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The Development of an Electron Capture Gas Chromatographic Method for the Assay of Catecholaldehydes in Tissues

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THE DEVELOPMENT OF AN ELECTRON CAPTURE GAS CHROMATOGRAPHIC METHOD FOR THE ASSAY OF CATECHOLALDEHYDES IN TISSUES

BY

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LIFE

Balbir Kaur Dhaliwal was born in Goniana, Punjab, India on January 1, 1947. She received a Bachelor of Science degree from Punjab University, India, in June, 1967. In 1967 she started her graduate work at Meerut University, India and received a Master of Science degree in January, 1970.

On May 6, 1970 she married Tehsel Singh Dhaliwal. On May 19, 1971 she gave birth to a baby girl, named Mandeep Kaur Dhaliwal.

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AcH.....Acetaldehyde

AN.....Acetonitrile

CA.....Catecholamine

CNS.....Central nervous system

COMT.....Catechol-O-methyl transferase

CSF.....Cerebro spinal fluid

DHPG.....3,4-dihydroxyphenyl glycol

DHPAcH.....3,4-dihydroxyphenyl acetaldehyde

DM.....Dopamine

DOPA.....3,4-Dihydroxyphenyl alanine

DNP hydrazine.....2,4-Dinitrophenyl hydrazine

E.....Epinephrine

EtOAc.....Ethyl acetate

EtOH.....Ethyl alcohol

GCQ.....Gas Chrom Q

HFBA.....Heptafluorobutyric anhydride

NE.....Norepinephrine

PNMT.....Phenylethanolamine-N-methyl transferase

PFPA.....Pentafluoropropionic anhydride

ppm.....parts/million

PFP hydrazine.....Pentafluorophenyl hydrazine

PPO.....2,5-Diphenyloxazole

PPO fluor.....5 gm PPO/liter of toluene containing 5% ethanol

Tc.....Column temperature

Td.....Detector temperature

Ti.....Injector temperature

MDQ.....Minimum detectable quantity

CHAPTER ONE

I. PURPOSE

The purpose of this research is to develop a method for the assay of catecholamine derived catecholaldehydes in brain tissue. Numerous workers have suggested that these endogenous aldehydes are of physiological importance in brain function (75). However, little or nothing is known about their *in vivo* concentrations in normal, pathological or drug states. Electron capture gas chromatography (EC/GC), which has the capability of detecting picogram quantities of particular catechol derivatives, will be utilized in this study.

II. INTRODUCTION

Natural catecholamines (CAs) like dopamine (DM) and norepinephrine (NE), as well as the indoleamine serotonin, are important neurotransmitters in the central nervous system (CNS) (1). The localization of these neurotransmitters in particular brain tracts has been established and their relationship to specific animal behaviour has in part been explained (2-6). NE is the transmitter released from the terminals of postganglionic neurons of the sympathetic nervous system (7). In addition, NE and DM occur in the brain and spinal cord of vertebrates (8-9). Histochemical fluorescence techniques have revealed the distribution of CA-containing neurons in the CNS (10). Histochemical and biochemical studies have also revealed the presence of separate neuronal systems in the CNS in which the predominant CA is DM rather than NE. NE-containing nerve terminals are widely distributed throughout the CNS with a particular abundance in the hypothalamus(8). DM-containing neurons are particularly abundant in the striatum (11). Epinephrine (E) is present in very small amounts in the mammalian CNS (12). These three CAs are very high in levels in the chromaffin tissue of the adrenal medulla (13).

A. BIOSYNTHESIS OF CATECHOLAMINES

CA-containing cells synthesize their endogenous amine content from the plasma amino acid L-tyrosine by a pathway(Fig.1) suggested by Blaschko in 1939 (14). The evidence for this pathway comes from studies of the synthesis of CAs in the adrenal medulla. In these studies it was shown that radioactivily-labeled tyrosine or 3,4-dihydroxyphenylalanine(L-DOPA) can be converted into DM, NE, & E both *in vivo* and *in vitro*(15-18). Tyrosine hydroxylase converts L-tyrosine to L-DOPA. The enzyme occurs in the adrenal medulla, brain and sympathetically innervated tissues(19-20). Tyrosine hydroxylase requires dihydrobiopterin as a cofactor(19). The hydroxylation of tyrosine by tyrosine hydroxylase is the rate limiting step in the biosynthesis of CAs(21). L-DOPA is decarboxylated to DM by the enzyme aromatic L-amino acid decarboxylase. It is named so, as the same enzyme apparently decarboxylates L-DOPA, L-5-hydroxytryptophane and L-histidine(22). Dopamine-beta-hydroxylase requires ascorbic acid as a cofactor (23,24). Phenylethanolamine-N-methyl transferase converts NE to E. This enzyme is not present in significant amounts in the CNS, but studies by Fuller suggest that it still may be important(68). It

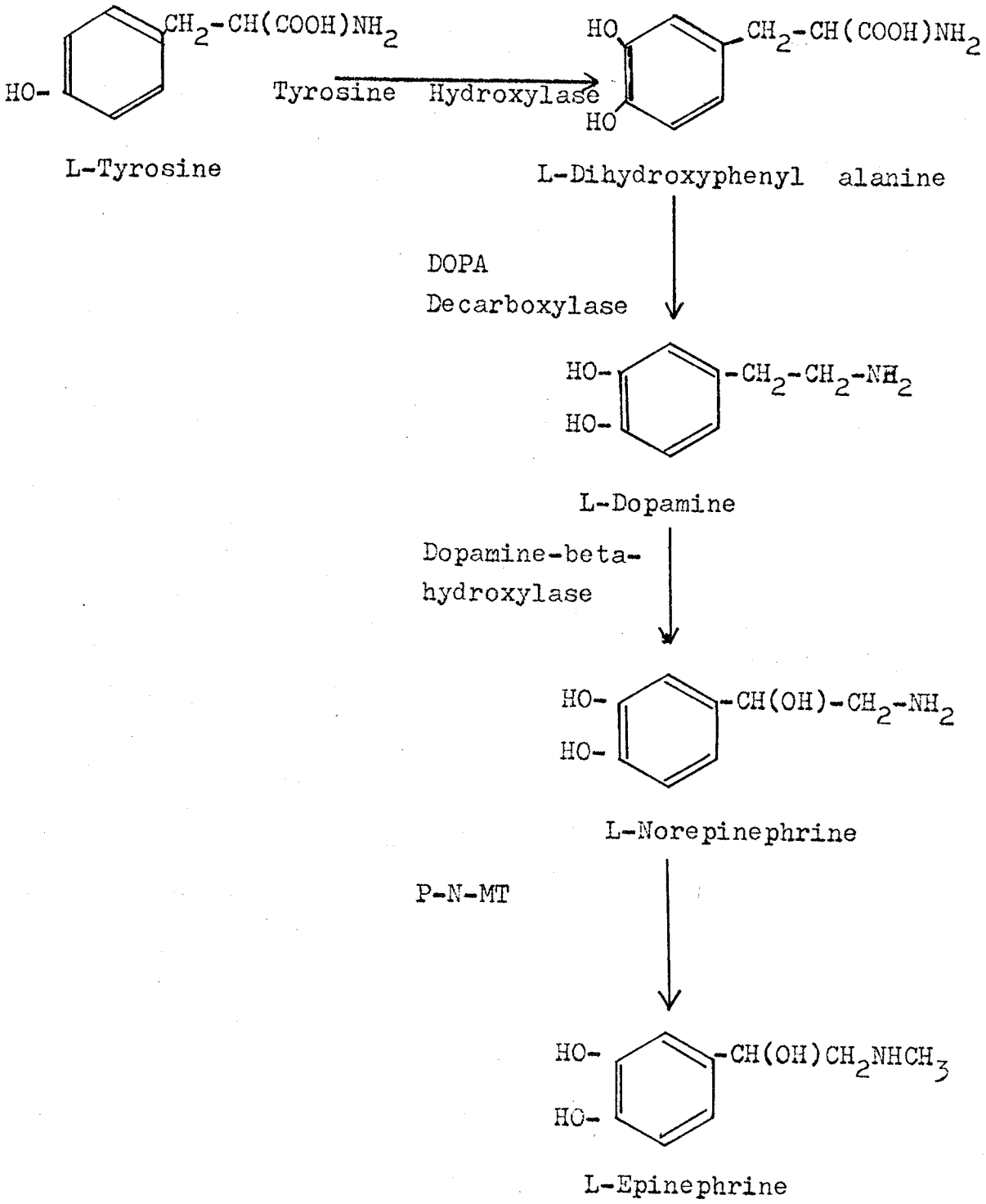


Fig. 1. Biosynthesis of catecholamines.

requires S-adenosyl L-methionine (SAMe) as a methyl donor and will N-methylate a variety of beta-phenyl-ethanolamines (25, 26).

B. METABOLISM OF CAs

The in vivo metabolism of CAs has been elucidated by the use of high specific activity tritium- labeled CAs (28, 29, 30, 31). NE, DM and E are metabolized in a similar fashion (Fig. 2). Monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) are the major enzymes for the metabolism of CAs. MAO oxidatively deaminates the CAs forming the corresponding aldehyde. COMT O-methylates the CAs by transferring a methyl group of SAMe to the meta-hydroxyl of catechol compounds. COMT is not very specific in its action. It can O-methylate aldehydes (32) and acids (32) formed from the action of MAO on CAs.

MAO is also non specific in its action and it will act on any monoamine including normetanephrine and 3-O-methyl dopamine forming the corresponding aldehyde. The aldehyde intermediate can be oxidized to the acid by aldehyde dehydrogenase or can be reduced to the alcohol by aldehyde reductase.

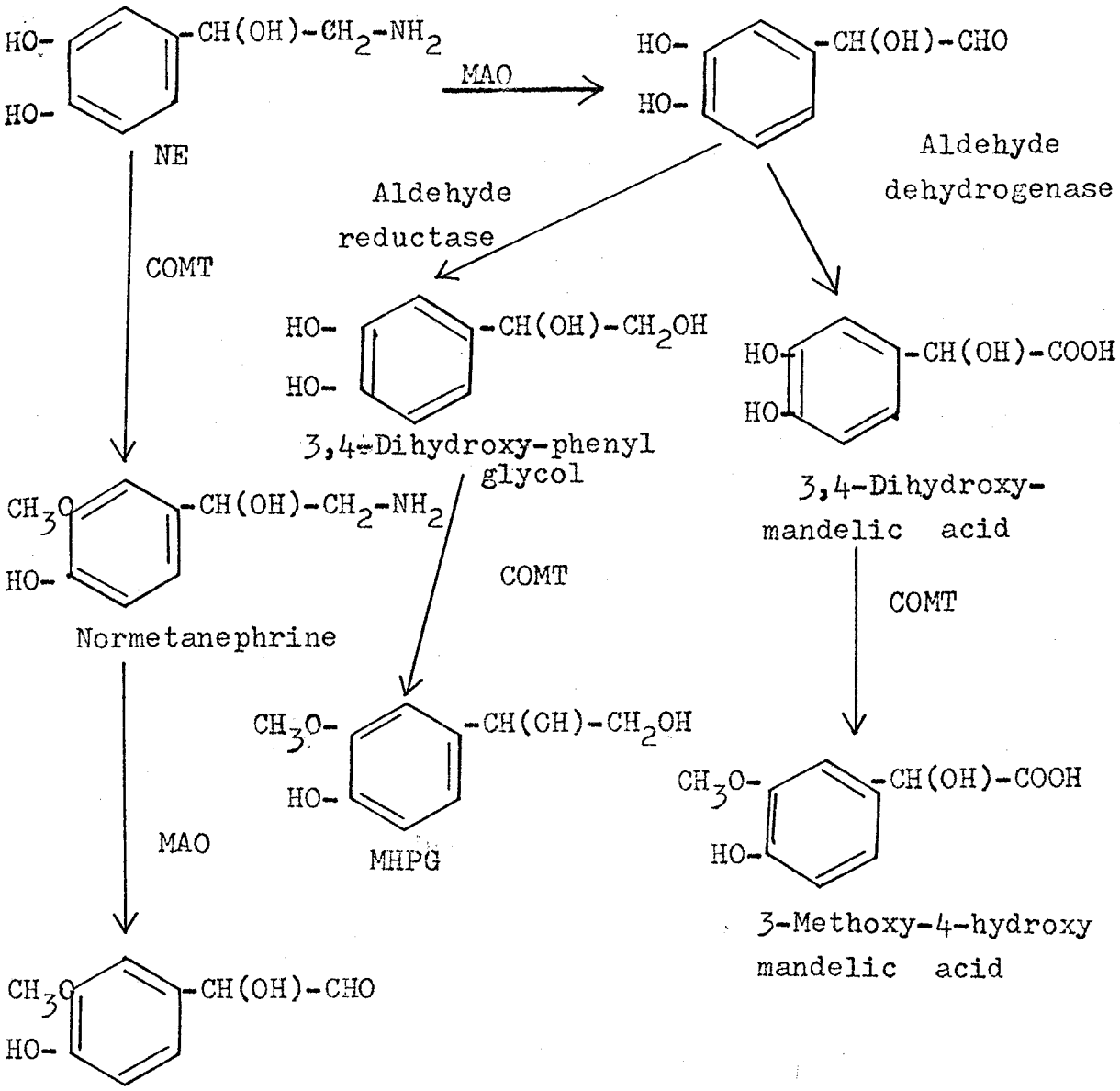


Fig. 2 Metabolism of catecholamines.

The studies of the metabolism of CAs in the CNS has been hampered by the existence of a blood brain barrier; which prevents circulating labeled CAs from entering the CNS. The metabolism of labeled CAs in the intact brain has been studied after injecting small amounts of labeled CAs into the CSF(29,30). The metabolism of NE and DM has also been studied in brain homogenates(31). These studies confirm that NE and DM are metabolized by O-methylation and deamination in all regions of the brain, as in the periphery. The major end products of NE metabolism in the brain are reduced alcohols, rather than acids. The alcohols are 3-methoxy 4-hydroxyphenylglycol(MHPG) and 3,4-dihydroxyphenylglycol (DHPG)(31). DM is metabolized principally to an acid both in intact brain and tissue slices. The major metabolites of DM are 3-methoxy,4-hydroxyphenylacetic acid(homovanillic acid) and 3,4-dihydroxyphenylacetic acid(31).

