B E N Z Y L A M I N E O X I D A S E

AN ENZYME IN SEARCH OF A FUNCTION

by

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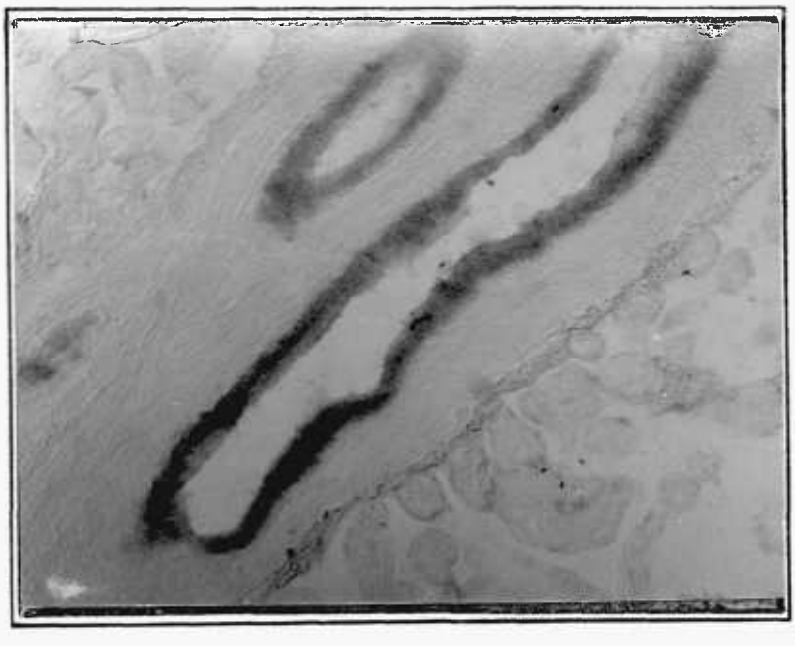
The first condition to be fulfilled by men of science, applying themselves to the investigation of natural phenomena, is to maintain absolute freedom of mind, based on philosophic doubt. Yet ... we must believe in a complete and necessary relation between things, among the phenomena proper to living beings as well as in all others; but at the same time we must be thoroughly convinced that we know this relation only in a more or less approximate way, and that the theories we hold are far from embodying changeless truths. When we propound a general theory in our sciences, we are sure only that, literally speaking, all such theories are false. They are only partial and provisional truths which are necessary to us, as steps on which to rest, so as to go on with investigation; they embody only the present state of our knowledge, and consequently they must change with the growth of science ...

Claude Bernard, An Introduction to the Study of Experimental Medicine, 1865. Translation by H. C. Greene. Dover, New York (1957).



A B S T R A C T

Benzylamine oxidase (BzAO) is one of a family of enzymes, the amine oxidases, which catalyse the oxidative deamination of biogenic amines in the living organism. BzAO shares a number of properties with the monoamine oxidases (MAOs), but is distinct from them in its prosthetic group, Cu, in substrate specificity and inhibitor sensitivity. Its function(s) and physiological substrate(s) are unknown. While working in Brazil, the author noted that BzAO activity is drastically reduced in the serum of patients with severe burns or cancer. In an attempt to answer some of the questions raised by these observations, a study was undertaken to determine the activity of BzAO in mammalian tissues (rat, man) and its sensitivity to amine oxidase inhibitors. This study showed BzAO to be widely distributed in mammalian tissues. Concomitantly, it was demonstrated that the distribution of MAO B activity, also widespread in man and rat, is different from that of BzAO. Further studies on human tissues at three stages of development (fetal, neonatal and adult) confirmed and extended these observations. Activity of BzAO, MAO A and/or B, studied in a wide range of tissues, was present in every tissue examined, apart from MAO B in placenta and vascular smooth muscle. From these studies, it became clear that BzAO is located in the wall of blood vessels. Histochemical studies have shown the enzyme to be localized in the vascular tunica media, not the endothelium as claimed by other authors. As for substrate specificity, a study on human placenta and lung, with liver as control, showed that phenylethylamine may be deaminated by MAO A in certain tissues. A large-scale survey of BzAO in the plasma of pregnant women was carried out to determine variation of enzyme activity during pregnancy, in the post-partum and postnatal period. Puerperal tissues and amniotic fluid were also examined and yielded interesting results. The author also reports on her studies of blood vessels, non-vascular smooth muscle and cultured cells. A tentative interpretation of these observations is presented, as well as an outline of further projects to follow up the line of research into BzAO.

A C K N O W L E D G E M E N T S

The work described in this thesis, based on research done in Brazil, was performed in London at the Bernhard Baron Memorial Research Laboratories. It would be impossible to name all those, both in the U.K. and abroad, who have contributed to my work with help, advice, criticism and encouragement. My heartfelt thanks are due to all my colleagues in these Laboratories, as well as the Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London, U.K.

It is my pleasant duty to thank Dr. Sylvia Lawler (Royal Marsden Hospital Fetal Tissue Bank), Dr. John Pryse-Davies and Dr. Gillian S. Gau (**) for assistance in obtaining fetal and neonatal specimens. I should also like to thank the medical and nursing staff of Queen Charlotte's Maternity Hospital and, in particular, Mr.John Shepherd, for their splendid cooperation in the collection of specimens for the study of benzylamine oxidase in pregnancy, as well as Dr. Ewa Brookes (South London Blood Transfusion Centre) for providing blood samples from normal donors, and Dr. Karl Blau (**) for samples of amniotic fluid. I am grateful to Professor Sir John Dewhurst (*) for providing office space and facilities for the preparation of this thesis. Dr. Brian L. Goodwin's (**) patient and perceptive discussion and criticism of the draft have been of inestimable help to me; Dr. T. A. Ryder (**) has kindly read the manuscript; to both of them I am deeply grateful.

No words can express the debt of gratitude I owe to Professor Merton Sandler (**), whose kindness and generosity have made my work in his laboratory both a pleasure and an unforgettable experience. To him this thesis is dedicated in admiration and friendship.

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ABBREVIATIONS

BAPN	β-Aminopropionitrile fumarate
Bz	Benzylamine
BzAO	Benzylamine oxidase
COMT	Catechol-O-methyltransferase
Cu-AO(s)	Copper-dependent amine oxidase(s)
DA	Dopamine
DAO	Diamine oxidase
D*Bz	Deprenyl-sensitive moiety of Bz deamination
DDTC	Diethyldithiocarbamate
FAD-AO(s)	Amine oxidase(s) containing flavin
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine (serotonin)
LO	Lysyl oxidase
MAO(s)	Monoamine oxidase(s)
NA	Noradrenaline
PEA	β-Phenylethylamine
P-5-P	Pyridoxal-5'-phosphate
RMA	Radiochemical microassay
SPO	Spermine oxidase
TA	Tyramine
Trypt	Tryptamine

I N D E X

T A B L E S

TABLES (cont'd) Page

F I G U R E S

Frontispiece: Histochemical localization of benzylamine oxidase activity in cryostat section of human term placenta (from Ryder et al, 1979).

CHAPTER 1: INTRODUCTION

1.1

Historical

Any investigation of the amine oxidases must trace its origins back to the seminal work of Schmiedeberg (1877). It is the discovery of tyramine oxidase (Hare, 1928), however, that marks the beginning of the history of the amine oxidases proper; the following year, Best (1929) described histaminase. The first great flowering of the study of the amine oxidases occurred in 1937, when Blaschko and his co-workers demonstrated the action of Hare's tyramine oxidase on the deamination of catecholamines (Blaschko et al, 1937 a, b) and Pugh and Quastel (1937 a, b) showed the enzyme to be present in the brain and other tissues of rat and guinea-pig. Further important studies of that memorable year were those of Kohn (1937) and Richter (1937). In 1938, Zeller showed that pig kidney histaminase also catalyses the oxidative deamination of putrescine and cadaverine.

This and other early work is dealt with in many excellent reviews (e.g., Zeller, 1951; Blaschko, 1952, 1962, 1974; Davison, 1958; Blaschko and Bonney, 1962; Buffoni, 1966; Costa and Sandler, 1972; Kety, 1976) and will therefore not be discussed here.

For purposes of this study, which deals with benzylamine oxidase (BzAO) as its main theme and monoamine oxidase (MAO) A and B as a counterpoint, the discovery of spermine oxidase (SPO), the first of the so-called plasma amine oxidases to be described, will be taken as a starting point. Hirsch (1953) discovered SPO in the blood plasma of sheep and cattle, and found that it also acts on spermidine, as well as a number of other substrates which are also oxidized by MAO.

In 1957, Bergeret and Blaschko showed that SPO is present in goat serum. Two years later Blaschko and Hawes (1959) described the occurrence of the enzyme in four other ruminants, but showed that in a number of non-ruminant species (man included), spermine is not oxidized at a significant rate.

Some earlier work had contained references to the occurrence of a deaminating oxidase in the blood plasma of various mammals (see Werle and Roewer, 1952; Kolb, 1956, 1957 a, b). The enzyme from bovine plasma was purified about 200-fold by Tabor et al (1954), who found the purified preparation still to be active against all the substrates oxidized by crude plasma. In 1957, Bergeret et al described an enzyme found in horse serum, with properties reminiscent of SPO, except that it lacked the rapid action on spermine and spermidine. Blaschko (1962) wrote: "The provisional name 'benzylamine oxidase' was proposed for this enzyme, because benzylamine was rapidly oxidized, and because it became apparent at once that the properties of the horse plasma enzyme differed fundamentally from those of the intracellular amine oxidase. It was shown that plasma amine oxidase had a much wider distribution in mammals than had been known (Blaschko and Hawes, 1959; Blaschko et al, 1959)." At the time of writing (Blaschko, 1962), the enzyme had not been demonstrated in the serum of man and several other primate species, but three species of primates were shown to have BzAO activity.

The following year brought the discovery (McEwen and Cohen, 1963) of a soluble amine oxidase in human plasma and serum which catalyses the conversion of benzylamine (Bz) to benzaldehyde, and is distinct from both caeruloplasmin (McEwen and Cohen, 1963) and diamine oxidase (DAO) (McEwen, 1964). 1965 saw the purification and identification (McEwen, 1965a) and the study of the kinetics of the enzyme (McEwen, 1965b). About the same time, pig plasma amine oxidase was purified

and crystallized by Buffoni and Blaschko (1964). Rabbit serum amine oxidase was described by McEwen et al (1966). Abnormalities of the plasma or serum enzyme in chronic congestive heart failure and in chronic liver disease were reported by McEwen and Harrison (1965) and McEwen and Castell (1967), respectively. The purification of human placental histaminase was first described by Kapeller-Adler (1965).

Three further milestones, concerned with MAO inhibitors, are important in these studies. The first was the discovery by Zeller et al (1955) that iproniazid is an "irreversible" inhibitor of amine oxidase. Although it was later shown that iproniazid is neither very potent nor selective (Pletscher, 1966), the report (Zeller et al, 1955) had the most far-reaching effect on subsequent research into MAO inhibition.

Another discovery was to exert a profound influence on all investigation of the amine oxidases: Johnston's demonstration (1968) that clorgyline irreversibly inhibits MAO, but that the concentration required for inhibition depends on the substrate used to assay enzyme activity. With 5-hydroxytryptamine (5-HT, serotonin) as substrate, considerably lower concentrations of clorgyline were shown to inhibit activity of rat brain MAO than are required to block activity towards tyramine (TA) and tryptamine (Trypt). From these results, Johnston concluded that rat brain contains two types of MAO: one, sensitive to inhibition by clorgyline and active against 5-HT and TA, which he called MAO "A"; the other, which he called type "B", is relatively insensitive to inhibition by clorgyline, and actively deaminates TA (*), but not 5-HT.

(*) It should be noted that the description of Bz as a substrate for MAO B, on the basis of its resistance to clorgyline, is erroneously ascribed to Johnston (1968). The amine is not mentioned in his paper.

In 1964 deprenyl, another irreversible inhibitor of MAO, had been discovered by Knoll and his co-workers (Knoll et al, 1968). The inhibitory pattern produced by deprenyl (e.g., 10^{-7}M), which also depends on the substrate employed, is complementary to that of clorgyline: whereas, with 5-HT as substrate, deprenyl produces little or no inhibitory effect in tissues such as rat brain or liver, it completely blocks activity when Bz or β-phenylethylamine (PEA) is used. Thus, while clorgyline (10^{-7}M) selectively inhibits MAO A, the activity of the clorgyline-resistant enzyme, which Johnston called MAO B, is effectively blocked by deprenyl. This attractive picture of differentiation of MAO into A and B forms by the selective action, respectively, of clorgyline and deprenyl in the presence of their "specific" substrates, 5-HT for the A form and Bz or PEA for the B form, has unfortunately become somewhat clouded by recent developments. It has since been demonstrated that both substrate specificity and inhibitor selectivity are less clear-cut than had been assumed (for reviews of this problem, see Neff and Yang, 1974; Houslay et al, 1976; Fowler et al, 1978; see also Lewinsohn et al, 1980 a, b; and Section 3.2.5, Inhibitors).

Early on in the study of the amine oxidases, the multiplicity of MAO had been suspected (Alles and Heegaard, 1943; Werle and Roewer, 1952). A vast literature bears witness to the interest the subject has engendered over the years; review articles alone make up a sizable roster (see Costa and Sandler, 1972; Sandler and Youdim, 1972, 1974; Tipton et al, 1976; Houslay et al, 1976; Wolstenholme and Knight, 1976; Jain, 1977; Singer et al, 1979, to name but a few. See also Gorkin's (1973) discussion of the versatility of catalytic properties and possible biological functions of the MAOs.).

The question of enzyme multiplicity is, of course, inherent in most if not all the attempts at characterization of the MAOs; inextricably linked with it is the search for specific substrates and for inhibitors

able to act as differentiators. Purification and solubilization of the enzyme(s), separation of enzymically active entities by electrophoresis and other methods, immunological, cytochemical and histochemical techniques, and other approaches have been developed in addition to the study of substrate specificity and inhibitor selectivity, to provide some insight into the localization, properties and functions of the amine oxidases (see Section 3.1.1, Methods for the study of amine oxidases).

1.2

Brazilian beginnings

In the spring of 1974, while working as an instructor and clinician at Santa Casa da Misericordia, the main teaching hospital of the Federal University of Rio de Janeiro, I was shown two abstracts in Gastroenterology by my colleague, Dr. Fernando W. Portella. The reports (Kirchner et al, 1973; Kirchner and Castell, 1973) described raised serum amine oxidase levels as an index of clinical and experimental liver cirrhosis. Since hepatic fibrosis (as distinct from cirrhosis) resulting from Schistosoma mansoni infection is extremely common in Brazil (Barbosa, 1966; Freitas, 1972), Dr. Portella suggested that a study of the activity of this enzyme in patients with schistosomiasis might yield interesting results, and that the assay might prove a valuable adjunct in the diagnosis of the disease. Although schistosomiasis is not hard to diagnose, a small proportion of "atypical" cases may progress to liver cirrhosis; many patients go on to develop a very severe form of the disease with gross hepato-splenomegaly and oesophageal varices leading to uncontrollable bleeding and early death.

Schistosomiasis is not endemic in Rio de Janeiro, but patients were plentiful and after a period of preparation, I began the study of BzAO in human serum. Bench space and technical guidance were

provided by Professor Maury Miranda, head of the Laboratory of Molecular Biology, Institute of Biophysics, of the Federal University of Rio de Janeiro, in whose laboratory I worked until the end of 1976.

As a preliminary, the range of normal activity was established in 77 individuals without any known disease process. Apart from schistosomiasis and hepatic cirrhosis, various disorders liable to result in marked fibrosis such as chronic congestive heart failure, fibrotic lung disease and others were investigated. The results of the American workers (McEwen and Harrison, 1965; McEwen and Castell, 1967) and those of Ito et al (1971) were confirmed by my findings in hepatic cirrhosis and chronic congestive heart failure, and high values were found in one case of chronic fibrotic lung disease. However, schistosomiasis and other fibrotic non-cirrhotic conditions gave readings on the low side of the normal range (Lewinsohn, 1977b). It is also interesting that normal values in Brazil (mean, 18.3 ± 0.6 S.E.of mean; range, 8.6 - 32.3 McEwen units; see Lewinsohn, 1977b) were lower than those observed by the American workers (mean, 27.1 ± 0.8 S.E.of mean; range, 10 - 43 McEwen units; see McEwen and Cohen, 1963; McEwen, 1972).

The quest for pathological conditions with marked primary or secondary fibrosis, which I then believed to be at the root of alterations in BzAO activity such as described by McEwen and co-workers, finally led me to measure serum BzAO in patients who had sustained severe burns; such injuries invariably result in large areas of permanent fibrosis. To my surprise, I found BzAO activity to be strikingly decreased in patients who had lost 18 per cent or more of body surface, within a period of six months before assay. Detailed investigation of the phenomenon confirmed my initial observations in every respect, as reported in my first papers on the subject (Lewinsohn, 1977 a, b).

The loss of large areas of skin in severe burns caused me to speculate on the role of the skin in BzAO activity, and suggested the possible use of "cosmetic" plastic surgery as a model for further study. Surgical removal of skin and subcutaneous tissue from grossly obese patients (mainly female) seemed to some extent to reproduce the situation in severe burns. One advantage of such a model was the possibility of determining pre-operative BzAO values, and the use of every patient as her own control, a possibility which evidently does not exist in the case of severe burns. Moreover, the skin removed at surgery could be measured, another advantage over the situation in severe burns, where the exact extent of skin loss is extremely difficult to assess (Artz, 1968). Eleven patients in this class were studied. Serial assays showed a moderate to severe loss (but never total disappearance) of serum BzAO activity within 72 hours after surgery, with a return to normal after a period of 2 - 4 weeks. There was no correlation between the area of skin resected and post-operative enzyme levels (Lewinsohn, unpublished data).

The use of this model immediately raised a host of questions, the foremost being whether any observed alteration in BzAO activity was due to loss of skin and subcutaneous tissue, or to surgical trauma <u>per se</u>. A further group of patients was therefore selected, scheduled to undergo surgery for various pathological conditions, but without substantial loss of skin or other tissue. This group consisted of 15 patients, including ten with cancer. The results of sequential determinations of serum BzAO activity in those with non-malignant disease were essentially similar to the findings in "cosmetic" plastic surgery: after minor reparative interventions such as herniorrhaphy, no alteration in activity was seen, while more extensive surgery was followed by a moderate fall and relatively rapid return to pre-operative values (Lewinsohn, unpublished data).

Admittedly, the numbers of patients in these groups were small, and the results would have warranted investigation on a larger scale. However, the finding that half the patients with cancer had significantly low serum BzAO values overrode all other considerations. I obtained permission to work at the National Cancer Institute, Rio de Janeiro, where in 1976 I studied 125 patients (Lewinsohn, 1977 b). Once again, the results of the detailed investigation confirmed the initial observation. 52% of the untreated patients and 41% of those treated had values significantly below normal; the mean activity of all patients with cancer was 42% less than that of normal individuals ($p \ll 0.0001$). Serial determinations on 50 patients yielded results which made it desirable to continue and widen the investigation. Extremely low values had been found only in patients with solid tumours; those with disseminated non-solid neoplastic disease such as the leukaemias, lymphomata, etc. showed normal serum BzAO activity. On the other hand, treatment with cytotoxic drugs without known inhibitory action on the amine oxidases (e.g., 5-fluorouracil, methylnitrosourea, methotrexate) was seen to be followed in some cases by a steady rise in activity from almost non-detectable to within the normal range. Furthermore, in one case (a patient with cervical carcinoma in situ), the very low serum BzAO activity seen pre-operatively returned to normal after removal of the tumour.

These findings demanded elucidation at various levels. From a study of the literature available to me, it appeared that both localization and function of BzAO were unknown; and its behaviour in fibrotic conditions such as liver cirrhosis, as well as my own observations, clearly raised more questions than they answered. The existence of isoenzymes in normal human circulating blood, and one abnormal component in patients with haemochromatosis, had been shown (Lin and Castell, 1973, 1974, 1975; Lin, Davis and Castell, 1976).

Kirchner et al (1973) had experimentally produced liver cirrhosis in rats by treating them with nialamide, a known MAO inhibitor (Knoll and Magyar, 1972). The significance of all these observations was obscure, however. In order to arrive at an evaluation of my findings in severely burnt patients and those with cancer, it was necessary to answer such questions as:

- the localization of the enzyme (?) in tissues
- the relationship between circulating and (?) tissue BzAO
- the physiological substrate of BzAO in plasma and (?) tissues
- the physicochemical properties of the enzyme in (?) tissues
- the presence of an endogenous inhibitor (?) in health or disease.

1.3

London

On my arrival in Europe at the beginning of 1977, it became clear to me that none of my questions had been answered by previous research. I also realized that work on these problems could be done more profitably in Europe, and was fortunate enough to be offered bench space, facilities and technical guidance by Professor Merton Sandler, Chairman of Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London. A large body of information on the MAOs had been produced at these laboratories, though no work had hitherto been done on BzAO. Under the guidance of Dr. V. Glover, I began to elaborate a radiochemical micro-assay for BzAO, based on extraction methods already in use for MAO A and B. In Brazil, I had used the spectrophotometric assay of McEwen and Cohen (1963), based on that developed by Tabor et al (1954). However, though highly specific, this method is relatively insensitive; moreover, only 18 samples could be processed in a day, and the amount of material required

(1.6 ml serum/tube) was relatively large. Our first attempts at adapting the MAO extraction method to BzAO in human plasma or serum proved successful, and I immediately started using ^{14}C-labelled Bz as substrate in the radio-microassay, having established at the outset that there was good correlation between the results obtained with this method and that of McEwen (Lewinsohn, unpublished data).

For my first inquiry into tissue BzAO, I studied a large number of tissues in four rats. Though activity was present in all the tissues investigated, there were wide differences between them. Aorta and lung showed considerable BzAO activity, while liver and brain were virtually inactive. It was also instructive that activity in those rat tissues rich in BzAO was many times greater than that found in plasma (Lewinsohn et al, 1978). I then graduated to human tissues. Once again, almost every tissue was found to contain BzAO, to a greater or lesser extent; brain and liver values were among the lowest. One important result of these studies was the observation that, although brain and liver parenchyma is virtually without activity, cerebral and portal vessels are rich in BzAO, the former strikingly so (Lewinsohn et al, 1978).

These results suggested further questions, such as the role of BzAO in growth and in pregnancy: might not embryo and fetus in utero represent a model for the situation in cancer, in that the presence of the, admittedly non-malignant, proliferating tissue might be "represented" in lower BzAO values in the maternal blood? Is there any correlation, (a) between fetal growth and maternal plasma BzAO? (b) between BzAO activity in placenta, placental vessels, umbilical vessels, cord blood and maternal plasma at term? Is BzAO activity present in all blood vessels?

Out of these questions grew three projects:

(1) a study of the activity of BzAO (and, to round off the picture, MAO A and MAO B) in a wide range of human tissues, at three stages of development, fetal, neonatal and adult;

(2) a study of the activity of the three enzymes in blood vessels;

(3) a survey of the activity of BzAO in human pregnancy. Maternal blood from all stages of gestation and at parturition was used, as well as cord blood; and placenta, placental vessels and umbilical vessels in 12 cases out of forty in whom serial determinations were carried out. Amniotic fluid was also studied for activity of the three enzymes.

In the course of study n^{o} (1), I found that in human placenta, MAO A deaminates PEA in an identical fashion to 5-HT; this led to a further project, in which human placenta, liver and lung were investigated (Lewinsohn et al, 1980a).

Since the work on severely burnt patients and patients with cancer was done in Brazil prior to my registration for a Ph.D. degree, it is here dealt with only summarily. It is my work in London that forms the body of this thesis, and will be presented in detail.

The studies described in reports already published have not been written up again, and are submitted as reprints forming an integral part of this thesis; they constitute a substantial portion of the experimental work performed in fulfilment of the requirements for the degree of Doctor of Philosophy.

1.4

Literature review

> "The formidable efforts devoted by pharmacologists to adrenergic agents foredooms to failure any attempt at a comprehensive review of this field... (This section) is, therefore, presented as a compromise between the impossible and the unavoidable." (Somlyo and Somlyo, 1970)

Far from abating in the 1970's, the wave of interest in biogenic amines carried over from the previous two decades (Fuller, 1972) has turned the stream of pertinent publications into a torrent. In the past few years, the literature on the amine oxidases, particularly the MAOs, has grown to such vast proportions that any attempt at comprehensive review is a daunting task indeed. Even reviewing a part of this area of study, such as BzAO, is fraught with difficulties, largely because of the ragged dividing line between research into the MAOs and BzAO. Often it is doubtful precisely what is being measured; but the indiscriminate use of the term MAO (see Nomenclature) and a strange reluctance on the part of researchers to employ the term "benzylamine oxidase" coined by Bergeret, Blaschko and Hawes (1957), combine to confound an already confused state of affairs. Further uncertainties stem from insufficient data and differences in methodology, so that no two experiments are entirely comparable. Only in recent years have more sensitive methods of assay and the availability of selective inhibitors enabled researchers to devise new approaches in their attempts to clarify these problems.

The major milestones in our knowledge of the amine oxidases are noted in the introductory pages of this thesis. In addition, the reviews of Buffoni (1966), Blaschko (1974), Sandler and Youdim (1974), Neff and Yang (1974), Murphy and Donnelly (1974), Tipton (1975), Houslay *et al* (1976), Goodwin (1976), Yasunobu *et al* (1976), Murphy (1978) and

Fowler et al (1978) should be consulted for recent work and an appreciation of the present state of the art. All these reviews deal mainly with the MAOs; except for those of Buffoni (1966), Blaschko (1974), Murphy and Donnelly (1974) and Yasunobu et al (1976), they contain only passing references, at best, to BzAO. On the subject of mammalian blood plasma amine oxidases, Blaschko (1962) reviewed the information available just prior to the discovery of BzAO in human serum (McEwen and Cohen, 1963). Blaschko's (1974) discussion of "amine oxidases containing copper and a carbonyl group (pyridoxal)" deals mainly with DAO, lysyl oxidase (LO) and the role of amine oxidases in connective tissue, but his brief comments on "plasma amine oxidase" proper are of major importance (see p. 185). Yasunobu et al (1976) reviewed the molecular, mechanistic and immunological properties of the amine oxidases, and dealt with the copper-dependent amine oxidases in considerable detail.

The earliest report on a BzAO-like enzyme in mammalian plasma is that of Werle and Roewer (1952) on a "MAO" active against tyramine (TA) in dog serum. In 1953, Hirsch noted SPO in sheep and bovine plasma. Beef plasma amine oxidase was purified 150- to 200-fold by Tabor et al (1954). The substrates most rapidly deaminated were spermine, spermidine, Bz and certain other aromatic and aliphatic amines. Their purified amine oxidase preparations were sensitive to cyanide, hydroxylamine and semicarbazide, confirming the findings of Hirsch (1953) in sheep serum. In the course of this work, Tabor et al (1954) developed the spectrophotometric assay method subsequently used by McEwen (see below) and a host of other workers. Kolb (1956, 1957a,b) describes an amine oxidase in the blood plasma of pig, ox and horse; in the opinion of Blaschko (1962), the bovine enzyme is probably identical with the SPO studied by Tabor et al (1954).

After Bergeret and Blaschko's discovery (1957) of SPO activity in goat serum, the plasma of many other mammalian species was investigated

for activity (Blaschko and Hawes, 1959). Blaschko and Chruściel (1959) reported that in the dog, unlike other species examined, there exist wide individual differences in the rate of Bz oxidation from animal to animal. Further studies on the BzAO activity of mammalian plasma are those of Blaschko et al (1959). The connecting thread in all these observations is the rapid deamination of Bz compared with other substrates studied, and the sensitivity of the enzyme to inhibition by cyanide and carbonyl reagents.

Buffoni (1963) and Buffoni and Blaschko (1964) purified and crystallized an amine oxidase from porcine plasma. In her review on histaminase and related amine oxidases, Buffoni (1966) avers that the histaminase activity of pig plasma is an intrinsic property of the enzyme that acts on Bz and other monoamines, but differs from pig kidney histaminase. Studies of substrate specificity and inhibitor sensitivity of pig plasma BzAO (Buffoni and Blaschko, 1964; Buffoni, 1966) show the enzyme to be most active against Bz and mescaline, and most sensitive to inhibition by cyanide, carbonyl reagents, cuprizone, diethyldithiocarbamate, aminoguanidine and p-chloromercuribenzoate.

The demonstration of BzAO in human plasma or serum (McEwen and Cohen, 1963), one of the most important events in the study of this enzyme, was followed by a profusion of careful papers by McEwen and his co-workers. In 1964, McEwen investigated BzAO and DAO in human pregnancy; a year later, he described the purification, identification and kinetics of BzAO in human plasma (McEwen, 1965a,b) and found abnormalities of BzAO activity in chronic congestive heart failure (McEwen and Harrison, 1965). Rabbit serum "MAO" was described by McEwen et al (1966) and McEwen and Sober (1967), and the abnormalities of human serum BzAO in chronic liver disease by McEwen and Castell (1967). McEwen and his colleagues then turned to human liver (mitochondrial) MAO and investigated the kinetics of this enzyme (McEwen et al, 1968)

as well as its substrate and inhibitor specificities and the kinetics of its time-dependent inhibition (McEwen et al, 1969 a, b).

Robinson et al (1968, 1971, 1972) studied the effects of drugs on human platelet and plasma amine oxidase activity in vivo and in vitro, and the relationship of sex and aging to activity in human brain, plasma and platelets. Tryding et al (1969) reported on the effect of age, sex, contraceptive steroids and diabetes on human serum amine oxidase levels. Several accounts deal with serum BzAO in hepatic fibrosis, e.g. Ito et al (1971), Kirchner et al (1973), Kirchner and Castell (1973), Borthakur et al (1973). Erickson et al (1973) report on serum amine oxidase activity in experimental schistosomiasis japonica in rabbits. Nies et al (1973) compared mono- and dizygotic twins with pairs matched for age and sex in an investigation of the genetic control of platelet and plasma amine oxidase activity. Multiple forms of human plasma BzAO were studied spectrophotometrically and by hydroxyapatite column chromatography by Lin and Castell (1974, 1975) and Lin, Davis and Castell (1974, 1976). Three forms of the enzyme were isolated from normal human plasma, and a fourth, abnormal form from the plasma of a patient with hepatic fibrosis and haemochromatosis. Yasunobu et al (1976) also described 3 forms of amine oxidase, purified and crystallized from bovine plasma. Further important studies on human amine oxidase in platelets, plasma and other tissues were published by Murphy and Donnelly (1974) and Murphy et al (1976). Murphy et al (1977) investigated relationships between plasma amine oxidase activity and age, sex and genetic factors in normal rhesus monkeys. Properties of amine oxidases in normal human platelets, plasma, lymphocytes and granulocytes were described by Bond and Cundall (1977), and those in platelets, plasma and liver of cirrhotic patients by Buffoni et al (1977).

Localization and physicochemical properties of BzAO. An early paper by Thompson and Tickner (1951) dealt with the occurrence and distribution

of "MAO" in mammalian blood vessels, but its interpretation is complicated by the relatively crude methods (e.g., homogenizing procedures) then employed. The publications of McEwen and Cohen (1963), McEwen (1965 a) and McEwen and Harrison (1965) contain some speculative comments on the possible derivation of mammalian serum amine oxidase from an intracellular MAO, on the identity of BzAO with a component of mitochondrial MAO, and on the elaboration of human serum amine oxidase by a specific human tissue. Since the early 1950's, cell fractionation had been used to study the intracellular localization of MAO (Cotzias and Dole, 1951; Hawkins, 1952). Interpretation of results achieved with this and other methods, however, took on a new dimension with the development of a radiochemical microassay (Wurtman and Axelrod, 1963; McCaman et al, 1965) and the discovery of two inhibitors highly selective against MAO B and MAO A, deprenyl and clorgyline, respectively, the former discovered in 1964 (Knoll et al, 1968), the latter described by Johnston in 1968.

Jarrott and Iversen (1968) studied the subcellular distribution of MAO in rat liver and vas deferens, and found a significantly higher proportion of all measured enzyme activities in the microsomal fraction of the vas deferens. Hall, Logan and Parsons (1969) studied MAO inhibition by clorgyline, in rat liver and brain, using 5-HT, TA and Bz as substrates. They concluded that 5-HT and Bz are specific substrates for each component of a binary enzyme system (MAO A and B, respectively), whilst TA is deaminated by both, thus confirming and extending the experiments of Johnston (1968). Tipton (1969 b) found that after pretreatment with 2-bromo-2-phenylacetaldehyde, a powerful inhibitor of pig brain MAO (Tipton and Spires, 1968), part of rat liver MAO activity towards Bz becomes insensitive to inhibition by clorgyline. Jarrott (1971) also found some clorgyline resistance in preparations of normal and denervated vas deferens of rat, with Bz as substrate. Reports on amine oxidase activity and biogenic amines in blood vessels, published by

Spector et al (1969, 1972), Spector (1971), Berkowitz (1970), Berkowitz et al (1971, 1974), Howland and Spector (1972), Lai et al (1975), and Lai and Spector (1977, 1978 a, b) are dealt with in detail in Section 4.3 (p. 165).

In perfusion experiments on normal and denervated rabbit ear artery preparations, de la Lande and Waterson (1968) and de la Lande et al (1970) investigated MAO activity against TA biochemically and histochemically. In the latter study, activity was seen to be localized in the vessel media and adjacent mixed nerve bundles, both in denervated and control preparations. No staining was observed in the endothelium.

The connective tissue amine oxidase in chick bone studied by Rucker et al (1969) is distinct from BzAO in molecular weight and other physicochemical properties, and appears to be lysyl oxidase (LO). Rucker and O'Dell (1971) purified bovine aorta amine oxidase about 170-fold and compared it with bovine plasma amine oxidase, purified about 60-fold. They found similar properties in both, except that peptidyl lysine was oxidized by the aorta enzyme (but see also Yasunobu et al, 1976 on unpublished work by Minamiura and Yasunobu), but not by the plasma amine oxidase. In view of the similarities between the two enzymes, these authors suggest that connective tissue may be the source of the plasma enzyme. The properties of amine oxidases in bovine brain and connective tissue (dental pulp) were described by Nagatsu et al (1972), who concluded that the soluble amine oxidase of dental pulp may deaminate peptidyl lysine and participate in the cross-linking formation of collagen.

Houslay and Tipton (1975 a) investigated the effect of clorgyline on purified beef plasma amine oxidase, and found clorgyline to be a reversible inhibitor of the enzyme. Riceberg et al (1975) reported on the effects of aminoacetonitrile and propargylamine on mescaline metabolism in various tissues of the rabbit.

The first suggestion that BzAO may originate in the vessel wall

(as distinct from connective tissue) appears in the report of Coquil et al (1973) on their study of MAO activity in rat arteries, with TA, 5-HT and noradrenaline (NA) as substrates and pargyline, clorgyline, cyanide and carbonyl reagents as inhibitors. Cell fractionation yielded a "soluble" (authors' quotation-marks) fraction with properties similar to those of BzAO; MAO A was also found to be partially soluble. However, the arterial amine oxidase was not inhibited by cyanide.

From experiments with perfused rabbit lung (Gillis, Roth and Baker, 1975; Roth and Gillis, 1975; Gillis and Roth, 1976, 1977) with PEA and 5-HT as substrates and pargyline and semicarbazide as inhibitors, these authors concluded that, besides MAO A and B, rabbit lung contains a third form of amine oxidase, similar to that found in plasma and several large arteries, and suggested that this enzyme is localized in vascular endothelium. Roth and Venter (1978), however, found no BzAO-like activity in cultured endothelial cells from rabbit aorta. They used PEA, 5-HT, NA, dopamine (DA) and TA as substrates, and inhibitors selective for MAO A. Ability to deaminate PEA, far more active than that towards any other substrate, and the pattern of enzyme activity after clorgyline and harmaline treatment, strongly suggest the predominance of MAO B in endothelial enzymic activity. Perfused lung was also used by Bakhle and Youdim (1976) to study the uptake and deamination of PEA and 5-HT in rat lung preparations. The results suggest that MAO A and B operate independently in the organized tissue. For a review of the pharmacokinetic functions of the pulmonary circulation, see Bakhle and Vane (1974).

Experiments with perfused rat brain preparations (with special reference to monoamine metabolism), led Woods et al (1976) to conclude that their histological and metabolic properties are close to those observed in vivo. Tyce et al (1968 a,b) employed isolated perfused rat liver to study the uptake and metabolism of 5-HT in that organ. The effect of some MAO inhibitors on gluconeogenesis was investigated by Triner et al

(1969) in *in vivo* and *in vitro* experiments with perfused rat liver. Phenelzine was shown significantly to decrease alanine incorporation into glucose, whilst pargyline had no effect on alanine uptake.

Lyles and Callingham (1974, 1975) found evidence for the presence of Bz-oxidizing activity, both resistant and sensitive to clorgyline, in rat heart. The clorgyline-resistant moiety, predominant in the high-speed supernatant, was inhibited by semicarbazide, but not by cyanide. The authors suggest that MAO A may contribute to Bz-oxidation in rat heart, but do not exclude the possibility that the Bz-metabolizing, clorgyline-sensitive activity may derive from membrane fragments that resealed around cytoplasm in the course of preparation of the fractions, while vascular elements contaminating the homogenate may account for the clorgyline-insensitive component of enzymic activity against Bz. However, the resistance of this component to cyanide inhibition would seem to preclude its identification as BzAO. In experiments with rat heart and mesenteric artery, Fuentes and Neff (1977) found some resistance (greater in vascular tissue than in heart) to inhibition by pargyline, using 2-PEA as substrate, in preparations sensitive to inhibition by semicarbazide and cuprizone. The authors concluded that the pargyline-resistant amine oxidase activity of rat mesenteric artery may play a role in the cross-linking of collagen and elastin in blood vessels.

Recent work by Lyles and Callingham (1979) confirms their own previous findings (Lyles and Callingham, 1974, 1975) in respect of a clorgyline-sensitive Bz-oxidizing component of amine oxidase activity in the rat heart. No such component was found in the human heart, however (Parkinson and Callingham, 1979).

Purified pig plasma BzAO (Buffoni and Della Corte, 1972) was investigated for substrate and inhibitor specificities and other physicochemical properties. The "true substrate" of BzAO was found to be protonated Bz; lysine was not deaminated by the pig plasma enzyme.

Buffoni et al (1975, 1976) partially purified two amine oxidases from porcine aorta, both sensitive to carbonyl reagents and copper-chelating agents but with different kinetic properties; one was found to be similar to pig plasma BzAO, the other to LO (see below).

The review of Yasunobu et al (1976) contains a summary of unpublished results of immunological investigations on "Cu-amine oxidases" carried out by these authors. Three forms of the purified beef plasma enzyme were identified by immunoelectrophoresis, all of which reacted in an identical manner with rabbit antisera in the double immunodiffusion test of Ouchterlony. The antisera inhibited the oxidation of Bz by the soluble enzyme(s) of beef, Aspergillus niger and pea seedlings, but failed to react with "FAD-amine oxidases" (MAO). Antibody to the porcine enzyme has also been raised by Della Corte (unpublished results, quoted in Yasunobu et al, 1976); immunofluorescence techniques yielded results pointing to the reaction of this antibody with LO present in several porcine tissues. Yasunobu et al (1976), however, found LO to be immunologically different from the "Cu-amine oxidases". Immunofluorescence-histochemical techniques were utilized by Buffoni et al (1977) to test the reactivity of various porcine tissues with rabbit antibodies to pig plasma amine oxidase. The results showed a similar immunological determinant in pig plasma BzAO and porcine connective tissue, thus establishing a close relationship between plasma and connective tissue amine oxidases, "although not necessarily proving their identity".

Two further lines of research cross with BzAO investigation and need to be mentioned, albeit briefly, in this context: (a) the role of LO in the chemistry of collagen cross-linking and connective tissue maturation; and (b) the study of body fluids and tissues during pregnancy. Several researchers (e.g., Rucker and O'Dell, 1971; Nagatsu et al, 1972) considered LO to be identical with BzAO, a point of view not shared by others (e.g., Yasunobu et al, 1976, whose review has already been mentioned). Siegel et al (1970) compared the activities

of LO from chick embryos and pig plasma amine oxidase in allysine formation and collagen cross-linking, and concluded that LO from connective tissue participates directly in the production of cross-links in collagen and elastin, whereas the plasma amine oxidases "appear to be a different enzyme system that serves functions which are as yet undefined". Reports by Bailey and Peach (1971), Bird et al (1966) and Harris et al (1973), respectively, describe the chemistry of collagen cross-links, the effect of Cu-deficiency and inhibitors on chick amine oxidase, and the purification and properties of chick aorta LO. Two forms of LO, isolated from beef aorta by Vidal et al (1975), showed distinct antigenic determinants and differed in net charge and in double immunodiffusion (Ouchterlony) tests with the respective antisera. The substrate specificity and synthesis of collagen cross-links in vitro was studied with highly purified LO by Siegel (1976) and Siegel et al (1970), and the in vitro inhibition of lysyl hydroxylase and collagen cross-links by catechol analogues, by Murray et al (1977 a,b). Arem and Misiorowski (1976) and Arem et al (1977) respectively reported on the lathyritic activity of isoniazid and MAO inhibition during control of scar formation in experimental animals. Simpson et al (1968) induced aortic rupture in turkeys by the use of β-aminopropionitrile (BAPN); mortality from the lesions was prevented by reserpine and enhanced by MAO inhibitors. Peacock and Madden (1978) described the beneficial effects of BAPN-induced lathyrism in patients with urethral strictures. Blaschko's (1974) review of Cu-dependent amine oxidases, including LO, has already been mentioned.

Amniotic fluid has been little studied for enzymic activity against monoamines. Butterworth et al (1972) investigated lysosomal enzymes, Scott et al (1972) the changes in concentration of amino acids in amniotic fluid during the first half of pregnancy, and Sutcliffe and Brock (1972) the origin of amniotic fluid enzymes. Specific activity

patterns of enzymes in amniotic fluid were described by Sutcliffe et al (1972). One chapter in Sutcliffe's review (1975) on the nature and origin of the soluble protein in human amniotic fluid deals with enzymes present in amniotic fluid supernatant. DAO levels in human amniotic fluid were investigated by Tufvesson (1978 a,b).

The following studies are concerned with the amine oxidases (mainly MAO and DAO) of human placenta and/or pregnancy plasma: McEwen (1964); Southren et al (1966 a,b; 1968); Youdim and Sandler (1967); Bardsley and Crabbe (1973); Bardsley et al (1974); Crabbe and Bardsley (1974); Tufvesson (1978 b); Lin et al (1978). Blood histamine and plasma histaminase levels during human pregnancy were studied by Dubois et al (1977). Pertinent studies, moreover, are those of Sadowsky et al (1963) on 5-HT metabolism in habitual abortion; Urban and Zasztowt (1964) on post-partum values of serum 5-hydroxyindole-acetic acid (5-HIAA); Tryding and Willert (1968) on DAO in pregnancy plasma; Southgate and Sandler (1968) on 5-hydroxyindole metabolism in pregnancy (review), and Robson and Sullivan (1968) on the effect of 5-HT on maintenance of pregnancy, congenital abnormalities, and the development of toxaemia. Sandler and Coveney (1962) first described decreased levels of placental MAO in toxaemia of pregnancy. Recently Illei and Morgan (1979 a,b) found an amine oxidase similar to spermine oxidase in the plasma of pregnant women, which was not detectable in the plasma of men or non-pregnant women, nor in the plasma of patients with cancer. Activity of this enzyme was also found in the decidua, less in the placenta, and none in umbilical vessels. The substrate employed was ^{14}C-spermine. Further investigations are in progress (G. Illei, personal communication).

Literature on specific topics is discussed in detail in the corresponding section, e.g., reports on methodology, listed in Section 3.1 (materials and methods) and Tables A-1 (a)-(h) (Appendix).

TABLE 1. NOMENCLATURE

Hare (1928) Tyramine oxidase.

Best (1929) Histaminase.

Blaschko et al (1937 a, b) Amine oxidase, active against adrenaline (adrenaline oxidase) and related monoamines, also aliphatic monoamines, but not against histamine, putrescine or cadaverine.

Zeller (1938) proposed monoamine oxidase (MAO) for amine oxidase, and diamine oxidase (DAO) for histaminase, having found their substrate specificities to be complementary.

Hirsch (1953) Spermine oxidase (SPO), also active against spermidine and a number of other substrates also oxidized by MAO.

Tabor et al (1954) Beef plasma amine oxidase, probably identical with bovine spermine oxidase.

Bergeret et al (1957) Benzylamine oxidase, different from intracellular amine oxidase (MAO) and SPO.

McEwen and Cohen (1963) Soluble amine oxidase in human plasma and serum; serum amine oxidase.

McEwen (1965 a, b) Human plasma monoamine oxidase (MAO)

McEwen and Castell (1967) Soluble monoamine oxidase (SMO)

Johnston (1968) Monoamine oxidase (MAO) type A and B, based on sensitivity towards selective inhibitor ("differentiator"), clorgyline.

Rauch and Rauch (1973) Plasma amine oxidase (PAO)

Blaschko (1974) Oxidases containing flavin and amine oxidases containing copper and a carbonyl group (pyridoxal).

Yasunobu et al (1976) Cu-amine oxidases and FAD—amine oxidases.

Lewinsohn et al (1978) Benzylamine oxidase (BzAO).

Illei and Morgan (1979 a, b) Polyamine oxidase (PAO).

CHAPTER 2: NOMENCLATURE AND CLASSIFICATION

It is, perhaps, opportune at this point to introduce briefly the problem of nomenclature. Zeller (1938), finding the substrate specificities of intracellular amine oxidase and histaminase to be complementary, proposed the term "monoamine oxidase" (MAO) for the former, and "diamine oxidase" (DAO) for the latter. The distinction between the two enzymes, based on their substrate specificities, was underlined by their differing and complementary sensitivity towards cyanide, semicarbazide and other carbonyl reagents, which are strong inhibitors of DAO, but have no effect on MAO.

Hirsch (1953) described "spermine oxidase" (SPO), a soluble plasma enzyme of ruminants active, in addition, against spermidine and some other substrates also oxidized by MAO.

2.1 BzAO

As shown in Table 1, the term "benzylamine oxidase" (BzAO*) was coined for the soluble amine oxidase found in plasma and serum, distinct from the spermine oxidase of ruminant and some non-ruminant species (Bergeret et al, 1957). The designation was provisional (Blaschko, 1962) but in the absence of data on the localization or function of the enzyme, its introduction was opportune, particularly since Bz was seen to be more actively metabolized by it than any other substrate. It is true that the substrates BzAO acts upon are mainly monoamines, but the appellation "monoamine oxidase" had been pre-empted for the mitochondrial, non-soluble enzyme of tissues. It is thus regrettable that, after

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(*) "BzAO", introduced by the present author (Lewinsohn et al, 1978) was thought preferable to "BAO", which might have led to some confusion with MAO B.

using the term "serum amine oxidase" in their first two papers (McEwen and Cohen, 1963, McEwen, 1964), McEwen and his co-workers later changed to "plasma monoamine oxidase" (McEwen, 1965a,b; McEwen and Harrison, 1965; McEwen et al, 1966; McEwen and Castell, 1967; McEwen, 1972; Lin and Castell, 1974, 1975; Lin et al, 1976). "Plasma MAO" was also used, among others, by Yamada and Yasunobu (1962 a,b; 1963) and even by Blaschko (1972), though in a later review this author employed the terms "flavin-containing oxidase" for MAO and "copper-containing amine oxidases" for the group of enzymes which includes BzAO (Blaschko, 1974). In my own preliminary communication written in Brazil in 1976 (Lewinsohn, 1977 a), I used the term "serum MAO". It was Blaschko who pointed out to me that the appellation was misleading, and in my second paper (Lewinsohn, 1977 b), I used the term "serum amine oxidase" (SAO). However, while the use of "MAO" for BzAO should be strongly discouraged, neither "serum-" nor "plasma-amine oxidase", nor "soluble amine oxidase" is without ambiguity. In several mammalian species, the blood plasma and tissues contain more than one soluble amine oxidase. Thus, Lyles and Callingham (1975) found both clorgyline-resistant and clorgyline-sensitive activities in the soluble fraction of rat heart. Besides BzAO, which in the human is virtually inactive against histamine (McEwen, 1965 a), human plasma also contains histaminase, which rises sharply in pregnancy (McEwen, 1964) and is quite distinct from BzAO. In species such as the pig, which possess a BzAO active against Bz as well as histamine, "plasma amine oxidase", "BzAO" and "histaminase" can be used interchangeably; but statements such as "The histaminase activity of human blood plasma is like that of pig blood plasma..." (Buffoni, 1966) may give rise to confusion. In man the activity of histaminase is different from that of BzAO while, as shown by Buffoni (1966), the histaminase activity of pig plasma is an intrinsic property of the enzyme that acts on Bz and other monoamines.

Thus, in order to avoid ambiguity, it would be essential to define not only mammalian species and tissue but also substrate and inhibitor employed in the experiment when using the term "soluble" or "plasma" (serum) amine oxidase -- a cumbersome and highly unsatisfactory procedure.

Although benzylamine, an "artificial" substrate, has not, so far, been shown to exist in the mammalian organism, it is felt (see Lewinsohn et al, 1978) that "benzylamine oxidase" is preferable to any other term proposed (e.g., "soluble", "extra-cellular" enzyme) so long as the physiological substrate of the enzyme remains unknown. It is certainly more appropriate than "plasma" (or serum) amine oxidase, not only for the reasons given above, but, most importantly, because it is now clear (see Lewinsohn et al, 1978, 1980 b, and Chapter 4, below) that the activity found in mammalian tissues is far higher than that in the corresponding plasma or serum. Thus, the use of "plasma" (or serum) amine oxidase for BzAO becomes an outright misnomer. Up to the time of writing, no other substrate has been identified which is as actively oxidized by BzAO as benzylamine; and the use of deprenyl (see Section 3.2.5, Inhibitors) clearly distinguishes BzAO from MAO B, which also deaminates Bz.

