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MITOCHONDRIAL MONOAMINE OXIDASE

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Thesis

Submitted to the Faculty of Medicine, in partial fulfilment of the requirements for the degree of Doctor of Philosophy .

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INTRODUCTION

The oxidative deamination of monoamines in the mammalian organism has been attributed to the enzyme monoamine oxidase since Blaschko (10) in 1937 concluded that the different amine oxidizing systems which had been described were probably one and the same enzyme . This conclusion has been quite generally accepted, although indirect evidence against it has emerged from different sides . More recently the multiplicity of mitochondrial monoamine oxidase has been suspected, mainly on the grounds of indirect evidence gathered more or less accidentally in the course of investigations on other aspects of this enzyme system . In the few cases where this problem was attacked directly, encouraging though non-conclusive evidence was found for the multiplicity of mitochondrial monoamine oxidase .

The present work was started with the hypothesis of the presence of different enzymes for the oxidative deamination of monoamines in rat liver mitochondria . If two enzymes catalysing the same reaction are present in a mixture, this has special implications for the kinetics of the reaction . Three different experimental approaches were used by us to test the conclusions

of this hypothesis with experimental data . These experiments are described and the results discussed in Part I of the section on experimental work .

The first type of experiment involved the use, in a mixture, of two substrates, one ^{14}C-labelled and the other one cold, in order to determine the apparent Km and the apparent Ki for both substrates . If two enzymes with different Km were present, one would expect the apparent Km to be different from the apparent Ki . The second experiment had a similar setup, only now the effect of the concentration of serotonin on the slope of the reciprocal plot for the benzylamine-^{14}C reaction was evaluated. If two enzymes are present, there should be a hyperbolic inhibition on the slopes . In the third experiment, one substrate (serotonin) was used in a simple assay of the rate of oxygen consumption at different levels of concentration of the substrate, and the reciprocal plot was made . If two enzymes with different Km for serotonin were present a hyperbolic reciprocal plot would be found .

In Part II of the experimental work, an attempt was made to separate the two enzymes . Since monoamine oxidase is known to be bound very tightly to the mitochondrial membrane, the problem of its solubilization had to be solved first . Indeed it was thought that the best chances of obtaining a separation of the two enzymes would be after as good a solubilization as possible . So we first investigated a variety of methods for solubilizing the enzyme . Some work in that direction had already been done, but with

limited success, and the criteria used to decide if a preparation was soluble were often less than rigorous . We therefore decided to study the solubilization using besides the common differential centrifugation technique, electron microscopy and density gradient centrifugation, hoping that the results of these methods would complement each other . When a suitable method for solubilization was found, the separation was performed using the disc gel electrophoresis technique . The enzyme activity was detected directly on the gel which was cut into thin slices . When the existence of two bands of monoamine oxidase activity was established in this way, artifacts were ruled out by eluting the enzyme from the gel prior to the assay .

The section on experimental work is preceded by a review of the literature on monoamine oxidase, in which chapter IX (on the multiplicity) gives a detailed description of the concepts and evidence which prompted the start of this work, and of which this work is only an attempted continuation .

SECTION I : REVIEW OF THE LITERATURE

Chapter I : HISTORY

In their review of the history of monoamine oxidase, both Blaschko (1) and Pletscher, Gey and Zeller (2) mention Schmiedeberg (3) as the first to carry out experiments on amine metabolism . He namely gave an oral dose of benzylamine to a dog and recovered benzoic acid in the urine . He concluded that the amine had been metabolized with loss of the amine group and oxidation . This was in the year 1877 . Mosso (4) in 1889 gave a quantitative basis to these findings : from a subcutaneous injection of benzylamine, he recovered 90% as benzoic acid in the urine . In 1883 Minkowski had shown that in vitro also benzylamine could be metabolized to benzoic acid . He was using minced rabbit tissue . Perfusion experiments performed by Ewins and Laidlaw (6) in 1910 gave essentially similar results : in a perfused liver they found that tyramine was metabolized to p-hydroxy-phenylacetic acid at a ratio of 70% . In a perfused rabbit liver tryptamine was

metabolized to indole-acetic acid . In all of this work it was assumed the first reaction was a hydrolysis, which was followed by oxidation .

(1) $R\text{-}CH_2\text{-}NH_2 + H_2O \longrightarrow R\text{-}CH_2\text{-}OH + NH_3 \longrightarrow R\text{-}COOH$

amine alcohol acid

It was Hare (7) who in 1928 discovered "tyramine oxidase" and made the first mention of oxidative deamination . In other words she showed that tyramine added to an incubating minced tissue increases its oxygen consumption, and she concluded that oxygen was necessary for the metabolism of tyramine . Bernheim-Hare in 1931 showed that the reaction liberated ammonia and hydrogen peroxide (8) . She also postulated the formation of aldehyde . In 1937 Blaschko, Richter and Schlossmann (10) described an adrenaline oxidase and they concluded that the tyramine oxidase of Hare, the amine oxidase described by Pugh and Quastel (9) and their enzyme were identical . Zeller in 1938 (11) proposed to call it monoamine oxidase to distinguish it from diamine oxidase . The value of these names and classification will be discussed later .

Chapter II : DEFINITION

1.4.3.4 monoamine : O_2- oxidoreductase (deaminating)

Monoamine oxidase deaminates alkyl- and aralkylamines with primary or secundary aminogroups while liberating ammonia or methylamine respectively .

(2) $R\text{-}CH_2\text{-}\overset{+}{N}H_3 + O_2 + H_2O \longrightarrow R\text{-}CHO + \overset{+}{N}H_4 + H_2O_2$

In the presence of catalase (e.g. whole homogenates or even washed mitochondria) :

(3) $H_2O_2 \longrightarrow H_2O + \frac{1}{2} O_2$

Sum of reactions 2 and 3 :

(4) $R\text{-}CH_2\text{-}\overset{+}{N}H_3 + \frac{1}{2}O_2 \longrightarrow R\text{-}CHO + \overset{+}{N}H_4$

H.Aebi et al. (12) showed that in the presence of catalase there are two possibilities for the further metabolism of hydrogen peroxide, depending on the presence or the absence of hydrogen donors :

- when no hydrogen donors are present :

(5) $H_2O_2 = \frac{1}{2} O_2 + H_2O$

In this case the molar ratio of oxygen consumption over ammonia production equals $\frac{1}{2}$.

- when hydrogen donors are present (e.g. formiate) :

(6) $H_2O_2 + \text{Donor-}H_2 \longrightarrow 2\ H_2O + \text{Donor}$

In this case the ratio of oxygen consumption to ammonia production equals 1 . In equation 5 we have a catalase-type reaction, in equation 6 a peroxidase-type reaction . The importance of these reactions is obvious, since one of the main assay methods for monoamine oxidase is based on the measurement of the rate of disappearance of oxygen, and the presence or absence of catalase and of hydrogen donors can greatly influence the oxygen consumption . According to Aebi (12) lyophylized mitochondria, without added catalase reacted 40 % as in reaction 6 .

Monoamine oxidase can be classified as a 2 electron transferase : 2 electrons are transferred directly or indirectly to oxygen, which is reduced to hydrogen peroxide, while the aminogroup is oxidized to the iminogroup or the aldehyde . Theoretically there are three possibilities (2) :

- monoamine oxidase reacts directly with oxygen
- it reacts with the aminogroup and dehydrates it to the

iminogroup, while oxygen is reduced to H_2O_2 . The ammonia is then split off by hydrolysis .

(7) $R\text{-}CH_2\text{-}\overset{+}{N}H_3 + O_2 \longrightarrow R\text{-}CH{=}\overset{+}{N}H_2 + H_2O_2$

(8) $R\text{-}CH{=}\overset{+}{N}H_2 + H_2O \longrightarrow R\text{-}CHO + \overset{+}{N}H_4$

The electrons released by the dehydration of the amine must be transferred, over the respiratory chain or an equivalent mechanism, to oxygen .

- the dehydration of the amine is coupled directly with a hydrogen consuming reaction (e.g. the reduction of pyruvic acid) .

In favor of the direct reaction with oxygen, the authors mention: monoamine oxidase does not use the respiratory chain in vitro ; no experimental evidence has yet been presented for the formation of an iminogroup in the reaction . In favor of the indirect reaction is the reduction of certain indicators (o-bromindophenol, o-cresolindophenol, tetrazolium salts (2,3,4) . But - the reduction of indicators requires a lag period which is not observed in the manometric measurement of the enzyme (15) ; - and the reduction of indicators is inhibited by carbonyl reagents whereas the disappearance of substrate is not . It must therefore be concluded

that the reduction of redox indicators is due to further oxidation of the aldehyde, not to the deamination reaction itself (2) .

The reaction described in equation 2 and 4 has always been found to be essentially irreversible . Proof that the deamination really happens according to equation 2 has been the detection of the reaction products and the stoichiometry . Hare (7) already detected hydrogen peroxide and ammonia . Many other authors have demonstrated the production of the aldehydes corresponding to the different substrates : benzaldehyde, phenylacetaldehyde, p-hydroxy-phenylacetaldehyde, 3,4,-dihydroxy-phenylacetaldehyde, etc...(2) .

Since it became more apparent when the substrate specificity of the amine oxidases was studied, that the classification by substrates was less than adequate, Blaschko et. al. (17) in 1959 proposed a new classification . Monoamine oxidase also attacks long chain diamines (e.g. histamine) and diamine oxidase is active on some monoamines as well . Therefore Blaschko proposed to base the new classification on the more typical and better defined inhibition characteristics :

- monoamine oxidase is inhibited by octyl alcohol but not by carbonyl reagents (e.g. semicarbazide) nor cyanide .
- diamine oxidase is inhibited by semicarbazide and cyanide but not by octyl alcohol .

As a conclusion one can define monoamine oxidase as an enzyme which deaminates oxidatively monoamines, but also certain diamines, is not inhibited by carbonyl reagents, but is inhibited by octyl alcohol and a series of specific inhibitors . These criteria enable us to distinguish it from diamine oxidase (1.4.3.6) and spermine oxidase (an enzyme which occurs in plasma of ruminants, is inhibited by carbonyl reagents and cyanide and is mainly active on spermine but also on benzylamine) .

Chapter III : PROPERTIES

Numerous studies have been devoted to the exploration of possible substrates for this broad specificity enzyme, and to the classification of those substrates according to their ability to be broken down by it . But although an impressive list of substrates was drawn up, the classification was quite difficult due to the great differences in specificity between the enzymes from different organs and species . Some general rules could be established however . Werle (16) enumerates them :

- of the aliphatic monoamines the ones with short chain length are the better substrates, with an optimum at butylamine ;
- the enzyme is not active on the typical diamine oxidase substrates putrescine and cadaverine, but longer chain diamines are substrates with an optimum chain length of thirteen

carbon units ;

- the arylalkyl- and the indolylalkylamines include several physiologically important amines which can be classified in the following order according to their oxidation rates :

good substrates are dopamine and tyramine ;

fair substrates : 3-methoxy-tyramine, tryptamine, 5-hydroxy-tryptamine, isoamylamine ;

poor substrates : phenylethylamine, normetanephrine, metanephrine, epinephrine, norepinephrine, N-methyl-histamine, kynuramine ;

Substitution of atoms in the substrate molecule has different effects when performed in different places in the molecule . Substitution of one hydrogen on the amine group has little effect on the ability as a substrate ; two or three hydrogens replaced by methyl groups e.g. markedly decreases the reactivity . Substitution of a hydrogen on the α-carbon atom completely prevents attack by the enzyme (isopropylamine, amphetamine, ephedrine). By deuterium isotope studies, Belleau and Moran (44) showed that substraction of the α-carbon-hydrogen is the rate limiting step in the reaction mechanism . Substitution of a hydrogen on the β -carbon causes a decrease in the reactivity (norepinephrine) .

That the substrate specificity depends on species and organ was shown by many different workers in the field, and,

as shall be seen further, this was one of the reasons for questioning the homogeneity of the enzyme . Werle (16) cites the example of cat liver where tryptamine and 5-hydroxytryptamine are oxidized at a slower rate than tyramine, and hog liver where tyramine and tryptamine are oxidized at equal rate, but 5-hydroxytryptamine more slowly . Hope and Smith (17) studied the monoamine oxidase of mouse tissues and found marked differences in substrate specificity . Whereas in brain and liver the enzyme was more active on tyramine than on serotonin, kidney, spleen and lung enzymes were more active on serotonin than on tyramine .

Blaschko (10) found that, contrary to oxidation rates, Km values were rather similar for different substrates : he gave as value for this constant $10^{-2.75}$ and $10^{-2.15}$, for tyramine and adrenaline respectively . Kinetic data from the literature are difficult to compare, due to differences in experimental conditions and also, as remark Alles and Heegaard (18), to the variations between different preparations and even more different species . These authors found an increase in Km for aliphatic amines with increasing chain length, and at the same time an increase in Vmax . The great variability between preparations, they ascribed to the possible existence of different enzymes . The optimum substrate concentration lies, according to Werle (16) around 2×10^{-3} to 10^{-2} M for tyramine, 4×10^{-2} M for adrenaline,

4×10^{-3} for tryptamine, 10^{-2} to 10^{-3} M for serotonin . The optimum pH is also different for different substrates . Werle gives values ranging from 6.9 to 8.4 .

Kinetic studies by McEwen et al. (19) demonstrate the great dependence of the Km on the pH . He explains this by the theorythat only the non-ionized form of the amine is attacked by the enzyme . When the Km is corrected for that fraction of the substrate which is ionized by the equation :

$$Km_{(apparent)} = Km \cdot (1 + \text{antilog } (pKa - pH))$$

then it is constant over a range of at least 6.5 to 9.0 . Some corrected Km values : 5 micromolar for benzylamine (pKa 9.37), 204 micromolar for veratrylamine (pKa 9.39), 4.1 micromolar for N-methyl-benzylamine (pKa 9.54) . These Km values are not real Kms in the sense that they were not measured at infinite oxygen concentration . Oxygen tension is one of the factors that can influence monoamine oxidase activity . In general when the enzyme is measured in a pure oxygen athmosphere, there is 50 % more activity than when it is measured in air (16) . Novick (20) found that the increase varies with tissue,species and substrate . The ratio of activity measured in an oxygen athmosphere to the activity in air varied as follows :

			ratio O_2/air
liver	rat	tyramine	3.3
"	guinea pig	"	2.0
"	rat	serotonin	1.4
"	guinea pig	"	1.4

The influence of oxygen tension on monoamine oxidase activity was also shown by Aebi et al. (12) . When the production of ammonia was measured at 20 % oxygen 0.3 micromoles were produced per 10 mg in 30 minutes, whereas at 100 % oxygen 4.7 micromoles of ammonia were produced . Interestingly the authors found a heavy inhibition of monoamine oxidase activity in the presence of pyruvate, which they explained by a competition for oxygen . The same paper describes a dependence of the enzyme activity on the morphological state of the mitochondria : swollen (as measured by light scattering) mitochondria, oxidizing pyruvate at a high rate, have a low monoamine oxidase activity . When the mitochondria are lysed (by any method : osmotic lysis, freezing and thawing, or the detergent Triton-X-100) the monoamine oxidase activity lies 5 to 10 times higher . This is also explained by the authors on the basis of a competition for oxygen .

Chapter IV : MEASUREMENT OF THE ACTIVITY

From equation 2 it is obvious that the activity of monoamine oxidase can be assayed in any of the following ways :

- the rate of oxygen consumption ;
- the rate of disappearance of the substrate amine ;
- the rate of formation of the product aldehyde ;
- the rate of ammonia formation ;
- the rate of appearance of hydrogen peroxide ;

All of these parameters have been used in various routine assay methods .

In classic experiments as those of Hare (7), the MANOMETRIC assay of the oxygen consumption in a Warburg apparatus was most often used . It is still very useful where the activity on different substrates is to be compared . As we mentioned above some precautions have to be taken to prevent side reactions (which also consume oxygen) from occurring, and also to limit the error which might be caused by the possibly different fates of the hydrogen peroxide formed during the reaction . In his studies on fresh mitochondria, Creasey (21) kept the stoichiometry in agreement with equation 4 (see chapter II) in the following way : addition of cyanide (10^{-3} M) and semicarbazide (10^{-2} M) blocks the

respiratory chain and traps the formed aldehyde, preventing its further oxidation ; no catalase was added, as it was present in excess in this preparation and not inhibited by cyanide at 10^{-3}M: no peroxidatic reaction was possible due to the lack of substrates in this preparation .

The liberation of AMMONIA was measured by the Conway microdiffusion technique in the method of Cotzias and Dole (22) . They had to dialyse the homogenates in order to decrease the high blank values due to the ammonia content and production of tissues. The assay was performed at a temperature of 20 degrees, so the reaction was linear over a period of two hours . Nagatsu and Yagi (23) used a colorimetric method for the estimation of ammonia . The color reaction with phenolreagent performed on a protein free supernatant was inhibited by some of the common substrates ; in fact the method was only satisfactory for an assay with serotonin as substrate . Since volatile amines also interfere with the microdiffusion method, Pugh and Quastel (16) used yellow mercuric oxide to separate the aliphatic volatile amines from ammonia . After microdiffusion and adsorption on yellow mercuric oxide, ammonia was titrated or estimated by Nesslerization .

The different SPECTROPHOTOMETRIC methods are simple and very useful . Their only drawback is that only certain substrates can be used and no comparison between the activity on different substrates is possible . Weissbach et al. (25) described

a spectrophotometric method based on the use of kynuramine as a substrate . By monitoring the reaction at360 millimicron the rate of disappearance of substrate could be determined . This was done at room temperature (30^0C), and the initial substrate concentration was 10^{-4} M . The fact that at this concentration the enzyme is probably not saturated with substrate makes the method less than ideal . But alternately the rate of formation of product can be followed at 310 to 335 millimicron, or even better by fluorometry . The product of the deamination cyclizes spontaneously to give the highly fluorescent 4-hydroxyquinoline . An assay method has been based on this property (see below) . Other spectrophotometric methods have been based on the use of benzylamine or its derivatives as substrates . The oldest of these methods was described by Tabor et al. in 1954 (26) . It was developed as an assay for beef plasma oxidase, but it is also applicable to monoamine oxidase . The enzyme is incubated at 30 degrees in the presence of 10 micromoles of benzylamine . The rate of the change in absorbance at 250 millimicron was followed in a 1 cm cuvette . The spectrophotometric unit was defined as the amount of enzyme which under those conditions caused a change in absorbance of 0.001 per minute . 250 millimicron is the peak of absorbance spectrum of benzaldehyde . The disadvantage of this method is that proteins too have a strong absorbance at this wavelength, which makes the method rather impractical, especially with low specific

activity preparations . Methods based on the use of different derivatives of benzylamine as substrates do not have this drawback, because they have their peak of absorbance in a different region of the spectrum . In 1964 Brusova, Gorkin et al. (27) used 3-nitro-4-hydroxy-benzaldehyde production as a measure of monoamine oxidase activity . The substrate was used at 3×10^{-2}M and the change in absorbance was followed at 315 millimicron . The sensitivity was lower than with benzylamine as substrate, but there was no interference of protein absorption at this wavelength . In 1965 Brusova, V'yugova and Gorkin (28) used p-nitrophenylethylamine at a concentration of 5×10^{-5} M . The absorbance was monitored at 450 millimicron in a cuvette at 37 degrees . The Km of this substrate was found to be 1.5×10^{-5} M . Zeller et al. (29) used m-iodo-benzylamine at 3.3×10^{-4} M . The absorbance was followed at 38 degrees at a wavelength of 253 millimicron . They found that this was a far better substrate than benzylamine or even than tyramine .

Among the most sensitive methods for assay of the enzyme are the FLUOROMETRIC ones . Some are based on the rate of disappearance of the substrate ; these are not entirely specific since monoamine oxidase is not always the only possible catabolic pathway for the substrate . Product formation as such is seldom measured since most tissues contain aldehyde dehydrogenase which may oxidize some of the product aldehyde .

Preferably aldehyde dehydrogenase is added in excess and the rate of formation of the carboxylic acid is followed . An excellent method is the one described by Krajl (30) in 1965 . It is a modification of the spectrophtometric method of Weissbach et al.(25). The aldehyde formed when monoamine oxidase is incubated with kynuramine cyclizes spontaneously to form the highly fluorescent 4-hydroxy-quinoline, so eliminating the problem of further oxidation by aldehyde dehydrogenase . The 4-hydroxy-quinoline can be measured in a deproteinized supernatant at an exciting wavelength of 315 millimicron and a fluorescence wavelength of 380 millimicron . In those conditions kynuramine does not fluoresce . Krajl used trichloroaceticacid to deproteinize his samples . This is a strong quenching agent . The efficiency of the measurement can be improved by replacing the 10 % TCA by other methods for precipitating proteins . Two papers describing such improvements were published . Century (31) replaced the TCA by two ml of 0.6 M perchloric acid . Drujan and Diaz-Borges (32) determined the fluorescence directly in the alcoholic supernatant, after an incubation in tris buffer medium and precipitation of the proteins with alcohol . They found that this was four times more efficient than the TCA method . Other fluorometric assays were described by Takahashi and Takahara (33), who used 5-hydroxy-kynuramine as substrate ; Udenfriend et al. (34), who measured the decrease in

serotonin (not entirely specific, see above) ; Udenfriend (35) measured the production of 5-hydroxy-indole acetic acid after the addition of excess aldehyde dehydrogenase, or the production of indole acetic acid from tryptamine ; Hidaka (36) separated the product of serotonin oxidation on a Dowex 50 anion exchanger column .

Monoamine oxidase assays can also be performed by the COLORIMETRIC determination of the aldehyde formed . Pugh and Quastel (37) first mentioned the qualitative assay of the aldehyde by reacting it with 2,4-dinitro-phenylhydrazine . Green and Haughton (38) based their method on this reaction . The enzyme was incubated in the presence of tyramine and semicarbazide, which traps the aldehyde as the semicarbazone . Following the incubation period 2,4-dinitro-phenylhydrazine is added . The hydrazone is extracted into benzene and returned to an alkaline aqueous medium . The absorbance is then measured at 450 millimicron.

RADIOISOTOPE assays of monoamine oxidase are, together with the fluorometric ones, the most sensitive . McCaman (40) published a micromethod using an ethylacetate extraction to separate the products from the ^{14}C-labelled substrates (5-hydroxy-tryptamine, 3-hydroxy-tyramine, tyramine) . The incubation was done in a 10 microliter volume at pH 7.2, the extraction with 50 microliter of ethyl acetate . The ethyl acetate layer was washed

once with 0.3 N HCl to improve the product/substrate extraction ratio . 35 microliter of the ethyl acetate phase was then counted in a liquid scintillation medium containing alcohol . The medium contained alcohol to dissolve the water which was extracted into the ethyl acetate . This is a disadvantage of the method since alcohol reduces the counting efficiency through quenching . Furthermore the product/substrate extraction ratio is not very good, so that at least one backwash is necessary . Wurtmann and Axelrod et al. (39) used toluene as an extraction solvent and tryptamine-^{14}C as substrate but they did not give any detailed evidence for the validity of their method as a monoamine oxidase assay . Otsuka and Kobayashi (41) describe an essentially similar method, with ample evidence supporting its validity as monoamine oxidase assay . They studied two solvents for the extraction : anisole and toluene . They determined the efficiency of the extraction at various pH's . They established the linearity with time and substrate concentration . They also checked the specificity of the assay for monoamine oxidase by using specific inhibitors and substrates for monoamine oxidase and diamine oxidase .

Several HISTOCHEMICAL staining techniques have been described, which allow demonstration of monoamine oxidase in tissue slices and also on strips of supporting medium after electrophoresis . Oster and Schlossmann (42) incubated tissue slices for 24 hours at 37 degrees with bisulfite to eliminate endo-

geneous aldehydes, then for a further 24 hours at the same temperature with tyramine and then stained them with Schiff reagent for aldehydes . This assay was criticized for its lack of accuracy and specificity . Francis (43) introduced the use of neotetrazolium (an electron acceptor dye) for the detection of monoamine oxidase in tissues . This reaction was studied further in vitro by Lagnado and Sourkes (13,14) who describe a requirement for a heat stable supernatant factor . Koelle and Valk (46) criticized the histochemical method of Francis because in their experiments the blank also showed a coloration, and they could not detect any inhibition by specific monoamine oxidase inhibitors (e.g. 0.05 M amphetamine) . Weissbach et al. (15) by their in vitro studies on the tetrazolium reduction as a measure of monoamine oxidase activity, observed a lag phase, which was also observed by Lagnado and Sourkes, and which does not occur when the enzyme is assayed with the manometric method . Pletscher, Gey and Zeller (2) explain this anomaly by the fact that the tetrazolium reduction may be due to the oxidation of the product of the monoamine oxidase reaction : the aldehyde . Cohen (47) also used tetrazolium salts and tryptamine in his histochemical assays of monoamine oxidase . But the caution which should be exercised when using this kind of non-specific staining procedures is emphasizedagain in two recent papers . Brooke warns against the use of phenazine methosulfate and

nitroblue tetrazolium in the same reaction medium : " A tissue section incubated with phenazine methosulfate, cyanide and nitroblue tetrazolium at pH 7.2 will develop a well marked stain in the absence of any substrate ." Falkenberg (49) says the unspecific staining by tetrazolium salts on gel slabs after enzyme electrophoresis, which has been observed by different people and has been called by some " nothing dehydrogenase ", is due to the presence of lactic dehydrogenase isoenzymes catalysing the dehydrogenation of endogeneous substrates . Blaschko and Hellmann (50) showed that the dark brown pigment, formed when monoamine oxidase is incubated in the presence of tryptamine, could be used in a histochemical assay . This method is rather insensitive according to Koelle and Valk (46), who publish their own method using 2-hydroxy-3-naphtoic acid hydrazide . This traps the product aldehyde as its hydrazone and it is then coupled with tetrazotized dianisidine . They show the validity of their method by using blanks (no substrate) and specific inhibitor (Marsilid) .