C. ENZYMES OF CATECHOLAMINE METABOLISM

a. CATECHOL-O-METHYL TRANSFERASE

COMT from rat liver utilizes S-adenosyl-L-methionine as the methyl donor and has an absolute

requirement for magnesium or other divalent cations (32). CAs are mostly methylated on the meta position but the para position can also be methylated to a certain extent depending on its nucleophilic characteristics. DM yields 90% meta and 10% para O-methylated products in in vitro experiments(33). There is no evidence that 3,4-dimethoxy products can be formed to any significant degree by this enzyme(34).

b. MONOAMINE OXIDASE

MAO is a poorly characterized enzyme. Partial chromatographic separation has been reported and it seems that MAO exists as isoenzymes(35). Purified preparations of MAO contain enzyme bound to flavin groups(36,37,38). MAO catalyzes the oxidative deamination of a wide variety of monoamines(Fig.3) (27). Oxidative deamination of monoamines takes place in two steps (Fig.4)(27). In the anaerobic step, flavin, bound to MAO, is reduced and in the aerobic step the flavin groups are oxidized to give MAO(FAD) which can again take part in oxidative deamination. The aldehydes produced are further

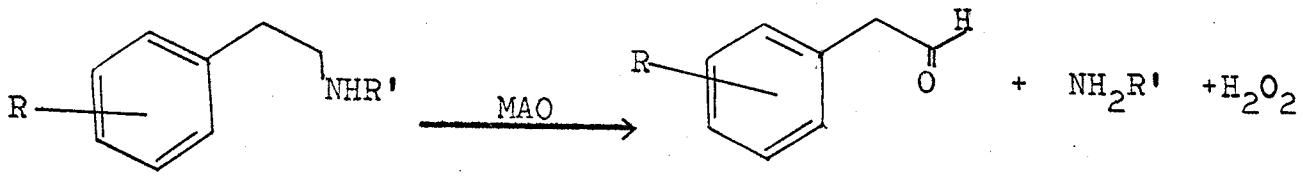


Fig. 3 Oxidative deamination of monoamines by MAO.

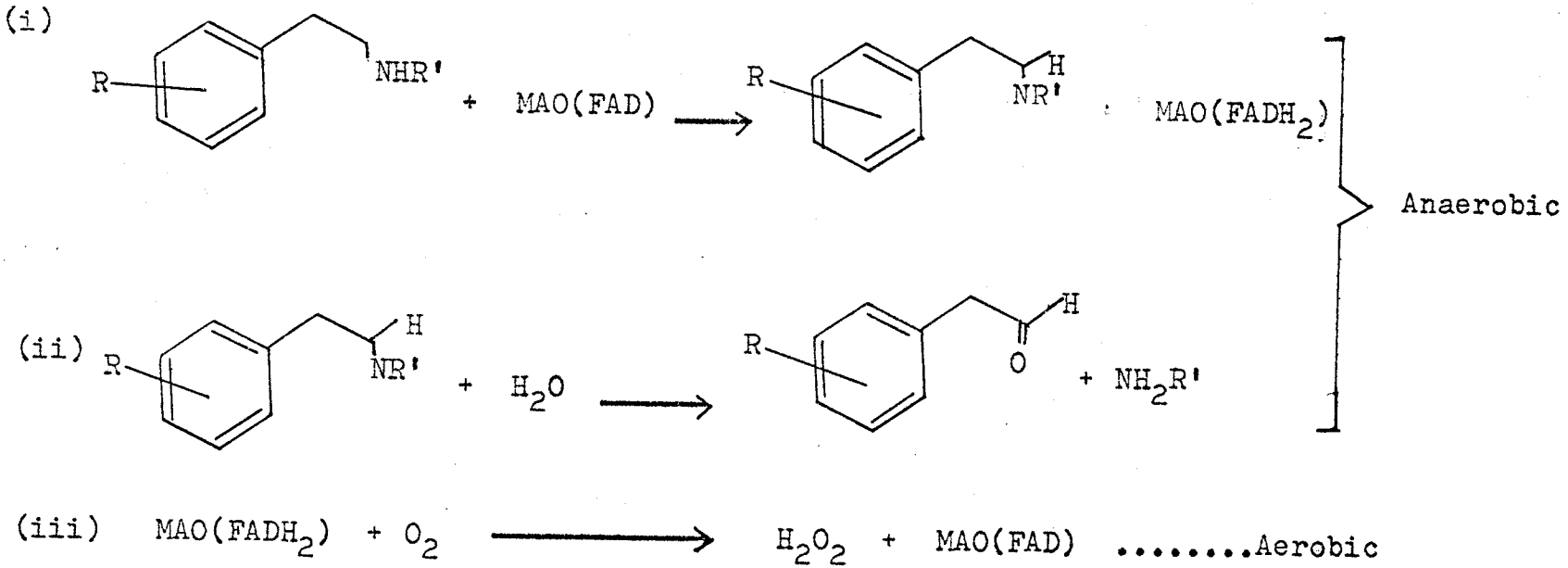


Fig. 4 Two steps of MAO action

metabolized(vide supra). MAO exhibits stereochemical selectivity in favor of the naturally-occurring L-anantiomers of optically active amines such as NE and E (40).

In vitro studies have been done for the measurement of monoamine oxidase activity. In these studies either the disappearance of O_2(as measured with an oxygen electrode), the appearance of NH_3, or the appearance of products(methyl amine or hydrogen peroxide) have been followed(43). In histochemical studies brain slices are incubated for several hours at 37°C in O_2 in presence of serotonin and traces of heavy metals(Co,Fe,Cu,Mo, etc.) which accelerate pigment formation. The slices are washed and embedded in paraffin, after which sections are cut, deparaffinized, dehydrated in alcohol and mounted in cedar oil. MAO activity is measured by the formation of dark brown pigment. The pigment is produced from 5-hydroxyindolacetaldehyde(44,45).

Microfluorimeteric determination of MAO activity is based on the rate of disappearance of serotonin in tissue homogenate,incubated at 37°C in presence of oxygen; and the amount of serotonin is measured by a fluorescence technique

(46,47). A colorimetric method for the measurement of MAO activity is based on the treatment of amine samples and MAO with alkaline 2,4-dinitrophenyl hydrazine. The aldehyde derived from the amine reacts with hydrazine to produce a characteristic color(48).

c. ALDEHYDE DEHYDROGENASE & ALDEHYDE REDUCTASE

Recently Tabakoff and co-workers have reported that NAD^+-dependent aldehyde dehydrogenase carries out the oxidation of biogenic aldehydes in brain(41). NAD^+ is loosely bound to the enzyme in most tissues for example kidney and liver in rat(39). Rat liver aldehyde dehydrogenase exists in two forms, one present in the mitochondrial matrix and another form present in the soluble portion of the cytoplasm(53). A third form of aldehyde dehydrogenase has been reported which is present in the microsomes(54).The mitochondrial enzyme has unequal distribution in the brain, with highest concentration in the caudate nucleus(55). With regard to the mechanism of action of aldehyde dehydrogenase, the mammalian enzyme from both liver and brain bind to NAD^+, followed by binding of the aldehyde to give a ternary complex(56).

The aldehyde reductase reduces the biogenic aldehydes to their corresponding alcohols. NADPH-dependent aldehyde reductase has been isolated and characterized from bovine brain (57).

D. BIOGENIC ALDEHYDES

As stated, biogenic aldehydes are formed by the oxidative deamination of biogenic amines by the action of MAO. Some amines like mescaline are deaminated by diamine oxidase or mescaline oxidase to form an aldehyde (50). Another path for the formation of biogenic aldehydes is by transamination. The neuroamine precursor amino acids, 5-hydroxytryptophan and DOPA can be transaminated and then decarboxylated to form the respective aldehydes (51).

E. IMPORTANCE OF BIOGENIC ALDEHYDES

Available information concerning the biogenic aldehydes indicate the possible *in vivo* participation of biogenic aldehydes in (a) the regulation of tissue respiration and oxidative

phosphorylation; (b) binding to macromolecular cell components; (c) stimulation of glucose oxidation by pentose phosphate shunt; (d) alteration in NAD^+/NADH levels; (e) condensation with parent amines and other products to form tetrahydroisoquinoline or carboline derivatives and (f) regulation of citric acid cycle.

Deamination products of a number of biogenic amines (i.e. NE, E, DM & serotonin) inhibit the cytochrome oxidase activity of rat liver mitochondria (62). Treatment of mitochondria with inhibitors of MAO, such as pargyline, prevents the inhibition of cytochrome oxidase activity of rat liver mitochondria. From these observations it seems that biogenic aldehydes could be regulatory factors in the electron transport chain; in other words, biogenic aldehydes could be controlling tissue respiration and oxidative phosphorylation. Binding of aldehydes to $-NH_2$ and -SH groups in cell components has been suggested to be of physiological importance in states such as sleep (63). Aldehydes of serotonin and E stimulate the oxidation of glucose by pentose phosphate shunt in beef pituitary.

This effect was also noted with benzaldehyde and it requires the presence of NADPH. As glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase both depend on the presence of $NADP^+$ for their catalytic activity, the reduction of aldehydes by NADPH-dependent aldehyde reductase with the simultaneous production of $NADP^+$ would probably act as a control mechanism for channeling glucose through this metabolic pathway (64, 65). Only 5-8 % of glucose is metabolized by pentose phosphate shunt in brain, but this pathway might be particularly important in the synaptic region (66). The metabolism of aldehydes by aldehyde dehydrogenases present in neural tissue would vary the NAD^+/NADH ratio in cytosol as well as in mitochondria. Hence the activity of regulatory enzymes of glycolysis i.e. phosphofructokinase might be effected by feed back inhibition due to high NADH concentration.

The aldehyde reductases are incapable of utilizing the short chain aldehydes, formaldehyde or acetaldehyde (AcH). AcH is primarily metabolized by aldehyde dehydrogenase in brain tissue. This type of exogenous aldehyde would compete with the dehydrogenase oxidation of natural biogenic aldehydes.

A shift in the disposition of biogenic aldehydes has been observed in tissues of animals and human subjects during ethanol metabolism and has been explained partly by this inhibition by AcH (58). Along with this change in metabolic pattern after ethanol (EtOH) or AcH, there are changes which take place in the CNS. The alcohol derivatives produced in the CNS would be circulated through the liver and kidney, where these alcohol derivatives are exposed to alcohol dehydrogenase,which may convert the alcohols to aldehydes and again make them available for oxidation. The Km's and maximal velocity for biogenic aldehydes favor their oxidation and the inhibition of oxidative pathway would most probably lead to an increase in the steady state level of these aldehydes (67)

Due to these increased levels of aldehyde intermediates, side reactions may occur. One such reaction is the condensation of aldehydes, as in the case of DM derived biogenic aldehyde with the parent amine, to form a 1,2,3,4,-tetrahydroisoquinoline derivative i. e. tetrahydropapaveroline(THP) (59). Direct condensation of biogenic amines with acetaldehyde leads to the formation of 1-methyl tetrahydroisoquinolines(60). The theory has been

advanced that the formation of the alkaloids after EtOH administration might be associated with EtOH addiction(60,69,70). The condensation of acetaldehyde with a catecholamine to form a tetrahydroisoquinoline probably proceeds through a schiff base intermediate(60). It has been suggested that mild reducing agents such as ascorbate or glutathione can reduce this schiff base, thus preventing the cyclization product(70).

Finally in support of the regulation of citric acid cycle by biogenic amines; the deamination products of a number of biogenic amines(NE,E,DM & serotonin) inhibit the activity of succinate dehydrogenase(71,72), This suggests that biogenic aldehydes might be a controlling factor in citric acid cycle.

Nevertheless, while the functions of biogenic amines are quite well established, the roles of biogenic aldehydes are still very speculative. There is much to learn about the biogenic aldehydes, particularly the catecholaldehydes, and their biological implications. The steady state catecholaldehyde levels are low but as the catecholaldehydes are no doubt very active metabolites, the determination of

these low levels of catecholaldehydes is of great importance. A sensitive and reliable method which can measure catecholaldehydes in the subnanogram range in brain and nerve tissue is required. Therefore the purpose of this study is to explore various trapping and derivatization procedures, combined with gas chromatography with the very sensitive electron capture detector for catecholaldehydes.

CHAPTER TWO

I. MATERIALS

A. CHEMICALS

L-NE, L-DM, L-E, L-epinine, L-DHPG were purchased from Regis Chemical Company. Ethyl acetate, acetonitrile(sequanal grade) and derivatizing reagents HFBA(heptafluorobutyric anhydride),PFPA(pentafluoro propionic anhydride) and TFAA(trifluoroacetic anhydride) were purchased from Pierce Chemical Company, and were stored at a temperature below or equal to $-10^{o}C$. Eastman Organic Chemicals supplied 2,4-dinitrophenyl hydrazine. Sodium cyanoborohydride was obtained from Ventron Alfa Inorganics Inc. Methylamine and hydroxylamine hydrochloride were purchased from Matheson, Coleman and Bell. Phosphoric acid (85%), glacial acetic acid and methanol were purchased from Mallinckrodt Chemicals. DL-NE-7-^{3}H in 0.2N acetic acid, specific activity 500mC/mM was purchased from ICN Tracerlabs. Pentafluorophenyl hydrazine was purchased from PCR Inc. Nitrogen (zero grade), air(zero grade), hydrogen(zero grade) and nitrogen(99.9%) were purchased from Liquid Carbonic.

B. G.C. EQUIPMENT AND CONDITIONS

A Varian 2100 gas chromatograph equipped with ^{63}Ni electron capture and H_2 flame ionization detectors was used. Carrier gas flow rates between 20-30 ml/min were routinely used. U shaped glass columns, 2 mm i.d. X 6 ft length were packed with GEXF-1105, SE-30, or OV-17 on Gas Chrom Q, 80/100 mesh.

II. METHODS

A. PREPARATION OF ACTIVATED ALUMINA

Activated alumina (Woelm neutral activity grade I) was prepared by Anton and Sayre's method (73). In all catechol extraction experiments activity grade I alumina was used. An outline for the preparation of activated alumina is as follows:

100 gm Al_2O_3 was added to 500 ml of 2N HCl in a large beaker. The contents of the beaker were heated to 90-100°C with continuous stirring for 45 min. The supernatant was discarded and residue was washed twice with fresh 250 ml of 2N HCl at 70°C. The alumina was washed with distilled water until pH of washings was 3-4. The alumina was

stored in an incubator under vacuum.

B. PREPARATION OF COLUMN PACKINGS

Column packings are mostly prepared by slurry method or filtration method.

1. Slurry method is used to prepare packing when more than 5% liquid phase loadings are required. General outline for this method is as follows:

Volume of coated packing required to fill a column is given by the formula:

$$\text{Volume (cc.)} = 154 \times \text{Length of column in feet} \times (\text{Inside diameter of column in inches})^2$$

The volume of the solid support was measured to fill column and an extra 10-15 cc was added to compensate for handling losses. It was weighed and enough appropriate solvent was added to wet the solid support. The weight of the liquid phase was calculated from the following formula:

$$\text{Weight of liquid phase} = \frac{\%\ \text{loading} \times \text{Weight of solid support}}{100 - \%\text{loading}}$$

The liquid phase was dissolved in solvent. This dissolved liquid phase was then added to the solid support. The whole solution was stirred to mix it uniformly. The excess solvent was removed on a vacuum rotary evaporator. The packing was dried at 45°C overnight and the columns were packed as usual.

2. The filtration method is used to prepare packing with less than 5% liquid phase loading. The whole procedure for this method is as follows: The volume of solid support was measured to fill the column and an extra 10 cc of solid support was added to compensate for handling losses. The solid support was weighed and the required liquid phase weight was calculated from the weight of the solid support. The liquid phase was dissolved in solvent(volume of solvent is twice the volume of solid support).The solid support was added to the liquid phase with constant stirring. The excess solvent was removed on a vacuum rotary evaporator. Final traces of solvent were removed by leaving overnight at 45°C. The dry packing was packed in glass columns. The fresh packed columns were conditioned for 24 hr with a small nitrogen

flow at appropriate temperatures, unattached to the detector. During the conditioning period, the EC detector inlet was capped to prevent any diffusion of gases into the EC detector.