Finally, the designation, BzAO cannot be subsumed with the DAOs under EC 1.4.3.6, which covers a group of pyridoxal-phosphate-dependent, copper-containing enzymes "oxidizing primary monoamines and diamines, including histamine" (Enzyme Nomenclature - Recommendations (1972) of the International Enzyme Commission, 1973). It has, of course, never been claimed that BzAO belongs among the DAOs, since there are more dissimilarities than resemblances between BzAO and this group of enzymes. Moreover, though it is undoubtedly a copper-enzyme (see p. 107), the claim that it is pyridoxal-phosphate dependent (Buffoni, 1966) is no longer universally accepted (Yasunobu et al, 1976).

Thus, while it is obvious what BzAO is not, the enzyme awaits

recognition as a separate physicochemical and functional entity graced with its own EC number.

2.2

MAO

From the discussion above it is clear that, so far, the nomenclature of BzAO hinges on the striking preference of the enzyme for Bz as substrate, and the matter must rest there until further studies establish its physiological function(s). As for the MAOs, the problem is more complex. Localization, function, substrate specificity and inhibitor selectivity are all inextricably linked with one another and with the vexed question of the multiple forms of the mitochondrial enzyme. Since multiplicity of forms will be briefly touched upon, but lies outside the scope of this thesis, I do not propose to discuss it here in any detail, except insofar as it impinges upon nomenclature.

Between 1943, when Alles and Heegaard suggested that amine oxidase is not a homogeneous enzyme but a mixture of enzymes and the latest, but inevitably not the last, reports on the subject (Fowler et al, 1980 a, b; Lewinsohn et al, 1980 a,b), the question, explicit or implicit, asked by every researcher has been concerned with the nature of the multiple forms of MAO. Are we dealing with isoenzymes or multiple forms as defined by the Enzyme Commission (1973), or with entirely different enzymes, i.e., quite different proteins? These questions transcend the confines of mere semantics. It had been hoped that once solubilized and purified preparations became available, an unequivocal answer would follow; but, as so often happens in biology, attainment of the goal has moved, not removed the boundaries of uncertainty. Surprisingly, only one enduring suggestion for the classification of MAO, after Zeller's, has emerged from the immense literature accumulating over the years: Johnston's (1968) differentiation between the two major forms of MAO, which he called A and B. Designations proposed

for further types, e.g., MAO C, BBO (for brain benzylamine oxidase) (Williams et al, 1975, 1979) have been received with less than enthusiasm. The same applies to "MADH" (monoamine dehydrogenating system) proposed by Guha and Ghosh (1970), which may or may not be identical with MAO. Since activity both of MAO and BzAO has been found in the microsomal fraction of tissues subjected to differential centrifugation (see p. 81), the appellation "microsomal" enzyme (Stjärne et al, 1968) is also inadequate for purposes of classification.

Although the classification of MAO A and B on the sole basis of inhibitor sensitivity, even when supplemented by substrate specificity studies, may be open to criticism, the very survival of the terminology introduced by Johnston (1968) attests to its usefulness. It will no doubt continue to be employed by all researchers in the field, until the molecular basis (genetic or otherwise) of the multiple forms of MAO is unravelled or other information educed to permit the introduction of a new nomenclature.

For a brief discussion of the problems of nomenclature concerning the amine oxidases, see Blaschko (1974).

CHAPTER 3: BIOCHEMISTRY

3.1

Materials and methods

In our first experiments (Lewinsohn *et al*, 1978), human and rat tissues were examined; subsequent work, except for the study of cultured cells (p. 175), was done on human tissues exclusively. Sources and treatment of tissues prior to homogenization have been described in full detail (Lewinsohn *et al*, 1978, 1980 a, b), and where appropriate in this thesis.

Homogenization procedures are described in Section 3.1.2, chemicals in Section 3.1.3, and in published reports (see above).

3.1.1

Methods for the study of the amine oxidases

$$RCH_2NH_2 + O_2 + H_2O \underset{AO}{\overset{AO}{\rightleftharpoons}} RCHO + NH_3 + H_2O_2$$

Since all the amine oxidases, whether flavin or copper dependent, catalyse the same deaminating reaction shown above, similar methods are employed to study the oxidizing activity of BzAO and the MAOs. No basic procedure has become universally accepted to assay amine oxidase activity in any one, let alone in more than one tissue or species. Thus, the data from various laboratories in respect of any one tissue, enzyme or substrate are seldom directly comparable. One method that has come close to the desideratum of universality is the radiochemical microassay developed by Wurtman and Axelrod (1963) and McCaman *et al* (1965), which has become virtually indispensable for the *in vitro* study of the amine oxidases. Its advantages lie in its great sensitivity and the fact that large numbers of samples can be processed simultaneously.

The usefulness of the radiochemical microassay has been enhanced by, and has in its turn multiplied that of, the selective inhibitors developed during the last fifteen years. Depending on the substrate employed, resin exchange or solvents may be used for extraction. There is no standard radiochemical assay in general use, however, and variations introduced by each worker to study a specific problem inevitably result in data which are, strictly speaking, not comparable.

In the experiments to be described in this thesis, the solvent-extraction approach has been employed to the exclusion of all others, and is the only method here described in detail (Section 3.1.3). A list of the most important methods employed in the investigation of the amine oxidases is given in Tables A-1 (a)-(h) (Appendix). No attempt has been made at comprehensiveness; methodology has been divided into major basic techniques and the first workers in the field to use them are shown, as well as a representative sample of those following.

Reviews on methodology were published by Levine and Sjoerdsma (1963), Kapeller-Adler (1970, 1971), Tipton (1975), Youdim (1975), and Tipton and Youdim (1976).

General problems related to radiochemical microassay methodology are dealt with by Browne et al (1973, 1978) in respect of the influence of substrates and buffer solutions on the estimation of MAO activity, and the effects of homogenization of rat liver in different buffer solutions on yield and kinetic properties of MAO. Fowler et al (1977) report on the effect of tris buffers on MAO activity in rat liver with TA, 5-HT, PEA and Bz as substrates, and Youdim and Woods (1975) on the influence of tissue environment on rates of metabolic processes and properties of enzymes. Comments on post-mortem stability of MAO in frozen tissues are to be found in Robinson et al (1971). This important problem was studied by Parkinson and Callingham (1978) in rat tissues;

previous reports on the study of post-mortem stability in other species are also discussed by these authors. Tipton et al (1977) describe a kinetic method for the detection of inhibitory contaminants in radioactive substrates,

Only passing reference will be made here to in vivo studies. Early investigations of amine oxidase activity in animals were mainly concerned with gauging the action of amine oxidase inhibitors. The advent of selective inhibitors for different types of amine oxidase (see above) intensified inquiry, in vivo as well as in vitro, into specificities both of the inhibitors themselves, and of substrates, for the different forms of MAO. The discovery of the behavioural effects of MAO inhibitors (see Kety, 1976) prompted many workers to investigate their pharmacological and biochemical aspects; in particular, their implications for psychiatry and neurology have been thoroughly scrutinized (Wolstenholme and Knight, 1976). In vivo studies have, of course, been prominent in all these inquiries. In another approach to in vivo studies, organ or tissue perfusion has been employed to investigate uptake, inactivation and substrate metabolism catalysed by the amine oxidases (see references throughout this thesis). Most if not all of these investigations have been concerned with the mitochondrial MAOs. As for in vivo studies of BzAO, these obviously include assay of plasma activity in health and disease. In vivo studies other than these, i.e., of an experimental nature, have been mostly either accidental or incidental. One notable exception is an experiment with human volunteers (Robinson et al, 1968) in which the effects of various amine oxidase inhibitors on the activity of the plasma and platelet enzymes were investigated. A fortuitous discovery is described in the same paper (Robinson et al, 1968), where pronounced plasma BzAO inhibition was observed in a patient medicated with isoniazid and a return to normal activity noted after the drug was discontinued. Incidental

observations on BzAO activity in the course of *in vivo* experiments are, for example, those of Lyles and Callingham (1974) in studies on the effects of thyroid hormones on MAO in rat heart.

3.1.2

Homogenizing procedures

3.1.2.1

Introduction

The first step among the many prerequisites for determining enzyme activity in any solid tissue is the preparation of a homogenate capable of giving reproducible results. The procedures employed by various workers shown in Table A-2 (Appendix) give an idea of the multiplicity of attempts to achieve a homogeneous suspension, a relatively easy task in the case of liver or brain, the tissues most often used in assaying MAO activity. When tougher tissues are studied, however, the task becomes far more difficult. The problem hinges on reducing the tissue to particles so small that a homogeneous suspension may be obtained without clots or clumps which would impede uniform pipetting. In reducing the tissue to a smooth brei, drastic measures resulting in denaturation of protein, e.g. through production of heat, cannot be employed, since the enzymes would be inactivated in the process. Many workers have resorted to the centrifugation of a crude homogenate, discarding the cellular debris contained in the solid sediment of the first spin. This procedure, which is the first essential step in studies involving cell fractionation, solubilization and others used to characterize enzymes, has its drawbacks when specific ("absolute") activity is investigated. Since it is impossible to estimate the amount of active enzyme discarded with the debris (as shown, e.g., by Collins *et al*, 1970), which is likely to be different for each tissue (see p. 82-83), the readings on various tissues treated in this fashion are not comparable. Assaying the pellet is no real solution, since in its turn, it

would need to be reduced to a smooth homogenate, not always an easy task; moreover, the work and cost would be doubled, both factors of considerable importance in large-scale projects.

The preparation of a homogenate from solid tissue may be divided into two parts: 1) breaking down the larger tissue pieces into the smallest possible particles; 2) homogenization proper, i.e., the preparation of a homogeneous suspension by mixing the tissue particles with buffer or water. Sometimes it is possible to do both at once by using a blender (e.g., with liver or brain), but with tissues such as aorta or skin, the result is invariably poor. The investigation of a large number of tissues, for example the study of amine oxidase distribution in the tissues of man or rat (Lewinsohn et al, 1978, 1980 a,b; see also Chapter 4), called for a technique appropriate to the homogenization of all tissues in a similar fashion. Obviously, the procedure suitable for the toughest tissues was the one adopted for all. Other important considerations were ease and speed of performance.

Before deciding on the technique of freezing and pulverizing at -196° (N_2-method) described below, and subsequent homogenization in an Ultra-Turrax blender, several other approaches were tried. By far the best homogenates were achieved with cryostat sections (10-20 µm) of tissues quick-frozen in CO_2 (see cryostat method, below). Unfortunately, this technique can only be used for a few tissues at a time.

3.1.2.2

"Mincing"

This method, which involves freezing tissue on a bed of dry ice, then scraping, shaving or paring thin sections with a scalpel blade, is less satisfactory, because the resulting sections or particles are not uniform. Mincing is, however, a good technique for reducing placenta or liver to small particles before homogenizing, and permits the separation of parenchymal tissue from visible blood vessels present in the tissue section.

3.1.2.3

N_2-method

Freezing tissues in liquid nitrogen and pulverizing them in a pre-frozen steel mortar and pestle with several blows of a hammer, was found to be the easiest method for processing large numbers of samples in the shortest possible time. The technique is described by Williamson et al (1967), Sassoon et al (1967) and in our previous communications (Lewinsohn et al, 1978, 1980 a, b). In our first project, homogenization proper was carried out at 0° in 1ml or 5ml glass homogenizers with ground-glass or Teflon-tipped pestles (Lewinsohn et al, 1978). For the preparation of all homogenates used in our subsequent studies (Lewinsohn et al, 1980 a, b, and those described in this thesis) an Ultra-Turrax homogenizer with a 10-N shaft (Sartorius Instruments, Surrey, U.K.) was employed. Frozen tissues were weighed in pre-weighed plastic tubes, care being taken to avoid thawing; an appropriate volume of chilled 0.1M K_2HPO_4/KH_2PO_4 buffer (pH 7.2) was then added to make up a 10% (w/v) homogenate. In the case of tissues known to be rich in amine oxidase (aorta, lung, liver), 2% to 5% (w/v) suspensions were prepared. After adding chilled buffer to the plastic tube containing weighed tissue, the latter was homogenized for 5-10 sec at top speed. At this point, the homogenate was still cold enough to be divided into aliquots without further chilling; these aliquots were immediately frozen in solid CO_2 and stored at -20° until required.

3.1.2.4

Cryostat method

This method was developed in the course of a series of experiments designed to study the distribution of BzAO and MAO A and B activity in subcellular fractions of human tissues. I here present the results of the cryostat treatment compared with a standard homogenizing procedure.

Experimental

Human aorta (two series of experiments), lung, and tumour tissue, obtained at autopsy, were treated as described (Lewinsohn et al, 1978, 1980 a,b) up to the actual homogenizing steps. After coarse mincing with a scalpel, the fragments were mixed to avoid treating various parts of the tissue by different procedures. One half of the coarsely minced tissue was immersed in liquid nitrogen, pulverized and homogenized (see N_2-method, above). The other was quick-frozen in solid CO_2 (-80°), and 10-20 µm sections cut on a Teddington Type QR cryostat (through the kindness of Mr. K. A. Napier, Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London). These sections were collected in a Petri dish kept frozen on a bed of CO_2, and placed in a pre-weighed plastic container. Sucrose buffer (see below) was added immediately in an appropriate volume to make up a 10% (w/v) suspension, and the container capped firmly. The cryostat-sectioned tissue visibly disintegrated in the buffer; after mixing for 1 min at top speed on a Vortex mixer, a smooth suspension was obtained which was not further homogenized.

Subcellular fractionation followed the method described by Lyles and Callingham (1975), except for the use of a "relaxing" buffer, which was omitted. The buffer employed for making up the homogenates and resuspending the pellets contained sucrose, 0.5M; KCl, 0.05M; EDTA, 0.001M; Na_2HPO_4, 0.004M and KH_2PO_4, 0.004M; the pH was adjusted to 7.2. Differential centrifugation was carried out as outlined by Lyles and Callingham (1975). The supernatant resulting from centrifugation of the crude homogenate at 800 g for 10 min was used as the "low-speed supernatant" (LS-SN). Subsequent centrifugation of the LS-SN at 9000 g for 10 min gave a mitochondrial pellet (P_1) and the medium-speed supernatant (S_1), which was further centrifuged at 105 000 g for 60 min, producing a "high-speed supernatant" (S_2) and a "microsomal" pellet (P_2). All pellets were resuspended in a similar volume of sucrose

buffer as the suspensions from which they were derived. For assay, the radiochemical procedure (p. 55) was employed. For aorta and lung, enzyme, buffer, inhibitor and substrate concentrations were as described (Lewinsohn et al, 1980 b). A preliminary assay had shown enzymic activity in tumour tissue to be exceedingly low; the volume of enzyme was therefore increased to 100 µl. Concentrations of all other reagents were similar to those used with lung and aorta. Since the main purpose of this study was to compare methods rather than determine "absolute" values, no intracellular marker was employed.

To differentiate between BzAO and MAO B, 4×10^{-4}M (-)-deprenyl (100 µl) was used with benzylamine as substrate. The deprenyl-sensitive moiety of Bz oxidation (D*Bz) was interpreted as the activity catalysed by MAO B, and the deprenyl-resistant activity against Bz as BzAO. Activity of MAO A was determined with 5-HT as substrate, without inhibitor (see Lewinsohn et al, 1980 a, b).

Radioactive substrates and all other reagents are described below (p.55). The method of Lowry et al (1951) was employed to estimate the protein content of tissue fractions, with bovine serum albumin as standard. For the calibration curve, 0.5M sucrose buffer was used instead of water, in order to avoid the pitfalls described by Schuel and Schuel (1967) associated with the use of sucrose buffer in the preparation of homogenates.

All assays were carried out at least in duplicate. Values shown are the means of these replicate determinations, after subtraction of blank values and correction for extraction efficiency.

Results and comments

The results of the present study are shown in Tables 2-4 and Figures 1 and 2. Figure 1 illustrates the differences in recovery of activity between subcellular fractions from four human tissues, comparing the cryostat method and N_2-homogenizing procedure. The recovery in

the high-speed supernatant fractions (S_2) and the "microsomal" pellets (P_2) was significantly different with the two methods, and gave *p* values between 0.002 and 0.005 for both fractions (Table 2). Table 3 shows that there was no such difference between any of the fractions in respect of MAO B (O*Bz). Figure 2 and Table 4, which illustrate a typical experiment of this sort for MAO A, using 5-HT as substrate, show the relatively higher recovery of soluble enzyme in the N_2-homogenate, but the percentage of recovery of fractions P_1, S_2 and P_2 from the 9 000 *g* supernatant (S_1) is about the same for both procedures.

Comparison of these results with those of others (Table 5) shows standard homogenizing procedures to produce broadly similar recovery. The total absence of activity in the high-speed supernatant of the preparations of Jarrott and Iversen (1968) is puzzling. Coquil *et al* (1973) report remarkably high S_2 activity in a single experiment on rat femoral artery, but do not comment on this observation. As for the absence of activity in the high-speed supernatant of rat brain, I have made a similar observation, using Bz as substrate in a cell fractionation experiment on the same tissue (p. 79).

In analysing the results of this study, it is tempting to describe the effect of the cryostat treatment as a "solubilization" of BzAO, although the percentage of recovery, both in respect of cpm and specific activity, is not uniform in all the tissues examined (Tables 2 and 3). Indeed, absolute values are lower for the cryostat- than the N_2-treated tissues. It is possible that the milder treatment represented by the former accounts for the lower absolute recovery values as compared with the latter. Nevertheless, there can be little doubt as to the superiority of the cryostat treatment in the investigation of soluble BzAO in subcellular fractions. For MAO B, the only difference approaching statistical significance was found in the medium-speed supernatant (S_1) fraction (Table 3), where the N_2-treatment proved better than the cryostat method, but there was no significant difference between the other

fractions with either method. As for MAO A, although higher values, both relative and absolute, are recovered with the standard homogenizing procedure, the pattern of distribution of activity in subcellular fractions is sufficiently alike for both procedures to warrant the use of cryostat sections in such studies (Fig. 2).

The reasons for such discrepancies must at present remain speculative. Apart from obvious differences in solubility, binding to membranes and subcellular localization of the three enzymes, one possible explanation has been suggested by Dr. B. L. Goodwin. The prolonged exposure of the tissues to a temperature of -80^{o} produces ice crystals within organelles which, on thawing, may release more enzyme protein than in the case of shorter exposure to the much lower temperature of liquid nitrogen (-196^{o}), where no ice crystals are formed.

Whatever the reason for the superiority of the cryostat treatment as a homogenizing procedure in the study of BzAO may be, one fact is indisputable. In processing tissue samples in the manner described here, no particles are dispersed into air, as inevitably happens when tissues are pulverized by the N_2-method or minced by hand, and again when they are homogenized by hand or in an apparatus such as the Ultra-Turrax or Waring blender. In the experiments described here, for the reasons given, tissues were coarsely minced before cryostat- or homogenizing treatment. When the cryostat method is used exclusively, the mincing step is not essential and may be omitted, thus reducing the handling of the tissue and possible dispersion of particles to a minimum. This clearly gives the cryostat treatment a unique place among homogenizing procedures of human tissues, and particularly of malignant tissues. Lower recovery in terms of "absolute" values is thus more than compensated for by the ease and safety of the cryostat method.

Table 2. Subcellular distribution of BzAO in fractions of human tissues. Comparison of cryostat-treated and N_2 - homogenized tissue preparations. Substrate: benzylamine. Recovery values shown are percentages of cpm for LS-SN (= 100%). Specific activity = nmoles/mg protein/30 min. For assay conditions, see text.

Preparation	LS-SN	Fraction S_1		P_1		S_2		P_2	
Tissue	Specific activity	Recovery cpm (%)	Specific activity	Recovery cpm (%)	Specific activity	Recovery cpm (%)	Specific activity	Recovery cpm (%)	Specific activity
Cryostat									
Aorta-1.	3.2	95	3.9	8	(1.0)*	60	2.2	33	(4.1)
Aorta-2.	2.2	107	2.3	4	(0.7)	79	2.3	5	(0.5)
Lung	6.1	90	5.1	9	12.7	63	4.5	17	28.8
Tumour	0.05	128	0.08	8	0.2	95	0.08	19	0.3
N_2-homogenate									
Aorta-1.	15.7	99	15.9	8	(10.9)	19	3.9	49	(39.6)
Aorta-2.	11.4	95	11.1	3	(3.5)	31	4.7	60	(35.5)
Lung	13.4	84	12.2	12	37.2	37	7.8	41	95.0
Tumour	0.22	95	0.25	26	1.0	38	0.1	49	1.1
Mean (± S.E.of mean) Cryostat		105 (7.3)		7.25 (0.96)		74.5 (7.2)		18.5 (5.0)	
N_2-homog.		93.2 (2.8)		12.25 (4.25)		31.25 (3.8)		49.75 (3.4)	
Student's t		1.5		1.15		5.33		5.2	
P		N.S.		N.S.		< 0.005		< 0.005	

*Figures between brackets represent approximate estimations based on protein ostimations from recombined values. All assays carried out at least in duplicate; individual values shown are means of replicate determinations. N.S. = not significant.

Table 3. Subcellular distribution of MAO B (D*Bz) in fractions of human tissues. Comparison of cryostat-treated and N_2-homogenized tissue preparations. Substrate: benzylamine. Recovery values shown are percentages of cpm for LS-SN (= 100%). Specific activity = nmoles/mg protein/30 min. For assay conditions, see text.

Preparation / Tissue	LS-SN	Fraction S_1		P_1		S_2		P_2	
	Specific activity	Recovery cpm (%)	Specific activity	Recovery cpm (%)	Specific activity	Recovery cpm (%)	Specific activity	Recovery cpm (%)	Specific activity
Cryostat									
Aorta-1.	0.50	86	0.55	86	(1.65)*	40	0.2	0	0
Aorta-2.	0.2	36	0.1	37	(1.2)	54	0.3	30	(0.6)
Lung	0.31	20	0.06	31	2.2	0	0	51	4.3
N_2-homogenate									
Aorta-1.	4.3	96	4.2	28	(10.9)	34	1.9	35	(7.6)
Aorta-2.	0.7	205	1.5	8	(0.5)	43	0.4	170	(6.1)
Lung	3.9	159	6.8	28	25.3	25	1.5	65	43.6
Mean (± S.E. of mean) Cryostat		47.3 (16.2)		51.3 (14.2)		31.3 (13.2)		27 (12.1)	
N_2-homog.		153.3 (25.8)		21.3 (5.4)		34 (4.2)		90 (33.4)	
Student's t		3.5		1.97		0.19		1.77	
P		< 0.05		N.S.		N.S.		N.S.	

Tumour tissue showed no detectable MAO B activity in the cryostat preparation, and only trace activity in the N_2-homogenate.

* See foot-note to Table 1.

Table 4. Subcellular distribution of MAO A in fractions of human lung: comparison of cryostat-treated and homogenized tissue preparations. Substrate: 5-HT. Specific activity is expressed as nanomoles/mg protein/30 min. For experimental and assay conditions, see text.

Fraction	CRYOSTAT PREPARATION		N_2 - HOMOGENATE	
	Specific activity	Recovery cpm (%)	Specific activity	Recovery cpm (%)
LS-SN	10.2	100	22.8	100
S_1	3.6	38	19.5	79
P_1	70.6	31	82.1	16
S_2	0.8	7	5.2	15
P_2	49.1	17	153.2	39

Table 5. Subcellular distribution of amine oxidase activity in fractions of rat tissues and bovine aorta (other authors). Recoveries express percentages of total activity recovered in $P_1 + S_2 + P_2$ combined.

Species / Tissue	Homogenizing procedure	Substrate	Recovery (%) P_1	S_2	P_2	Reference
Rat Heart	Conical glass homogenizer (*)	Tyramine	40	8	52	Lyles and Callingham (1975)
		Benzylamine	33	23	44	
Rat Liver	All-glass Duall homogenizer	Tryptamine		2	18	de Champlain, Mueller and Axelrod (1969)
Heart		"		20	48	
Salivary gland		"		20	37	
Vas deferens		"		2	34	
Rat Liver	All-glass hand homogenizer	Tyramine	76	0	24	Jarrott and Iversen (1968)
Vas deferens		"	58	0	42	
Liver		5-HT	76	0	24	
Vas deferens		"	58	0	42	
Rat Arteries (pooled)	2-ml glass homogenizer, Teflon	Tyramine	27	33	41	Coquil, Goridis, Mack and Neff (1973)
Mesenteric artery	postle	"	43	57		
Mesenteric artery		5-HT	56	44		
Femoral artery (**)		Tyramine	30	70		
Brain (**)		"	100	0		
Salivary gland (**)		"	100	0		
Bovine aorta	N_2-method followed by 2 homogenizing steps in Waring blender	Benzyl-amine		20		Rucker and O'Dell (1971)

(*) Minced tissue stirred at 0-4° during several changes of "relaxing" buffer, which was decanted; muscle washed in sucrose buffer, then homogenized in sucrose buffer in conical glass homogenizer.

(**) Duplicate determinations on one preparation.

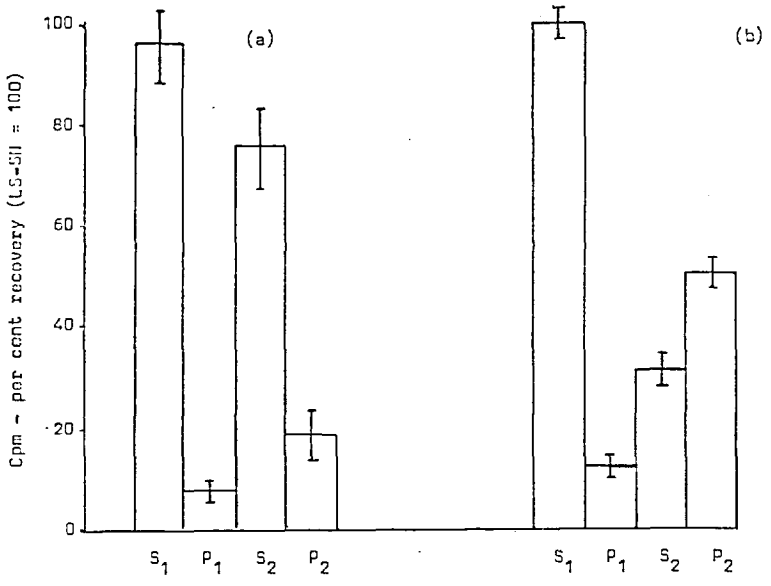


Figure 1. Recovery of BzAO activity in subcellular fractions of four human tissues: comparison of cryostat treatment (a) and N_2-method (b). Values express recovery in cpm (LS-SN = 100%), and are means of values shown individually in Table 2; vertical bars are the standard error of mean. LS-SN = low-speed supernatant; S_1 = 9000 g supernatant; P_1 = mitochondrial pellet; S_2 = 105 000 g supernatant; P_2 = "microsomal" pellet. For experimental and assay conditions, see "Experimental".

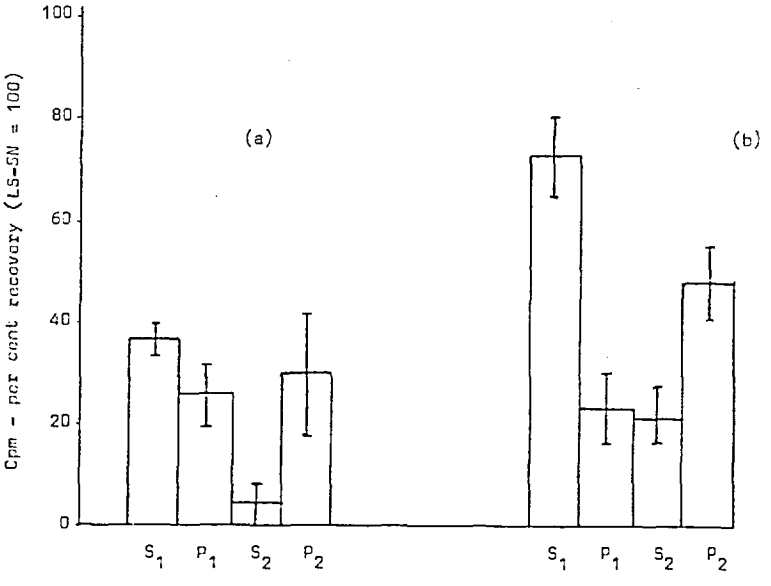


Figure 2. Recovery of MAO A activity in subcellular fractions of two human tissues, comparing cryostat treatment (a) with N_2-method (b) Values shown are means of the two experiments; vertical bars express the range of the two determinations. Remaining legend as in Figure 1.

3.1.3

Radiochemical microassay

Chemicals. All reagents used were analytical grade. ^{14}C-labelled substrates were purchased from various sources (New England Nuclear Corporation, Boston, U.S.A.; Radiochemical Centre Ltd., Amersham, U.K.; ICN Pharmaceuticals, Inc., Irvine, U.S.A.). The following compounds were kindly donated: (-)-deprenyl by Professor J. Knoll and the Chinoin Company, Budapest; clorgyline by May & Baker Ltd., Dagenham, U.K.; procarbazine hydrochloride (Ro 4-6467) and benserazide (Ro 4-4602) by Roche Products Ltd., Welwyn Garden City, U.K.; carbidopa by Merck, Sharp & Dohme Ltd., Hoddesdon, U.K.; and phenelzine by William R. Warner & Co. Ltd., Eastleigh, U.K. Isoniazid, penicillamine and β-aminopropionitrile fumarate (BAPN) were purchased from Sigma Chemical Co., St.Louis, U.S.A., pargyline from Abbott Laboratories, North Chicago, U.S.A., and semicarbazide hydrochloride, 99.5 per cent pure (AnalaR grade) from BDH Chemicals, Poole, U.K. Benzylamine, purchased as free base from Sigma London Chemical Co. Ltd., Kingston-upon-Thames, U.K., was converted to its hydrochloride by treatment with hydrochloric acid, and recrystallized (Dr. B. L. Goodwin). All other reagents were obtained from commercial sources.

Substrates. Substrate solutions were made up in 0.01M HCl and unlabelled amine, divided into aliquots and stored at -20° until required.

Inhibitors. Stock solutions of inhibitors, stored at -20°, were thawed and diluted as required, immediately before use.

Buffer solutions were made up at frequent intervals, and stored at 4° between assays. Except where otherwise stated, K_2HPO_4/KH_2PO_4 buffer, pH 7.2, was used with PEA and 5-HT as substrates, and tris buffer, pH 9.0, with Bz as substrate. The strength of all buffers employed was 0.1M.

Assay procedure. This method has been described and discussed in detail by Tipton and Youdim (1976). The basic procedure was similar for plasma and tissues. Volumes and concentrations of buffers, substrates, inhibitors and enzyme employed by us varied, as shown in published reports, and Tables and legends to Figures throughout this thesis. Buffer, enzyme (tissue homogenate, plasma, amniotic or other fluid), and inhibitor solution or water were mixed in 75 x 12 mm open polypropylene tubes and preincubated at room temperature for 20 min before substrate solution was added. Water blanks were similar to samples but contained water instead of enzyme; in enzyme-plus-acid blanks, 2M citric acid was added to the assay mixture before incubation. Incubation of samples and blanks proceeded for 30 min at 37° in a constant-temperature water bath. The reaction was stopped by addition of 0.1 ml of 2M citric acid. 3 ml of organic solvent (toluene/ethyl acetate 1:1 v/v in 5-HT assays; toluene in all others) was added to all the tubes, which were capped, shaken vigorously in a mechanical shaker for 5 min, spun in a clinical centrifuge for 5 min at 800 g, and placed upright in racks at -20° until the lower (aqueous) phase was frozen solid. The supernatant was then decanted, essentially quantitatively, into polypropylene tubes, 4 ml scintillation fluid (Insta-gel or 1% Butyl-PBD in toluene) added and radioactivity measured for 1 min in a liquid scintillation counter (Packard Model 3320 Tri-Carb Scintillation Spectrometer).

Experimental. All assays were carried out at least in duplicate; results express means of replicates, which varied < 5%. Reaction products gave readings accounting for 5-20% of the total labelled substrate used.

Blanks. Tubes containing no enzyme and those with an enzyme-plus-acid mixture gave similar blank values, which were as follows: Bz < 5%, PEA < 3%, 5-HT < 0.01% of total labelled substrate radioactivity. All readings were corrected for the corresponding blank value.

Extraction efficiency of radioactivity, tested as described (Lewinsohn et al, 1978) with three substrates in 4-6 different human tissues, varied less than 1% among all tissues with each substrate (cf. also Jarrott, 1971). Mean extraction efficiency values were 95, 96.2 and 91.2 per cent, respectively, for Bz, PEA and 5-HT and have been corrected for in all specific enzyme activities quoted.

Specific enzyme activity, expressed as nanomoles/mg protein/30 min, was calculated from the following formula:

$$\frac{AC \times n \times 1000 \times 100}{TC \times P \times V \times E}$$

where TC = total count (cpm) in the volume of substrate used;

n = nanomoles of substrate in the same volume;

AC = actual count (cpm) of reaction product;

P = protein content of enzyme (mg/ml) in test tube;

V = volume of enzyme solution in test tube (µl);

E = extraction efficiency (per cent).

Protein was measured by the method of Lowry et al (1951) in aliquots of the homogenates or fluids employed in the assay.

Linearity with time and enzyme concentration. Incubation time up to 60 min and enzyme concentrations as described (Lewinsohn et al, 1978) served to confirm the linearity of the reaction in the experimental conditions employed.

Variability of the method is discussed in the next Section, together with stability of the enzyme.

Statistical methods. For evaluation of statistical significance of results, "Student's" t test and regression analysis have been applied. In some tables, standard deviation (S.D.) as well as standard error (S.E.) of the mean are shown, to permit rapid calculation of significance, where not given.

3.2

Properties

3.2.1

Stability of enzyme and variability of method

3.2.1.1

Stored tissues

A measure of the stability of rabbit liver MAO is found in Blaschko (1952), who observed that acetone-dried powder of rabbit's liver prepared in November 1945 and kept standing in the laboratory without any precautions had retained more than one third of its initial activity six years later.

Post-mortem stability of BzAO and MAO in human tissues (cf. Epps, 1945) was similar to that shown during the first 24 h after death for rat tissues (Parkinson and Callingham, 1978). Changes in enzyme activity observed in tissues homogenized after storage of the tissues at -20° for up to five months, when compared with homogenates of the same tissues prepared within 24 h of death, could be accounted for by experimental error. Activity was observed in tissues stored at -20° for more than twelve months (one human tissue showed considerable activity after 24 months of storage), but no comparative studies were carried out to determine the rate of variation after such long intervals. Generally speaking, BzAO activity tended to give higher and MAO A lower readings after lengthy storage, while MAO B showed little change. Since the protein content of the tissues also showed substantial variation not correlated with changes in cpm, such altered readings of enzymic activity are difficult to interpret.

3.2.1.2

Tissue homogenates

Variability of the method was tested in two human tissues over

a period of 5 months, and also gave a measure of enzyme stability in frozen homogenates of solid tissues kept at -20°. On 11 different occasions, one previously unthawed aliquot of neonatal lung and liver homogenate was assayed for BzAO, MAO A and MAO B, together with a series of tissues. The results (Table 6) show the variability of the method and of enzyme stability to be well within the limits of experimental error.

Table 6. Stability of enzymes and variability of method for determination of amine oxidase activity. Means express specific activity (nanomoles/mg protein/30 min); standard error of the mean (SE of mean) is expressed as percentage of the mean. For experimental and assay conditions, see Lewinsohn et al (1980 b).

Tissue	BzAO	MAO B (D*Bz)	MAO B (PEA)	MAO A
Lung (n = 11)				
mean	22.7	2.5	5.3	25.8
SE of mean (%)	6	6	5	8
Liver (n = 10)				
mean	4.1	77.4	22.9	56.3
SE of mean (%)	5	5	5	6

D*Bz = deprenyl-sensitive Bz-oxidizing activity.

3.2.1.3

Plasma

Stability of BzAO and variability of the method with time of storage and repeated thawing and freezing were also tested in human plasma and serum. In experiment (1), aliquots of human blood serum and plasma from two individuals were compared for stability at room temperature and 4°, during a period of 24 h. Immediately after separation of serum and plasma, half the aliquots were placed in a refrigerator at 4°; the other half were kept on the bench at room temperature.

At hourly intervals up to 10 h and at 24 h, one aliquot from the bench and one from the refrigerator were placed in the deep freezer at -20°. All aliquots were assayed together 24 h after preparation. The results of this experiment are shown in Figure 3. For a period of up to 2 h, at either room temperature or 4°, both serum and plasma are entirely stable. Although the drop in activity is slight at 4 and even 6 h, at either temperature, the protocol adopted for all subsequent experimental work on plasma was to spin and freeze specimens within 2 h of blood collection.

In experiment (2), plasma from 3 individuals was divided into two parts. One was stored in one tube (P) and the other further divided into individual aliquots (A). Over a period of nine months, 9 assays were performed on individual aliquots (A), thawed immediately before assay, and on the plasma stored in the (P) tubes, which were thawed and refrozen on each occasion. As shown in Figure 4, loss of activity was greater in the plasma subjected to repeated thawing and freezing. Up to about 20 weeks, variability was slight in all specimens; only after 36 weeks was there a substantial drop in activity, greater in the (P) than in the (A) tubes, as might have been expected.

A third test of variability of BzAO activity measured in human plasma was carried out as part of the pregnancy project (Section 4.2). Seven months after the start of the project, 145 plasmas had been collected and were assayed for BzAO activity. Three months later, when 336 plasmas had been collected for the project, previously unthawed aliquots of the first 145 plasmas were assayed with the rest and compared with the earlier readings. A straightforward comparison of specific activity and standard error of the mean found in the two assays is shown below.

N = 145	Assay (a)*	Assay (b)*	Variation (a)/(b) (%)
Specific activity (mean)	0.114	0.121	6.0
Standard error of mean	0.0023	0.0025	8.0

(* = nanomoles/mg protein/30 min)

The variability of the difference between assays (a) and (b) was calculated in 15 random samples and gave the following results:

N = 15	Assay (a) *	Assay (b) *	Calculated mean of individual differences *	Calculated variability of difference (%)
Specific activity (mean)	0.126	0.129	0.014	10.87
Standard error of mean	0.0084	0.01	0.00246	1.84

(* = nanomoles/mg protein/30 min)

Thus it would appear that BzAO in plasma prepared and frozen within 2 h of blood collection is stable for at least nine to ten months. In the case of the pregnancy project, it was essential to determine activity in all specimens simultaneously, and 9-month-old plasmas, treated as described, were assayed along with specimens of more recent date. In general, however, I consider six months' storage at -20^{o} as the maximum allowable in order to remain within the narrowest limits of reproducibility.

For other observations on stored plasma or serum, see Thermal stability, Section 3.2.7.2.

Normal variation in plasma amine oxidase activity over time was studied by Murphy et al (1976) 1-2 and 8-10 weeks apart in 21 and 41 individuals, respectively. Activities agreed closely (r = 0.97 and 0.84, respectively). These results are in close agreement with my own observations (Lewinsohn, unpublished data) and those of others (e.g., McEwen, 1971, 1972) and confirm that in the normal individual, plasma BzAO levels are stable over long periods of time. For variation of activity with sex, age and disease states, see Section 3.2.8.

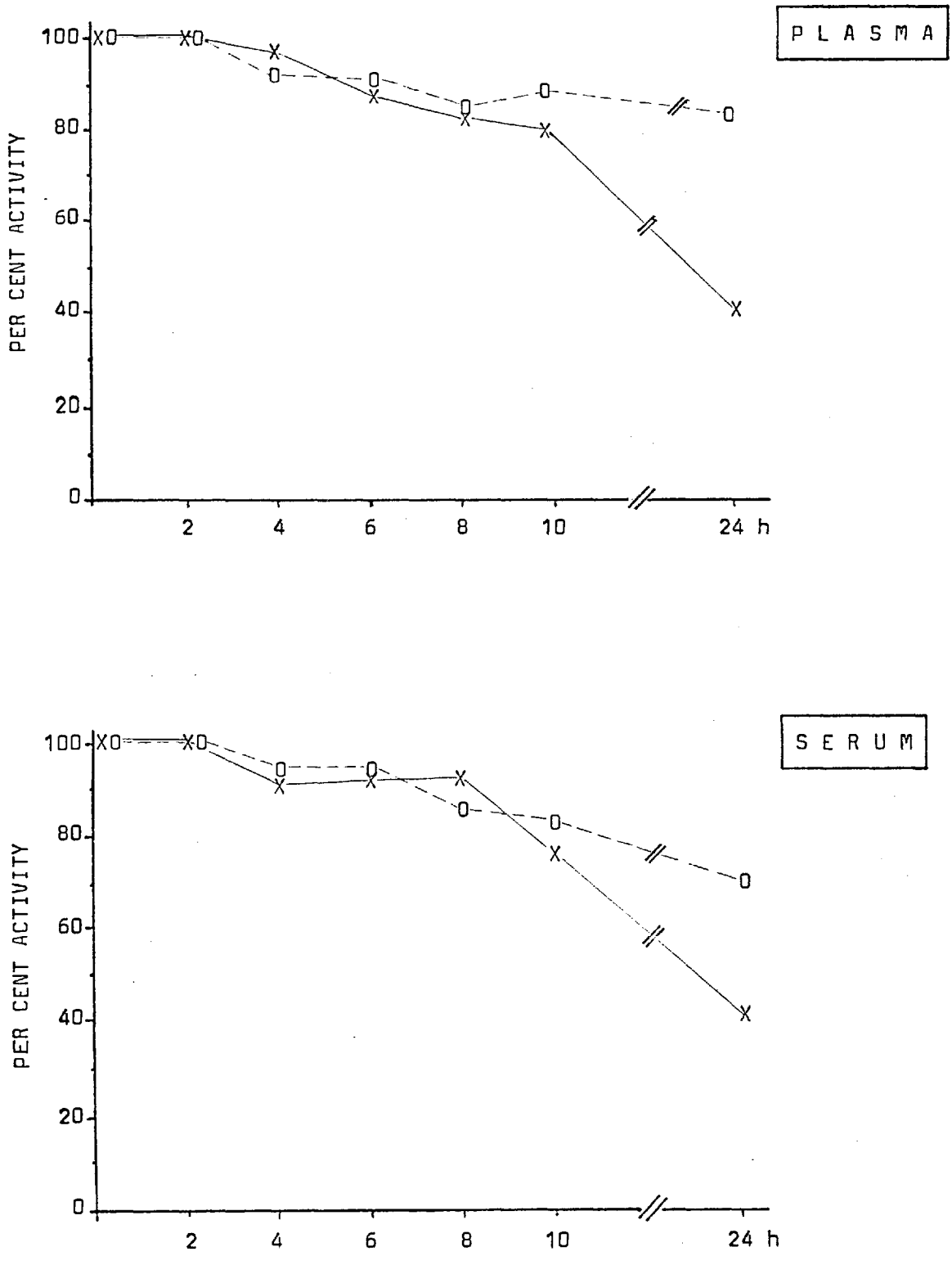
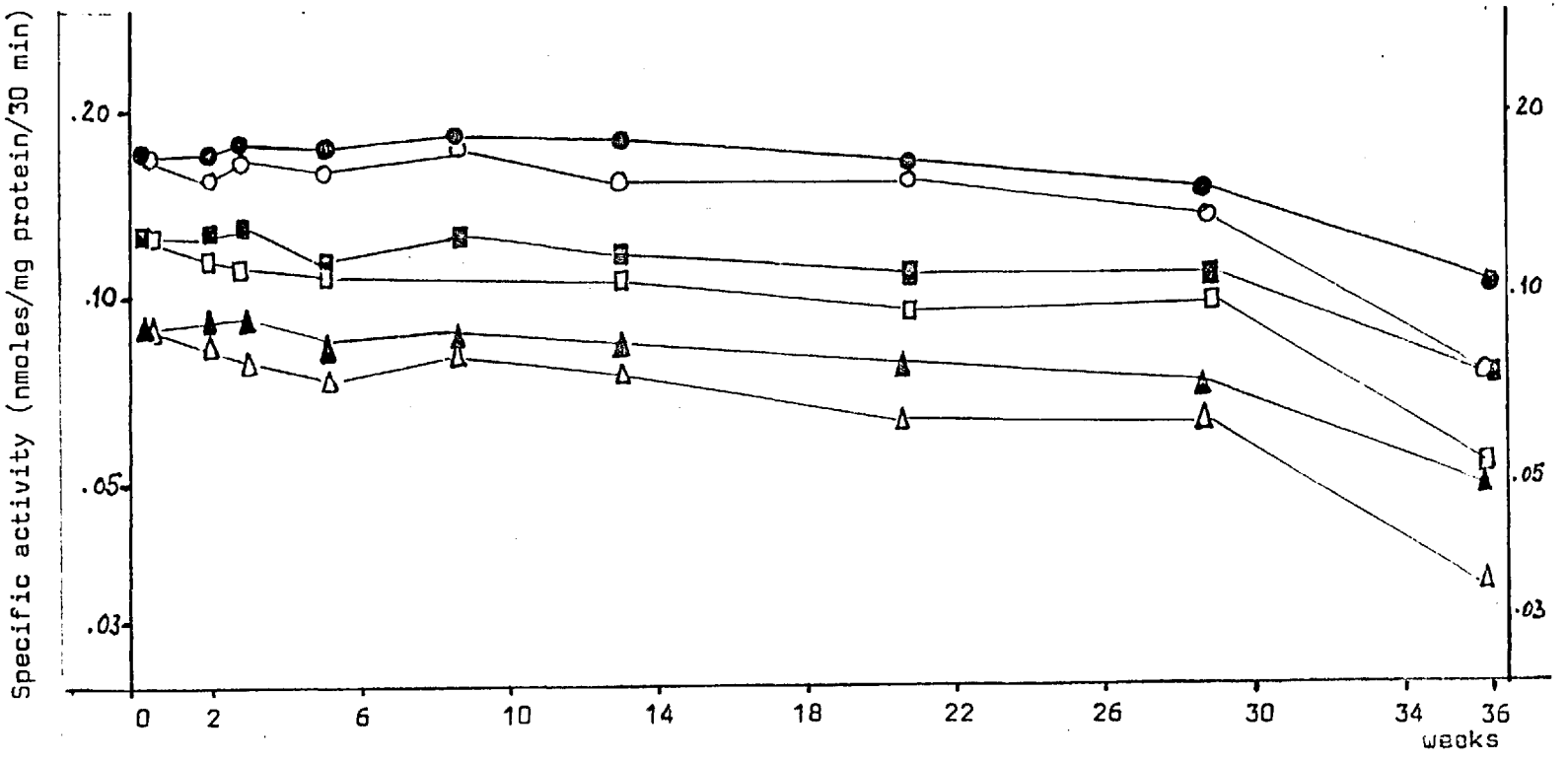


Fig.3 STABILITY OF PLASMA AND SERUM BzAO AT ROOM TEMPERATURE (RT) AND 4°C. X = RT; O = 4°. For experimental and assay conditions, see text.

Fig. 4 STABILITY OF BzAO IN HUMAN PLASMA. VARIABILITY OF METHOD.



Plasma from three normal individuals, with high (circles), medium (squares) and low (triangles) BzAO activity. Full symbols = A (single aliquots); open symbols = P (repeated freezing and thawing). For assay procedure, see text.

3.2.2

Stoichiometry

Comparing their findings on purified beef plasma amine oxidase with the data of Hare (1928), Zeller (1951), Blaschko (1952) and others, Tabor et al (1954) showed that hydrogen peroxide and ammonia are formed during the degradation of the plasma amine oxidase substrates, as with TA and other monoamines in the liver preparations used in earlier experiments. Similar results were reported, among others, by McEwen and Cohen (1963), and McEwen (1965 a) for human plasma amine oxidase, and by Tipton (1968 a, 1969 a) for rat and pig brain preparations. In all these studies, it was shown that the conversion of amine to aldehyde in crude tissue preparations, as well as in serum or plasma, requires molecular oxygen. In the presence of catalase, 1 atom of oxygen is consumed and one mole of ammonia produced in the overall reaction, per mole of substrate.

Hydrogen peroxide formation could only be demonstrated by coupling the amine oxidase reaction with the enzymatic peroxidation of o-dianisidine, since catalase activity is present even in the most purified enzyme preparations. With n-decylamine as substrate, 31 nanomoles of H_2O_2 were measured after oxidation of 30 nanomoles of amine (McEwen, 1965 a).

In a study of partially purified amine oxidase from chick bone, Rucker et al (1969) found activity in all cases to be proportional to incubation time and protein concentration. Rucker and O'Dell (1971) compared purified amine oxidase from bovine aorta with beef plasma enzyme. In the presence of catalase, the rates of O_2 consumption, ammonia liberation and H_2O_2 production by the aorta preparation were equal to the rate at which Bz was converted to benzaldehyde. The purified aorta and plasma preparations were also tested against peptidyl lysine. When lysine-vasopressin (approx. 4 mg) was added to

the reaction mixtures, the aorta enzyme (but not the plasma amine oxidase) oxidized peptidyl lysine, as judged by the peroxidase-coupled reaction: H_2O_2 was produced at a rate equal to 20% of that obtained with Bz.