Chapter V : INHIBITION

Werle (16) classifies the inhibitors of monoamine oxidase in two big groups : competitive and non-competitive inhibitors . The competitive inhibitors include the hydrazine derivatives, the alkylated arylalkylamines, cholinethers and certain alkaloids . The non-competitive inhibitors include the monoamidines, monoisothiourea derivatives, diisothiourea derivatives and certain antihistaminics . One problem in the study of these inhibitors is that the in vivo action does not always match the in vitro findings .

I . COMPETITIVE INHIBITORS

1.Hydrazine derivatives

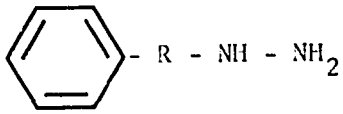
In 1952 Zeller et al. (51) discovered that the tuberculostatic Iproniazid was a very effective monoamine oxidase inhibitor . Since then many hydrazines have been tested and some were found to be superior to Iproniazid .

a) alkyl- and arylalkylhydrazines :

$$R - NH - NH_2$$

hydrazine itself (R = H) does not affect monoamine oxidase . The most effective inhibitors of this class have an alkyl radical (R) consisting of two to four carbon units . Arylhydrazines,e.g. phenylhydrazine, are only mild inhibitors .

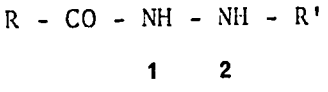
b) arylalkylhydrazines :



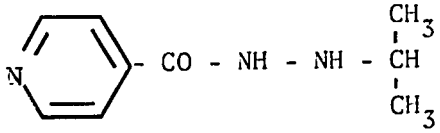
are very good inhibitors, the strongest of which, β -phenylisopropylhydrazine (PIH, Catron), has an in vitro action 50 times stronger than Iproniazid .

c) dialkylhydrazines

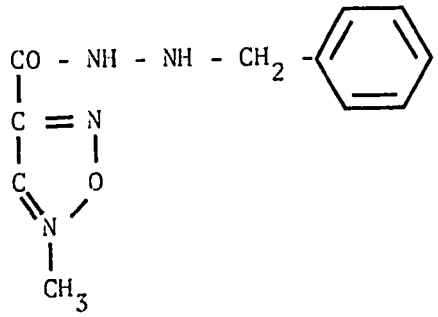
d) hydrazides :



Two well known drugs belong in this class : Iproniazid, and Isocarboxazid . Iproniazid (1-isonicotinyl-2-isopropylhydrazin, IIH, Marsilid) :



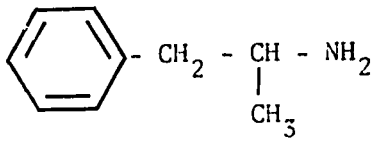
has a pI_{50} of 5.28 for the rat liver mitochondrial monoamine oxidase . Isocarboxazid (1-benzyl-2-(5'-methyl-3'-isoxazolyl-carbonylhydrazin Marplan) :



is much stronger in vivo and in vitro than Iproniazid .

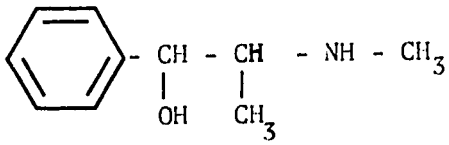
2.α -Alkylated arylalkylamines

- Amphetamine (β-phenylisopropylamine) :

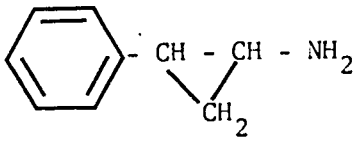


is active only in vitro, and has a pI_{50} of 3.05 (rat liver mitochondria) .

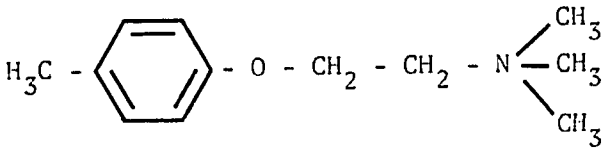
- Ephedrine :



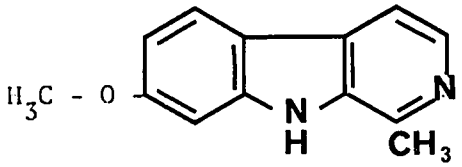
- Phenylcyclopropylamine (Parnate) :



3.p-Tolylcholinether :



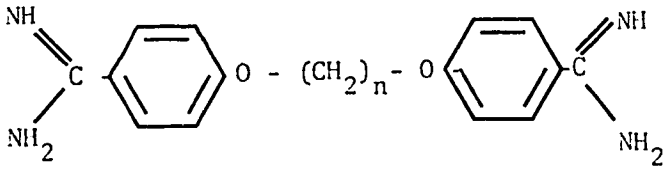
4.Harmaline :



5.Alkaloids as piperidine, nicotine, and chlortetracycline .

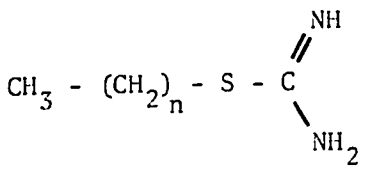
II . NON - COMPETITIVE INHIBITORS

1.Amidines :



a) propamidine (n = 3) and pentamidine (n = 5) are especially active (80 - 100 % inhibition at 2×10^{-4} to 10^{-3} M)

b) monoisothiourea derivatives :



Maximum inhibition occurs when n = 9 (90 % inhibition at 10^{-3} M) .

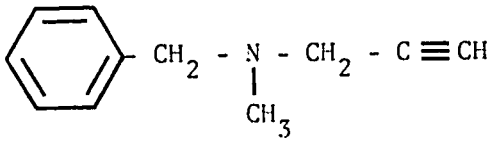
c) diisothiourea derivatives give maximum inhibition when n = 9 - 12 (90 - 100 % at 10^{-3} M) .

2.Antihistaminica

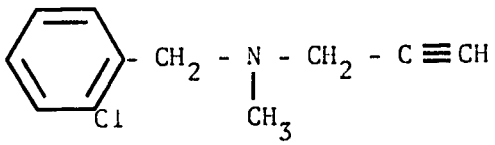
e.g. Phenergan (Promethazine) gives an inhibition of 48 % at 10^{-3}M).

3.The Pargyline series

In 1960 Tabor et al. (52) discovered a new type of monoamine oxidase inhibitor : Pargyline (Eutonyl) or N-benzyl-N-methyl-2-propylamine :



It has an I_{50} of 9×10^{-7} . Swett et al. (53) showed that different substituents on the benzene ring (Cl-, Br-) improved the inhibitory activity :



I_{50} 5×10^{-8}

Chapter VI : PURIFICATION

Tissue monoamine oxidase is always bound to membrane structures : mitochondria and microsomes . Plasma monoamine oxidase (benzylamine oxidase) is soluble . Not surprisingly the first purification was done on this enzyme : Tabor et al. (26) purified the amine oxidase from beef plasma in 1954 . In 1962 the same enzyme was crystallized by Yamada and Yasunobu (54) . In 1964 Buffoni and Blaschko crystallized a similar enzyme from pig plasma (56) . In 1969 McEwen et al. purified an enzyme from rabbit serum about 450-fold (57) . Plasma monoamine oxidase is not the same enzyme as the one found in mitochondria . For the mitochondrial enzyme, the problem was the insolubility . According to Weissbach et al. (58) the liver and kidney enzyme from guinea pig is partially soluble . However they were homogenizing the tissues in distilled water so that their method may in fact be equated with an osmotic lysis of the mitochondria . Verevkina, Gorkin et al. (59) studied the solubilization of mitochondrial monoamine oxidase by ultrasound . They found that this treatment did break down the mitochondria to small particles, which could however still be sedimented by spinning at 100,000 x g for 1 hour .

Coq and Baron (60) compared the action of digitonin, saponin, Tween 20, sodium laurylsulphate, on the solubility of mitochondrial monoamine oxidase . They concluded that 0.2% sodium laurylsulphate at pH 7 and 2 degrees during one hour gave the best results . After one hour of centrifugation at 135,000 x g however, only 30 % of the activity was found in the supernatant . In some cases where reasonably good solubilization was obtained, the preparation was very unstable . Nagatsu (60 b) used a detergent (Cutscum, 1.2 %) and sonication (20 Kc, 4 minutes, twice) to get 70 % of the original activity in the supernatant after 3 hours of centrifugation at 100,000 x g . Their preparation lost its activity at a very high rate . The difficulty of the purification of mitochondrial monoamine oxidase appears also from the different preliminary communications published, which were not followed by the complete papers .

That a certain degree of purification can be achieved without complete solubilization of the enzyme is shown by the successful purifications obtained in different laboratories . Though several of the reports hereon mention that "solubilized" preparations were used, the data provided usually indicate that the preparations were omly partially solubilized . The first report on a significant purification was by Guha and Krishna Murti (61) . They achieved a 350-fold purification of the rat liver mitochondrial enzyme (over the homogenate) by two successive steps

of chromatography on DEAE cellulose . The solubilization was obtained by sonication and 90 % of the activity was found in the supernatant after 4,000,000 g-minutes centrifugation . No data were given on the yield of the purification . In 1966 Nara et al. (62) published the method for a purification of 58 times over the mitochondrial activity by treatment of the mitochondria with Triton-X-100, ammonium sulphate fractionation, alumina gel treatment and chromatography on DEAE cellulose . In most of their experiments, detergent had to be added all through the procedure to keep the enzyme in solution . This method was later modified slightly : calcium phosphate gel was used instead of alumina gel and chromatography on hydroxylapatite followed by starch gel electrophoresis were added as the last two steps (Yasunobu et al., 63) . In this procedure the enzyme became soluble after the sixth step (hydroxylapatite chromatography) . The overall yield was 5 % and a purification of 61 x was obtained .

Also in 1966 Klyashtorin, Gridneva and Gorkin (64) used the non-ionic detergent OP-10 for their purification of rat liver mitochondrial monoamine oxidase . The procedure involved, after "solubilization" of the mitochondria, chromatography on calcium phosphate gel . Overall purification was 26 x over the homogenate with a yield of 4 % . A short note on purification of rat liver mitochondrial monoamine oxidase was published by Youdim

and Sourkes in 1966 (65) . A purification of 34 x (over the mitochondrial extract) was achieved by Erwin and Hellerman (66) by a procedure consisting of ammonium sulphate fractionation of the digitonin treated mitochondria, followed by three successive steps of calcium phosphate gel chromatography . The yield was 10 % .

Tipton (67) purified monoamine oxidase from pig brain mitochondria 1000 x over the mitochondrial activity . The enzyme was solubilized by up to six successive steps of sonication . Purification involved a precipitation of inactive protein at low pH, treatment with DEAE cellulose and fractionation with ethanol . Final yield was 22 % . Gabay and Valcourt (68) purified the enzyme from rabbit liver mitochondria 240 x over the original homogenate . They used sonication and detergent to solubilize and chromatography on DEAE cellulose, Biogel P-300, DEAE Sephadex A-50, and hydroxyl-apatite columns . The yield was approximately 0.3 per thousand . Monoamine from a special source (thyroid gland) was purified by Fisher et al. (69) 17 x over the mitochondrial activity with a yield of 30 % . This latter monoamine oxidase was found to have a very high specificity for tyramine as substrate .

Chapter VII : THE PROSTHETIC GROUP

Until at least some degree of purification of mitochondrial monoamine oxidase had been attained, the nature of the prosthetic group(s) could only be deduced from comparison with similar enzymes whose prosthetic group is known, and from indirect evidence such as inhibition by metal chelators etc... In 1962 Yamada and Yasunobu (70) discovered that copper was one of the prosthetic groups of plasma monoamine oxidase, and one year later the same authors (71) described the other prosthetic group : pyridoxal phosphate . Blaschko et al. (72) showed that animals on a copper deficient diet have very low plasma amine oxidase levels . Mills et al. (73) showed that there is a good correlation between the plama copper content and plasma amine oxidase levels . However the regression equation showed that some non copper containing amine oxidase must be expected to be present in plasma . Later Otsuka and Kobayashi (41) using their sensitive radioassay, showed the presence in human serum of some monoamine oxidase which could not be inhibited by semicarbazide or aminoguanidine, but was inhibited by different specific monoamine oxidase inhibitors .

Thus people came to expect that copper might play a similar role in the mitochondrial monoamine oxidase . On the other hand mitochondrial monoamine oxidase is quite different from plasma amine oxidase : it is not inhibited by cyanide nor semicarbazide . It is perhaps more closely related to D-amino acid oxidase, which is also cyanide insensitive and carries out a similar reaction : oxidative deamination of D-amino acids . Hawkins (74) found that liver homogenates of riboflavin deficient rats had only 50 % of the monoamine oxidase activity of that of the control animals . D-amino acid oxidase contains FAD . The first purifications by Nara et al. (62) yielded preparations that contained 0.07 % copper, and the copper content was proportional to the specific activity of the enzyme at different steps of the purification . The authors conceded however that one specific test was needed to definitely establish the role of copper in the enzyme activity : the reactivation of the apoenzyme by the prosthetic group. This evidence was missing . One report by a different group of workers (75) has described the reactivation of mitochondrial monoamine oxidase by copper after prolonged dialysis (4 days) . But studies on the further purified enzyme by Yasunobu et al. (63) showed that the relationship between copper content and specific activity was reversed during the later steps of the purification . Indirect evidence showed the presence of a flavin group (difference

spectrum of native and reduced enzyme) . But the flavin was apparently covalently bound, since it could not be removed (76) . A microbiological assay also detected the presence of a flavin group . In a later paper (77) the same group of authors mention that they did not succeed in separating the prosthetic group from the apoenzyme . Erwin and Hellerman (66) also found that the spectrum of their purified bovine kidney mitochondrial enzyme was very similar to that of FAD, but they could not remove it from the apoenzyme . Finally in 1968 Tipton (67) was able to extract a fluorescent material from his purified pig brain monoamine oxidase and to identify it with FAD . Furthermore he was able to reactivate the apoenzyme by the addition of exogeneous FAD. It can thus be concluded that, whereas plasma amine oxidase is a pyridoxal-copper enzyme, mitochondrial monoamine oxidase is an FAD containing enzyme . It is not excluded that a small amount of this type of monoamine oxidase is also present in plasma, and that copper might play a role in some of the mitochondrial enzyme . Recently, a possible role of iron was suggested by experiments of Sourkes et al. (45).

Chapter VIII : DISTRIBUTION

1.Species and organ distribution

Practically all organs of humans and mammals have been shown to contain monoamine oxidase activity . Similar enzymes have been found in other vertebrates (birds, reptiles and amphibians) as well as in invertebrates, plants and bacteria (16) . In different vertebrates the liver and salivary glands always have high activity . The kidney from guinea pig has high activity, rat kidney has low activity . High activity preparations can also be obtained from pancreas and intestine . Some activity is found in arteries, lung, muscle, uterus, testis, brain, adrenals, heart, thyroid gland (1) . Comparison of the activity reported for different species and organs is difficult due to the different assay systems used by different authors .

2.Subcellular distribution

That the major part of the monoamine oxidase in the cells of rat liver was associated with mitochondria was shown by Cotzias and Dole in 1951 (78) . Hawkins (79) found that 56-77 % of rat liver monoamine oxidase was localized in the mitochondria . The rest of the activity resided in the microsomes . The same results were also obtained by de Duve et al. (80) .

In brain all the monoamine oxidase activity seems to be confined to the mitochondria according to Weiner (81) . Oswald and Strittmatter (82) confirmed the intracellular distribution of monoamine oxidase in rat liver as 75 % in the mitochondria and 13 % in the microsomes . Recently de Champlain, Mueller and Axelrod (55) described a different distribution of monoamine oxidase in heart and salivary glands : in these organs most of the activity is associated with the microsomal fraction in contrast with rat liver where it is associated with the mitochondrial fraction .

3.Submitochondrial distribution

In the past two years attention has focused on the submitochondrial distribution of monoamine oxidase, specially since it was claimed to be useful as a marker enzyme of the outer mitochondrial membrane by Schnaitman et al. (83) . This aroused some controversy . It had been attempted before to separate the inner and outer membrane of mitochondria by different methods and the criteria used for characterization of the outer membrane fraction were the absence of the respiratory chain enzymes and the absence of the morphologically distinct structures of the inner mitochondrial membrane as observed with the electron microscope . The use of an enzymatic marker for monitoring the enrichment of a submitochondrial fraction in outer membrane would facilitate the separation .

Schnaitman et al. found monoamine oxidase to be a good marker for the outer membrane . Although the fractions obtained by two different methods were not claimed to be pure inner and outer membrane, there was found a considerable enrichment of the specific activity of monoamine oxidase in one fraction and of cytochrome oxidase in the other . At the same time 82 % of the total cytochrome oxidase activity was recovered in the fraction identified as containing mainly inner membrane, and 62 % of the total monoamine oxidase activity was found in the fraction labelled outer membrane fraction . These findings were contradicted by Green et al. (84), who presented their own results which would demonstrate that monoamine oxidase is an enzyme of the inner membrane . Green and coworkers also contended that the rotenone-insensitive DPNH cytochrome c reductase (RIDCR) activity, which had been used by others as an external membrane marker (85,86) was in fact due to microsomal contamination of the mitochondrial fraction . Green et al. explained the contradiction by the presumed unreliability of the monoamine oxidase assay used by Schnaitman et al. and the insufficient purity of the preparations used . However, both these allegations were disproved in papers by Beattie (87) and Schnaitman (88) . Furthermore a paper by Tipton (89) who used different methods, confirms the conclusions of Sottocasa, Schnaitman and Beattie . Recently Rendon and Waksman (98 b) showed a difference in distribution of aspartate

aminotransferase between the membranes of the mitochondria, depending on the energetical state of the mitochondria . This migration of activity between membranes did not occur with monoamine oxidase though . Monoamine oxidase was localized by these authors mainly in the inner membrane . The greatest specific activity however they found in the outer membrane .

Though the different research groups all rely on different methods for the separation of the membrane fractions, as well as for the assay of monoamine oxidase activity, all rely ultimately on the appearance of the different fractions in electron microscopy for the assignment of a fraction to one membrane or the other . The difficulty of recognizing the characteristic features of inner and specially of outer membranes, which are hard to preserve intact even in carefully isolated whole mitochondria, may help to explain the present confusion . Possibly the detection of the enzyme in situ by cytochemical techniques at the electron microscopic level might provide a more definite answer .

Chapter IX : ON THE MULTIPLICITY OF MONOAMINE OXIDASE

As mentioned under "History" (chapter I), it was Blaschko et al. (10) who in 1937 came to the conclusion that monoamine oxidase was an enzyme with broad specificity and that the adrenaline oxidase which they described, the tyramine oxidase of Hare (7) and the amine oxidase of Pugh and Quastel (9) were probably to be considered as one and the same enzyme . But they did remark: " There is no known method by which the identity of any two enzymes can be conclusively established ; but it can be shown that they are so similar in their properties that their identity becomes increasingly probable . " So they concluded that the three enzymes were one because they were similar in the reactions they catalyse, their distribution in the animal kingdom, and their behaviour towards inhibitors, and because their substrates compete with each other . This conclusion has been widely accepted ever since, although over the years indirect evidence suggesting the possible multiplicity of monoamine oxidase has been accumulating .

In two papers in 1943 (18,19), Alles and Heegaard studied the distribution of monoamine oxidase activities in different species and the inhibitor specificity of the enzyme . They

found marked differences in the relative activity on different substrates between species and also between the Ki for one inhibitor when different substrates were being used . Whereas in rabbit liver extract the maximum rate for hydroxytyramine oxidation is 65 % of the maximum rate for phenylethylamine, in guinea pig liver it is 200 % . Phenylisopropylamine is 30 to 40 times as effective in inhibiting amylamine oxidation than in inhibiting phenethylamine oxidation . These and similar findings led Alles and Heegaard to conclude that, although the different amines were attacked by one type of enzyme (as Blaschko had proposed), the enzyme from different species or organs was probably different . Hagen and Weiner (91) discussed the differences in substrate and inhibitor specificity between various species and organs . Their conclusion was similar : " it is probable that the primary active center to which the amine attaches is identical or very similar in the various tissues and species, but it is likely that adjacent groups on the molecule do differ, and that these adjacent structures serve to reinforce or diminish the enzyme-substrate interaction, depending on the nature of the substrate " .

Neither the previous papers nor the next one do consider the possibility of two different enzymes being present in the same organ at the same time . Hope and Smith (17) found more evidence of the difference between the substrate specificity of monoamine oxidase from various organs in a study of the distribution of the enzyme in mouse tissues .

They concluded that the substrate specificity allows to distinguish between the monoamine oxidase from each tissue . The occurrence of two enzymes in the same organ was rejected on the basis of multiple substrate studies, already by Blaschko in 1937 (10) . These multiple substrate studies could provide data only on the sum of the activities on the different substrates, since the techniques available at that time did not permit the separate measurement of the oxidation of each of the substrates in a mixture . In 1952 Kobayashi and Schayer (92) were able to do just that, by using a mixture of isotope-labelled substrates . These authors did come to the same conclusion though : the competition between the substrates suggested the presence of only one enzyme, but they did admit that their data cannot provide conclusive evidence . In 1961 Hardegg and Heilbronn (93) reported evidence for the multiplicity of monoamine oxidase in rat liver mitochondria . Iproniazid inactivates the enzymatic activity on serotonin at a different rate than the oxidation of tyramine . When tyramine and Iproniazid are added to the enzyme at the same time, there is a fast inactivation of most of the activity, but some activity then remains and is disappearing much more slowly . This is compatible say the authors, with the presence of two different enzymes acting on tyramine, one of which is markedly better protected by the presence of tyramine against the action of Iproniazid . The authors consider the following possibilities : in rat liver

there are at least two oxidases ; - two monoamine oxidases, one of which oxidizes tyramine, the other serotonin or else both oxidizing tyramine, and only one serotonin ; - or there is besides monoamine oxidase still an other oxidase which also attacks tyramine .

Oswald and Strittmatter (82) studied the monoamine oxidase activity on four different substrates in various tissues of the rat . These showed quite different relative rates of activity with different substrates . Km values were also different . From this they concluded that various tissues might a) contain a different single monoamine oxidase ; b) contain a different group of monoamine oxidases ; or c) contain different proportions of several monoamine oxidases common to several tissues . In multiple substrate experiments, the authors found apparent competition for the enzyme by four substrates, a finding which they call suggestive for only a single enzyme being present in each tissue . But, as Severina and Gorkin (94) state : " Competitive interactions between amines observed by the method of mixed substrates cannot be considered as simple evidence for the presence in a preparation of a single enzyme, since it is known that there are cases of change in enzymatic activity under the influence of different compounds which do not react directly with active centers (so-called allosteric or conformation factors) . "*

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* Different enzymes with overlapping specificity could also explain competition between substrates (see discussion) .

The same athors then present their own indirect evidence for the multiplicity of the enzyme in rat liver . They performed two types of experiments : heat inactivation of the enzyme and measuring the activity at various pH . In the heat inactivation experiments the enzyme was preincubated at various temperatures for a set time ; this caused a different degree of inhibition of the activities on different substrates . For example after 7 minutes at 40 degrees the activity on tyramine was inhibited 45 % whereas no inhibition of the activity on benzylamine was observed. After 45 minutes at 48 degrees the activity on tyramine was completely inhibited, while the activity on benzylamine was still 54%. At pH 7.45 the ratio of the activity on tyramine to the activity on benzylamine was 2.6, at pH 9.3 this ratio was 0.5 . What may have been the first separation of two distinct monoamine oxidase activities from the same preparation (rat liver mitochondria) was reported by Gorkin in 1963 (95) . After solubilization with hypotonic phosphate and the detergent OP-10, a rat liver mitochondria preparation was chromatographed on a calcium phosphate gel column. The effluent was monitored for monoamine oxidase activity on two substrates : m-nitro-p-hydroxy-benzylamine (PHB) and p-nitrophenylethylamine (PN) . Two bands of activity were separated : one was active only on PN, the other one deaminated both PN and PHB . Unfortunately only 6 % of the activity on PN was recovered (versus 90 % for PHB) . After their report on selective heat inactivation of the monoamine oxidase activity on two different

substrates, Severina and Gorkin (96) looked for and found other examples of selective inhibition . They found 8-hydroxy-quinoline to inhibit the oxidation of tyramine, while at the same concentration it had no effect on the oxidation of benzylamine . Gorkin (97) in 1967 confirmed his findings on the partial separation of monoamine oxidase activity on PN and PHB, but he still did not obtain more than 25 % recovery of the activity on PN .

Another indication of the possible multiplicity of mitochondrial monoamine oxidase is found in the fact that in partially purified preparations a different inhibitor specificity was observed than in crude preparations . Burger and Nara (98) found that for their purified preparation of rat brain monoamine oxidase, harmine and harmaline were far less potent inhibitors than for the crude homogenate (I_{50} was 1.2×10^{-7} M for the crude homogenate ; in the purified preparation, even at 10^{-3}M only 46 % inhibition was observed) .

Fuller and Walters (99) did some interesting observations on the inhibition of kynuramine oxidation by other substrate amines . They found that the oxidation of kynuramine was inhibited by dopamine, noradrenaline, serotonin, tyramine and others . They reasoned, correctly, that this is compatible with a single monoamine oxidase acting on all these substrates or different monoamine oxidases with broad, overlapping substrate specificity . More important, they found that there was no

relationship between the degree of inhibition by any one of these substrates and its efficiency as a substrate .