C. GENERAL PREPARATION OF FLUOROACYL DERIVATIVES

Fluoroacyl derivative was prepared by treating 1 mg of the catechol compound with 0.1 ml of acetonitrile and 0.2 ml of HFBA. The mixture was allowed to stand at room temperature for 20 min. The solution was evaporated to dryness with a N_2 stream. The residue was dissolved in EtOAc and subjected to EC/GC analysis.

D. ESTABLISHING OPTIMUM EC/GC CONDITIONS

The electron capture (EC) detector is a very sensitive part of the gas chromatograph. The sensitivity of the EC detector can be affected by a number of factors. If the carrier gas (N_2) has more than 5ppm oxygen or water as contaminants, the detector sensitivity and standing current decrease. The sensitivity of EC detector is directly related to standing current. The base line on the chromatogram is the result of a standing current of the order of 10^{-9} amperes or higher. For

measuring the standing current the range on the electrometer is adjusted to 10^{-9} and attenuation is adjusted to 2. The pen is adjusted to zero and the EC cell is turned to OFF position on the electrometer. As there is no current the pen moves. If the deflection is 60% or higher the detector is ready for EC/GC analysis. If the true standing current is 60% or more the sensitivity of the detector is considered to be adequate.

The EC/GC response to a compound is checked with standard solutions of lindane in EtOAc or another appropriate solvent. A sharp single peak for lindane, with the minimum detectable amount of about 1-5 pg, and a single peak for solvent assures that the instrument is in good working condition.

E. OBTAINING STANDARD CURVES

A known amount of catecholamine is derivatized by treating with HFBA and AN according

to the procedure described already. The fluoroacyl derivative is dissolved in EtOAc and various dilutions are injected into the GC. Peak heights are directly proportional to the amount of substance injected in the linear range of that compound. The standard curves are drawn in the linear range of the detector for each particular substance. The concentration is plotted against peak height on a graph paper to get the standard curve.

F. PREPARATION OF DHPAcH FROM E OR NE(74)

To 5 microliter of ^{3}H-NE(500mC/mM), 1 ml of 1 N $HClO_4$, containing 5 mM $Na_2S_2O_3$, was added. The pH was adjusted to 6 with 0.2 N NaOH. Activated Al_2O_3 (500 mg) was added and the pH was further adjusted to 8-8.2 with 0.2 N NaOH. Following 10 min of mechanical shaking, the supernatant was discarded and the Al_2O_3 was successively suspended in three 2-ml portions of distilled H_2O, and finally was shaken 10 min at room temperature in 2 ml 0.2 N HCl. The HCl supernatant, containing eluted NE, was decanted. The total volume of the eluate was 3 ml. A 25 microliter aliquot of the acid supernatant was added to

10 ml of PPO fluor in a counting vial and counted thrice for 5 min in a Beckman Scintillation Spectrophotometer.

Purified ^{3}H-NE and 150.2 mg of NE were added to 3 ml of 85% H_3PO_4. The mixture was heated to 125°C and then immediately poured into 30 ml of distilled H_2O. The solution was allowed to stand at room temperature for 90 min. The aqueous acid solution was extracted twice with 15 ml of EtOAc. The combined EtOAc extracts were washed twice with 5 ml of distilled H_2O. The volume of EtOAc was reduced to 13 ml with N_2.

Counting efficiency of the instrument was determined using an internal standard. ^{3}H-Toluene (2.06×10^{6} dpm/ml) was used as the internal standard. The fraction of the activity left at the time of the experiment was 0.813. Accurately measured 24 microliter of ^{3}H-toluene was added to the vials containing 25 microliter of purified ^{3}H-NE in 10 ml PPO. It was counted three times for 5 min each. After preparation of DHPAcH(total volume 13 ml), a 20 microliter aliquot was added to 10 ml PPO in glass vials and counted three

times for 5 min each. Accurately measured 20 microliter of ^{3}H-toluene was added to the vials and counted three times for 5 min each. From these observations the % recovery was calculated.

In other experiments DHPAcH was made without any addition of radioactive carrier. This solution of aldehyde in EtOAc was used for the preparation of various derivatives which follow.

An attempt was made to purify DHPAcH by crystallization or by distillation. Efforts to get a pure oil of DHPAcH by vacuum distillation were unsuccessful. A similar problem was faced by Fellman and the aldehyde could only be obtained in the impure form(74).

G. FORMATION OF 2,4-DNP HYDRAZONE OF DHPAcH & ITS DERIVATIZATION FOR EC/GC ANALYSIS.

An estimated 0.33 mM of DHPAcH in EtOAc was treated with 0.50 mM of 2,4-dinitrophenyl hydrazine dissolved in 2 ml of methanol, and 1 ml of glacial acetic acid. The mixture was allowed to stand at room temperature. A yellow precipitate appeared slowly(addition of a small amount of H_2O

increased the rate of precipitation) and within 30 min the precipitation appeared to be complete. The yellow crystals of 2,4-DNP hydrazone of DHPAcH were separated by suction filtration, washed with absolute alcohol and dried under vacuum. The M.P. was found to be 170°C, in agreement with the literature(74).

An accurately weighed 1 mg of 2,4-DNP hydrazone of DHPAcH was treated with HFBA and AN for 20 min and dried with a N_2 stream. The dry residue was dissolved in EtOAc and based on 100% reaction, various dilutions in the pg range were made. The EtOAc solutions were subjected to EC/GC analysis.

An attempt was made to reduce the DNP hydrazone of DHPAcH with sodium cyanoborohydride by stirring for 6 hr at room temperature. The pH of the reaction mixture was kept constant at 6 with 1M acetate buffer. The substituted hydrazine was extracted with ether. The extract was evaporated in vacuum rotary evaporator and the dry residue was fluoroacylated according to the procedure described before. The dry residue was dissolved in EtOAc, and dilutions in the pg range

were injected into GC for EC/GC analysis.

H. REACTION OF PENTAFLUOROPHENYL HYDRAZINE WITH DHPAcH & DERIVATIZATION OF THE PRODUCT FOR EC/GC ANALYSIS

Approximately 0.33 mM of DHPAcH(estimation based on the starting NE precursor) in 2 ml of EtOAc was treated with 0.60 mM of pentafluorophenyl hydrazine dissolved in 2 ml of methanol and 1 ml of glacial acetic acid. Total volume of reaction solution was 5 ml. The reaction was allowed to proceed over a period of 12 hr. Samples(0.5 ml) were taken at 3 hr, 8 hr, and 12 hr reaction time. These samples were immediately evaporated to dryness with a N_2 stream . The residue was treated with 0.5 ml of HFBA and 0.25 ml of AN for 20 min. The reaction mixture was evaporated to dryness with a N_2 stream. Dilution ($1:10^5$) of this residue was made in EtOAc and subjected to EC/GC analysis on 5% GEXF 1105 (GCQ 80-100). The carrier gas flow rate was 25 ml/min. The detector, column and injector temperatures were 240°C, 170°C and 200°C respectively.

I. REACTION OF NH_2OH WITH CRUDE DHPAcH & DERIVATIZATION OF THE PRODUCTS FOR EC/GC ANALYSIS

Approximately 0.2 mM of DHPAcH(estimation based on the starting NE precursor) in 1.5 ml of EtOAc was treated with 0.90 mM NH_2OH and 28 mg sodium acetate in 1 ml H_2O. A small amount (2 ml of 95%) ethanol was added to effect solution. The whole solution was stirred vigorously for 3 min. Then it was cooled in ice. The sides of the test tube were scratched with a glass rod. After 5 min the crystals were filtered, washed with absolute EtOH and dried. Fluoroacylation was performed by treating 1 mg of the crystals with 0.2 ml HFBA and 0.1 ml AN. The mixture was allowed to stand for 20 min at room temperature and evaporated to dryness with a N_2 stream. Dilutions in pg range in EtOAc were injected into GC for EC/GC analysis.

J. REDUCTIVE TRAPPING METHODS WITH $NaBH_3CN$

1. Attempt to reduce endogenous DHPAcH in extracts of whole rat brain:

A standard curve was obtained with commercially available DHPG. Fluoroacylation was done by treating 1 mg DHPG with 0.2 ml HFBA and 0.1 ml AN at room temperature for 20 min. The solution was evaporated to dryness with N_2

and the dry residue was dissolved in EtOAc. Dilutions of pg range were analysed by EC/GC.

Whole rat brain tissue (2.253 gm) was homogenized for 10 min in 3 ml of 1N $HClO_4$, containing 5 mM $Na_2S_2O_3$ and 500 mg of $NaBH_3CN$. The homogenate was transferred to a conical flask and the solution was stirred with a stirring bar for 2 hr at room temperature. During this period the pH was kept at 3-4 by adding 2N HCl dropwise. The homogenate was centrifuged at 2000 rpm for 20 min. The residue was discarded and the supernatant was treated with 1 gm activated alumina. The pH was adjusted to 8-8.4 with 0.5 N NaOH. The mixture was shaken for 10 min with a mechanical shaker. The supernatant was discarded and alumina was washed twice with distilled H_2O. The catechol compounds were eluted with 0.1 N HCl and the acid eluant was freezedried. The residue was treated with 0.5 ml AN and 1 ml HFBA for 20 min. The reaction mixture was evaporated to dryness with N_2. The residue was dissolved in EtOAc and $1:10^5$ dilution was injected into the GC for EC/GC analysis.

A control experiment was done where 2.3 gm of whole rat brain tissue were homogenized in a similar manner, without $NaBH_3CN$. The column used for EC/GC analysis was 5% SE-30 on GCQ 80-100 mesh. The carrier gas flow rate was 30 ml/min. The detector, injector and column temperatures were 250°C, 230°C, and 170°C respectively.

2. Reductive amination(52) of DHPAcH with CH_3NH_2 and $NaBH_3CN$(epinine formation) and HFB derivative formation for EC/GC analysis.

An estimated amount of 2.07 mM DHPAcH in 2 ml of EtOAc was added to a mixture of 3 ml methylamine(40% solution in H_2O) and 10 ml 1 N $HClO_4$ containing 5 mM $Na_2S_2O_3$. Immediately 4 mM $NaBH_3CN$ and an excess of 1 M acetate buffer (35 ml) were added to maintain the pH at 6 during the reduction process. The total volume of the reaction solution was 50 ml. The reaction solution was stirred at room temperature throughout. Samples of 5 ml were taken out at 10 min, 1 hr and 2 hr intervals. To each sample, 0.5 gm activated alumina was added, followed by a pH adjustment to 8- 8.2 with 0.5 N

NaOH. The mixture was subjected to 10 min mechanical shaking. The supernatant was discarded and alumina was successively suspended in 3-5 ml portions of distilled H_2O, and finally was shaken 10 min at room temperature in 2 ml 0.1 N HCl. The HCl supernatant containing eluted epinine was decanted, lyophylized and treated with 1 ml HFBA and 0.5 ml AN for 20 min at room temperature. The reaction mixture was evaporated to dryness with N_2. The residue was dissolved in EtOAc ($1:10^5$ dilution) and injected into the GC. The column used for analysis was 5 % GEXF 1105 on GCQ 80-100 mesh. Carrier gas flow rate was 30ml/min. The detector, injector and column temperatures were 280°C, 240°C and 175°C respectively.

3. Reductive amination of phenylacetaldehyde with isopropylamine and $NaBH_3CN$ & GC analysis with FID.

To establish the optimum conditions for the formation of an amine by reductive amination of an aldehyde, phenylacetaldehyde was treated with isopropylamine and $NaBH_3CN$ under four different sets of conditions and the extent of formation of presumed product N-isopropyl phenylethylamine was

determined in each case.

(a) <u>One step procedure(pH 6) at room temperature or at 0°C.</u>

An accurately measured 2 mM of phenylacetaldehyde was suspended in 5 ml of absolute EtOH, followed by the addition of 15 ml 1 N acetate buffer pH 6 and 9 ml 1 N $HClO_4$, containing 5 mM $Na_2S_2O_3$. The total volume of the mixture was 30 ml. The pH of the mixture was 6. Finally 6 mM of $NaBH_3CN$ was added and the mixture was stirred throughout at room temperature. Aliquots(2 ml) were removed at 0,5,15,30,45,60 and 120 min of reaction time. The pH of each aliquot was adjusted to 10-11 with 4 N NaOH followed by the extraction of the product with 2 ml ether twice. The ether extracts were evaporated to 0.5 ml, stored in the freezer until analysis, and were analysed directly with FID on 3% OV-17 on GCQ (80-100 mesh). The column, detector and injector temperatures were 110°C, 240°C and 200°C respectively.

After dissolving 2 mM phenylacetaldehyde in absolute alcohol at room temperature, the rest of the experiment was carried out at 0°C in a similar fashion as before.

(b) Two step (pH 3-4 & pH 6) procedure at room temperature or at 0°C

Accurately measured 2 mM of phenylacetaldehyde was suspended in 5 ml of absolute EtOH, followed by the addition of 9 ml 1 N $HClO_4$, containing 5 mM $Na_2S_2O_3$. Finally 20 mM isopropylamine was added. The contents were mixed and allowed to stand at pH 3-4 at room temperature for 5 min. Then 15 ml 1M acetate buffer, pH 6, was added to the reaction solution in order to keep the pH constant at 6, followed by the addition of 6 mM $NaBH_3CN$. The reaction solution was stirred throughout and samples were taken out at 0,5,15,30,45,60 and 120 min of reaction time. The pH of each sample was adjusted to 10-11 with 4N NaOH. Accurately measured 0.5 ml solution(H_2O), containing 4 mg of 1-methylisoquinoline, was added to each sample as an external standard. The reaction product was extracted with 2 ml of ether twice. The extract was reduced to 0.5 ml with a N_2 stream and was analysed by GC with FID on 3% OV-17 on GCQ(80-100 mesh) column. The carrier gas flow rate was 21ml/min. The column, the detector and

injector temperatures were 110°C, 240°C and 200°C respectively.

For the reaction at 0°C, 2 mM phenyl-acetaldehyde was dissolved in 5 ml absolute EtOH at room temperature and rest of the reaction was carried out at 0°C (ice bath) in a similar manner as above.