3.2.3

Kinetic studies

In the first Report of the Enzyme Commission, published in 1961 (see Kapeller-Adler, 1970), enzymologists were urged to base enzyme assays, wherever possible, on measurements of initial rates of reaction and not on amounts of substrate changed by the end of a certain period of time, unless the velocity of the enzymic reaction was known to remain constant throughout that period. From a review of the techniques employed in the early stages of amine oxidase research, it would appear that the introduction of better analytical methods, such as the study of kinetics, awaited not only the isolation of the enzyme(s), but also the development of assay methods more sensitive than manometry or fluorimetry, which dominated the field during the first two decades of amine oxidase history.

The soluble enzyme was purified from beef plasma by Tabor et al (1954), who commented on the effect of pH on enzymic activity with Bz as substrate. It was McEwen (1965b) who noted that the oxidation of aliphatic amines by human plasma amine oxidase had "a number of distinctive kinetic features ... (consisting) chiefly of inhibition at high substrate concentrations, strict dependence of apparent Michaelis and inhibitor constants upon pH, and contributions of nonpolar residues to their affinities for the enzyme", none of which appeared to have been recognized in earlier studies with other plasma amine oxidases.

The dependence of the Michaelis constant of amine oxidase on pH is illustrated in Table 7, which shows the values given by several workers, compared with my own results (Table 8) for K_m and V_{max} of

Bz deamination, with deprenyl as inhibitor. Table 9 shows K_m and V_{max} values for oxidation of 5-HT, and is included to provide a basis for discussion of my results and those of others, in respect of MAO A. Data on studies with other substrates and inhibitors have been deliberately omitted, mainly because, at present, Bz is beyond doubt the substrate of choice for the assay of BzAO activity (see p.104, and references throughout this thesis).

It is clear from Tables 7-9 that the Michaelis constant depends on species and tissue; it may be influenced, moreover, by other factors such as temperature, degree of purification and solubilization of the preparation, etc. Thus, the data shown, particularly in Table 7, need to be analysed individually in order to arrive at a criterion of comparability of the different values from various sources.

There was good agreement between my values for the K_m of Bz oxidation in human plasma and those found by others (McEwen, 1965b; Lin and Castell, 1974; Murphy et al, 1976; Bond and Cundall, 1977) in experimental conditions similar to those employed by me. In some of my experiments with plasma at pH 7.2, the K_m varied between 1.2×10^{-4}M and 4.2×10^{-5}M (Table 8). The fact that the plasma samples came from different individuals may have contributed in some small measure to the variation. It is possible, however, that the larger volumes of plasma used in some experiments (5- to 10-fold) may have raised the pH of the solution in the assay tube (which was not checked). While the pH of plasma is normally about 7.4, 200 µl of plasma in a total volume of 340 µl, containing 0.7M potassium phosphate buffer, pH 7.2, measured after 2 weeks' storage at 4^{o}, gave a pH of 8.3 - 8.4, whereas 20 µl samples of rat tongue, lung and liver homogenate in a total volume of 240 µl, treated similarly, gave a pH of 7.4 - 7.5. The V_{max}, however, is remarkably constant in all the plasma experiments, and does not seem to be affected by change in pH. The only difference noted was in the V_{max} for the deprenyl-sensitive moiety of Bz oxidation, which I inter-

pret as due to MAO B, possibly owing to some slight contamination of the plasma with platelets, which are very rich in MAO B.

The most important question asked in these experiments concerns the identification of the enzymes investigated. Rucker et al (1969) obtained a partially purified amine oxidase from chick bone; a substantial amount of activity was found in the solubilized preparation, which gave a K_m of 1.5×10^{-3}M at pH 7.4, with Bz as substrate. From this and other experimental data, it seems unlikely that the enzyme involved was BzAO. Although some of the properties described by the authors are shared by the latter, the catalytic activity in this case was probably due to lysyl oxidase, which is not identical with BzAO (see pp. 32 and 109).

Murphy and Donnelly (1974) and Murphy et al (1976) show the K_m for Bz deamination in human blood platelets, at pH 7.2 and 9.0, to be 1.8×10^{-4}M and 8.0×10^{-5}M, respectively. Bond and Cundall (1977), working with the same tissue, found a K_m of 7×10^{-5}M for Bz oxidation at pH 7.4, about half the value given by Murphy and co-workers for the same substrate and pH. Both groups incubated the preparations at 37^{o} for 30 min. Edwards and Chang (1975) used solubilized preparations of human and rabbit platelets; with Bz as substrate and an incubation time of 60 min at 37^{o}, they found the K_m for human platelets to be 1.3×10^{-4}M, and for rabbit platelets 6.8×10^{-5}M. Unfortunately the pH at which the assay was carried out is not stated, but potassium phosphate buffer, pH 7.4, was used for the solubilization procedures, and the value given for human platelets is closely similar to that found by Murphy and co-workers at pH 7.2. Bond and Cundall (1977) also give a K_m of 7.5×10^{-5}M for 5-HT deamination in crude plasma (0.5 ml), but I have been unable to determine activity with 5-HT at any concentration between 36 and 570 μM.

From my experiments (Table B) it would seem that the K_m of Bz

deamination is very similar for BzAO and MAO B, but the V_{max} is unquestionably different. This is particularly clear in liver, with a K_m for BzAO about one-half that for MAO B, but a V_{max} for MAO B about 100 times that of BzAO.

Working with preparations of rat kidney mitochondria and Bz, 5-HT, DA, TA and PEA as substrates, Lyles and Shaffer (1979) show that 10^{-4}M deprenyl inhibits all deaminating activity, whatever the substrate employed (p. 101). It may therefore be assumed that 10^{-4}M deprenyl acts as a true "differentiator" and inhibits both MAO A and B in the tissues studied by me, and that the residual Bz-oxidizing activity after deprenyl (10^{-4}M) inhibition is catalysed by BzAO. As pointed out (Lewinsohn et al, 1980 b), I interpret BzAO activity in tissues poor in this enzyme (e.g., liver) as due to contamination with vascular fragments, which it is almost impossible to eliminate entirely from the preparation.

The question remains whether the moiety of Bz-oxidation sensitive to deprenyl at the concentration employed, is metabolized by MAO B or A since, as shown by Lyles and Shaffer (1979), 10^{-4}M of this inhibitor blocks all deaminating activity. A clorgyline-resistant activity was found in rat heart by Lyles and Callingham (1975) with Bz as substrate, suggesting that in this tissue, some deamination of Bz may be catalysed by MAO A. As pointed out previously, however (Lewinsohn et al, 1980a,b) Bz is a very poor substrate for MAO A, but an excellent one for MAO B. The present study, as well as the total lack of correlation between K_m or V_{max} values for deamination of Bz and 5-HT in any tissue examined so far, would seem to lend support to this view.

This problem is further discussed in Section 3.2.5, Inhibitors.

Table 7. K_m AND V_{max} OF BENZYLAMINE OXIDATION (VARIOUS AUTHORS)

SPECIES	TISSUE, PREPARATION	BUFFER, pH	°C	INCUB. (min)	INHIBITOR (M)	K_m (M)	V_{max} (μmoles/mg protein)	ASSAY METHOD	OBS.	REFERENCE
Pig	Plasma, purified 760x	Na.K.P 7.4	37.5	5-15	-	9×10^{-5}	-	Sp		Buffoni & Blaschko, 1964
Man	Plasma, purified 3000x	P 8.2	25	180	-	3.3×10^{-5}	1.63×10^{-4}/h/unit	Sp	(1)	McEwen, 1965b
Rabbit	Plasma, purified 450x	P 7.2	25	60	-	8.4×10^{-5}	-	Sp		McEwen et al, 1966
Rat	Skin, part.purif. (10x)	P 7.5	37	60	-	2×10^{-5}	-	RMA		Lovenberg et al, 1968
Rat	Liver mitochondria	P 7.5	37	60	-	5×10^{-4}	-	RMA		" " " "
Man	Crude plasma, 0.6ml	P 7.2	37	60	-	3.3×10^{-4}	2.37/h	RMA	(2)	Robinson et al, 1968
Man	Platelets, 0.1ml	P 7.2	37	60	-	1.5×10^{-4}	4×10^{-2}/h	RMA		" " " "
Rat	Liver mitoch.,part.purif.	P 7.2	37	60	-	4.5×10^{-4}	3.65×10^{-1}/h	RMA		" " " "
Chick	Bone, part.purif.	P 7.4		30-60	-	1.5×10^{-4}	-	Sp		Rucker et al, 1969
Rabbit	Aorta, purified 20x	P 7.8	25		-	9×10^{-5}	-	Sp		Rucker & Goettlich-Riemann, 1972
Pig	Plasma, purified 760x	Na.K.P 7.99	37		-	1.1×10^{-4}	39.1 moles/min/ mole of enzyme	Sp	(3)	Buffoni et al, 1972
Beef	Brain mitochondria	P 7.4	37	60	-	2×10^{-3}	-	Sp		Nagatsu et al, 1972
Beef	Dental pulp, part.purif.	P 7.4	37	60	-	7×10^{-4}	-	Sp		" " " "
Rat	Liver, part.purif.	Tris-HCl 8.2	30		-	8.0×10^{-5}	-	Sp	(4)	Houslay & Tipton, 1973a
Rat	Liver mitoch.outer memb.	K.P 7.2	30		-	2.45×10^{-4}	-	Sp	(4)	Houslay & Tipton, 1974
Man	Brain, part.purif.	Tris-HCl 8.2	30		-	9.1×10^{-5}	-	Sp	(4)	Tipton et al, 1973
Man (N)	Plasma, purif.100-200x	P 7.2	37	90	-	2.6×10^{-4}	-	Sp	(5)	Lin & Castell, 1974
Man (N)	Plasma, purif.100-200x	P 7.2	37	90	Semicarbazide 1.33×10^{-4}	2.6×10^{-3}	-	Sp	(5)	" " "
Man (A)	Plasma, purif.100-200x	P 7.2	37	90	-	2.7×10^{-4}	-	Sp	(6)	" " "
Man (A)	Plasma, purif.100-200x	P 7.2	37	90	Semicarbazide 1.33×10^{-4}	1.63×10^{-3}	-	Sp	(6)	" " "

Table 7. K_m AND V_{max} OF BENZYLAMINE OXIDATION (VARIOUS AUTHORS) (cont'd)

SPECIES	TISSUE, PREPARATION	BUFFER, pH	°C	INCUB. (min)	INHIBITOR (M)	K_m (M)	V_{max} (μmoles/mg protein)	ASSAY METHOD	OBS.	REFERENCE
Beef	Aorta, purified 150x	Na.P 7.6	25		-	6.5×10^{-4}	-	Sp		Rucker & O'Dell, 1971
Beef	Plasma, purified 60x	Na.P 7.6	25		-	1.4×10^{-3}	-	Sp		" " "
Rat	Heart, homog. 600g SN	K.P 7.8	37	10-60	-	3.12×10^{-5}	-	RMA		Lyles & Callingham, 1974
Rat	Heart mitochondria, "high affinity"	K.P 7.8	37		- clorgyline 10^{-7} semicarbaz. 10^{-3}	2.1×10^{-5} 1.1×10^{-5} 6.4×10^{-5}	- - -	RMA	(7)	Lyles & Callingham, 1975
Rat	Heart mitochondria, "low affinity"	K.P 7.8	37		- clorgyline 10^{-7} semicarbaz. 10^{-3}	3.1×10^{-4} 4.6×10^{-4} 6.6×10^{-4}	- - -	RMA	(8)	" " "
Rat	Heart, low-speed SN "high affinity"	K.P 7.8	37		- clorgyline 10^{-3}	6.4×10^{-6} 6.0×10^{-6}	- -	RMA	(9)	" " "
Rat	Heart, low-speed SN "low affinity"	K.P 7.8	37		- clorgyline 10^{-3}	6.8×10^{-4} 1.8×10^{-3}	- -	RMA	(10)	" " "
Rat	Liver mitochondria	Na.P 7.4	35	30	-	1.06×10^{-4}	-	Sp		Mantle et al, 1975b
Man	Crude plasma	P 7.2	37	30	-	2.4×10^{-4}	-	RMA		Murphy et al, 1976
Man	Crude plasma	Tris 9.0	37	30	-	1.6×10^{-5}	-	RMA		" " " "
Beef	Plasma, purified 200x	K.P 7.4	30		-	9×10^{-4}	3.4×10^{-2}/min	Sp		Houslay & Tipton, 1975a
Beef	Heart mitochondria	Na.P 7.2	30		-	1.6×10^{-4}	0.5nanomoles/min/mg	RMA		Mantle et al, 1976
Pig	Aorta, part.pur. "soluble"	Na.K.P 8.4	37	30	-	6.4×10^{-4}	-	RMA	(3)	Buffoni et al, 1976
Pig	Aorta, part.pur. "insoluble"	Na.K.P 7.8	37	30	-	1.4×10^{-3}	-	RMA	(3)	" " " "

Table 7. K_m AND V_{max} OF BENZYLAMINE OXIDATION (VARIOUS AUTHORS) (cont'd)

SPECIES	TISSUE, PREPARATION	BUFFER, pH	°C	INCUB. (min)	INHIBITOR (M)	K_m (M)	V_{max} (μmoles/mg protein)	ASSAY METHOD	OBS.	REFERENCE
Man	Lymphocytes 0.25ml	P 7.4	37	30	-	5×10^{-5}	-	RMA	(11)	Bond & Cundall, 1977
Man	Crude plasma 0.5ml	P 7.4	37	30	-	1.1×10^{-4}	-	RMA	(11)	" " "
Man	Platelets 0.25ml	P 7.4	37	30	-	7×10^{-5}	-	RMA	(11)	" " "
Man	Granulocytes 0.25ml	P 7.4	37	30	-	1.8×10^{-4}	-	RMA	(11)	" " "
Rat	Kidney mitochondria	K.P 7.8	37	10	-	9.8×10^{-5}	17.1nanomoles/ mg protein/h	RMA		Lyles & Shaffer, 1979
Man	Heart mitochondria	K.P 7.8	37	60	-	1.51×10^{-4}	-	RMA		Parkinson & Callingham, 1979
Man	Heart mitochondria	K.P 7.8	37	60	d-Amphet. K_i (M)	2.12×10^{-4}	-	RMA	(12)	" " "
Man	Heart mitochondria	K.P 7.8	37	60	d-Amphet. K_i (M)	1.24×10^{-6}	-	RMA	(13)	" " "

Table 7: Notes.

Data are shown, as far as possible, in the form and notation used by authors in their publications.

Buffers: P = phosphate; K.P = potassium phosphate; Na.P = sodium phosphate.

Assay methods: Sp = spectrophotometry; RMA = radiochemical microassay.

(1) 1 unit is defined as the amount of enzyme catalysing a change in optical density of 250 mμ of 0.001/min in 3.0-ml reaction mixtures containing 0.2M Na.K.P buffer, pH 7.2, and 3.3mM Bz.

(2) "Plasma values calculated from data of McEwen (1965 b) on purified enzyme"; but McEwen (1965 b) gives 33 μM = 3.3×10^{-5}M, not as shown by Robinson et al (1968).

(3) Buffoni et al (1972) give range of K_m and V_{max} variation with pH, 5.3 to 9.5; and 5.3 to 8.4 (1976). (See also Buffoni et al, 1977.)

(4) Coupled assay, MAO + aldehyde dehydrogenase.

(5) Pooled plasma from normal individuals (N).

(6) Plasma from patient with hepatic fibrosis and haemochromatosis (A).

(7) Suspension made by resuspending twice-washed mitochondrial pellet was used for assay. "High affinity": substrate concns. 6.25 - 31.25 μM.

(8) Preparation as in (7). "Low affinity": substrate concns. 31.25 μM - 5.0 mM.

(9) Low-speed supernatant (SN) from homogenate centrifuged at 800 g x 10 min. "High affinity": substrate concns. 6.25 - 31.25 μM.

(10) Preparation as in (9). "Low affinity": substrate concentrations 1.0 - 5.0 mM.

(11) For product extraction these authors used the resin-exchange method.

(12) K_i value calculated according to McEwen et al (1969).

(13) K_i' = pH-independent K_i value.

Table 8. K_m AND V_{max} OF BENZYLAMINE OXIDATION

SPECIES TISSUE	BUFFER	pH	INHIBITOR (μM)	K_m (M)	V_{max} (μmoles/mg protein/30 min)
RAT					
Lung	K.P	7.2	-	2.3×10^{-5}	4.24×10^{-3}
Caecum	K.P	7.2	-	2.1×10^{-5}	5.45×10^{-3}
Liver	K.P	7.2	-	1.0×10^{-4}	3.0×10^{-2}
MAN					
Aorta	K.P	7.2	-	1.1×10^{-4}	2.3×10^{-2}
Plasma, 20 μl	K.P	7.2	-	1.2×10^{-4}	1.72×10^{-4} (*)
Plasma, 100 μl	K.P	7.2	-	3.0×10^{-5}	2.26×10^{-4} (*)
Plasma, 100 μl	K.P	7.2	-	3.45×10^{-5}	2.26×10^{-4} (**)
Plasma, 200 μl	K.P	7.2	-	4.2×10^{-5}	2.15×10^{-4} (**)
Plasma, 20 μl	Tris	9.0	-	1.5×10^{-5}	2.5×10^{-4} (*)
Plasma, 20 μl	Tris	9.0	-	1.5×10^{-5}	2.0×10^{-4} (***)
Plasma, 20 μl	Tris	9.0	Resistant to deprenyl 10^{-4}	1.9×10^{-5}	1.9×10^{-4} (***)(R)
Plasma, 20 μl	Tris	9.0	Sensitive to deprenyl 10^{-4}	1.8×10^{-5}	1.6×10^{-5} (***)
Liver	K.P	7.2	-	1.7×10^{-4}	2.24×10^{-2}
Lung	K.P	7.2	-	1.1×10^{-4}	2.3×10^{-2}
Brain	K.P	7.2	-	5.0×10^{-5}	1.2×10^{-2}
Lung	Tris	9.0	Resistant to deprenyl 10^{-4}	4.3×10^{-5}	2.6×10^{-2} (R)
Liver	Tris	9.0	" "	2.7×10^{-5}	9.2×10^{-4} (R)
Kidney	Tris	9.0	" "	3.5×10^{-5}	2.3×10^{-3} (R)
Umbil.vessels	Tris	9.0	" "	3.3×10^{-5}	2.5×10^{-2} (R)
Lung	Tris	9.0	Sensitive to deprenyl 10^{-4}	2.9×10^{-5}	2.6×10^{-3}
Liver	Tris	9.0	" "	4.3×10^{-5}	7.6×10^{-2}
Brain	Tris	9.0	" "	7.9×10^{-5}	3.1×10^{-2}
Kidney	Tris	9.0	" "	2.6×10^{-5}	7.4×10^{-2}

Notes. First 5 entries from Lewinsohn et al (1978); all others, unpublished work by the author.

(cont'd)

Table 8: Notes (cont'd)

(*) = Plasma specimens from different individuals; (**) = plasma from same individual, different from (*) and (***). (***) = plasma from same individual, different from (*) and (**).
Lung, liver, brain and kidney from same (adult) individual.
Umbilical vessels pooled from three different homogenates.
(R) = residual activity after inhibition by 10^{-4}M deprenyl.
Brain preparations were totally sensitive and umbilical vessels totally resistant to inhibition by 10^{-4}M deprenyl.

Determinations were performed in duplicate by radiochemical microassay, on crude plasma, or crude tissue homogenates prepared as described (Lewinsohn et al, 1978, 1980 b). Final concentrations of benzylamine were 2.6 - 210 µM. Incubation time was 30 min, temperature 37^{o}, in all cases. Inhibitors, when used, were preincubated for 20 min at room temperature. For other details concerning assay procedure, see published reports and Section 3.1.3.

Values shown are means of duplicate determinations. K_m and V_{max} values were determined graphically from Lineweaver-Burk plots, by calculating the line of best fit by linear regression, and by computer-programmed analysis of Lineweaver-Burk plots. Correlation coefficients (r) determined by computer programme were between 0.95 and 0.99.

Table 9. K_m AND V_{max} OF 5-HT OXIDATION IN HUMAN TISSUES

TISSUE	BUFFER	pH	K_m (M)	V_{max} (μmoles/mg protein/30 min)
Lung	K.P	7.2	1.6×10^{-4}	7.4×10^{-3}
Liver	K.P	7.2	1.5×10^{-4}	3.6×10^{-2}
Brain	K.P	7.2	2.1×10^{-4}	4.2×10^{-3}
Kidney	K.P	7.2	2.1×10^{-4}	9.8×10^{-3}
Umbilical vessels	K.P	7.2	1.2×10^{-4}	7.0×10^{-4}

Notes. See Notes to Table 8 for source of tissues, and remarks concerning assay procedures. No inhibitors were used in these determinations. Final concentrations of 5-HT were 36 - 570 μM. See Notes to Table 8 for observations regarding determination of K_m and V_{max} values.

3.2.4

Subcellular fractionation. Solubility of amine oxidases.

Differential centrifugation (for references, see Zak et al, 1970) was carried out on a number of solid tissues of man and rat, with a view to determining the presence and distribution of soluble amine oxidase in subcellular fractions. In addition, human plasma and amniotic fluid were submitted to differential centrifugation, both as a control of the experiments on solid tissues and for the information the experiments might yield on the enzymes present in fractions of these fluids. Experiments described earlier (p.46) complement the present study.

3.2.4.1

Experimental

The sources and treatment of human and rat tissues used in these experiments have been described (Lewinsohn et al, 1978, 1980a,b). In addition, full-term amniotic fluid was obtained during normal delivery from patients in the Labour Ward, Queen Charlotte's Maternity Hospital, London. The fluid, centrifuged for 10-15 min at low speed (800 g) to remove mucus and cellular debris, was stored at -20° up to the time of differential centrifugation and assay. Plasma used in these experiments was my own, prepared from blood taken on the day differential centrifugation was carried out, and treated as described (Lewinsohn et al, 1978). Sources and treatment of umbilical vessels and rat aorta smooth muscle cells are described on pp. 130 and 175, respectively.

Subcellular fractions were prepared according to the method of Lyles and Callingham (1975), and as described earlier (p.46). Plasma, diluted 1:1 (v/v) in 0.1M potassium phosphate buffer, pH 7.2, and undiluted amniotic fluid were used as low-speed supernatants (LS-SN); pellets from the low-speed centrifugation of these fluids were discarded. As described (p.46), crude tissue homogenates were centrifuged at 800 g for 10 min, yielding the low-speed supernatant (LS-SN), as well as a

low-speed pellet (LS-P). Centrifugation of the LS-SN at 9000 g for 10 min produced the mitochondrial supernatant (S_1) and pellet (P_1). The S_1 fraction was then centrifuged at 105 000 g for 60 min to yield the high-speed supernatant (S_2) and "microsomal" pellet (P_2). Centrifugation at 9000 and 105 000 g was carried out at 4°. Pellets were resuspended in the same volume of buffer as that used in the suspensions from which they were derived. All other procedures and reagents have been described in detail (see p. 55).

3.2.4.2

Results

The results of these experiments are shown in Tables 10-12 and Figures 5-6. Protein content was not determined in early work (Table 10), whilst in more recent experiments several fractions contained only traces of protein, too low for estimation by the method employed (Table 11). To allow for overall comparison of percentages of recovery, I have used the cpm as a basis for calculations; the cpm for the LS-SN fraction was taken as 100 per cent.

BzAO. As seen in Table 10, brain in man and rat contained no BzAO in any fraction. The pattern of BzAO activity (*) in subcellular fractions of all other solid human tissues examined (Tables 10, 11; Figure 5) was remarkably similar (cf. Table 2, N_2-homogenate, and Figure 1, p. 50 and 54 respectively). Values for BzAO activity recovered in fractions of rat aorta smooth muscle also agree closely with those for human aorta, umbilical vessels and lung (Tables 10, 11), but except for the P_1 fraction the distribution pattern of BzAO recovery in rat lung differs from the human (Table 10, cf. Table 2, p.50). As anticipated, BzAO activity in plasma and amniotic fluid was found almost

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(*) As used in this Section, "activity" is synonymous with recovery (cpm), not specific activity.

exclusively in the soluble fractions; only the "microsomal" pellet (P_2) of amniotic fluid showed some activity against Bz. Slight deprenyl-sensitivity was detected only in the LS-SN and P_1 fractions of plasma (platelet contamination?) and in the high-speed supernatant (S_2) of amniotic fluid.

MAO B. Table 10 shows that in human and rat brain, Bz oxidation was catalysed by MAO B exclusively, but except for the "mitochondrial" pellet (P_1), fractional distribution of MAO B activity was totally different in the two species. Activity of this enzyme in the two tissues of man and rat compared in Table 10 was found mainly in the pellets, with the notable exception of human brain, in which a relatively large proportion (43%) of activity was recovered in the high-speed supernatant (S_2). In the experiment comparing human umbilical vessels with cultured cells from rat aorta smooth muscle, the contribution of MAO B to Bz oxidation was low; in the latter tissue, D*Bz (deprenyl-sensitive) activity was found only in the low-speed fractions, LS-SN and LS-P. In human umbilical vessels, some MAO B activity was seen in the high-speed fractions, S_2 and P_2 (Table 11). In the two human fluids examined, D*Bz activity was low; but 10% of Bz oxidation registered in the S_2 fraction of amniotic fluid was catalysed by MAO B (Table 12).

MAO A. Deamination of 5-HT in subcellular fractions of human umbilical vessels and rat aorta smooth muscle is shown in Table 11 and Figure 6 (a, b). Comparison of these data with Figure 6 (c,d), which represents 5-HT oxidation in the tissues described previously (see p. 46), shows similar patterns of fractional distribution of MAO A activity in all the preparations examined. Except for the pooled values for the N_2-preparations (p. 46) illustrated in Figure 6 (d), where recovery in the S_2 fraction was higher, little activity was observed in the high-speed supernatant. No MAO A activity was detectable in any fraction of the plasma or amniotic fluid examined.

3.2.4.3

Comments

Earlier comments (p. 47), comparing results of the cryostat and N_2-homogenizing procedures with tissue fractionation experiments of other workers, obviate reiteration. Some reports on subcellular fractionation by various authors are also discussed in Section 3.2.5 (Inhibitors) of this thesis.

Like the use of inhibitors, subcellular fractionation has long been a favourite method for the investigation of the amine oxidases (see Table A-1 (e), Appendix) and many workers have employed it, for example to study the multiple forms of MAO. In this brief survey, only those studies will be discussed which have a bearing on BzAO or the use of Bz as substrate, and in which cell fractionation procedures were similar to those described above; experiments involving solubilization procedures will not be discussed.

From the data presented here, it appears that BzAO activity is preferentially located in the cell sap and microsomes (S_2 and P_2 fractions) of solid tissues, whereas MAO B is found mainly in the P_1 fraction, corresponding to the mitochondrial pellet (Table 10). Although substantial MAO B activity was found in the S_2 fraction of human brain, no soluble MAO B was found in rat brain with Bz as substrate, a finding identical with that of Coquil et al (1973), who used TA as substrate. Human aorta, lung and umbilical vessels show similar fractional distribution patterns of BzAO activity. In rat lung, however, recovery of BzAO activity in the soluble (S_2) fraction is lower and that in the microsomal pellet (P_2) considerably higher, than in human lung, no doubt reflecting inter-species differences. It is therefore the more remarkable to observe the similarity in the S_2 and P_2 fractions of BzAO in cultured smooth muscle cells from rat aorta, and human solid tissues (Table 11 and Figure 5; cf. p. 47 ff).

Also worthy of comment is the pattern of distribution of BzAO activity in the soluble fraction of the four cryostat-treated solid tissues (Table 10 and Figure 5(c)), which closely resembles the pattern observed in plasma (Table 12), making it appear as if the enzyme had been "solubilized" in the tissues treated by the cryostat method. Distribution of MAO A activity in rat aorta smooth muscle is similar to the pattern seen in the cryostat-treated human lung (Table 11, Figure 6(c); cf. Table 4, p.52). There is also similarity in the patterns of MAO A distribution in human lung (N_2-procedure, Table 4) and umbilical vessels (Table 11).

As no mitochondrial marker such as lactic or succinic dehydrogenase was assayed together with the amine oxidases, it cannot be stated with certainty that the high-speed supernatant (S_2 fraction) contained no insoluble material. Nevertheless, the results presented here (see also p.47) are consistent, and in fair agreement with the findings of most workers in respect of the S_2 fraction, while there is close agreement between values for the P_2 fraction shown by most workers and mine. Although, strictly speaking, no comparison is possible, the physical principle involved in differential centrifugation is the same for any enzyme; it can therefore be stated at the very least that the method employed produced no glaring discrepancies.

There is no published report on subcellular fractionation experiments on human tissues to compare my results with; the only experiments on vascular tissues of rat (Coquil et al, 1973) and rabbit lung (Roth and Gillis, 1975) were done with substrates other than Bz. It is, however, interesting to note that Student and Edwards (1977), using PEA and 5-HT as substrates in a study of rat brain subcellular fractions, found a distribution pattern very similar to that shown here for the same tissue, with Bz as substrate (Table 10). Differential centrifugation by these workers at 1000 g x 10 min, 14000 g x 15 min

and 70000 *g* x 60 min respectively gave fractions roughly corresponding to LS-SN, $S_1 + P_1$ and $S_2 + P_2$ described above. Taking the LS-SN values for MAO B and MAO A recovered from homogenate (22.5 and 25.1%, respectively) as 100 per cent, fractional recovery in the preparation of Student and Edwards (1977) was as follows:

	Recovery (per cent)				
	LS-SN	S_1	P_1	S_2	P_2
MAO B (substr.PEA)	(100)	15.6	62.2	1.6	11.1
MAO A (substr.5-HT)	(100)	12.0	65.7	0.1	9.2

Elsewhere (p. 97) I discuss the conclusions of Coquil *et al* (1973) in respect of their inhibitor studies on subcellular fractions of rat arteries (cf. also Table 5). Here I should like to call attention to the high recovery of activity, with TA and 5-HT as substrates, in the "soluble" fractions of their preparations, which was probably catalysed by both MAO A and B. Since MAO A contributes to oxidation in the soluble fraction to a relatively minor extent, the contribution of MAO B to total activity in these preparations is very high indeed. The only tissue in which I have found a comparable activity of MAO B in the S_2 fraction is human brain (Table 10).

The localization of BzAO activity predominantly in the soluble (S_2) and microsomal (P_2) fractions of solid tissues comes as no surprise. Nor is the localization of MAO A and B in the mitochondrial fraction other than expected. Moreover, both forms of MAO have been shown by other workers to exist in the high-speed supernatant and microsomal fractions (cf. Table 5), and the present findings are in agreement with such observations. As for soluble MAO B in amniotic fluid, a fair number of samples surveyed (see p. 149) have been seen to contain the enzyme in varying proportion.

Table 10.

SUBCELLULAR DISTRIBUTION OF BzAO AND MAO B IN TWO HUMAN AND RAT TISSUES

	MAN				RAT			
Tissue	BzAO		MAO B		BzAO		MAO B	
Fraction	Cpm	Recovery (%)	Cpm	Recovery (%)	Cpm	Recovery (%)	Cpm	Recovery (%)
Lung								
LS-SN	3480	(100)	40	-	18950	(100)	4350	(100)
S_1	3530	100	90	-	5180	27	700	16
P_1	820	24	190	-	2880	15	1880	43
S_2	1310	38	10	-	2270	12	370	9
P_2	1030	30	260	-	12430	66	380	9
LS-P	2270		680		3150		3540	
Brain								
LS-SN	0		4760	(100)	0		1510	(100)
S_1	0		6650	140	0		100	7
P_1	0		3570	75	0		1000	66
S_2	0		2030	43	0		0	0
P_2	0		210	4	0		340	23
LS-P	170				200		5600	

Substrate: Bz (42 uM). Inhibitor: deprenyl (4×10^{-4}M final concentration. Assay for MAO A (substrate: 5-HT) was not done on these preparations. BzAO is defined as the deprenyl-resistant, MAO B as the deprenyl-sensitive (D*Bz) moiety of Bz oxidation. All assays in duplicate; values shown are means of replicate determinations. For explanation of symbols for fractions, experimental and assay procedures, see text.

Table 11.

SUBCELLULAR DISTRIBUTION OF BzAO AND MAO A IN FRACTIONS OF HUMAN UMBILICAL VESSELS AND CULTURED CELLS FROM RAT AORTA SMOOTH MUSCLE

Tissue / Fraction	BzAO Cpm	BzAO Recovery (%)	BzAO Specific activity	Deprenyl inhibition (%)	MAO A Cpm	MAO A Recovery (%)	MAO A Specific activity
Umbilical vessels							
LS-SN	46750	(100)	36.6	0	2185	(100)	1.7
S_1	46100	99	16.1	0	1420	65	1.0
P_1	1840	4	51.5	3	260	12	14.3
S_2	20950	45	11.3	8	190	9	0.3
P_2	15200	33	63.8	12	1340	61	11.0
LS-P	21800		34.3	0	8850		26.8
Rat aorta smooth muscle							
LS-SN	520	(100)	2.0	9	3320	(100)	22.6
S_1	410	71	1.4	0	1140	34	7.7
P_1	56	10	(*)	0	460	14	(*)
S_2	240	43	1.7	0	120	4	0.8
P_2	220	39	(*)	0	560	17	(*)
LS-P	1160		8.1	13	3510		10.6

(*) Specific activity not calculated because protein concentration of samples was too low for determination by the method employed. Specific activity is expressed as nanomoles/mg protein/30 min. For remaining legend and explanation of symbols for fractions, see foot-note to Table 10.

Table 12.

SUBCELLULAR DISTRIBUTION OF BzAO AND MAO B IN HUMAN PLASMA AND AMNIOTIC FLUID

Tissue	BzAO			MAO B		
Fraction	Cpm	Recovery (%)	Specific activity	Cpm	Recovery (%)	Specific activity
Plasma						
LS-SN	6350	(100)	0.11	320	(100)	0.005
S_1	8110	128	0.15	0	0	0
P_1	150	2	0.08	135	42	0.03
S_2	5920	93	0.10	0	0	0
P_2	186	3	0.09	0	0	0
Amniotic fluid						
LS-SN	1740	(100)	1.1	210	(100)	0.13
S_1	1860	107	1.3	90	43	0.56
P_1	50	3	-	0	0	0
S_2	1520	87	1.1	170	81	0.89
P_2	670	38	4.7	0	0	0

Low-speed pellets of plasma and amniotic fluid discarded.
Specific activity is expressed in nanomoles/mg protein/30 minutes.
With 5-HT as substrate, no activity was detected in any of the fractions of plasma or amniotic fluid.
For remaining legend, see foot-note to Table 10.

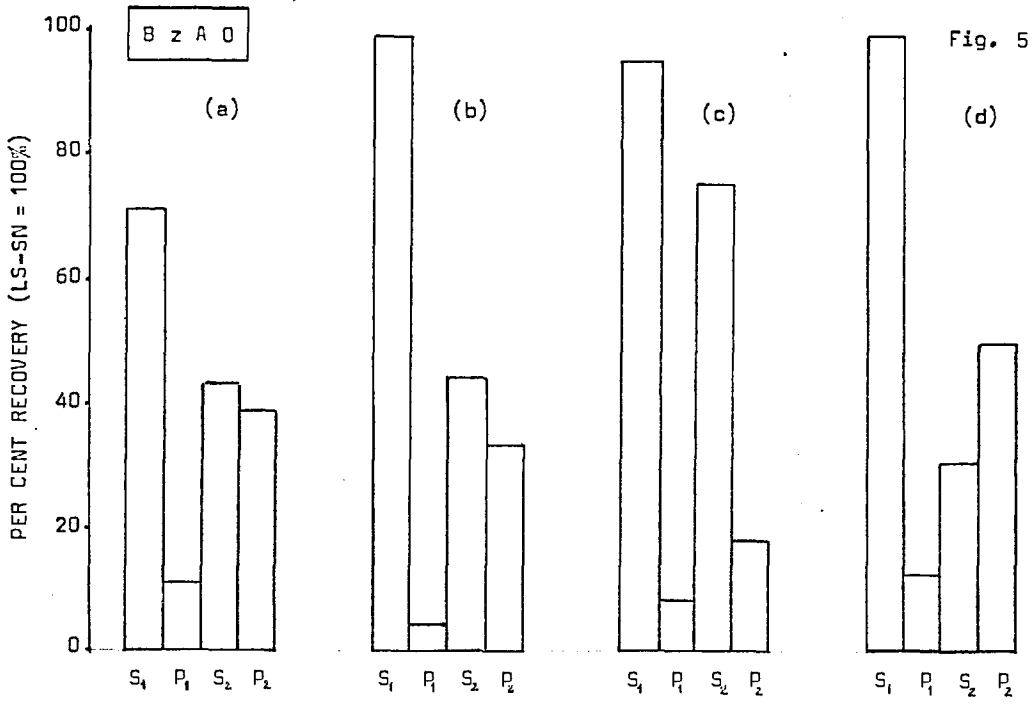

Fig. 5

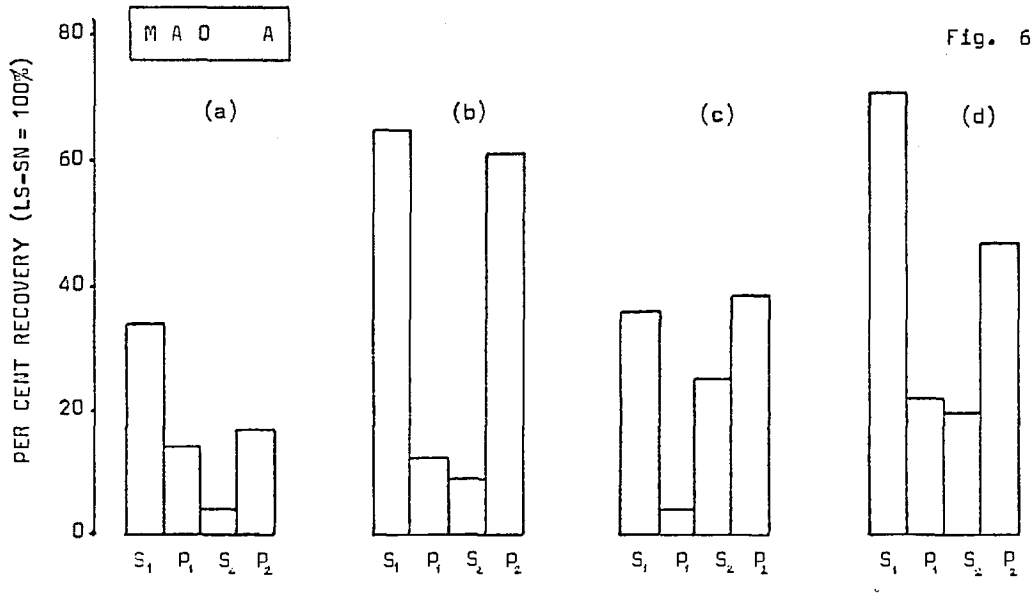

Fig. 6

Figures 5 and 6. Recovery of BzAO and MAO A activity in subcellular fractions of human and rat tissues: (a), cultured smooth muscle cells from rat aorta; (b) human umbilical vessels; (c) 4 human tissues, treated by the cryostat-method; (d) 4 human tissues, treated by the N_2-method. For experimental and assay conditions, see text.

3.2.5

Inhibitors

3.2.5.1

Introduction

Research into BzAO activity would be greatly facilitated if we had an inhibitor as selective against this enzyme as clorgyline and deprenyl are against MAO A and B, respectively. Although not without their pitfalls (see below, 'How selective is "selective"?'), these two compounds have become powerful tools in the investigation of the MAO variants. Carbonyl reagents and cyanide, allegedly without effect on MAO activity (see below), might have filled the role of "differentiators" for BzAO, but several paradoxical situations have recently been described where these compounds were used. Phenelzine, at low concentrations/a strong contender for the role of selective inhibitor of BzAO, also acts on DAO (Bardsley et al, 1974) and appears to lack, to some extent, the discriminating ability of clorgyline and deprenyl (see below); however, further studies may yet reveal it to be the BzAO inhibitor of choice.

3.2.5.2

Experimental, and comments

I have examined a number of compounds in the course of my work on human and rat plasma and tissues. Although considerations of a pharmacological or therapeutic nature have not, so far, played any significant part in these studies, the choice of certain compounds for experimentation has been prompted by the fact that they are used in the treatment of human disease, e.g., procarbazine in cancer, penicillamine in rheumatoid arthritis, benserazide in parkinsonism. Some of the results are to be found in published reports (Lewinsohn et al, 1978, 1980 a), where the inhibitory effect of semicarbazide,

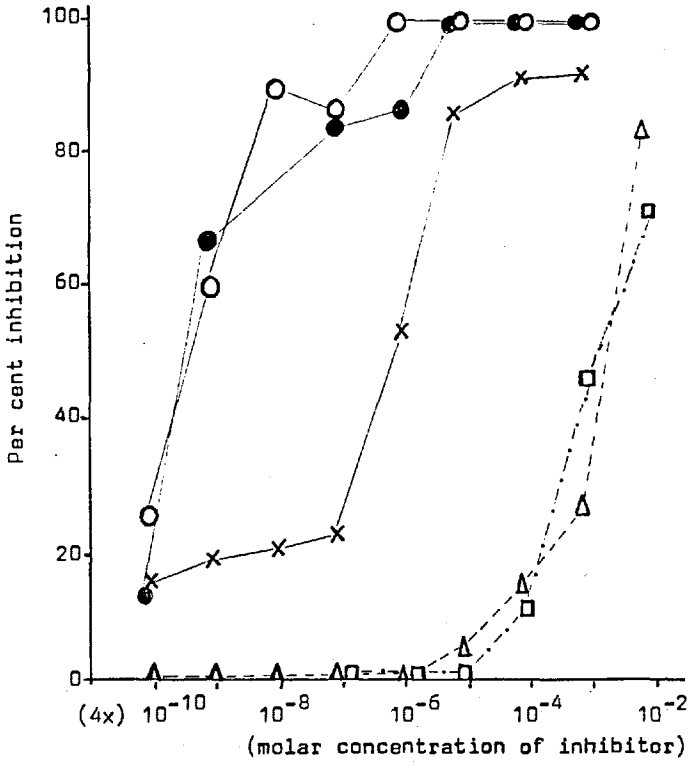

Fig. 7.
Inhibition of Bz oxidation in human tissues.

Tissue	Inhibitor	Symbol
Plasma	Deprenyl	Δ---Δ
Plasma	Clorgyline	□···□
Plasma	Phenelzine	●—●
Lung	Phenelzine	O—O
Brain	Phenelzine	X—X

For assay conditions, see text.

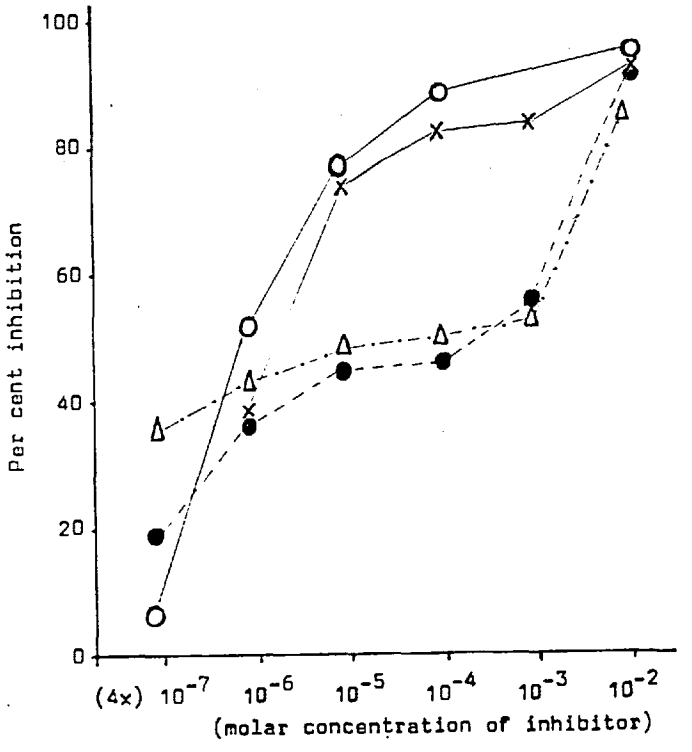

Fig. 8.
Inhibition of Bz oxidation in human plasma and rat lung.

Tissue	Inhibitor	Symbol
Plasma	Penicill-amine	●---●
Plasma	Pargyline	Δ···Δ
Plasma	Benserazide	X—X
Rat lung	Benserazide	O—O

For assay conditions, see text.

carbidopa, procarbazine, deprenyl, clorgyline, isoniazid and phenelzine on Bz deamination is described. Studies of some of these compounds and others, hitherto unpublished, are presented below.

Figure 7 illustrates the inhibition of Bz oxidation in human plasma, lung and brain by serial concentrations of phenelzine; for the sake of comparison, published data on deprenyl and clorgyline inhibition of Bz deamination in human plasma (Lewinsohn et al, 1978) are also included. This work, which shows the selectivity of the three inhibitors in the tissues studied, with Bz as substrate, provided the basis for our decision to use deprenyl and phenelzine, at the concentrations employed, as "differentiators" of BzAO and MAO B activity in human tissues at three stages of development (Lewinsohn et al, 1980 b).

Benserazide (Figure 8) inhibits Bz metabolism in human plasma and rat lung in a manner similar to, but less active than, phenelzine. On the other hand, human plasma BzAO is inhibited by both penicillamine and pargyline in a biphasic manner which suggests the existence in the sample of more than one enzyme (possibly because of platelet contamination *). An alternative explanation (Dr. B. L. Goodwin) suggests that, at low concentrations, the inhibitors produce a conformational change in the enzyme molecule, such as that suggested by Fowler et al (1977, 1978) in respect of the effect of tris buffers causing partial inhibition of enzyme activity, whilst higher concentrations of the inhibitor block all activity. It should also be noted that my experiments with pargyline were carried out at pH 7.2, whereas Murphy et al (1976), who found no inhibition of human plasma BzAO with 2×10^{-4}M pargyline, used the optimum pH for plasma activity (9.0) which I later employed in all my BzAO studies.

(*) To rule out platelet contamination, it may be preferable to use serum rather than plasma. Comparative assays of serum and plasma from the same blood samples showed similar activities (Lewinsohn, unpublished data).

Table 13. INHIBITION OF Bz OXIDATION IN HUMAN TISSUES BY 4×10^{-7}M DEPRENYL

TISSUE	FETAL		NEONATAL		ADULT	
	MEAN	S.E. OF MEAN	MEAN	S.E. OF MEAN	MEAN	S.E. OF MEAN
Brain	92.3	0.9	93.7	0.3	97.3	0.5
Cerebral vessels	-	-	-	-	0	0
Heart	94.3	0.9	91.0	1.2	93.0	0.8
Aorta	44.4	7.9	28.5	5.5	13.0	2.8
Lung	37.8	4.0	7.7	3.5	20.4	7.5
Liver	92.6	0.7	94.7	0.7	93.3	0.8
Pancreas	85.0	2.8	56.0	7.6	44.0	n=1
Kidney	94.3	1.2	93.0	1.2	86.3	1.7
Adrenal	78.2	6.5	81.7	2.9	82.0	(90, 74)
Spleen	71.0	4.0	35.5	(30, 41)	55.0	(65, 45)
Tongue	57.0	8.7	71.7	7.1	66.3	1.9
Oesophagus	18.0	4.7	38.7	8.0	35.3	9.3
Stomach	44.3	16.2	23.0	7.4	29.0	5.1
Ileum	52.8	4.3	36.7	8.5	52.7	8.7
Colon	56.1	6.7	15.7	2.3	40.0	(46, 34)
Mesentery & mesocolon (*)	61.4	5.0	43.0	6.3	29.6	3.6
Skeletal muscle (**)	41.7	6.3	63.0	4.8	85.0	2.5
Diaphragm	55.6	7.6	80.5	(80, 81)	75.5	(84, 67)
Skin & scalp (*)	58.6	5.5	51.6	7.1	51.0	(45, 57)

(*) Assayed individually; values shown are means. In fetus, scalp and skin were assayed individually (values = means). In neonate and adult, only skin was assayed. (**) In fetus and neonate, means of pectoral and thigh muscles; in adult, means of pectoral and psoas muscles, assayed individually. Values are percentage inhibition of specific activity (not shown) of samples containing inhibitor compared with uninhibited controls from same aliquots. For assay conditions, see text. Fetal tissues, n = 7; neonatal, n = 3; adult, n = 3-6. (Data based on Lewinsohn et al, 1980 b)

Table 14. INHIBITION OF Bz OXIDATION IN HUMAN TISSUES BY 2×10^{-6} M PHENELZINE

TISSUE	FETAL MEAN	FETAL S.E. OF MEAN	NEONATAL MEAN	NEONATAL S.E. OF MEAN	ADULT MEAN	ADULT S.E. OF MEAN
Brain	50.4	8.5	73.7	8.7	49	18.9
Cerebral vessels	-	-	-	-	86.3	2.2
Heart	15	5.6	33.3	10.6	16.3	8.4
Aorta	76.7	4.1	78.8	5.2	84.6	2.2
Lung	73.5	1.3	83.3	3.3	73	4
Liver	16.7	3.2	36	11.1	24.2	7.8
Pancreas	34	7	59.3	4.5	40	n = 1
Kidney	22.5	4	41	11.9	16.3	4.3
Adrenal	26.4	3.8	49	9.4	32	(11, 52)
Spleen	49.3	4.9	67	(67, 67)	49	(36, 62)
Tongue	65.8	6.8	57.7	9.3	50.3	9.9
Oesophagus	62	1.2	76.3	4.5	69.7	10.5
Stomach	74.4	3.3	83.7	0.7	63	4.5
Ileum	51	4.7	67.3	5.2	41.3	6.3
Colon	45.3	4.5	87.6	1.9	62	(50, 74)
Mesentery & mesocolon (*)	41	4.0	63	3.5	80	3.6
Skeletal muscle (**)	74	2.0	46.6	3.7	43	13.2
Diaphragm	59	4.0	48	(48, 48)	42.5	(19, 66)
Skin & scalp (*)	50	3.2	72	0.8	49	(55, 43)

(*) (**) See foot-note to Table 13 for explanation of symbols, and remaining legend.