In this regard it is important to mention a new theory, proposed by Zeller in 1963 before the New York Academy of Science, in order to explain his findings with substituted benzylamines (100) . His studies on the activity of monoamine oxidase on such substituted monoamines (e.g. o-iodo, m-iodo,p-iodo) led to the following concept : substrates of monoamine oxidase would bind to the active site in different ways forming "eutopic" and "dystopic" complexes . A eutopic complex is one that leads to degradation of the substrate, a dystopic complex would not lead to degradation or the formation of a product . Most substrates would form both eutopic and dystopic complexes (e.g. benzylamine could form 1 eutopic and 5 dystopic complexes) . Because of the steric effects of their substituents, some substrates would be able only to form eutopic complexes (m-iodo-benzylamine), some only dystopic complexes (o-iodo-benzylamine) . The value of this theory in providing an explanation for other experimental data is worth considering, since it could also explain some differences between the efficiency as substrate and as inhibitor respectively of some amines .

Selective inhibition has also been observed by Van Woert and Cotzias (101), who studied the effect of anions on monoamine oxidase . Not only did they find that the inhibition by anions was different for different substrates, but the effect of changing the temperature on the inhibition was also different .

Actually the effect was reverse for tyramine, where rising temperature increased the inhibition by salts, than for serotonin, where rising temperatures decreased the inhibition .

Not only inhibitors can selectively change the activity of monoamine oxidase on various substrates, but a change in oxygen tension affects the activity on tyramine in a different way than the activity on serotonin, as Novick (20) has shown .

Then in 1967 came the first reports on the electrophoretic separation of "isoenzymes" of monoamine oxidase from rat liver mitochondria, in a communication by Kim and D'Iorio (102), and later a paper by the same authors (103) . Similar results were obtained by Youdim and Sandler (104,105) . However the methods used for solubilization of the enzyme (sonication and detergents), as well as the relatively non-specific staining method (with the electron acceptor dye nitroblue tetrazolium) left some doubts as to the significance of the stained bands which were observed .

Further evidence of selective inhibition of the deamination of serotonin was reported by Gorkin (106, 107) .

In 1968 Ragland (108) reported a partial separation of the activity of beef, rabbit and rat liver mitochondrial monoamine oxidase on various substrates . The separation was per-

formed with mitochondria solubilized by detergent, by chromatography on a Sephadex-G-200 column equilibrated with the same detergent . Only relative activities were given, and no data on the recovery of the enzyme .

In contrast with the above mentioned preparations, pig brain mitochondrial monoamine oxidase was found to consist of only one enzyme by Tipton and Spires (109) . This conclusion was based on the fact that the time course of the inhibition for various substrates was the same when 2-bromo-2-phenylacetaldehyde was used as inhibitor . Also, mixed substrate experiments indicated the presence of only one enzyme and partial heat denaturation caused the same loss of activity on all substrates . Harmine was a competitive inhibitor and the Ki was the same whatever the substrate used .

Fuller (110) described a new group of inhibitors, N-substituted N-cyclopropylamines, which are especially capable of selectively inhibiting the activity on various substrates . With some of these inhibitors, the concentration required to block tyramine oxidation by 50 % is several hundred times the one for inhibition of serotonin or tryptamine . A different type of inhibitor was studied in the following two papers . Observing the effect of the new inhibitor M & B 9302 Johnston (111) found, in a plot of % inhibition versus log (I), a double sigmoidal curve

instead of the simple sigmoidal curve one would expect in the case of a single enzyme . Similarly, in mouse tissues, Squires (112) found a 2-phased inactivation curve with increasing doses of harmine or pargyline . Even the heat denaturation curve was 2-phased . In mouse liver mitochondria one enzyme had a half-life of 2 minutes at 50 degrees, the other had a half-life of 10 minutes at the same temperature .

Kinetic studies on monoamine oxidase in rat liver mitochondria led Coq and Baron (113) to accept the presence of at least two enzymes in this preparation .

Hall et al. (114) studied the substrate specificity of the new inhibitor M & B 9302 in a variety of species and organs . They concluded that two enzymes are present in the brain of rat, man, rabbit, ox, dog and cat and also in the liver of rat and man . Only one monoamine oxidase would be present in pig brain and the liver of rabbit, ox, dog and cat .

Yasunobu et al. obtained at one point in the purification of beef liver mitochondrial monoamine oxidase, three fractions of enzyme activity . They called them fraction A and component 1 and 2 . Using immunochemical techniques, they were able to demonstrate the antigenic identity of the three fractions (115) . Further identification of components 1 and 2 showed that they had a molecular weight of 405,000 and 1,280,000 respectively.

Component 1 contained 4 and component 2 contained 12 moles of FAD per mole of enzyme . They respectively contained 24 and 106 moles of phospholipid (116) . From this they deduced that component 2 may well be a trimer of component 1 and represent a larger fragment from the outer mitochondrial membrane, specially since no difference in substrate specificity was found between the different fractions . It should be noted however that this separation was performed on a partially purified enzyme, representing only about 15 % of the original activity .

Tipton (116 b) recently studied the effect of the inhibitor 2-bromo-2-phenylacetaldehyde on the deamination of benzylamine and serotonin by rat liver mitochondria . The time course of the inhibition was a 2-phased curve in the case of benzylamine, but no such effect was seen for serotonin . He concluded that, while there were probably two enzymes for the deamination of benzylamine, only one was active on serotonin .

Chapter X : BIOLOGICAL SIGNIFICANCE

The biological role of monoamine oxidase has been subject to a lot of speculation, and mainly four possible functions have been proposed for it : detoxification of exogeneous amines, metabolism of endogeneous amines, regulation of the biosynthesis of catecholamines through its effect on dopamine levels, and the production of unknown metabolites with an effect on carbohydrate metabolism .

The detoxification function of monoamine oxidase, or in other words the metabolism of exogeneous amines, was suggested by Blaschko (1) as a distinct possibility, since the high concentration of the enzyme in liver and intestine would be an effective barrier against any amines taken up from the alimentary tract . Since the conditions in the intestinal lumen do not normally favor the bacterial formation of biogenic amines, this role would be necessary only in exceptional conditions . But recently clinical and experimental findings on the effects caused by monoamines (e.g. from cheese) introduced in conditions of almost complete blockade of oxidative deamination by a monoamine oxidase inhibitor, supported this protective role of the enzyme (117) .

The possible importance of monoamine oxidase for the metabolism of endogeneous amines was recognized by Blaschko (1) . But not until more recently was it becoming clear what the respective roles of monoamine oxidase and catechol-O-methyltransferase were in the metabolism of catecholamines . Kopin (118) reviewed the subject and summarized the current concept as follows . Norepinephrine is present mainly in intracellular vesicles in sympathetic nervous tissues . There is an equilibrium between norepinephrine in the vesicles and the unbound norepinephrine in the cell fluids, which is available to destruction by monoamine oxidase . Drugs which deplete norepinephrine in tissues, without much sympathomimetic effect (like reserpine) cause a decrease in the binding of the norepinephrine and thus expose it to monoamine oxidase . Nerve stimulation or sympathomimetic amines cause a release of active norepinephrine from the cell into the circulation . There catechol-O-methyltransferase is the main enzyme responsible for the destruction of norepinephrine . For indolealkylamines such as serotonin, deamination by monoamine oxidase must still be considered the first step in the metabolic pathway (90 b) . The resulting aldehyde is further oxidized to the carboxylic acid or it can take several alternate pathways .

Possibly monoamine oxidase is not only involved in catecholamine metabolism, but also in the regulation of their synthesis, namely in the destruction of the precursor dopamine . Since dopamine is an important amine in nervous tissue which may have a function of its own, Hagen and Weiner (91) observed that monoamine oxidase action on dopamine may play two possible roles : the destruction of a biological agent with an as yet unknown function, or the destruction of a precursor of a biological agent to prevent its overproduction . Speculation about other possible roles of monoamine oxidase was prompted by the unexplained effects of monoamine oxidase inhibitors on carbohydrate metabolism in vivo, and also by the suppression of the radioprotective action of indolylalkylamines by monoamine oxidase inhibitors (117) .

SECTION II : EXPERIMENTAL

PART I : Kinetic experiments

A. Materials and Methods

All chemicals used in this work were purchased from Fisher, except where otherwise stated .

Preparation of rat liver mitochondria .

Male Sprague-Dawley rats weighing between 200 and 300 g were killed by decapitation and a portion of the liver was weighed and homogenized immediately in cold 0.25 M sucrose (pH 7.4) . The mitochondria were isolated according to the method of Schneider and Hogeboom (119), washed twice in 0.25 M sucrose and resuspended in the same solution .

Radioassay of monoamine oxidase .

The assay method which we used was described by Otsuka and Kobayashi (41) . It was adapted for very small incu-

bation volumes as follows . Polyethylene microcentrifuge tubes (A. Thomas Scientific Co.) with a capacity of 0.4 ml were used for the incubation . The transfer of solutions was done with micropipettes of the Lang-Levy type . Some of these pipettes were of glass (Fisher brand) and presented some difficulty in delivering solutions from the glass tip to the non-wettable wall of the polyethylene microfuge tube . This problem was solved by applying a thin film of silicone stopcock grease (A. Thomas) to the tip of the pipette . This prevented the solution from flowing around the tip, and it could be delivered as a little drop against the wall of the microfuge tube . Polyethylene micropipettes were also used (B & B brand, Bio-Rad) and did not present this problem . The tubes were kept in ice at all times except during the incubation . The incubation was carried out at 37 degrees for 30 minutes in a shaking water bath . The reaction medium consisted of the following :

0.2 M phosphate buffer (pH 7.4).....20 µl

^{14}C-substrate..... 5 µl

enzyme suspension..... 5 µl

final volume.....30 µl

The different solutions were brought into contact by a short (5 seconds) centrifugation in the Coleman microfuge . After the incubation 10 µl of 5 N hydrochloric acid were added . This stops the reaction and renders the medium acid, which is necessary for

the selective extraction of the product . For this purpose 100 µl of toluene were then added and the contents of the capped tube thoroughly mixed using a vortex mixer . The phases were then separated by a 2 minute centrifugation . A 100 µl syringe (The Hamilton Co)with Chaney adaptor was used to pipet off 60 µl of the toluene phase . This was transferred to a counting vial containing 10 ml of an Omnifluor solution (4 g/l ; New England Nuclear).

The counting was done in a Mark 1 liquid scintillation counter (Nuclear Chicago), operated at balance point for ^{14}C . Two channels were monitored in order to detect any quenching effects by the channels ratio method . A quench correction curve (figure 1) was made, using a set of quenched ^{14}C standards (New England Nuclear) . Since practically no quenching was detected in any sample, no correction had to be applied to the results .

^{14}C-labelled substrates were obtained from New England Nuclear . Benzylamine-^{14}C (specific activity 2.03 mCi per mmole) was dissolved into 0.2 M sodium phosphate buffer, pH 7.4, to a concentration of 9 nmoles/5 µl or approximately 40,000 cpm/5 µl . This substrate was used in concentrations ranging from 50 to 300 µM by making the appropriate dilutions from the stock solution, which was stored in the freezer . 5-hydroxy-tryptamine-^{14}C-bioxalate (serotonin ; specific activity 2.8 mCi/mmole) was

dissolved to a concentration of 3 µmole/ml . Thus 5 µl contained 15 nmoles and approximatively 100,000 cpm .

Using these substrates the reaction was found to be linear with different amounts of enzyme (figures 2, 3) . The specificity of the reaction was tested using three specific inhibitors of monoamine oxidase : Marsilid and Marplan (Hoffman-Laroche) and Catron (Lakeside Laboratories) . These inhibitors were all three used at 100 µM final concentration . Table 1 summarizes the results obtained . As the efficiency of the extraction was not known, all velocities are reported as counts per minute in the 60 µl of extraction solvent, and they thus have only a comparative value .

- EXPERIMENT 1

In this experiment it was attempted to assess separately the efficiency of the same amine as a substrate and as an inhibitor respectively . This was possible through the use of the same compound in a ^{14}C-labelled form and then in its non-labelled form ("cold") .

a. Experimental setup and assay .

The radioassay as described above was used to determine the apparent Km for benzylamine, using

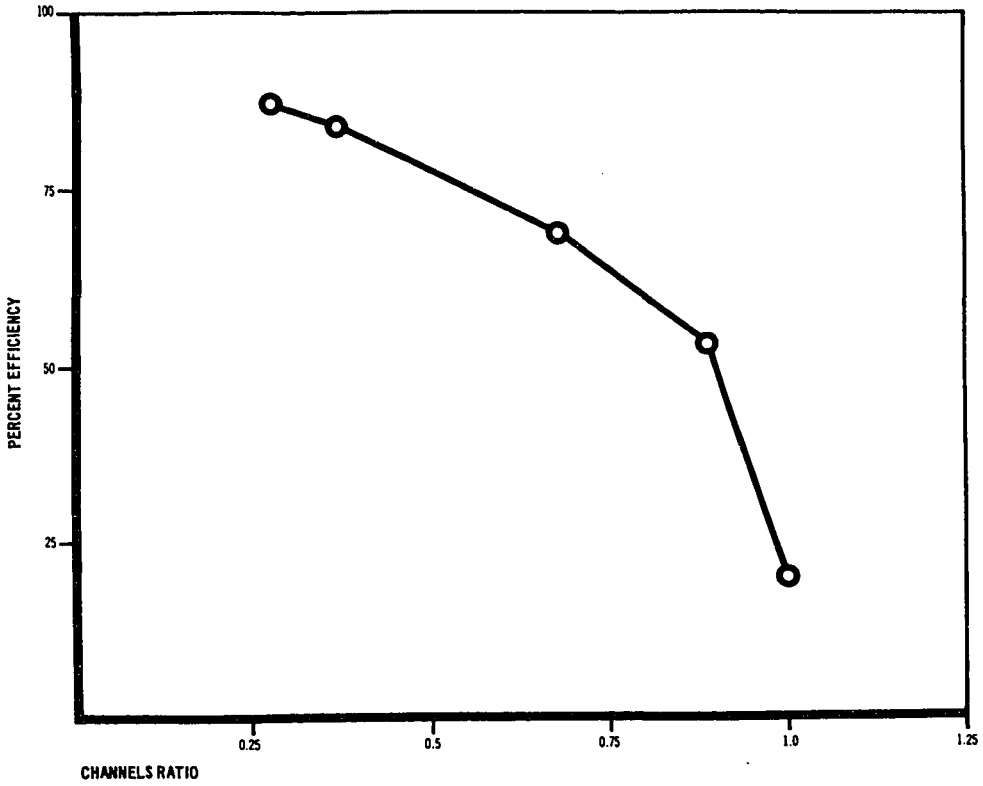

Figure 1 . Correction curve for quenching .

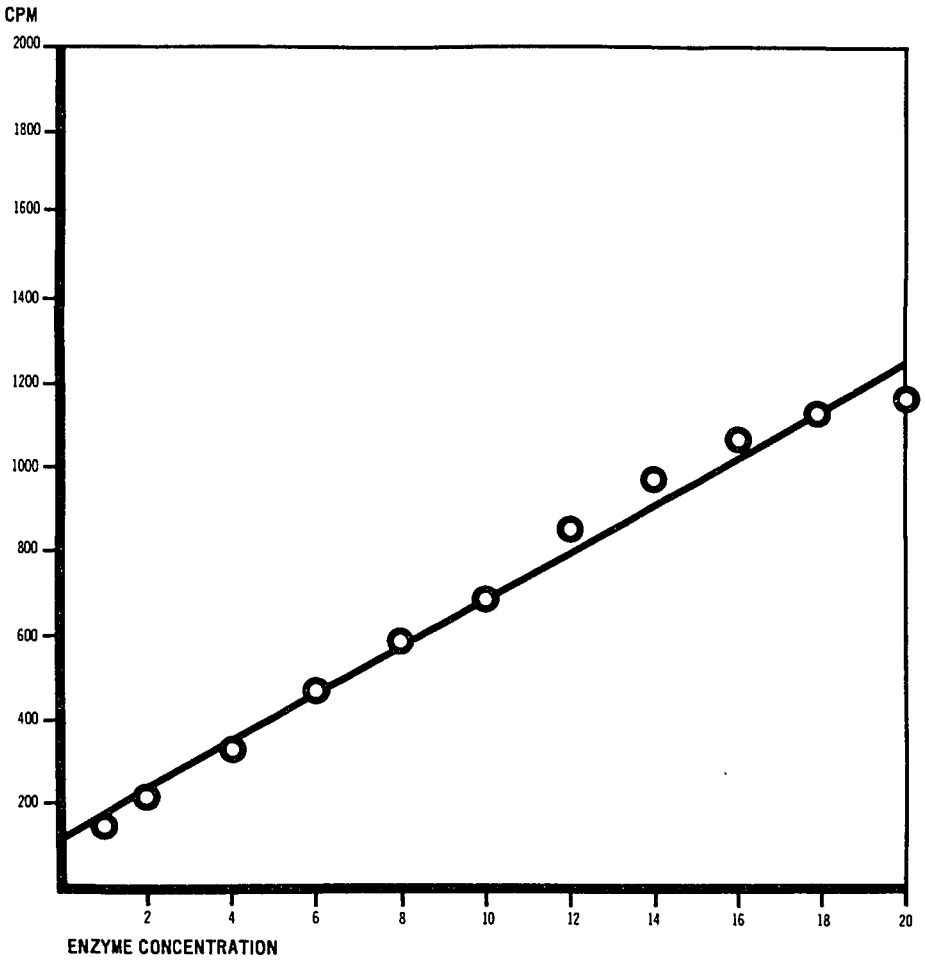

Figure 2 . Monoamine oxidase activity on benzylamine-^{14}C .
Activity expressed as cpm in 60 µl of the toluene phase (no blank substracted) .
Enzyme concentration in arbitrary units .

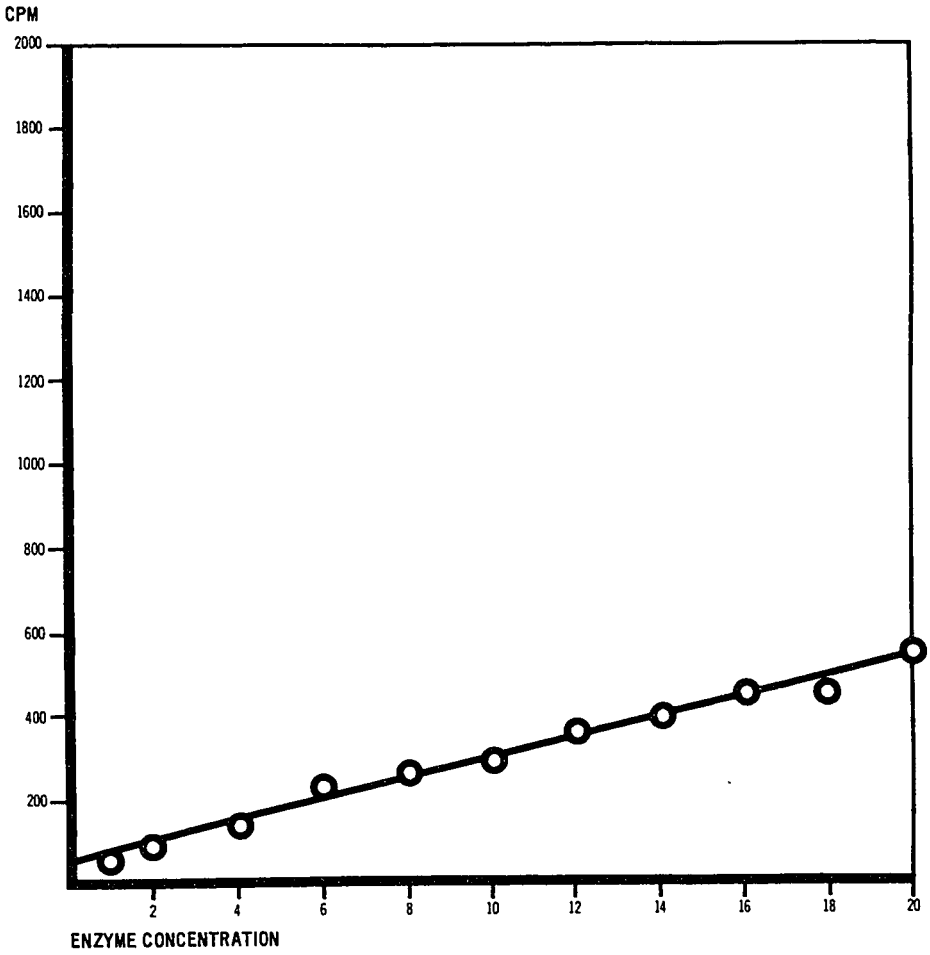

Figure 3 . Monoamine oxidase activity on serotonin-14C . Activity expressed as cpm in 60 µl of the toluene phase (no blank substracted) .

Enzyme concentration in arbitrary units .

TABLE I

Monoamine oxidase radioassay : benzylamine-^{14}C and serotonin-^{14}C. Effect of specific monoamine oxidase inhibitors . †

	Benzylamine-^{14}C		Serotonin-^{14}C	
	activity cpm	% inhibition	activity cpm	% inhibition
blank	80	-	40	-
control	494	-	155	-
Marsilid	294	48	74	70
Catron	78	100	49	92
Harmalin	558	0	47	94

† The inhibitors were used at a concentration of 0.1 mM .

benzylamine-^{14}C at the following concentrations : 50, 60, 75, 100, 150, and 300 µM . The same concentrations of benzylamine-^{14}C were used to determine the apparent Ki for serotonin, using a concentration of cold serotonin of 16.66 mM . Similarly the apparent Km for serotonin was determined using serotonin-^{14}C at 83.33, 100, 125, 166.66, 250, and 500 µM . To determine the apparent Ki for benzylamine the same concentrations were used and cold benzylamine at 500 µM .

b. Theoretical - calculations - program .

Cleland (120) described in 1967 a method for the statistical analysis of enzyme kinetic data . This method makes use of a computer program in FORTRAN and has the advantage over the usual graphical methods that it gives more accurate results and, more important, that it provides an estimate of the reliability of the fitted constants .

The Michaelis-Menten equation :

$$(1) \qquad v = \frac{V \cdot S}{K + S}$$

represents a rectangular hyperbola passing through the origin . Since this is not a convenient form for graphical analysis, the equation is usually rearranged into a linear form such as the Lineweaver-Burk plot :

$$(2) \qquad \frac{1}{v} = \frac{K}{V} \cdot \frac{1}{S} + \frac{1}{V}$$

The parameters K and V can then be obtained graphically from the slope and the intercept of the resulting line .

Least squares fitting to the reciprocal equation.

The best fit to this linear equation is the one that minimizes the expression :

$$(3) \qquad \sum \left(\frac{1}{v_i} - \left(\frac{K}{V} \cdot \frac{1}{S} + \frac{1}{V} \right) \right)^2$$

This can be done simply by taking the partial derivative of this expression with respect to $\frac{K}{V}$ and $\frac{1}{V}$ and setting them equal to zero, yielding a set of two linear equations : *

$$(4) \qquad \begin{aligned} \frac{K}{V} \sum \left(v_i^4 \cdot \frac{1}{S_i^2} \right) + \frac{1}{V} \sum \left(v_i^4 \cdot \frac{1}{S_i} \right) &= \sum \left(v_i^4 \cdot \frac{1}{S_i v_i} \right) \\ \frac{K}{V} \sum \left(\frac{v_i^4}{S_i} \right) + \frac{1}{V} \sum \left(v_i^4 \right) &= \sum \left(\frac{v_i^4}{v_i} \right) \end{aligned}$$

If this set of equations is to be solved by matrix methods, the matrix positions are given by :

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* The factor v_i^4 , present in each term, is a weighting factor (see below) .

$$(5)\qquad \begin{vmatrix} \sum\left(v_i^4 \cdot \frac{1}{S_i^2}\right) & \sum\left(v_i^4 \cdot \frac{1}{S_i}\right) & \sum\left(v_i^4 \cdot \frac{1}{S_i v_i}\right) \\ \sum\left(v_i^4 \cdot \frac{1}{S_i}\right) & \sum\left(v_i^4\right) & \sum\left(v_i^4 \cdot \frac{1}{v_i}\right) \end{vmatrix}$$

Solution of this matrix (e.g. by Gaussian elimination) yields the values for $\frac{K}{V}$ and $\frac{1}{V}$.

It should be noted that the variance of $\frac{1}{v}$ is not constant . If the variances are known, then a weighted fit can be made, and the expression which is minimized is :

$$(6)\qquad \sum w_i \left(\frac{1}{v_i} - \left(\frac{K}{V}\frac{1}{S_i} + \frac{1}{V} \right) \right)^2$$

where w_i is the weight attributed to $\frac{1}{v_i}$ and is proportional to the reciprocal of the variance of $\frac{1}{v_i}$. It can be shown that :

$$(7)\qquad \mathrm{var}\left(\frac{1}{v}\right) \equiv \frac{\mathrm{var}(v)}{v^4}$$

Where reaction rates are measured at constant enzyme concentration the variance of v will be essentially constant and w equals v^4 . However, if the experimental velocity deviates from the calculated velocity, the percentage error in the weighting factor is four times as great . It is therefore better to eliminate the need for these variable weights entirely by fitting the data directly to

The hyperbolic rate equation .

In the case of non-linear equations the above procedure does not yield a set of solvable equations . The equation can be put into a linear form in the following way . Equation 1 is linear in V, non-linear in K . It can be shown that if K_1 is the value that gives the best fit, and a rough estimate K_o is known, then :

$$(8) \qquad v \equiv \frac{V \cdot S}{K + S} + (K_1 - K_o) \left[\frac{\partial \left\{ \frac{V \cdot S}{K_o + S} \right\}}{\partial K} \right]$$

This function is linear in two constants : V and $V(K_1 - K_o)$. Fitting by least squares yields values for V and for $V(K_1 - K_o)$ which are easily solved for K_1 . This new value can then be used as K_o and the process repeated until $K_1 - K_o$ is reduced to nearly zero .