STANDARD CURVE

The reaction of phenyl acetaldehyde with isopropylamine was carried out in one step at pH 6 and at room temperature for 12 hr. The products were extracted with ether and extract was evaporated under vacuum. The oily material remaining was vacuum distilled. The first fraction came over at 37°C at 2 mm pressure and second fraction came over at 56°C. By comparison and elimination process, it was determined that N-isopropylphenylethylamine has a retention time of 7 min at 110°C, column temperature. The carrier gas flow rate was 21ml/min and injector and detector temperatures were 200°C and 230°C respectively. The first fraction gave a similar major peak (Fig 5). The fraction was almost pure and this

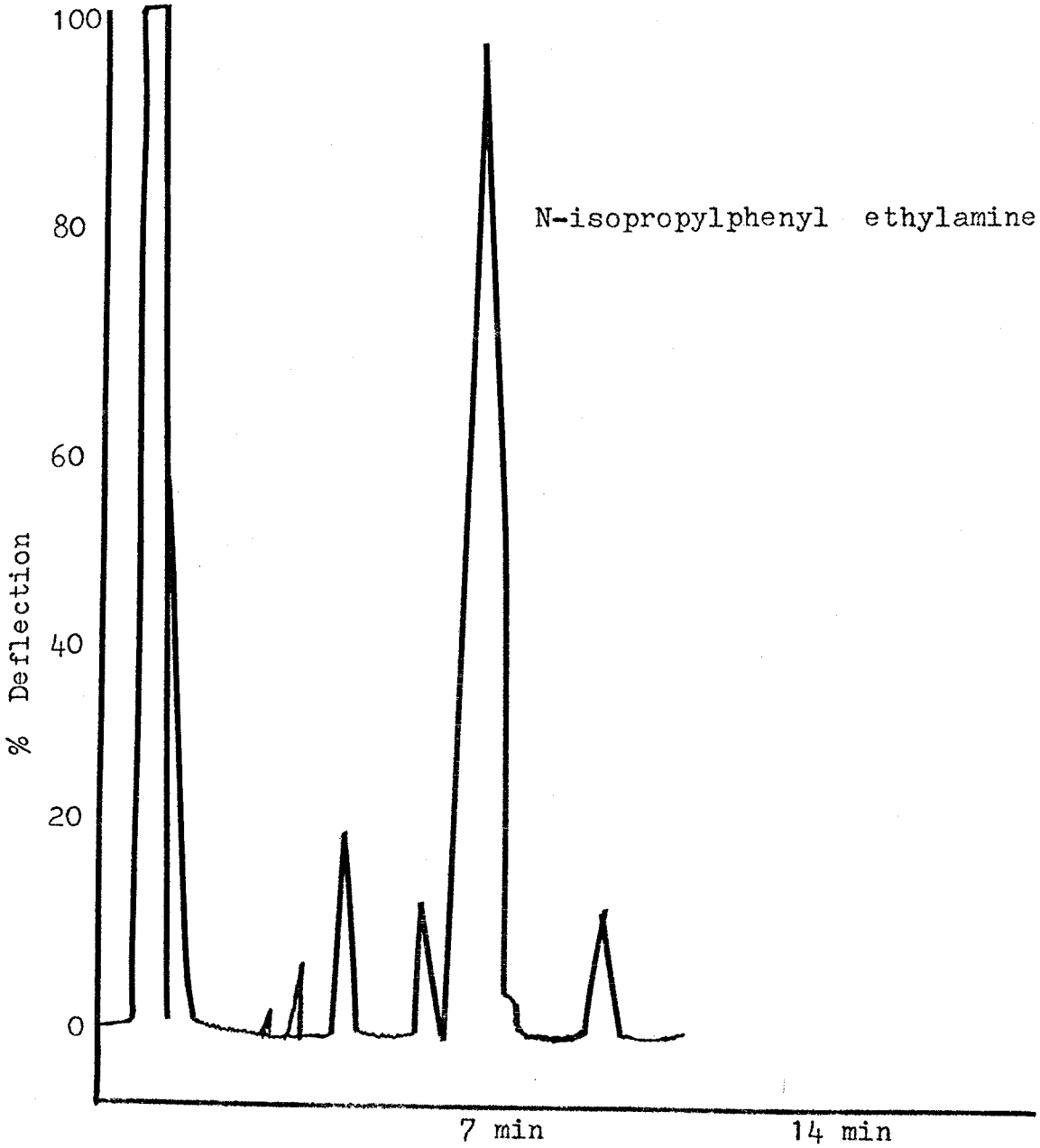


Fig. 5 Chromatogram showing suspected N-isopropyl-phenylethylamine peak with retention time of 7 min in first fraction.
GC Conditions: FID, 3% OV-17 on 80-100 GCQ, Tc = 110°C, Ti = 200°C, Td = 240°C, N_2 flow rate = 21ml/min, Att =4 x 10^{-11}.

oil was used to make standard curve by injecting it directly.

PRELIMINARY REDUCTIVE AMINATION OF CATECHOLALDEHYDES IN WHOLE RAT BRAIN

Whole rat brain tissue(2.319 gm) was homogenized in 2 ml of 5 mM $Na_2S_2O_3$/1N $HClO_4$. During homogenization 500 mg pargyline(MAO inhibitor), 20 mM(1260 mg) $NaBH_3CN$ and 20 mM(620 mg) methylamine were added. After homogenization the pH was adjusted to 6 with 1M acetate buffer. The mixture was stirred for 2 hr at room temperature followed by centrifugation(2000 rpm) for 20 min at 4^oC. The supernatant was treated with 2 mg activated alumina and the pH was adjusted to 8-8.2 with 0.2N NaOH, followed by mechanical shaking for 10 min. The supernatant was discarded. The alumina was successively suspended in three 5 ml portions of distilled H_2O, which were discarded and finally was shaken 10 min at room temperature in 4 ml 0.1N HCl. The HCl supernatant, containing eluted catechols, was decanted and lyophylized. The residue was treated with 1 ml HFBA and 0.5 ml acetonitrile for 20 min at room temperature. The reaction mixture was

evaporated to dryness with a N_2 stream and was dissolved in EtOAc and $1:10^5$ dilution was injected into GC for EC/GC analysis.

A similar control experiment was done in which 2.411 gm whole rat brain tissue were homogenized in 2 ml 5 mM $Na_2S_2O_3$/1N $HClO_4$, containing 500 mg pargyline and 20 mM(1260 mg) $NaBH_3CN$. Catechols were extracted with activated alumina and prepared for EC/GC analysis as above.

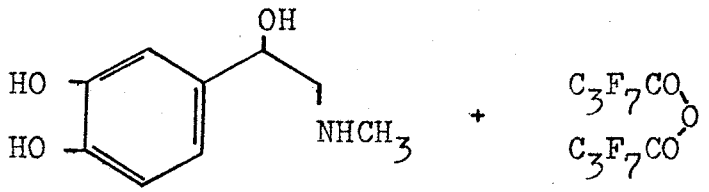
CHAPTER III

RESULTS

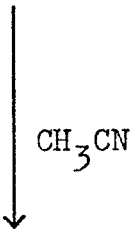
A. FLUOROACYLATION OF CATECHOLAMINES AND DERIVED CATECHOLALDEHYDES

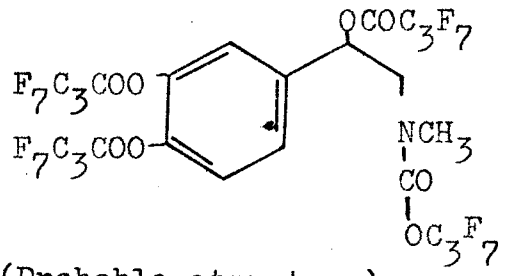
Most of the work for making a suitable derivative for electron capture detection has been done with CAs. Heptafluorobutyrate derivatives of CAs were found to be quite stable and suitable for electron capture detection (Fig. 6).

HFB derivatives of E, NE and DM each separate as single peaks on column packed with 5% GEXF-1105(on Gas Chrom Q, 80-100 mesh). E and DM give a single peak on columns packed with 3% OV-17 or 5% SE-30. The HFB derivatives of all CAs are stable for 2-3 days at -20°C, after this time the HFB derivative decomposes as judged by the multiple peaks obtained on chromatogram. Relative detector responses for E,NE and DM are shown in fig 7. The detector response for DM is very high. As little as 1-2 pg of DM can be detected. The response for NE and E



Epinephrine HFBA





(Probable structure)

Fig. 6 Fluoroacylation of epinephrine with HFBA & acetonitrile.

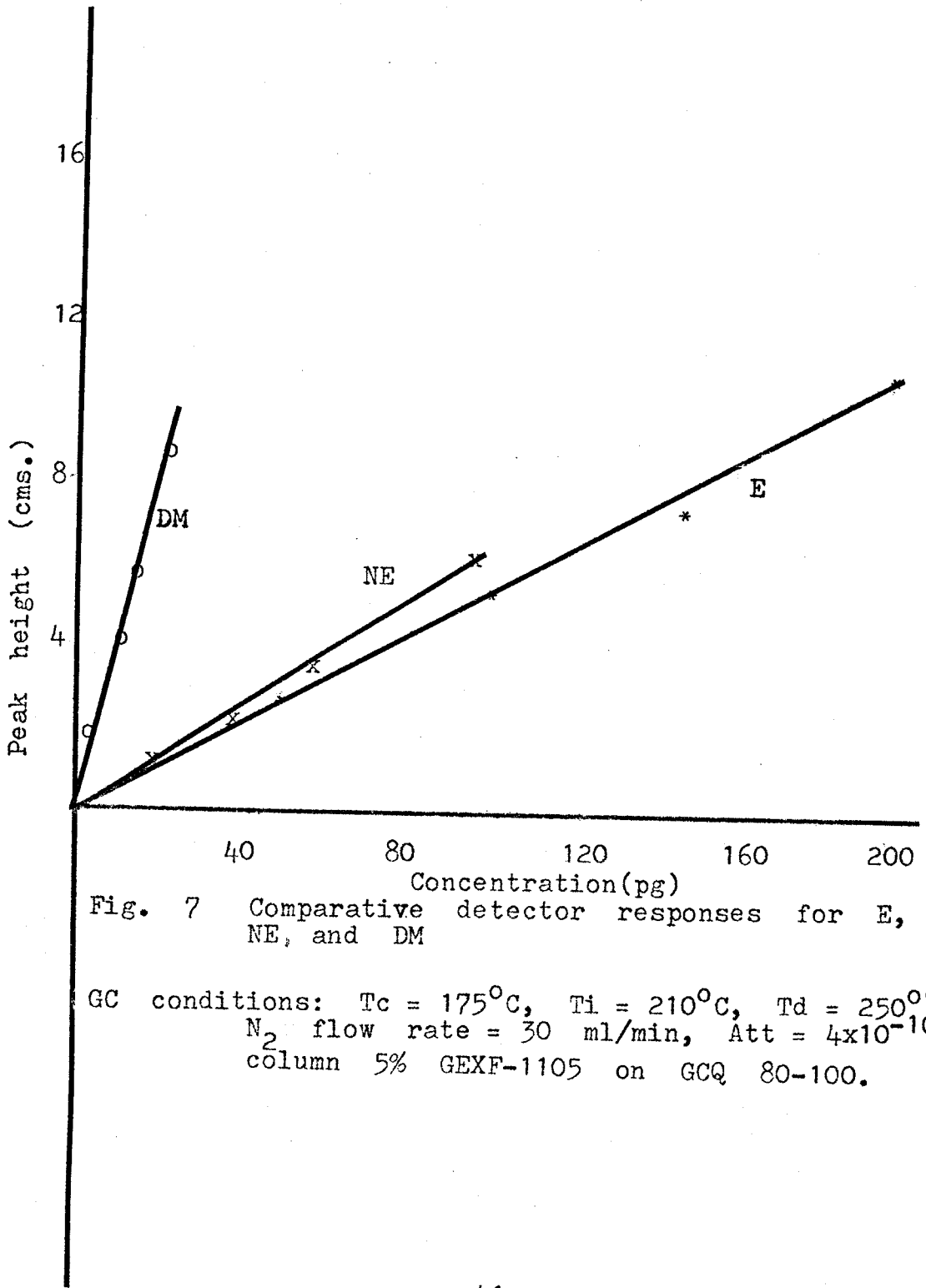


Fig. 7 Comparative detector responses for E, NE, and DM

GC conditions: Tc = 175°C, Ti = 210°C, Td = 250°C. N_2 flow rate = 30 ml/min, Att = 4×10^{-10}, column 5% GEXF-1105 on GCQ 80-100.

is not as high but it is acceptable.

Similarly the HFB derivative of DHPG and epinine each gave a single peak. The HFB derivative of the product from the reaction of pentafluorophenyl hydrazine with crude DHPAcH, and the HFB drivative of the product from reaction of hydroxylamine with crude DHPAcH each gave one major peak and one minor peak on 5% GEXF-1105. The two peaks from the reaction product of DHPAcH with pentafluorophenyl hydrazine can be explained on the basis that the hydrazone of DHPAcH has both cis and trans isomers. Similarly the reaction product of hydroxylamine and DHPAcH, e.g. oxime of DHPAcH, also has cis and trans isomers. HFB-DHPG gave a single peak on 5% SE-30. HFB-epinine gave a single peak on 3% OV-17 as well as on 5% GEXF-1105.

B. <u>% RECOVERY OF DHPAcH WHEN PREPARED BY FELLMAN'S METHOD (74)</u>

Total activity added in the beginning can be calculated as follows:

Total activity present in 24 microliter of toluene

$= 2.06 \times 10^6$ (dpm/ml) $\times$ 0.024 (ml) $\times$ 0.813 = 40195 dpm

$$C.E. = \frac{(\text{Net count rate of sample + standard}) - (\text{Net rate of sample})}{\text{Disintegration rate of standard}}$$

$$= \frac{30105 - 9668}{40195} = 0.15$$

$$\text{dpm sample} = \frac{9668}{0.51} = 18959$$

$$\text{Total activity } (^3H \text{ NE}) \text{ added} = \frac{18959 \times 3000}{25}$$

$$= 2275080 \text{ dpm}$$

Total activity recovered at the end of experiment can be calculated as follows:

Total activity present in 20 micro litre of toluene = 2.06×10^6 (dpm/ml) $\times$ 0.02 (ml) $\times$ 0.813 (fraction of activity) = 33495 dpm

$$C.E. = \frac{10118 - 300}{33495} = 0.29$$

$$\text{dpm sample} = \frac{300}{0.29} = 1035$$

$$\text{Total activity recovered} = \frac{1035 \times 13000}{20}$$

$$= 672750 \text{ dpm}$$

$$\% \text{ Recovery} = \frac{672750 \times 100}{2275080} = 30\%$$

C. EC/GC ANALYSIS OF 2,4-DINITROPHENYL HYDRAZONE OF DHPAcH

The HFB and PFP derivative of 2,4-dinitrophenyl hydrazone of DHPAcH gave no peaks at 3% OV-17, 5% GEXF 1105 and 5% SE-30 on GCQ 80-100 mesh at any column temperature between 130°C and 210°C.

An attempt was made to reduce the hydrazone with $NaBH_3CN$ at pH 6. The HFB derivative of reduced hydrazone gave no peak on 5% GEXF-1105 from 130°C to 210°C. The whole reaction can be written as shown in fig 8.