Several experiments on human plasma and a range of human and rat tissues (cf. Lewinsohn et al, 1978) gave equivocal results using Bz as substrate, and BAPN (β-aminopropionitrile fumarate), a powerful inhibitor of lysyl oxidase (LO) (Lewinsohn, unpublished data). Oxidation of Bz in plasma was inhibited to a varying degree (67 to 81%, n = 3), but no clear pattern emerged from determination of the effect of BAPN on Bz oxidation in solid tissues of man or rat such as was observed with deprenyl and phenelzine. Human and rat aorta and lung showed substantial inhibition, but the effect of BAPN on Bz deamination in brain tissues (3 different regions) was of the same order of magnitude. Thus it is unlikely that BAPN selectively inhibits BzAO, or that BzAO is identical with LO, as has been claimed by some (see p. 28, and 31-32).

The effect of 4×10^{-7}M deprenyl and 2×10^{-6}M phenelzine on Bz oxidation in fetal, neonatal and adult human tissues is shown in Tables 13 and 14. In most tissues the total inhibition achieved with the two compounds is 100 per cent, which lends support to our contention (Lewinsohn et al, 1978, 1980a,b) that Bz is exclusively deaminated in the tissues studied by the two enzymes, BzAO and MAO B, and justifies our identification of the D*Bz (deprenyl-sensitive) moiety of the activity against Bz as MAO B, and of the deprenyl-resistant moiety as BzAO. Also worthy of note is the low variability of inhibition by either compound (cf. standard error of the mean) in those tissues where either MAO B or BzAO is clearly predominant. Deprenyl, in particular, shows remarkably little variation in tissues with high MAO B activity, but the consistently high inhibition by this compound in such tissues is not paralleled by the effect of phenelzine on BzAO, e.g. in cerebral vessels, aorta or lung (cf. Robinson et al, 1968). The difference in sensitivity toward the inhibitory effect of deprenyl on Bz oxidation at different stages of

Table 15. INHIBITION OF Bz (42 uM) OXIDATION IN SUBCELLULAR FRACTIONS OF HUMAN AND RAT TISSUES, BY DEPRENYL (4×10^{-4} M) AND PHENELZINE (4×10^{-7} M)

Tissue / Fraction		HUMAN		RAT	
		Deprenyl	Phenelzine	Deprenyl	Phenelzine
Aorta	Homogenate	27	96	25	98
	LS-SN	19	98	17	100
	LS-P	19	93	19	100
	S_1	15	95	65	100
	P_1	38	91	0	100
	S_2	24	97	0	100
	P_2	10	96	6	100
Lung	Homogenate	16	92	23	85
	LS-SN	0	96	19	94
	LS-P	23	90	53	89
	S_1	2	90	10	95
	P_1	19	93	50	82
	S_2	0	100	14	99
	P_2	0	100	3	93
Brain	Homogenate	100	33	99	20
	LS-SN	100	54	98	60
	LS-P	99	41	97	14
	S_1	100	52	100	82
	P_1	100	33	100	55
	S_2	100	0	N.D.A.	N.D.A.
	P_2	N.D.A.	N.D.A.	97	63

Homogenates prepared by N_2 method (see "Homogenizing procedures"). Values for human aorta are means of two experiments on different individuals. For experimental and assay conditions, see Section 3.2.4 (Subcellular fractionation). N.D.A. = no detectable activity in control (no inhibitor) or inhibited samples. Values are per cent inhibition of specific activity (not shown) of samples containing inhibitor compared with uninhibited controls from same aliquots. All assays in duplicate. For notation of fractions, see Section 3.2.4.

development seems to be more pronounced in tissues rich in BzAO; notable exceptions are skeletal muscle and diaphragm (Table 13). Table 15 shows the highly selective inhibition of BzAO and MAO B by phenelzine and deprenyl, respectively, in subcellular fractions of three human and rat tissues.

3.2.5.3

Literature review

Inhibition plays so prominent a part in every aspect of enzyme research that its importance can hardly be exaggerated. A review of the literature on MAO inhibition would virtually be a review of the entire literature on MAO, and clearly lies outside the scope of this thesis (for a brief survey, see Section 1.4, Literature review, and Costa and Sandler, 1972; Wolstenholme and Knight, 1976). I shall therefore confine myself to published reports on, or relevant to, BzAO inhibition and investigations in which Bz was employed as substrate.

Inhibitor studies with purified beef plasma amine oxidase were reported by Tabor et al (1954), with spermine as substrate. Blaschko et al (1959) used dialysed pig serum, and Bz as substrate; the preparation was totally inhibited by 10^{-3}M concentrations of both isoniazid and aminoguanidine. Like Tabor et al (1954), these authors point out that cyanide and carbonyl reagents are potent inhibitors of the plasma amine oxidase, but do not affect the deamination catalysed by mitochondrial MAO. In the study of Buffoni and Della Corte (1972) on pig plasma amine oxidase, the I_{50} of sodium diethyldithiocarbamate (DDTC) is shown to be similar to that of sodium cyanide, but McEwen (1965 a,b) reports that DDTC has virtually no inhibitory effect on human plasma amine oxidase and points to cuprizone as the most potent inhibitor of this enzyme. Yasunobu and Smith (1971) list metal chelators and carbonyl reagents in descending order of inhibitory effective-

ness against beef plasma amine oxidase purified approximately 430-fold, as follows:

Metal chelators	Carbonyl reagents
cuprizone	hydrazine
o-phenanthroline	phenylhydrazine
sodium diethyldithio-carbamate	hydroxylamine
	semicarbazide
2,2'-bipyridyl	isonicotinic acid hydrazide
neocuproine	sodium cyanide
sodium cyanide	sodium bisulfite

The PI_{50} varied from 1.0×10^{-7} to 1.0×10^{-3}M for these compounds when tested by the spectrophotometric assay of Tabor et al (1954).

Buffoni et al (1975) obtained a soluble and an insoluble fraction of the amine oxidase from purified pig aorta; histamine, a substrate for the soluble pig plasma amine oxidase, was found to be a competitive inhibitor of the insoluble fraction, which the authors called "benzylamine oxidase", but in view of the properties described by them, the designation is questionable. Houslay and Tipton (1975 a) demonstrate the reversible inhibition of purified beef plasma amine oxidase ("MAO") by clorgyline; the enzyme had a K_m for Bz of 0.9mM, and was shown to be irreversibly inhibited by 1mM potassium cyanide.

Inhibition of Bz deamination by high substrate concentrations has been described by various authors (e.g., McEwen, 1965 b; Jarrott, 1971; Murphy et al, 1976). In previous work (Lewinsohn et al, 1978), we found substrate inhibition by 690 µM Bz in rat but not human tissues. Product inhibition by ammonia is reported by McEwen (1965 b) in human plasma, by Buffoni and Blaschko (1971) in pig plasma, and by Houslay and Tipton (1973 b) in the deamination of Bz by purified rat liver MAO; the latter workers also demonstrated inhibition by benzaldehyde produced in the reaction. Since the concentrations of NH_3 employed by McEwen (1965 b) and Houslay and Tipton (1973 b) exceed any likely to be found in physiological conditions, the significance of these observations with regard to the situation in vivo is obscure and must await

further studies. On the other hand, McEwen and Castell (1967), searching for correlations between high plasma BzAO activity and ammonia levels in patients with liver cirrhosis, found that ammonia, administered orally to 42 patients, produced no inhibition of plasma BzAO activity. Increased enzyme activity was more clearly associated with elevated ($>$ 235 ug/100 ml) peak ammonia concentrations, but although chi^2 analysis yielded a p of < 0.001, regression analysis showed $r = 0.51$ only.

Fowler et al (1977, 1978) demonstrate that the oxidation of TA, 5-HT and PEA is inhibited by tris buffer, with a K_i of 15-25mM, but Bz oxidation is not. Apart from 5-HT, the inhibitory effect in each was completely reversible. The authors suggest that such effects are produced by conformational changes in the structure of MAO.

Although a dialysable endogenous inhibitor of MAO has been reported to exist in the plasma of some schizophrenic patients (Berrettini and Vogel, 1978), such claims are disputed by others. Murphy et al (1976) could not demonstrate any reversible endogenous inhibition of MAO following dialysis for 12 h or after the mixing of platelet and plasma samples from individuals with high and low MAO activities. My own experiments with serum from severely burnt patients and from cancer patients, dialysed overnight against buffered normal saline, and the incubation of sera from such patients, mixed with samples showing normal or high BzAO activity, did not produce any evidence for the existence of an endogenous inhibitor of BzAO in human serum (Lewinsohn, 1977).

On the other hand, Lyles and Callingham (1975) comment on the curious effect of low concentrations of clorgyline which frequently produce a "slight but not significant increase in MAO activity". I have often observed a similar enhancement of activity with both clorgyline and deprenyl (Lewinsohn, unpublished data). Considerable

activation of platelet MAO by human plasma is reported by Yu and Boulton (1979). This effect was found to be substrate-selective. While samples assayed with MAO substrates, PEA and Trypt, showed little enhancement of activity, the deamination of Bz was substantially increased. No explanation can be offered at present for any of these phenomena.

3.2.5.4

How selective is "selective"?

According to Zeller (1951), Tabor et al (1954), Blaschko et al (1959) and many others, cyanide, an inhibitor selective for the copper-containing amine oxidases, BzAO and DAO, has no effect on MAO activity. However, Lyles and Callingham (1975) found a Bz-oxidizing activity in rat heart which was sensitive in part to clorgyline and semicarbazide (see below), but resistant to cyanide. A similar sensitivity pattern was demonstrated by Coquil et al (1973) in rat artery amine oxidase with TA as substrate (see below). This raises the interesting possibility as to whether rat tissues in general might be insensitive to cyanide. Houslay and Tipton (1973b), however, demonstrated KCN inhibition, freely reversible by gel filtration or dilution, of enzyme purified from the outer membranes of rat mitochondria, solubilized by Triton X-100 and treated with perchlorate.

Lyles and Callingham (1974) describe a biphasic inhibition curve obtained with clorgyline in rat heart preparations, using Bz as substrate. They exclude blood contamination as a possible cause of the clorgyline-resistance seen in the inhibition curve; no mention is made in this paper of the possibility of contamination of the homogenates with vascular fragments. In a later paper the same authors (Lyles and Callingham, 1975) describe a component of total MAO activity assayed with Bz as substrate in subcellular fractions of rat heart,

which was found to be resistant to clorgyline, relatively sensitive to semicarbazide and isoniazid, but insensitive to KCN. Blood contamination was again ruled out, but the authors state that "although the hearts used ... had the larger blood vessels dissected away before homogenization, it is likely that vascular elements would still be present within the homogenate". Except for the property of resistance to cyanide, the clorgyline-resistant moiety of Bz oxidation might, of course, be interpreted as BzAO activity. Equally puzzling is the relative insensitivity to semicarbazide of the clorgyline-resistant activity against Bz. Both inhibitors were used at a high concentration, 10^{-3}M, which in the case of semicarbazide might have been expected to cause total inhibition of BzAO. The authors suggest that membrane components which resealed around cytoplasm during the preparation of the subcellular fractions might account for the presence of mitochondrial MAO in each fraction, and this might well explain the relative insensitivity to semicarbazide observed in the high-speed supernatant.

In experiments on subcellular fractions of rat arteries and brain, with TA as substrate, Coquil et al (1973) found 42% inhibition by 10^{-3}M semicarbazide in the "soluble" fraction (authors' quotation marks) of rat mesenteric artery; 10^{-4}M clorgyline and 5×10^{-5}M pargyline produced 44 and 54% inhibition, respectively, whilst 10^{-3}M NaCN had no inhibitory effect. Inhibition of the "mitochondrial" fraction was 21, 74, 86 and 0% for semicarbazide, clorgyline, pargyline and NaCN, respectively (concentrations as above). Conversely, activity in a crude homogenate of brain, with the same substrate and inhibitors, was blocked to the extent of 0, 96, 100 and 0%, respectively. TA, a very poor substrate for BzAO (McEwen, 1965a; Lin and Castell, 1973, 1974, 1975), is deaminated in varying degree by MAO A and B (Johnston, 1968); it is thus probable that the activity recovered in the "soluble"

fraction was catalysed predominantly by the two forms of MAO. The inhibition of this fraction by semicarbazide is therefore suggestive of a selectivity far less marked than had previously been assumed (see above). Similar conclusions may perhaps be drawn from the report of Fuentes and Neff (1977) on a semicarbazide-resistant activity against PEA in rat heart and mesenteric artery: 1mM semicarbazide produced 30 and 87% inhibition, respectively, in the heart and mesenteric artery preparations; 0.1mM cuprizone blocked 36% and 70% of activity, respectively, in the same preparations, with PEA as substrate. Since PEA is a poor substrate for BzAO (Tabor et al, 1954), it is probable that the results with this substrate, particularly when compared with those obtained with noradrenaline (NA) as substrate and clorgyline and pargyline as inhibitors, can be interpreted as activities catalysed by MAO A and B. Indeed, in the light of recent work, my own included, I suggest that some results of our experiments on rat tissues (Lewinsohn et al, 1978) may admit of an interpretation somewhat different from that given in our paper, which was based mainly on the relative sensitivities of Bz and PEA deamination towards deprenyl inhibition. The contribution of MAO A to PEA oxidation, which has been demonstrated in various tissues (Lewinsohn et al, 1980 a, and references in that paper), and the loss of selectivity of MAO inhibitors when employed at high concentrations, make it likely that some proportion, at least, of deprenyl-sensitive as well as deprenyl-resistant PEA deamination was catalysed by MAO A, rather than BzAO. Comparison of the inhibitory effect of 4×10^{-4}M deprenyl on oxidation of Bz, PEA and dopamine (DA) employed in these experiments bears out this interpretation (data from Lewinsohn et al, 1978; tissues: rat).

Substrate	PER CENT INHIBITION				
	Diaphragm	Lung	Liver	Psoas	Aorta
Bz	8	10	98	11	9
PEA	45	47	97	58	16
DA	90	88	99	99	19

Reporting on their experiments with crude homogenate and perfused rabbit lung, with PEA and 5-HT as substrates, and pargyline and semicarbazide as inhibitors, Roth and Gillis (1975) describe three forms of amine oxidase in rabbit lung, two with the characteristics of "classical" MAO A and B with respect to substrate and inhibitor specificity, and a third form, "similar to the enzyme found in plasma and several large arteries". The authors suggest that "all these forms of MAO are associated with vascular endothelium". Although it is more than likely that rat lung is rich in BzAO (Lewinsohn et al, 1978), such conclusions cannot be drawn from experiments with PEA or 5-HT as substrates. Certainly the inference that BzAO activity is localized in vascular endothelium seems unwarranted. If this were the case, the crude homogenate preparation might be expected to be more sensitive to semicarbazide than the perfusion effluent; such differential inhibition was observed with 10^{-3}M pargyline (57.8 and 73.4% inhibition of effluent and homogenate, respectively), pointing to the predominance of MAO B in the latter preparation. 10^{-3}M semicarbazide, however, inhibited the crude homogenate to a lesser extent (21.1%) than the perfusion effluent (27.8%). With Bz as substrate, we have shown (Lewinsohn et al, 1978) that 10^{-3}M semicarbazide inhibits amine metabolism in human tissues rich in both MAO A and B (49, 36 and 34.5% respectively in kidney, brain and liver); 96% inhibition was recorded in crude homogenate of human lung. With PEA as substrate, the similarity of values for the inhibition of effluent and crude homogenate shown by Roth and Gillis (1975) suggests that 10^{-3}M semicarbazide inhibited MAO non-selectively, in both preparations. As for the endothelial location of BzAO, this is virtually ruled out by the study of Roth and Venter (1978) demonstrating the marked predominance of MAO B in cultured intimal endothelial cells from rabbit aorta, with PEA, TA, 5-HT, DA and NA as substrates and clorgyline and harmaline as

inhibitors. This agrees with our histochemical study of human placenta (Ryder et al, 1979), which showed BzAO to be highly localized to the tunica media of placental vessels, while vascular endothelium showed no staining for this enzyme. Inhibitors used in these experiments, with Bz and TA as substrates, were deprenyl (10^{-6} to 10^{-8} M), clorgyline (10^{-7} to 10^{-9} M), pargyline (10^{-3} M) and phenelzine (5×10^{-5} M); only phenelzine had an effect, abolishing the reaction, whilst none of the other inhibitors had any effect whatsoever, at any concentration (see also p. 159).

With Bz as substrate, both deprenyl and clorgyline gave biphasic inhibition curves in crude homogenates of lung; in this preparation, clorgyline inhibition was also biphasic with PEA as substrate (Lewinsohn et al, 1980 a). This observation raises the question, already suggested by the work of Lyles and Callingham (1974, 1975, 1979) as to the selectivity of clorgyline for MAO A or, alternatively, the possible role of MAO A in Bz deamination, a question not easily answered from present experimental data. There can be no doubt concerning the high selectivity of deprenyl against MAO B, and of clorgyline against MAO A, particularly when used with the appropriate substrates. At high concentrations, however, most amine oxidase inhibitors tend to become non-selective. Lyles and Shaffer (1979) studied substrate specificity and inhibitor sensitivity of MAO in rat kidney mitochondria with clorgyline and deprenyl (among others) as inhibitors, and a range of substrates. With Bz as substrate and 2×10^{-4} M clorgyline, 20% of enzymic activity in the mitochondrial preparation was resistant to inhibition, a finding comparable to that of Jarrott (1971) in a crude homogenate of rat vas deferens, which showed a 20% resistance to inhibition of Bz deamination by 10^{-3} M clorgyline. Resistance to the (reversible) inhibition of Bz oxidation in purified beef plasma amine oxidase by 10^{-3} M clorgyline was

shown by Houslay and Tipton (1975 a) to be of the same magnitude. When plotted as a semi-log. "dose-response" curve, the data of the latter experiment gave a biphasic curve; Dixon analysis, however, yielded results inconsistent with the presence of two enzymes in the preparation.

The inhibitory effect of a relatively low concentration (2×10^{-5} M) of deprenyl, which abolished Bz oxidation in rat kidney mitochondria (Lyles and Shaffer, 1979), closely agrees with my experiments on the effects of serial dilutions of deprenyl on crude homogenates of human tissues: in kidney, all Bz deamination was suppressed by 4×10^{-6} M deprenyl (Lewinsohn, unpublished data). Indeed, at 2×10^{-4} M, deprenyl is shown by Lyles and Shaffer (1979) to block all enzymic activity in rat kidney mitochondria, whatever the substrate employed. However, 20% resistance to 10^{-3} M deprenyl, with 5-HT as substrate, was shown by Ekstedt and Oreland (1976) in pig liver mitochondria; in the same preparation, they found an 8% resistance to the same concentration of deprenyl, with TA as substrate.

Unlike PEA which, in certain tissues, may act as a substrate for MAO A and B (Edwards and Chang, 1975; Egashira, 1976; Ekstedt, 1976; Dial and Clarke, 1978; Suzuki et al, 1979; Lewinsohn et al, 1980a), Bz has consistently been shown to be a very poor substrate for MAO A. A case apart is the deamination of Bz and PEA by mouse neuroblastoma cultured cells (Donnelly et al, 1976), which was almost totally inhibited by 10^{-7}M clorgyline, suggesting that oxidation of these two substrates in the cultured tumour cells may be largely due to a form of MAO sensitive to clorgyline, probably type A. In tissues extremely rich in MAO A, e.g., human placenta and lung, this enzyme may contribute to some slight extent to the deamination of Bz (Lewinsohn et al, 1980 a; see also p. 142). Further blocking of Bz oxidation in these tissues by clorgyline is probably the effect of non-selective inhibition of BzAO, as indicated above. There can be little doubt, however, that

such cases are the exception, not the rule. Examination of large numbers of tissues has failed to yield any correlation between deamination of Bz and the activity of MAO A (Lewinsohn et al, 1980 b), or between the inhibitory effect of clorgyline and the activity of BzAO.

All these observations would seem to corroborate my interpretation of the slight clorgyline-sensitivity of Bz oxidation found in homogenates of some solid tissues as an expression, in most cases, not of inhibition of MAO A, but of the presence of traces of BzAO, inhibited in a non-selective manner by clorgyline(*). Trace activity of BzAO in tissues rich in MAO A or B is easily explained by the fact that it is virtually impossible to prepare a mammalian tissue homogenate which does not contain fragments of vascular tissue. The presence of vascular fragments is suggested by Lyles and Callingham (1975) as a possible explanation of their findings in rat heart (see above) and such elements may well have been present, for example, in the mitochondrial pellet employed by Lyles and Shaffer (1979). The striking effect of aminoguanidine, a potent inhibitor of the copper-enzymes, on Lyles and Shaffer's (1979) preparations of rat kidney mitochondria, might provide indirect support for such an interpretation, despite the fact that semicarbazide had virtually no inhibitory effect on the preparation. Conversely, the very high inhibitory effect of aminoguanidine strongly suggests that MAO activity also was blocked, in a non-selective manner.

3.2.5.5

Conclusions

Many of the comments and conclusions of the preceding paragraphs are explicit or implicit and require no elaboration. Sensitivity to

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(*) It cannot, of course, be ruled out that BzAO itself consists of multiple forms, one or more of which might be sensitive to inhibition by clorgyline (see next Section, p. 104).

semicarbazide or cyanide, even with Bz as substrate, can no longer by itself be interpreted as evidence for the activity of a copper-containing enzyme, whether soluble or insoluble. In this context, it is worth noting the low rating for inhibitory effectiveness given to both compounds, but particularly cyanide, by Yasunobu and Smith (1971) (see p. 94).

From my own observations and those of others, discussed above, it would appear that deprenyl and phenelzine are excellent differentiators for the deamination of Bz by MAO B and BzAO respectively, in the tissues examined. The action of clorgyline on BzAO, however, is by no means clear, and awaits further work for interpretation.

In this chapter, I have dealt almost exclusively with compounds I have used in my experiments. However, several substances (e.g., aminoguanidine), which I have never tested, show considerable promise as selective BzAO inhibitors. Two compounds, aminoacetonitrile and propargylamine (see Riceberg et al, 1975), seem to be extremely effective against TA and mescaline oxidation in various rabbit tissues, including serum; the former appears to be the more selective of the two for the plasma-type amine oxidase in the rabbit, using mescaline as substrate. I have not seen any studies with these compounds where Bz was used as substrate, and suggest that this line of investigation might well prove rewarding.

The caution recommended by Fowler et al (1978), Murphy (1978) and countless others, in planning and interpreting experiments, particularly as regards the choice of substrates and inhibitors, is of course a cardinal point in the study of BzAO as well as the two forms of MAO; and no review of this subject would be complete without the emphatic re-enunciation of the First Commandment of Enzymology:

" Thou Shalt Not Extrapolate ! "

3.2.6

Substrate specificity and multiplicity of forms

Multiple forms and substrate specificity of the mitochondrial flavo-enzyme(s) have been the subjects of innumerable studies and reviews (see Section 1.4, Literature review, and references throughout this thesis). Although some aspects are touched upon elsewhere (see, for example, the preceding Section), a detailed discussion of the problems involved clearly exceeds the scope of this thesis.

3.2.6.1

Substrate specificity of BzAO

Studies of substrate specificity of BzAO by many workers (see Section 1.4, Literature review, and Chapter 2, Nomenclature) have invariably shown Bz to be deaminated at a much higher rate than any other substrate examined. I have compared PEA, 5-HT, TA, DA, NA and adrenaline oxidation with that of Bz in human plasma, with similar results (Lewinsohn, unpublished data). It cannot, of course, be ruled out that any of the physiological monoamines examined may be oxidized by BzAO *in vivo* and may indeed be its "true" substrate, but much further work is required before any hypothesis can be advanced, let alone a conclusion formulated.

As pointed out earlier (p. 37), Bz has not been demonstrated in the mammalian organism. A search of the literature for a possible exogenous source of Bz in plants, foodstuffs or drugs consumed by man has been fruitless. Schmiedeberg's historic experiment (1877) with dogs was repeated recently (Wood *et al*, 1978) with two male volunteers, who were given oral Bz. The fate of benzoic acid in various species was studied by Bridges *et al* (1970), while urinary hippuric acid excretion as an index of toluene exposure has been investigated by Pagnotto and Lieberman (1966), and Ikeda and Ohtsuji (1969 a,b). Perry *et al* (1966, 1972) described the urinary excretion of amines

in normal and chronically hospitalized patients, and benzoic aciduria in chronic psychoses. Whilst much information has come from these studies, none of them has shed new light on the problem of the "true" substrate of BzAO, which remains obscure.

3.2.6.2

Multiple forms of BzAO

Although many authors have postulated the existence of multiple forms of BzAO, investigation of this subject, so far, is restricted to the work of Rauch and Rauch (1973), Lin and Castell (1973, 1974, 1975) and Lin et al (1976).

Lin and Castell (1973, 1974) compared BzAO, purified 200 to 300-fold, from the plasma of a patient with haemochromatosis and liver fibrosis, with that from pooled normal plasma. Substrate specificity studies revealed that for both the normal and the abnormal amine oxidase, Bz was the best substrate, while TA, Trypt and kynuramine were deaminated at a much slower rate. Activity ratios of the latter 3 substrates compared with Bz showed distinct differences in specificity between normal and abnormal preparations. The oxidation rate of Trypt and TA was about 15% of Bz deamination in normal plasma, but only 2-3% of that in abnormal plasma. While the optimum pH was 7.2 for both preparations, abnormal BzAO lost more activity when the pH was either acidic or basic. Sensitivity towards semicarbazide was higher in the abnormal than the normal plasma at all inhibitor concentrations employed, and the K_m was significantly different for the two preparations in the presence of semicarbazide (see Table 7, p. 69). Kynuramine was found to inhibit Bz oxidation non-competitively; at optimum substrate concentrations, when the inhibitor concentration was held constant, both preparations were inhibited in a similar manner, but the abnormal enzyme reflected its dependence on substrate concentration when low Bz concentrations were employed. The authors

conclude that the differences observed represent different intrinsic molecular properties of the enzyme in the preparations, pointing to the presence of isoenzymes of BzAO.

These observations were extended by the same authors (Lin and Castell, 1975). Three forms of BzAO, which they designated alpha, beta and gamma, were isolated from normal human plasma by hydroxyapatite column chromatography; the alpha form was further purified 10,000-fold by DEAE-Sephadex A50 column chromatography. All three forms had a molecular weight of 150,000. Specificity toward Bz, TA and Trypt, sensitivity toward semicarbazide, and thermal stability were all found to be different for the three forms. Bz was by far the best substrate. Deamination of the beta form, which had the lowest ratio of Bz-oxidation compared with Trypt, was 29.5 times more rapid with Bz, while the ratio for the alpha and gamma forms was 72.5 and 44.8, respectively. The Bz:TA ratio was about the same as that for Trypt. The critical temperature was 55° for the alpha form and 60° for the beta and gamma forms; at 65.5°, the alpha form lost its activity according to third-order kinetics; the other two followed first-order kinetics.

Further work by Lin et al (1976) showed the isoenzyme composition of normal human plasma to be different from that in the plasma of patients with haemochromatosis. The latter showed a decreased proportion of the alpha form and an additional form ($alpha_1$), not seen in normal plasma, as well as an increase in the proportion of the beta form compared with normal plasma.

Rauch and Rauch (1973) used starch gel electrophoresis and column chromatography to determine patterns of enzyme or isoenzyme activity in human plasma and various human tissues. With Bz as substrate and tetrazolium salts as reducing agent, electrophoresis of human plasma revealed four areas of enzyme activity with patterns

which chromatography indicated might correspond to isoenzymes of different molecular weights (not given). No such pattern was found in any of the solid tissues examined.

3.2.7

Other properties

3.2.7.1

Copper content and pyridoxal dependence

While the presence of copper in BzAO has been generally accepted (Blaschko, 1974; Grant et al, 1978), the dependence of activity of this enzyme on pyridoxal phosphate (P-5-P) has been the subject of some controversy. Both questions are reviewed in great detail by Yasunobu et al (1976), especially the mounting evidence against P-5-P dependence of the copper-enzyme. McEwen and Cohen (1963) had found no stimulation of enzyme activity on addition of 0.15 µM pyridoxal-5'-phosphate or pyridoxamine-5-phosphate to standard incubation mixtures of normal human serum. I have used P-5-P (10^{-4}M) in a single experiment on human lung and aorta, with Bz as substrate and three inhibitors. P-5-P in uninhibited samples caused a drop in activity of about 40%, compared with controls without P-5-P. In inhibited samples, P-5-P lowered the effectiveness of phenelzine against BzAO to that of clorgyline and deprenyl, so that the same degree of inhibition was observed for all three compounds, as shown below.

Effect of 10^{-4}M pyridoxal-5'-phosphate on Bz oxidation in human lung and aorta. 0.1M K_2HPO_4/KH_2PO_4 buffer, pH 7.2. Bz, 42 µM. Inhibitors as shown. Homogenates prepared by N_2-method. Assay conditions, standard. Values: cpm, means of duplicate determinations.

Tissue	Control		4×10^{-3}M	4×10^{-4}M	2×10^{-6}M
	No P-5-P	P-5-P	Deprenyl	Clorgyline	Phenelzine
Lung	14600	10530	8260	8340	8540
Aorta	2920	2260	1820	1900	2000

3.2.7.2

Thermal stability

Thermal inactivation has been used by many researchers as a method to characterize multiple forms of mitochondrial MAO in a range of species and tissues (see Table A.1 (h), Appendix; for reviews, see Sandler and Youdim, 1972, 1974).

Little work seems to have been done on the determination of thermal stability of the copper-amine oxidases, in plasma or solid tissues. Kapeller-Adler and MacFarlane (1963) showed pig kidney histaminase to be stable in a wide range of temperatures between -20° and 62°. Tufvesson (1969) found BzAO activity in crude serum reduced by 4% after 30 days' storage at -20°, while McEwen (1972) described the purified plasma enzyme as exceedingly stable if stored in the frozen state. In experiments with purified pig plasma, Blaschko and Buffoni (1965) demonstrated that the enzyme from this source is stable for months in the dark, under ammonium sulphate, at 0 to 4°. Lin and Castell (1975) found different critical temperatures for 3 forms of amine oxidase isolated from normal human plasma (see p. 106).

My only experiments involving thermal stability were concerned with the effect of freezing tissues at various temperatures. Just before homogenization by hand (in ground-glass homogenizers), by "mincing", or by the liquid nitrogen method (see Section 3.1.2, Homogenizing procedures), human aorta and placental vessels were frozen at -20°, -80° or -196°, respectively for the three homogenizing procedures, and were assayed immediately afterwards. Mean values observed in both tissues for the three temperatures were 100, 82 and 75 per cent, respectively. Amniotic fluid and plasma, frozen at the same temperatures and thawed just before assay, gave comparative mean readings of 100, 98 and 96 per cent, respectively (Lewinsohn, unpublished data).

For comments on stability of enzyme in stored tissues, see Section 3.2.1, Stability of enzyme and variability of method.

3.2.7.3

Molecular weight of BzAO and other Cu-enzymes

Table 16, below, shows the values given by various authors for the molecular weight of BzAO and other copper-dependent enzymes.

Species, tissue	Enzyme	Molecular weight	Reference
Beef, aorta	BzAO (?)	255,000	Yamada & Yasunobu (1962a)
Pig, plasma	BzAO (*) (histaminase)	195,000	Buffoni & Blaschko (1964)
Beef, plasma	BzAO	170,000	Yamada et al (1964)(**)
Hog, kidney	DAO	185,000	Yamada et al (1966)(**)
Chick, bone	LO (?)	250,000	Rucker et al (1969)
Beef, dental pulp	BzAO	170,000	Nagatsu et al (1972)
Chick, aorta	LO (? subunit)	61,000	Harris et al (1974)
Man, placenta	DAO (? subunit)	90,000	Bardsley et al (1974)
Pig, plasma	BzAO (*)	190,000	Lindström & Pettersson (1974); Lindström et al (1974)
Beef, aorta	BzAO	180,000	Shieh et al (1975)
Man, plasma	BzAO	150,000	Lin & Castell (1975)
Chick, cartilage	LO (? subunit)	62,000	Siegel & Fu (1976)

(*) See observations on identity of pig BzAO with histaminase (DAO), in Buffoni (1966) and Chapter 2 (Nomenclature, p. 36), this thesis.

(**) Quoted in Yasunobu et al (1976).

3.2.8

Influence of sex, age, drugs and disease states on amine oxidase activity in man

3.2.8.1

Age and sex-linked variation in circulating BzAO activity in man

Tryding et al (1969) found highly significant correlations between age and serum amine oxidase levels in 210 normal children and 550 adults. Activity was highest in prepuberal children; it is not stated whether sex-linked differences were found in this group. In puberty, the enzyme activity fell abruptly to adult level, at which it persisted in females up to 50 years and in males up to 65; thereafter, a moderate increase was noted in both. The reports of Robinson et al (1971, 1972) confirm these findings in every detail for human adults between 25 and 85 years of age. Murphy et al (1976), however, found no significant correlation between plasma amine oxidase activity, sex and age in 680 normal adult subjects up to 60 years of age. Children, and subjects over 60, were not included in their frequency distribution plots. The authors point out that their failure to find a statistically significant sex difference in mean BzAO activities, when individuals from all age groups between 18 and 60 were combined, may have resulted from the large representation in their sample of young adults; when early and late adolescents were considered separately from adult subjects, sex differences for plasma amine oxidase activity were statistically significant for the 11-17 and 31-40 age groups. My own observations concerning plasma BzAO activity in healthy adults (Lewinsohn, 1977b; see also Section 4.2, p. 130) agree closely with these findings. The significantly higher BzAO values in severely burnt children compared with adult, and the closely similar levels in male and female adult patients with severe burns, mainly young adults, further corroborate the reports mentioned above. See also Murphy et al (1977a) on the effect of age, sex and genetic factors on enzyme activity in rhesus monkeys.

3.2.8.2

Genetic control of platelet and plasma amine oxidase activity

A study of enzyme activity in platelets and plasma of mono- and dizygotic twins (Nies et al, 1973) suggests that genetic factors influence the activity of amine oxidase in both tissues. The intra-pair (mono-/dizygotic) difference for platelets was $0.05 < p < 0.10$, but for plasma it was highly significant ($p < 0.005$), whilst the difference between dizygotic twins and controls was not significant for either tissue. The correlation coefficient of heritability, on the other hand, was high both for platelet and plasma amine oxidase activity.

The finding of very high BzAO activity (ca. 6 times the mean for its group) in one sample of human amniotic fluid in which a chromosomal abnormality was demonstrated (see p. 149), suggests that further work on this aspect of amine oxidase activity may be rewarding.

3.2.8.3

Influence of disease states and drugs on human plasma BzAO

McEwen and Harrison (1965) were the first to note increased plasma amine oxidase activity in patients with chronic congestive heart failure, which could not be ascribed to myocardial infarction, hepatocellular damage or renal insufficiency. Highly significant increases in serum BzAO activity in chronic liver disease were shown by McEwen and Castell (1967) to be associated with hepatic fibrosis, increased hepatic "sinusoidal" pressure, and signs of portal hypertension. Activity in one of their patients, with untreated diabetes mellitus, was slightly raised and returned to normal after hyperglycaemia was controlled (cf. Tryding et al, 1969). Elevated levels of serum BzAO were also found in two out of 9 patients with metastatic liver disease, but activity was normal in patients with primary hepatic tumours, a hamartoma and a hepatoma not associated with cirrhosis; normal levels were seen, moreover, in Hodgkin's disease, reticulum cell sarcoma, lymphosarcoma, acute lymphoblastic

leukaemia, chronic myelogenous leukaemia, myelofibrosis and idiopathic thrombocytopenic purpura. It will be noted that all but the last-mentioned are diffuse neoplastic conditions; as shown (Lewinsohn, 1977 b), I found decreased serum BzAO activity in cancer to be associated with solid tumours (see below).

Significant increases in human serum BzAO activity were demonstrated by Tryding et al (1969) in children and adults with diabetes mellitus, whilst first-degree relatives of such patients, and subjects with borderline glucose tolerance tests, showed normal serum levels. An earlier report (Wickström and Pettersson, 1964) had pointed to the hypoglycaemic effect of mebanazine, an inhibitor of MAO, in patients with different types of diabetes. In the majority of a series of 35 patients with both diabetes and depressive illness who were treated with a combination of mebanazine and a sulphonylurea, the anticipated hypoglycaemic effect was exceeded.

The studies of Ito et al (1971) and Borthakur et al (1973) on hepatic fibrosis and portal hypertension confirmed the observations of McEwen and Castell (1967); the former also reported slightly increased serum BzAO activity in some patients with acromegaly, hyperthyroidism and progressive systemic sclerosis, but not in phaeochromocytoma. Buffoni et al (1977), however, found no significant difference between circulating BzAO levels in normal subjects and cirrhotic patients. Abnormal isoenzymes of plasma BzAO, demonstrated by Lin and Castell (1973, 1974) in a patient with haemochromatosis and liver fibrosis, are discussed on p.105 (Multiple forms of BzAO). Experimental Schistosoma japonicum infection in rabbits was described by Erickson et al (1973), who found highly significant increases in enzyme activity one to five weeks after eggs were first detected in the faeces; after a further 4 weeks, the increase became less significant. In human infection with Schistosoma mansoni, however, I found serum BzAO activity to be on the

low side of normal (Lewinsohn, 1977 b); the discrepancies may reflect inter-species differences (of host and parasite) and different stages of the disease.

My own studies on serum BzAO in patients with severe burns, and cancer patients, have been described in a published report (Lewinsohn, 1977 b). Briefly, both groups showed decreases in serum BzAO activity which were highly significant. In 20% of the former, no activity was demonstrable, and the mean value for the entire group was only 15% of the normal. Low values persisted for several months even in patients in good physical condition who had had skin autografts. Patients with solid tumours, treated and untreated, had mean activity 42% below the normal; in several, chemotherapy with cyclophosphamide, 5-fluorouracil, methylnitrosourea, methotrexate and bleomycin (singly or in various combinations) was followed by a notable increase in serum BzAO levels; radiotherapy produced no apparent change in enzyme activity.

The effects of drugs on human blood platelet and plasma amine oxidase activity *in vitro* and *in vivo* are variable, according to Robinson *et al* (1968). Platelet and plasma amine oxidase inhibition and changes in urinary amine excretion during phenelzine treatment were observed by Murphy *et al* (1977), who found maximum reductions in enzyme activity after 7-14 days of treatment; after therapy was stopped, an average of 14 days and 6 weeks, respectively, was required for recovery of enzyme activity in platelets and plasma. Schmutzler (1968) reported an increase in plasma BzAO and DAO in man and guinea-pig, induced by intravenous injection of 500 iU heparin/kg body weight. Enhancement in the guinea-pig was remarkable and reached its maximum within 5 min of injection; in man, the increase was less pronounced and slower to appear (60-120 min), making it likely that it was secondary to the release by heparin of some other substance(s) which, in turn, affected amine oxidase activity. Heparin-induced increase of blood and lymph DAO was also shown, among others, by Hansson (1973), whose paper should be consulted

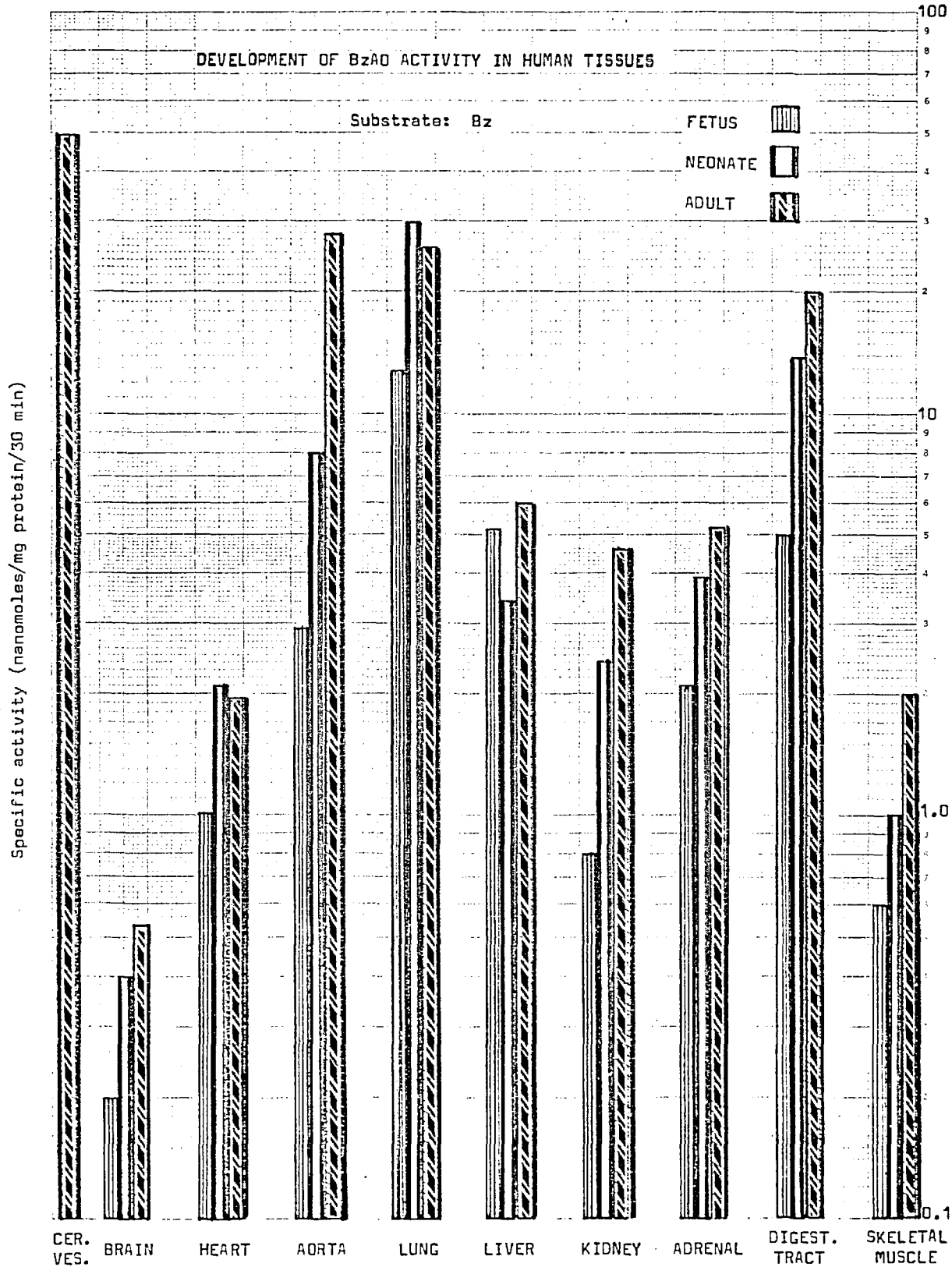

DEVELOPMENT OF BzAO ACTIVITY IN HUMAN TISSUES
Substrate: Bz
FETUS
NEONATE
ADULT
Specific activity (nanomoles/mg protein/30 min)
100
10
1.0
0.1
CER. VES.
BRAIN
HEART
AORTA
LUNG
LIVER
KIDNEY
ADRENAL
DIGEST. TRACT
SKELETAL MUSCLE

Figure 9.

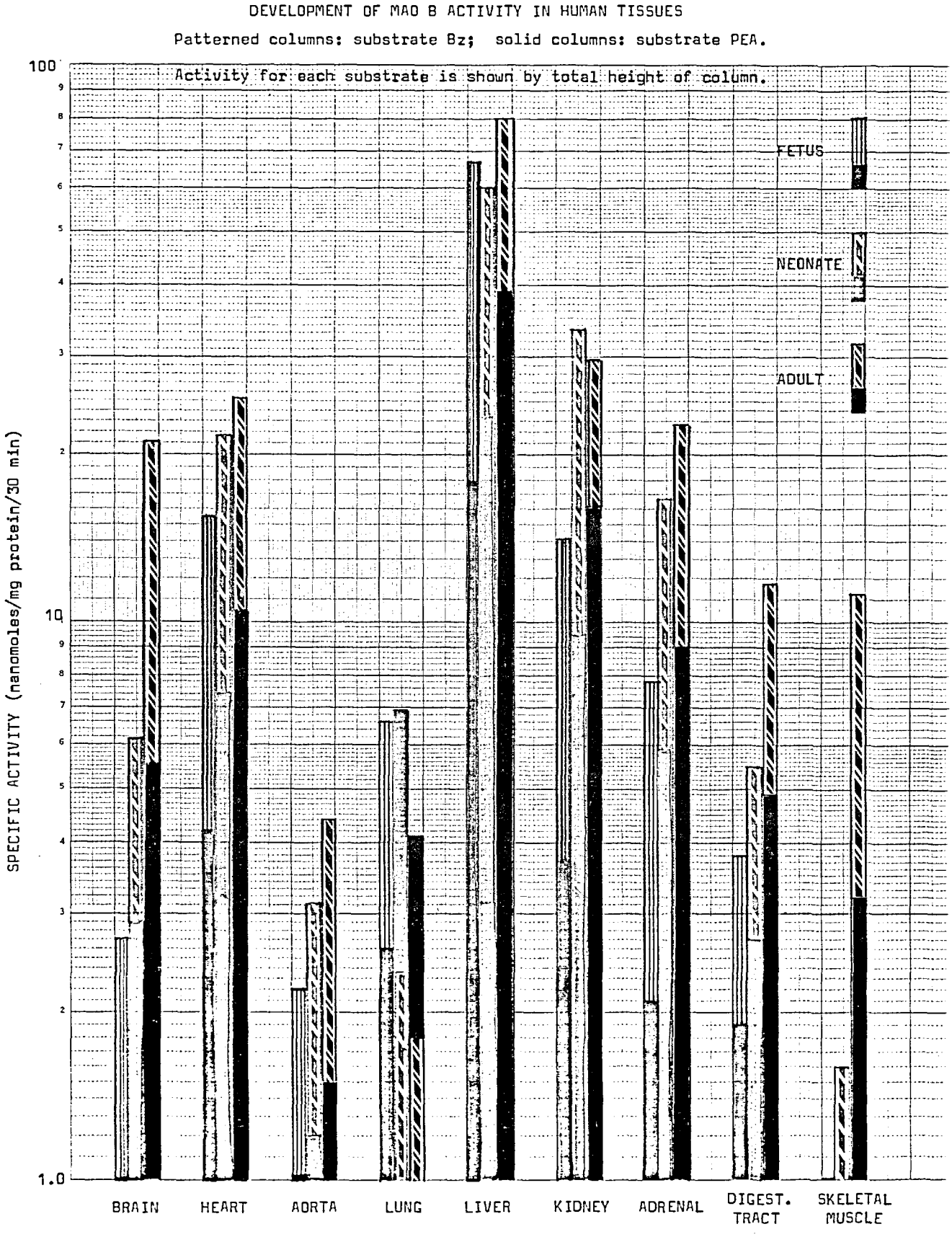

DEVELOPMENT OF MAO B ACTIVITY IN HUMAN TISSUES
Patterned columns: substrate Bz; solid columns: substrate PEA.
Activity for each substrate is shown by total height of column.
SPECIFIC ACTIVITY (nanomoles/mg protein/30 min)
100
10
1.0
FETUS
NEONATE
ADULT
BRAIN
HEART
AORTA
LUNG
LIVER
KIDNEY
ADRENAL
DIGEST. TRACT
SKELETAL MUSCLE

Figure 10.