Standard deviations of the kinetic constants are easily obtained if a matrix method is used to solve the set of equations : if the matrix is inverted and multiplied by σ^2, it gives the variances and covariances of the constants . σ^2 is the average square of the difference between the experimental velocity and the corresponding calculated velocity :

$$(9) \qquad \sigma^2 \equiv \frac{\sum w_i \left[v_i - f(S_i ; V, K) \right]^2}{P - n}$$

where P - n is the number of degrees of freedom .

FORTRAN program KINET

In the same paper (120) Cleland publishes a worked out program for fitting to equation 1 . We have used this program with only the minor modifications necessary to use it on the IBM 360 system, which uses a different level FORTRAN . The printout of this program is found in Appendix 1 . It consists of the following parts :

-PRELIMINARY FIT : a least squares fit to the reciprocal equation as described above, to give the preliminary estimates of the constants V and K, which are used in the iterative fit that follows .

-ITERATIVE FIT TO HYPERBOLA : a three cycle iterative least squares fit as described above, provides the fine adjustments of the values for V and K .

-CALCULATION OF STANDARD DEVIATIONS AND WEIGHTS : the standard deviation is defined as the square root of the variance * and the weight as the reciprocal of the variance .

-Ki, SD Ki, AND T-TEST : this section of the program was only used for experiment 1 and was left out for experiment 2 .

-MATRIX SOLUTION SUBROUTINE : referred to in various parts of the program .

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* Cleland calls it standard error, contrary to common usage .

The value of the apparent Ki in experiment 1 is found by the following relationship :

$$(10) \qquad Kmi \equiv Km \left(1 + \frac{[I]}{K_i} \right)$$

$$\text{or :} \qquad Ki \equiv \frac{Km\ [I]}{(Kmi - Km)}$$

where Kmi is the apparent Km as determined in the presence of cold substrate (" inhibitor") at concentration [I] . According to Wilkinson (121) the variance of a function can be derived from the variance of its parts :

$$(11) \quad Var(Ki) \simeq F^2Km \cdot Var(Km) + F^2Kmi \cdot Var(Kmi) + 2\ F\ Km \cdot F\ Kmi \cdot Cov(Kmi \cdot Km)$$

where F is the partial derivative to the respective parameter . Since in this case Kmi and Km are statistically independent, the covariance term drops (121) .

For the t-test t is given by :

$$(12) \qquad t \equiv \frac{Km - Ki}{\sqrt{SD^2_{Km} + SD^2_{Ki}}}$$

The program takes as input : the number of points in the plot (NP), the concentration of cold substrate (CI), the various concentrations of ^{14}C-labelled substrate (A(I)), and the respective velocities (V(I)), usually the mean value of five determinations .

- EXPERIMENT 2

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a. Experimental setup and assay

This experiment consisted of the measurement of the slope (" Km/V ") of the reaction with benzylamine-^{14}C at various concentrations of cold serotonin . As will be seen in the discussion, it was expected that in the case of two enzymes being present at the same time, the plot of the slopes versus the concentration of serotonin would fit the following equation :

$$(13) \qquad y \equiv \frac{(1 + b\,x + c\,x^2)}{1 + d\,x}$$

where y represents the slope of the reciprocal plot for the benzylamine-^{14}C reaction, and x the concentration of cold serotonin .

This is the equation for a hyperbola with a non-horizontal asymptote .

The different parameters of this 2/1 hyperbola, as Cleland (120) calls it, can be derived graphically . If the slope and the vertical intercept of the asymptote (S_∞ and I_∞) and the slope when $x \equiv 0$ (S_0) and the actual vertical intercept (I_0) are measured, then :

$$(14) \qquad d \equiv \frac{(S_o - S_\infty)}{(I_\infty - I_o)}$$

$$(15) \qquad b \equiv \frac{S_o}{I_o + d}$$

$$(16) \qquad c \equiv \frac{d \cdot S}{I_o}$$

$$(17) \qquad a \equiv I_o$$

This graphical method provides only a rough estimate of these parameters, which can be used as preliminary etimates in the iterative fit described below .

The radioisotope assay as described above was used, with the same concentrations of benzylamine as in experiment 1 . The concentrations of cold serotonin were the following : 0, 0.166, 0.333, 0.833, 1.666, 3.333, 8.333, 16.666 mM . All the assays were done with the same concentration of enzyme and in triplicate .

b. Theoretical - calculations - program

The value of "K/V" for each concentration of serotonin was obtained by the program KINET described above (without its section for calculation of Ki and related values) . The

resulting data were then plotted : "K/V" versus serotonin . This allowed the graphical estimation of the constants, necessary for the iterative fit to equation 13 . This iterative fit was done by the program 2/1 HYPERBOLA which we developed after the method of Cleland (120) .

FORTRAN PROGRAM 2/1 HYPERBOLA

As seen above non-linear equations can be fitted by an iterative fit if preliminary estimates of the constants are known . Therefore equation 13 was put into the following linear form :

$$(18)\quad y \equiv \frac{a\,(1 + bx + cx^2)}{1 + dx} + (b_1 - b_0)\left\{\frac{\partial\,(f(x;a,b,c,d))}{\partial b}\right\}_{b_0,c_0,d_0} + (c_1 - c_0)\left\{\frac{\partial(f(x;a,b,c,d))}{\partial c}\right\}_{b_0,c_0,d_0} + (d_1 - d_0)\left\{\frac{\partial(f(x;a,b,c,d))}{\partial d}\right\}_{b_0,c_0,d_0}$$

This expression is linear in a, $a(b_1 - b_0)$, $a(c_1 - c_0)$, $a(d_1 - d_0)$ and can be written :

$$(19)\qquad y \equiv p\Omega_1 + q\Omega_2 + r\Omega_3 + s\Omega_4$$

The most probable fit is the one that minimizes the expression :

$$(20) \qquad \sum \left[y - f(x;\, p,\, q,\, r,\, s)\right]^2$$

This is done by taking the partial derivatives of this expression with respect to the four parameters and setting them equal to zero . This yields a set of four equations, linear in the four constants :

$$(21)\quad p\sum(Q_{1,i})^2 + q\sum(Q_{1,i}Q_{2,i}) + r\sum(Q_{1,i}Q_{3,i}) + s\sum(Q_{1,i}Q_{4,i}) = \sum(Q_{1,i}y_i)$$

$$(22)\quad p\sum(Q_{2,i}Q_{1,i}) + q\sum(Q_{2,i})^2 + r\sum(Q_{2,i}Q_{3,i}) + s\sum(Q_{2,i}Q_{4,i}) = \sum(Q_{2,i}y_i)$$

$$(23)\quad p\sum(Q_{3,i}Q_{1,i}) + q\sum(Q_{3,i}Q_{2,i}) + r\sum(Q_{3,i})^2 + s\sum(Q_{3,i}Q_{4,i}) = \sum(Q_{3,i}y_i)$$

$$(24)\quad p\sum(Q_{4,i}Q_{1,i}) + q\sum(Q_{4,i}Q_{2,i}) + r\sum(Q_{4,i}Q_{3,i}) + s\sum(Q_{4,i})^2 = \sum(Q_{4,i}y_i)$$

where $Q_1 = \dfrac{(1 + bx + cx^2)}{1 + dx}$ $\qquad$ $Q_2 = \dfrac{x}{1 + dx}$

$Q_3 = \dfrac{x^2}{1 + dx}$ $\qquad$ $Q_4 = \dfrac{(1 + bx + cx^2)\, x}{(1 + dx)^2}$

The matrix positions are given by (if we put $0_5 \equiv y_i$) :

$$(25) \qquad \left|\begin{array}{cccc|c} \sum(0_{1,i})^2 & \sum(0_{1,i}0_{2,i}) & \sum(0_{1,i}0_{3,i}) & \sum(0_{1,i}0_{4,i}) & \sum(0_{1,i}0_{5,i}) \\ \sum(0_{2,i}0_{1,i}) & \sum(0_{2,i})^2 & \sum(0_{2,i}0_{3,i}) & \sum(0_{2,i}0_{4,i}) & \sum(0_{2,i}0_{5,i}) \\ \sum(0_{3,i}0_{1,i}) & \sum(0_{3,i}0_{2,i}) & \sum(0_{3,i})^2 & \sum(0_{3,i}0_{4,i}) & \sum(0_{3,i}0_{5,i}) \\ \sum(0_{4,i}0_{1,i}) & \sum(0_{4,i}0_{2,i}) & \sum(0_{4,i}0_{3,i}) & \sum(0_{4,i})^2 & \sum(0_{4,i}0_{5,i}) \end{array}\right|$$

When weights are available, expression 20 becomes :

$$\sum w_i \left[y_i - f(x;\ p,\ q,\ r,\ s)\right]^2$$

and the resulting matrix contains w_i at every position :

$$\sum(w_i 0_{k,i} 0_{j,i})$$

Solving the matrix yields values for a, $a(b_1 - b_0)$, $a(c_1 - c_0)$ and $a(d_1 - d_0)$ which are easily solved for b, c, and d . The new values of the parameters are then used as preliminary estimates and the process is repeated . Usually more iterations are required here because the preliminary estimates are less accurate than in the program KINET . Also the intermediate results are

printed out as a check on convergence . A minimum of five iterations was used .

The program 2/1 HYPERBOLA then consists of the following parts :

- READ IN DATA AND PRELIMINARY ESTIMATES : the program reads in the number of points (NP), the values for X(I), Y(I) and W(I) (if any); also the preliminary estimates of the constants a, b, c, d .
- ITERATIVE FIT : as described above, minimum five cycles .
- STANDARD DEVIATION, WEIGHT : calculated as in the program KINET .
- MATRIX SOLUTION SUBROUTINE .

The printout of the program appears in appendix 2 . In order to visualize the curve and to see how well it fitted the data, the equation was plotted by computer using the program PLOT HYPERBOLA 2/1 . This program consists of a main program generating the function and the FORTRAN subroutine (NPLOT) (see printout in appendix 3) .

- EXPERIMENT 3

This experiment consisted in the determination of the oxygen consumption by rat liver mitochondrial monoamine oxidase at different concentrations of serotonin . The oxygen

consumption method was selected because it allows the use of high substrate concentrations, only limited by the solubility of the substrate . The purpose of this was to observe the shape of the reciprocal (Lineweaver-Burk) plot at these concentrations .

a. Assay of oxygen consumption .

Mitochondria were prepared as described above . A 0.045 M solution of serotonin was made in freshly distilled boiled water and kept at 37 degrees to prevent any crystallization . Dilutions were made from this stock solution to give a final concentration in the incubation mixture of 0.375, 0.500, 0.750, 1.500, 3.000, and 7.500 mM . All these dilutions were made with distilled water except the last one, which was made with 0.5 M sodium phosphate buffer (pH 7.4) because at this concentration the serotonin started to affect the pH of the incubation mixture . The other concentrations did not change the pH of the incubation medium as checked with a pH meter . The incubation mixture was constituted as follows :

0.4 ml buffer (0.5 M sodium phosphate, pH 7.4)

0.2 ml KCN (0.01 M)

0.2 ml semicarbazide (0.1 M)

2.0 ml substrate solution

0.2 ml enzyme suspension

3.0 ml final volume

This composition is based on the method for monoamine oxidase assay by oxygen consumption described by Creasey (21) . The oxygen consumption was followed on an oxygen monitor (Yellow Springs Instrument Co) .

After proper testing and calibration of the oxygen electrode, all the ingredients of the reaction mixture except the enzyme were added to the cuvette at 37 degrees and were equilibrated with athmospheric air for 3 minutes (while stirred with the magnetic stirrer) . At that time the enzyme was added, the electrode introduced, and the measurement started immediately . The electrode response was recorded on a Varicord recorder (Photovolt Corporation) . The initial velocity of the oxygen consumption was estimated by drawing the tangent to the recorded line through its origin . It was expressed as % oxygen consumed in 5 minutes . The oxygen consumption of a blank (same reaction mixture, no substrate) was substracted . In general the reaction was linear at least over the first five minutes . The expression of the rate of oxygen consumption was then transformed into µmoles O_2 consumed per hour per ml of enzyme, using the appropriate factors ; it was assumed that at 37 degrees and 760 mm Hg air pressure, 1 ml water dissolves 0.217 µmoles of oxygen (122) . A previous test had shown that the solubility of oxygen in the reaction mixture as it was used in this assay was not appreciably different from the solubility in distilled water under the same conditions .

b. Calculations.

A plot of 1/v versus 1/ S was made . This allowed visual inspection of the curve and it was also used for the graphical determination of the preliminary estimates of the constants for a 2/1 hyperbola (same procedure as under experiment 2, equations 14-17) . With these constants an iterative fit to a 2/1 hyperbola was done using the same program 2/1 HYPERBOLA as described above .

B. Results

Experiment 1

Table 2 summarizes the results from which the following constants were obtained :

"Km (serotonin)".....0.140 ± 0.043 mM

"Ki (serotonin)".....12 ± 4.4 mM

The t-test on the difference between "Km" and "Ki" for serotonin gives a t-value of 2.6 ($p<0.05$ for 10 degrees of freedom) : a significant difference . For benzylamine the difference between "Km" and "Ki" was not found to be significantly different .

"Km (benzylamine)"....0.25 ± 0.033 mM

"Ki (benzylamine)"....0.21 ± 0.096 mM

$t = -0.078$ $(p>0.9)$

TABLE 2

Experiment 1 . Apparent Michaelis and inhibitor constants for substrate amines with rat liver mitochondrial monoamine oxidase. (values given are mean of six determinations)

benzylamine-^{14}C µM	v cpm	serotonin mM	v cpm
300	2836	166	821
150	1969	166	451
100	1380	166	343
75	1140	166	293
60	1033	166	224
50	950	166	195

serotonin-^{14}C µM	v cpm	benzylamine µM	v cpm
500	972	50	598
250	769	50	410
166	575	50	299
125	536	50	253
100	611	50	194
83.3	436	50	172

Km(benzylamine) 256 ± 33.8 µM

Ki(benzylamine) 218 ± 96.2 µM

t : 0.08. ($p > 0.9$)

Km(serotonin) 144 ± 43.3 µM

Ki(serotonin) 12800 ± 4430 µM

t : 2.63 ($p < 0.05$)

Experiment 2

The assay with benzylamine-^{14}C at various concentrations of cold serotonin gave the results shown in table 3a. When the slopes (K/V) of the different reciprocal plots are replotted versus the concentration of serotonin, a non-linear curve is obtained which bends down near the vertical axis, and approaches a non-horizontal asymptote at the higher concentrations of serotonin (figure 4) . On such a plot the preliminary estimates of the constants a, b, c, d were made graphically (table 3b) .

Using these preliminary estimates an iterative fit to equation 13 was performed by the program 2/1 HYPERBOLA . Table 3b shows the convergence of the iterations and the fitted constants with their standard deviation . Figure 4 shows a plot of the equation using the fitted constants with the experimental points added in . The conclusion is that a good fit is obtained to equation 13, in other words that cold serotonin caused a hyperbolic inhibition on the slope of the reciprocal plot of the benzylamine-^{14}C reaction .

Experiment 3

Table 4a shows the rates of oxygen consumption obtained at different concentrations of serotonin . The reciprocal plot is shown in figure 5 . Assuming a hyperbolic relationship, the constants were derived graphically and used as preliminary estimates for the iterative fit to equation 13 by the

TABLE 3a

Experiment 2 . Effect of the serotonin concentration on the slope of the reciprocal plot for the benzylamine-^{14}C reaction .

benzylamine-^{14}C µM	activity (cpm) at serotonin concentration mM							
	0	0.166	0.333	0.833	1.666	3.333	8.333	16.666
300	2770	2918	3079	2503	2392	2023	1601	1107
150	1946	1970	2035	1708	1159	1362	989	665
100	1581	1492	1450	1322	1191	978	737	427
70	1415	1570	1481	1102	1042	869	648	476
60	1292	1259	1187	970	873	707	536	384
50	1115	1095	1144	843	819	606	434	321

serotonin mM	slope ("K/V") ±S.D. x 10^8	weight x 10^{-17}
0	3.46 ± 0.277	1.3
0.166	3.64 ± 0.449	0.493
0.333	4.05 ± 0.463	0.466
0.833	5.03 ± 0.176	3.23
1.666	6.25 ± 1.30	0.0586
3.333	7.08 ± 0.261	1.46
8.333	10.1 ± 0.499	0.401
16.666	15.1 ± 1.87	0.0285

TABLE 3b

Least squares fitting of the data relating the slopes of the reciprocal plot of the benzylamine-^{14}C reaction and the concentration of serotonin to a 2/1 hyperbola .

parameter / iteration	a $\times 10^{-8}$	b $\times 10^{-3}$	c $\times 10^{-5}$	d $\times 10^{-3}$
0 +	3.46	1.31	1.34	0.771
1	3.39	2.21	2.19	1.29
2	3.40	1.80	1.46	0.932
3	3.39	2.00	1.76	1.08
4	3.40	1.98	1.72	1.06
5	3.40	1.98	1.73	1.07

Fitted constants ± standard deviation

a : $3.40 \times 10^{-8} \pm 2.17 \times 10^{-10}$

b : $1.98 \times 10^{-3} \pm 4.37 \times 10^{-6}$

c : $1.73 \times 10^{-5} \pm 5.90 \times 10^{-4}$

d : $1.07 \times 10^{-3} \pm 2.95 \times 10^{-6}$

+ Preliminary estimate .

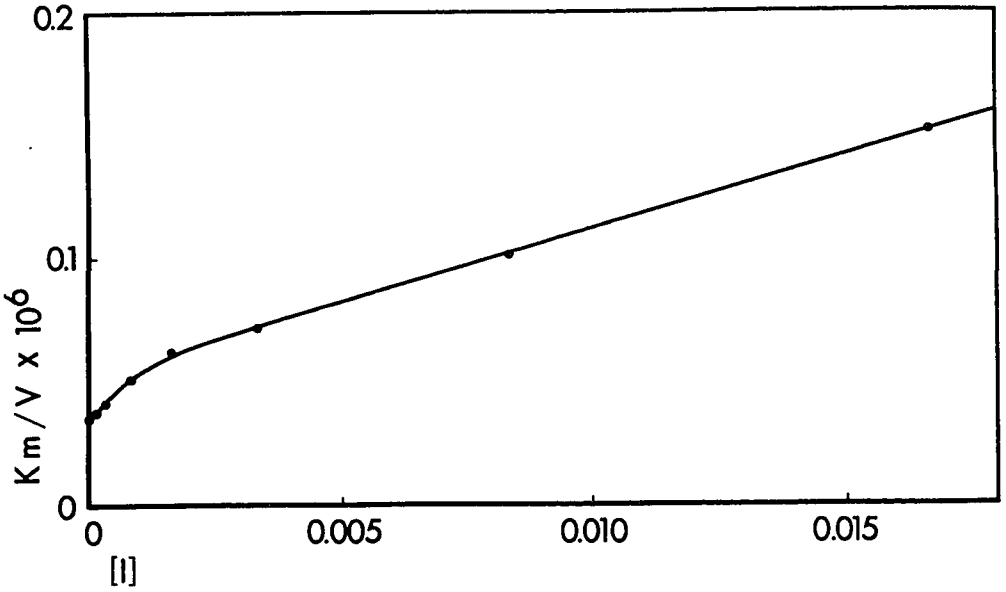


Figure 4 . Plot of the slopes ("K/V") of the reciprocal plot of the benzylamine-^{14}C reaction vs. serotonin concentration . The plot was generated by the computer using the fitted constants from table 3b . The points represent experimental data .

TABLE 4a

Oxygen consumption by rat liver mitochondria at different concentrations of serotonin .*

serotonin mM	1/[S]	v µmoles O_2/hr.ml	1/v
7.500	133	7.20	0.138
3.000	333	6.38	0.156
1.500	666	5.68	0.176
0.750	1333	4.90	0.204
0.500	2000	4.58	0.218
0.375	2666	4.39	0.228
0.300	3333	4.19	0.238

* Oxygen consumption was measured by oxygen electrode . The values for v represent the net consumption after substraction of a blank (: no substrate) .

TABLE 4b

Least squares fitting of the reciprocal plot of the oxygen consumption of rat liver mitochondria in the presence of serotonin to a 2/1 hyperbola .

parameter / iteration	a x 10	b x 10^3	c x 10^8	d x 10^4
0 †	1.27	1.87	14	11.9
1	1.24	1.53	-3.83	5.40
2	1.24	1.81	1.53	8.15
3	1.24	1.95	2.89	9.18
4	1.24	1.94	2.83	9.18
5	1.24	1.94	2.83	9.18

Fitted constants ± standard deviation

a : $1.24 \times 10^{-1} \pm 2.54 \times 10^{-2}$

b : $1.94 \times 10^{-3} \pm 4.76 \times 10^{-4}$

c : $2.83 \times 10^{-8} \pm 4.16 \times 10^{-8}$

d : $9.18 \times 10^{-4} \pm 2.87 \times 10^{-4}$

† Preliminary estimates .

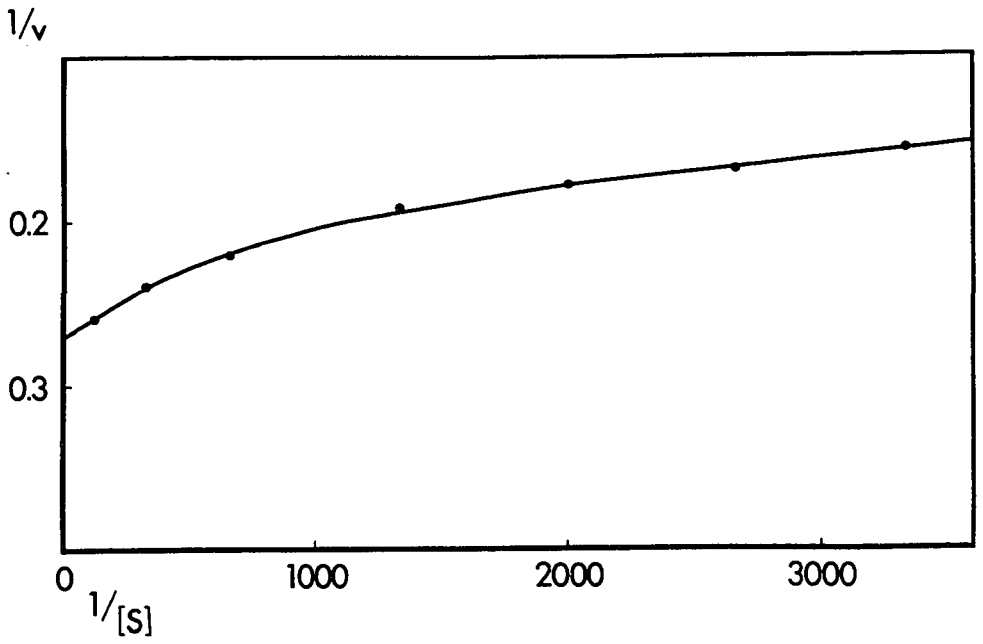


Figure 5 . Reciprocal plot for mitochondrial oxygen consumption vs. serotonin concentration . The plot was generated by the computer using the fitted constants from table 4b . The points represent experimental data .

program 2/1 HYPERBOLA . The iterations were convergent and the constants with their standard deviations (table 4b) show that a good fit was obtained . This is further illustrated by the computer-generated plot (figure 5) which used the fitted constants and on which the experimental points were superimposed .

C. Discussion

From the review of the literature it is clear that there are many reasons to believe that there may be more than one monoamine oxidase in rat liver mitochondria . The indirect evidence falls into two categories : inhibition studies and multiple substrate experiments . The selective inhibition of the activity on certain substrates can be caused by specific agents : monoamine oxidase inhibitors (93, 106, 107, 110, 111, 112, 114) . Or it can be caused by non-specific agents : heat denaturation (94), pH changes (94), anions (101), oxygen tension (20) . Selective inhibition is by itself only a weak argument for the multiplicity of the enzyme, since it could also be explained by alterations of the active site and the surrounding groups which would affect the activity on various substrates in a different way . Multiple substrate experiments on the other hand would be conclusive only if they showed that substrates do not compete with each other .

Since monoamine oxidase substrates do show competition, this is compatible as well with the presence of one enzyme as with the presence of several enzymes with overlapping substrate specificity (10, 82, 92) .

These indications for the existence of multiple monoamine oxidases were mostly accidental findings in the course of studies on the inhibition or other features of this enzyme system . We decided to start from the hypothesis that there actually was more than one enzyme in rat liver mitochondria, and we designed the kinetic experiments to test this hypothesis .

The first experiment was a comparison between the ability of an amine as substrate and as inhibitor respectively. It was possible to determine the apparent Km and the apparent Ki for each amine using the mixed substrate method described above . The fact that a significant difference was found between Km and Ki for serotonin is compatible with the two enzyme model . The finding that Km and Ki for benzylamine were not significantly different is also compatible with this model . The kinetic behaviour in experiment 1 of the monoamine oxidase activity on serotonin is also compatible with other models e.g. one enzyme having an active site and an "allosteric" site capable of activating the enzyme, or else the theory of eutopic and dystopic complexes proposed by Zeller (100) . This latter theory however would require a significant difference between Km and Ki for benzylamine : this

is not the case , so that this theory can be eliminated .

