q.2.12

2.11.2 Employee / Self-employed

2.11.3 Type of job

Manager / Foreman / Other

2.11.4 Type of business

2.11.5 Medical surveillance for occupational lead exposure in present job

YES	NO	Don't know
0	1	2

2.11.6 Length of time in present job

Less than six months	More than six months
0	1

If more than six months go to Q 2.14

2.12

All those not currently working

Have you ever worked? YES NO

0 1

All those in current job less than 6 months.

Different job within

last 12 months ? YES NO

0 1

IF NO TO EITHER QUESTION

GO TO Q 2.14

2.13 Previous occupation

2.13.1 Employee Self-employed

2.13.2 Type of job

Manager Foreman Other

2.13.3 Type of business

2.13.4 Medical surveillance for occupational exposure to lead?

YES NO Don't Know

0 1 2

2.14 Exposure of others in household to occupational lead

	YES	NO	DONT KNOW	Not applicable
Spouse				
Father				
Mother				
Other adult				
	0	1	2	3

2.15 Smoking habits

2.15.1 Cigarettes

Never smoke these days	0
Less than 5 a day	1
5-20 a day	2
More than 20 a day	3

2.15.2 Pipe or Cigars

YES	NO
0	1

2.16 Predominant drinking habits

Please tick all which apply

- Tap water
- Bottled water
- Milk
- Canned juices
- Beer
- Wine
- Spirits

PART 3: RESULTS OF BLOOD LEAD ANALYSIS
(TO BE RETURNED TO DOE)

3.1 Blood sampling

3.1.1 Date of collection

day month year

3.1.2 Method

capillary 0

venous 1

3.1.2 Anti-coagulant

EDTA

3.2 Blood analysis

3.2.1 Date of analysis

day month year

3.2.2 Laboratory

3.2.3 Blood lead (ug/100ml)

3.2.4 Internal quality control

Orange

Green

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EPILOGUE

EPILOGUE

Certain important observations have emerged from the collected data presented in the various chapters throughout this thesis. Firstly, there is adequate evidence in the results section of each chapter to show that lead is affecting brain neurochemistry; and secondly, this neurotoxic action is not uniform throughout the brain. Investigation of the cholinergic, catecholaminergic and tetrahydrobiopterin systems within the brain has demonstrated a selective effect of lead in only two of the four anatomical brain regions studied in this research.

The results obtained in the cholinergic (chapter 4), catecholaminergic (chapter 5), and tetrahydrobiopterin (chapter 6) systems, shows that it is in the midbrain and the diencephalon that lead is having the greatest effect. Although several parameters were measured in the telencephalon region of rat brain following lead exposure at periods of between one and seven months post-weaning in addition to a period of exposure in utero, only one significant change was observed by comparison with control animals; namely when rats were exposed to 480μM lead for one month post-weaning, there was a significant elevation in dopamine in this brain region, but only when the results were expressed per unit of tissue and not protein.

In the cerebellum no significant lead-related changes were observed in choline acetyltransferase activity, tetrahydrobiopterin concentrations, or dihydropteridine reductase activity in any group of lead exposed animals. There were

however alterations in various parameters of the catecholaminergic system in this brain region. Noradrenaline levels were reduced in the group of rats exposed in utero and for one month post-weaning to 480μM lead while a reduction in dopamine was noted following seven months post-weaning exposure to 48μM lead. The rate controlling enzyme of the catecholamine synthetic pathway, tyrosine hydroxylase was also increased in those lead exposed rats which exhibited raised noradrenaline levels in the cerebellum.

However, as stated above, the midbrain and diencephalon exhibited the majority of lead related changes. Within the cholinergic nervous system lead exposure resulted in a significant reduction in choline acetyltransferase activity in the midbrain following three months exposure to 24μM lead, whilst the same enzyme was elevated in the diencephalon. Investigation of the metabolism of tetrahydrobiopterin in the four brain regions showed significant changes both in cofactor concentration and dihydropteridine reductase activity only in the diencephalon. In the catecholaminergic nervous system (chapter 5), several lead related effects were noted in both the midbrain and the diencephalon. Considering all the lead exposure groups, noradrenaline levels were noted to be reduced by lead exposure in the midbrain, as were dopamine concentrations. The only change in tyrosine hydroxylase activity in the midbrain was observed in the seven month (48μM) group of animals in which enzymatic activity was significantly reduced. Three months exposure to 480μM lead caused an elevation in phenylethanolamine N-methyl transferase (PNMT) activity

while lengthening the time to seven months resulted in PNMT activity falling below control values. While dopamine concentrations were observed to fall in the diencephalon as a result of lead exposure and noradrenaline levels were also altered, the direction of change of noradrenaline was variable being dependant on the length and degree of lead exposure. Tyrosine hydroxylase activity was also noted to be reduced by lead, as was PNMT activity in the diencephalon.

These results suggest that the effect of lead is regionally specific with little or no effect in the telencephalon. There is a modest influence on the catecholaminergic nervous system in the cerebellum, and numerous effects on the catecholaminergic nervous system, tetrahydrobiopterin metabolism and choline acetyltransferase activity in the midbrain and diencephalon.

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