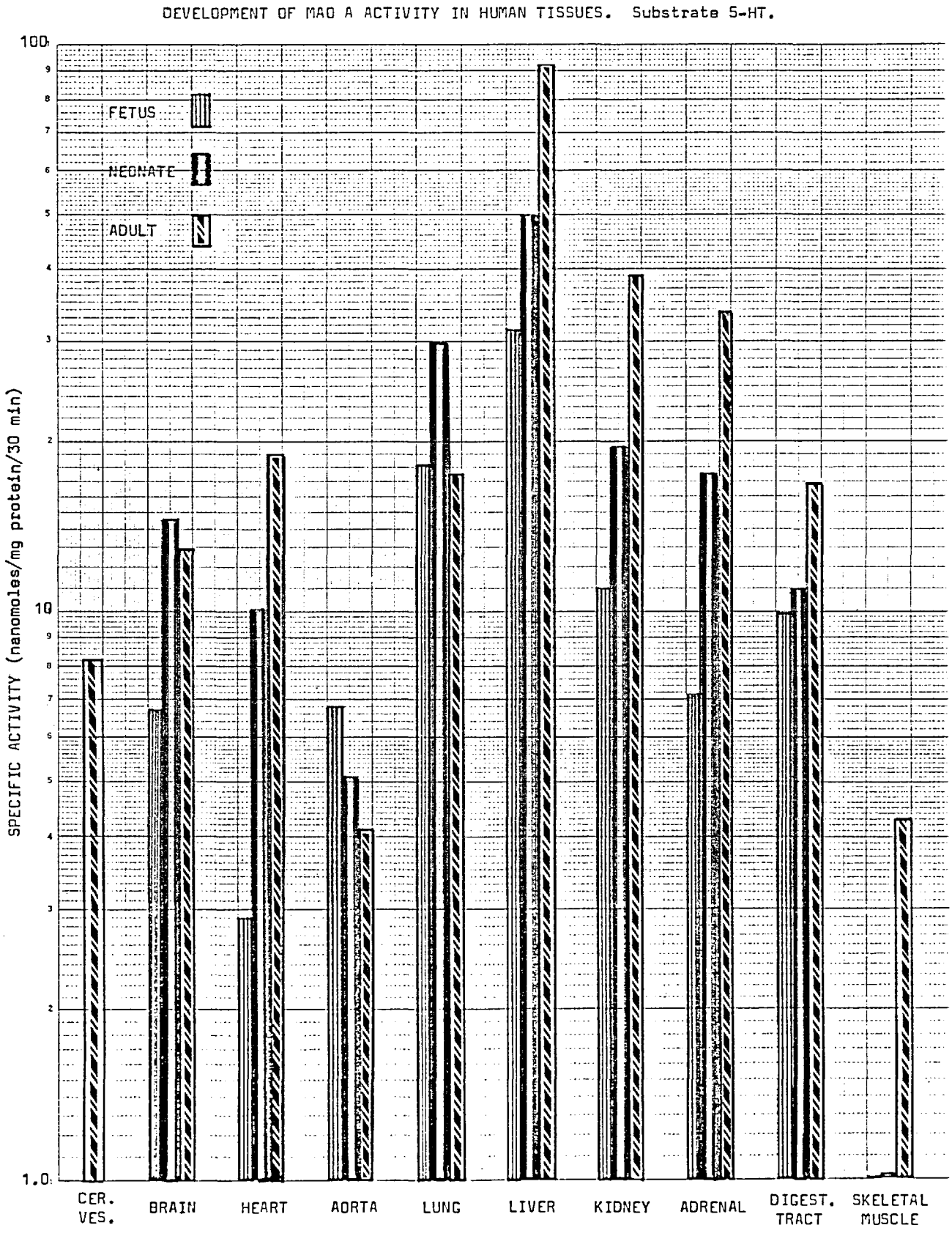

DEVELOPMENT OF MAO A ACTIVITY IN HUMAN TISSUES. Substrate 5-HT.
SPECIFIC ACTIVITY (nanomoles/mg protein/30 min)
100
10
1.0
FETUS
NEONATE
ADULT
CER. VES.
BRAIN
HEART
AORTA
LUNG
LIVER
KIDNEY
ADRENAL
DIGEST. TRACT
SKELETAL MUSCLE

Figure 11.

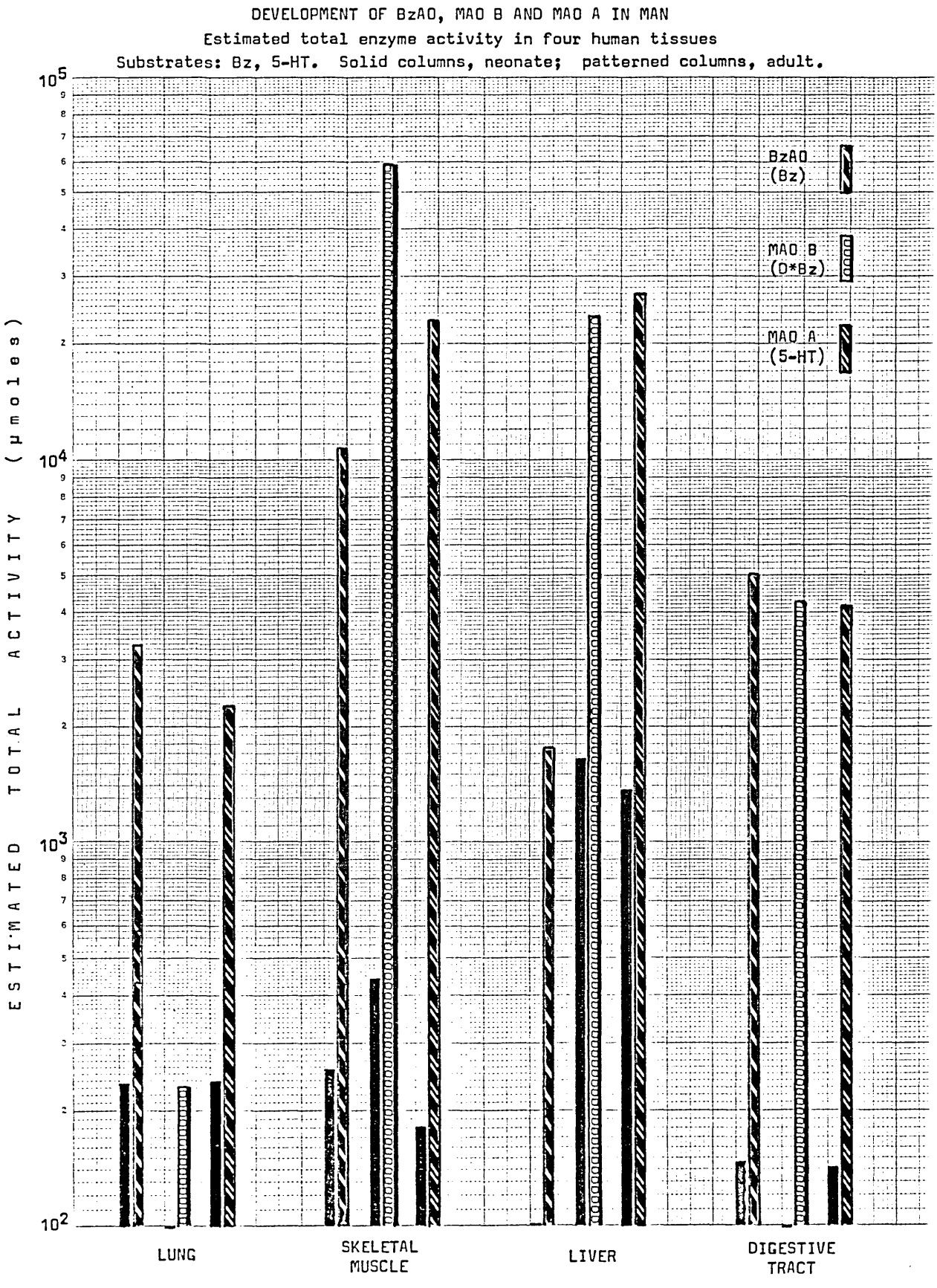

DEVELOPMENT OF BzAO, MAO B AND MAO A IN MAN
Estimated total enzyme activity in four human tissues
Substrates: Bz, 5-HT. Solid columns, neonate; patterned columns, adult.
ESTIMATED TOTAL ACTIVITY (μmoles)
BzAO (Bz)
MAO B (D*Bz)
MAO A (5-HT)
LUNG
SKELETAL MUSCLE
LIVER
DIGESTIVE TRACT

Figure 12.

for references on earlier work in the field. The significance of these findings is obscure. I have assayed human plasma with EDTA and lithium-heparin, and found no significant difference in vitro between BzAO activity in these preparations and serum from the same blood (Lewinsohn, unpublished data).

Changes in plasma amine oxidase activity in human pregnancy are described in Section 4.2 (p. 133).

3.2.8.4

Influence of age on amine oxidase activity in tissues other than plasma

Insight into the varied patterns of age-linked activity of these enzymic systems is provided by our study of development of BzAO and the two forms of MAO in man (Lewinsohn et al, 1980 b). Complementing this paper, Figures 9-11 illustrate the development of specific activity of each enzyme in major human tissues from the fetal state to the adult. The overall increase in specific activity between fetus and adult is clearly seen in these histograms, except only for aortic MAO A and MAO B in lung; specific activities of BzAO and MAO B in fetal liver, however, resemble adult values. Estimated total activity for the three enzymes, in 4 neonatal and adult organs (fetal values being too low to appear in this histogram) is shown in Figure 12, which illustrates part of the data in Table 5 of our report (Lewinsohn et al, 1980 b). A full discussion of these observations is found in our paper (see also Chapter 5, Discussion).

Major reports by other authors on the development of amine oxidase activity in mammalian tissues are reviewed in our paper (Lewinsohn et al, 1980 b). Studies on age-dependent changes in amine oxidase activity in rat vessels (Lai et al, 1975) and rat heart (Callingham and Laverty, 1973; Lyles and Callingham, 1975, 1979) are discussed elsewhere in this thesis. On the latter subject, see also Callingham and Lyles (1975), and Horita (1967), who described age and sex-linked changes in rat heart. Age-linked changes in human platelet and brain MAO and the relation of hindbrain MAO

A to mean concentrations of NA and 5-HIAA have been shown by Robinson et al (1971, 1972). Murphy et al (1976) found statistically significant differences dependent on sex but not age for platelet MAO activity in the entire age range studied.

3.2.8.5

Influence of drugs and disease states on amine oxidase activity in human tissues other than plasma

I have performed one experiment on human skin and granulation tissue obtained (through the kind offices of Mr. B. D. G. Morgan, University College Hospital, London) during an autograft operation on a female 35-year-old patient who had suffered severe burns one month earlier. The assay was performed at pH 7.2 (potassium phosphate buffer); all other procedures and assay conditions have been described (Lewinsohn et al, 1978, 1980 b). The results are shown below.

Tissue	BzAO (*)	Per cent inhibition (4×10^{-4} M)		
		Isoniazid	Deprenyl	BAPN
Normal skin	1.81	84	12	55
Granulation tissue (**)	0.09	78	82	67
Scar tissue (skin)	1.01	91	19	67

(*) Residual specific activity (nanomoles/mg protein/30 min) after inhibition of MAO B by 4×10^{-4} M deprenyl.

(**) Heavily contaminated with blood.

Comments. Although deprenyl-sensitivity in normal skin and scar tissue was roughly similar, BzAO activity in the former was almost twice as high as in the latter; the discrepancy may be due both to the fact that the specimens came from different regions, and to different vascularization of these regions. Granulation tissue, however, had much lower BzAO activity, and was five to six times more sensitive to deprenyl

inhibition, than normal skin or scar tissue. Since granulation tissue is a spongy mass of capillaries and connective tissue elements, which it is impossible to free from blood, both the deprenyl-sensitive and insensitive moieties of Bz deamination probably represent enzymic activity of the blood with which the preparation was heavily contaminated, with the possible contribution of capillary endothelium to the former. This interpretation is confirmed by the activity of BzAO in the preparation, which is on the low side of normal plasma values, whilst the high deprenyl-sensitivity probably represents the effect of this inhibitor on MAO B in platelets and, perhaps, vascular endothelium (cf. Roth and Venter, 1978). It is also worthy of note that only deprenyl proved selective in its inhibitory effect; both isoniazid and BAPN inhibited normal skin, scar tissue and granulation tissue activities to a similar degree (*).

To my knowledge, there is no published report on BzAO activity in abnormal human tissue.

For the effect of disease states and drugs on MAO levels in platelets and solid tissues, see reviews by Costa and Sandler (1972), Wolstenholme and Knight (1976), Sandler et al (1979), Ruthven and Sandler (1979) and Sandler (1980); see also Sandler (1978) on the implications of reduced platelet MAO activity during migraine attacks, and Anselmi et al (1976) on abnormal platelet MAO activity in human arterial hypertension.

Work on DAO activity in human tumours (Baylin et al, 1970, 1972, 1975; Baylin, 1977) lies outside the scope of this thesis.

(*) For a description of structure and function of granulation tissue, see Gabbiani et al (1972); see also p. 159 for discussion of vascular endothelium and activity of MAO B.

CHAPTER 4: DISTRIBUTION AND LOCALIZATION OF AMINE OXIDASES IN HUMAN TISSUES

4.1

Distribution of amine oxidases in tissues

As shown by Blaschko (1952, 1962, 1963), Davison (1958), Blaschko and Bonney (1962), Weiner (1960) and many others (for reviews, see Tipton, 1975; Goodwin, 1976), the amine oxidases are widely distributed throughout the animal and vegetable kingdoms. One or more of the enzymes, BzAO, MAO A and MAO B, have been found in virtually every species and tissue assayed for activity, varying from species to species, and from tissue to tissue in the same species, and differing at each developmental stage of a tissue (Lewinsohn et al, 1980 b; see also pp. 114-117 (Figures 9-12), and p.118).

4.1.1

Intracellular distribution

According to Blaschko (1952), the most characteristic property of MAO is its localization in insoluble cell constituents. Cotzias and Dole (1951) and Hawkins (1952) first studied the intracellular distribution of MAO in rat liver; since Schnaitman et al (1967) localized it in the outer membrane of the mitochondrion, innumerable studies have confirmed and extended this observation, which has come to be universally accepted (see, e.g., Wolstenholme and Knight, 1976). It is claimed, however, that part of MAO activity is localized in microsomes (Jarrott and Iversen, 1968; Blaschko, 1974; see also p.172), and activity of MAO in soluble fractions has been demonstrated, among others, by Lyles and Callingham (1975), de Champlain et al (1969), Coquil et al (1973) and the present author (p.79). The nature and locations of the multiple forms of MAO A and B in rat tissues are discussed by Tipton et al

(1976), to name but one among many papers on the subject. Tipton (1975) comprehensively reviews the literature up to 1975 on the intracellular localization of MAO in organs and tissues of vertebrates and invertebrates.

4.1.2

Distribution of amine oxidases in glandular and other tissues of man

4.1.2.1

Introduction

As shown earlier (Lewinsohn et al, 1980 a,b), human tissues, like those of the rat (Lewinsohn et al, 1978), contain varying amounts of BzAO, and/or MAO A or B. Our published reports contain some description of enzymic activity in glandular tissues, e.g. parotid and testis in rat (Lewinsohn et al, 1978), adrenal and pancreas in man (Lewinsohn et al, 1980 b). Additional data on enzyme activity in sundry glandular, connective and other tissues from the latter series, hitherto unpublished, are presented in this Section. Further studies on amine oxidase distribution in human pregnancy plasma and tissues, in vascular tissue, the human uterus and oesophagus, and in cultured cells, are dealt with in subsequent Sections.

4.1.2.2

Experimental

Sources, treatment and assay of the tissues employed in these studies have been described (Lewinsohn et al, 1980 b). Where two individuals are shown, e.g., for thyroid, values for each individual appear in the same order for each enzyme (for example, thyroid BzAO, MAO B and MAO A for Adult n^0 1 are 2.5, 1.6 and 17.7, respectively).

4.1.2.3

Results and comments

(a) Glandular tissue. Table 17 shows the activities of the three

Table 17. DISTRIBUTION OF BzAO, MAO B AND MAO A IN HUMAN TISSUES

Tissue	Devel. stage	Number	BzAO	MAO B (D*Bz)	MAO A
Pituitary	N	1	2.2	53.0	14.6
Thymus	F	5	0.16 (0.07)	0.08 (0.01)	0.24 (0.04)
Thymus	N	3	0.51 (0.05)	0.31 (0.08)	0.2, 0.94
Thyroid	A	2	2.5, 10.0	1.6, 24.5	17.7, 40.8
Submaxillary gland	A	1	7.3	33.2	58.9
Testis	N	1	2.4	0.8	0.4
Cartilage	F	5	0.46 (0.04)	0.58 (0.17)	0
Cartilage	N	2	0.98, 0.56	0.48, 0.14	0.38, 1.4
Intestinal serosa	F	1	13.4	19.3	29.3
Renal capsule	F	1	0.4	3.5	1.8
Gall bladder	F	3	2.93 (1.13)	4.84 (0.92)	15.1 (4.3)
Appendix	F	2	8.36, 13.54	15.5, 5.0	67.6, 8.34
Para-aortic lymph nodes	N	1	5.1	12.5	11.0

Devel. stage = developmental stage; N = neonate; F = fetus; A = adult.

D*Bz = deprenyl-sensitive moiety of Bz oxidation.

Values express specific activities (nanomoles/mg protein/30 min) and are means of replicate determinations. Values between brackets represent standard error of mean. For experimental and assay conditions, see text.

enzymes in various glandular, connective and other tissues (cf. Tables 1-3, Lewinsohn et al, 1980 b). In the neonatal pituitary, MAO B activity is remarkably high, comparable only to activity in human fetal liver. MAO A activity is similar to that in neonatal brain. BzAO activity is somewhat lower than that found in neonatal adrenal; the difference may well reflect the different vascularity of the two organs: Bradbury (1975) comments on the remarkably thick walls of the larger adrenal veins, "containing much smooth muscle". High activity of BzAO and both forms of MAO was found in one adult thyroid, but not in the other preparation. The former (highly active) came from a male subject aged 64, the latter from a female aged 68 years. BzAO activity in both is probably associated with vascular components present in the homogenates.

Adult submaxillary gland preparations also showed high activity of all three enzymes (Table 17). This agrees well with the findings of other workers in various species (see Literature review, p. 127), though quantitative data are rarely quoted, and are generally not comparable because of differences in species examined and assay techniques employed.

The pattern of enzymic activity shown by the neonatal testis is notable for the value for BzAO compared with MAO B and A. Activity of BzAO in this organ is similar to that of the neonatal pituitary, and may reflect similar vascularization.

(b) Connective tissue. Cartilage in fetus and neonate showed uniformly low values; in the fetal renal capsule, MAO B was moderately active, whilst activity of BzAO and MAO A was low. In fetal intestinal serosa, however, all three enzymes were remarkably active. Comparison of these values with those reported earlier (Tables 1 to 3, Lewinsohn et al, 1980 b) for the digestive tract (including oesophagus, stomach, ileum, colon and mesenteries) reveals that similar values for BzAO were seen only in the muscular coat of oesophagus and stomach of

the most active fetal preparations. MAO B activity comparable to that shown here was recorded in neonatal and adult ileum only, whilst MAO A in this fetal intestinal serosa was more active than in any other tissue of the digestive tract, at any stage of development. The preparation was not contaminated with tissue from any other intestinal region, as can be seen from a comparison of the values for enzymic activity of other parts of the digestive tract of this fetus with those for its intestinal serosa (Table 18). Nor is it likely that the activity found in this tissue can be explained either by contamination with liver, which showed the normal fetal pattern, or by the presence of vascular tissue, since no fetal blood vessel examined showed a comparable pattern.

Table 18. Amine oxidase activity in digestive tract of fetus n° 7

Tissue	Specific activity (nanomoles/mg protein/30 min)		
	BzAO	MAO B (D*Bz)	MAO A
Oesophagus	6.8	2.4	12.5
Stomach	4.1	5.0	8.8
Jejunum	1.8	2.9	5.0
Ileum	1.6	2.6	2.7
Mesentery	1.8	5.1	6.7
Colon	4.6	3.1	8.8
Mesocolon	3.9	10.5	14.7

It is true that one fetal carotid (see p. 160) had high MAO A activity, but both BzAO and MAO B values were much lower than those of the fetal intestinal serosa, in which no major blood vessel was discernible at dissection. Activity in lymphatic elements which might have been present in the tissue is also unlikely to account for any part of the pattern described here, since the spleen of this fetus showed low values for all three enzymes.

(c) Other tissues. Even higher MAO A activity is seen in one fetal appendix (fetus n° 4), which was also very active for BzAO and MAO B.

Comparison of these values with activities in other regions of the digestive tract of the same fetus was as unrevealing as that shown above for fetus n° 7. Again the pattern of enzyme activity is completely different from that of any other tissue; MAO A activity, for example, is twice as high in this appendix as the mean for fetal liver, and higher than the most active fetal liver preparation in this series. Specific activities of BzAO, MAO B and MAO A in the spleen of this fetus were 0.8, 1.3 and 3.1 respectively, which makes it unlikely that lymphoid tissue was the active component in this appendical preparation. As for specific activity of BzAO, the comments on intestinal serosa (above) apply equally to both appendices shown here. Explanation of all these findings must await further work.

It is not surprising that the pattern of enzymic activity of the fetal gall bladder is totally different from that of fetal liver. The ratio of BzAO/MAO A activity is about the same in the two organs, but the ratios of BzAO/D*Bz and MAO B/A are totally different (respectively 1:13 and 2.1 : 1 in liver; 0.6 : 1 and 0.32 : 1 in gall bladder). Yet another pattern is seen in the neonatal para-aortic lymph nodes: in this case, BzAO activity is somewhat lower, MAO B somewhat higher and MAO A well within the range of most regions of the neonatal digestive tract.

The only investigation in the literature on MAO, relevant to these studies, is that of Gennser and v.Studnitz (1969), who found high activity in paraganglia of the mid-term human fetus. These authors employed a microfluorimetric assay method; their results, converted to nanomoles/mg protein/30 min, appear to agree with mine. Reports by other workers on glandular tissues of other mammalian species are briefly discussed below.

4.1.2.4

Literature review

Bovine thyroid MAO in subcellular fractions was studied by Fischer et al (1966, 1968 a,b), who measured O_2 uptake and production of H_2O_2

with a range of substrates coupled with o-dianisidine. Oxidation was inhibited by flavin analogues, but not by carbonyl reagents. The preparations showed considerable substrate specificity: only TA and PEA were significantly oxidized; no activity was noted against other substrates, Bz included. About 55 and 35 per cent of activity were found, respectively, in the mitochondrial and microsomal fractions. The authors comment on the significance of their findings, the possible role of thyroid MAO in iodothyronine synthesis (Fischer et al, 1966), and the contribution of MAO to the self-regulatory system of the thyroid (Fischer et al, 1968 b). The studies of Lyles and Callingham (1974, 1979) on the effect of thyroid hormone on rat heart have been mentioned elsewhere (p. 130).

Harada et al (1971) studied the submaxillary gland in man, cat, rat and ox, with kynuramine as substrate. In man, about 25% of activity was found in the microsomal fraction and 75% in mitochondria; intracellular distribution was different for each species. No activity was demonstrated in human parotid secretion. Jarrott (1971) measured MAO activity in the submaxillary gland of rat and rabbit, with TA and Bz as substrates; values for Bz oxidation in rabbit were comparable to those shown here, in man. In rat salivary gland, de Champlain et al (1969) found a substantial proportion of MAO activity to be localized in the microsomal fraction (cf. Jarrott and Iversen, 1968, on rat vas deferens, p.172). Sympathetic denervation of the salivary gland produced partial disappearance of MAO activity from all subcellular fractions. Histochemical and biochemical techniques were employed by Fujiwara et al (1966) to demonstrate the localization of MAO, NA and acetylcholinesterase in salivary glands of dogs. With TA or 5-HT as substrate, MAO activity was highest in the sublingual gland, followed by parotid and submaxillary. This was the reverse of the relative concentrations of NA. Histochemistry showed even staining for MAO in the cells of secretory acini and excretory ducts; there was no selective staining in or around cell membranes. For earlier work on the subject, see references in Fujiwara et al (1966).

Other workers have studied glandular tissues not investigated by me. Zeller (1941) found considerable activity of MAO in human prostate and seminal vesicles. Yang et al (1972) studied the oxidation of TA in rat pineal gland and superior cervical ganglion, and found the pineal preparation to be the least active. MAO A accounted for about 15% of the activity in the pineal, the remaining activity was catalysed by MAO B. As for adrenal (cf. Lewinsohn et al, 1978, 1980 b), MAO activity in the mammalian gland is reported by Davison (1958); bovine adrenal medulla was studied for MAO activity by Werle and Roewer (1952) and Laduron and Belpaire (1968). The former group found that adrenal medulla oxidized butylamine twice as rapidly as TA, whilst the latter showed that approximately 57 per cent of MAO activity was localized in the mitochondrial fraction, 25% in the microsomes, and 3% in the final supernatant. Recent work is reviewed by Youdim and Holzbauer (1976). Most of these studies, as well as investigations into the effect of adrenalectomy on MAO in rat heart (Callingham and Laverty, 1973; Della Corte and Callingham, 1977) and other organs of rat (Youdim and Holzbauer, 1976) lie outside the scope of this thesis.

4.2

Amine oxidases in human pregnancy

4.2.1

Introduction

The reasons behind the design and execution of a project on amine oxidase activity in human pregnancy have been set out in the Introduction (see p. 21). A considerable body of work has been performed by many workers, on a wide range of problems related to enzyme activity in pregnancy. The majority of publications dealing with such problems are concerned with compounds other than the amine oxidases, and lie outside the scope of this thesis. Of the amine oxidases, DAO (variously called plasma (or other tissue) diamine oxidase, histaminase) has been more widely studied than any other; very few studies deal with MAO. Any papers relevant to the present study are discussed in the following pages together with related topics. Some mention must be made, however, of investigations of 5-HT in pregnancy which have a bearing on the present report on MAO A activity in puerperal tissues.

Sandler and Coveney (1962) first described decreased levels of placental MAO in toxaemia of pregnancy. Early literature on 5-hydroxyindole metabolism in pregnancy was reviewed by Southgate and Sandler (1968). Robson and Sullivan (1963, 1968) discuss the mechanisms of the lethal action of 5-HT on the mouse fetus, and the effect of 5-HT on maintenance of pregnancy, congenital abnormalities, and the development of toxaemia. They suggest that the mechanism by which 5-HT, accumulating in placenta, is most likely to affect placental function is by constricting the uterine blood vessels immediately adjacent to and supplying the placenta. Diffusion of this 5-HT to the adjacent uterine endometrial area would cause powerful vasoconstriction, resulting in placental deprivation of oxygen and other nutrients, thus leading to the production of pharmacologically active substances responsible for the

systemic manifestations of toxaemia. Decreased nutrition of placenta and, perhaps, constriction of umbilical vessels would, in turn, affect the fetus. Vasoconstriction by 5-HT as a factor in the physiological post-partum occlusion of umbilical vessels was demonstrated by Schmermund et al (1959). Sadowsky et al (1963) examined nearly 7,000 urine specimens from 141 pregnant women for excretion of 5-hydroxyindoleacetic acid (5-HIAA) and found that habitual abortion, "psychogenic in origin" (sic), may be associated with high 5-HT production. Klinge et al (1964) studied the 5-HT content of human placentae and uteri obtained at Caesarean section and vaginal delivery from normal and toxaemic patients, and observed no differences between the various groups. Hazra et al (1965) described 5-HT levels in normal full-term infants; low at birth, concentrations rose to adult values by 4-6 weeks of life, though blood platelet counts remained unchanged.

4.2.2

Experimental

(a) Normal controls. 27 blood samples from normal healthy individuals were kindly supplied by Dr. Ewa Brookes (South London Blood Transfusion Centre) immediately after collection. Nineteen donors were female and 8 male, aged 18-56 (mean, 34.3) and 18-58 (mean, 34.5) years, respectively. Within 2 h of collection, the samples were centrifuged at 800 *g* for 15 min, divided into aliquots and stored at -20° until assay. Mean specific activity of BzAO was identical in plasma of male and female donors.

(b) Patients. All the women under investigation were normal out-patients without known disease process, attending Queen Charlotte's Maternity Hospital for ante-natal care and eventual delivery. Some were seen in the Family Planning Clinic several weeks after parturition, when blood was collected by the writer. The blood from out-patients at the Ante-Natal Clinic was an aliquot of that collected for routine

laboratory tests. Informed consent was obtained from all the women in the longitudinal study (see (ii), below). At parturition, blood was collected by the attending midwife or the writer, except at Caesarean section, when the attending obstetrician collected it. All specimens were treated as above. A total of 324 samples of maternal blood (including 30 obtained at parturition) were assayed; they were made up as follows:

(i) 148 random samples ("vertical", i.e., cross-sectional,study) from patients at various gestational ages, as well as 6-72 h post-partum and 5-52 days post-natal samples of maternal blood.

(ii) 176 specimens from 40 patients, who had 2-6 (mean, 4.1) blood determinations of BzAO up to (and/or including) parturition, post-partum and/or post-natally ("horizontal", i.e., longitudinal,study). Further assays of BzAO and MAO A and B activity were carried out on placenta (12), placental vessels (12) and umbilical vessels (10) from twelve patients in this group, who had 3-6 (mean, 4.7) plasma BzAO determinations up to and including parturition.

In addition, 29 corresponding samples of cord blood were studied.

Fifty-three per cent of all patients were primiparae and 47% multiparae. Their age range was 16-39 years (mean, 27.04 ± 4.92 SD); that of the 40 patients on the longitudinal study was 16-36 years (mean, 27.85 ± 5.21); the mean for primiparae in this group was 26.4 (± 5.0) and that of multiparae 27.9 (± 5.6) years. Except for three patients, whose gestational ages were 35, 37 and 37 weeks, respectively, all were delivered at term (39-41 weeks), six by Caesarean section; the remaining were vaginal deliveries, twelve by forceps.

(c) Plasma. Fresh whole blood from non-pregnant and pregnant individuals was collected by puncture of the antecubital vein with disposable 40 mm 8/10 needles into plastic syringes, and transferred to plastic vials containing lithium heparin (5 units/ml) or K_2-EDTA (1 mg/ml). Immediately

after delivery of the placenta, cord blood was collected from the severed umbilical vein into similar vials. Within 2 h of collection, the blood was centrifuged at 800 *g* for 15 min, divided into aliquots in polypropylene vials and stored at -20° until use.

(d) Tissues. Placenta, placental vessels and umbilical vessels obtained from 12 deliveries at the Labour Ward of Queen Charlotte's Maternity Hospital were dissected within 6 h of delivery of the placenta. Between delivery and dissection, the tissues were kept in the refrigerator at 4°. Placental tissue was taken from four regions of the maternal and fetal sides, care being taken to avoid blood vessels. The tissue was rinsed in cold running water, and dried between layers of filter paper. Placental vessels were dissected by freeing a large vessel which was lifted with forceps; the surrounding tissue was then scraped from the branches until a portion of the vascular tree could be removed. This was thoroughly washed in cold running water until all visible traces of placental tissue and blood had been removed. The vessels were then split open lengthwise, washed again and dried between layers of filter paper. Umbilical vessels were treated similarly. After drying, vessels were coarsely minced with scissors. All tissues were placed in polypropylene vials and stored in the deep-freeze at -20°. Homogenization was carried out by the N_2-method (see p.45) in K_2HPO_4/KH_2PO_4 buffer (1:10 w/v); the homogenate was divided into aliquots, and stored at -20° until assay.

(e) Amniotic fluid was kindly supplied by Dr. Karl Blau (Bernhard Baron Memorial Research Laboratories) from stored specimens obtained at amniocentesis; some specimens came from normal deliveries at Queen Charlotte's Maternity Hospital. All specimens were clear, uncontaminated by blood or meconium. The fluid supplied by Dr. Blau had been spun down and was cell-free; that obtained from the Labour Ward was centrifuged for 15 min at 800 *g* to remove mucus and cellular debris. All specimens were divided into aliquots and stored at -20° for future use.

(f) Assay. The radiochemical microassay procedure employed has been fully described (p. 55). Determination of BzAO activity in plasma (50 µl) was carried out with ^{14}C-Bz as substrate (final concentration, 83 µM) and 0.1M tris buffer, pH 9.0 (50 µl) in a final volume of 120 µl. Tissue BzAO and MAO B were assayed with Bz as substrate (42 µM), water or 4 x 10^{-7}M deprenyl (100 µl) and 0.1M tris buffer, pH 9.0 (100 µl); enzyme volume was 50 µl for placenta, and 20 µl for vessels. MAO A activity was determined with ^{14}C-5-HT as substrate (371 µM), and K_2HPO_4/KH_2PO_4 buffer, 0.1M, pH 7.2 (100 µl); enzyme volumes were as above. For determination of amine oxidase activity in amniotic fluid, 100 µl of enzyme was used; all other volumes and concentrations were as described above. Preparation of water blanks and all other procedures were as shown (Lewinsohn et al, 1978, 1980 a,b; see also p. 56). For description and sources of reagents, see p. 55. Protein content of plasma, amniotic fluid and tissues was estimated by the method of Lowry et al (1951), with bovine serum albumin as standard.

4.2.3

Results and comments

4.2.3.1

BzAO activity in plasma of pregnant women

Variation of BzAO with gestational age was compared for the cross-sectional and longitudinal groups and proved to be identical in both. Mean activity in cord blood was similar to that in maternal blood. No correlation was found between BzAO values in maternal and the corresponding cord blood; nor was there any correlation between activity of maternal or cord blood BzAO on the one hand, and maternal age, parity, birth weight, sex of infant, or amine oxidase activity in placenta, placental vessels or umbilical vessels, on the other. Values

for cord and maternal blood BzAO activity from patients who underwent Caesarean section or forceps delivery were not different from those of patients who had normal vaginal deliveries. Gestational age, anaesthesia (general or epidural), analgesics, oxytocin or other medication had no apparent effect on maternal perinatal or cord blood BzAO activity.

Highly significant variations in maternal plasma BzAO activity were seen (Table 19, Figure 13):

(a) at 8-12 weeks gestational age, a drop of 18% compared with normal non-pregnant plasma activity ($p < 0.005$);

(b) at 13-17 weeks and parturition, increases of similar magnitude ($p < 0.005$) compared with the preceding groups (8-12 and 34-39 weeks, respectively), but not significant when compared with non-pregnant plasma;

(c) at 6-72 h post-partum, a drop of 31% ($p \ll 0.001$) relative to the preceding group.

Between the post-partum and post-natal (5-52 days) groups, a rise of 35% ($p < 0.005$) was recorded; however, when the latter activity was compared with that of normal non-pregnant plasma, the difference was not significant ($p < 0.20$).

A comparison of the values shown in Table 19 with estimated figures for fetal growth (Table 20) makes it fairly obvious that the variations in maternal blood BzAO activity are not related to growth of the fetus. As shown earlier (Table 5, Lewinsohn et al, 1980 b), estimated total activity of BzAO in the 19-21 week fetus is very low; it is thus very unlikely that the fall in maternal plasma BzAO at 8-12 weeks' gestation might be accounted for by fetal requirements. On the other hand, this period possibly coincides with the time of most intense development of the vascular network in both uterus and placenta. Unfortunately (though perhaps not surprisingly), a search for specific

Table 19. BzAO ACTIVITY IN PLASMA OF NON-PREGNANT AND PREGNANT WOMEN, AND CORD BLOOD AT TERM.

	N	Gest. age (wks)	Spec. act. (mean)	SE of mean	Student's t for	D.F.	t	p
(a)	27	Non-pregnant	0.13	0.005				
(b)	28	8-12	0.106	0.006	(a)/(b)	53	3.16	<0.005
(c)	73	13-17	0.123	0.003	(b)/(c)	99	2.68	<0.005
(d)	9	18-19	0.124	0.01	-			NS
(e)	66	20-30	0.129	0.004	-			NS
(f)	68	34-39	0.13	0.004	-			NS
(g)	46	40-41 Partu-rition	0.153	0.006	(f)/(g)	112	3.18	<0.005
(h)	20	Post-partum 6 - 72 h	0.106	0.006	(g)/(h)	64	4.48	$\ll 0.001$
(i)	14	Post-natal 5 -52 days	0.143	0.012	(h)/(i)	32	3.09	<0.005
					(h)/(a)	39	1.208	<0.20 (NS)
(j)		Cord (term)	0.13	0.007	-			NS

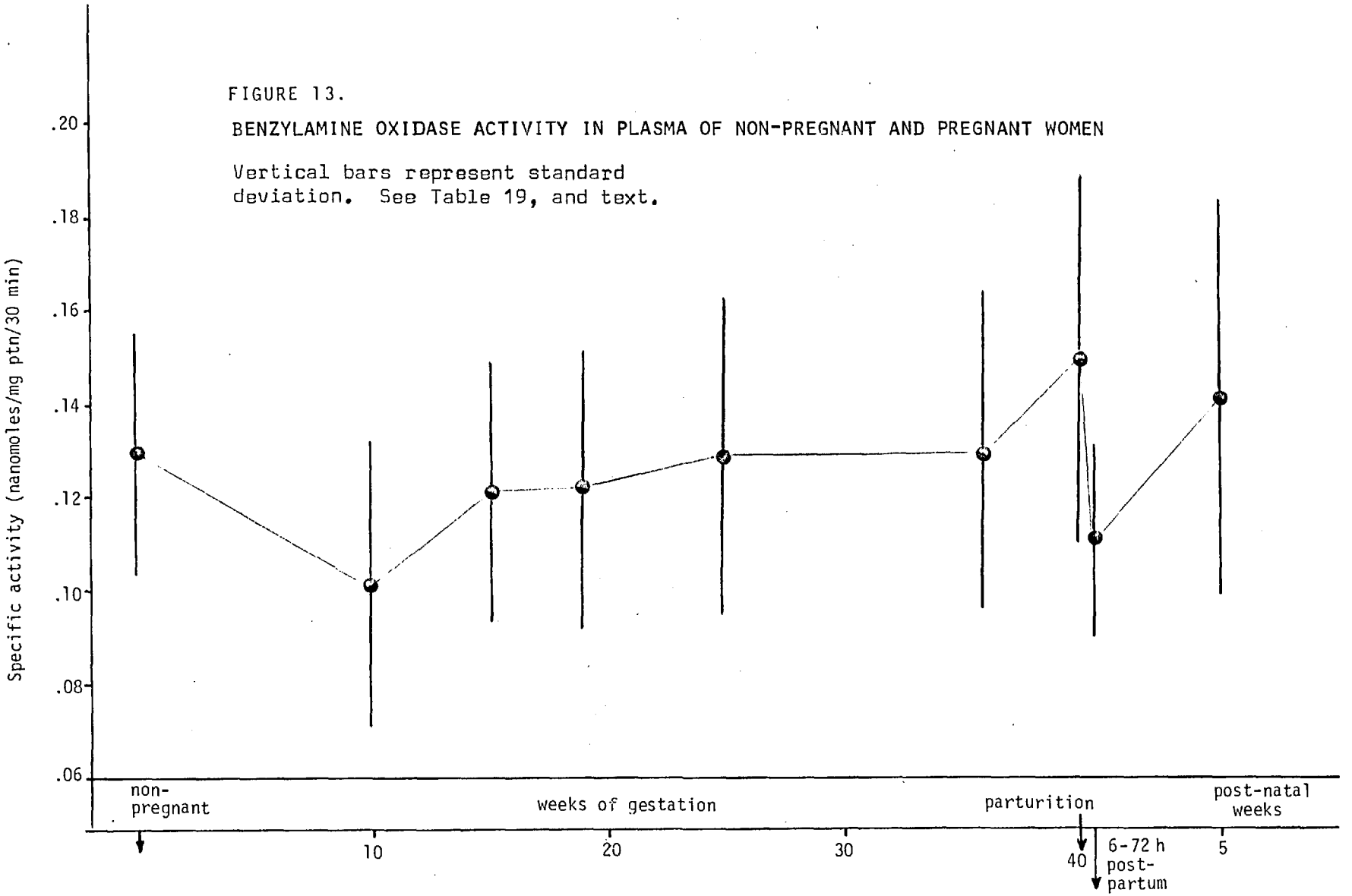
SE of mean = standard error of mean; D.F. = degrees of freedom; NS = not significant.

Specific activity expressed as nanomoles/mg protein/30 min. Values are means of replicate determinations. For experimental and assay conditions, see text.

FIGURE 13.

BENZYLAMINE OXIDASE ACTIVITY IN PLASMA OF NON-PREGNANT AND PREGNANT WOMEN

Vertical bars represent standard deviation. See Table 19, and text.



morphometric data on the development of uterine and placental vessels has proved fruitless. Inferences drawn from morphological studies are obviously insufficient to lend adequate support to such claims; nevertheless, a few studies permit conclusions of this sort (e.g., Brosens et al, 1967; Boyd and Hamilton, 1970). Ramsey (1977) states that "endometrial spiral arteries grow even after communication with the placenta has been established and they have come to merit the designation uteroplacental arteries. Indeed, they continue to grow until the placenta has attained its definitive form late in the first trimester." As for the placenta, it reaches its maximum thickness during the first half of pregnancy; in the latter half it further increases its surface area, doubling in diameter, but does not increase in thickness (Boyd and Hamilton, 1970; Warwick and Williams, 1973).

Table 20. Total weight of human uterus, placenta and fetus (Hytten and Leitch, 1964)

Weeks of pregnancy	Total weight (g)		
	Uterus	Placenta	Fetus
Non-gravid	50	-	-
10 weeks	135	20	5
20 weeks	585	170	300
30 weeks	810	430	1500
40 weeks	900	650	3300

Thus it is fairly certain that the most significant development of the placental vascular network, like that of the gravid uterus, occurs early in pregnancy. It is this development of uterine and placental vessels which, in my opinion, accounts for the first drop in BzAO activity in maternal plasma (a, above). Conversely, at the time of maximal fetal growth, maternal plasma activity is similar to the non-pregnant.

The sharp drop in activity recorded post-partum (c, above) agrees

closely with my observations in patients who underwent general surgery (see p. 18), and may probably be accounted for by enzyme requirements for tissue repair (see Discussion, p. 184), whilst the increases seen at 13-17 weeks (b) and in the post-natal period may represent "overshoot" phenomena.

So long as the function of BzAO remains obscure, it is impossible even to venture a guess as to the intricate mechanisms underlying these changes. The explanation of the sharp increase in BzAO activity at parturition (b, above) is likely to be even more complex. Total plasma protein concentration declines as gestation advances, while total volume of plasma and body water increases. Aldosterone, cysteine aminopeptidase and prostaglandin $F_{2\alpha}$ in maternal plasma rise sharply towards term (Hytten and Leitch, 1971). Profound changes take place in the "milieu intérieur" at term and during labour, mediated by all manner of circulating substances. Whilst the influence of some of them (e.g., hormones) on normal or abnormal metabolism of MAO has been widely demonstrated (see, e.g., Wurtman and Axelrod, 1963; Southgate, 1972; Lyles and Callingham, 1974, 1979; Aleyassine and Gardiner, 1975; Youdim and Holzbauer, 1976; Sourkes, 1979), virtually nothing is known of the interaction of such substances with BzAO (cf. Tryding et al, 1969). All that can be said at present is that it is more than likely that an interaction of this kind exists and is probably influenced by hormonal and other changes occurring at parturition. Speculation may, perhaps, be permitted on the bearing such changes may have, for example, on alterations in vascular permeability, which might lead to the appearance of larger amounts of BzAO in the maternal peripheral circulation. Increase in venous distensibility due to increased pressure in the femoral veins, mechanical obstruction at the level of the inferior vena cava, and consequent obstruction of the outflow of the renal veins (Hytten and Leitch, 1971) may contribute to altered patterns of appearance and flow of

soluble or particulate substances in circulating maternal blood. The roles in human pregnancy and/or parturition of vasopressor substances (review by Weir, 1975), the prostaglandins (Terragno et al, 1974, 1976; comprehensive review by Keirse, 1979), plasma bradykininogen (McDonald and Perks, 1976) and the kallikrein-kinin system (reviews by Kellermeyer and Graham, 1968; Roberts and Mashford, 1972; see also Litorowicz and Malofiejew, 1978) have been studied in some detail; thus far, investigation of their interaction with the amine oxidases is a virgin field of research. Another promising approach might be the study of the role of human chorionic gonadotropin (HCG) in amine oxidase behaviour. HCG levels in plasma rise sharply between $3\frac{1}{2}$ and 9 weeks' gestation, then decline rapidly. A smaller and slower increase and fall occur at the beginning of the third trimester (Brody, 1969; Tulchinsky and Hobel, 1973; Dattatreyamurty et al, 1975).

The only other studies of BzAO in human pregnancy that I know of are those of McEwen (1964, 1972). In his study of 45 pregnant women, primiparous and multiparous (mean age 29 years), he found no change in serum BzAO between normal pregnancy levels and those of non-pregnant individuals. Determinations were carried out at weeks 7-13 (N = 31), 14-20 (N = 28), 21-27 (N = 24), 28-34 (N = 23) and 35-40 (N = 24), the latter group presumably at parturition, though this is not stated. No post-partum determinations are mentioned. Only the mean and standard error are given for the total of 120 determinations and no table is shown, but it is obvious from the graph illustrating the paper that no fall in BzAO activity was observed at 7-13 weeks, nor is there any increase in serum activity at parturition. I find no explanation for this absence of variation at either gestational age, and indeed the values for the entire study of BzAO in 120 serum specimens are remarkably uniform. McEwen employed the spectrophotometric assay, which is less sensitive than the radiochemical microassay used by us, though

in my initial experiments I found good correlation between results recorded with both (Lewinsohn, unpublished data). McEwen (1972) found no difference between BzAO levels in cord blood and mean adult activity.

4.2.3.2

Activity of amine oxidases in puerperal tissues

Table 21 shows the activity of BzAO and MAO A in placenta, placental vessels and umbilical vessels, compared with D*Bz (deprenyl-sensitive) activity in the solid tissues, and BzAO activity in maternal and cord plasma from the same individuals. As stated above, no correlation was found between any two sets of values. Mean deprenyl-sensitivity with Bz as substrate in placenta, placental vessels and umbilical vessels was 31 (± 4.3 SE of mean), 8.7 (± 1.9) and 4 (± 1.4) per cent, respectively. Relative deprenyl inhibition of Bz oxidation in placenta was thus considerably higher than in placental vessels, which in its turn was higher than in umbilical vessels. All 12 placental preparations showed some effect of deprenyl inhibition, whilst 3 out of 11 preparations of placental vessels and five out of ten umbilical vessels were unaffected.

Deprenyl inhibition of Bz deamination in these preparations may be interpreted as expressing the presence of either MAO B, blocked selectively by deprenyl, or MAO A deaminating Bz and being inhibited non-selectively by deprenyl (see Section 3.2.5, Inhibitors, p. 101). For a better examination of the problem, the three tissues will be considered separately.

Placenta. Except for n° 16, which showed lower inhibition, the effect of deprenyl on Bz deamination in the 12 specimens was fairly similar. The mean of inhibition in all twelve specimens agrees closely with the value shown in Figure 1 (c) of our paper (Lewinsohn et al, 1980a).

Table 21. ACTIVITY OF BzAO AND MAO A, AND DEPRENYL-SENSITIVE Bz OXIDATION IN HUMAN PUERPERAL TISSUES

Ptt. N°.	Age (yrs) Parity	Birth weight (g)	Sex	Maternal blood BzAO	Cord blood BzAO	Placenta			Placental vessels			Umbilical vessels		
						BzAO	D*Bz	MAO A	BzAO	D*Bz	MAO A	BzAO	D*Bz	MAO A
4	29 Pr	3420	F	0.11	0.17	1.5	0.66	46.	31.	4.3	11.	28.	2.5	7.
7	36 Mu	3860	F	0.17	-	1.2	0.68	72.	29.	4.9	22.	-	-	-
9	34 Mu	2800	F	0.11	0.10	0.8	0.32	55.	27.	0	3.	27.	3.2	4.
16	25 Pr	3770	F	0.19	0.20	1.6	0.03	35.	35.	3.8	3.	38.	2.7	8.
21	20 Mu	3370	M	0.20	0.13	0.9	0.20	30.	30.	0	17.	22.	0	6.
22	26 Mu	4020	M	0.12	0.14	0.9	0.14	43.	55.	0	2.	22.	1.5	3.
48	25 Pr	3755	F	0.11	0.08	1.6	0.35	67.	22.	3.7	34.	33.	0	4.
52	21 Mu	3680	M	0.08	0.14	0.7	0.17	36.	50.	6.0	16.	19.	0	4.
62	35 Pr	3350	M	0.11	0.13	0.9	0.29	45.	41.	5.3	9.	53.	2.6	11.
68	29 Pr	3860	F	0.17	0.09	0.8	0.26	60.	38.	2.7	2.	28.	0	5.
72	26 Pr	4470	M	0.27	0.11	1.0	0.28	46.	39.	1.9	11.	-	-	-
84	26 Mu	4120	M	0.13	0.09	0.5	0.27	51.	-	-	14.	12.	0	5.
	N			12	11	12	12	12	11	11	12	10	10	10
	Mean			0.15	0.12	1.03	0.30	48.8	36.0	3.0	12.0	28.2	1.25	5.7
	Standard error of mean			0.015	0.01	0.1	0.05	3.5	2.8	0.64	2.6	3.4	0.4	0.7

Pr = primipara; Mu = multipara. F = female; M = male. N° 7, gestational age = 37 weeks. N°s 9, 16, 21 = Caesarean section; all others, vaginal delivery: 4, 7, 52, 84 = normal; 22, 48, 62, 68, 72 = forceps. Values shown are means of replicate determinations, and express specific activity (nanomoles/mg protein/30 min) of BzAO (residual activity after 4×10^{-7}M deprenyl), D*Bz (deprenyl-sensitive moiety of Bz oxidation) and MAO A (uninhibited 5-HT oxidation). For experimental and assay conditions, see "Experimental".

In my opinion, both MAO B and MAO A contributed to Bz oxidation in all 12 placental preparations shown here. In a "spongy" tissue such as placenta, it is impossible, despite all precautions, to avoid the presence of vascular fragments (which account for BzAO activity); it is equally impossible to eliminate all traces of blood. Reverting to Figure 1 (c) of our paper (Lewinsohn et al, 1980 a), I suggest that the first inhibitory plateau of Bz deamination (25-30%), between 10^{-7} and 5×10^{-5} M deprenyl, corresponds to MAO B of blood platelets trapped in the tissue, and, perhaps, vascular endothelium. The second plateau, between 10^{-5} and 4×10^{-4} M deprenyl, probably represents non-selective inhibition of MAO A. A similar situation appears to obtain in the present study. Nevertheless, from a comparison of the absolute values for MAO A with those for BzAO and D*Bz, the conclusion is inescapable that, whatever the explanation of the latter activity, the contribution of MAO A to Bz deamination, or MAO B activity in the preparation with Bz as substrate, is clearly negligible: MAO A is beyond doubt the predominant, if not the only, active amine oxidase in human placenta. Regression analysis of the relationship between D*Bz and 5-HT oxidation in the placental preparations, which gave r = 0.47, underlines this conclusion.