If one accepts the two enzyme model as hypothesis, some characteristic kinetic behaviour can be deduced . Dixon and Webb (123) describe the rate equation for two enzymes attacking the same substrate . The total activity will be the sum of the two activities :

$$(26) \qquad v_t \equiv \frac{V_1 \cdot S}{K_1 + S} + \frac{V_2 \cdot S}{K_2 + S}$$

In reciprocal form this equation becomes :

$$(27) \qquad \frac{1}{v_t} \equiv \frac{1 + \frac{K_1 + K_2}{S} + \frac{K_1 \cdot K_2}{S}}{V_1 + V_2 + \frac{V_1 \cdot K_2 + V_2 \cdot K_2}{S}}$$

or :

$$(28) \qquad \frac{1}{v_t} \equiv \frac{1}{V_t} \cdot \frac{(1 + (K_1 + K_2)\, X + K_1 \cdot K_2\, X^2)}{1 + \left(\frac{V_1 \cdot K_2 + V_2 \cdot K_1}{V_t} \right) X}$$

where $X \equiv 1/[S]$

This equation is of the same form as equation 13 : a hyperbola with non-horizontal asymptote . It should be noted that if K1 = K2 the equation reduces to a simple Michaelis-Menten equation and the reciprocal plot is a straight line . Also

since the slope of the straight part of the hyperbola is given by equation 16, it follows :

$$(29) \qquad S\infty = \frac{c \cdot a}{d}$$

Replacing by the corresponding values from equation 28 :

$$(30) \qquad S\infty = \frac{K_1 \cdot K_2}{V_1 \cdot K_2 + V_2 \cdot K_1}$$

If there is competitive inhibition on the slopes for the two enzymes :

$$(31) \qquad K_1 = Km_1 \left(1 + \frac{[I]}{K_{i_1}} \right)$$

where $[I]$ is the concentration of inhibitor .

And similarly :

$$(32) \qquad K_2 = Km_2 \left(1 + \frac{[I]}{K_{i_2}} \right)$$

If we combine equations 31,32 with 30 and work out :

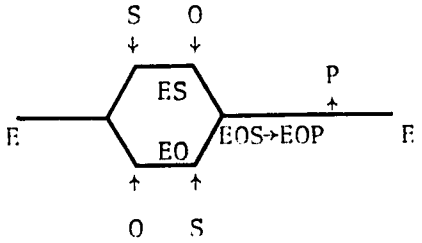
$$(33) \quad S_\infty = \frac{Km_1 \cdot Km_2}{V_1 \cdot Km_2 + V_2 \cdot Km_1} \cdot \frac{\left(1 + \left\{ \frac{1}{Ki_1} + \frac{1}{Ki_2} \right\} [I] + \frac{1}{Ki_1 \cdot Ki_2} [I]^2 \right)}{1 + \left(\frac{Ki_1 V_1 \cdot Km_2 + V_2 \cdot Km_1 \cdot Ki_2}{Ki_1 \cdot Ki_2 \ (V_1 \cdot Km_2 + V_2 \cdot Km_1)} \right) [I]}$$

This is of the same form as equation 13 if $X = [I]$.

In the experiments with isotope-labelled substrates, the substrate concentrations are in the lower range and the reciprocal plots show only straight lines : it is not possible to distinguish them from the regular reciprocal plots due to a Michaeli-Menten kinetic behaviour . But it can be seen from equation 33 that competitive inhibition in both enzymes would express itself by a hyperbolic inhibition on the slope S_∞ of the reciprocal plot .

This is exactly what was observed in experiment 2, where the addition of cold serotonin produces a hyperbolic inhibition on the slope of the reciprocal plot . The results of the second experiment are thus in agreement with the two enzyme model . They could alternately be explained by a single enzyme with an allosteric site ; only, in this case the allosteric site would have to be inhibitory rather than activating . Another possible explanation for the results of experiment 2 lies in the fact that oxidative deamination is a two-substrate reaction which involves, beside the substrate amine, oxygen as the second substrate . Cleland (124) discusses the kinetics of some multisubstrate enzymatic reactions . For certain mechanisms it is theoretically possible to find non-linear inhibition :

(34)



where E ≡ enzyme
S ≡ substrate
O ≡ oxygen
P ≡ product

In this reaction mechanism, there would be two enzyme forms with which the alternate substrate could combine : EO or E . But it should not be forgotten that in experiment 2 the inhibition was caused by cold serotonin, which is a normal substrate of the enzyme . To explain the results it would be necessary to accept that EO differs in affinity from E for serotonin but not for benzylamine, which seems rather unlikely .

The third experiment uses a different method for the assay of monoamine oxidase activity (oxygen monitor) . This permits the use of much higher substrate concentrations than in the radioisotope assay . With the radioisotope assay all the reciprocal plots were linear . This was to be expected, since the substrate concentrations were in such a range that one is merely looking at the straight part of the hyperbolic curve . From equation 28 it appears that the two enzyme model, in case the two Km's are different, requires a hyperbolic reciprocal plot, at least if the substrate concentrations used are high enough : the plot becomes non-linear near the vertical axis with downward concavity. Since the reciprocal plot would be non-linear only when the two Km's are different, serotonin was chosen as substrate for this experiment, and the concentrations used were in the higher range . The results of the experiment showed the expected hyperbolic curve.*

---

*Interestingly, similar results were observed by others (17) for mouse liver mitochondrial monoamine oxidase activity on serotonin. Only a logarithmic relationship between velocity and serotonin concentration was assumed, without explanation .

This could also be explained by substrate activation through an allosteric mechanism, but experiment 2 showed a requirement for an inhibitory allosteric site .

This means that the simplest model which can explain all the experimental results is the two enzyme model . The experimental results are also in agreement with the evidence in the literature for the multiplicity of monoamine oxidase in rat liver mitochondria .

PART II : Separation of two rat liver mitochondrial monoamine oxidases

Introduction

Since mitochondrial monoamine oxidase is a membrane bound enzyme and is difficult to remove from the membrane (59), it seemed that success in the separation of the enzymes was likely to depend on a thorough solubilization . This prompted us to study different methods of solubilization before attempting to separate the enzymes . Previous workers usually considered this enzyme soluble if it remained in the supernatant after a centrifugation of arbitrarily chosen speed and duration .

Though differential centrifugation is an easy and quick method, useful in screening different techniques of solubilization, we thought it would be useful to study some of the promising methods in more detail by using the tecniques of electron microscopy and density gradient centrifugation . The combination of these two techniques would allow us to appreciate better what a particular method did to the mitochondrion and our enzyme . Then the method that was most satisfactory for solubilization was chosen to prepare the samples for electrophoresis .

I. Solubilization

A. Materials and methods

§ 1. Differential centrifugation

Chemicals and enzyme preparation . Chemicals were purchased from Fisher, unless another source is indicated .

Sonication . The mitochondria (prepared as described in Part I) were suspended in a volume of 30 ml sodium phosphate buffer (0.2 M, pH 7.4) and sonicated for 1 hour in a Bronwill Biosonik ultrasound device . During sonication the suspension was cooled by a circulation of coolant which kept the temperature below 5 degrees .

Assays . The spectrophotometric assay of monoamine oxidase was based on the method of Tabor et al. (26) . The incubation mixture consisted of 0.1 ml of 0.1 M benzylamine (Eastman Organic Chemicals), 1 ml of phosphate buffer 0.2 M, pH 7.4 and enzyme in a total volume of 3 ml . The incubation was carried out in a B & L Spectronic 505 spectrophotometer with 1 cm cuvettes kept at 37 degrees . The reference cell contained the same mixture except the substrate . The reaction was started by addition of the enzyme suspension and recording started immediately on the external recorder (Varicord, Photovolt) . The increase in absorbance at 250 mμ was measured during the first 10 minutes of reaction : it was usually linear in that interval . The activity was expressed in spectrophotometric units (SU), one unit being the amount of enzyme causing a change in absorbance of 0.001 per minute under the described conditions .

Centrifugation . The samples were centrifuged for two hours at 100,000 x g in a Spinco L 2 preparative ultracentrifuge, using rotor # 40 .

§ 2. Electron microscopy

Thin sections - positive staining . A pellet containing the monoamine oxidase activity was obtained when some of the preparations were spinned at 100,000 x g . This pellet was fixed for 2 hours in a 1 % solution of osmium tetroxide in 0.25 M sucrose . The fixed pellets were then dehydrated and embedded in

the acrylic resin Vestopal W (Martin Jaeger, Geneva, Switzerland). Thin sections were made, which were stained in lead citrate (Reynolds solution) and uranyl acetate (1 % in water) . The sections were observed in a Philips 100-B electron microscope . This method produces positively stained pictures, this means that the stained structures appear dark on the prints .

Direct negative staining . On some preparations a direct negative staining procedure was followed . For this an aliquot of the enzyme preparations was mixed directly on a grid for the electron microscope with some 0.5 % phosphotungstic acid . This procedure yields prints in which the structures appear white on a dark stained background .

§ 3. Density gradient centrifugation .

Sucrose gradient . A gradient making device as described by Martin and Ames (126) was used to produce a gradient which was practically linear between 5 and 20 % (w/v) in sucrose . The total volume of the gradient was 4.55 ml . It was made in the cellulose nitrate tubes of 5 ml volume and centrifuged in the SW 65 rotor of the Spinco L2-65 B ultracentrfuge at 5 degrees.

Monoamine oxidase assay . The method of Tabor et al. (26) described above was used on the samples being prepared for centrifugation . On the fractions of the gradient, the radio-

assay after Otsuka and Kobayashi (41), also described above, was applied .

Sucrose concentration in the gradient fractions was determined by refractometry, using an American Optical T/C Goldberg type refractometer . The instrument was calibrated directly in percent sucrose (% w/w) and was compensated for variations in temperature .

Catalase was assayed by the Sigma method . The substrate, hydrogen peroxide, was diluted in 0.05 sodium phosphate buffer (pH 7.0) to an absorbance of between 0.550 and 0.520 at 240 mμ . For the assay, a silica cuvette with 1 cm light path, at 25 degrees was filled with 2.9 ml of the substrate solution . The enzyme (0.1 ml) was added and the contents of the cuvette mixed . The B & L Spectronic 505 was used to monitor the absorbance at 240 mμ , the signal being recorded on a Photovolt Varicord external recorder . The time required for the absorbance to decrease from 0.450 to 0.400 was measured . This corresponds to the decomposition of 3.45 μmoles of H_2O_2 in 3 ml solution . One sigma unit (ΣU) is the amount of catalase decomposing one micromole of H_2O_2 per minute in the described conditions .

Estimation of the sedimentation coefficient .

Martin and Ames (126) described a method for the estimation of sedimentation coefficients of enzymes by centrifugation on a sucrose gradient in a preparative ultracentrfuge . By definition the sedimentation coefficient in a medium at temperature T is :

(35) $$s_{T,m} \equiv \frac{dx/dt}{\omega^2 x}$$ where ω is the angular velocity (rad/sec) and x is the distance from the rotor centre.

This coefficient can be multiplied by a correction factor to extrapolate to the standard state (water at 20 degrees) :

(36) $$s_{20,w} \equiv s_{T,m} \frac{\eta_{T,m}(\rho_p - \rho_{20,w})}{\eta_{20,w}(\rho_p - \rho_{T,m})}$$

where η is the viscosity and ρ is the density of the respective media ; ρ_p is the density of the particular enzyme or protein .

In a sucrose gradient this is still valid, only now the density of the solution as well as the viscosity are function of the sucrose concentration and thus of the distance from the rotor centre:

(37) $$\omega^2 s_{20,w} dt \equiv A \frac{\eta_{T,m}}{(\rho_p - \rho_{T,m})} \cdot \frac{1}{x} dx$$

where $A \equiv \frac{(\rho_p - \rho_{20,w})}{\eta_{20,w}}$, a constant at any given protein density.

Equation 37 can be integrated using a trapezoidal approximation :

$$(38)\quad \omega^2 s_{20,w} t = \sum_{o}^{x_{i+1}} F(x_i)\Delta x + \tfrac{1}{2} \sum_{o}^{x_{i+1}} \left(F(x_{i+1}) - F(x_i)\right)\Delta x$$

This can be done by tabulating arbitrary distances (x_i) from the rotor centre, starting at the meniscus and ending at the bottom of the tube . If the gradient is assumed to be linear between 5 and 20 %, then standard tables can provide the values for viscosity and density at the start of each fraction . The problem with this method is that the gradient is not always the same, nor linear . Also it is often not possible to find standard tables for the concentrations of sucrose and the temperature of the centrifugation that were actually used . These problems can be eliminated by measuring the sucrose concentration in each fraction by refractometry, and then calculating the corresponding viscosity and density . Empirical equations relating these parameters to the sucrose concentration and the temperature were developed by Barber (127) . The 9 constant equation 39 fits the empirical data for density over a range of 0 to 75 % (w/w) sucrose and 0 to 30 degrees with a maximum deviation of 7 parts in 10 thousand :

(39) $$\rho_{T,m} \equiv (B_1 + B_2T + B_3T^2) + (B_4 + B_5T + B_6T^2)\ Y + (B_7 + B_8T + B_9T^2)\ Y^2$$

where $\rho_{T,m}$ ≡ density of the sucrose solution

T ≡ temperature (degrees centigrade)

Y ≡ weight fraction sucrose

B_{1-9} ≡ empirical constants (see ref 127)

A set of equations relates the viscosity of sucrose solutions to their concentration and the temperature . The maximum deviation of the calculated data from the empirical values is 3 parts per thousand between 5 and 40 degrees and 5 to 40 % (w/w) sucrose .

(40) $$\log \eta_{T,m} \equiv A + \frac{B}{T + C}$$

where $\eta_{T,m}$ ≡ viscosity of the sucrose solution and A, B and C are constants defined below .

(41) $$A \text{ (or } B) \equiv D_0 + D_1y + D_2y^2 + D_3y^3 + D_4y^4 + D_6y^6 + D_7y^7 + D_8y^8$$

D_{0-7} are empirical constants (127)

y is the mole fraction of sucrose (see below)

(42) $$y \equiv \frac{Y/M_1}{Y/M_1 + (1 - Y)/M_2}$$

where M_1 ≡ molecular weight of sucrose

M_2 ≡ molecular weight of water

Y ≡ weight fraction sucrose

(43) $$C \equiv G_1 - G_2(1 + (y/G_3)^2)^{\frac{1}{2}}$$

G_{1-3} are empirical constants (127)

$y \equiv$ mole fraction sucrose

Since the concentration measured in each fraction is the average concentration in that fraction, the second term of equation 38 drops : (132)

(44) $$\omega^2 s_{20,w} t \equiv \sum_{o}^{x_{i+1}} F(x_i)\, \Delta x$$

The position of the peak after the centrifugation was determined by the method of Schumaker (128) . The center of gravity of the peak is derived from the activity in the different fractions and the dimensions of the rotor bucket by :

(45) $$r_i \equiv r_m + \left(\frac{2V' - (V'_i + V'_{i-1})}{2V'}\right) \frac{V}{R_L^2}$$

and : $$(r) = \frac{\Sigma r_i c_i}{\Sigma c_i}$$

where V' = total volume recovered from the gradient (drops)

V'_i = volume recovered up to and including the i^{th} fraction (drops)

V = initial volume placed into the tubes (ml)

r_m = radius position of the meniscus (cm)

r_L = radius of the tube (cm)

r_i = radius position of the i^{th} fraction

(r) = center of gravity of the peak

All of the above computations were programmed on the Olivetti Programma 101 desk top computer . The program (table 5) consisted of seven parts, each recorded on one side of a magnetic card . A printout of the actual programs is presented in Appendix 4 . Parts 1 to 3 were used to calculate viscosity, based on the weight fraction sucrose and temperature . This was repeated for every fraction of the gradient . Similarly part 4 was used to calculate the density of all the fractions . The center of gravity of the peak is calculated in part 5 and it is stored in memory until part 6 uses it to calculate the integral (equation 44) from the data on density and viscosity of the gradient. This integral is also stored in memory for part 7 which calculates and prints out the sedimentation constant in Svedberg units . This part includes an automatic correction for the acceleration and deceleration of the rotor .

Since the centrifuge requires a substantial time to reach the dialed speed setting, and also continues spinning for some time after the drive has been turned off, even when the brake is on, a correction has to be made for this . The acceleration and deceleration of the SW 65 rotor were recorded for a typical run at speed setting of 60,000 rpm (figure 6) . In terms of the distance travelled by a particle in the gradient the following equivalence is valid :

TABLE 5

Sedimentation constant estimation by centrifugation on a sucrose gradient : summary of the calculations as programmed on the Olivetti Programma 101 .

	part	Input (keyboard)	Output (printer)	Remarks
Viscosity	1	y(sucrose weight fraction)	coeff. A	for all fractions of the gradient .
	2	y	coeff. B	
	3	y,T,A,B	viscosity η	
Density	4	T, y	density ρ	idem
Sedimentation rate (Svedberg)	5	sample nr. activity	-	for all fractions of the peak (starting with fastest)
	6	η , ρ	-	all fractions, from top of the gradient until computer prints intermediary result .
	7	rpm,duration	$s^{0.725}_{20,w}$	

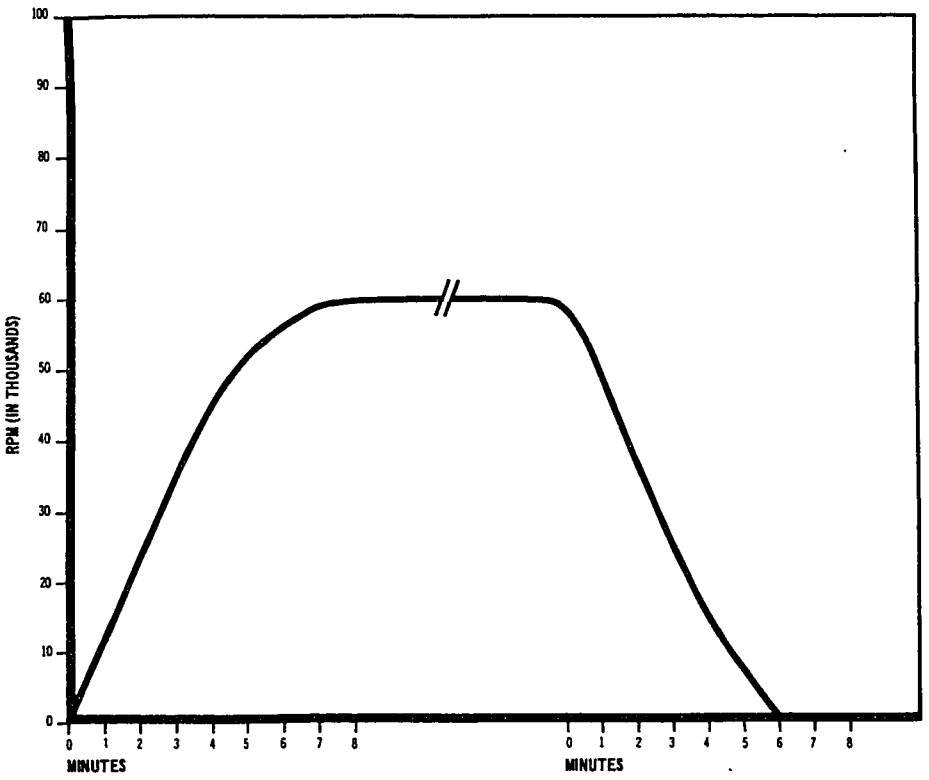


Figure 6 . Acceleration and deceleration of rotor SW 65 .

(46) $$(rpm_1)^2 \cdot t_1 \equiv (rpm_2)^2 \cdot t_2$$

For acceleration and deceleration the equivalent time is :

(47) $$\int_0^{59,500} (rpm_i) \cdot t_i \equiv (38,000) \cdot t_{38,000}$$

Solution of the integral by trapezoidal approximation gives, for the acceleration period :

(48) $$\sum (rpm_i)^2 \cdot t_i + \tfrac{1}{2} \sum \left((rpm_i)^2 - (rpm_{i-1})^2 \right) \cdot t_i$$

Similarly for the deceleration :

(49) $$\sum (rpm_i)^2 \cdot t_i + \tfrac{1}{2} \sum \left((rpm_i)^2 - (rpm_{i+1})^2 \right) \cdot t_i$$

When these equations were worked out with the data of figure 6, a correction factor of -3.627 minutes equivalent time at 38,000 rpm was obtained . As a test of the program, viscosities and densities were calculated for various sucrose concentrations and compared with the empirical data given in a table by de Duve et al. (129) . In another test of the program as well as of the whole experimental setup the sedimentation coefficient of catalase was determined . This experiment is described here as an example of the use of the method .

A gradient of 5 - 20 % (w/v) sucrose with a total volume of 4.55 ml was prepared . A catalase solution (Sigma Chemical Company) was diluted to approximately 15,000 ΣU/ml and 0.05 ml of this solution was carefully layered on top of the sucrose gradient . The gradient was then centrifuged at 60,000 rpm during 120 minutes . The odometer reading and the time were recorded at the moment the centrifuge was started, and again when the centrifuge was stopped manually after the rquired time . The gradient was then fractionated by puncturing the bottom of the tube and collecting drops, 11 per fraction . In this way 24 fractions were collected . The enzyme activity and sucrose concentration in the fractions were assayed as described . The activity appeared in a sharp peak in the upper part of the gradient (figure 7) . Table 6 shows the calculated values for density and viscosity in the various fractions . The $s_{20,w}$ obtained was 11.6 Svedberg, assuming a specific volume for the enzyme of 0.725, as compared with the value of 11.3 given by Martin and Ames .

B.Results

§ 1. Differential centrifugation

In a screening experiment different methods for solubilization of mitochondria were tried, and the resulting

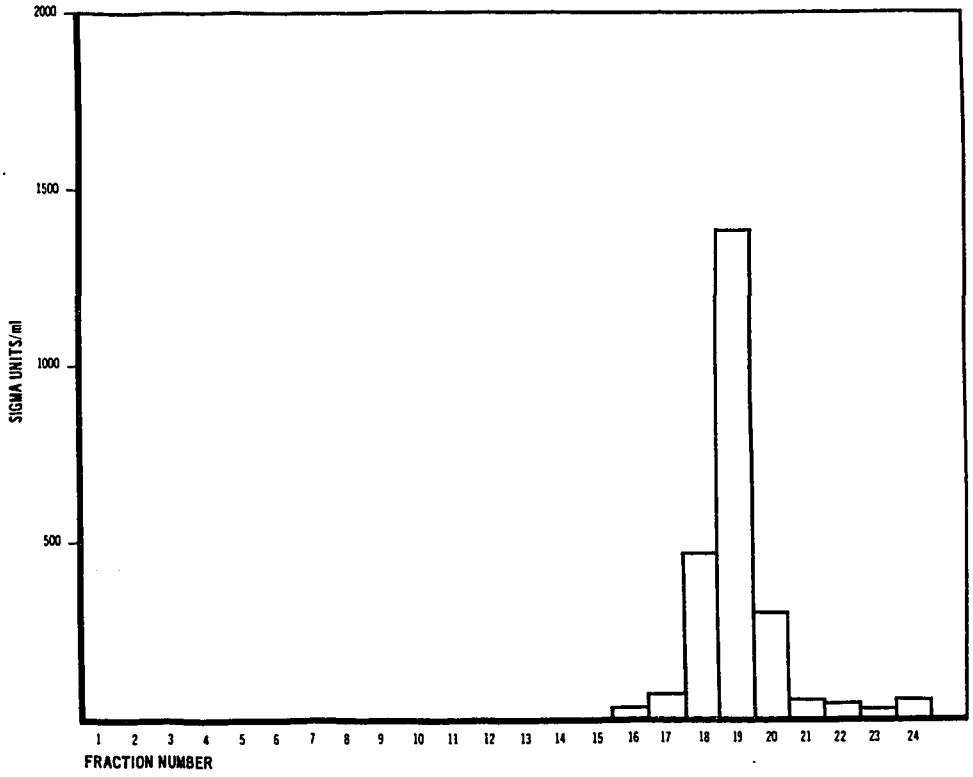


Figure 7 . Centrifugation of a pure catalase sample on a sucrose gradient . Conditions as in table 6 .

TABLE 6

Sedimentation rate of pure catalase : a test of the experimental setup.

fraction	sucrose % w/w	density	viscosity	activity ΣU/ml
16	10.6	1.043	2.109	27
17	9.9	1.040	2.058	69
18	9.2	1.037	2.007	460
19	8.7	1.035	1.975	1380
20	8.2	1.033	1.941	300
21	7.7	1.031	1.908	46
22	7.1	1.025	1.872	38
23	6.5	1.026	1.834	34
24	6.3	1.025	1.825	62

Odometer initial : 87610 th duration : 120 min. speed : 60,000 rpm
94568 th T : 5 degrees

$s^{0.725}_{20,w}$: 11.6 Svedberg

preparations were centrifuged . Table 7 shows the activities remaining in the supernatant after 45 minutes and 2 hours 45' of centrifugation respectively . The effect of the concentration of the buffer and of the presence of the substrate benzylamine on the activity of monoamine oxidase and its solubilization were assessed in the same way, as shown in table 8 .

The effect of the concentration of deoxycholate on the degree of solubilization is shown in figure 8 . It appears that to obtain solubilization a critical concentration has to be exceeded . When a preparation dissolved in this way with deoxycholate is dialysed against buffer containing no deoxycholate, the solution, which was clear, becomes cloudy . The precipitate contains a high proportion of the monoamine oxidase activity . This is shown in table 9 .

During these experiments, it was found that after deoxycholate treatment, the enzyme had to be kept on ice, as it became much more sensitive to heat denaturation . This is demonstrated in an incubation experiment, the results of which are seen in table 10 .

§ 2. Electron microscopy

Three methods of solubilization were further studied using electron microscopy : sonication, treatment with Lubrol and treatment with deoxycholate .

TABLE 7

Solubilization of rat liver mitochondrial monoamine oxidase : screening of different methods by differential centrifugation .

Preparation	activity SU/ml	activity recovered in supernatant after centrifugation at 100,000 x g			
		45 minutes		2 hours 45 min.	
		SU/ml	% *	SU/ml	% *
mitochondria	730	-	-	-	-
sonicated m.	540	230	31.5	0	0
1%deoxychol.	520	520	71	520	71
1%Tween 20	500	220	30	50	7
1%Tween 40	530	100	13.5	0	0
1%Tween 80	510	220	30	50	7
1%Tween 81	490	290	40	0	0
1%Tween 85	†	-	-	-	-
5%Lubrol	610	160	22	150	20

* Percent of the total initial activity of the mitochondria .