D. EC/GC ANALYSIS OF THE REACTION OF PENTAFLUOROPHENYL HYDRAZINE WITH DHPAcH

A single HFB derivative of the reaction product of pentafluorophenyl hydrazine with DHPAcH (tentatively assumed to be the HFB-pentafluorophenyl hydrazone of DHPAcH) could be detected

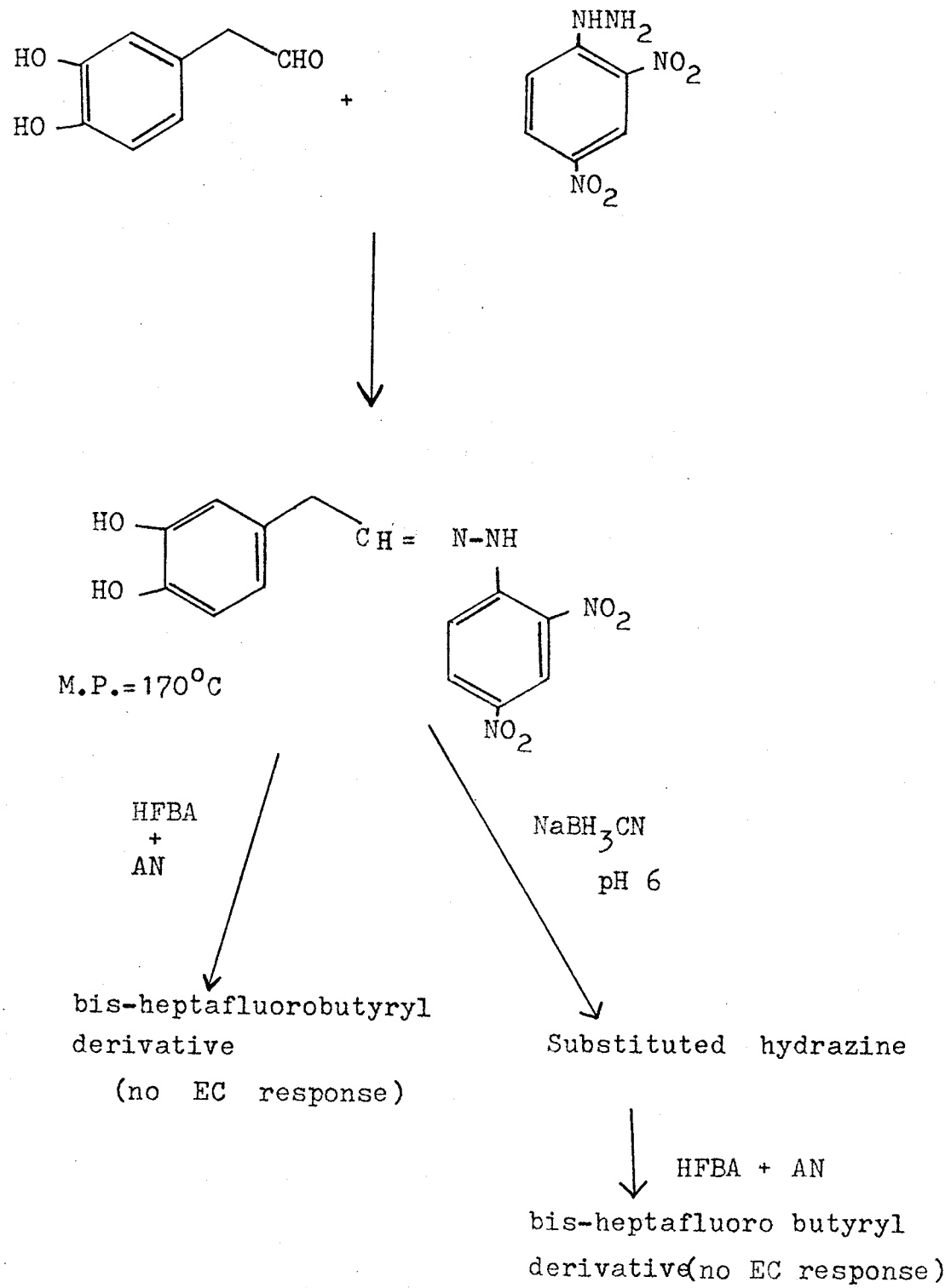


Fig.8 Reaction of 2,4-dinitrophenyl hydrazine with DHPAcH reduction &/or HFB derivative formation.

by electron capture detector. The standard curve for this HFB-pentafluorophenyl hydrazone of DHPAcH, is shown in Fig. 9. The MDQ was 5 pg at the conditions shown. The standard curve was drawn after running the reaction for 24 hr and assuming that 70% of reaction has been completed. The melting point of this hydrazone was not in literature and it was not crystallised for elemental analysis and melting point determination.

The reaction progress was determined for the formation of pentafluorophenyl hydrazone of DHPAcH by following the reaction for 12 hr. The reaction progress curve is shown in Fig.10.

E. EC/GC ANALYSIS OF THE REACTION PRODUCT OF HYDROXYLAMINE AND DHPAcH AFTER HFB-DERIVATIVE FORMATION

The reaction of DHPAcH with NH_2OH is very fast, and crystallization of the reaction product(3,4-dihydroxyphenyl acetaldoxime) seemed to be complete in 15-20 min. HFB derivative of 3,4-dihydroxyphenyl acetaldoxime gave a single peak on

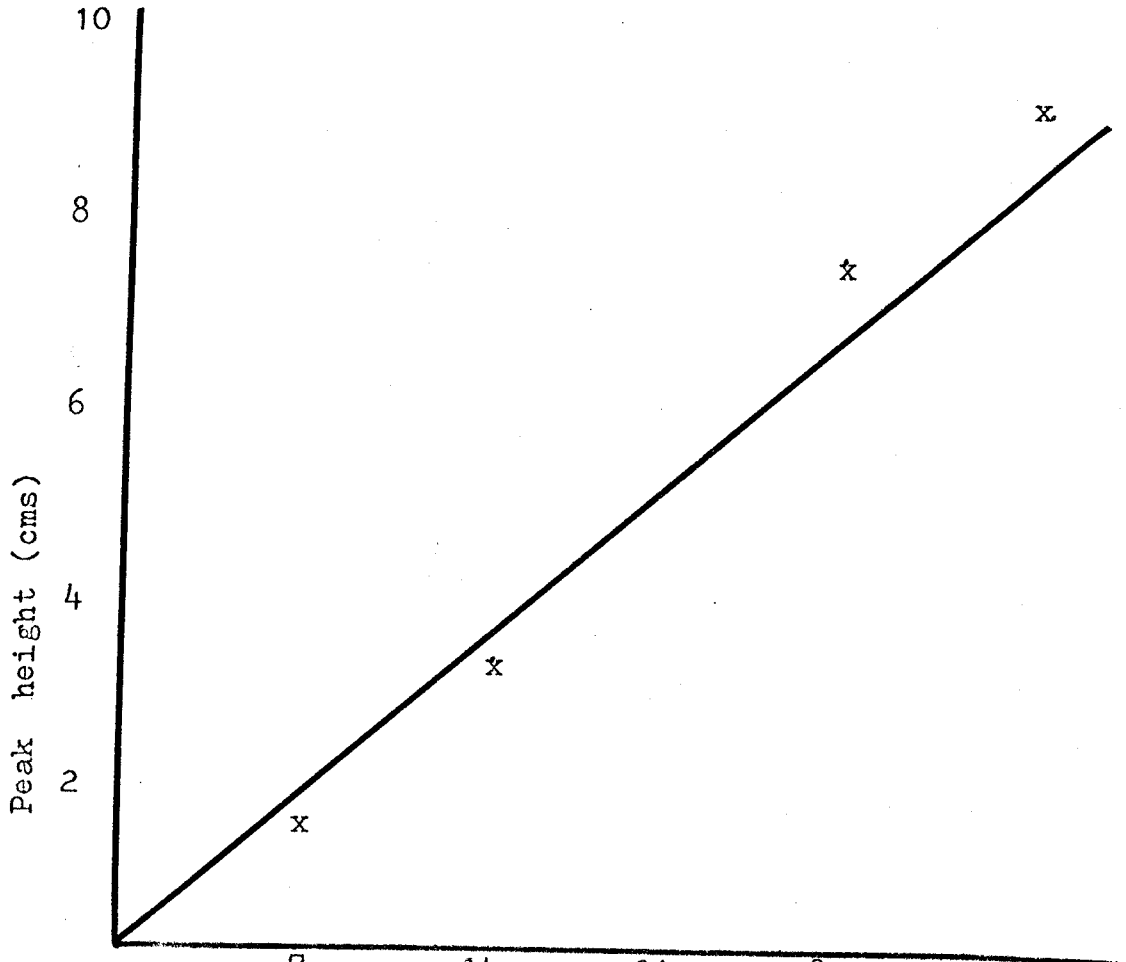


Fig.9 Standard curve for the HFB derivatized reaction product of pentafluorophenyl hydrazine with DHPAcH.

GC Conditions: Tc = 170°C, Ti = 200°C, Td = 240°C, N_2 flow rate = 25ml/min, Att. = 4×10^{-10}
RT = 6 min 15 sec.

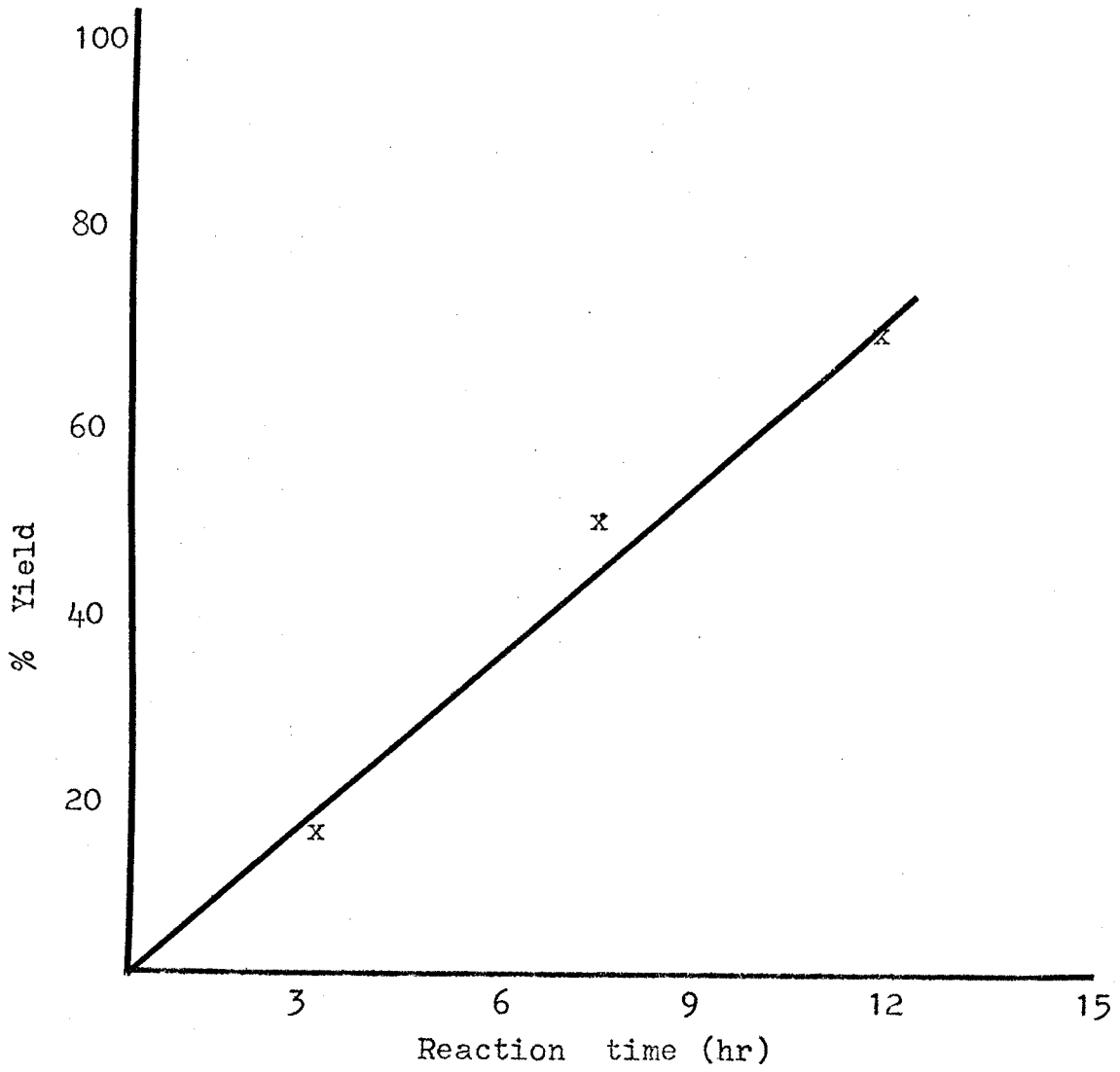


Fig.10 Reaction progress curve for the formation of reaction product of pentafluorophenyl hydrazine and DHPAcH.

GC Conditions: Tc =170°C, Ti = 200°C, Td = 240°C, Att = 4×10^{-10}, N_2 flow rate = 25ml/min, RT = 6 min 15 sec, Column 5% GEXF-1105.

5% GEXF-1105 on GCQ 80-100 mesh. The retention time was 5 min 30 sec at a N_2 flow rate of 23ml/min and injector, detector and column temperatures of 200°C, 240°C and 180°C respectively. The MDQ was 500 pg. The standard curve for HFB-3,4-dihydroxyphenyl acetaldoxime is shown in fig.11. The probable reaction of NH_2OH with DHPAcH is shown in fig. 12.

F. REDUCTION OF DHPAcH WITH SODIUM CYANOBORO-HYDRIDE & EC/GC ANALYSIS OF HFB-DHPG.

Commercial DHPG was used to obtain the standard curve. The HFB-DHPG standard curve is as shown in fig.13. The MDQ was 40 pg at 170°C on a 5% SE-30 column. The injector and detector temperatures were 230°C and 250°C respectively. Higher temperatures were tried and up to 200°C the sensitivity was about the same; but at 210°C up to 1-2 pg of DHPG can be detected. The only drawback at 210°C is that, there is no resolution between CAs and DHPG. The reduction of DHPAcH can be written as in fig. 14.