As for the differences in absolute values elicited in the various specimens, these may be due mainly to individual variation. The fact that the various regions of maternal and fetal placenta assayed were probably not always the same is not likely to be very important. The placenta of patient n° 62 was examined for differences between the maternal and fetal sides; activity of both BzAO and MAO A was closely similar. A previous experiment on another specimen, in which six samples were taken from the edge and mid-placenta on the maternal and fetal sides as well as the middle portion, showed negligible differences (Lewinsohn, unpublished data). The latter results were

confirmed by histochemical examination of the same specimens (T. A. Ryder, unpublished observations).

Histochemical studies in these laboratories (Ryder et al, 1979) have localized MAO A to the trophoblastic layer of the chorionic villi in human placenta. With TA, PEA and Trypt as substrates, activity was sensitive to 10^{-7}M clorgyline, but unaffected by 10^{-7}M deprenyl (cf. also placental vessels, p. 144).

There is close agreement between the present results and the study of Egashira (1976), who showed the highest activity of human placenta to be elicited with 5-HT as substrate, but Bz was also oxidized to a slight extent. The conclusions reached by this author are identical with mine, viz. that human placental MAO is likely to be a single enzyme, probably MAO A. Youdim and Sandler (1967), however, separated two bands of enzymic activity from human placental mitochondria by polyacrylamide gel electrophoresis, suggesting the presence of two isoenzymes in the preparation.

Of the two soluble amine oxidases purified from human placenta by Bardsley and Crabbe (1973) and Bardsley et al (1974), one was a DAO resembling pig kidney DAO in its properties; the other was a "typical" MAO, except that it was found to be soluble, cytoplasmic in origin and an oxidant of many diamines. Its effect on the latter, however, was in no way comparable to that on catecholamines. In this respect, it resembles MAO A in placenta reacting with Bz as compared with 5-HT, its specific substrate. The authors discuss the possible functional significance of the DAO-MAO enzyme system in pregnancy as a protection for the feto-placental unit against excesses of biogenic amines. In a histochemical study of human placentae at 19-40 weeks gestation, Jones et al (1974) demonstrated MAO in syncytiotrophoblast and cytotrophoblast (cf. Ryder et al, 1979). According to these authors, failure to identify 5-HT in placental tissues suggests that the human placenta

is incapable of producing 5-HT. They propose that MAO may protect the placental vasculature from 5-HT produced by the developing fetus.

Placental vessels. Compared with umbilical vessels, placental vessels showed higher BzAO and MAO A activity, far greater variability, and a greater degree of inhibition by deprenyl. All three values varied independently, and no correlation was found between any two parameters. However, the more than twofold inhibitory effect of deprenyl on Bz oxidation of placental vessels compared with umbilical vessels clearly points to contamination of some of the former with placental parenchyma, despite careful washing. On the other hand, the far higher absolute values for D*Bz in placental vessels compared with those for placenta suggest that some of the preparations contained some MAO B. It is also noteworthy that of the 3 preparations of placental vessels in which deprenyl had no inhibitory effect on Bz oxidation, two had extremely low MAO A activity, although the third showed substantial activity of this enzyme. Conversely, specimen n^{o} 68 had very low MAO A activity, whilst Bz deamination was substantially inhibited by deprenyl (Table 21).

The presence of BzAO in human placental vessels is strikingly demonstrated in the histochemical study of Ryder et al (1979). With Bz as substrate, intense staining was seen in the tunica media of the blood vessels in the larger villi, and appeared to be associated with smooth muscle (cf. also Section 4.3, Amine oxidases in vascular tissues of man, and Chapter 5, Discussion). No reaction was seen in the endothelium, in the study of Ryder et al (1979) (cf. p. 159).

Umbilical vessels. While BzAO activity in umbilical vessels was only slightly lower than in placental vessels (Table 21), activity of MAO A was significantly different ($t = 2.34$ for 19 DF; $0.025 < p < 0.05$). A difference of the same magnitude was found between the D*Bz values of umbilical and placental vessels ($0.025 < p < 0.05$); as in the case of

the latter, values for the three parameters varied independently, and no relationship was found between any two.

One specimen of Wharton's jelly (patient n° 62) was examined for specific amine oxidase activity. Readings for BzAO and MAO A were 0.4 and 4.8, respectively; 4×10^{-7}M deprenyl had no effect on Bz oxidation. It will be seen that only the value for MAO A is similar to that for umbilical vessels, whereas the specific activity of BzAO in Wharton's jelly clearly distinguishes it from that of the vessels it contains (cf. Section 4.3, Amine oxidases in vascular tissues of man, and Chapter 5, Discussion).

Dyer and Weber (1971) examined 5-HT concentrations and MAO activity (with kynuramine as substrate) in sheep tissues. The umbilical vein had about three times the 5-HT concentration found in the umbilical artery. MAO was demonstrated in all blood vessels examined; activity was similar in fetal and umbilical blood vessels, whilst that in maternal vessels was greater than in either. The authors conclude that MAO may play a role in the termination of the action of 5-HT in sheep umbilical vessels, as well as in the uptake mechanism described earlier by Dyer (1970).

4.2.3.3

Activity of amine oxidases in amniotic fluid

Thirty-seven specimens of amniotic fluid, gestational ages 16-40 weeks, were assayed for BzAO and MAO A; in 31, MAO B was assayed with Bz and PEA as substrates, and deprenyl as inhibitor. BzAO activity was found in all. MAO B, defined as D*Bz activity (Bz oxidation sensitive to deprenyl), was present in 23 specimens, but no inhibitory effect of 4×10^{-7}M deprenyl was found in eight samples. With PEA as substrate, no detectable activity was recorded in any. As for MAO A, no activity was elicited with 5-HT as substrate in any of the specimens examined.

BzAO activity is illustrated in Table 22 and Figure 14. Although

Table 22. BzAO ACTIVITY IN HUMAN AMNIOTIC FLUID

	N	Gest. age	So.activity Mean	SE	Student's t for	DF	t	p
(a)	6	17	0.26	0.067				
					(a)/(b)	11	1.86	NS
(b)	7 *	18-21	0.92	0.35				
					(b)/(c)			NS
					(a)/(c)			NS
(c)	12	27-36	0.89	0.20				
					(c)/(d)			NS
(d)	12	37-40	1.14	0.19				
					(a)/(d)	16	3.125	$0.005 < p < 0.01$

Gest. age = gestational age (weeks). SE = standard error of the mean. DF = degrees of freedom. NS = not significant.

Specific activity expressed as nanomoles/mg protein/30 min. Values are means of replicate determinations. For experimental and assay conditions, see text.

(*) This group included one high value at 20 weeks' gestation (specific activity = 2.96). Since no other amniocentesis was performed on this patient, further investigation was impossible. The patient was delivered at term of a normal, healthy baby. Another 20-week amniotic fluid had extremely high BzAO activity (specific activity = 6.0). This value was not included in the Table, since cytogenetic study showed a chromosomal defect (see text).

the total number of specimens examined was small, a significant increase in the activity of this enzyme is seen as gestation progresses, particularly when the earliest specimens are compared with those towards term. Group (b), at 18-21 weeks' gestation, included one specimen with high BzAO activity (sp.act. = 2.96); when this sample was excluded from computation, the mean for the group fell to 0.583, but analysis showed no significant difference between this value and that for the preceding group. Individual variation in BzAO activity of amniotic fluid is high, however, as shown in Table 23. In two individuals whose amniotic fluid was assayed for BzAO on two occasions, specific activity rose with advancing gestational age, whereas in a third case, it fell. It is well known that protein concentration in amniotic fluid varies considerably during gestation (Sutcliffe, 1975), and since specific activity is related to the total protein content of the material examined, this variable adds to the difficulties in interpreting results.

Table 23. Variation in protein content and activity of BzAO in three human amniotic fluids.

AF N°	Gest.age weeks	Protein mg/ml	BzAO sp.act.	Gest.age weeks	Protein mg/ml	BzAO sp.act.
11/17	20	4.8	0.14	27	4.5	0.80
15/23	27	7.5	0.79	37	3.2	0.26
18/22	27	4.9	0.15	36	2.4	0.74

Despite these drawbacks, the difference between values for early and late liquors suggests that, eventually, assay for BzAO might prove a useful adjunct in determination of fetal maturity, though much more work will have to be done before BzAO can be included in the roster of amniotic fluid components routinely assayed for such determination.

Figure 14 shows one amniotic fluid with extremely high BzAO activity

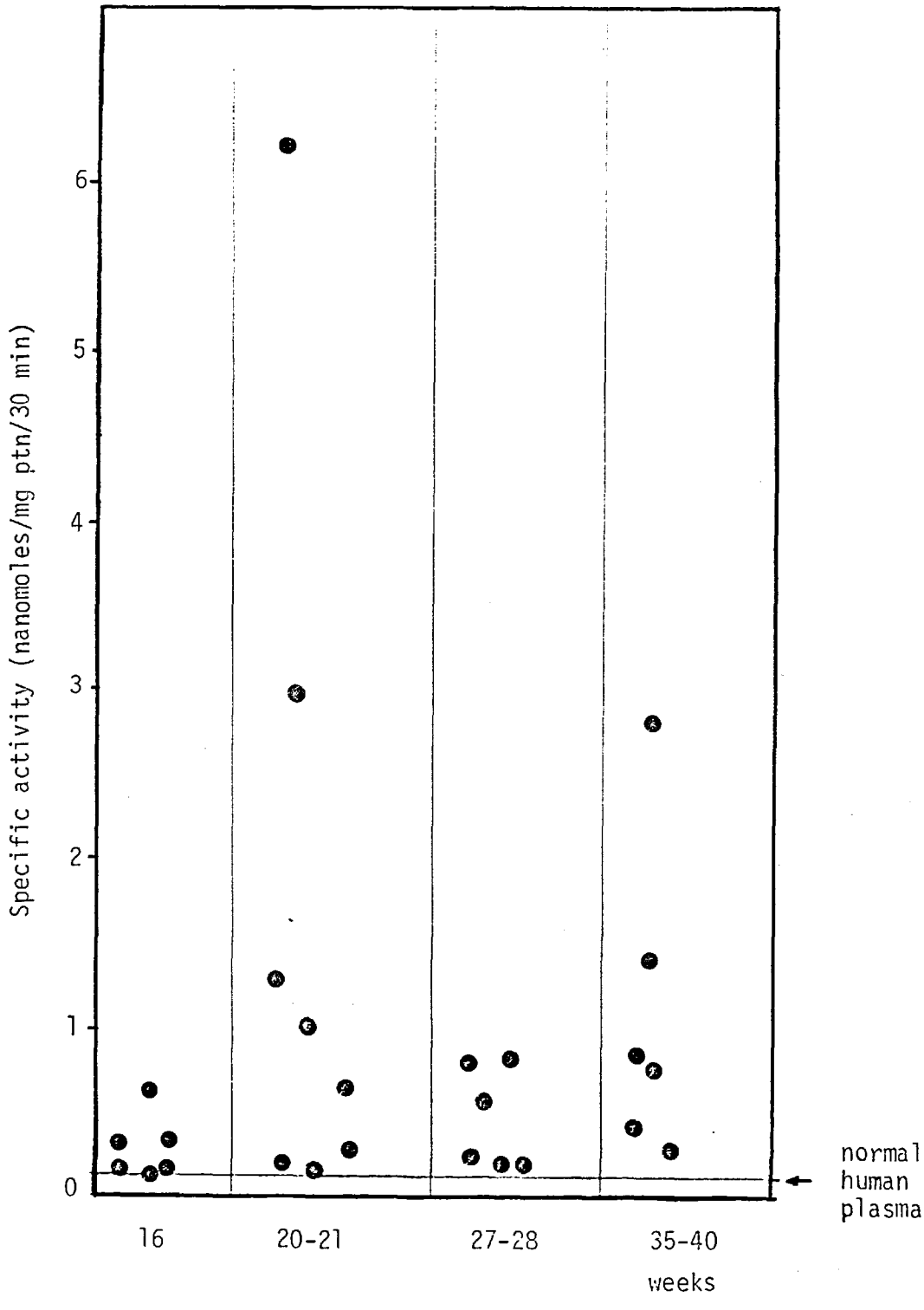


Figure 14. BENZYLAMINE OXIDASE ACTIVITY IN HUMAN AMNIOTIC FLUID

(sp.act. = 6.0). Bz deamination in this sample was insensitive to deprenyl, and, like all other liquors, it did not react with 5-HT as substrate. Cytogenetic examination revealed a chromosomal defect in the cells cultured from this amniotic fluid; it was therefore excluded from computation. (It should be noted, however, that when I received the specimens from Dr. Blau, neither he nor I knew of the cytogenetic study on this specimen.)

Two other liquors in Figure 14 had high BzAO activity; they have, however, been included in the calculations because no clinical abnormality was recorded in the further course or outcome of these pregnancies, which were successfully completed and produced normal babies at term.

MAO B activity (Table 24) in early amniotic fluid was not significantly different from that at term, though intervening stages showed some statistically significant differences. Its presence in amniotic fluid, however, like that of BzAO, is important per se, although the functions and physicochemical properties of both amine oxidases in amniotic fluid have yet to be established. The significance of these results lies in the finding of a soluble MAO in a normal human fluid of physiological importance. The presence of mitochondrial fragments in liquor, which might account for MAO B activity, is an unlikely explanation, in view of my observations on amniotic fluid submitted to differential centrifugation (see p. 78). Moreover, several filtered samples of amniotic fluid were sensitive to deprenyl inhibition, with Bz as substrate (Lewinsohn, unpublished data).

Salafsky and Nadler (1971) could detect no activity of MAO, cytochrome C oxidase, succinic dehydrogenase or glutamic dehydrogenase in 5 amniotic fluids or in any of the fractions obtained by ultracentrifugation, despite the presence of mitochondria in the 600 g fraction. Electron micrographs of liquor early in the second trimester showed

Table 24. EFFECT OF DEPRENYL INHIBITION ON Bz OXIDATION IN HUMAN AMNIOTIC FLUID

	N	Gest. age	Per cent inhibition Mean	SE	Student's t for	DF	t	p <
(a)	6	17	11.5	2.51				
(b)	7	18-21	3.3	1.71	(a)/(b)	11	2.7	0.025
(c)	8	27-36	5.1	1.72	(b)/(c)	13	0.76	NS
(d)	10	37-40	15.1	4.22	(d)/(c)	16	2.19	0.05

Gest. age = gestational age (weeks). SE = standard error of the mean. DF = degrees of freedom. NS = not significant.

Final concentration of deprenyl was 4×10^{-7}M. For experimental and assay conditions, see text. Values are means of replicate determinations.

the presence of various organelles and intact amniotic fluid cells, but the authors were unable to trace the source of the organelles. For assay of MAO activity, they employed m-iodo-Bz (in phosphate buffer, pH 7.2), which Zeller et al (1965) had shown to be a good substrate for the mitochondrial enzyme in homogenates of solid tissues with high MAO activity; it may, perhaps, be less effective to demonstrate the presence of soluble MAO when present in minute amounts. This may account, in part, for the failure of Salafsky and Nadler (1971) to detect MAO in amniotic fluid. Moreover, the number of liquors examined was very small. In my sampling of 31 specimens for MAO B activity, with Bz as substrate and deprenyl as inhibitor at pH 9.0, eight liquors were totally insensitive to deprenyl inhibition, and some of the 23 specimens positive for MAO B had very low activity. When PEA (supposedly a good substrate for MAO B) was employed, no activity was recorded with any of the specimens examined.

There is, to my knowledge, only one published report (Brzezinski et al, 1962) which claims that MAO is present in amniotic fluid, and is quoted to this effect by others (e.g., Salafsky and Nadler, 1971 (see above); Sutcliffe, 1975). From a perusal of the paper, it is clear that Brzezinski et al (1962) did no experimental work on this specific problem. In their own words, these authors present "circumstantial evidence of the existence of MAO in amniotic fluid". No sources are quoted to support their claims, "it appears that the amniotic fluid contains MAO in fairly large quantities", and "it can be safely assumed that the MAO which is produced in the placenta is also passed in large quantities into the amniotic fluid." In the light of the results presented here, the latter statement is untenable. "The MAO which is produced in the placenta" is form A, of which no activity was detectable in the amniotic fluid, whilst MAO B is present in amniotic fluid, but not in placenta.

From their observations on the origin of amniotic fluid enzymes, Sutcliffe and Brock (1972) conclude that the majority of liquor enzymes studied are probably not of fetal or maternal serum origin. As shown by these authors, the complex patterns of enzyme activity at 13-18 weeks and in the last two months of pregnancy suggest that significant quantities of tissue protein are released into the liquor. However, the sources of non-serum protein in amniotic fluid remain in doubt, and may possibly be identified by immunological studies.

Investigation of specific activity patterns of enzymes in liquor (Sutcliffe et al, 1972) shows complex changes of enzymic specific activities in amniotic fluid as gestation proceeds. No significant correlations were found in this study between protein concentration in the amniotic fluid and enzyme specific activity. In the authors' opinion, whatever the biological implications of the complex factors controlling the composition of amniotic fluid (see Sutcliffe and Brock, 1972), the trends observed during the latter half of pregnancy suggest that measurement of enzymes in liquor may be useful in assessing fetal maturity.

Sutcliffe (1975) reviews the nature and origin of soluble protein in human amniotic fluid. DAO and histaminase are quoted separately, and various authors give different ratios for titres of these enzymes in liquor, maternal serum and fetal serum. Sutcliffe's only reference to MAO activity in amniotic fluid is based on Brzezinski et al (1962), mentioned above.

Other studies, such as those of Setnikar et al (1959) on the fetal lung as a source of amniotic fluid, Wharton et al (1971), who investigated total hydroxyproline in amniotic fluid and its effect on intrauterine growth, and Scott et al (1972) on changes in amino acid concentrations during the first half of pregnancy, are beyond the scope of this work.

4.3

Distribution and localization of amine oxidases in vascular tissues of man

(See also: Kinetic studies, Section 3.2.3; Subcellular fractionation, Section 3.2.4; Inhibitors, Section 3.2.5; Amine oxidases in human pregnancy, Section 4.2; and Lewinsohn et al, 1978, 1980 b.)

4.3.1

Introduction

From my earliest observations on amine oxidases in man and rat (Lewinsohn et al, 1978), evidence has pointed to blood vessels and richly-vascularized tissues as the main sites of BzAO activity. It was therefore natural that in the course of mapping out human amine oxidase activity at different developmental stages, vascular tissues were explored with special interest, which was heightened when BzAO activity was shown histochemically to be localized to the tunica media of human blood vessels (Ryder et al, 1979). One fetal carotid (not shown) displayed the highest specific activity for BzAO (49.2) recorded in any human vessel, exceeding even that in adult cerebral vessels; MAO B (D*Bz) activity was moderate (8.7), but higher than in comparable tissues, whilst deamination of 5-HT was within the range of fetal aortic values. All other tissues of this fetus (n^o 5 in the series, Lewinsohn et al, 1980 b) gave readings for the three enzymes which were well within the normal fetal range. Histochemical studies had shown the presence of MAO in the carotid body of rabbit, cat and dog (Thybusch, 1968), and of catecholamines and 5-HT in the human fetal carotid body (Hervonen and Korkala, 1971). I therefore decided to take a closer look at amine oxidase activity in the fetal and neonatal carotid. An attempt was also made to localize activity, by examining proximal and distal portions, as well as media and adventitia, separately. Since the fetal carotid arteries were too small for this treatment, only the neonatal vessels were so examined.

Although choroid plexus, meninges and Wharton's jelly are not vessels, they are included in this Section to permit comparison with values for adjoining tissues and vessels.

4.3.2

Experimental

Sources and treatment of the tissues dealt with in this Section have been fully described (Lewinsohn et al, 1980 b; see also Section 4.2.3.2, p.140). In addition, fetal and neonatal carotid arteries were obtained, respectively, from the Royal Marsden Fetal Tissue Bank (by the kindness of Dr. Sylvia Lawler) and from cases of early neonatal death autopsied at Queen Charlotte's Maternity Hospital (by the kindness of Dr. Gillian S. Gau and Dr. J. Pryse-Davies). The fetal vessels came from therapeutic abortions at 20 and 17 weeks, respectively. The neonates' sex was M, F and F; their gestational ages were 33, 40 and 40 weeks, their birth weights 2110, 2800 and 2280 g and crown-rump lengths 32, 34.5 and 32 cm, respectively. Immediately after dissection, the neonatal carotids were divided into proximal and distal portions; each portion was split longitudinally into media and adventitia. Values for whole-wall thickness of proximal and distal regions shown in Table 26 are the arithmetical means of media and adventitia for each individual.

All other procedures were as described (Lewinsohn et al, 1980; see also p. 55).

4.3.3

Results and comments

In human blood vessels, the predominant amine oxidase is clearly BzAO, as shown in Table 25. However, MAO A predominates in fetal aorta and in the vessels surrounding the adult mesenteric lymph node (not lymphatic vessels), whilst both forms of MAO show higher activity than BzAO in neonatal hepatic vessels. The latter result is probably due

Table 25. DISTRIBUTION OF AMINE OXIDASE ACTIVITY IN VASCULAR TISSUE OF MAN

Tissue		BzAO	MAO B		Ratio	MAO A	Ratio MAO A/B
			D*Bz	PEA	BzAO/D*Bz	(5-HT)	(5-HT/D*Bz)
Aorta (*)	F	2.9	2.2	0.9	1.32	6.8	3.15
Aorta (*)	N	8.0	3.1	1.2	2.58	5.1	1.65
Aorta (*)	A	27.9	4.4	1.5	6.34	4.1	0.9
Aorta (**) - media	A	20.6	4.5	1.2	4.58	2.0	0.44
Aorta (**) - adv.	A	9.9	2.5	0.7	3.96	2.7	1.1
Aorta (**) - ath.pl.	A	2.4	2.1	0.24	1.14	0.14	0.07
Hepatic vessels	N	4.0	6.7	2.6	0.6	11.2	1.7
Hepatic vessels	A	17.2	15.9	7.0	1.08	5.7	0.36
Mesenteric vess.	A	31.6	7.1	5.5	4.45	5.2	0.7
Mesent.lymph node vessels (***)	A	14.3	2.9	3.0	4.93	19.8	6.8
Pulmonary artery	A	44.0	5.9	5.0	7.46	9.7	1.65
Splenic artery	A	31.5	7.9	8.8	3.99	21.4	2.7
I. Vena cava	A	24.0	19.6	6.2	1.22	7.7	0.4
Coeliac trunk	A	29.9	11.6	2.3	2.56	6.6	0.6
Cerebral vess.(*)	A	47.8	0	1.9	-	8.3	-
Choroid plexus	A	1.8	33.7	6.8	0.05	3.0	0.09
Placental vess.(†)		36.0	3.0	ND	12.0	12.0	-
Umbilical vess.(†)		28.2	1.2	ND	23.5	5.7	-
Wharton's jelly (†)		0.4	0	ND	-	4.8	-
Meninges	F	0.5	0.7	0	0.71	1.5	-
Meninges	N	3.5	1.4	0.9	2.5	3.8	2.6

Except for ratios, values express specific activities (nanomoles/mg protein/30 min). For experimental and assay conditions, see text. D*Bz = deprenyl-sensitive moiety of Bz oxidation. ND = not done. F = fetus; N = neonate; A = adult.
(*) Values from Lewinsohn et al (1980b). All other adult values, except placental and umbilical vessels and Wharton's jelly, from one individual (see text).
(**) Different regions taken from same specimen; ath.pl. = atheromatous plaque.
(***) Vessels surrounding mesenteric lymph node (not lymphatic vessels).
(†) See Table 21 (Section 4.2.3.2).

to contamination of the homogenate with liver parenchyma, and, perhaps, MAO activity in hepatic sinusoidal endothelium (see below).

BzAO activity in adult aortic adventitia was about half that found in the tunica media, but the ratio of BzAO/D*Bz activity was approximately the same in both regions. MAO A activity in the aortic media was slightly lower than in the adventitia. The atheromatous plaque, however, showed only 12 and 7 per cent, respectively, of BzAO and MAO A activity in the media. The mesenteric lymph node, from which the surrounding vessels were taken, was completely calcified, defying all attempts at homogenization. BzAO activity in these vessels was relatively low for adult vascular tissue, but deamination of 5-HT was high, second only to that in the splenic artery in this series. One explanation might be that MAO A may be very active in lymphatic tissue, and that the splenic artery preparation might have been contaminated with parenchymal tissue. This is hardly likely, however, since the spleen of this patient showed only 5% of the MAO A activity seen in the artery (cf. observations on fetal appendix, p. 126). MAO B was relatively active in the inferior vena cava and coeliac trunk; at present I do not know how to interpret this finding. Deprenyl-resistant Bz oxidation in cerebral vessels has already been shown (Lewinsohn et al, 1980 b). The pattern of enzymic activity of the choroid plexus is remarkable for its high MAO B value, the more so as deamination of Bz in the cerebral vessels of the same individual (sp.act. 33.1) was totally insensitive to deprenyl inhibition. This is reminiscent of the histochemical demonstration of MAO B in rat ependyma (Williams et al, 1979), as well as in the structures surrounding the fourth ventricle of rat (Figure 15). As expected, the meninges, both fetal and neonatal, showed low values throughout, though the ratio of fetal/neonatal Bz oxidation points to an increase in activity which might progress to adult values perhaps comparable to other highly-vascularized structures. Comparison of the patterns of

enzymic activity in Wharton's jelly and umbilical vessels is instructive. In the former, there is hardly any BzAO, and no detectable activity of MAO B, but MAO A is moderately active and may derive from the fibroblasts in which the tissue abounds (Bradbury, 1975; cf. p. 179).

Whereas BzAO is the predominant enzyme in the blood vessels shown in Table 25, in fetal and neonatal carotid arteries (Table 26) MAO A was far more active than either BzAO or MAO B. In one fetal carotid, the latter two enzymes were seen to be active to a similar degree,

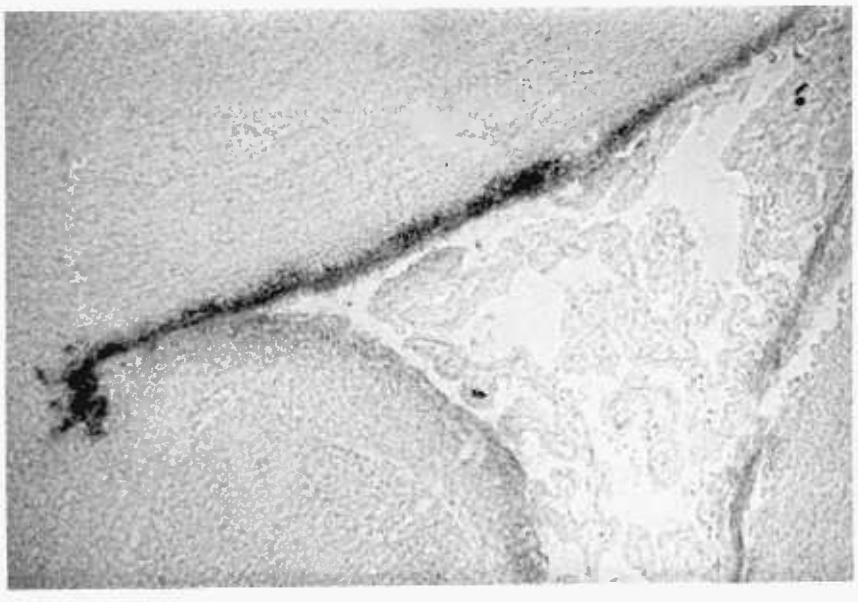


Figure 15. Histochemical localization of MAO B (with Bz as substrate) in structures surrounding fourth ventricle of rat. (By the kindness of Dr. T. A. Ryder.)

while in the other, MAO B was about three times as active as BzAO. In neonatal carotid, the pattern of distribution of BzAO and MAO B varied in the three specimens examined; mean values showed BzAO to be somewhat more active in the media, whilst in the adventitia MAO B was the more active of the two. In fetal vessels the relatively high activity of MAO B may, perhaps, be attributed to a high proportion of endothelial cells compared with more mature vessels; the changes which the subendothelial space undergoes with development and aging (cf. Sandler and

Table 26. DISTRIBUTION OF AMINE OXIDASE ACTIVITY IN HUMAN FETAL AND NEONATAL CAROTID ARTERY

Tissue fraction	Specific activity							
	BzAO		MAO B (D*Bz)		MAO B (PEA)		MAO A	
	Proximal	Distal	Proximal	Distal	Proximal	Distal	Proximal	Distal
Whole vessel - F.1	2.2		1.3		5.2		32.1	
Whole vessel - F.2	1.1		2.4		3.5		19.1	
Whole wall - N.1	8.4	11.3	17.7	11.0	8.2	7.3	37.0	31.3
Whole wall - N.2	5.3	5.3	10.5	10.3	4.2	4.8	8.3	15.0
Whole wall - N.3	9.4	7.4	10.6	10.4	5.6	5.9	18.3	20.9
Whole wall - mean	7.9		11.8		6.0		21.8	
S.D.	2.15		2.7		1.4		9.7	
S.E.of mean	0.9		1.1		0.6		4.0	
Media - N.1	4.0	13.7	4.0	6.2	3.3	6.1	9.6	33.8
Media - N.2	8.0	5.3	5.5	8.2	3.0	4.0	8.2	10.6
Media - N.3	12.3	5.6	5.3	4.9	3.3	5.0	10.7	12.1
Media - mean	8.1		5.7		4.1		14.2	
S.D.	3.6		1.3		1.1		8.9	
S.E. of mean	1.5		0.5		0.5		3.6	
Adventitia - N.1	12.8	8.9	31.4	17.9	13.2	8.4	64.4	28.8
Adventitia - N.2	2.7	5.3	5.4	12.4	5.3	5.6	8.3	19.4
Adventitia - N.3	6.5	9.3	16.0	15.9	8.0	6.8	25.9	29.6
Adventitia - mean	7.6		16.5		7.9		29.2	
S.D.	3.2		7.8		2.6		17.2	
S.E. of mean	1.3		3.2		1.0		7.0	

Specific activity expressed as nanomoles/mg protein/30 min. F = fetus; N = neonate. Numbers after symbols identify individuals (see text). For experimental and assay conditions, see "Experimental".

Bourne, 1963; Elias, 1961; Cliff, 1967, 1970; Gerrity and Cliff, 1972) may also be reflected in varying patterns of enzymic activity of the cellular elements in the vessel wall.

It is true that no staining of vascular endothelium has been seen in any of the preparations examined in these laboratories (Ryder et al, 1979, 1980) with any substrate or inhibitor employed. Histochemical demonstration of endothelium is fraught with difficulties, largely because endothelial cells appear to be far less stable than other vascular elements. When endothelial structure in cord vessels was studied in time sequence (0 - 24 h) by scanning electron microscopy in these laboratories, endothelium was seen to "strip off" within one hour; within 6 h, endothelial cells were no longer demonstrable in the preparation (T. A. Ryder, personal communication). A second point of some importance is that different structures may require differential histochemical treatment (incubation time, strength of reagents). For example, the staining reaction for MAO in the trophoblastic layer of human placenta (Ryder et al, 1979) was so strong that incubation time had to be cut short in order to avoid "swamping" the entire preparation. "Streaks" of clorgyline-sensitive activity, with TA as substrate, appeared in myometrial smooth muscle only when incubation time was substantially increased; after extended incubation with TA, staining was also observed in areas (such as the tunica media of myometrial blood vessels) which had previously shown no reaction (Ryder et al, 1980). Thus, whilst extrapolation of the biochemical studies of Roth and Venter (1978) on cultured cells of rabbit vascular endothelium (cf. also de la Lande et al, 1970) to human vascular endothelium is not warranted, it may be asserted that failure, so far, to demonstrate MAO in human endothelium by histochemical techniques is no evidence for its absence, whereas many biochemical results may be interpreted as suggesting the presence of MAO B in vascular endothelium (see pp. 120, 142) and, possibly, that of hepatic sinusoids.

The high BzAO activity described above (p. 153) has not been found in any of the other carotid arteries studied (Table 26). In one fetal carotid (F.1), however, 5-HT deamination was second only to that in fetal liver and to that demonstrated in the proximal adventitia of one neonatal carotid (N.1). The division of the vessels into proximal and distal portions produced no great differences when whole-wall thickness was assayed. On splitting each region into media and adventitia, however, differences in activity were striking, both between proximal and distal portions, and between media and adventitia. Nevertheless, whilst the differences for MAO B between whole wall and media, and between media and adventitia, were statistically significant (Table 27), that for MAO A was not, despite the extremely high value found in the proximal adventitia of subject N.1. The reason for this discrepancy lies in the high variability and small number of specimens examined.

Table 27. Student's t test for comparison of results shown in Table 26. (DF = 10)

	t	p
D*Bz - whole wall/media	5.048	$\ll 0.001$
D*Bz - media/adventitia	3.356	$0.005 < p < 0.01$
PEA - whole wall/media	2.43	$0.025 < p < 0.05$
PEA - media/adventitia	3.4	$0.001 < p < 0.005$
MAO A - media/adventitia	1.93	$0.05 < p < 0.10$

Pharmacologically heterogeneous smooth muscle cell distribution in blood vessels (Somlyo et al, 1965), and differentiated response patterns to vasoactive substances in various regions of the intact circulation (Mellander and Johansson, 1968) may well contribute to differences in patterns of enzymic activity throughout the vascular tree such as noted in Tables 25 and 26.

In view of the high BzAO activity observed in blood vessels, I scanned the literature for morphometric data on the actual mass of

vascular tissue present in organs or systems. However, such information does not exist even for small animals. Most of the literature on this subject, from Spalteholz (1888) to the most recent studies (Cervós-Navarro et al, 1978; see also Myrhage, 1977 for review on early and recent work), is concerned with the morphology of the microcirculation. The few morphometric studies available refer to capillaries only (Myrhage and Hudlická, 1976; Myrhage, 1977, Bär, 1978; see the two last-mentioned for further references). Studies dealing with pre- and post-capillary vessels, though extremely important for information on anatomical, physiological and physiopathological aspects, contain no morphometric data (Rhodin, 1967, 1968; Elias, 1961; Zweifach, 1961; Cliff, 1976; Elliott and O'Connor, 1978; see also references throughout Discussion, Chapter 5).

4.3.4

Literature review

The literature on MAO abounds with reports on functional aspects of enzymic activity as well as the presence of catechol- and other amines in vascular tissue, demonstrated by a variety of techniques (see Tables A.1 and A.2, Appendix). It is not proposed to present a comprehensive review of work done on MAO in vessels, which would exceed the scope of this study. Besides the handful concerned with BzAO or deamination of Bz, only the more important studies on MAO and related subjects will be mentioned here, and should be consulted for further references.

Using a manometric technique and TA as substrate, Thompson and Tickner (1951) measured O_2 uptake in the vessels of rabbit, guinea-pig and rat, and in the human popliteal artery. "MAO" in the great veins and brachial, femoral and ear arteries of the rabbit was found to be less active than in aorta, carotid, pulmonary and renal arteries. It should be noted, however, that the amine oxidase of rabbit aorta and plasma actively deaminates TA (see Rucker and Goettlich-Riemann, 1972, quoted

below). With the techniques employed by Thompson and Tickner (1951) both to homogenize tissues (see Table A.2, Appendix) and to assay enzymic activity, identification of the enzymes involved must remain doubtful.

De la Lande and Waterson (1968) described the action of TA on the rabbit ear artery, and found the vasoconstrictor potency of extraluminal TA on the isolated perfused artery to be considerably greater than that of intraluminal TA. The role of MAO in the response of the isolated central artery of the rabbit ear to TA was studied by de la Lande et al (1970). Amine oxidase activity was demonstrated histochemically throughout the media of the artery but not in the adventitia at its border with the media, where sympathetic nerve terminals are concentrated. Neither intensity nor distribution of enzyme activity was perceptibly altered up to 60 days after sympathetic denervation. Inhibitors (iproniazid, nialamide) reduced the difference between the potencies for intra- and extraluminal administration of TA. The results indicate that substrate inactivation by MAO in the tunica media is a factor contributing to the relatively low effect of intraluminal TA on the rabbit ear artery. De la Lande and Jellett (1972) reported on the relationship between the roles of MAO and sympathetic nerves in the vasoconstrictor response of the rabbit ear artery to NA.

Rucker and O'Dell (1970, 1971) used preparations of bovine aorta, purified ca. 150-fold, which actively deaminated Bz and spermidine, whilst histamine and TA were oxidized at distinctly lower rates. The most important difference noted in the substrate specificity of the aorta preparations compared with plasma, was the active oxidation of peptidyl lysine by the former, but not by the plasma enzyme.

Rucker and Goettlich-Riemann (1972) found patterns of substrate specificity, inhibitor sensitivity and pH optima for purified rabbit aorta amine oxidase similar to those described by McEwen et al (1966) for rabbit plasma. Unlike human BzAO, rabbit aorta and plasma amine

oxidase actively deaminates TA; unlike beef aorta amine oxidase, it does not react with spermine or spermidine. Lysine-vasopressin was not used as substrate in these experiments.

Dyer and Weber (1971) and Dyer (1970 a,b) studied the pharmacology of isolated sheep vessels, the mechanism of potentiation by cocaine of responses to 5-HT in umbilical cord blood vessels, and endogenous 5-HT and MAO in maternal, umbilical and fetal vessels of sheep. The conclusions presented by the authors are discussed elsewhere (p. 145).

Some aspects of the experiments of Coquil et al (1973) on subcellular fractions of rat artery have been commented upon (p. 81; 97). Whilst chemical sympathectomy serves to establish the intra- or extraneuronal location of enzyme activity, the use of TA, 5-HT and NA as substrates precludes interpretation of results in terms of BzAO activity. The authors reached their conclusions concerning the presence of BzAO in rat artery by extrapolation of their own results and those of others, based on relative sensitivities of their preparations towards cyanide, semicarbazide and clorgyline. No homogenate or subcellular fraction was inhibited by 10^{-3}M semicarbazide to such a degree as to warrant interpretation of enzyme activity as due to BzAO. Bz was not employed as substrate nor were concomitant experiments performed on plasma with the same reagents as used in the experiments on rat arteries. It is probable, of course, that these vessels contained BzAO (cf. Lewinsohn et al, 1978, for data on rat aorta), but as in the case of Roth and Gillis's (1975) experiments on rabbit lung (p. 99), such conclusions cannot be drawn from the results presented, which strongly suggest high activity of MAO A and B in the preparations.

Buffoni et al (1975 a,b) found amine oxidase activity of purified pig aorta to be dependent on the presence of more than one enzyme. They describe two enzymes in the partially purified preparation: one was a soluble enzyme, very similar to pig plasma BzAO in inhibitor sensitivity

and pH dependence (see Buffoni et al, 1972); the other, insoluble at pH 6-7, was inhibited by cupric copper-chelating agents and by carbonyl reagents in the same way as the plasma enzyme, but with a completely different kinetic response to pH changes. Bz oxidation by this enzyme was competitively inhibited by histamine, and at pH 6.8, its activity optimum, it was more active against histamine than Bz. During the purification procedure it appeared to be insoluble or linked to some insoluble protein, and the authors concluded that it might be identical with lysyl oxidase. In the studies of Buffoni et al (1977) employing immunofluorescence histochemistry to investigate porcine tissue amine oxidases (see p. 31), evidence is presented for the existence of a protein in the connective tissue of all the organs studied, possessing the same immunological determinant as pig plasma amine oxidase. A close relationship is also shown to exist between the plasma and the connective tissue amine oxidases in the pig. Buffoni herself (1966) demonstrated that the histaminase activity of porcine plasma is an intrinsic property of the enzyme which, in this species, acts on Bz and other monoamines, although, unlike pig kidney histaminase, it does not act on putrescine or cadaverine. The immunofluorescence studies (Buffoni et al, 1977) thus denote wide distribution of histaminase in porcine tissues. The intense staining seen in blood vessels, glomerular basement membranes and interstitial tissue shows that both the plasma and renal enzymes react with the same antibody preparations, corroborating biochemical evidence for high histaminase activity, e.g. in porcine renal cortex (cf. Zeller, 1951, 1972).

Harris et al (1973, 1974) describe lysyl oxidase purified from chick aorta; evidence for two forms of this enzyme in bovine aorta is presented by Vidal et al (1975). On the other hand, Shieh et al (1975), who purified an amine oxidase from bovine aorta, concluded that it was neither a plasma enzyme nor a lysyl oxidase. Antibody to bovine plasma

amine oxidase reacted with the bovine aorta amine oxidase but not with partially purified aorta LO; moreover, the aorta enzyme, but not the plasma enzyme, oxidized lysyl-vasopressin. From these and other observations, the authors suggest that the preparation described by Rucker and O'Dell (1971) may have been contaminated with LO.

Vascular smooth muscle reactivity in normotensive and hypertensive rats was studied by Spector et al (1969), who found that aortic strips from spontaneously hypertensive rats were less responsive than normal controls to the contractile effects of noradrenaline, 5-HT, and potassium chloride, but more reactive to the relaxant effects of isoproterenol. The effect of 6-OH-Dopa and L-Dopa on NA metabolism in rat brain, heart, adrenal and mesenteric artery was described by Spector (1971). Daily administration of L-Dopa (800 and 1000 mg/kg) caused a marked reduction in vascular tyrosine hydroxylase, and a striking increase in MAO activity; 400 mg/kg of the drug caused a substantial fall, similar for both enzymes. Catecholamines in blood vessels were investigated by Tarver and Spector (1970) and Berkowitz (1970), and NA in blood vessels of rat, rabbit and guinea-pig by Berkowitz et al (1971). The same group (1974) reported on the disposition of 5-HT in rat blood vessels and heart. Concentration of the amine doubled rapidly after administration of pargyline 2 - 24 h before the rats were killed, suggesting that MAO regulates the content of 5-HT in blood vessels and heart. Howland and Spector (1972) and Spector et al (1972), respectively, described the disposition of histamine in blood vessels of rat and rabbit, and the effects of drugs and physiological factors in the disposition of catecholamines in vascular tissues of rat, rabbit and guinea-pig. Lai and Spector (1978 a) measured COMT and MAO A and B in mesenteric artery, cerebral microvessels and brain filtrate of rat, using TA and 5-HT as substrates, and clorgyline as inhibitor. They found that COMT and MAO activity was greatest in the cerebral microvasculature; chemical sympathectomy with 6-hydroxy-

dopamine had no effect on total MAO, MAO A or COMT activity in any of the preparations examined. These results suggest that COMT and both forms of MAO in rat brain microvessels are extraneuronal in origin. In two further studies, the same authors (Lai and Spector, 1977, 1978b) compared MAO activity in vessels of normotensive and hypertensive rats, and found no difference in central and peripheral blood vessels of both groups. Age-linked changes in rat MAO activity were studied by Lai *et al* (1975), with TA as substrate. Cardiac MAO was seen to increase with age; mesenteric artery and vein, as well as aorta, failed to exhibit this change. Maximum MAO activity was reached in these vascular tissues within 3 weeks of birth, then remained constant. While half-life of MAO was increased (about 50% higher) in older rats, that of the vascular tissues was the same for both age groups.

The effect of copper deficiency and a dietary lathyrogen (β-aminopropionitrile, BAPN) on the amine oxidase activity of various tissues in growing chicks was investigated by Bird *et al* (1966). BzAO activity of aorta, as well as heart and kidney, was decreased in copper-deficient chicks, while liver activity was not affected. Copper added *in vitro* partially restored the activity of deficient aortic tissues. Dietary BAPN had no effect on aortic BzAO activity, but *in vitro* addition of BAPN competitively inhibited the enzyme. Ascorbic acid *in vitro* also inhibited the activity of aortic BzAO, but in a non-competitive manner. It should be noted that "BzAO" activity in these experiments probably includes some contribution of MAO B to Bz oxidation, since no selective MAO B inhibitor was employed. Thus, "BzAO" activity of Cu-supplemented aorta was similar to that of Cu-deficient or Cu-supplemented liver, while semicarbazide inhibition of the crude aortic homogenate was only 30%. At 10^{-3}M, BAPN inhibited only 42% of the aortic enzyme, which makes it unlikely that enzyme activity was due solely to LO (cf. Arem and Misiorouski, 1976, and Harris *et al*, 1974, on the effects of BAPN on lysyl

oxidase preparations). The interrelationship of dietary copper and amine oxidase (LO) in the formation of elastin in chick aorta was studied by Kim and Hill (1966).

No review of the literature on blood vessels would be complete without passing reference, at least, to atherosclerosis and its effects on vascular smooth muscle (comprehensive reviews by Sandler and Bourne, 1963, and Somlyo and Somlyo, 1968). The platelet MAO deficit observed in migraine (for review, see Sandler, 1978) raises the question as to the behaviour of BzAO in this condition, the more so because activity is higher in cerebral vessels than in any other human tissue (see above). The effect of disease on amine oxidase activity is dealt with in Section 3.2.8 and in the next Chapter, in which altered vascular permeability is discussed in some detail.

Observations on enzymes other than the amine oxidases are scattered throughout the vast literature on blood vessels and vascular smooth muscle. Only a few will be mentioned here. Comprehensive reviews by Somlyo and Somlyo (1968, 1970) deal, respectively, with normal structure, pathology, biochemistry and biophysics of vascular smooth muscle, and the pharmacology of normal and hypertensive vessels. The symposia on MAO (Costa and Sandler, 1972) and inhibitors of MAO (Wolstenholme and Knight, 1976), as well as Cliff's monograph on blood vessels (1976) and the proceedings of a symposium on cerebrovascular smooth muscle (Elliott and O'Connor, 1978) contain a wealth of information and references. Some of these and further references will be discussed in the wider context of the next Chapter.

4.4

Distribution and localization of amine oxidases in human non-vascular smooth muscle

4.4.1

Introduction

As described elsewhere, the finding that BzAO is active in all blood vessels examined was followed by the histochemical demonstration of enzymic activity in the tunica media of blood vessels (Ryder et al, 1979). No staining was observed in endothelial cells (see p.159); nor do I think it likely, for reasons to be discussed below (see Chapter 5, Discussion), that BzAO activity is localized in connective tissue cells. It appears, therefore, that the localization of this enzyme is confined to the smooth muscle layer of the vascular tunica media. This hypothesis raises the question of whether BzAO activity may be found in smooth muscle other than vascular, or whether it is localized in vessels exclusively. The investigation in these laboratories of variations in uterine enzymic activity dependent on the menstrual cycle (Ryder et al, 1980), in which I participated, provided an opportunity for the study of two adjacent tissues sharing the same vascular network, one rich in smooth muscle whilst the other is virtually devoid of such cells. The human oesophagus is another organ with distinct muscle populations in its different regions: in man, striated muscle is limited to the upper third, whereas the lower third contains non-striated muscle only (Warwick and Williams, 1973). I therefore decided to assay these regions separately.

4.4.2

Experimental

Endometrial and myometrial tissues were resected and treated as described by Ryder et al (1980). Oesophageal tissues from a female adult were obtained at autopsy (Lewinsohn et al, 1980 b). Homogenization and assay procedures have been fully described (Lewinsohn et al, 1980 b; p. 55).

4.4.3

Results and comments

The distribution of the three amine oxidases in the human uterus at different stages of the menstrual cycle is shown in Table 28. Owing to the small number of samples examined and the great variability in BzAO activity of specimens at the same menstrual stage, it is difficult to estimate the influence of the cycle on activity of this enzyme in either myometrium or endometrium. It is clear, however, that BzAO activity in myometrium is markedly higher than in endometrium; in the latter, vascular elements undoubtedly account for this enzymic activity. D*Bz oxidation in endometrium is remarkably constant throughout the cycle, while two high values stand out in myometrium, one in the early, the other in the late secretory phase. The early secretory specimen also shows relatively high myometrial MAO A activity, as compared with all but one of the other samples. However, only endometrial MAO A activity is clearly correlated with the menstrual cycle, as evinced by the striking increases recorded in the late secretory and premenstrual phases, which appear to be progesterone-dependent (see literature review). "Streaks" of clorgyline-sensitive activity, with TA as substrate, were observed by Ryder et al (1980) in myometrial smooth muscle and, according to these authors, may indicate MAO A activity in nerve tissue.

It is interesting to compare BzAO activity in myometrium with that in other adult human tissues. Table 1 of our previous report (Lewinsohn et al, 1980 b) shows a distinct division of the tissues into groups with high and low BzAO activity. Vessels, lung and digestive tract (oesophagus to mesocolon) belong in the former group, all others in the latter. Myometrium appears to occupy an intermediate position. In the histochemical study of Ryder et al (1980), myometrium stained evenly for BzAO, but less intensely than vascular tissue. Although it is possible

Table 28. DISTRIBUTION OF BzAO, MAO B AND MAO A IN THE HUMAN UTERUS

Specimen N°	Phase of menstrual cycle	BzAO		MAO B (D*Bz)		MAO A	
		Myo.	Endo.	Myo.	Endo.	Myo.	Endo.
269-E	Prolif.	-	0.2	-	4.0	-	1.1
320-E	Early secret.	-	0.4	-	6.3	-	4.5
612-E/M	Early secret.	4.6	0.6	15.3	6.6	8.0	4.7
622-E/M	Early secret.	6.7	0.1	6.9	5.7	1.3	6.9
376-E/M	Late secret.	10.9	2.1	10.1	9.0	3.9	18.9
621-E/M	Late secret.	14.0	0.3	5.5	6.1	1.2	20.0
559-E/M	Premenst. late secret.	7.4	1.1	4.2	9.0	8.2	118.0

All determinations at least in duplicate. Values express specific activity (nanomoles/mg protein/30 min) and are means of replicate determinations. For experimental and assay conditions, see "Experimental".