† This treatment yielded a thick emulsion which could not be assayed .

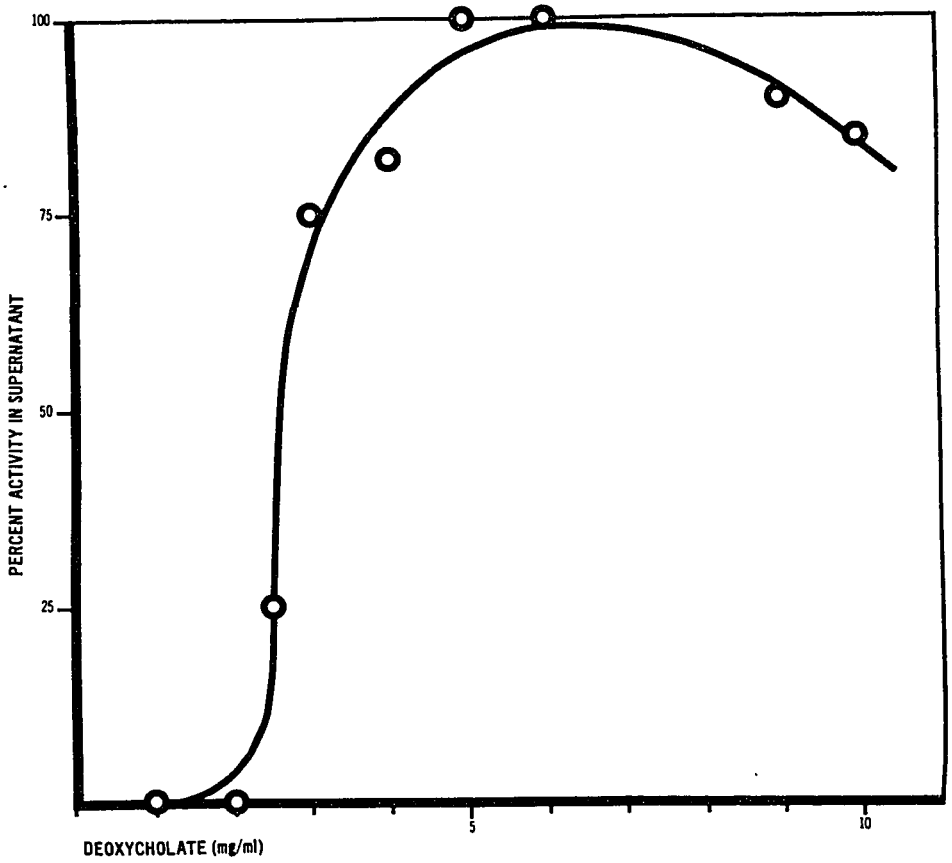


Figure 8 . Solubilization of mitochondrial monoamine oxidase by deoxycholate . Percent of the original activity remaining in the supernatant after centrifugation for 2 hours at 100,000 x g.

TABLE 8

Solubilization by deoxycholate : effect of buffer concentration and the presence of benzylamine . Activities in SU/ml .

Buffer (pH 7.4) M	benzylamine M	control	1%deoxychol.	supern.*	% †
0.2	0	264	190	150	58
0.15	0	220	180	160	73
0.1	0	190	280	160	85
0.2	0.003	244	280	240	98

* Activity in the supernatant after centrifugation for 2 hours at 100,000 x g .

† Percent of the activity of the untreated (control) mitochondria in the same buffer .

TABLE 9

Deoxycholate solubilized mitochondrial monoamine oxidase : effect of dialysis on the solubility .

preparation	activity SU/ml	protein mg/ml	specific activity SU/mg protein
mitochondria	235	9	26.1
same with 1% deoxycholate	265	8.15	32.5
100,000 x g * supernatant	100	4.9	20.5
dialysate ** (supernatant)	50	4.25	11.4
dialysate ** (precipitate)	270	4.9	55

* the deoxycholate dissolved mitochondria were centrifuged for 2 hours.

** the dialysate (12 hours against 0.02 M sodium phosphate buffer) was centrifuged at 8000 x g for 10 minutes .

TABLE 10

Resistance to denaturation by heat of mitochondrial monoamine oxidase before and after treatment with deoxycholate . Activities in SU/ml .

Preparation	Incubation time (minutes) 0	2	5	10	60
mitochondria*	100	105	115	120	55
deoxycholate supern.	75	65	55	35	15

* Mitochondria were isolated as described and resuspended in 0.2 M buffer (pH 7.4), containg benzylamine 0.003 M . An aliquot of this suspension was made 1 % in deoxycholate and centrifuged at 100,000 x g for 2 hours . The two preparations were then incubated at 37 degrees .

When mitochondria were sonicated under the conditions described above, the activity was spinned down by centrifugation at 100,000 x g for 20 minutes . The pellet containing the monoamine oxidase activity was fixed and thin sections were made and positively stained as described under Methods . The appearance of the particles is shown in the electron micrograph A in figure 9 . An estimate of their size was obtained by measuring on the print, and dividing this dimension by the combined magnification of the electron microscope and the photographic enlargement . The thus obtained dimensions varied between 300 Å and 700 Å.

Lubrol treated mitochondria were handled in the same way . Their appearance is shown in figure 9, B . They consist mainly of ruptured membranes, with an estimated length of up to 2800 Å .

The deoxycholate treated mitochondria did not yield an activity containing pellet, even after prolonged centrifugation . Therefore the supernatant which contained the monoamine oxidase activity was stained using the negative staining procedure described above . The particles, appearing white on a dark stained background (figure 10, A) were estimated to have a diameter of approximately 100 Å . The precipitate which formed after dialysis of such a deoxycholate solubilized preparation appeared to consist of vesicles (figure 10, B) of oval shape with an estimated long diameter of up to 1800 Å and a short diameter of about 700 Å .

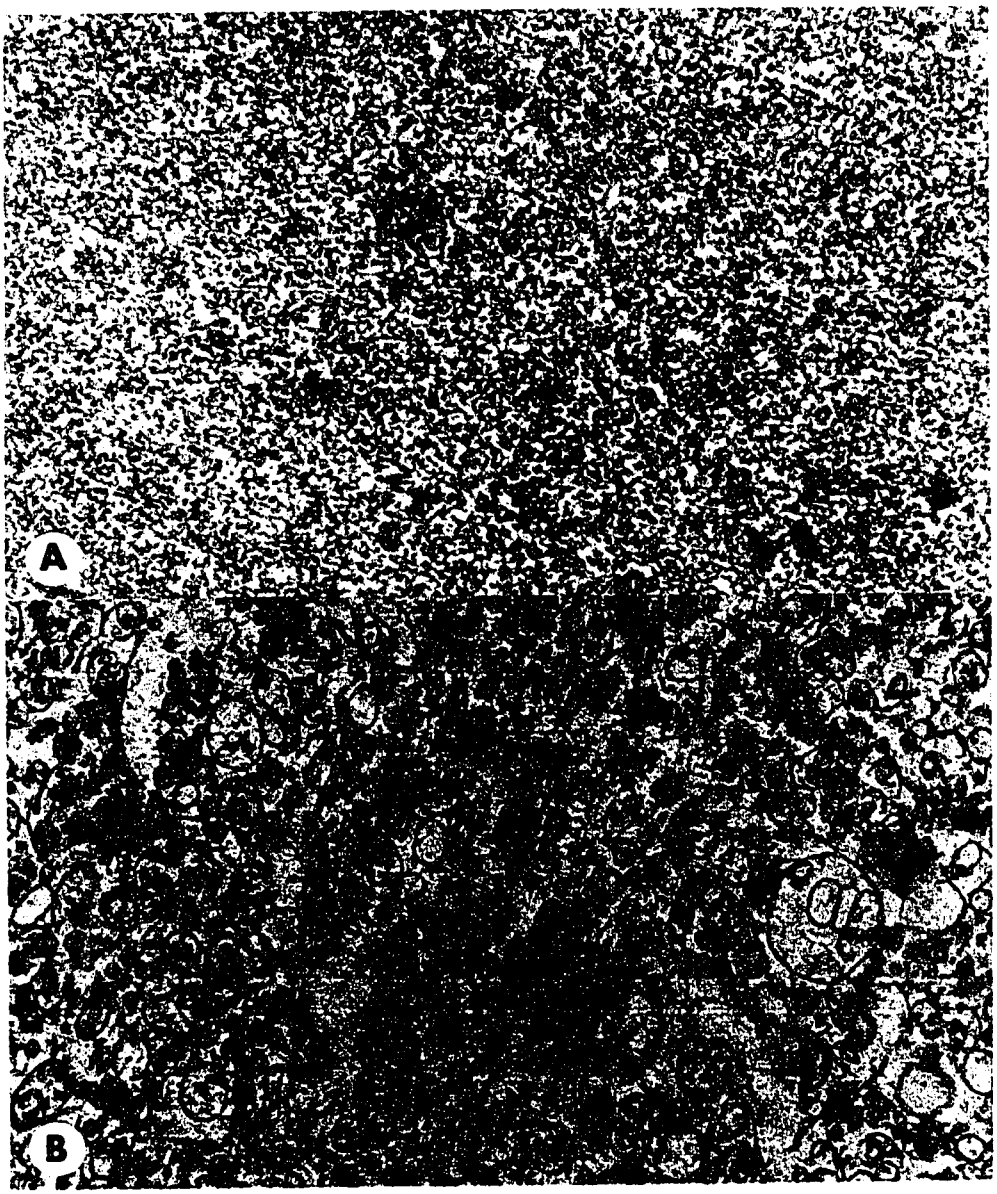


Figure 9 . Electron micrographs prepared as described in text .
A. Sonicated mitochondria : 15,000 x .
B. Lubrol treated mitochondria : 8,000 x .

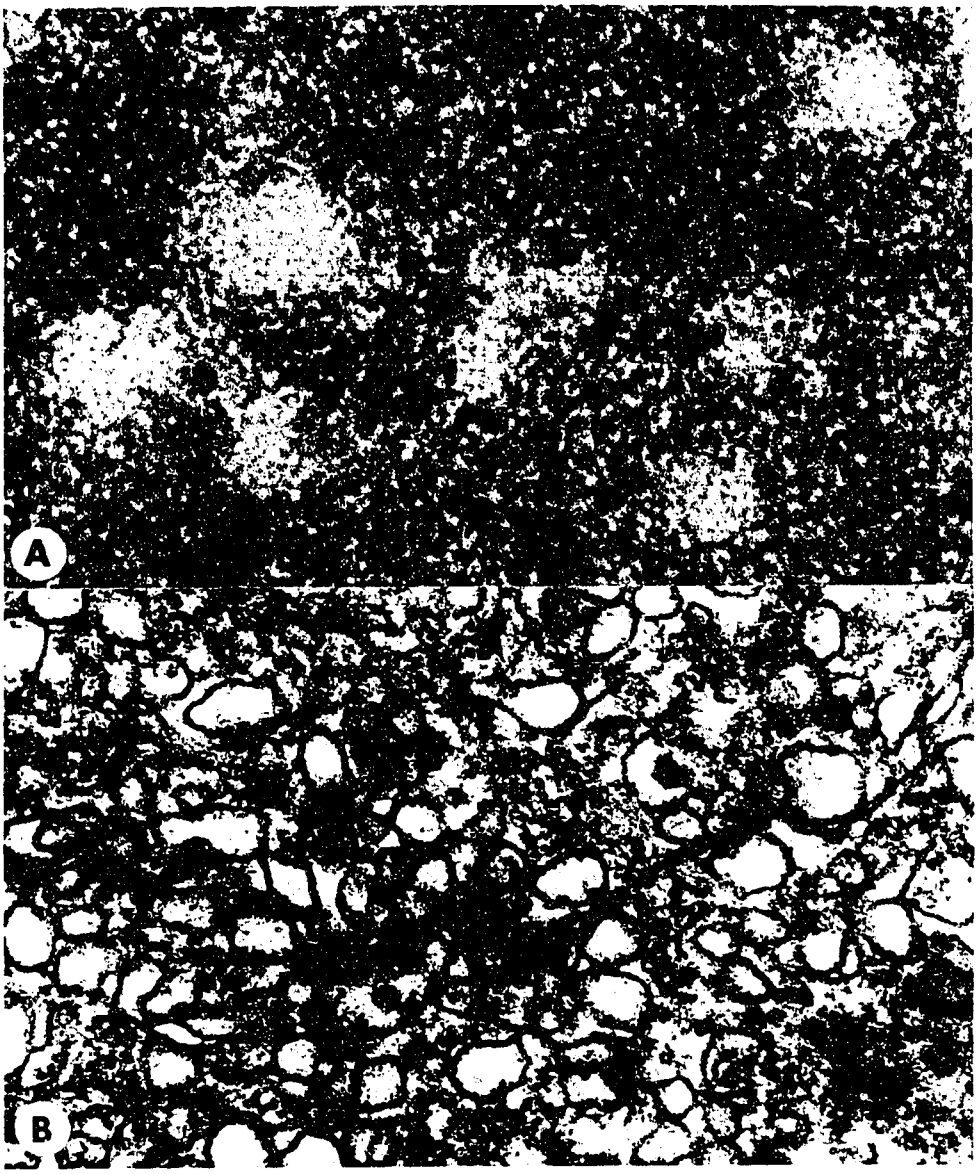


Figure 10 . Electron micrographs prepared as described in text .
A. Deoxycholate treated mitochondria (negatively stained) 400,000 x .
B. Same preparation after dialysis : 90,000 x .

§ 3. Gradient centrifugation

A mitochondrial suspension, made 5 % in Lubrol and stored in the refrigerator for 3 days, was layered on a sucrose density gradient and centrifuged as described under methods . The speed setting of the centrifuge was 60,000 rpm and the duration of the centrifugation was 15 minutes . Tables 11 and 12 show the results of the refractometry and monoamine oxidase assays in the two experiments and also the calculated values for $s^{0.725}_{20,w}$ of monoamine oxidase in this preparation : 148 and 133 . A histogram shows the distribution of the activity in the gradient after one of the centrifugations (figure 11) . From this figure as well as from figures 12 and 13 (for the other preparations it appears that the population of particles observed was far from homogeneous, if compared to the catalase experiment e.g. Since the particle population is so polydisperse, the sedimentation coefficients should be regarded as average values for a broad range of particles .

When sonicated mitochondria were centrifuged on the same 5 - 20 % gradient, all the activity was centrifuged down and found as a pellet at the bottom of the gradient, even after very short centrifugations . Therefore a higher concentration gradient was used in was used in this case : 15 - 40 % sucrose,

TABLE 11

Sedimentation rate of monoamine oxidase from Lubrol treated mitochondria.

fraction	sucrose % w/w	density	viscosity	activity cpm
12	12.1	1.049	2.228	199
13	11.4	1.046	2.173	236
14	10.7	1.043	2.117	307
15	10.1	1.041	2.074	348
16	9.4	1.038	2.022	442
17	9.0	1.036	1.994	609
18	8.5	1.034	1.961	617
19	8.1	1.033	1.934	672
20	7.7	1.031	1.908	1035
21	7.4	1.030	1.891	705
22	7.0	1.028	1.865	638

22 fractions of 13 drops & 1 fraction of 6 drops

Odometer initial : 508,048 th
508,772 th

duration : 15 minutes
T : 5 degrees
$s^{0.725}_{20,w}$: 148.6

TABLE 12

Sedimentation rate of monoamine oxidase from Lubrol treated mitochondria.

fraction #	sucrose % (w/w)	density	viscosity	activity cpm
14	11.0	1.045	2.141	179
15	10.1	1.041	2.074	283
16	9.4	1.038	2.022	348
17	8.7	1.035	1.975	508
18	8.2	1.033	1.941	587
19	7.9	1.032	1.921	641
20	7.6	1.031	1.904	703
21	7.1	1.028	1.872	687
22	6.9	1.028	1.859	486

22 fractions of 13 drops
& 1 fraction of 2 drops

Odometer initial : 508048 th.
final : 508772 th.

duration : 15 minutes
T : 5°C

$s^{0.725}_{20,w}$: 133.6

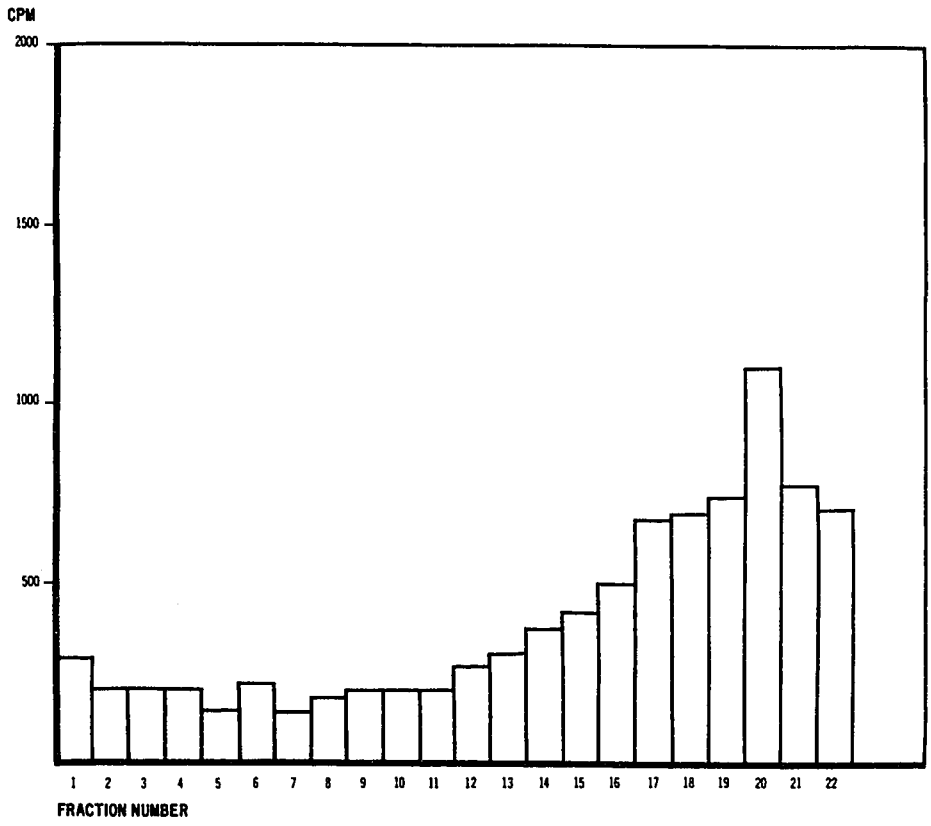


Figure 11 . Centrifugation on a sucrose gradient of Lubrol treated mitochondria .Data from table 11 .

and the speed of centrifugation was reduced to 10,000 rpm . The duration of the centrifugation was 10 minutes . Table 13 shows the results of the monoamine oxidase assay on the different fractions, while figure 12 is a histogram of the distribution of the activity over the gradient after the centrifugation . In this case the sucrose concentrations were estimated by interpolation because the refractometer was not usable in this range . The $s^{0.725}_{20,w}$ for the monoamine oxidase in this preparation was found to be 68,000 (table 13) .

The deoxycholate treated mitochondria presented a different problem in these experiments : even after a short centrifugation on the gradient, a cloudy precipitate was formed, similar to that after dialysis of this preparation . When the enzyme was first precipitated between 5 and 20 % ammonium sulfate saturation, and then redissolved in 0.02 M sodium phosphate buffer (pH 7.4), a clear solution was obtained which no longer precipitated while being centrifuged on the gradient . This was the preparation used in the gradient centrifugations reported below . The gradient was the usual 5 - 20 % sucrose . The speed of centrifugation was 60,000 rpm . The centrifugations lasted 45, 62, or 70 minutes . The results are shown in tables 14 to 22 . The values for $s^{0.725}_{20,w}$ obtained varied between 23.0 and 33.1 . These were all determined with benzylamine as substrate, and since the population of particles here was also polydisperse, the results should be considered as the average sedimentation rate for a broad range of

TABLE 13

Sedimentation rate of monoamine oxidase from sonicated mitochondria .

fraction #	sucrose * % (w/w)	density	viscosity	activity cpm
2	38.32	1.173	8.901	146
3	37.20	1.167	8.207	200
4	36.09	1.162	7.599	250
5	34.97	1.156	7.041	236
6	33.85	1.150	6.539	446
7	32.73	1.145	6.095	376
8	31.61	1.139	5.685	440
9	30.50	1.134	5.322	430
10	29.38	1.128	4.984	420
11	28.26	1.123	4.676	473
12	27.15	1.118	4.402	426
13	26.03	1.112	4.144	360
14	24.91	1.107	3.908	200
15	23.79	1.102	3.697	264
16	22.67	1.096	3.497	239
17	21.55	1.091	3.314	176
18	20.44	1.086	3.149	119
19	19.32	1.081	2.992	100
20	18.20	1.076	2.848	45
21	17.08	1.071	2.714	-
22	15.97	1.066	2.592	-

22 fractions of 11 drops
& 1 fraction of 4 drops

Odometer initial : 811236 th.
final : 811330 th.

duration : 10 minutes
T : 5°C
speed : 10000 rpm
$s^{0.725}_{20,w}$: 68,157

* by interpolation .

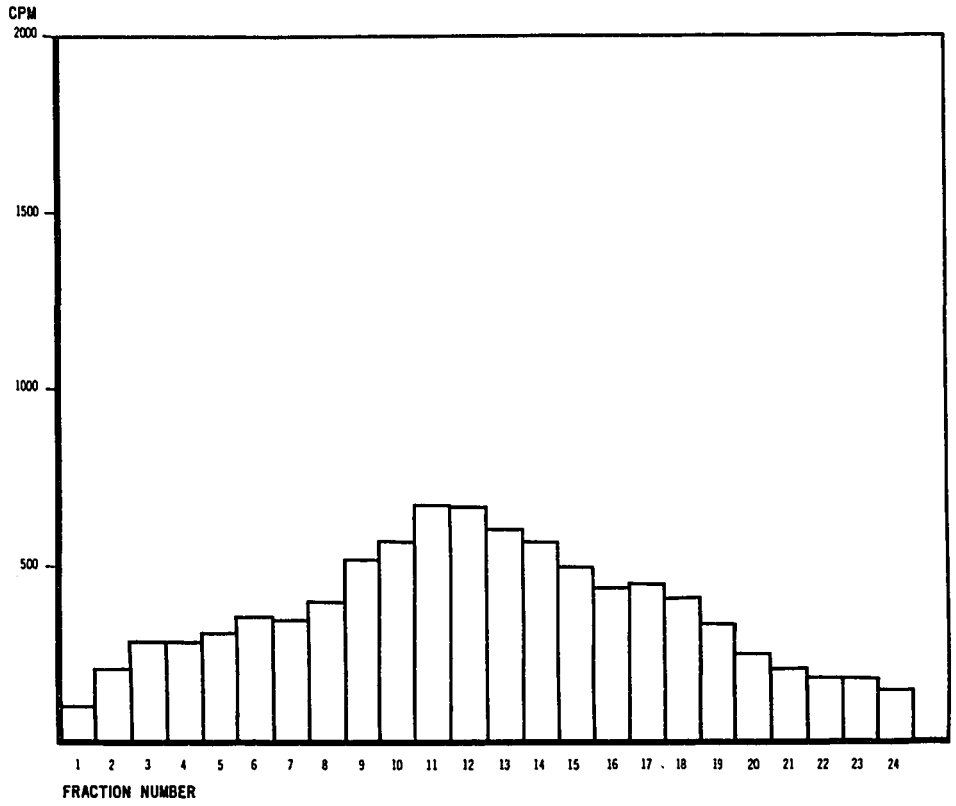


Figure 12 . Centrifugation on a sucrose gradient of sonicated mitochondria . Conditions as in table 13 .