G. MEASUREMENT OF IN VIVO CATECHOLALDEHYDE LEVELS AS DHPG

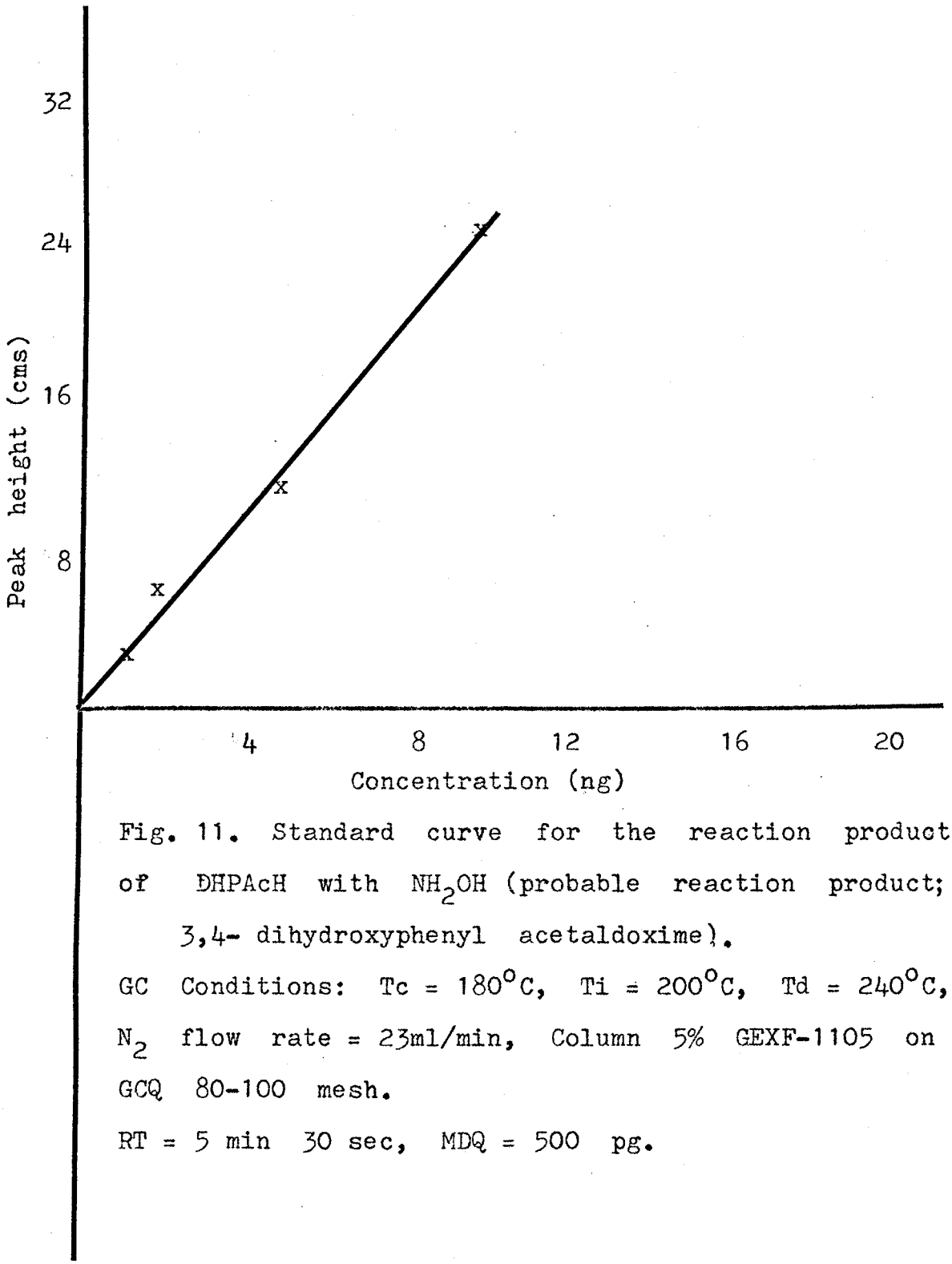


Fig. 11. Standard curve for the reaction product of DHPAcH with NH_2OH (probable reaction product; 3,4- dihydroxyphenyl acetaldoxime).

GC Conditions: Tc = 180°C, Ti = 200°C, Td = 240°C, N_2 flow rate = 23ml/min, Column 5% GEXF-1105 on GCQ 80-100 mesh.

RT = 5 min 30 sec, MDQ = 500 pg.

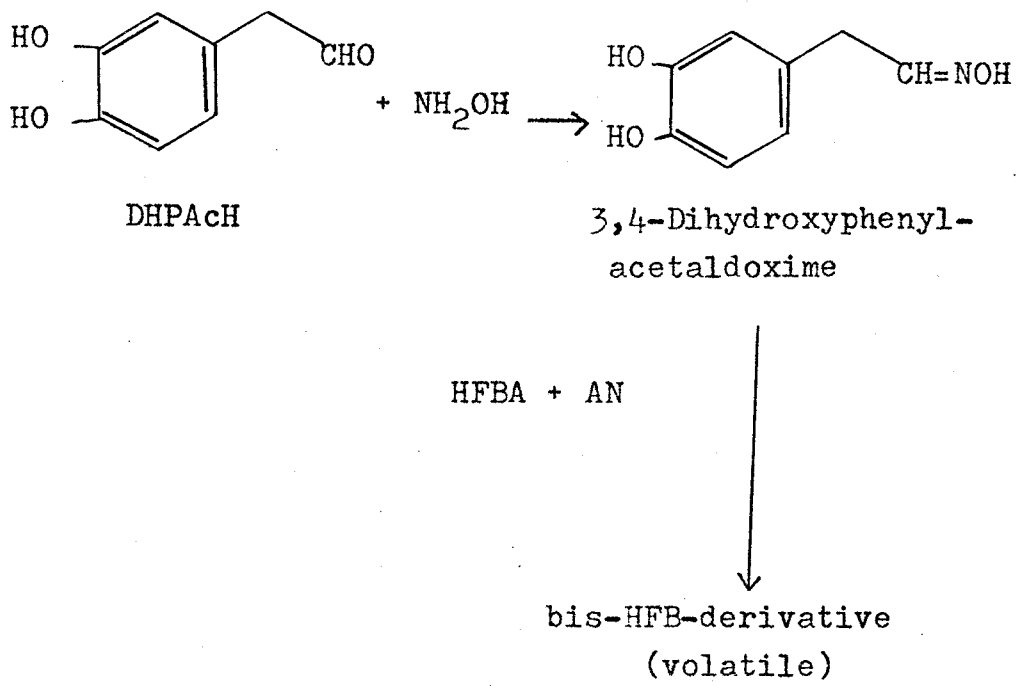


Fig. 12. Probable reaction of NH_2OH with DHPAcH, and fluoroacylation of expected 3,4-dihydroxyphenylacetaldoxime (product of reaction).

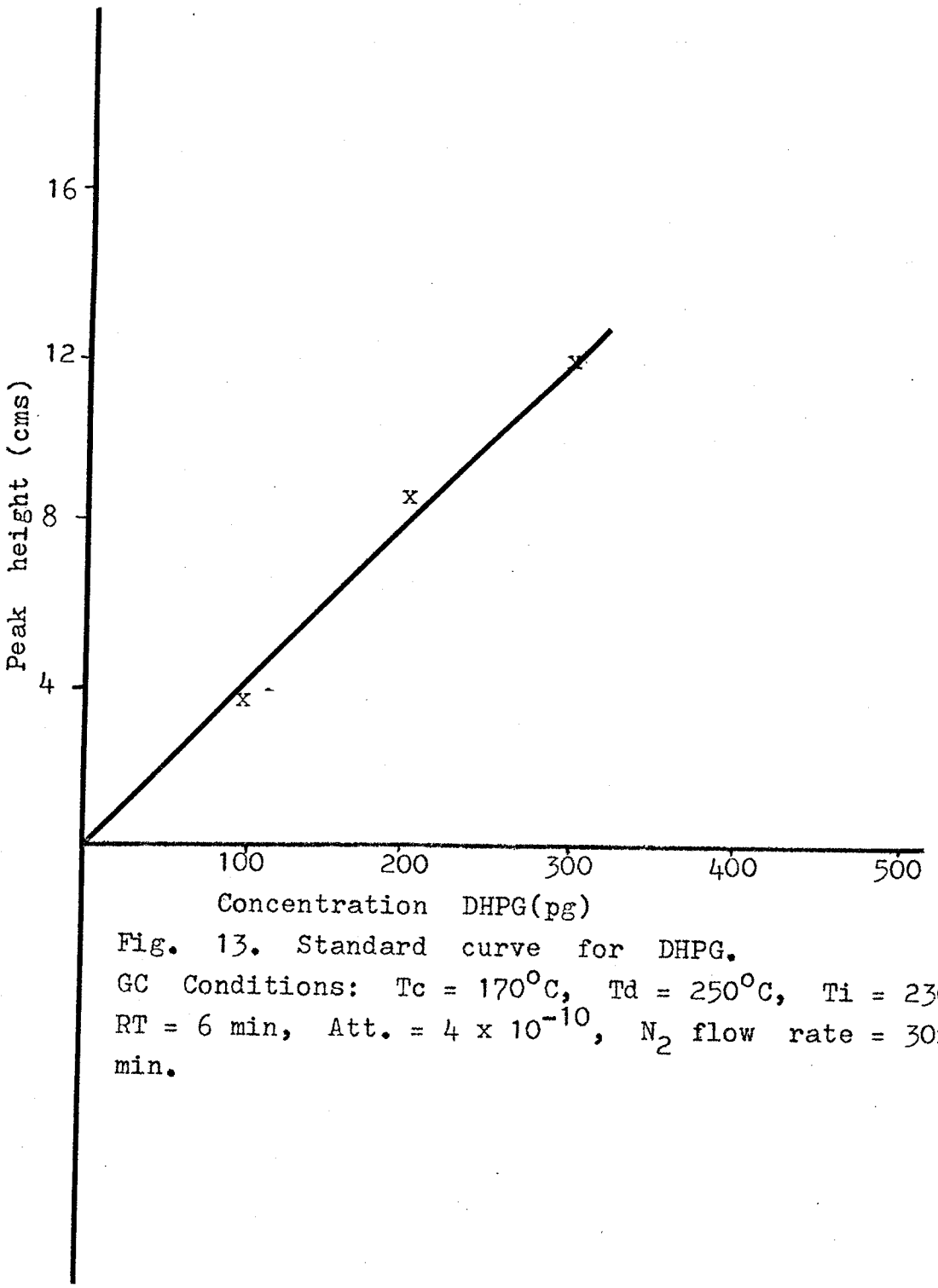


Fig. 13. Standard curve for DHPG.
GC Conditions: Tc = 170°C, Td = 250°C, Ti = 230°C, RT = 6 min, Att. = 4 x 10^{-10}, N_2 flow rate = 30ml/min.

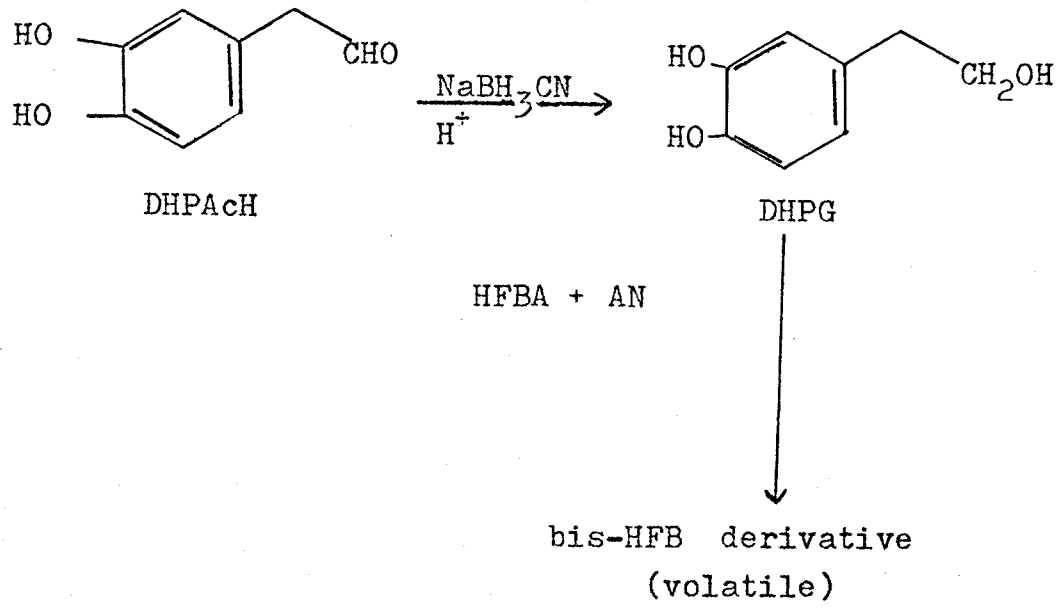


Fig. 14. Reduction of DHPAcH with $NaBH_3CN$ at pH 3-4.

In whole rat brain(control without any addition of $NaBH_3CN$), a peak for DHPG was observed by EC/GC analysis. In experimental whole brain the DHPG peak was obtained but it was some what smaller than control.

The DHPAcH level = DHPG level after reduction with $NaBH_3CN$ -(DHPG level in control)

By this calculation the DHPAcH level in brain comes out to be negative which is impossible as there is enough indirect evidence for the positive catecholaldehyde levels in brain.

H. TRAPPING SYNTHETIC DIHYDROXYPHENYL ACETALDEHYDE OR PHENYLACETALDEHYDE VIA REDUCTIVE AMINATION

The reaction conditions for the formation of an amine by reductive amination of aldehyde, were established by treating isopropylamine with phenyl acetaldehyde under four different sets of conditions, as discussed in materials and methods. The standard curve for the product,

tentatively called N-isopropylphenyl ethylamine, was drawn as shown in fig. 15 (obtained on the flame ionization detector). The % yield of N-isopropylphenyl ethylamine in one step preparation and two step preparation at room temperature and $0^{o}C$ were plotted in each case as shown in fig. 16 and fig. 17 respectively. The most probable reaction for one step and two step processes are as shown in fig. 18.