Prolif. = proliferative; secret. = secretory; premenst. = premenstrual.

that the behaviour of BzAO is not the same in different populations of smooth muscle cells, I think it more likely that the ratio of smooth muscle to other cells in the tissue determines the intensity of the enzymic reaction. Albeit rich in smooth muscle cells, myometrium contains a considerable amount of interstitial connective tissue (Bradbury, 1975). Thus, there would appear to be close agreement between histological, histochemical and biochemical observations concerning myometrium; it is worthy of note that substrate specificity and inhibitor sensitivity of the histochemical preparations closely paralleled those observed biochemically.

Table 29. Distribution of BzAO, MAO B and MAO A in upper and lower regions of human oesophagus

Enzyme	Upper third	Lower third	Ratio L/U
BzAO	9.0	24.0	2.7
MAO B (D*Bz)	12.4	7.6	0.6
Deprenyl sensitivity (%)	58	24	0.4
MAO B (PEA)	3.2	3.8	1.2
MAO A (5-HT)	10.2	21.6	2.1

Except for deprenyl sensitivity (= inhibition of Bz oxidation), values express specific activity (nanomoles/mg protein/30 min), and are means of replicate determinations. See text for experimental and assay conditions.

Table 29 illustrates the marked differences between amine oxidase activities in two regions of the oesophagus. The ratio of activity in the lower third compared with the upper is somewhat higher for BzAO than MAO A, whilst that for MAO B (D*Bz) is quite distinct from either. The pattern of enzymic activity in the upper third is somewhat similar to that of myometrium in the late secretory phase, except that MAO A activity is higher in oesophagus. On the other hand, the pattern in

the lower third clearly resembles that of the rest of the adult digestive tract (cf. Tables 1-3, Lewinsohn et al, 1980 b), where regional differences undoubtedly reflect the histologically different mural structure of the digestive tube.

4.4.4

Literature review

Early histochemical work on amine oxidase activity in the female genital tract was reviewed by Southgate (1972). The effect of oral contraceptives on endometrial MAO activity was described by Grant and Pryse-Davies (1968). Variations in this activity and tissue with phases of the menstrual cycle were observed by Southgate et al (1968). High activity of human and rat endometrial MAO A provoked by progesterone is also reported by Mazumder et al (1980); for sundry related observations, see also Wolstenholme and Knight (1976). There have been a number of investigations on amine oxidase activity in another urogenital organ rich in smooth muscle (not studied by me), the vas deferens. The study of Jarrott and Iversen (1968) has already been mentioned briefly (p.127). The use of TA and 5-HT as substrates with subcellular preparations of rat vas deferens produced closely similar results. An appreciable proportion of the total MAO activity was recovered in the microsomal fraction; compared with liver, a significantly higher proportion of all measured enzyme activities was found in the microsomal fraction of the vas deferens. In agreement with previous studies, the authors found only negligible activity of MAO in the supernatant fraction; they concluded that MAO activity recovered in the microsomal fractions might arise as an artefact of mitochondrial disruption during tissue homogenization (cf. Lyles and Callingham, 1975). Alternatively, "microsomal" MAO might arise from enzyme activity present in endoplasmic reticulum membranes, representing the truly microsomal portion of total enzyme activity. In crude

homogenates of mouse and rat vas deferens studied by Jarrott (1971) with TA, 5-HT and Bz as substrates, the inhibitory effect of clorgyline suggested the presence of multiple forms of MAO. Differences in inhibitor sensitivity, substrate specificity and thermal inactivation of MAO in normal and denervated vas deferens were found, suggesting distinct properties of neuronal and extraneuronal MAO. Rat vas deferens was also studied by de Champlain et al (1969); with tryptamine as substrate, about 35% of MAO activity could be detected in the microsomal fraction, and about 15% in the supernatant. In vitro studies of guinea-pig vas deferens by Thoa et al (1969) showed an accumulation of ^{14}C-labelled 5-HT after incubation of the amine with tissue slices and subcellular fractions. Dial and Clarke (1977) used whole tissue homogenates of rat vas deferens, with kynuramine and tryptamine as substrates. Clorgyline and deprenyl inhibited kynuramine oxidation to a similar degree; in mixed substrate experiments, the effect of clorgyline was greater than that of deprenyl. From these results, the authors concluded that both MAO A and B were present in their preparations.

Wakade et al (1975) investigated the effect of castration on smooth muscle cells of internal sex organs in the rat. Histological examination of normal and castrate rats indicated that, along with a reduction in epithelial cells, smooth muscle cells of vas deferens, seminal vesicles and coagulating glands were markedly reduced in size; atrophy of internal sex organs following castration appeared to be the combined effect of reduction in size and number of smooth muscle cells. The authors suggest that any alteration in size of smooth muscle cells or loss of such cells of internal sex organs indirectly influences their sympathetic nerves in such a manner that NA concentrations, and thereby the density of innervation, are maintained at normal levels. Crude homogenates of rat vas deferens were studied by Dial

and Clarke (1978) with PEA as substrate. Clorgyline inhibition produced a biphasic curve, suggesting that in this preparation, PEA was oxidized by both MAO A and B (cf. Lewinsohn et al, 1980 a).

I do not know of any published studies on amine oxidase activity in myometrium or oesophagus.

For a review of work on catecholamines in bronchial smooth muscle, see Widdicombe (1975).

4.5

Amine oxidase activity in cultured cells

4.5.1

Introduction

These experiments constitute a further attempt at localization of BzAO activity. Subcellular fractionation of rat aorta cultured smooth muscle cells has already been described (p.76). In the experiments reported here, I compare cultured smooth muscle cells and fibroblasts obtained from the same rat aorta for activity of the three enzymes investigated. Cultured fibroblasts from human skin are also examined. The advantages and disadvantages of such experiments are obvious: on the one hand, assay of activity in the cell which I consider to be the site of BzAO activity; on the other, the artificial conditions of growth and environment of the cultured cell. The former clearly outweigh the latter, however, and the assay of crude homogenates as well as subcellular fractions yielded information of considerable interest.

4.5.2

Experimental

Human fibroblasts cultured from cells obtained from normal skin were the generous gift of Mr. G. Rein, of these laboratories. Rat aorta smooth muscle cells and fibroblasts, kindly donated by Mr. G. Barrett (Department of Anatomy and Embryology, University College, London), were obtained from 10-week old animals, kept on a standard diet. The cells were cultured in Standard Eagle medium, supplemented with amino acids, vitamins, fetal calf serum and sodium bicarbonate. Microscopic examination before harvesting showed no contamination of either cell line by the other. Cells were washed twice in phosphate-buffered saline and suspended in 0.1M potassium phosphate buffer,

pH 7.2, kept overnight at -20°, then thawed and refrozen three times. Homogenization of the semi-frozen suspension was by hand in a 1 ml ground-glass homogenizer, followed immediately by assay. Human fibroblasts, suspended in 0.1M sodium phosphate buffer, pH 7.2, were treated similarly. Since very little material was available in the latter case, 20 µg protein in 80 µl buffer was used for each tube. The protein content of the rat cell preparations was 0.28 and 0.78 mg/ml for aortic fibroblasts and smooth muscle, respectively; 100 µl of the homogenate was used for each tube. The rat aorta smooth muscle homogenate was the same material as that used in the cell fractionation experiments described on p.76. The final concentrations of deprenyl and phenelzine were 4×10^{-7} and 2×10^{-6}M, and those of Bz, PEA and 5-HT were 42, 150 and 371 µM, respectively. For all other procedures, see p. 55 ff.

4.5.3

Results and comments

In these experiments, illustrated in Table 30, activity of BzAO could be demonstrated only in rat aorta smooth muscle. Trace activity was present in human skin and rat aorta fibroblasts, whilst MAO B deamination of both Bz and PEA was only slightly higher. Comparison of BzAO activity in the cultured cells of rat aorta smooth muscle with that estimated in crude homogenate of rat aorta (Lewinsohn et al, 1978) shows the latter to be substantially higher (specific activity = 14.2). If the value for the crude homogenate, which was assayed at pH 7.2, is converted to the value corresponding to pH 9.0, which may be greater by a factor of at least 5 (Lewinsohn, unpublished data), the discrepancy between cultured cell and crude homogenate becomes even wider. One explanation for the difference in activity may be that the aortic smooth muscle cells were obtained from growing rats. Cells do not "mature" in culture; on the other hand, cells from older animals replicate less well than those from younger ones (G. Barrett, personal communication).

Taking into account that a large proportion of BzAO is soluble, another equally plausible explanation is that enzyme molecules present in intercellular spaces as well as in the cells of organized tissue will also be present in the crude homogenate and may contribute substantially to the enzymic activity measured by assay. Neither factor excludes the other; both (and many others) may contribute to the difference noted (for a review of the current state of knowledge on smooth muscle in cell and tissue culture, as well as further references, see Chamley-Campbell et al, 1979). MAO A, on the other

Table 30. Distribution of amine oxidases in cultured cells

Enzyme	Substrate	pH	Human skin fibroblast	Rat aorta fibroblast	Rat aorta smooth muscle
BzAO	Bz	7.2	0	ND	ND
BzAO	Bz	9.0	0.09 (*)	0.015	5.3
MAO B (D*Bz)	Bz	9.0	0.11	0.04	0
MAO B	PEA	7.2	0.14	ND	ND
MAO A	5-HT	7.2	0.68	33.1	111.4

Two separate experiments on different aliquots of the rat cell preparations gave similar results. Values express specific activities (nanomoles/mg protein/30 min), and are means of replicate determinations. For experimental and assay conditions, see text. (*) Phenelzine inhibition was 3%. ND = not done.

hand, is far less soluble than BzAO: a relatively small proportion of its total activity is found in the microsomal fraction and even less in the supernatant (see p. 77), so that it is likely that the activity in the cultured cell corresponds more closely to that in the organized tissue. Even so, the activity of MAO A in rat aorta fibroblasts is remarkably high, and strikingly so in the aortic smooth muscle cells. Comparison of this activity with that recorded in my cell fractionation

Table 31. MONOAMINE OXIDASES IN CULTURED CELLS

Species, tissue, cell	Enzyme demonstrated	Substrate *	Inhibitor	Reference	Note
Guinea-pig ventricle, fibroblast	MAO (form?) (also COMT)	TA	Tranylcypromine	Jacobowitz, 1972	
Rat glial cells	MAO A (B)	TA, 5-HT, DA, Trypt, PEA, Bz	Clorgyline, deprenyl	Murphy et al, 1976	(1)
Mouse neuroblastoma cells	MAO A (B)	TA, 5-HT, DA, Trypt, PEA, Bz	Clorgyline, deprenyl	Donnelly et al, 1976	(1)
Human skin fibroblasts	MAO A, B	5-HT, PEA	Clorgyline	Roth et al, 1976	(2)
Human skin fibroblasts	MAO A, B (also COMT)	5-HT, Bz, Trypt	Clorgyline, deprenyl	Groshong et al, 1977	(2)
Human skin fibroblasts	MAO A, B	Trypt	-	Edelstein et al, 1978	(3)
Rabbit aorta endothelial cells	MAO B (A)	PEA, TA, 5-HT, DA, NE	Clorgyline, harmaline	Roth & Venter, 1978	(4)

Note (1) MAO A activity strongly predominant, MAO B negligible.

(2) MAO A predominant.

(3) Authors state that MAO A activity was predominant, but Trypt was used as substrate, and no selective inhibitor was employed.

(4) MAO B strongly predominant.

(*) Substrates are only those employed for determination of MAO activity, not COMT.

experiments (on the same cultured cells; see p. 76) shows that the total activity of the mitochondrial supernatant and pellet was about one-third of that measured in the crude cell homogenate shown here. I do not know why so much activity was lost in the initial steps of cell fractionation, in which overall recovery of MAO A activity was poor, whereas results for BzAO were excellent.

Specific activities of BzAO and MAO B (D*Bz) were about the same in the human fibroblast preparation; PEA was oxidized at a slightly higher rate than D*Bz. Deamination of 5-HT, however, was five times that of PEA. These results are at variance with those of Groshong et al (1977), who show a ratio of 10:1 for uninhibited 5-HT to deprenyl-sensitive Bz oxidation, whilst specific activities of PEA and 5-HT oxidation are identical. However, their assay procedure differed from mine, precluding comparison of quantitative results. Table 31, therefore, shows the qualitative findings of these and other workers. An interesting observation by Donnelly et al (1976) is the almost total inhibition by 10^{-7}M clorgyline of Bz and PEA deamination in mouse neuroblastoma, suggesting that oxidation of these two substrates in the mouse tumour cells may be largely attributed to a clorgyline-sensitive MAO of the A type.

> In the purely biological ... aspects of science, we are, strictly speaking, outside the pale of logic, and there can be no question of certainty or proof. We cannot even reach probabilities expressible in mathematical terms; we can look only for psychological probability.
>
> Agnes Arber, The Mind and the Eye
> Cambridge University Press, 1953.

CHAPTER 5: DISCUSSION

The studies presented here shed some light on the distribution, localization and certain of the physicochemical properties of BzAO, and expand our knowledge of its activity in human pregnancy and development. More important than the answers they provide, however, is the new series of questions arising from them.

Before going on to discuss results, conclusions and further problems, I must clear up one essential point. My interpretation of results, past and present, is based on the working hypothesis that deprenyl-resistant Bz oxidation in human tissues and plasma is catalysed by the same enzyme. I further postulate, as part of the same working hypothesis, that the Bz-oxidizing activity in normal human plasma is similar to that in various disease states in which increased or reduced plasma levels have been observed, though demonstration of an abnormal isoenzyme in hepatic cirrhosis makes it likely that different isoenzymic patterns of plasma BzAO exist in other pathological conditions. I am, of course, aware that conclusive experimental evidence for all such assumptions must await further work, the most important being identification of the physiological substrate and function of BzAO. Nonetheless, I suggest that the information available to date from studies of kinetics, solubility, substrate and inhibitor specificity, as well as the close

agreement between histochemical and biochemical observations, lend a measure of support to these assumptions that warrants their use as a working hypothesis.

There can no longer be any doubt as to the widespread distribution of BzAO throughout the human organism (Table 32), nor about its localization in vascular tissue (*) and, in particular, vascular smooth muscle. Non-vascular tissue rich in smooth muscle also shows BzAO activity, though less than that found in vessels. The discrepancy is probably due, in large part, to the relative proportions of connective tissue elements (**) and smooth muscle cells in the various organs examined. Tissues such as Wharton's jelly and endometrium, which consist of connective tissue elements exclusively, showed very low BzAO activity, compatible with vascular fragments in the preparations. On the other hand, the major - and in many cases the only - cell type described in the walls of small and medium-calibre mammalian vessels, which make up almost half the cross-sectional area of the entire blood-vascular network in man (Ganong, 1975; Gregg, 1966) is the smooth muscle cell (Elias, 1961; Sandler and Bourne, 1963; Rhodin, 1967, 1968; Somlyo and Somlyo, 1968; French, 1970; Cliff, 1976). The amount of smooth muscle is inversely proportional to the calibre of the vessel (Elias, 1961). Needless to say, structure varies widely throughout the vascular tree; a universal feature, however, is the presence of one smooth muscle cell layer in the smallest arterioles and venules, which is in direct contact with endothelium on the inner (luminal) surface

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(*) "Vascular tissue" in the context of this thesis includes all vessels except those of the capillary and sinusoidal microcirculations; lymphatic vessels have not been examined.

(**) As used throughout this thesis, the term is defined as "connective tissue proper" (Bradbury, 1975), made up of intercellular matrix, collagen and elastic fibres, fibroblasts and other cellular elements in varying proportion, but few if any smooth muscle cells.

Table 32. BzAO ACTIVITY IN HUMAN PLASMA AND TISSUES

Age group	Plasma or serum	Vascular tissue (ɵ)	Other tissue	Reference
Fetus	?	++	+	(1)
Neonate (plasma = cord)	*	++/+++	+	(1)(5)
Children - normal	**			(2)
Children - diabetes	***			(2)
Adults - normal	*	++++	+/++	(1)(2)×
Adults - diabetes	**			(2)
Adults - cirrhosis	***/****			(3)(4)
Adults - pregnancy at term	**			(5)
Children - severe burn	Ø			(4)
Adults - severe burn	O/Ø			(4)
Adults - cancer	Ø			(4)
Amniotic fluid	**			(5)

ɵ = Vessels, or tissue rich in vessels or smooth muscle.

Plasma * = normal adult activity; ** = increased; *** = high; **** = very high; O = no detectable activity; Ø = very low.

Tissues + = very low; ++ = low; +++ = high; ++++ = very high.

References (1) Lewinsohn et al, 1980b

(2) Tryding et al, 1968 (x = serum only)

(3) McEwen and Castell, 1967

(4) Lewinsohn, 1977b

(5) This thesis.

and with the surrounding connective tissue on the outer (non-luminal) side (Elias, 1961). In general, the fine structure of vascular smooth muscle cells is similar to that of smooth muscle in other sites (Cliff, 1976). Inner muscle cells in cerebral vessels have no direct nerve supply, but are much more sensitive than outer cells to NA and other vasoconstrictor agents (Keatinge, 1978). The inner muscle, which is the only part of the artery wall that is sensitive enough to respond to common circulating concentrations of vasoactive substances, probably also contributes to the vessel's response to vasoconstrictor nerves, because its high sensitivity enables it to respond to small amounts of transmitter that diffuse to it from the innervated outer region. The major part of the vessel's response to substances derived from clotting blood is produced by the highly sensitive inner muscle (Keatinge, 1978).

Although, at present, all hypotheses regarding the function of BzAO in smooth muscle can only be speculative, evidence from clinical observations suggests that it is related to the secretory rather than the vasomotor activity of the cell. In view of the negligible plasma levels of BzAO, compared with high activity in the vessel wall, it is probable that degradation of the physiological substrate of BzAO does not occur in the circulating blood but at the cellular level within tissues. If BzAO were concerned with vasomotor action or changes in peripheral resistance, the large increase in plasma activity in cirrhosis or diabetes, or the virtual disappearance of the enzyme from the circulating blood of severely burnt patients, might be expected to produce some clinical signs or symptoms. This does not occur, however. In the case of severely burnt patients, the fall is relatively rapid; it is unlikely that in this short time, adjustment to new levels of homeostasis takes place (cf. Murphy et al, 1977). On the other hand, such patients may have very low peripheral activity for many months, without any evidence of circulatory disturbances.

As suggested in Section 4.2, variations of BzAO levels in the circulating blood seem to be related, at least in part, to phenomena of vascular permeability, to which both increased and decreased plasma activity may well be related. Vascular permeability, in its turn, is influenced by alterations in circulating substances such as catecholamines, sex steroids and vasoactive substances (Mellander and Johansson, 1968; Somlyo and Somlyo, 1970; Walter and Israel, 1974; Mier and Cotton, 1976; Cliff, 1976). Increased levels of plasma BzAO have been demonstrated in cirrhosis, diabetes and late pregnancy; vascular permeability, which is known to be altered in these conditions, added, perhaps, to direct interaction between the substances mentioned above and BzAO, may lead or contribute to the appearance of abnormal amounts of enzyme in the circulation. In cirrhosis, moreover, high circulating ammonia levels have been described; whether they contribute to, or are a consequence of, high plasma BzAO in this disease, is not known. A fall in activity has been shown in severely burnt patients, in patients with cancer and in the early puerperium, conditions which, in addition to altered vascular permeability, share another feature: in all of them, new vessels are formed, for purposes of repair (severe burns, post-partum) or nutrition of tumour tissue. As shown in my paper on the subject (Lewinsohn, 1977 b), only patients with solid tumours had reduced serum BzAO activity.

Other factors which may have a bearing on the appearance or fall of the plasma enzyme and which, to my knowledge, have never been investigated, are the effects of anoxia, hypoxia and acid-base balance on Bz deamination. The optimum pH for measuring plasma and tissue BzAO activity *in vitro* (see p. 88) provides no clue as to the situation *in vivo*; it is, however, likely that oxygen supply and acid-base balance affect enzymic patterns in health and disease both directly, by their effect on the oxidative reaction, and indirectly, by their influence on vascular permeability.

So far, of course, we have no indication whatsoever of a relationship between circulating BzAO and activity in tissues *in vivo*; thus it cannot be assumed that increase or decrease of the one is positively or negatively proportional to the other. Nevertheless, existing evidance points to such an association. "If the plasma enzymes are a product of the degradation or secretion of the cells of the connective tissue, one could understand that they are not released when the formation of new collagen and elastic fibres is at its maximum" (Blaschko, 1974). Except for "connective tissue", which I would replace by "smooth muscle", this statement neatly sums up my interpretation of the fall in plasma BzAO. In addition to increased or diminished release of enzyme into the circulation, there may be active uptake of molecules from the peripheral blood at sites of intense repair, in which the state of permeability of the vessel wall may also play a role. A third factor which may account for diminished peripheral levels in the conditions described above (as well as low values in fetal tissues) concerns the relative BzAO activities in fibroblasts and smooth muscle cells. It is not proposed to enter into the controversy regarding dedifferentiation of smooth muscle cells (Hoff, 1970; Gabbiani *et al*, 1972; Cliff, 1976; Jackson, 1977). The interrelationship between vascular smooth muscle and the connective tissue fibroblast was studied by Poole *et al* (1970), who described these two cells as modulations of a basic cell type; this view is shared by Cliff (1976). Whilst smooth muscle is the major or only cellular element in normal vessels, in injury repair or disease "the involvement of smooth muscle cells in fibroblastic activities in the walls of blood vessels has been suggested or inferred by numerous writers. ... In mature animals vascular smooth muscle cells largely lose their fibroblastic regions and their cytoplasm is occupied almost exclusively by myofibril tracts and associated mitochondria. *However, they can be stimulated during repair processes to revert to their earlier type of structure* (my italics). ...

Similar changes can be found in smooth muscle cells present in the arterial intima in spontaneous ... and experimental atherosclerosis ... There are other instances where smooth muscle cells are fibroblastic: in the hormonally-stimulated myometrium ... and in the distended ureter above an experimental obstruction" (see Cliff, 1976, for the above quotations and other references).

A large proportion of the literature on blood vessels is concerned with vascular injury and wound repair (Ross, 1968; Hoff, 1970; Poole et al, 1970, to name but a few); in general, there seems to be fair agreement among authors on the views quoted above. Changes such as those described are bound to have a profound effect both on the smooth muscle/fibroblast ratio and the activity of BzAO in any given tissue. It seems clear, moreover, that the character of the cell population in the vessel wall and in organs such as myometrium and ureter is subject to considerable physiological variation; consequent changes in enzymic activity may or may not be reflected in the amount of enzyme released into the circulation. Thus, myometrial and vascular repair related to the menstrual cycle is probably too transient and circumscribed to find expression in peripheral blood levels of BzAO, which may explain why I have found no changes from normal serum values in women studied during this cycle (Lewinsohn, unpublished data).

Precisely what part of the secretory function of smooth muscle BzAO is involved in, is another open question. Human arterial elastin is remarkably constant (Lansing, 1954; Cliff, 1976); the amount of collagen present within the walls of arteries, however, shows a general increase with advancing age (Cliff, 1976) as well as redistribution of collagen fibrils (Cliff, 1970) described in various laboratory animals as well as man. Apart from aging, pregnancy, disease and injury, some remodelling of vascular tissue must be going on constantly in the normal individual, and BzAO may well be a link in the chain of reactions. The importance

of smooth muscle in the pathogenesis of atherosclerosis is well documented (Lansing, 1954; Sandler and Bourne, 1963; Somlyo and Somlyo, 1968, 1970; Cliff, 1976). Gabella and Yamey (1977) showed an increase in collagen synthetized by smooth muscle in experimentally induced intestinal hypertrophy in guinea-pig. Large numbers of myofibroblasts located in close proximity to vessels have been described in hypertrophic scars in man (Baur et al, 1975). Several oxidative enzymes were demonstrated histochemically in the cytoplasm but not in nuclei or ground substance of cells in healing fractures (Balogh and Hajek, 1965). Their activity was high during the proliferative phases of both osteogenesis and chondrogenesis. This report is especially interesting for the demonstration of changing patterns of enzyme activity as cell maturation and healing proceeded (see also Ross, 1968).

As for solid tumours, I have assayed a few samples of neoplastic and normal adjacent tissue obtained at surgery and found the tumour tissue far more active against Bz than the normal (Lewinsohn, unpublished data). Such observations and reports, which could easily be multiplied, point to the manifold opportunities for research in a field that waits to be explored.

The foremost problem requiring elucidation is unquestionably the physiological substrate of BzAO (see p. 104). It is obvious that its solution will be the key to a new approach to amine oxidase investigation. It is a prerequisite in the search for an inhibitor truly selective for BzAO, and for the exploration in depth of relationships between circulating and tissue BzAO in health and disease, as well as the other problems set out in Table 33. Paramount among these, apart from the basic issues, are the clinical implications of this line of research. It seems to me that relatively little work is now required to produce conclusive evidence for the relationship of BzAO to smooth muscle. Once this is accomplished, the way seems clear for research

Table 33. F U R T H E R P R O B L E M S

1. Physiological substrate of BzAO in man.
2. Purification of BzAO (i) from plasma; (ii) from amniotic fluid; (iii) from a solid tissue, e.g. aorta or lung; characterization and comparison of enzyme from 3 different sources by isoelectric focusing and other methods, including immunological techniques.
3. Inhibitor highly selective for BzAO.
4. Functions of BzAO: similar in smooth muscle and fibroblast? in different smooth muscle cell populations? in health and disease?
5. Functions of BzAO in smooth muscle:

   (?) in peripheral resistance, vascular tone, vasomotor activity

   (?) in secretory activity:

   - collagen fibres) growth of vessels
   - elastic fibres) repair of vessels
   - mucopolysaccharides) wound healing
   - others) disease processes

   - collagenolysis: resorption, remodelling of vascular and/or connective tissue

6. Relationships (quantitative and qualitative):

   (a) between circulating and tissue BzAO

   (b) between tissue BzAO and MAO A, B; DAO, LO; other amine oxidase(s)(?)

   (c) between BzAO in smooth muscle and fibroblast

   (d) between BzAO (peripheral and in tissue) and circulating substances, e.g. vasoactive, steroid hormones, products of metabolism, others

   (e) between amniotic fluid BzAO and MAO B

   (f) between BzAO in amniotic fluid and in fetal tissues

   (g) effect of oxygen supply, acid-base balance, disease states on 6. (a)-(f)

   (h) between tissue or circulating BzAO, and endothelial enzymes.

into the effect, for example, of BzAO inhibition on the development of vessels in experimental animals, in health as well as in experimentally produced solid tumours. The ultimate aim, of course, is the use of such knowledge as we have acquired in the diagnosis, monitoring and, possibly, therapy of various human disease states. I have already outlined my ideas in respect of the assay of BzAO in severely burnt patients (Lewinsohn, 1977 b). In view of recent research, and the results presented here, it is conceivable that therapeutic lathyrism through inhibition of BzAO might also contribute to the prevention of hypertrophic scar formation. How far our present knowledge might be helpful in early diagnosis of cancer is a moot point, although in one case (Lewinsohn, 1977 b), after removal of a small cervical tumour in situ, serum BzAO rose to normal from a very low pre-operative level. The use of BzAO inhibitors to prevent new development of a vascular network to nourish primary or secondary tumours, suggested above, would be a totally new approach to cancer therapy; many prerequisites must, however, be fulfilled before its application in man can be envisaged. An interim goal, i.e., monitoring cancer patients on chemotherapy, could be attempted at an earlier stage, in view of the promising results described (Lewinsohn, 1977 b) and the non-invasive procedure involved in assaying peripheral BzAO activity.

Other human pathologies in which smooth muscle is implicated, such as broncho-pulmonary disease or intestinal fibrotic conditions, may become fruitful fields of research.

As for atherosclerosis, it is difficult to say where the study of BzAO (and other amine oxidases) may lead. Extrapolating from the work described here (and for once, perhaps, extrapolation may be permitted), it is highly probable that relationships will be found once we start looking for them. Further insight into the properties of BzAO is likely to help define its role and interaction with other enzyme systems (for

example, LO) in connective tissue as well as vessels at all stages of development, in health and disease. Thus it is bound to add to our basic knowledge, though its specific importance in the study of atherosclerosis is impossible to estimate at present.

A final word must be said about the importance of BzAO in the human body. Although all opinions must be tentative so long as its physiological substrate is unknown, published data (Lewinsohn et al, 1980 b) and Figure 12 (p. 117) suggest that total activity of the enzyme in man is considerable. Even the vascular fragments which "contaminate" liver homogenates produce a very substantial activity. The estimated value for the total mass of skeletal muscle is very high indeed. Thus I have devoted much time and effort to a search for morphometric data which might provide a clue to the actual mass of vessels present in tissues. As pointed out earlier (p.161), however, no such information exists even for small animals, let alone the human body. It has thus proved impossible to produce even a gross estimate as to the proportion of BzAO activity attributable to vascular elements in the total mass of organ or tissue. Since they are ubiquitous, however, it follows that an enzyme more active in blood vessels than in any other tissue is likely to perform an important function in the living organism. I suggest that the search for this function is only a beginning, the first strains of our theme, so to speak; and I hope that more researchers will join in the effort to develop the theme to its fullest, to bring out the best of the music, which is yet to come.

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A P P E N D I X

TABLE A.1

METHODS FOR THE STUDY OF THE AMINE OXIDASES

(*) Paper contains detailed description of method.

A.1 (a)

Oxygen uptake, microdiffusion, coupled peroxidase reaction

Manometry

All early workers (see reviews)
Creasey (1956)
Buffoni & Blaschko (1964)
McEwen et al (1966)
Tipton (1968b)
Tipton & Dawson (1968)
Tipton (1969a)
Williams (1974, 1977)

Oxygen polarography

Symes et al (1969)
Wiberg & Oreland (1976)

Microdiffusion and acid absorption

Cotzias & Dole (1951) *
Bragança et al (1954)
Cotzias & Greenough (1958)
McEwen (1965 a,b)
McEwen et al (1966)
Nagatsu & Yagi (1966) *

Coupled peroxidase reaction

McEwen (1965a)
Snyder & Hendley (1968)
Tipton (1969 a)

A.1 (b)

Spectrophotometry, colorimetry

Tabor et al (1954) *
Weissbach et al (1957, 1960) *
Green & Haughton (1961)
Yamada & Yasunobu (1962 a,b,c)
Brzezinski et al (1962)
McEwen and Cohen (1963) *
Gorkin et al (1964)
Zeller et al (1965)
McEwen (1965 a,b)*
McEwen et al (1966)
Bird et al (1966)
Buffoni (1966)
Huszti & Borsy (1966)
Huszti et al (1969)
Tryding et al (1969)
Ito et al (1971)
Christ et al (1973) *
Houslay & Tipton (1974)
Mantle et al (1975 a,b)

A.1 (c)

Fluorimetry

Lovenberg et al (1962 *, 1968)
Kraml (1965)
McCaman et al (1965)*
Tipton (1968 b,c)
Squires (1968)
Takahashi & Takahara (1968) *
Snyder & Hendley (1968)*
Tipton (1969 a)
Guibault et al (1969) *
Tufvesson (1969, 1970 a,b)
Suzuki et al (1976; 1978 a,b)
Zaitsu et al (1977)

A.1 (d)

Radiochemical microassay

Wurtman & Axelrod (1963) *
Otsuka & Kobayashi (1964) *
McCaman et al (1965) *
Southren et al (1966 a,b)
Southgate et al (1968)
Tryding & Willert (1968)
Tufvesson & Tryding (1969)
Tufvesson (1969, 1970 a)
Southgate & Collins (1969) *
Iverson & Jarrott (1970)
Sierens & D'Iorio (1970)
Yang et al (1972)
Yang & Neff (1973, 1974)
Youdim et al (1974)
Houslay & Tipton (1974, 1975 a,b)
Lyles & Callingham (1974, 1975, 1979)
Mantle et al (1975 a,b)
White & Wu (1975)
Wise (1976) *
Buffoni et al (1976)
Arem & Misiorowski (1976)
Murphy et al (1976, 1977)
Marshall & Campbell (1977)
Sullivan et al (1977)
White & Glassman (1977)
Murray et al (1977 a,b)
Nakano et al (1978) *
Prozialeck & Vogel (1978)
Waldmeier & Felner (1978)
Sawyer & Greenawalt (1979)
Illei & Morgan (1979)

A.1 (e)

Cell fractionation, solubilization, purification, crystallization

Hogeboom et al (1948) *
Cotzias & Dole (1951)
Hawkins (1952)
Tabor et al (1954) *
Yamada & Yasunobu (1962 a,b,c) *
Flodin & Killander (1962)
Buffoni & Blaschko (1964) *
McEwen (1965 a,b)*
McEwen et al (1966)
Nara et al (1966)
Youdim & Sourkes (1966)
Erwin & Simon (1968)
Jarrott & Iversen (1968)
Laduron & Belpaire (1968)
Tipton (1968 a,b)
De Champlain et al (1969)
Rucker et al (1969)
Tipton & Dawson (1968)
Gomes et al (1969)
Oreland (1971)
Gillis (1971)
Nagatsu et al (1972)
Shih & Eiduson (1973)
Houslay & Tipton (1973a)
Lin & Castell (1974, 1975)
Lyles & Callingham (1974, 1975, 1979)
Shieh et al (1975)
Buffoni et al (1976)
Dennick & Mayer (1977)
Gallagher (1977)
Student & Edwards (1977)
White & Glassman (1977)
Fowler et al (1980 a,b) *

A.1 (f)

Electrophoresis, chromatography, mass spectrometry

Arvidsson et al (1956) *
Uspenskaja & Goryachenkova (1958) *
Yamada & Yasunobu (1962 a,b)
Youdim & Sandler (1967)
Aures et al (1968) *
Kim & D'Iorio (1968)
Teuchy & Van Sumere (1969) *
Shih & Eiduson (1969)
Youdim et al (1969, 1970)
Murali & Radhakrishnan (1970)
Nagatsu et al (1972)
Collins et al (1972)
Tipton et al (1973)
Eldjarn et al (1974)
Shah & Shah (1976)
Lin et al (1976)
Buess et al (1977)

A.1 (g)

Histochemistry, cytochemistry, electron microscopy, immunological techniques, radioautography, electron spin resonance

Hogeboom et al (1948)
Robinson & Gershon (1971)
Shannon et al (1974)
Lowe et al (1975)
Vidal et al (1975)
Yoo & Oreland (1976)
Buffoni et al (1977)
Müller & da Lage (1977)
Dennick & Mayer (1977)
Lin et al (1978)
Shannon (1978)

Snipes et al (1968)
Taxi & Droz (1969)
Strum & Junod (1972)
Partanen et al (1976)
Huan & Eiduson (1977)*

A.1 (h)

Thermal stability

Oswald & Strittmatter (1963) - rat liver mitochondria
Kapeller-Adler & MacFarlane (1963) - hog-kidney histaminase
Blaschko & Buffoni (1965) - pig plasma histaminase
Nara et al (1966) - beef liver mitochondria
Tipton & Spires (1968) - pig brain mitochondria
Squires (1968, 1972) - mitochondrial MAO, 8 species, various tissues
Youdim et al (1970) - isoenzymes of human and rat liver mitochondria
Yang et al (1972) - rat pineal gland and superior cervical ganglion
Oreland & Ekstedt (1972) - pig liver, membrane-bound mitochondrial MAO
Sandler & Youdim (1972, 1974) - REVIEWS
Yang & Neff (1973) - rat brain
Lyles & Callingham (1974) - rat heart, control and hyperthyroid
Lin & Castell (1975) - isoenzymes from normal human plasma
Egashira et al (1976) - rat liver mitochondria
Egashira (1976) - human placenta

TABLE A.2

DESCRIPTION OF SOME HOMOGENIZING PROCEDURES EMPLOYED IN PREPARING SOLID TISSUES FOR AMINE OXIDASE ASSAY

Philpot (1937) alternately froze and thawed rabbit liver, ground it in ice-cold Ringer's solution and strained resulting mash through muslin.

Richter (1937) ground liver with sand and phosphate buffer pH 7.2, centrifuged preparation and used supernatant as enzyme source.

Blaschko et al (1937) used tissue slices or extracts from isolated intestine and liver of rabbit; also liver extract purified by dialysis and freed from glycogen by digesting with takadiastase.

Epps (1945) used dog liver ground in mortar, suspended in M/15-phosphate buffer pH 7.5; part of mash was centrifuged and sediment suspended in original volume of buffer. Activity in supernatant after centrifugation, resuspended sediment and whole mash, respectively, gave uptake of 13, 11 and 33 µl O_2/10 min. In later experiments, whole mash was rendered homogeneous by straining through muslin. Dog kidney cortex and medulla were treated similarly. The mucous membrane of dog gut was scraped off and suspended in phosphate buffer; the muscular layer was also assayed (not clear how muscle was treated). Uptake of whole gut, mucosa and muscle layer, respectively, was 12, 19 and 8 µl O_2/10 min.

Thompson & Tickner (1951) minced blood vessels with fine scissors, after removal of adherent connective tissue and squeezing out of vessels while in saline, to remove contained blood.

Blaschko & Hawkins (1950) Rabbit liver, pig kidney. Acetone-dried powder was washed repeatedly with 0.067 M sodium-phosphate buffer, then centrifuged and supernatant discarded. This treatment considerably reduced oxygen consumption of enzyme blank.

Blaschko (1952) freeze-dried guinea-pig liver, and found activity in insoluble material; washing increased enzymic activity.

Werle & Roewer (1952) minced, then ground, animal and vegetable tissues with sea-sand; extraction was with water (1:2), 40 min; extract was centrifuged, and supernatant used as enzyme source.

Weissbach et al (1957) used guinea-pig, rat and rabbit liver and kidney, also rabbit lung and brain. Tissues were homogenized in water (1:5) in ground-glass homogenizer, or Waring blender. Tissue homogenate spun at 8000*g* x 20 min in refrigerated International angle-head centrifuge, residue was discarded. Supernatant spun in Spinco preparative ultracentrifuge, 10^5 *g* x 30 min. Supernatant (high-speed fluid) used for further purification of enzyme.

Wollenberger et al (1960) designed pair of tongs for quick-freezing large tissue pieces and whole organs of animals, which can be compressed *in situ* to a thin layer, and frozen in fraction of second.

Williamson et al (1967) removed rat liver within 10 sec of killing animal; pressed between metal clamps (see preceding reference); pulverized in a mortar to fine powder, with frequent additions of liquid N_2. Powder was transferred to weighed plastic centrifuge tube containing 2ml of frozen 30% (w/v) $HClO_4$. After rapid reweighing, tissue (1-2g) was mixed with the $HClO_4$, care being taken that no thawing occurred. Ice-cold distilled water (5 ml) was added and mixture immediately homogenized in the centrifuge tube with glass pestle, driven by low-speed motor; this was continued for ca. 2 min, until thawing was complete.

Sassoon et al (1967) Hug, human and rat aorta. Immediately after dissection, aorta was placed in ice-cold EDTA-saline (9g NaCl + 0.25 g EDTA sodium salt/litre H_2O), taken to cold temperature laboratory (4^o). Tissues were cleaned, examined for gross lesions, blotted, weighed and diced with scissors. Human or hog aortic samples were homogenized for 60 sec

in Omnimixer at approx. 16,000 rpm with chamber resting in crushed ice; blades were cleared with a rubber "policeman" type 14-110 (Fisher Scientific Co., Inc., Houston, Texas, U.S.A.). Repeated twice. Sample of resultant homogenate was quickly placed in cellulose nitrate Spinco tube, using $3\frac{1}{2}$" 13 gauge hypodermic needle, centrifuged at 105 000 g x 60 min. Fat-free supernatant was carefully withdrawn by syringe ($2\frac{1}{2}$" 20 gauge needle); yield was approx. 0.9 ml supernatant per ml of homogenate. (Alternative methods are described by authors.) LIQUID NITROGEN METHOD: after weighing, rat aorta dipped for 5 sec in liquid N_2 and quickly pulverized in a Plattner's steel mortar (Fisher Scientific Co., see above) with 3 taps of hammer. Powder transferred to ground-glass Potter-Elvehjem tube and homogenized in 3 ml EDTA-saline, with 4 cycles at 175 rpm. 105 000 g supernatant obtained as above.

Lovenberg et al (1968) minced human and rat skin (no details given). Whole homogenates of rat liver were used (no details given).

McEwen et al (1968) Human liver. Initial centrifugation 10000 g (time?); supernatant solution filtered through cheese cloth to remove superficial fatty gels and prevent contamination with loosely-packed "nuclear" pellet.

Tipton (1968a) Pig brain, kept in ice until dissection; then defatted and suspended in 9 vol. (w/v) 0.25M sucrose adjusted to pH 7.6 with 1.0M K_2HPO_4. Blended in Waring blender at medium speed x 1 min; pH readjusted to 7.6 with same buffer. This preparation was used for purification.

Tipton & Dawson (1968) Within 15 min of killing, pig brains were placed in ice, kept until dissection (approx. 1 h). Brains dissected at 5°, portions weighed and homogenized by hand in 9 vol. (w/v) of 0.25M sucrose adjusted to pH 7.6 with 1.0M K_2HPO_4 buffer. Brain regions homogenized in Potter-Elvehjem homogenizer with all-nylon pestle. Samples of homogenates removed and stored at -10° until assayed (mitochondrial preparation).

De Chamolain et al (1969) chilled rat liver, heart, salivary gland, vas deferens, on cracked ice, homogenized within minutes in 3-5 vol. ice-cold isotonic sucrose pH 7.4 containing $MgCl_2$ (0.001M) and sodium phosphate buffer pH 7.4 (0.005M). All-glass Duall homogenizer at 0^{o} for 1 min, with 10-12 pestle strokes. Unbroken cells, nuclei and cellular debris removed by centrifuging homogenate(600 g x 10 min) in refrigerated RC-2 Sorvall centrifuge. This preparation was then used for cell fractionation.

Coquil et al (1973) freed rat arteries from adhering connective or fat tissue, rinsed free of blood. Arteries were pooled and homogenized in 2ml glass homogenizer with Teflon pestle, in 50mM sodium-phosphate buffer pH 7.2 (150 µl/artery). Heart, brain or salivary gland: similar procedure, 1:20 (w/v) homogenate. Crude homogenate centrifuged at 750 g x 10 min; supernatant used as enzyme source, and for cell fractionation.

Callingham & Laverty (1973) Rat heart washed in 0.145M potassium-phosphate solution pH 7.8, homogenized in conical glass hand-homogenizer in 0.001M potassium-phosphate solution pH 7.8. Homogenates diluted to a 1:10 or 1:20 suspension of tissue, centrifuged at 2000g x 10 min to remove large debris. Supernatants stored for short periods at 4^{o} or deep-frozen for more prolonged storage.

Bashey & Mori (1973) Rat skin: epidermis, fat and muscle separated from skin by scraping and excision. Skin further cleaned, minced into small segments, weighed and used in enzyme assay.

Lyles & Callingham (1975) Rats killed by cervical dislocation, hearts removed, washed quickly in saline, blotted; larger blood vessels dissected away. After weighing, each heart homogenized in 0.001M potassium phosphate buffer, pH 7.4 as a 1:10 (w/v) suspension of tissue in buffer; centrifuged at 600 g x 10 min; supernatant stored on ice for immediate MAO assay or deep-frozen for longer storage. Subcellular fractionation

(method: Zak et al, 1970): minced heart tissue stirred at 0-4° during several changes of a "relaxing' buffer containing KCl 0.1M, $MgCl_2$ 5, EGTA 5, $Na_4P_2O_7$ 5mM, pH 6.8. The relaxing buffer was then decanted and the muscle washed in buffer containing sucrose 0.25M, KCL 0.05M, EGTA 5, $Na_4P_2O_7$ 1, MgCl 5mM, pH 6.8. Tissue then homogenized in this buffer at v/w ratio 10:1, using conical glass homogenizer. This homogenate centrifuged at 800 g x 10 min. Supernatant saved as "low-speed supernatant". Bulk of supernatant centrifuged at 9000 g x 10 min to sediment mitochondria. Mitochondrial pellet washed and resuspended in buffer, recentrifuged at 800 g x 10 min to remove contaminating debris, then at 9000 g x 10 min to resediment mitochondria. Suspension made by resuspending this pellet, in buffer, was used as mitochondrial fraction. Supernatant resulting from first sedimentation of mitochondria subjected to further spin at 9000 g x 10 min; supernatant decanted and centrifuged at 105 000 g x 60 min to produce microsomal pellet, which was resuspended in buffer and used as the microsomal fraction. Supernatant resulting from microsomal sedimentation was used as "high-speed supernatant".

Egashira (1976) Human placenta: initial steps not described. Partial purification of mitochondria: differential centrifugation in 0.1M phosphate buffer, pH 7.5, followed by sonication at 20kHz x 30 min. Triton added at final concentration of 1% (v/v). Preparation allowed to stand 60 min, then centrifuged at 10^5 g x 60 min.

Buffoni et al (1976) Excess tissue removed from pig aorta; rinsed with cold distilled water to remove blood; chopped in ice, using either quartz sand or stainless steel cutter; extracted with water in cold room for 12h (4°). Crude homogenates obtained by centrifugation at 1500 rpm x 30 min at 0-4°. Sonication with Blackstone apparatus.

Browne et al (1978) Procedure similar to that of Callingham & Laverty (1973).

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HUMAN SERUM AMINE OXIDASE. ENZYME ACTIVITY IN SEVERELY BURNT PATIENTS AND IN PATIENTS WITH CANCER

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Summary

During a study of serum amine oxidase (SAO) levels in patients with fibrotic disease processes, 127 patients (100 adults and 27 children) with moderate to severe burns were investigated. Of these, 55 adults and 20 children, classified as severe, had enzyme levels of 2.7 ± 0.3 and 4.6 ± 0.9 McEwen Units (McE.U) (mean ± S.E.) respectively, which, compared with those for normal healthy adults (18.3 ± 0.6 McE.U), gave a difference of striking significance (t = 21.05 for 130 d.f., and t = 10.33 for 95 d.f., respectively).

Subsequently, 125 patients with proven cancer were studied. 52% of the untreated and 41% of the treated showed remarkably low enzyme activity. The mean of the group of untreated patients was 9.6 ± 1.1 McE.U, that of the treated 11.6 ± 1.0 McE.U, and that of the entire group 10.7 ± 0.7 McE.U. The difference between these values and normal activity is highly significant ($p < 10^{-6}$).

Sequential SAO estimations on severely burnt patients and those with cancer established the time course of enzyme activity in these patients.

Our observations suggest that estimation of SAO activity may be useful not only for the classification, monitoring and prognosis of severe burns, but also in the diagnosis of cancer and the evaluation of its treatment.

Introduction

Human serum oxidase (SAO), a copper-containing amine oxidase [1] distinct from the flavine-dependent intracellular monoamine oxidases found in tissues, was first described in 1963 by McEwen and Cohen [2]. The demonstration of elevated levels of the serum or plasma amine oxidase in fibrotic liver disease [3] led us to investigate enzyme levels in patients with *Schistosoma mansoni*

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infection, a disease endemic in large parts of Brazil [4], which produces severe liver fibrosis in a high proportion of cases [4,5]. Groups studied included normal controls, patients with hepatic cirrhosis, and disorders not involving liver disease (Lewinsohn, R., unpublished data).

In the course of our investigation we decided to measure SAO levels in severely burnt patients (SBP), whose injuries invariably lead to the development of large areas of permanent fibrosis. To our surprise, we found activity to be strikingly decreased in all patients who had sustained severe burns, i.e., those who had lost 18% or more of body surface within a period of six months before assay. We therefore decided to investigate the problem in greater detail.

One of the control groups used in the study on SBP included a number of patients with cancer, whose pre- and post-operative SAO levels showed highly significant alterations in 50% of the cases. An investigation was therefore undertaken to determine SAO levels in the sera of patients with proven cancer. Sequential assays were also carried out, to establish the time course of SAO in these patients.

Patients and methods

Controls

(a) Normal, healthy individuals ("negative" controls) were picked at random among university, laboratory and hospital personnel, as well as a wide range of volunteers who offered their collaboration.

(b) Patients ("positive" controls) with proven schistosomiasis (all stages) [6], hepatic cirrhosis, fibrotic lung disease, chronic congestive heart failure, and various other disorders liable to result in marked fibrosis.

(c) The first cancer patients belonged to a further "positive" control group of patients waiting to undergo major surgery, on whom pre- and post-operative SAO estimation was performed.

Patients

(a) Severely burnt patients (SBP). All subjects investigated, hospitalized or ambulant, were residents of the State of Rio de Janeiro, Brazil.

Samples were taken from patients who, when first investigated, had suffered severe burns one day to weeks, months or years previously. Wherever possible, serial assays were performed. For the purpose of inclusion in this study, subjects were classified as SBP on the basis of: (i) extent of injury (estimated by the admitting clinician) according to the following criteria: deep second-degree or third-degree burns of 18% or more of body surface; (ii) time elapsed since injury: up to six months.

(b) Cancer patients. All cancer patients included in this study, hospitalized or ambulant, were registered at the Department of Clinical Oncology of the National Cancer Institute, Rio de Janeiro. Cancer had been diagnosed in these patients by one or more of the following methods: (i) Histopathology of biopsy specimen or resected tumour tissue; (ii) Cytology of cervical smears, bronchial washings, etc. (iii) Examination of blood and/or bone marrow smears; (iv) Radiology and/or scintiscanning.

The records of the National Cancer Institute were used for the clinical

history, physical examination and laboratory investigations. All patients were seen by the author, who collected the blood samples and carried out the estimations of SAO activity, total plasma proteins and SGOT. 200 assays were performed as a preliminary investigation on 125 patients, of whom 54 had had no treatment of any kind. 50 patients had two or more estimations in the course of 4 months.

No selection was attempted as to site or type of tumour, or treatment before or during the time of the study. Information as to staging was available in a small number of cases only, and was therefore not included in this study.