T A B L E 14

Sedimentation rate of monoamine oxidase from deoxycholate treated mitochondria .

fraction #	sucrose %(w/w)	density	viscosity	activity cpm
15	11.1	1.045	2.149	156
16	10.6	1.043	2.109	351
17	10.0	1.041	2.066	120
18	9.5	1.038	2.029	451
19	9.1	1.036	2.001	1328
20	8.6	1.035	1.968	1079
21	8.1	1.033	1.934	1287
22	7.4	1.029	1.891	1246
23	7.1	1.028	1.872	1205
24	6.7	1.027	1.847	768
25	6.2	1.025	1.819	713
26	5.8	1.023	1.795	286

26 fractions of 15 drops
& 1 fraction of 4 drops
Odometer initial : 482540 th.
final : 485018 th.

duration : 45 minutes
T : 5°C
speed : 60,000 rpm
$s^{0.725}_{20,w}$: 31.9

T A B L E 15

Sedimentation rate of monoamine oxidase from deoxycholate treated mitochondria .

fraction #	sucrose %(w/w)	density	viscosity	activity cpm
13	11.1	1.045	2.149	258
14	10.4	1.042	2.094	263
15	9.9	1.040	2.058	514
16	9.3	1.038	2.015	820
17	8.6	1.035	1.968	1346
18	8.0	1.032	1.927	1500
19	7.4	1.030	1.890	1244
20	6.8	1.027	1.853	642
21	6.3	1.025	1.825	526
22	6.0	1.024	1.806	307

22 fractions of 16 drops
& 1 fraction of 2 drops
Odometer initial : 482540 th.
final : 485018 th.

duration : 45 minutes
T : 5°C
speed : 60,000 rpm
$s^{0.725}_{20,w}$: 33.1

TABLE 16

Sedimentation rate of monoamine oxidase from deoxycholate treated mitochondria .

fraction #	sucrose %(w/w)	density	viscosity	activity cpm
15	9.9	1.040	2.059	393
16	9.3	1.037	2.017	679
17	8.7	1.035	1.975	1220
18	8.1	1.033	1.934	1373
19	7.4	1.030	1.890	1316
20	6.9	1.028	1.959	876
21	6.3	1.025	1.825	555
22	6.0	1.024	1.806	461

22 fractions of 16 drops & 1 fraction of 1 drop

Odometer initial : 482540 th.

final : 485018 th.

duration : 45 minutes

T : 5°C

speed : 60,000 rpm

$s^{0.725}_{20,w}$: 28.6

T A B L E 17

Sedimentation rate of monoamine oxidase from deoxycholate treated mitochondria .

fraction #	sucrose %(w/w)	density	viscosity	activity cpm
11	12.4	1.051	2.254	169
12	11.7	1.048	2.195	295
13	10.8	1.044	2.125	380
14	10.6	1.043	2.109	253
15	9.5	1.038	2.029	669
16	8.9	1.036	1.989	692
17	8.4	1.034	1.954	577
18	8.2	1.033	1.941	507
19	7.9	1.032	1.921	385
20	7.3	1.029	1.884	235
21	6.9	1.028	1.859	261

21 fractions of 16 drops
& 1 fraction of 9 drops
Odometer initial : 490799 th.
final : 494275 th.

duration : 62 minutes
T : 5 °C
speed : 60,000 rpm
$s^{0.725}_{20,w}$: 29.7

T A B L E 18

Sedimentation rate of monoamine oxidase from deoxycholate treated mitochondria .

fraction #	sucrose %(w/w)	density	viscosity	activity cpm
11	11.6	1.047	2.187	207
12	10.9	1.044	2.133	277
13	10.1	1.041	2.074	729
14	9.4	1.038	2.022	525
15	8.7	1.035	1.975	715
16	8.0	1.032	1.927	783
17	7.4	1.030	1.890	587
18	6.9	1.028	1.859	332
19	6.3	1.025	1.824	160
20	6.6	1.026	1.840	180

20 fractions of 17 drops & 1 fraction of 12 drops

Odometer initial : 490799 th.

final : 494275 th.

duration ; 62 minutes

T : 5°C

speed : 60,000 rpm

$s^{0.725}_{20,w}$: 29.0

TABLE 19

Sedimentation rate of monoamine oxidase from deoxycholate treated mitoc

fraction #	sucrose %(w/w)	density	viscosity	activity cpm
13	12.7	1.052	2.280	245
14	11.9	1.048	2.212	316
15	11.1	1.045	2.149	375
16	10.3	1.042	2.086	494
17	9.8	1.040	2.051	621
18	9.1	1.037	2.001	667
19	8.4	1.034	1.954	723
20	8.4	1.034	1.954	544
21	8.0	1.032	1.028	411
22	7.7	1.031	1.908	253
23	7.2	1.029	1.878	200
24	6.4	1.026	1.829	128

24 fractions of 16 drops
& 1 fraction of 12 drops
Odometer initial : 490799 th.
final : 494275 th.

duration : 62 minutes
T : 5°C
speed : 60,000 rpm
$s^{0.725}_{20,w}$: 31.0

TABLE 20

Sedimentation rate of monoamine oxidase from deoxycholate treated mitochondria .

fraction #	sucrose % (w/w)	density	viscosity	activity cpm
11	11.8	1.048	2.203	171
12	11.1	1.045	2.149	179
13	10.8	1.044	2.125	362
14	9.7	1.039	2,044	358
15	8.9	1.036	1.989	551
16	8.2	1.033	1.940	511
17	7.6	1.030	1.903	398
18	7.0	1.028	1.865	212
19	6.5	1.026	1.835	106
20	6.0	1.024	1.807	109

20 fractions of 17 drops & 1 fraction of 14 drops

Odometer initial : 494423 th.

final : 498360 th.

duration : 70 minutes

T : 5°C

speed : 60,000 rpm

$s^{0.725}_{20,w}$: 23.0

T A B L E 21

Sedimentation rate of monoamine oxidase from deoxycholate treated mitochondria .

fraction #	sucrose % (w/w)	density	viscosity	activity cpm
10	12.1	1.049	2.228	223
11	11.4	1.046	2.173	235
12	10.7	1.043	2.117	300
13	10.0	1.040	2.066	460
14	9.3	1.038	2.015	725
15	8.6	1.035	1.968	727
16	8.1	1.033	1.934	693
17	7.4	1.030	1.890	618
18	6.9	1.028	1.859	385
19	6.4	1.026	1.829	187
20	6.4	1.026	1.829	117
21	6.0	1.024	1.807	170

21 fractions of 16 drops	duration : 70 minutes
& 1 fraction of 10 drops	T : 5°C
Odometer initial : 494423 th.	speed : 60,000 rpm
final : 498360 th.	$s^{0.725}_{20,w}$: 29.6

T A B L E 22

Sedimentation rate of monoamine oxidase of deoxycholate treated mitochondria .

fraction #	sucrose %(w/w)	density	viscosity	activity cpm
11	12.0	1.049	2.220	106
12	11.4	1.046	2.173	162
13	10.8	1.044	2.125	201
14	10.2	1.041	2.079	390
15	9.5	1.038	2.029	696
16	8.7	1.035	1.975	815
17	8.2	1.033	1.941	661
18	7.6	1.030	1.903	410
19	7.1	1.028	1.872	245
20	6.6	1.026	1.841	213
21	6.2	1.025	1.819	184

21 fractions of 16 drops
& 1 fraction of 10 drops
Odometer initial : 494423 th.
final : 498360 th.

duration : 70 minutes
T : 5°C
speed : 60,000 rpm
$s^{0.725}_{20,w}$: 25.6

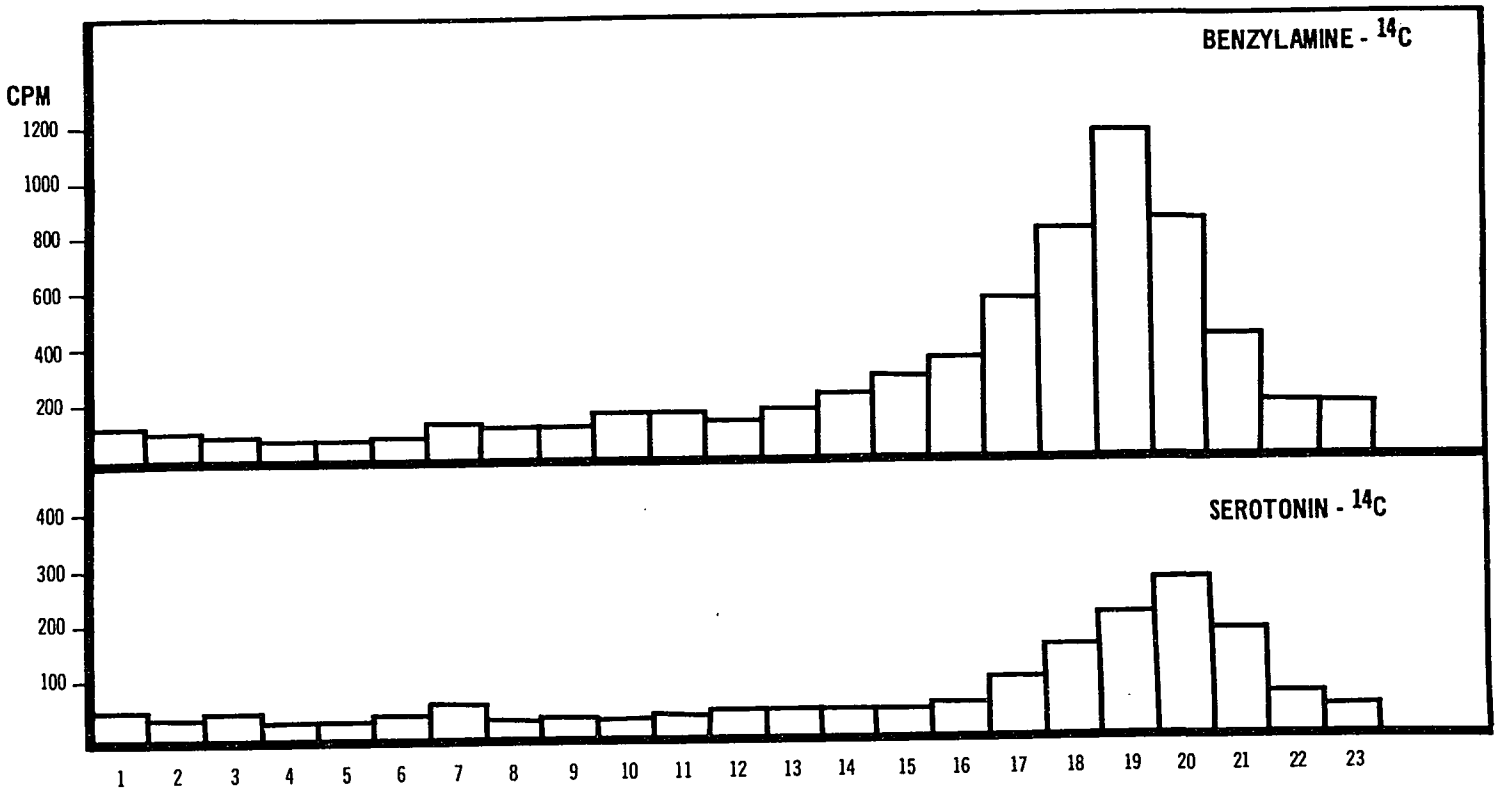


Figure 13 . Centrifugation on a sucrose gradient of deoxycholate treated mitochondria . The monoamine oxidase activity on two substrates was determined in the same gradient .

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of particles .

In order to see if there was any difference in sedimentation rate for the monoamine oxidase activity on other substrates, a similar centrifugation was performed (60 minutes at 60,000 rpm) . On the fractions of the same gradient, the activity for both benzylamine and serotonin was determined . The result in the form of a histogram is shown in figure 13 . There is a small, possibly significant difference in the position of the peak of the activity on benzylamine and the activity on serotonin . Unfortunately, when longer centrifugation times were used in an attempt to improve the resolution, too much of the activity was lost .

C. Discussion

The reports in the literature on the solubilization of monoamine oxidase from mitochondria are often confusing in that they use different criteria to determine that the enzyme was solubilized . Various authors consider as soluble the supernatant fraction after centrfugation at 12,000 x g for 30 minutes (68), 15,000 x g for 20 minutes (64), 160,000 x g for 90 minutes (65), 4,050,000 g-minutes (61), 144,000 x g for 2 hours (108) . Usually it is not clear if the indicated g-value is the average or the maximum centrifugal field . It must be said how-

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ever that in many cases the purpose of the work was purification of the enzyme and real solubilization was not essential, but the authors intended a mere extraction of the enzyme .

Since it was our intention to try and separate the multiple forms of monoamine oxidase, it was important first to obtain the enzyme as well solubilized as possible . Differential centrifugation as a criterion for the solubility of a protein is difficult to apply . Not only is it hard to compare the centrifugation in different instruments, even if the gravitational field is expressed in g or g-minutes (130) . But also, even if a particle is sedimenting at a fairly rapid rate, it may not be packed into a solid pellet and be confounded with the supernatant . It seemed that centrifugation on a sucrose gradient was easier to put into quantitative terms, and that an electron microscopic study would be a good complement and facilitate the interpretation of the results .

In a first screening experiment different methods of solubilization were compared, including sonication and different detergents (table 7) . Deoxycholate treatment immediately emerged as the most promising method . Therefore some additional experiments were done in order to determine the optimum conditions for this method . It was found that deoxycholate has a tendency to cause rather quick denaturation of the enzyme, but this effect could be slowed down at least partially by having benzylamine present in the medium when the deoxycholate was added

to the suspension of mitochondria (table 8) . Even then the enzyme was much more sensitive to heat denaturation than the native enzyme (table 10) . Therefore care was taken to keep all the solutions at 4 degrees or less in handling the preparation .

Three of the solubilization methods were further tested by density gradient centrifugation and electron microscopy : sonication, Lubrol treatment and deoxycholate treatment . Under the electron microscope the sonicated mitochondria appeared as particles of 300 - 700 Å. This would indicate that the sonication was not very effective in our hands, since Verevkina et al. (59) obtained particles of 50 - 100 Å . This impression is confirmed when we look at the density gradient centrifugation of the sonicated mitochondria . The value for $s^{0.725}_{20,w}$ for this preparation was 68,000 . This is of the order of magnitude of the sedimentation coefficient of whole mitochondria (131) . This would mean that the sonication technique as used by us was inefficient in that the particles obtained were still quite large, but also that after sonication the active monoamine oxidase was still largely confined to the unbroken mitochondria . Either a relatively minor fraction of the mitochondria were actually disrupted by the sonication or the enzyme was inactivated in those mitochondria that were disrupted .

The electron microscopic image of the Lubrol-treated mitochondria shows that this treatment was a very gentle

one : the mitochondrial membranes, though disrupted, still had a considersble size and integrity . The sedimentation coefficient for the monoamine oxidase in this preparation is relatively low . The explanation for this may be that the mitochondria by rupturing increased their surface and thus their resistance to sedimentation without changing their density . Or else the monoamine oxidase activity was not contained in the bigger membranes seen under the electron microscope, but in some smaller structures .

The sedimentation coefficient of the deoxycholate - treated enzyme was much lower . It is of the order of magnitude of that of big protein complexes . It should be realized of course that the density of the particles may be considerably lowered through micelle formation with the lower density deoxycholate . This would also lower the sedimentation rate and give the false impression of a smaller particle . When we look at the electron microscopic pictures however, the dimensions of the observed particles agree rather well with the measured sedimentation rates . We may thus conclude that deoxycholate treatment of all the methods studied, disrupts the mitochondria into the smallest fractions . Therefore this was the method chosen for the preparation of the sample for an attempt at the separation of the multiple forms of mitochondrial monoamine oxidase . The problem of the denaturation of the enzyme could be overcome (at least partially) by working at low temperature and by dissolving the mitochondria in the presence of benzylamine .

II. Separation

A. Materials and methods

§ 1. Preparations and electrophoresis

All chemicals used in this section were purchased from Fisher, unless indicated otherwise .

The enzyme solution was prepared from mitochondria isolated from fresh rat liver as described above . The mitochondria from 4 g of fresh rat liver were resuspended in 16 ml sodium phosphate buffer (0.2 M, pH 7.4) which was 0.003 M in Benzylamine (Eastman Organic Chemicals) . To this suspension 192 mg of deoxycholate, dissolved in 1 ml water, were added and the solution stirred for 10 minutes . During the whole procedure the enzyme was kept on ice . The preparation was then centrifuged in a refrigerated Servall centrifuge (4 degrees) at 30,000 x g for 15 minutes . From the supernatant 20 µl were applied per gel .

Of the methods used for the separation of proteins, electrophoresis on polyacrylamide gel is capable of relatively high resolution . This is due to the molecular sieving effect which adds on to the inherent resolving power of the electrophoresis technique . Furthermore the discontinuous gel and

buffer system which was described by Ornstein and Davis (133) gives an additional improvement in resolution because the protein sample is concentrated into a very thin starting zone before the actual separation . The technique as originally described was limited however to a certain range of protein molecules, because of the small pore size of the gel . This made it less suitable for lager proteins or protein complexes . A modification by Dingman and Peacock (134) provided a solution to this problem . By the incorporation of 0.5 % agarose into the polyacrylamide gel it was possible to lower the concentration of the acrylamide and still obtain solid, easy to handle gels . These gels produced an excellent resolution for higher molecular weight compounds . A set of preliminary experiments had determined the optimal concentration for our separation .

The following solutions were prepared, which could be stored in the refrigerator for weeks (except B, which had to be prepared every day, and I, which was prepared fresh every week) .

A. Acrylamide monomer

19 g acrylamide (Eastman Organic Chemicals)

1 g N,N'-methylene bisacrylamide (")

water to 100 ml .

B. Ammonium persulfate

0.5 g ammonium persulfate

water to 100 ml .

C. TEMED

0.287 ml N,N,N',N'-tetramethylethylene diamine
(Eastman Organic Chemicals)

water to 100 ml

D. separating gel buffer

60 ml 1 N HCl

45.375 g tromethamine (tris)

water to 100 ml .

E. acrylamide

5 g acrylamide

1.25 g N,N'-methylene bisacrylamide

water to 50 ml .

F. riboflavin

2 mg riboflavin

water to 50 ml .

G. stacking gel buffer

24 ml 1 N HCl

2.99 g tromethamine

0.23 ml TEMED

water to 50 ml .

H. eluting buffer

288 g glycine

60 g tromethamine

water to 2 l .

I. sucrose

40 g sucrose

water to 100 ml .

J. tracking dye

2.5 mg bromophenol blue

water to 50 ml .

A Canalco model 12 gel electrophoresis apparatus was used . To make the gels, 125 mg agarose (special grade, Mann Research Laboratories) was dissolved into 12.5 ml of the 40 % sucrose solution (I) by boiling for 10 to 15 minutes . The solution was then cooled to 40 degrees . Meanwhile, in a separate erlenmeyer were added together 2.5 ml buffer (D), 2.5 ml TEMED (C) and 2.5 ml acrylamide solution (A) . This mixture was warmed to 40 degrees and then both solutions were added together . Then 2.5 ml of the ammonium persulfate solution (B) were stirred in quickly and the solution was introduced by means of a syringe to the glass tubes sitting in their stand . The tubes were filled to within about 1 cm from the top . The solution was then overlayered with water to create a flat horizontal surface on top of the gel . The polymerization occurred in about 10 minutes . At the same time the cooling causes the agarose to gel and the tubes were left standing at room temperature for a total time of about 30 minutes .

Then the layer of water was removed and replaced by the stacking gel solution . This mixture was prepared by adding 1 part buffer (G) to 1part riboflavin (F) and 2 parts acrylamide (E) and 4 parts sucrose (I) . The tubes were filled to 0.5 cm from the top and water was layered on top . The polymerization of the stacking gel was triggered by light : the tubes were left in front of a fluorescent light (at ±10 cm) for 20 minutes . When the polymerization started the stacking gel solution became slightly cloudy . A additional 10 minutes were then allowed for completion of the polymerization . When the gels were ready for electrophoresis the tubes were taken out of their stand and fixed in the top buffer reservoir .

The two reservoirs were filled with eluting buffer (H, diluted 10 x) . To the top buffer reservoir 0.5 ml of tracking dye (J) was added . Care was taken to expel air bubbles from the top of the tubes . The enzyme solution (20 µl) was applied to each gel by introducing the tip of a micropipette below the surface of the buffer and gently layering the solution onto the top of the gel . The electrophoresis was then started at a constant current of 2 mAmp per gel, with the positive electrode connected to the bottom buffer reservoir . The progress of the electrophoresis could easily be followed by watching the disc of tracking dye form in the stacking gel and then move into the separating gel in front of the protein bands . When this tracking

disc had travelled about 3 cm into the separating gel (after about 1 ½ hours), the current was turned off and the gels were removed from the glass tubes by pushing them from the top end with a "Q-tip" .

§ 2 . Assays

The gels were cut, using the Canalco gel slicer, into discs of about 25 mg . Each slice was put into a microfuge tube ; a short centrifugation forced them down into the tip of the tube . The monoamine oxidase activity was detected using labelled substrates, as described in part 1 , except that no buffer was added . The extraction and counting were done as described in part 1 . Since the gel slices were not homogenized nor eluted for the assay, the mixing could not be very efficient, but it was hoped that the diffusion of substrate and products would be sufficiently fast to make the detection of the activity possible . Actually a preliminary experiment showed that this assay directly on the gel slices was about 50 % as effective as the one on enzyme dissolved in buffer .

In another experiment 50 µl was applied to each gel and twelve of them were run simultaneously . After the electrophoresis the gels were cut into two parts in such a way that the two peaks of activity detected by the direct assay each fell on one side of the division . The respective parts were then pooled .

The fraction containing the tracking dye and the fastest moving peak of activity was labelled fraction I, the other fraction II . Both fractions were homogenized in a glass homogenizer in 30 ml of 0.02 M sodium phosphate (pH 7.4) . The particles of the gels were then centrifuged off and the protein in the two eluates was then concentrated by ultrafiltration in an Amicon 401 cell . An UM-10 membrane with a cutoff at MW 10,000 was used at a pressure of 100 psi . When the volume of the solution was reduced to about 2-3 ml it was assayed for monoamine oxidase activity on the substrates benzylamine-^{14}C and serotonin-^{14}C by the method described in part 1 .

B. Results

The direct assay on the gel slices revealed two peaks of activity for benzylamine-^{14}C, and only one for serotonin-^{14}C . These results are shown in the form of a histogram (figure 14) . One of the enzyme peaks seems to attack both benzylamine and serotonin whereas the other one is essentially active on benzylamine alone . The assays after elution confirm this finding : fraction I is active on both benzylamine and serotonin, fraction II on benzylamine only (table 23) .

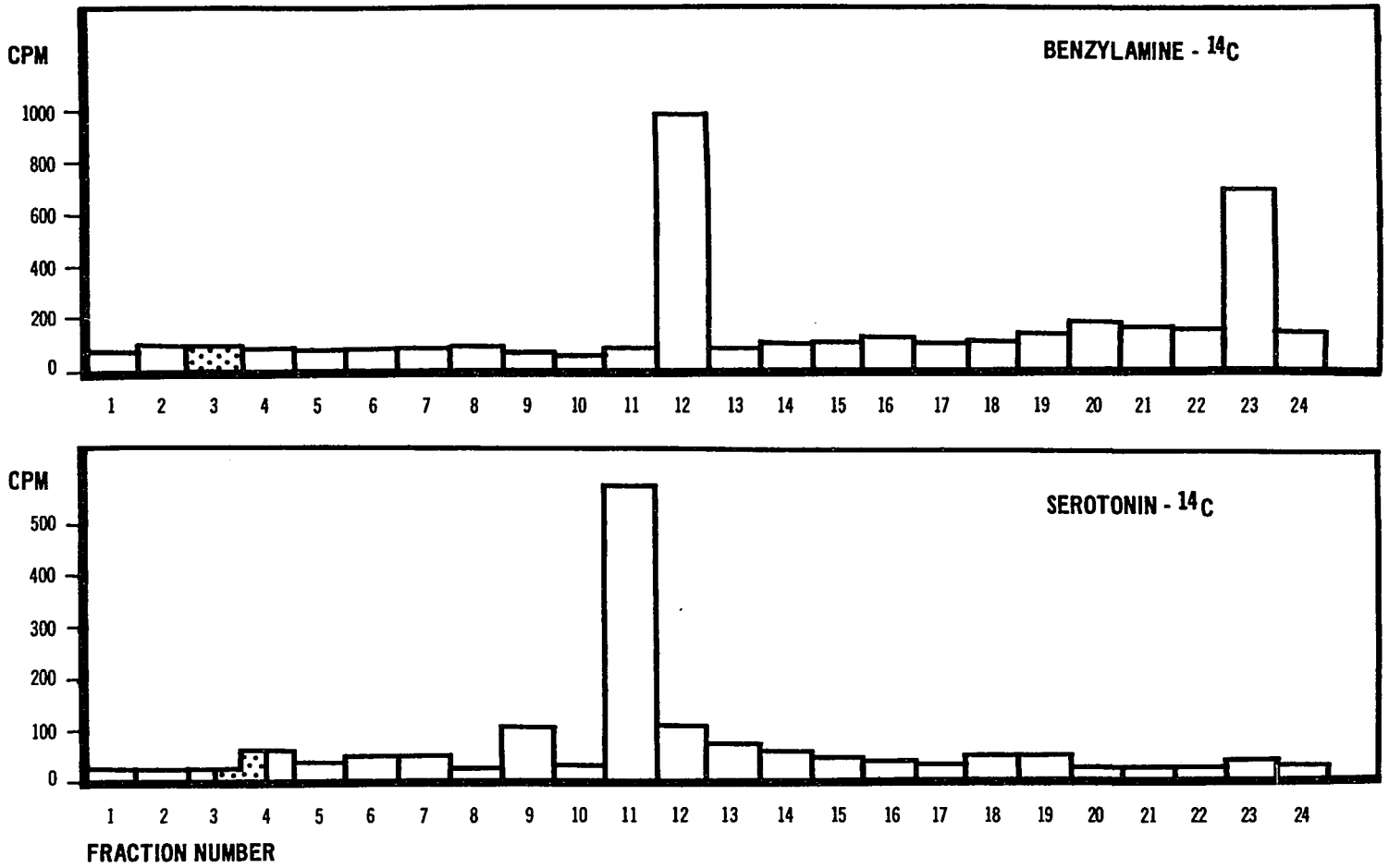

Figure 14 . Disc electrophoresis of mitochondrial monoamine oxidase . The activity was assayed directly on the gel fractions . The marked fractions contained the tracking dye .

TABLE 23

Disc electrophoresis of deoxycholate dissolved mitochondria : monoamine oxidase assay after elution .*

substrate	blank cpm†	fraction 1 cpm†	fraction 2 cpm†
benzylamine-^{14}C	30	76.5	451.5
serotonin-^{14}C	14.9	117	18

* Electrophoresis, elution and assay described in text .

† The activities shown are the mean of four determinations, and are expressed as the net cpm (background substracted) in 60 µl of extraction solvent .

C. Discussion

The problem of the separation of the two forms of mitochondrial monoamine oxidase was complicated by two factors : first the enzyme is very insoluble or very tightly bound to the membrane structure of the mitochondrion ; second, the existing methods for the detection of monoamine oxidase activity in situ after electrophoresis did not have the required specificity . In this work we have attempted to find a solution to these problems . The deoxycholate solubilization seems to be satisfactory in as far as it breaks the mitochondria into very small particles, but it has problems of its own . The enzyme becomes very labile after treatment with deoxycholate (e.g. increased sensitivity to denaturation by heat) and the removal of the deoxycholate (by dialysis, gel filtration or apparently even centrifugation on a density gradient) causes the enzyme to aggregate into big vesicle-like structures . Nevertheless this solubilization method permitted us to use the gel electrophoresis technique for separation of the multiple forms of the enzyme, leaving enough enzyme activity to be detected in the gel after electrophoresis, or even after elution from the gel .