Epinine was prepared by treating DHPAcH with methylamine at pH 6 and reducing with $NaBH_3CN$ at pH 6. The reaction yield was followed by taking out 5 ml samples at different time intervals. Yield of epinine was calculated by comparing peak heights to the HFB-epinine standard curve as shown in fig.19. The retention time for epinine was 3 min at conditions mentioned in experimental and MDQ was 25-30 pg. The yield of epinine was calculated as follows:

The 10 min sample(5 ml) diluted to $1:10^5$ times has epinine as read from standard curve = 30 pg

Epinine concentration in 5 ml sample = 30×10^5

Total epinine in 50 ml = $30 \times 10^5 \times 10$

= 30 mg

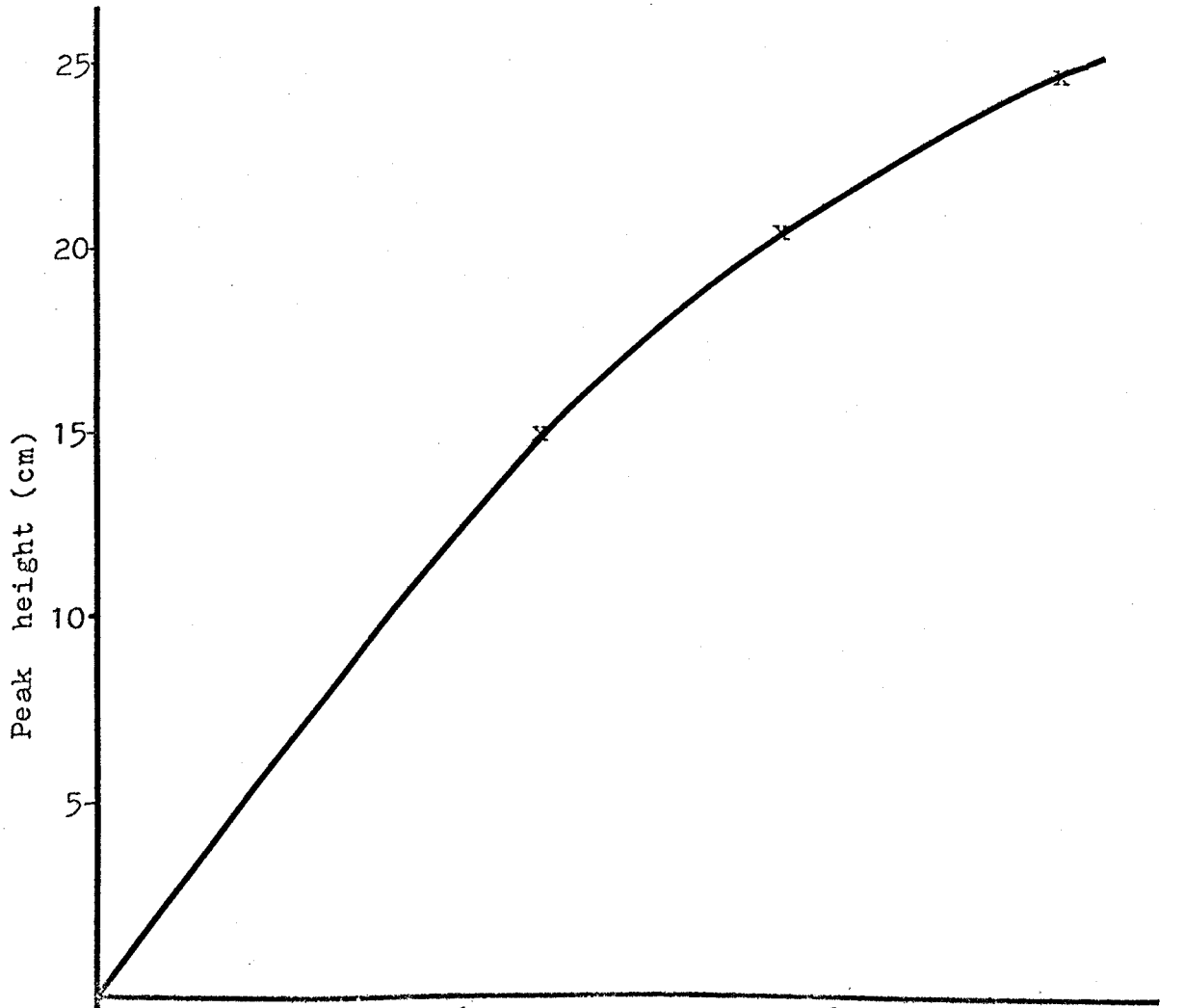


Fig.15 Standard curve for the product obtained by reductive amination of phenyl acetaldehyde with isopropylamine and $NaBH_3CN$ (the reaction product tentatively called N-isopropylphenyl ethylamine)

GC Conditions: 3% OV-17 On GCQ 80-100 mesh, Tc = 110°C, Td = 230°C, Ti = 200°C, N_2 flow rate= 21ml/min, Flame ionization detector.

RT = 7 min.

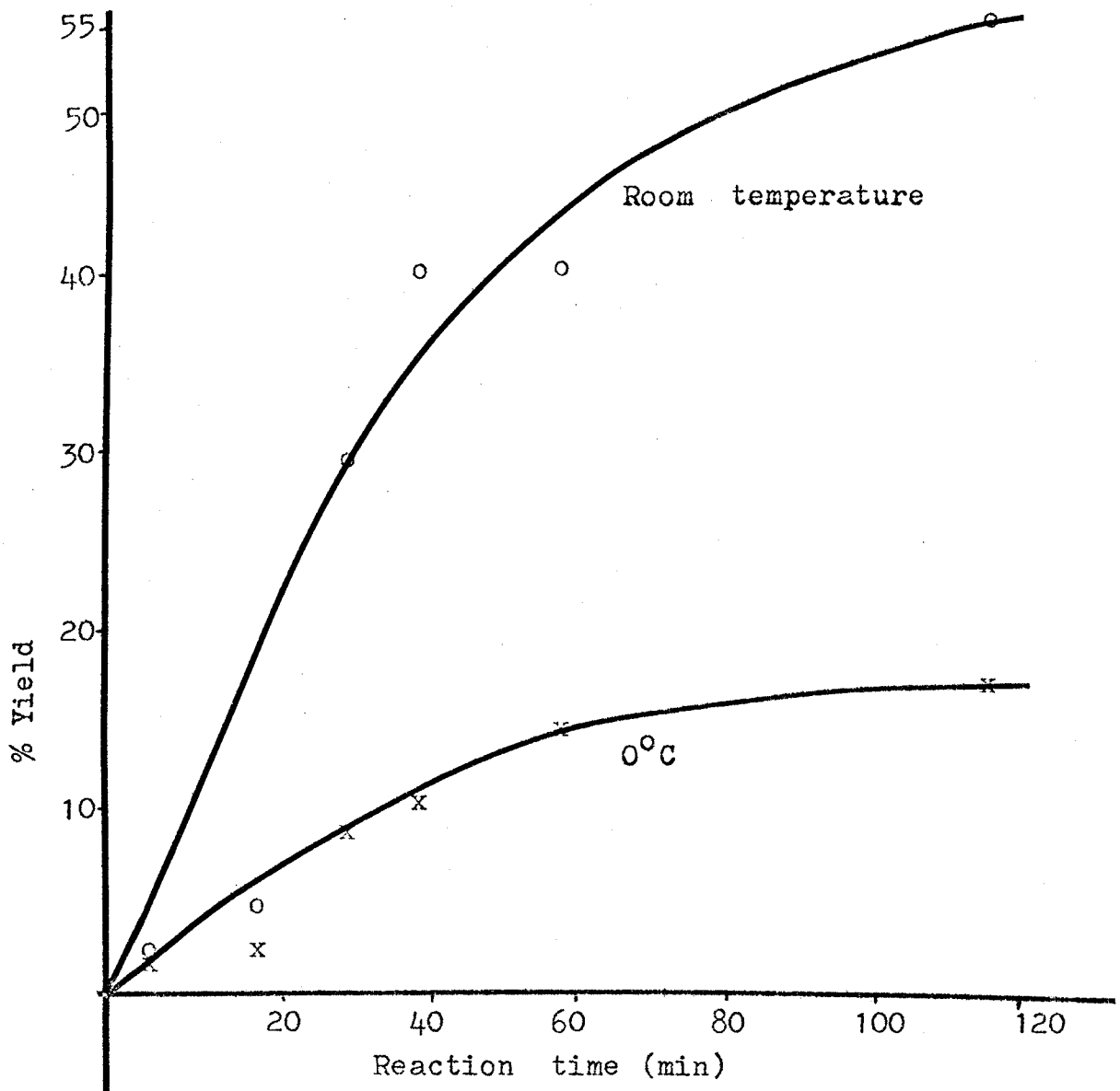


Fig. 16 The % yield of the product (obtained by reductive amination of phenyl acetaldehyde with isopropylamine and $NaBH_3CN$) in one step preparation at pH 6 at different reaction times.

GC Conditions: 3% OV-17 on GCQ 80-100 mesh, Tc = 110°C, Ti = 200°C, Td = 230°C, N_2 flow rate = 21ml/min, Flame ionization detector.

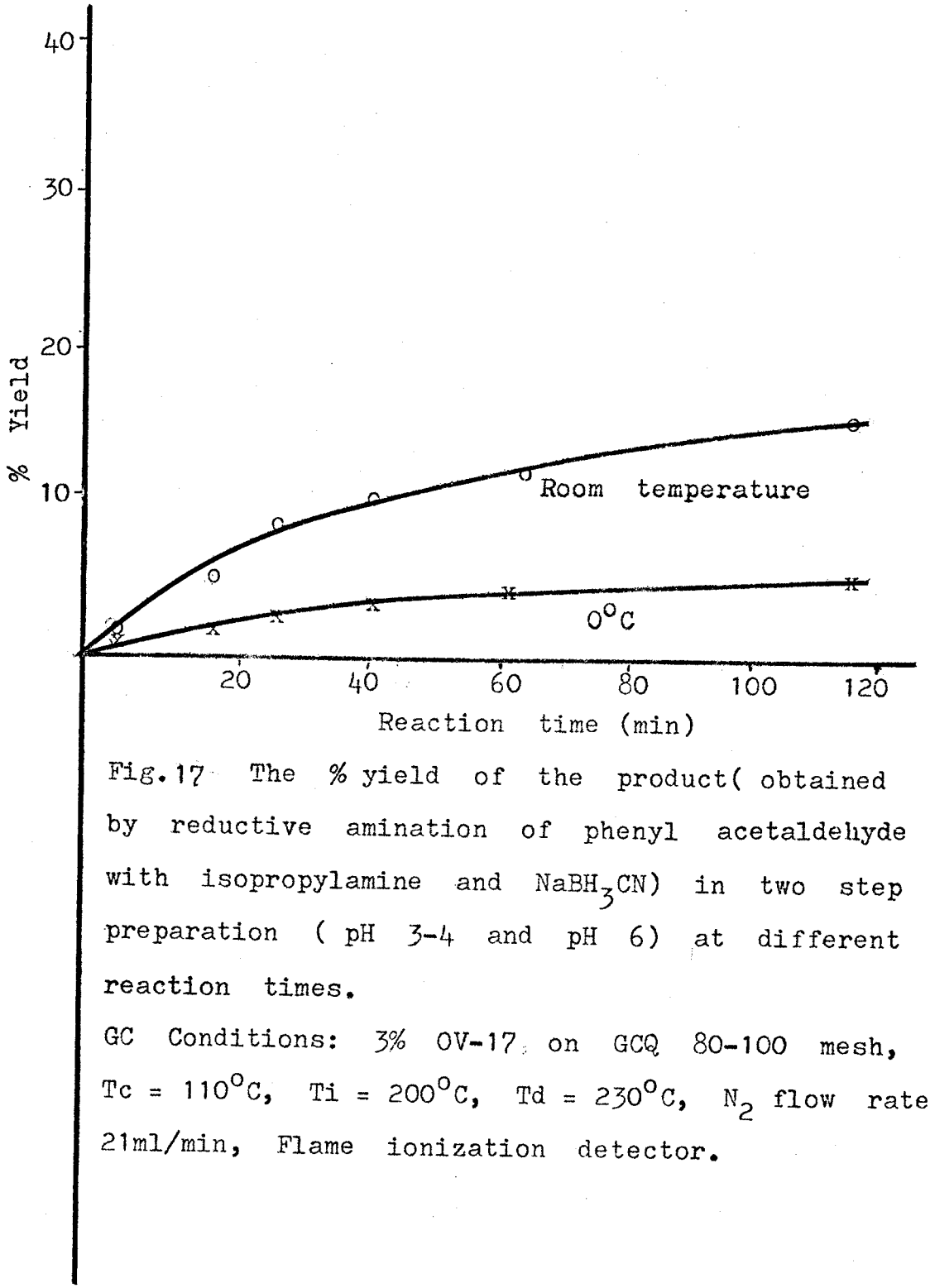
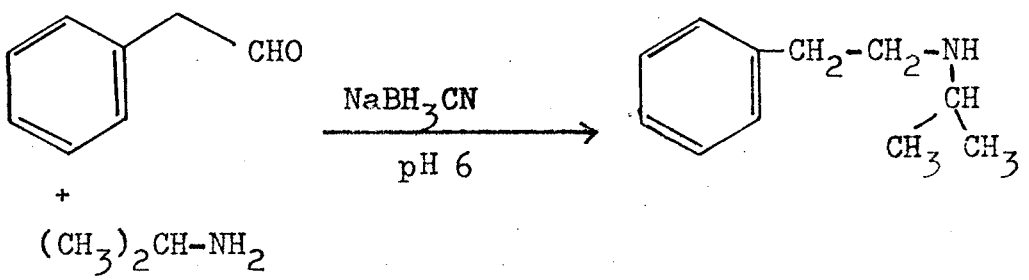
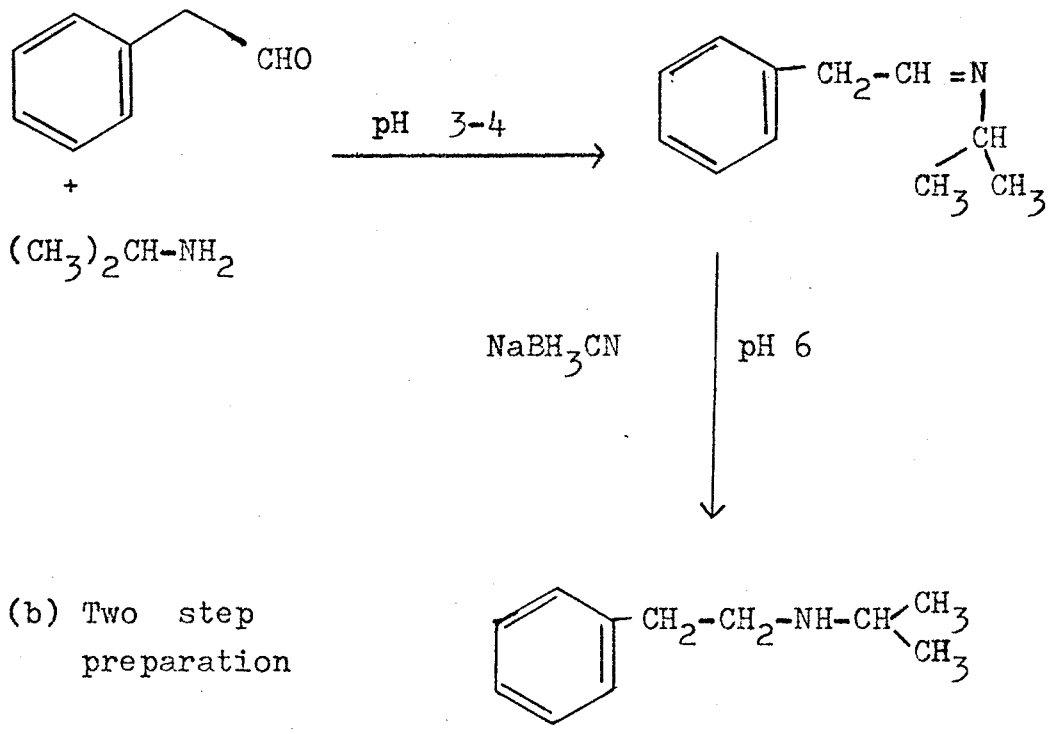


Fig.17 The % yield of the product(obtained by reductive amination of phenyl acetaldehyde with isopropylamine and $NaBH_3CN$) in two step preparation (pH 3-4 and pH 6) at different reaction times.

GC Conditions: 3% OV-17 on GCQ 80-100 mesh, Tc = 110°C, Ti = 200°C, Td = 230°C, N_2 flow rate = 21ml/min, Flame ionization detector.



(a) One step preparation.



(b) Two step preparation

Fig. 18 The most probable reaction for reductive amination of phenyl acetaldehyde with isopropylamine and $NaBH_3CN$ in one step and two step preparation.