The methods for detection of monoamine oxidase activity in situ or after electrophoresis that have been used were

mostly based on the use of nitroblue tetrazolium as electron acceptor (13, 43, 47, 103), usually with phenazine methosulfate as intermediary electron carrier . This dye reduction system has been used and found to be quite satisfactory for the detection of dehydrogenases like lactic dehydrogenase in blood plasma (135) . However when the same system is used in tissue slices or other preparations containing mitochondria, the problem of non-specific staining becomes important (48) . Furthermore when this system is applied for the detection of monoamine oxidase, there is some doubt wether the reduction of the dye is due to the monoamine oxidase activity itself or rather to the further oxidation of the product aldehyde by aldehyde dehydrogenase (15,2) . It has not been demonstrated conclusively that oxygen can be replaced by alternate electron acceptors for monoamine oxidase . Also the lag period which is observed when the tetrazolium reduction by monoamines is carried out in vitro and the necessary presence of NAD as cofactor (14), both in contrast with normal monoamine oxidase behaviour, would confirm the view that the tetrazolium reduction system is not a direct assay of this enzyme (2) .

It thus became one of our goals to find a sensitive, specific assay which could be conveniently applied to a number of fractions after electrophoresis . The method described by Otsuka and Kobayashi (43) was simple, highly specific and sensitive . It could easily be adapted to small volumes . This is the method we decided to use, with the modifications which we

described above .

The electrophoresis experiments showed that it was possible to separate two bands of monoamine oxidase activity from rat liver mitochondria . One band contains an enzyme which is active on benzylamine and serotonin, the other band consists of an enzyme which is active on benzylamine but not on serotonin . The fact that the deoxycholate treatment greatly decreases the resistance to denaturation of mitochondrial monoamine oxidase and that the total activity recovered after electrophoresis represents only a small fraction of the activity applied to the column, suggests the possibility that the two bands of activity are due to the same enzyme, altered in two different ways by the treatment . Then fraction II could be an aggregate of fraction I, in a similar way to that observed by Yasunobu et al. (115, 116) . It would be hard to explain however how the deoxycholate treatment would alter the substrate specificity in a different way in the two fractions . The simplest explanation is of course that the two bands of activity correspond to two different enzymes, each of which has its own substrate specificity .

Conclusion

We have discussed above the different possible explanations for the results of the experiments on the kinetics of mitochondrial monoamine oxidase as well as for the results of the separation experiments . It appears that one simple model is compatible with all our experimental data as well as with the data available in the literature : there are at least two different monoamine oxidases present in rat liver mitochondria . Each has its own substrate specificity, inhibitor specificity, resistance to denaturation, physicochemical properties and possibly different prosthetic group, and maybe even a different physiological function . This conclusion is not in disagreement with the literature, since it has never been shown that the mitochondrial monoamine oxidase consisted of only a single enzyme, and many authors have argued the possibility that multiple monoamine oxidases exist in mitochondria . But ever since Blaschko et al. in 1937 concluded that monoamines were probably oxidized by one type of enzyme, it has been considered as a single enzyme in many studies on the assay, inhibition, purification, physiological role etc... If our conclusion is confirmed, some of these studies may have to be reconsidered . We have not used the term "isoenzymes", though this term has been used rather freely (135), because the two enzymes

described here are quite different in substrate specificity . Isoenzymes would refer to enzymes which are different in physico-chemical properties, but whose differences in catalytic properties are small and incidental . Until the enzymes described here are better defined in terms of substrate specificity, enzymatic properties and prosthetic groups, it may be better to refer to them as multiple forms of mitochondrial monoamine oxidase .

To summarize our original work :

- We have compared the affinity as substrates of benzylamine and serotonin for rat liver mitochondrial monoamine oxidase with their affinity as inhibitors . This was possible through our use of a mixed substrate experiment where the substrates were alternately used in their ^{14}C-labelled form and in their non-labelled form . For the substrate serotonin the apparent Km was different from the apparent Ki, for benzylamine there was no difference . These findings are compatible with a two enzyme model .
- The fact that we found no difference between the apparent Km and the apparent Ki for benzylamine is in contradiction with the theory of Zeller (100) on the eutopic and dystopic complexes of substrates and inhibitors of monoamine oxidase .

- The kinetics of the monoamine oxidase activity (rat liver mitochondria) on benzylamine-^{14}C in the presence of various concentrations of cold serotonin can be explained by the two enzyme model .
- The kinetics of serotonin oxidation by rat liver mitochondria are also in agreement with the two enzyme model .
- Of the different methods used for solubilization of mitochondrial monoamine oxidase that were studied, deoxycholate treatment in the conditions described gives the best solubilization in terms of the size of the particles seen by electron microscopy or the sedimentation rate of the dissolved monoamine oxidase . Allthough this treatment reduces the stability of the enzyme, certain precautions permit the use of this preparation for small scale separation .
- Following solubilization, the enzyme activity can be separated into two bands by gel electrophoresis, as detected by a sensitive, specific radioassay . The substrate specificities of these enzyme bands are in agreement with the properties derived from the kinetic behaviour in whole mitochondria .

We hope that this work may lead to the separation in larger amounts and more stable form of the multiple mitochondrial monoamine oxidases . This would facilitate their characterization and further study .

APPENDIX I

```
FORTRAN IV G LEVEL 1, MOD 3            MAIN                DATE = 69251

      C
      C     PROGRAM KINET
      C
0001        DIMENSION V(110),A(100),W(100),S(3,4),
           CO(3),SM(3),SS(3),Y(4),VR(50)
0002        WRITE(3,100)
0003      100 FORMAT(1H1)
0004        JJ=0
0005       14 READ(1,11) NP,CI,DATE
0006       11 FORMAT(I2,F12.6,F6.2)
0007        IF (NP) 99,99,12
0008       12 M=1
0009        N=2
0010        P=NP-N
0011        N1=N+1
0012        N2=N+2
0013        READ(1,1) (A(I),I=1,NP)
0014        1 FORMAT(6F10.9)
0015        READ(1,70) (V(I),I=1,NP)
0016       70 FORMAT(6F10.2)
0017        GO TO 2
      C
      C     PRELIMINARY FIT
      C
0018       15 Q(I)=V(I)**2/A(I)
0019        Q(2)=V(I)**2
0020        Q(3)=V(I)
0021        W(I)=1
0022        VR(I)=1/V(I)
0023        GO TO 13
0024       16 CK=S(1,1)/S(2,1)
0025        JJ=JJ+1
0026        WRITE(3,61)JJ,CI,DATE
0027       61 FORMAT(1X,I3,F12.6,' M INHIBITOR      DATE  ',F6.2,'  1959'
0028        WRITE(3,65)(V(I),I=1,NP)
0029        WRITE(3,65)(VR(I),I=1,NP)
0030       65 FORMAT(1X,6E14.5)
0031        NT=0
0032        M=2
0033        GO TO 2
      C
      C     ITERATIVE FIT  TO HYPERBOLA
      C
```

```
FTRAN IV G LEVEL 1, MOD 3            MAIN                DATE = 69251          1

0034          17 D=CK+A(I)
0035             Q(1)=A(I)/D
0036             Q(2)=Q(1)/D
0037             Q(3)=V(I)
0038             GO TO 13
0039          18 CK=CK-S(2,1)/S(1,1)
0040             NT=NT+1
0041             IF (NT-5) 2,21,21
0042          21 S2=0
0043             DO 22 I=1,NP
0044          22 S2=S2+(V(I)-S(1,1)*A(I)/(CK+A(I)))**2*W(I)
0045             S2=S2/P
0046             S1=SQRT(S2)
0047             SL=CK/S(1,1)
0048             VINT=1./S(1,1)
0049             VK=1./SL
0050             DO 10 J=2,N1
0051             DO 10 K=1,N
0052          10 S(K,J)=S(K,J)*SM(K)*SM(J-1)
0053             SEV=S1*SQRT(S(1,2))
0054             SECK=S1*SQRT(S(2,3))/S(1,1)
0055             SEVI=SEV/S(1,1)**2
0056             S(1,3)=S1*SQRT(CK**2*S(1,2)+S(2,3)+2.*CK*S(1,3))
0057             SESL=S(1,3)/S(1,1)**2
0058             SEVK=S(1,3)/CK**2
0059             WCK=1./SECK**2
0060             WV=1./SEV**2
0061             WSL=1./SESL**2
0062             WVI=1./SEVI**2
0063             WVK=1./SEVK**2
0064             WRITE(3,30) CK,SECK,WCK
0065             WRITE(3,31) S(1,1),SEV,WV
0066             WRITE(3,32) SL,SESL,WSL
0067             WRITE(3,33) VINT,SEVI,WVI
0068          30 FORMAT(1X,'K    = 'E11.5,'   S.E.(K)    = ',E11.5,'   W = ',E1
0069          31 FORMAT(1X,'V    = ',E11.5,'   S.E.(V)    = ',E11.5,'   W = ',E
0070          32 FORMAT(1X,'K/V  = ',E11.5,'   S.E.(K/V)  = ',E11.5,'   W = ',E
0071          33 FORMAT(1X,'1/V  = ',E11.5,'   S.E.(1/V)  = ',E11.5,'   W = ',E
0072             GO TO 14
           C
           C
           C     MATRIX SOLUTION SUBROUTINE
           C
```

```
FTRAN IV G LEVEL 1, MOD 3            MAIN                DATE = 69251

0073            2 DO 3 J=1,N2
0074              DO 3 K=1,N1
0075            3 S(K,J)=0
0076              DO 4 I = 1,NP
0077              GO TO(15,17),M
0078           13 DO 4 J=1,N1
0079              DO 4 K=1,N
0080            4 S(K,J) = S(K,J)+Q(K)*Q(J)*W(I)
0081              DO 5 K= 1,N
0082            5 SM(K)=1./SQRT(S(K,K))
0083              SM(N1)=1.
0084              DO 6 J=1,N1
0085              DO 6 K=1,N
0086            6 S(K,J) = S(K,J)*SM(K)*SM(J)
0087              SS(N1)=-1.
0088              S(1,N2)=1.
0089              DO 8 L=1,N
0090              DO 7 K=1,N
0091            7 SS(K)=S(K,1)
0092              DO 8 J=1,N1
0093              DO 8 K=1,N
0094            8 S(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1)
0095              DO 9 K=1,N
0096            9 S(K,1)=S(K,1)*SM(K)
0097              GO TO (16,18),M
0098           36 FORMAT(32H PROGRAM COMPLETED - END OF DATA )
0099           99 WRITE(3,36)
0100              STOP
0101              END
```

APPENDIX II

```
FORTRAN IV G LEVEL 1, MOD 3            MAIN                DATE = 69269          1

      C  PROGRAM 2/1 HYPERBOLA
      C
      C                    FIT TO HYPERBOLA Y = A*(1+B*X+C*X**2)/(1+D*X)
      C
0001        DIMENSION X(20),Y(20),W(20),S(5,6),Q(5),SM(5),SS(5)
0002        WRITE(3,100)
0003      100 FORMAT(1H1)
0004        JJ=0
      C
      C  READ IN DATA AND PRELIMINARY ESTIMATES OF CONSTANTS
      C
0005       14 READ(1,11) NP,DATE
0006       11 FORMAT(I2,F6.2)
0007          IF (NP) 99,99,12
0008       12 READ(1,20) (X(I),I=1,NP),(Y(I),I=1,NP),
             Z(W(I),I=1,NP),(A,B,C,D)
0009       20 FORMAT (6E10.5)
0010          JJ=JJ+1
0011          N=4
0012          P=NP-N
0013          N1=N+1
0014          N2=N+2
0015          NT=0
0016          WRITE(3,25) JJ,DATE
0017       25 FORMAT (1X,' EXPERIMENT NO 'I3,',   DATE 'F5.2,'  1969'//)
0018          WRITE (3,26) (X(I),I=1,NP),(Y(I),I=1,NP),(W(I),I=1,NP)
0019       26 FORMAT(1X,11E11.4)
0020          WRITE(3,27) A,B,C,D
0021       27 FORMAT(1X,' PRELIMINARY ESTIMATES '/,
             Z ' A = 'E11.5,' B = 'E11.5,' C = 'E11.5,' D = 'E11.5//)
0022          GO TO 2
      C
      C  ITERATIVE FIT
      C
0023       17 DEN=1+D*X(I)
0024          Q(1)=(1+B*X(I)+C*X(I)**2)/DEN
0025          Q(2)=X(I)/DEN
0026          Q(3)=X(I)**2/DEN
0027          Q(4)=-(1+B*X(I)+C*X(I)**2)*X(I)/DEN**2
0028          Q(5)=Y(I)
0029          IF (W(I)) 19,19,22
0030       19 W(I)=1
0031       22 GO TO 13
```

```
RAN IV G LEVEL 1, MOD 3            MAIN                DATE = 69269

2           18 DIFB=S(2,1)/S(1,1)
3              DIFC=S(3,1)/S(1,1)
4              DIFD=S(4,1)/S(1,1)
5              A=S(1,1)
6              B=B+DIFB
7              C=C+DIFC
8              D=D+DIFD
9              NT=NT+1
0              WRITE(3,300) NT,A,B,DIFB,C,DIFC,D,DIFD
1          300 FORMAT(1X,' ITERATION NO 'I3/,
              Z1X,' A = 'E11.5,' B = 'E11.5,' DIF 'E11.5,' C = 'E11.5,
              Z' DIF 'E11.5,' D = 'E11.5,' DIF 'E11.5//)
2              IF (NT-5) 2,31 ,31
        C
        C  CALCULATE STANDARD ERRORS AND WEIGHTS
        C
3           31 S2=0
4              DO 32 I=1,NP
5           32 S2=S2+(Y(I)-S(1,1)*(1+B*X(I)+C*X(I)**2)/(1+D*X(I)))**2
6              S2=S2/P
7              S1=SQRT(S2)
8              DO 10 J=2,N1
9              DO 10 K=1,N
0           10 S(K,J)=S(K,J)*SM(K)*SM(J-1)
1              SEA=S1*SQRT(S(1,2))/S(1,1)
2              SEB=S1*SQRT(S(2,3))/S(1,1)
3              SEC=S1*SQRT(S(3,4))/S(1,1)
4              SED=S1*SQRT(S(4,5))/S(1,1)
5              WA=1./(SEA**2)
6              WB=1./(SEB**2)
7              WC=1./(SEC**2)
8              WD=1./(SED**2)
9              WRITE(3,40) A,SEA,WA
0              WRITE(3,41) B,SEB,WB
1              WRITE(3,42) C,SEC,WC
2              WRITE(3,43) D,SED,WD
3           40 FORMAT(1X,' A = 'E11.5,' SEA = 'E11.5, ' WA = 'E11.5)
4           41 FORMAT(1X,' B = 'E11.5,' SEB = 'E11.5,' WB = 'E11.5)
5           42 FORMAT(1X,' C = 'E11.5,' SEC = 'E11.5,' WC = 'E11.5)
6           43 FORMAT(1X,' D = 'E11.5,' SED = 'E11.5,' WD = 'E11.5)
7              WRITE(3,100)
8              GO TO 14
        C
```

```
FORTRAN IV G LEVEL 1, MOD 3            MAIN                DATE = 69269          1

      C
      C     MATRIX SOLUTION SUBROUTINE
      C
      C
0069        2 DO 3 J=1,N2
0070          DO 3 K=1,N1
0071        3 S(K,J)=0
0072          DO 4 I = 1,NP
0073          GO TO 17
0074       13 DO 4 J=1,N1
0075          DO 4 K=1,N
0076        4 S(K,J) = S(K,J)+Q(K)*Q(J)*W(I)
0077          DO 5 K= 1,N
0078        5 SM(K)=1./SQRT(S(K,K))
0079          SM(N1)=1.
0080          DO 6 J=1,N1
0081          DO 6 K=1,N
0082        6 S(K,J) = S(K,J)*SM(K)*SM(J)
0083          SS(N1)=-1.
0084          S(1,N2)=1.
0085          DO 8 L=1,N
0086          DO 7 K=1,N
0087        7 SS(K)=S(K,1)
0088          DO 8 J=1,N1
0089          DO 8 K=1,N
0090        8 S(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1)
0091          DO 9 K=1,N
0092        9 S(K,1)=S(K,1)*SM(K)
0093          GO TO 18
0094       36 FORMAT(32H PROGRAM COMPLETED - END OF DATA )
0095       99 WRITE(3,36)
0096          STOP
0097          END
```

APPENDIX III

```
FORTRAN IV G LEVEL 1, MOD 3            MAIN                DATE = 69272

              C  PROGRAM PLOT HYPERBOLA 2/1
0001              1 WRITE(3,100)
0002            100 FORMAT(1H1)
0003                READ(1,10) A,B,C,D,XIN,YMX
0004             10 FORMAT(6E10.5)
0005                IF(A) 99,99,2
0006              2 CONTINUE
0007                WRITE(3,11)
0008             11 FORMAT(5X,' X ',20X,' Y   (2/1 HYPERBOLA) '//)
0009                X=-XIN
0010                DO 120 I=1,110
0011                X=X+XIN
0012                Y=A*(1+B*X+C*X**2)/(1+D*X)
0013                CALL NPLOT(X,Y,1,YMX,0,100)
0014            120 CONTINUE
0015                GO TO 1
0016             99 RETURN
0017                END
```

```
FORTRAN IV G LEVEL 1, MOD 3            NPLOT                DATE = 69272          (
0001                  SUBROUTINE NPLOT(X,Y,NFCNS,YMAX,YMIN,NWIDTH)
0002                  DIMENSION Y(4),CHAR(109)
0003                  INTEGER CHAR
0004                  XN=NWIDTH
0005                  NEST = -1
0006                  N = NWIDTH+1
0007                  DO 120 I = 1,N
0008              120 CHAR(I) = 1077952576
0009                  IF(NFCNS - 1)200,200,140
0010              140 NEST= 1
0011              200 SCALE=XN/(YMAX-YMIN)
0012                  NZ=-YMIN*SCALE+.5
0013                  IF(NZ)300,220,220
0014              220 IF(NZ - NWIDTH)240,240,300
0015              240 NZ = NZ+1
0016                  CHAR(NZ) = -918536128
0017              300 DO 400 I = 1,NFCNS
0018                  NPOS=SCALE*(Y(I)-YMIN)+.5
0019                  IF(NPOS)400,320,320
0020              320 IF(NPOS - NWIDTH)340,340,400
0021              340 NPOS = NPOS+1
0022                  IF(I-1)350,350,355
0023              350 CHAR(NPOS) = 1262501952
0024                  GO TO 400
0025              355 IF(I-2)360,360,365
0026              360 CHAR(NPOS) = 1547714624
0027                  GO TO 400
0028              365 IF(I-3)370,370,375
0029              370 CHAR(NPOS)=1312833600
0030                  GO TO 400
0031              375 IF(I-4)380,380,385
0032              380 CHAR(NPOS) = -264224704
0033                  GO TO 400
0034              385 CHAR(NPOS) = 1530937408
0035              400 CONTINUE
0036                  IF(NEST)800,800,500
0037              500 WRITE(3,700)X,(CHAR(I),I = 1,N)
0038              700 FORMAT(3X,E17.8,2X,109(A1))
0039                  RETURN
0040              800 WRITE(3,900)X,Y(1),(CHAR(I),I = 1,N)
0041              900 FORMAT(3X,F8.4,E17.8,2X,101(A1))
0042                  RETURN
0043                  END
```

APPENDIX IV

Part 1

```
AV      D*
 S      BX
b↑      e*
 S      BX
c↑      E*
 S      BX
C↑      f*
AW      BX
/◊      F*
a↑      A◊
d↓       W
 ↓       S
 S
B↑
B-
a↑
RX
R↓
RS
r◊
D↓
 ÷
B↕
b÷
B↕
B*
B↕
B÷
B↕
c↓
BX
C*
BX
d*
BX
```

```
103233490.000000  d◊
-4692710.200000   D◊
  105041.370000   e◊
   -1143.574100   E◊
       9.411215   f◊
      -1.501832   F◊

Decimal setting 6

Procedure :

-Gen.Res.
-V
-enter : 342.3
-S
-enter :
  4592191100
-S
-enter :
  -1102898100
-S
→-enter : y
|   (sucrose weight fraction)
|-S
←-prints : A : coeff. A
```

Part 2

```
AV      E*
 S      BX
c↑      f*
 S      BX
C↑      F*
AW      a↑
a↑      rS
d↓      RS
 ↓      D↓
 S       X
B↑      A◊
B-       W
a↑
RX
R↓
RS
r◊
D↓
 ÷
B↕
b÷
B↕
B*
B↕
B÷
B↕
c↓
BX
C÷
BX
d*
BX
D*
BX
e*
BX
```

```
-129858340.000000  d◊
  6065477.500000   D◊
  -141643.710000   e◊
     1691.161100   E◊
       16.077073   f◊
        2.116990   F◊

Decimal setting 6

Procedure :

-Gen.Res.
-V
-enter : 342.3
-B/↑
-enter :
  -5497041600
-S
-enter :
  1353250700
-S
→-enter : y
|-S
←-prints : A : coeff. B
```

Part 3

```
AV     ÷
 S     S      146•06635000  d◊
B↑     •       25•25172800  D◊
 ↓    B↕        0•07067484  e◊
 ÷    a↑
 -    rS
a↑    D↓
RX    c↑
R↕    a↑
RS    d↓
r◊    C↑
D↓    AY
 ÷    c↓
B↕    A√
c↑    c↕
r↕    c-
R↑    A↕
R•    /Z
D↕     Z
 ÷    aZ
B↕    B↓
B•    /Y
B↕     Z
B÷    aY
e÷    A•
AX    /↕
a↑    B↑
d↓     -
 ÷    /V
A√     Y
DX    aV
 ↕    c↓
d↓    CX
 -    C↕
 S     Y
 •    AZ
 S    C◊
 ↕     V
```

Decimal setting 8

Procedure :

```
-Gen. Res.
-V
-enter : 146.06635
-D/↑
-enter : 25.251728
-D↑
-enter : 0.070874842
-E/↑
→-enter : y
 -S
 -enter : T
 -S
 -enter : coeff. B
 -S
 -enter coeff. A
 -S
←-prints C : n
```

Part 4

```
AV    a↑        0•0000123928  d◊
 S    R◊       -0•0010578919  D◊
C↑    R•   -58513•8923900000  e◊
Ak    R◊        0•0000396805  E◊
 S    R↕
b↑    dS
e↓     •
/↕    B•
 ↓    cX
a↑    B↕
R↓    e↓
RS    /↕
RS     -
RS    a↑
RS    R↓
dS    RS
 X    RS
bX    RS
C•    RS
bX    dS
a↑     X
R◊     X
R•    bX
RS    E•
R÷    bX
R↓    a↑
dS    R÷
 •    R↕
 S    RS
c↑    RS
 X    RS
B↕    d↓
d↓     ↓
bX    B•
D•    A◊
bX     W
```

Decimal setting 10

Procedure :

```
-Gen. Res.
-V
-enter : 0.0004753008
-S
→-enter T
 -S
 -enter y
 -S
←-prints A : p
```

Part 5

```
AV  Ak   3•6370000000 d◊
 S  e↓   5•0224000000 D◊
B↑  C÷
 S  b↕
E↑  a↑   Decimal setting 6
 S  d↑
 ↓   ÷
BX  B↕   Procedure :
E•  B÷
A•  dX   -Gen.Res.
b↕  B↕   -V
 S  E↕   -enter  nr. drops/fraction
 ↓  E÷   -S
BX  dX   -enter  nr. drops in last
c↕  C↕          fraction
AY       -S
 S       -enter  nr. fractions - 1
 ↓       -S
BX       -enter  fraction nr. preceding
c↕               peak (below)
c•       -S
 ↕       →-enter fraction nr.(starting
b↓                with first fraction of
 -                peak)
b÷       -S
dX       -enter activity for that fractior
D•       -S
 S       ←-repeat for all fractions of the
 X                peak
C↕       -W
 •          note : do not stop machine
C↕                 nor push Gen.Res.
e•
e↕
 Y
```

Part 6

```
AV       1•379310 d◊
a↑       5•022400 D◊
R-
dS
 ↓
BX   Decimal setting 6
D•
C•
D↕   Procedure :
d↓
 S   -NO Gen.Res. !
 -   -V
 S   →-enter   i  (starting
 ↕      with last or ton
 ÷      fraction)
D÷   -S
C↕   enter    i
D↓   ←-repeat until computer
a↑      prints intermediate
R•      result
RS
d-       note : do not stop
 -       machine nor push Gen.Res.
CX   b↓
C↕   D-
AZ   /V
d↓   B•
 S   cX
 -   C•
 S   A◊
 ↕    S
 ÷   aV
D↕   c↓
B•   BX
D↕   C•
D÷   C↕
c↕    Z
```

```
                Part 7
                ------

A V   a ↑       0.37742200000  D ◊
D X   r S                      e ◊
D ↕   D ↓       0.00001583520  E ◊
 S     ÷
 ↓    B ↕
 S    D ↓       Decimal setting 10
 ↕    E ÷
 -    B ÷       Procedure
a ↑   / ◊
R -   / ◊
R ◊   A ◊       -NO Gen.Res.
R ↓   / ◊       -V
R ↑   / ◊       -enter odometer initial reading (millions)
R ◊   / ◊       -S
R ↑             -enter odometer final reading (millions)
d X             -S
 X              -enter duration of centrifugation (minutes)
B ↕             -S
 S              -prints A : s(20,w)^0.725 (Svedberg)
 ↓
a ↑
r S
D X
 X
B ↕
B ÷
A X
B X
E ÷
a ↑
r ◊
R ↓
D ↑
 -
```

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