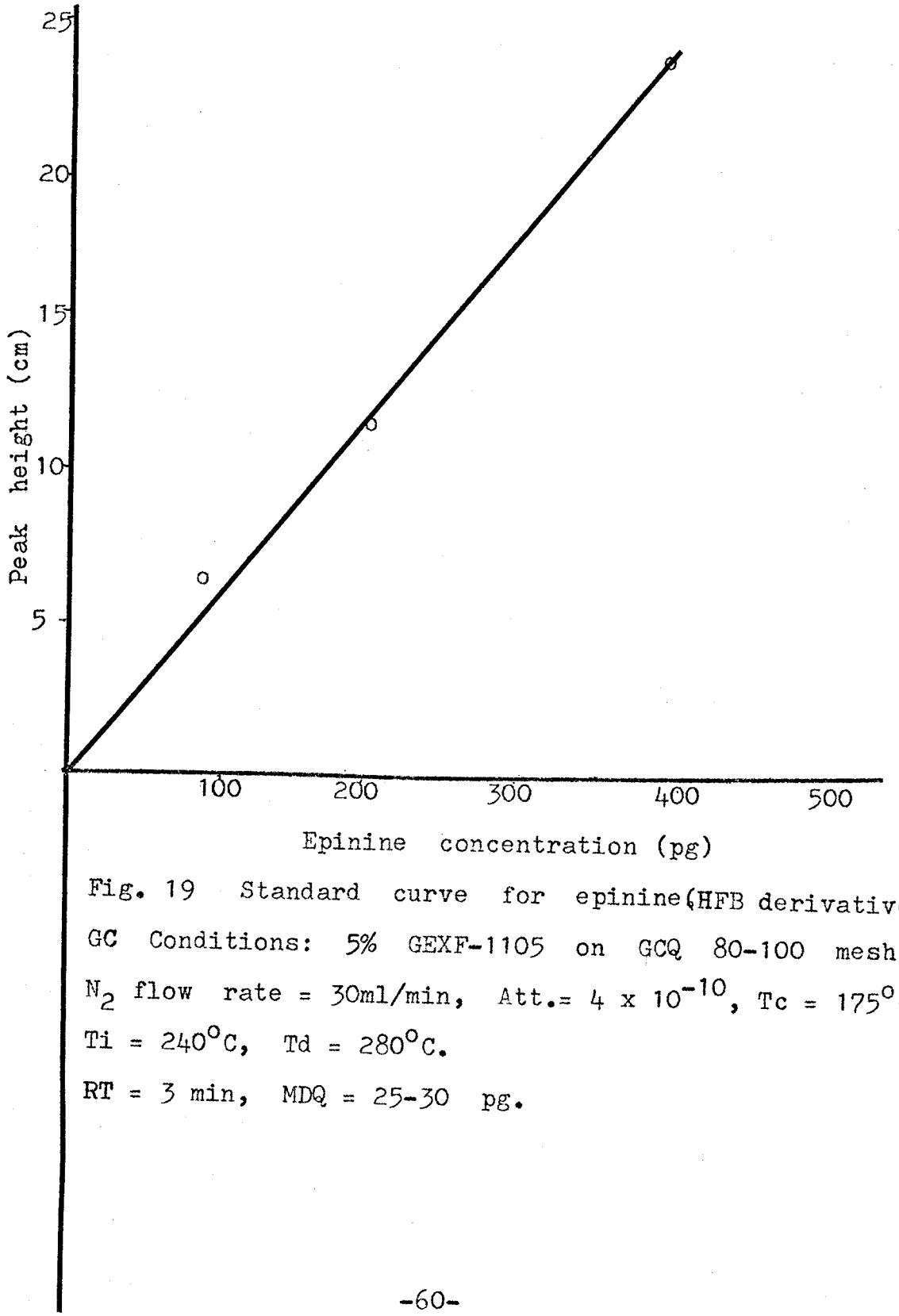


Fig. 19 Standard curve for epinine (HFB derivative).
GC Conditions: 5% GEXF-1105 on GCQ 80-100 mesh,
N_2 flow rate = 30ml/min, Att.= 4×10^{-10}, Tc = 175°C,
Ti = 240°C, Td = 280°C.
RT = 3 min, MDQ = 25-30 pg.

Similarly total epinine at 1 hr = 133 mg

and at 2 hr = 144 mg

The % recoveries of epinine at various reaction times are shown in table 1. The whole reaction for the formation of epinine is shown in Fig. 20.

I. TESTING THE REDUCTIVE AMINATION METHOD WITH BRAIN TISSUE

This method was tested by measuring epinine formation in whole rat brain. There is no epinine present in the rat brain; but there was a small peak for epinine in the experimental sample, which contained methylamine and $NaBH_3CN$. However, a control with methylamine only should be included in future studies. With this method it is possible that dopaldehyde levels can be read directly as epinine. The chromatogram of the HFB-catechol compounds from whole rat brain after reaction with methylamine and reduction with $NaBH_3CN$ at pH 6 for 2 hr is shown in Fig.21.

Initial amt. mM	Rx time min	Total volume ml	Sample volume ml	Sample dil	sample Epi-nine pg	Total epi-nine pg	Epi-nine mM	% Yield
2.07	10	50	5	$1:10^5$	30	30	0.19	9%
2.07	60	50	5	$1:10^5$	133	133	0.80	39%
2.07	120	50	5	$1:10^5$	144	144	0.84	41%

Table 1. % Recoveries of epinine at various reaction times.

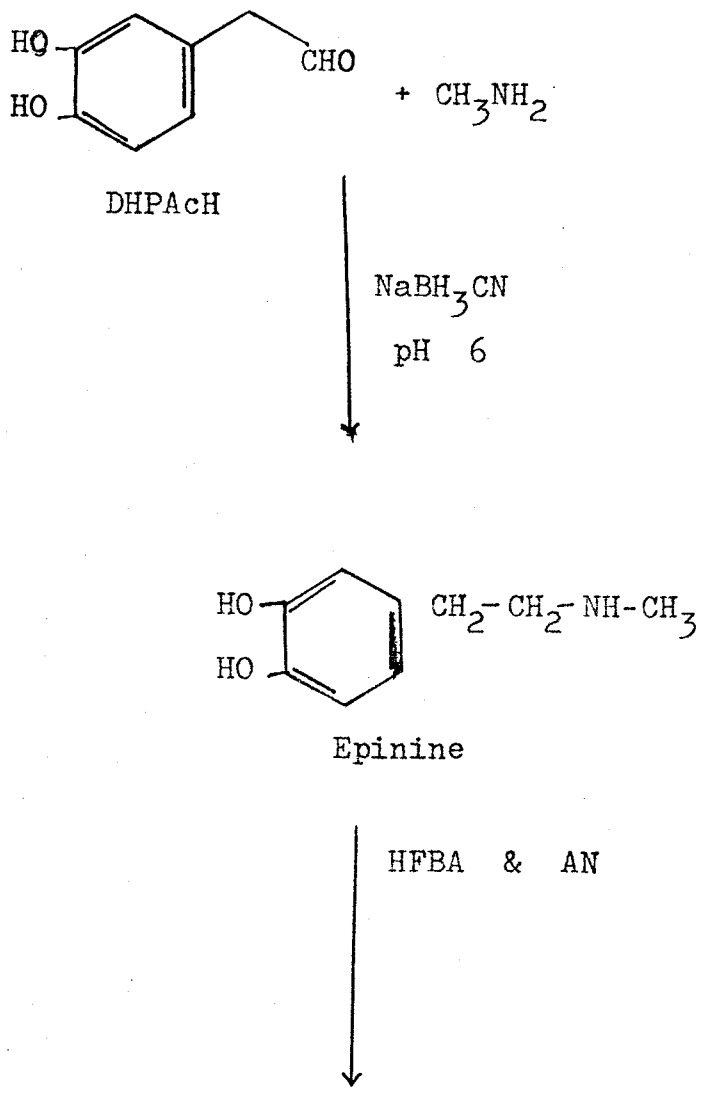


Fig. 20 Formation of epinine by reductive amination with $NaBH_3CN$ & CH_3NH_2 at pH 6.

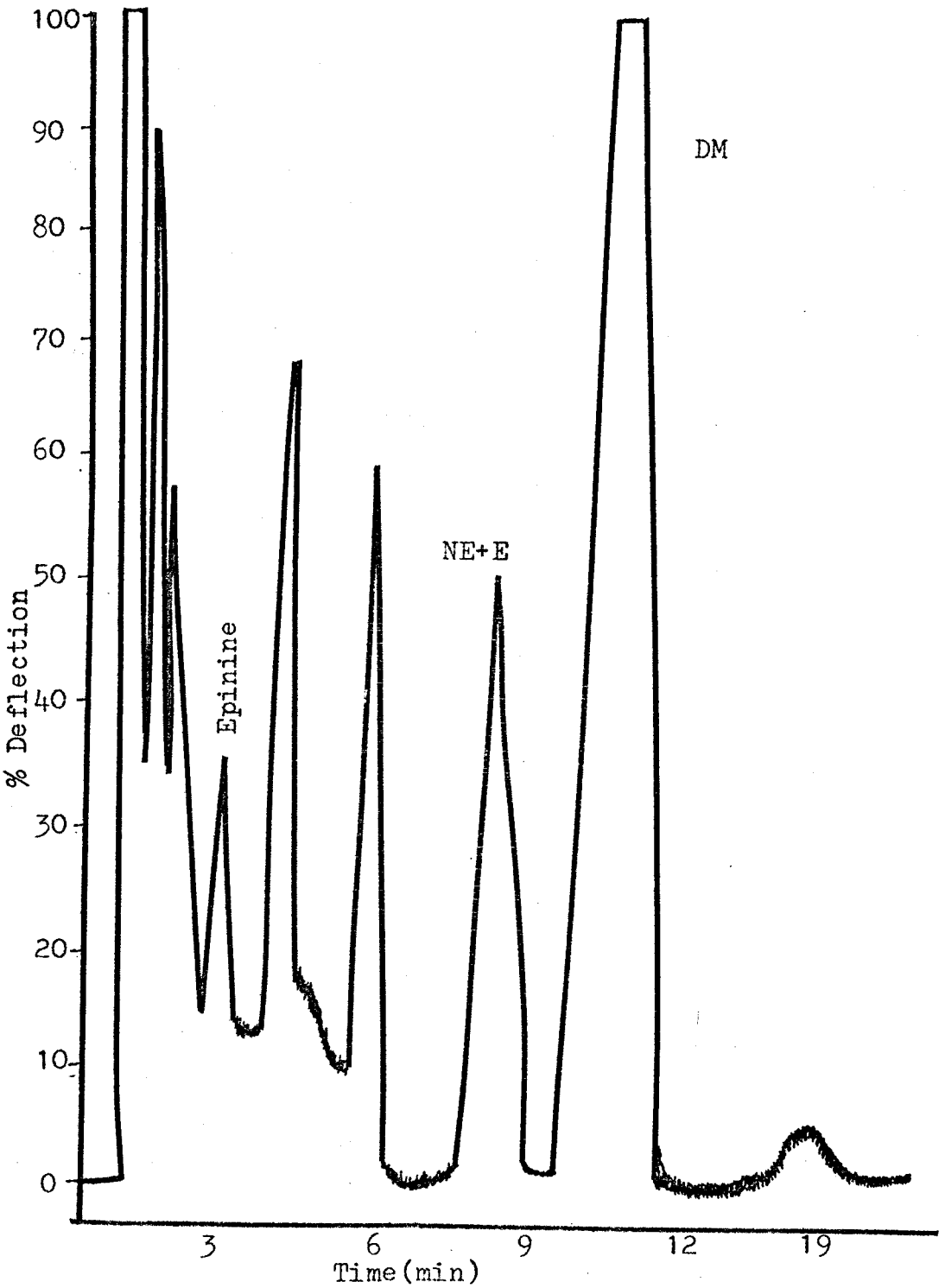


Fig. 21. EC/GC chromatogram of the HFB-catechol compounds from whole rat brain after reduction with $NaBH_3CN$ at pH 6 for 2 hr(CH_3NH_2 present). GC Conditions: 3% OV-17 on GCQ 80-100; Tc=160°C; Td=250°C; Ti=200°C; N_2 flow rate=30ml/min, Att.=8×10^{-10}.

CHAPTER FOUR

DISCUSSION & CONCLUSIONS

Four different approaches were tried to establish a reliable and sensitive method for the measurement of catecholaldehyde levels in brain tissue. The first approach was to measure catechol-aldehyde levels as the 2,4-dinitrophenyl hydrazone derivative. There was no electron capture response for HFB or PFP derivative on 3% OV-17, 5% GEXF -1105, or 5% SE-30. On the other hand HFB-pentafluorophenyl hydrazone derivative gave a very good electron capture response. The minimum detectable quantity was 5 pg on 5% GEXF 1105 (on GCQ 80-100 mesh) at 170°C and carrier gas flow rate of 25 ml/min. The only drawback with this method was that it takes 12 hr to get a fairly good yield (65%) of pentafluorophenyl hydrazone of DHPAcH. The 2,4-dinitrophenyl hydrazone preparation takes only 1/2 hr but it does not show any electron capture response. It was apparent that the HFB- or PFP- 2,4-dinitrophenyl hydrazone of DHPAcH was not volatile.

The second approach was to trap aldehydes as oxime derivative. The reaction time was only 15 min, but the electron capture response for this derivative was not very great. The minimum detectable quantity was 500 pg.

The third approach was to reduce DHPAcH with $NaBH_3CN$ and measure as DHPG. The minimum detectable quantity for HFB-DHPG was 40 pg. The method seemed quite sensitive. But when this method was applied to tissue there was no difference between DHPG level of control and reduced tissue (experimental tissue which has been reduced with $NaBH_3CN$ at pH 3-4) DHPG. In contrast by calculation one comes up with a negative number. Possible explanations for this observation are; (a) that may be the $NaBH_3CN$ is not reducing the catecholaldehydes as they are bound to tissue; (b) that catecholaldehydes might have been metabolised by aldehyde dehydrogenase or by aldehyde reductase <u>in vitro</u> during decapitation and homogenization.

The fourth approach was to trap catecholaldehydes as imines and reduce to an amine. It is very clear from the one step (Fig. 16) and two step (Fig. 17) preparations of N-isopropylphenyl ethylamine that the optimum condition for reductive amination of aldehydes is to carry on reductive amination in one step at pH 6 at room temperature.

The reductive amination method was established by preparing epinine from DHPAcH by treating with methylamine and $NaBH_3CN$ at pH 6 for 2 hr. The reaction mixture was stirred throughout at room temperature. The epinine formed was extracted by binding to activated alumina at pH 8-8.2 and then eluting the bound epinine with 0.2N HCl. The acid eluate was lyophilysed and treated with HFBA and AN for 20 min. The fluoroacylated epinine was dried under a N_2 stream. The HFB-epinine gives a single peak on 5% GEXF-1105. The minimum detectable quantity was 30 pg. There is no apparent epinine present in whole rat brain tissue under our conditions. Hence this

method has advantage that dopaldehyde levels can be read directly as epinine. The E and NE derived catecholaldehydes can be measured with some modifications of the basic method. The HFB-epinine derivative is stable for at least 2-3 days. at -20°C. Under the same conditions epinine is stable for weeks. The catecholaldehydes are very important metabolites and this method will help study the catechol-aldehyde levels in normal, disease or drug state.

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APROVAL SHEET

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The final copies have been examined by the director(Dr. Michael A. Collins) of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

5/15/75
Date

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