The majority of patients were ambulant and in good or fair physical condition. Those in whom no malignancy was found were excluded from the investigation. Data on patients who were on a therapeutic regime including a drug known to inhibit amine oxidase activity, were excluded from the calculations of means and from the tables and graphs of the present study.

Methods

Serum amine oxidase activity was determined by the spectrophotometric assay of McEwen and Cohen [2]. Sera were assayed on the day of collection or stored at −20°C for use within a few days. Results are expressed in McEwen Units (McE.U).

The benzylamine used as substrate was kindly supplied by Carlo Erba do Brasil S.A., São Paulo, and redistilled in our laboratory as required.

Certain serum samples were dialyzed overnight at 4°C in cellophane bags against physiological saline buffered with potassium phosphate buffer in a final concentration of 2 mM.

To detect the possible presence of inhibitors, mixed assays were performed. In a total volume of 3.2 ml, the assay tube contained 1.2 ml of active (normal or high-activity) serum, 1.2 ml of serum from a SBP or cancer patient (no activity detected), 0.6 ml of 0.2 M potassium phosphate buffer (pH 7.2) and 0.2 ml of 0.012 M (2.4 μmol) benzylamine in the same buffer. The sera used in mixed assays were assayed singly on the same day in a total volume of 3.0 ml, made up as follows: serum 1.2 ml, 0.2 M phosphate buffer (pH 7.2) 1.6 ml, and 0.012 M benzylamine in the same buffer, 0.2 ml (2.4 μmol). Control tubes were identical except for the benzylamine, which was added at the end of the 3-h incubation time. The rest of the procedure was as described by McEwen and Cohen [2], except that the volumes of 60% perchloric acid and cyclohexane were 0.3 ml and 3.0 ml, respectively.

Total plasma proteins were estimated by the biuret method [7]. Serum aspartate aminotransferase (SGOT) determination [8] was carried out with the Merckotest kit, kindly donated by Merck S.A. Industrias Quimicas, Rio de Janeiro.

Results

Mean SAO levels in normal individuals, SBP and cancer patients are shown in Table I.

While the ratio between the highest and lowest activity in normal subjects (about 4 : 1) is similar to that found by McEwen and Cohen [2], the mean and range in our series are lower.

TABLE I

SERUM AMINE OXIDASE (SAO) ACTIVITY IN NORMAL SUBJECTS, SEVERE BURNS (SBP), AND CANCER

	Normal	SBP	Cancer		
			Untreated	Treated	Total
Number of subjects	77	55	54	71	125
Age range (years)	19—63	14—75	18—85	10—82	10—85
SAO (McE.U)					
Mean	18.3	2.7	9.6	11.6	10.7
S.E. of mean	0.6	0.3	1.1	1.0	0.7
Observed range	8.6—32.3	0.0—8.2	0.0—31.0	0.0—30.2	0.0—31.0

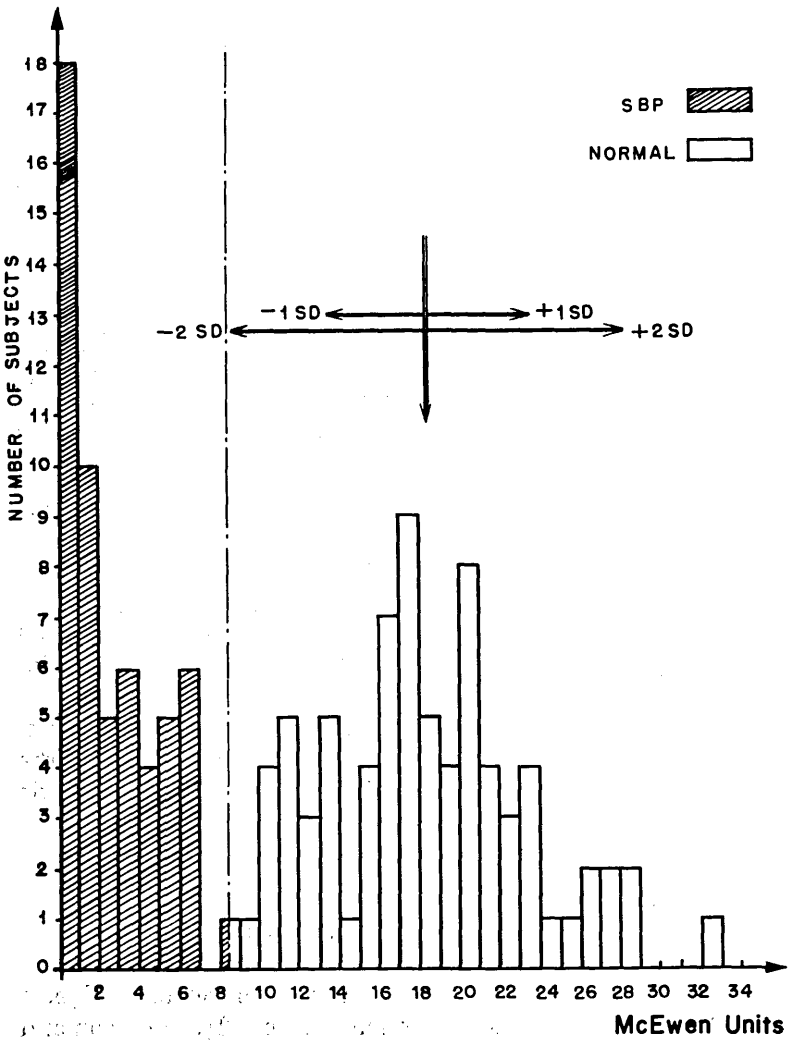

Fig. 1. Serum amine oxidase activity in normal subjects and severely burnt patients (SBP).

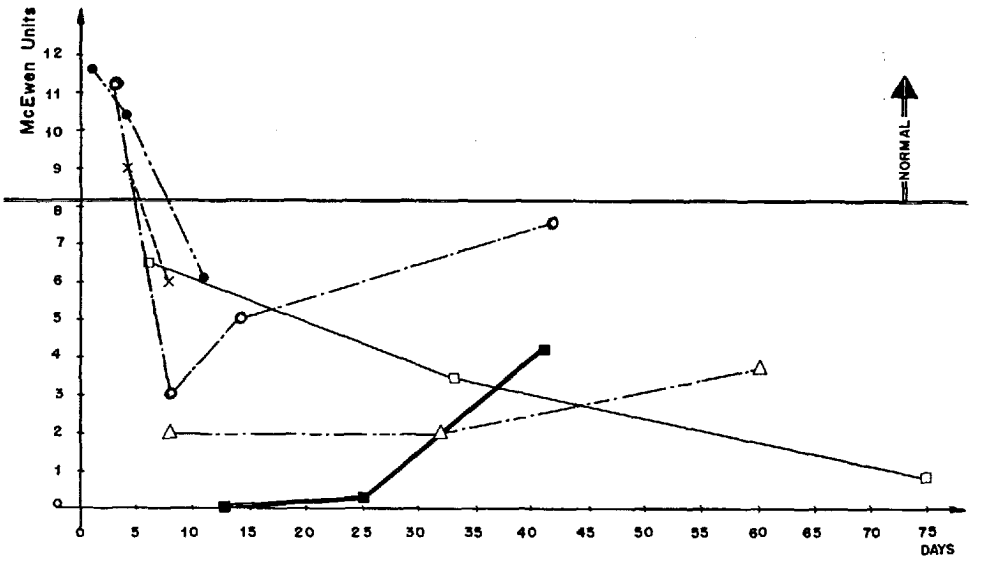

Fig. 2. SAO activity in 6 SBP, plotted against time elapsed since injury. Each point represents one determination. ●—. . —●, female, 43 years, skin loss greater than 30%; ○—. — . —○, male, 23 years, skin loss 20%; X— — — —X, female, 17 years, skin loss 20%; □————□, male, 39 years, skin loss 20%; △—. —. —△, male, 14 years, skin loss 20%; ■————■, male, 21 years, skin loss 54%.

Fig. 1 shows the distribution of enzyme activity in normal adult subjects, compared with adult SBP. There was no overlap between the two groups. 39 out of 55 patients (71%) showed activity less than 4 McE.U, and 28 (51%) less than 2 McE.U, whilst 11 out of 55 (20%) had no activity whatsoever by the method employed.

Values tended to be higher in burnt children compared with adults. Sex did not influence the results (Table II).

Serum samples were not available in any case prior to injury.

Serial determinations showed that enzyme activity starts to drop at the time of or immediately after injury, and continues to fall until about one week to ten days after injury (Fig. 2). Another feature observed in SBP is the persistence of low values or complete absence of SAO activity for several months after injury (Table III; Fig. 2).

TABLE II

SAO ACTIVITY IN SEVERELY BURNT ADULTS AND CHILDREN

	Number of patients	Mean (McE.U)	S.E. of mean	t for 73 d.f.
Adults				
Male	20	2.8	0.6	
Female	35	2.7	0.4	
Total	55	2.7	0.3	
Children				
Total	20	4.6	0.9	2.34 $0.01 < P < 0.0125$

TABLE III

TIME COURSE OF SAO ACTIVITY IN SBP (30–180 DAYS)

Serum No.	Days from injury	SAO (McE.U)
213	90	2.5
288	120	1.9
333	58	7.0
341	60	1.0
390	60	2.3
431	30	0.0
454	35	1.0
467	60	3.7
468	41	4.2
475	42	7.6
525	30	0.0
526	60	4.1
534	36	0.0
535	60	7.0
536	150	3.1
538	175	5.2
539	75	0.8
555	135	0.3
Mean	73.2	2.9
S.E. of mean	10.3	0.6
t (for 93 d.f.)		12.39

Fig. 3 shows the distribution of SAO activity in 54 untreated and 71 treated cancer patients. Analysis of the difference between the means of untreated (9.6 ± 1.1 McE.U) and treated patients in this series (11.6 ± 1.0 McE.U) gave a significant difference ($P < 0.015$).

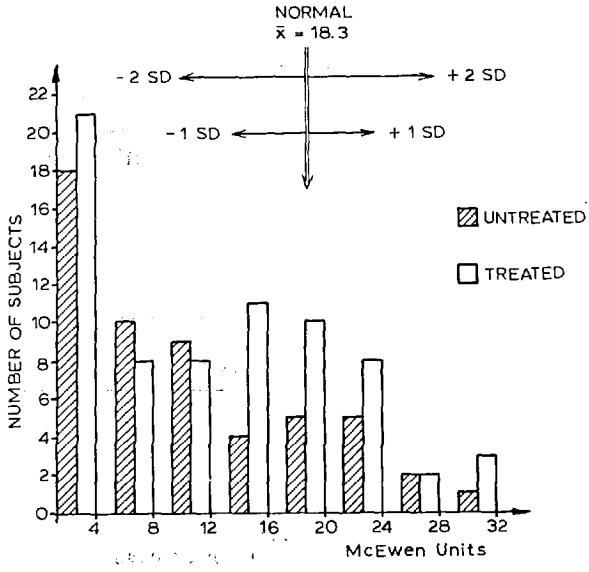

Fig. 3. SAO activity in 71 treated and 54 untreated patients with proven cancer.

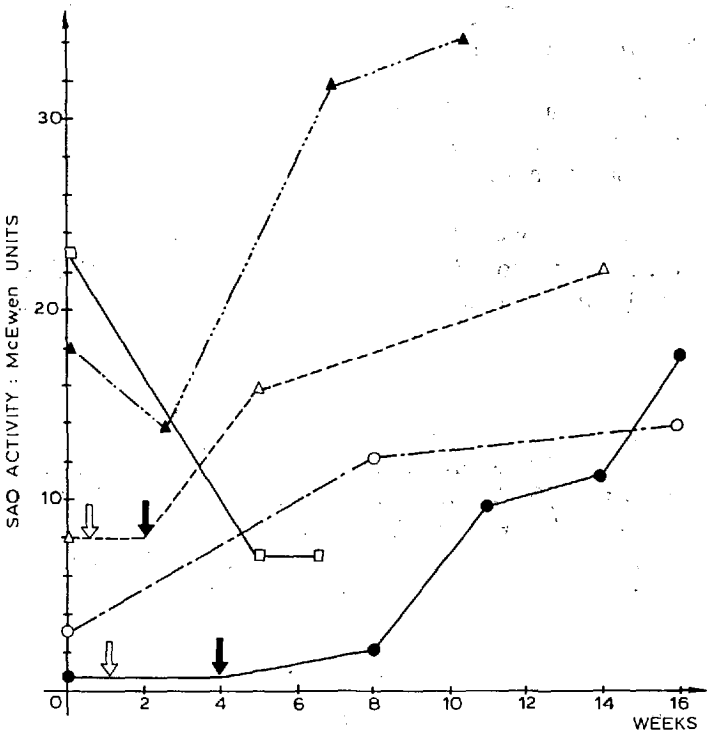

Fig. 4. Time course of SAO activity in 5 cancer patients on chemotherapy. All patients in good clinical condition. ○—·—·—○, carcinoma of oropharynx in 63-year-old male patient given 6600 rad 6 months before first assay. On CMB. △- - - - -△, 52-year-old male patient with adenocarcinoma of oesophagus, inoperable. Surgery: cholecystectomy, gastrostomy. On MeCCNU + 5FU. ●———●, 65-year-old male patient, with inoperable adenocarcinoma of stomach, secondaries to liver, pancreas, transverse mesocolon, duodenum; pre-operative ascites. On 5 FU. □———□, Oat-cell carcinoma of lung in 58-year-old male patient given 10 000 rad one month before first assay. On MeCCNU + 5FU + MTX. ▲— · · —▲, 47-year-old female patient with breast cancer. Oophorectomy. On CMF. At week 3 chemotherapy was suspended for short course of antibiotics, to be resumed immediately. By week 10 the patient had completed 3 courses of chemotherapy, the tumour and shrunk considerably and her physical condition was excellent. 5FU, 5-fluorouracil; MeCCNU, methylnitrosourea; MTX, methotrexate; CMB, cycliphosphamide + MTX + Bleomycin; CMF, cyclophosphamide + MTX + 5FU. Open arrows, surgery; black arrows, start of chemotherapy.

Radiotherapy produces no apparent alterations in enzyme activity. Some chemotherapeutic regimes, on the other hand, seem to have a profound effect on SAO values (Fig. 4).

The effect of chemotherapy on SAO activity complicates the interpretation of data on leukaemia and the lymphomas. Several of the schemes for these conditions include substances known to inhibit amine oxidase activity (e.g. procarbazine). It is not surprising, therefore, that SAO drops as soon as treatment is started; once the inhibiting drug is withdrawn, activity returns to pre-treatment levels [9,10].

No correlation could be established between SGOT and total plasma protein values and SAO levels.

SAO activity at any level was not affected by overnight dialysis against buffered normal saline (6 experiments with sera from SBP, 6 with sera from

cancer patients). Incubation of 8 SBP sera or those from ten cancer patients together with samples showing normal or high activity produced no change in the total from the expected value.

Activity in hepatic cirrhosis was significantly raised at 26.8 ± 3.1 McE.U ($P < 0.001$). On the other hand, SAO activity in 23 patients with proven schistomiasis (all stages) was somewhat lower than normal but not significantly so (16.4 ± 1.4 McE.U).

We found high values in several patients with fibrotic lung disease from a variety of causes. However, activity proved to be normal (19.3 ± 1.5 McE.U) in a group of 39 patients with chronic obstructive lung disease.

Discussion

To date, there have been few clinical studies on SAO.

In the normal individual, SAO levels are stable over long periods of time (Lewinsohn, R., unpublished data) [9,17]. Low values such as those in SBP and cancer are extremely rare (Lewinsohn, R., unpublished data).

Our findings in patients with hepatic cirrhosis parallel those of McEwen and Castell [3]. Levels in schistomiasis were not significantly different from normal, however.

A small number of patients with congestive heart failure gave readings similar to those shown by McEwen and Harrison [11]; activity in a few patients with scleroderma, systemic lupus erythematosus and keloid formation was within or slightly above normal levels (Lewinsohn, R., unpublished data).

No other group gave data as striking as those in SBP. Extremely low values were observed, a change perhaps affecting all three isoenzymes of SAO described in normal human serum [12], although it is not known whether each is proportionately decreased. Conversely, when increased SAO activity is observed [3,11,13–15], it is not clear whether the multiple forms are increased proportionally or whether isoenzymes other than those normally present are involved, as has been suggested by some [12,16].

Although the method of assay employed is not sufficiently sensitive to measure activity with any degree of accuracy at these low values, there can be little doubt that the fall in enzyme activity is a general phenomenon in patients with relatively recent severe burns. This observation applies even to burnt children, whose mean levels of activity, though higher than adult SBP values, are very significantly lower than those of the normal adult. The difference may merely reflect the fact that normal children have higher enzyme activity than adults [9,17,18].

Whilst it has not been easy to obtain material from adult burnt patients for serial assays, such work on severely burnt children may present even greater difficulty, so that most of our observations were made on the former. In so doing we were guided by several considerations. The difficulties of classifying severe burns are well known. Initial attempts at determining extent and depth of injury can only be approximate at best [19]. Estimates of the percentage of immediate skin loss admit of a wide margin of error; as time goes on, infection may transform superficial second-degree burns into deep dermal or third-degree lesions [19]. A "20%" skin loss may therefore in fact represent 15 or 25%.

Thus it has not been possible, perhaps for these reasons, to establish a correlation between decrease of SAO activity and the degree of injury sustained; nevertheless, two facts are worth mentioning:

1. The steeper the initial drop in SAO, the less favourable the prognosis: one patient with an estimated 50% skin loss 20 h after the accident had a value of 3.7 McE.U. He died the following day. Another with an estimated 33% injury, seen on the fourth day, had no detectable activity; she died the same day.

2. In a number of patients with a purported skin loss of 20% or more, seen within one to two weeks of injury, enzyme activity close to or within the normal range invariably confirmed the presence of lesser injuries than had originally been estimated.

Our study of the time course of SAO activity in SBP showed that low values persisted, even in patients in good physical condition who had had skin grafts. Several months, at least, seem to be required for activity to return to normal.

Since there is a substantial fall in total plasma protein concentration in SBP, with a slow return to normal levels, one possible explanation was that the drop in enzyme activity might be correlated with that of total plasma proteins. However, SAO values remained depressed long after total plasma proteins returned to normal.

The incubation of mixtures containing inactive and normal or high-activity sera did not evince the presence of an inhibitor of SAO in sera from SBP or cancer patients.

The significantly depressed values found in a large proportion of patients with proven cancer, both treated and untreated, suggest that the determination of SAO may be useful in dealing with neoplastic disease.

Since treatment may be reflected in SAO values, the sequential study of large groups is required to assess the value of the test in monitoring patients on chemotherapy. Investigation of larger numbers of untreated patients will also show its value in the diagnosis of the different types of cancer.

Acknowledgements

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A BENZYLAMINE OXIDASE DISTINCT FROM MONOAMINE OXIDASE B—WIDESPREAD DISTRIBUTION IN MAN AND RAT

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Abstract—Benzylamine oxidase (BzAO) and monoamine oxidase type B(MAO-B) both selectively catalyse the oxidative deamination of benzylamine (Bz). We define the former as that benzylamine-metabolizing activity insensitive to 4×10^{-4} M deprenyl, a concentration which totally inhibits all forms of MAO. Although both enzymes are widespread in human and rat tissues, their organ distribution differs. Liver and brain show highest MAO-B activity, whilst BzAO activity predominates in aorta and lung. Relatively low BzAO and no MAO-B activity is present in plasma. In the rat, phenylethylamine (PEA) and dopamine (DA) are both substrates for a deprenyl-resistant enzyme with a distribution similar to BzAO, but in man these amines are solely oxidized by MAO. At pH 7.2 the K_m of BzAO for benzylamine is 2.2×10^{-5} M in the rat; μn man, it is 1.1×10^{-4} M. The K_m of MAO-B for benzylamine is 1.0×10^{-4} M in the rat and 5×10^{-5} M in man. Semicarbazide, procarbazine and carbidopa are potent inhibitors of BzAO and inhibit it selectively, leaving MAO substantially unaffected.

Monoamine oxidase (monoamine: O_2 oxidoreductase (deaminating) EC 1.4.3.4.) (MAO) is a mitochondrial flavoprotein enzyme which oxidatively deaminates a wide range of monoamines[1]. Two forms have been distinguished by Johnston[2], although such a classification is at best approximate[3]; type A selectively deaminates 5-hydroxytryptamine (5-HT); type B prefers phenylethylamine (PEA) and benzylamine (Bz) as substrates[4]. Evidence has also accrued that a different enzyme, benzylamine oxidase (BzAO), is able to catalyse the oxidation of Bz[1]. It is not a flavoprotein, but is copper-dependent[1] and may use pyridoxal as cofactor[1]. This enzyme, which has been identified in the blood of many species, has been extensively purified from human plasma and characterized by McEwen[5, 6]. It bears a strong resemblance to a Bz-oxidizing activity distinct from MAO-B, which has been sporadically described in tissues as various as bovine aorta[7], rat heart[8], rabbit lung[9], rat artery[10], and many organs in the pig, particularly in connective tissue[11].

One of us has recently shown[12] that serum BzAO activity is significantly decreased in patients who have sustained severe burns, and in patients with cancer. In an attempt to elucidate the significance of these observations, we decided to embark on a study of BzAO activity and inhibitor sensitivity patterns in human and rat tissues. BzAO can be conveniently distinguished from MAO by selective inhibition with deprenyl, to which MAO-B is particularly sensitive but which inhibits all forms of MAO in higher concentration, leaving BzAO unaffected. Although the Bz-oxidizing MAO-B has aroused greater interest[1, 8–10, 13–17], no fully systematic attempt has so far been made to study the distribution of either enzyme in human or rat tissues.

MATERIALS AND METHODS

Chemicals. Benzylamine hydrochloride methylene[^{14}C] was purchased either from ICN Pharmaceuticals, Inc., Irvine, U.S.A. (sp. act. 12.5 mCi/m-mole), or from Radiochemical Centre Ltd., Amersham, U.K. (sp. act. 56 mCi/m-mole). The radiochemical purity of both was 99 per cent. [^{14}C]Phenylethalamine, sp. act. 50.98 mCi/m-mole, was purchased from New England Nuclear, Boston, U.S.A., and [^{14}C]dopamine, sp. act. 62 mCi/m-mole, from Radiochemical Centre Ltd., Amersham, U.K.

The following compounds were kindly donated: deprenyl by Professor J. Knoll, Budapest, by arrangement with the Chinoin Co., Budapest; procarbazine hydrochloride (Ro 4-6467) and benserazide (Ro 4-4602) by Roche Products Ltd., Welwyn Garden City, U.K.; carbidopa by Merck, Sharp & Dohme Ltd., Hoddesdon, U.K.; and clorgyline by May & Baker Ltd., Dagenham, U.K.

Isoniazid, penicillamine, and β-aminopropionitrile fumarate (BAPN) were purchased from Sigma Chemical Co., St. Louis, U.S.A., pargyline from Abbott Laboratories, North Chicago, U.S.A., and semicarbazide hydrochloride, 99.5 per cent pure (AnalaR grade) from BDH Chemicals, Poole, U.K. Benzylamine, purchased as free base from Sigma London Chemical Co. Ltd., Kingston-upon-Thames, U.K., was converted to its hydrochloride by treatment with hydrochloric acid, and recrystallized. All other reagents were obtained from commercial sources.

Tissues. (a) Rat. Male Wistar rats (200–250 g) were killed by decapitation. Tissues, freed from blood by rinsing in 0.9% saline and drying between layers of filter paper, were coarsely sliced or minced with scalpel or razor blade, quick-frozen in solid CO_2(−80°), and either homogenized immediately with 0.1 M sodium phosphate buffer (pH 7.4),

or stored at −20° for future use. For homogenization, tissues were immersed in liquid nitrogen (−196°) and pulverized by repeated hammer blows in a stainless steel mortar-and-pestle unit. All further steps were carried out at 0°. A 10% (w/v) homogenate in 0.1 M sodium phosphate buffer (pH 7.4) was prepared with ground-glass or Teflon-tipped pestles in 1 ml or 5 ml glass homogenizers, divided into aliquots and stored at −20° until required.

(b) Human tissues were obtained at autopsy from University College Hospital Medical School (kindly arranged by Dr. P. M. Sutton), and treated as described above.

(c) Plasma. Fresh whole blood was collected by venepuncture from normal human donors free of any known disease process, into plastic vials containing lithium heparin (5 units/ml) or K_2-EDTA (1 mg/ml). Within 1–2 hr of collection, it was spun for 10 min at 900 *g*, divided into aliquots in polypropylene vials and stored at −20° until use. When rats were killed by decapitation, blood was collected into vials containing appropriate amounts of anticoagulant, and dealt with as described above.

Tests were carried out on 4 series of rat tissues and two series of human tissues, unless otherwise stated in the Tables. Replicate assays were carried out (mostly duplicate). Results express the means of the series.

Assay procedures. (a) Radiometric microassay for BzAO in plasma or serum. Potassium phosphate buffer, 0.7 M, pH 7.2 (20 μl), water or inhibitor solution (100 μl), plasma or serum (100 μl) and substrate solution (20 μl) were incubated for 30 min in 75 × 12 mm open polypropylene tubes in a 37° shaking water bath. The final concentration of substrate in the assay tube was 50 μM (rat) or 690 μM (man); that of inhibitors is given in the Tables and graphs. Where inhibitors were used, the mixture was preincubated at room temperature for 20 min before labelled substrate was added. When substrate concentrations higher than 50 μM were used, unlabelled substrate (100 μl in an appropriate concentration) was added immediately before labelled substrate, without preincubation. Blanks were prepared as follows: enzyme and acid blanks, 2 M citric acid (100 μl) was added to the assay mixture before incubation; water blanks were prepared by substituting water for plasma or serum and incubated at 37°. The reaction was stopped by 2 M citric acid (100 μl). Toluene (3 ml) was added to each tube, which was capped, shaken vigorously for 5 min, spun for 5 min at 900 *g* and placed upright in racks at −20° until the lower phase was frozen solid. The supernatant was then decanted into polypropylene tubes, scintillation fluid (Insta-gel) (4 ml) added and radioactivity measured in a liquid scintillation counter.

Blanks gave radioactivity counts representing ≤0.5 per cent of total substrate activity.

(b) Radiometric microassay for BzAO in tissues. The assay mixture was made up as for plasma, except that the amount of tissue homogenate used instead of plasma was 20 μl for high-activity and 50 μl for low-activity tissue, made up with 80 or 50 μl water. The final concentration of substrate in the assay mixture was 50 μM (rat) or 690 μM (man); that of inhibitors is shown in the Tables and graphs.

(c) K_m. Linearity with time and enzyme concentration. Incubation times and concentrations of substrate and enzyme in the final assay mixture were varied as follows:

(1) K_m: (final concentration)
12.5, 25, 50, 100, 200 and 300 μM [^{14}C]Bz
12,5, 25, 50, 100, 250, 500 and 1000 μM [^{14}C]Bz

(2) Linearity with time and enzyme concentration: incubation times up to 60 min and enzyme concentrations of 5, 10, 20, 30, 40 and 80 μl tissue homogenate, and 5, 10, 20, 40, 100, 200 and 400 μl plasma or serum established the linearity of the reaction under the conditions of the assay described above.

(d) Efficiency of method. After the product of the reaction had been extracted and the supernatant decanted as described, the frozen phase was allowed to thaw, toluene added and the extraction procedure repeated twice. Extraction efficiency was found to be 95 per cent both for plasma and tissues.

(e) McEwen assay of BzAO in plasma or serum. The method of McEwen and Cohen[18], with slight modifications as described previously[12], was used to correlate spectrophotometric and radiometric assays.

(f) Assay of MAO in rat and human tissues, using[^{14}C]PEA and [^{14}C]DA. An extraction procedure similar to that described above was used for DA, and a cation exchange resin method for PEA. Volumes were: buffer 100 μl, inhibitor solution or water 100 μl, enzyme 20 μl and substrate 20 μl; final concentrations of substrate were 312 μM for DA and 27 μM for PEA. For inhibitor studies, mixtures were preincubated at room temperature for 30 min with freshly prepared inhibitor solutions. Blanks were prepared by incubating the buffer–enzyme mixture with 10^{-3} M clorgyline/deprenyl. Otherwise all procedures were as described by Glover *et al.*[19].

(g) The Lowry method[20] was used for determination of total plasma or tissue protein, with bovine serum albumin as standard.

RESULTS

The distribution of Bz and PEA-oxidizing activity in man and rat, considerable in several tissues, is shown in Table 1. It is apparent that more than one enzyme is reponsible for the distribution of activity using the two substrates, as the Bz/PEA ratio is quite dissimilar from tissue to tissue. Liver and brain are the major sources of PEA-oxidizing activity, whereas aorta and lung are most active against Bz. Bz-oxidizing activity in human tissues is many times greater than that of plasma; in the rat, trace activity only is detectable in serum or plasma.

Table 2 shows the effect of deprenyl on deamination of each substrate in different tissues. The two species differ: in the rat, both PEA and Bz oxidation are sensitive to deprenyl, to a varying

Table 1. Specific activities,* using benzylamine (Bz) and phenylethylamine (PEA) as substrates, of rat and human tissues (nmoles/mg protein/30 min)

Tissue	RAT		MAN	
	Bz	PEA	Bz	PEA
Aorta	15.6	7.1	17.5	1.2
Liver	7.4	22.8	14.9	15.2
Lung	6.0	3.5	11.4	1.5
Colon	5.1	2.9	5.8	0.9
Stomach	4.7	3.2	5.7	—
Ileum	4.3	5.3	6.5	0.7
Portal vein	—	—	6.4	—
Duodenum	4.0	3.6	—	—
Pancreas	3.5	21.0	—	—
Tongue	2.4	2.5	3.9	2.5
Adrenal	2.3	1.6	1.9	0.8
Forebrain	2.2	11.6	4.8	7.3
Skin	1.9	—	0.8	—
Oesophagus	1.6	2.8	3.1	1.6
Testis	1.5	2.4	—	—
Brainstem	1.5	8.2	4.1	5.3
Cerebellum	1.4	7.9	4.1	5.0
Diaphragm	1.3	1.3	2.7	—
Spleen	1.1	1.9	1.4	0.5
Parotid	1.1	3.0	—	—
Heart	0.9	3.5	10.3	19.4
Abdominal muscle	0.8	—	4.3	—
Psoas muscle	0.6	0.6	—	—
Kidney	0.6	2.8	10.2	12.1
Serum	0.01	—	0.1	—

*There was close agreement between individual values from human tissues. Rat tissues showed a narrow range of values in some examples, e.g. tongue (mean 2.4, range 2.0–2.8) and diaphragm (mean 1.3, range 1.1–1.4), but wide in others (stomach, mean 4.7, range 1.4–7.5; lung, mean 6.0, range 3.4–9.0). Duplicate assays on the same sample varied by less than 5 per cent.

Table 2. Effect of deprenyl on rat and human tissues, expressed as percentage of enzyme activity inhibited, using benzylamine (Bz) and phenylethylamine (PEA) as substrates

Tissue	RAT		MAN	
	Bz	PEA	Bz	PEA
Pancreas	98	99	—	—
Cerebellum	97	98	97	100
Liver	96	98	96	100
Forebrain	96	99	98	100
Brainstem	89	98	99	100
Kidney	60	91	91	100
Portal vein	—	—	55	—
Parotid	35	97	—	—
Oesophagus	—	—	26	—
Spleen	25	56	32	100
Serum	23	—	0	—
Adrenal	22	63	54	100
Stomach	20	39	15	—
Ileum	20	50	22	100
Skin	16	—	6	—
Tongue	16	52	78	—
Heart	13	90	93	100
Testis	12	62	—	—
Psoas muscle	11	58	—	—
Lung	10	47	12	100
Aorta	9	16	9	100
Duodenum	9	59	—	—
Diaphragm	8	45	65	100
Abdominal muscle	8	—	77	—
Colon	7	43	28	100

degree; in man, however, PEA oxidation is totally inhibited by deprenyl, whereas Bz-oxidizing ability is impaired to an extent varying with the tissue. It must therefore be inferred that in man, all PEA is deaminated by MAO-B, but in rat both PEA and Bz are oxidized by an enzyme or enzymes distinct from MAO-B. The pattern of sensitivity of rat tissues to deprenyl differs according to whether PEA or Bz is employed as substrate; this finding can be explained if the different relative activities of BzAO and MAO-B towards Bz and PEA are taken into account. In rat brain the Bz/PEA activity ratio was 0.18, whereas in aorta it was 2.2.

Table 3 shows the distribution of BzAO (deprenyl-resistant) Bz-oxidizing activity, and Table 4 that of MAO-B (deprenyl-sensitive) in different human and rat tissues. BzAO is most active in aorta, lung and digestive tract. Human plasma has relatively low activity. BzAO is absent from liver and brain, the most active sources of MAO-B.

The isoniazid inhibition pattern of human and rat tissues, employing Bz as substrate, is shown in Table 5. Isoniazid is a selective but weak inhibitor of BzAO and MAO-B in the rat, but in human tissues it is neither potent nor selective (Fig. 1).

In the rat DA-oxidizing activity was also identified, resistant to 4×10^{-4} M deprenyl, a concentration which inhibits both MAO-A and B (Table 6). This activity is likewise highest in aorta and lung and is inhibited by 4×10^{-4} M isoniazid in a manner similar to BzAO, suggesting that this enzyme may contribute to DA oxidation in the rat. In human tissues, however, all DA oxidation is inhibited by 4×10^{-4} M deprenyl, pointing to the converse.

Table 3. Distribution of BzAO activity in human and rat tissues (nmoles/mg protein/30 min). MAO-B was inhibited by 4×10^{-4} M deprenyl. Substrate: Bz, 50 μM (rat); 690 μM (man)

Tissue	Specific activity	
	Rat	Man
Aorta	14.2	15.9
Lung	5.7	10.0
Colon	4.7	4.2
Ileum	4.5	5.1
Stomach	3.9	4.8
Portal vein	—	2.9
Duodenum	3.6	—
Tongue	2.0	0.9
Adrenal	2.0	0.9
Skin	1.6	0.8
Testis	1.3	—
Diaphragm	1.2	0.5
Parotid	0.9	—
Liver	0.9	0.6
Spleen	0.8	1.0
Heart	0.8	0.7
Abdominal muscle	0.8	1.0
Psoas muscle	0.5	—
Kidney	0.2	0.9
Brainstem	0.2	0.04
Cerebellum	0.1	0.1
Forebrain	0.1	0.1
Pancreas	0.1	—
Serum	0.01	0.1

Table 4. Distribution of MAO-B in human and rat tissues (nmoles/mg protein/30 min). Substrate: PEA, 27 μM. For definition of MAO-B activity, see text

Tissue	Specific activity Rat	Man
Liver	22.3	15.2
Pancreas	20.8	—
Forebrain	11.5	7.3
Brainstem	8.0	5.3
Cerebellum	7.7	5.0
Heart	3.2	19.4
Parotid	2.9	—
Ileum	2.7	0.7
Kidney	2.5	12.1
Duodenum	2.1	—
Lung	1.7	1.5
Testis	1.5	—
Stomach	1.3	—
Tongue	1.3	2.5
Colon	1.2	0.9
Aorta	1.1	1.2
Spleen	1.0	0.5
Adrenal	1.0	0.8
Diaphragm	0.7	—
Psoas muscle	0.4	—

BzAO and MAO-B show different K_m values for Bz. In rat lung, BzAO gave a K_m of 2.3×10^{-5} M and in caecum 2.1×10^{-5} M, whereas the K_m for MAO-B in rat liver was 1.0×10^{-4} M. The K_m for Bz in human tissues also distinguishes between MAO-B (5.0×10^{-5} M in brain) and BzAO (1.1–1.2×10^{-4} M for aorta, lung and plasma). This difference results in a different relative distribution of Bz-oxidizing activity in the tissues, depending on substrate concentration used. Table 7 shows that in the rat, liver and brain are relatively more active at higher Bz concentrations, compared with aorta and lung. In addition, substrate inhibition of BzAO appears to supervene with higher substrate concentrations.

Figures 2, 3 and 4 show that carbidopa, procarbazine and semicarbazide are all selective inhibitors of BzAO in human tissues. With each, enzyme activity in tissues such as aorta, lung and colon, rich in BzAO, is selectively inhibited by concentrations which have little effect on brain, kidney and liver, predominantly MAO-B-containing tissues.

Of the other potential inhibitors tested, penicillamine had little effect on human plasma enzyme activity, clorgyline and pargyline none. In human tissues, BAPN proved potent but non-selective. In order of potency, carbidopa and procarbazine were the most effective and selective inhibitors of BzAO, followed by benserazide, semicarbazide and isoniazid.

DISCUSSION

Our studies in man and rat point to a close similarity if not identity of plasma and tissue BzAO in each species. Although it has, in the past, received most attention as a plasma enzyme, we demonstrate that an enzyme of closely similar physicochemical properties is widely distributed in the tissues, and that plasma is, in fact, a relatively poor source of activity. We therefore suggest that the term "plasma (or serum) amine oxidase" be abandoned, and that "benzylamine oxidase", first proposed by Bergeret, Blaschko and Hawes[21], be employed as a provisional name for the enzyme wherever it occurs, until its true function and physiological substrate(s) can be identified.

Table 5. Inhibition by isoniazid (4×10^{-4} M) of enzyme activity in human and rat tissues, using Bz as substrate. Residual activity is expressed as nmoles/mg protein/30 min

Tissue	Rat % inhibition	Rat Residual activity	Man % inhibition	Man Residual activity
Diaphragm	95	0.1	70	0.4
Aorta	93	1.1	79	3.7
Lung	91	0.6	70	3.4
Colon	83	0.9	81	1.1
Abdominal muscle	82	0.1	60	1.7
Psoas muscle	81	0.1	—	—
Tongue	81	0.5	74	1.0
Skin	81	0.4	92	0.1
Ileum	79	0.9	71	1.9
Adrenal	79	0.5	61	0.7
Serum	78	0.002	92	0.01
Duodenum	77	0.9	—	—
Spleen	77	0.2	83	0.2
Stomach	76	1.1	77	1.3
Testis	76	0.3	—	—
Heart	69	0.3	68	4.3
Kidney	55	0.3	47	5.4
Parotid	38	0.7	—	—
Forebrain	31	1.5	57	2.1
Pancreas	29	2.5	—	—
Brainstem	27	1.1	67	1.4
Cerebellum	26	1.0	73	1.1
Liver	19	6.0	21	11.8

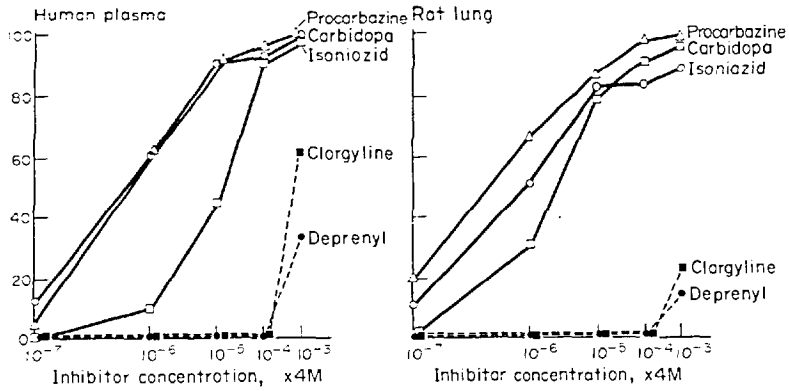

Fig. 1. Effect of selective inhibitors on *in vitro* benzylamine-oxidizing activity in human plasma and rat lung. Each point represents the mean of duplicate determinations at each concentration of inhibitor.

Table 6. Dopamine (DA) oxidizing activity of BzAO in human and rat tissues (nmoles/mg protein/30 min). Substrate: DA. 312 μM. Inhibitor: deprenyl, 4×10^{-4} M

Tissue	Rat			Man	
	Specific activity	Deprenyl % inhibition	Residual activity	Specific activity	Deprenyl % inhibition
Pancreas	72.7	99	0.7	—	—
Liver	64.5	99	0.6	71.3	100
Forebrain	33.4	99	0.3	9.8	100
Heart	31.7	98	0.6	30.3	100
Brainstem	22.6	98	0.4	6.5	100
Cerebellum	17.9	100	0.0	6.4	100
Ileum	14.2	43	8.1	2.2	100
Aorta	12.2	19	9.9	1.6	100
Adrenal	10.1	95	0.6	3.3	100
Colon	8.8	68	2.8	1.8	100
Duodenum	8.6	68	2.8	—	—
Kidney	8.4	96	0.3	36.0	100
Stomach	8.0	69	2.5	—	—
Lung	6.4	88	0.8	6.4	100
Oesophagus	5.9	72	1.6	0.9	100
Tongue	5.4	82	1.0	10.9	100
Testis	5.4	—	—	—	—
Diaphragm	3.9	90	0.4	—	—
Spleen	3.7	96	0.1	0.9	100
Parotid	3.6	—	—	—	—
Psoas muscle	2.2	99	0.02	—	—

BzAO is most highly active in aorta and lung. Recent work on lung and blood vessels of rat [10], rabbit [9, 22] and pig [11, 23] suggests that this semicarbazide-sensitive form of amine oxidase originates in the walls of blood vessels, particularly the endothelial lining. However, these data do not imply that its activity is invariably associated with blood vessels. Buffoni *et al.* [11] found fluorescent antibody cross reaction in connective tissue distinct from blood vessels. They did not identify any activity in liver parenchyma or brain; fluorescence was, however, present in interlobular connective tissue of the liver and in meninges and blood vessels of the brain. These findings agree closely with those of the present study; we were unable to detect BzAO in liver parenchyma or brain, the most active sources of MAO-B, although considerable BzAO activity was present in the portal vein. One fact stands clear. Because plasma BzAO activity is very low, blood contamination [13] can no longer be invoked to account for the high values present in certain tissues.

The inhibition pattern of BzAO with a number of different inhibitors did not appear to vary significantly from tissue to tissue. However, there are pointers in the literature to the existence of isoenzymes of plasma BzAO [24] and multiple forms which differ in their substrate and inhibitor specificities cannot yet be excluded.

Although the distribution patterns of MAO-B and BzAO activities in different tissues are fairly similar in man and rat, the properties of BzAO differ in the two species. In the rat, the K_m for Bz at pH 7.2 is approximately 2.2×10^{-5} M, whereas in man it is about 1.1×10^{-4} M. The rat enzyme also

Table 7. Distribution of Bz-oxidizing activity in rat tissues, employing two different substrate concentrations. Each value represents the mean of 3 series of tissues. Activity expressed as nmoles/mg protein/30 min

Tissue	Benzylamine 50 μM	Benzylamine 690 μM	Ratio
Aorta	15.6	2.2	1:0.1
Liver	7.4	17.6	2.4
Lung	6.0	4.3	0.7
Colon	5.1	2.5	0.5
Stomach	4.7	1.5	0.3
Ileum	4.3	2.3	0.5
Duodenum	4.0	5.2	1.3
Pancreas	3.5	8.4	2.4
Tongue	2.4	1.1	0.4
Forebrain	2.2	7.1	3.3
Oesophagus	1.6	0.5	0.3
Testis	1.5	0.5	0.4
Brainstem	1.5	4.9	3.3
Cerebellum	1.4	2.4	1.8
Diaphragm	1.3	0.5	0.4
Spleen	1.1	0.9	0.8
Parotid	1.1	1.0	0.9
Heart	0.9	1.0	1.1
Psoas muscle	0.6	0.3	0.5
Kidney	0.6	2.2	3.6

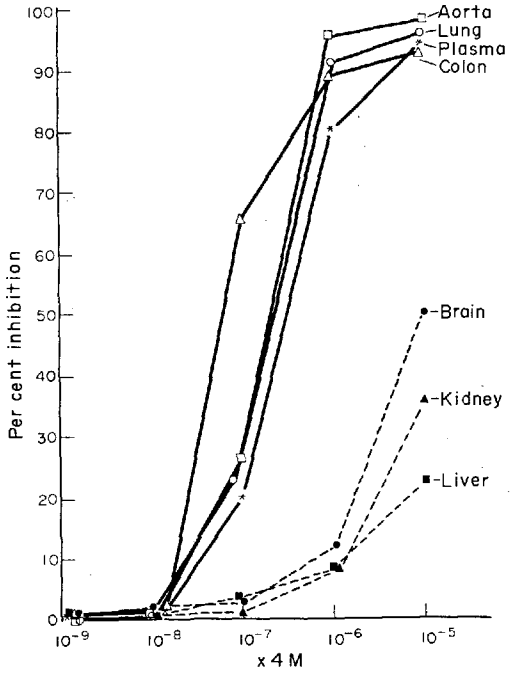

Fig. 2. Selective inhibition of benzylamine oxidation in various human tissues by carbidopa. Each point represents the mean of duplicate determinations at each concentration of inhibitor.

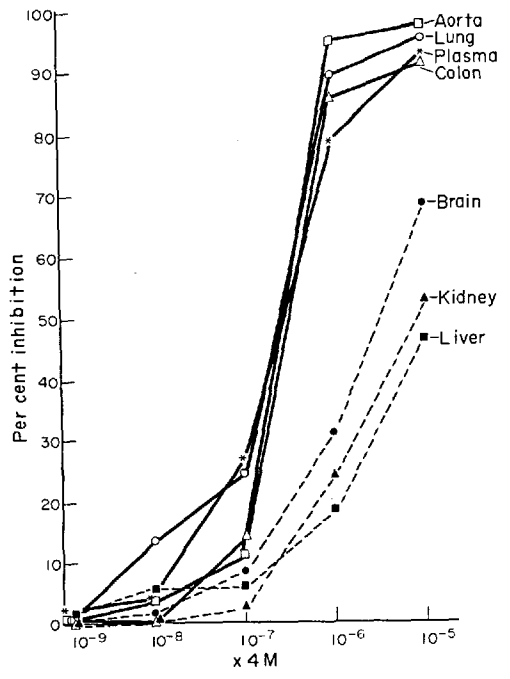

Fig. 3. Selective inhibition of benzylamine oxidation in various human tissues by procarbazine. Each point represents the mean of duplicate determinations at each concentration of inhibitor.

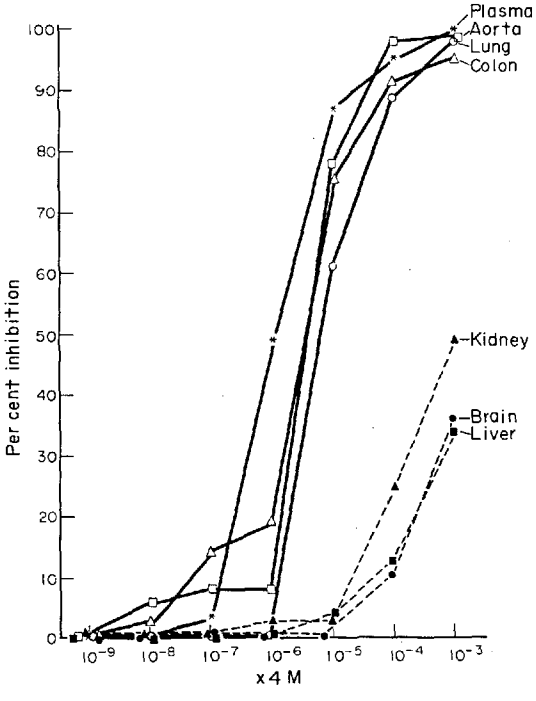

Fig. 4. Selective inhibition of benzylamine oxidation in various human tissues by semicarbazide. Each point represents the mean of duplicate determinations at each concentration of inhibitor.

deaminates PEA and DA, whereas these amines are not substrates of BzAO in man, in whom PEA and DA appear to be oxidized solely by MAO.

The K_m of BzAO for Bz is highly sensitive to changes in pH [6, 25], a property to be taken into account when comparing results from different laboratories. Nevertheless, our results agree well with those of others [8, 25]. Lyles and Callingham [8], distinguishing BzAO from MAO-B in the rat heart, noted K_m values of 10^{-5} M and 5×10^{-4} M respectively. They also observed, and we confirm,

that BzAO is inhibited by high concentrations of substrate, an observation which parallels that of McEwen on rabbit serum enzyme [26].

The function of BzAO is at present unknown. Bz itself has not so far been identified in man or rat, and seems unlikely to be the natural substrate. Lysyl oxidase is known to be present in connective tissue but differs from BzAO both chemically [27] and immunologically [13, 28]. Nevertheless, it is worth noting that two BzAO inhibitors, BAPN and benserazide, are respectively responsible for the connective tissue disease, lathyrism [1] and a condition not unlike it in rats [29].

Semicarbazide was previously known to be a potent selective inhibitor of BzAO [30]. It is of interest that procarbazine and carbidopa have now also been established as potent inhibitors of this enzyme and selectively inhibit it with respect to MAO-B. Carbidopa, a peripheral decarboxylase inhibitor, is used extensively with levodopa for the treatment of Parkinsonism [31], whilst procarbazine is administered for certain types of neoplasm [32]. Both drugs would be inhibitory to BzAO in the dosage regimens employed in clinical practice. Thus, once again, supposedly specific drugs are shown to possess a multiplicity of actions. Careful clinical observation in patients treated with these inhibitory drugs may well shed light on the function or functions of BzAO in man.

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β-PHENYLETHYLAMINE AND BENZYLAMINE AS SUBSTRATES FOR HUMAN MONOAMINE OXIDASE A: A SOURCE OF SOME ANOMALIES?

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Abstract—Monoamine oxidase (MAO) A predominates both in human placenta and lung. With 5-hydroxytryptamine (5-HT), β-phenylethylamine (PEA) and benzylamine (Bz) as substrates and clorgyline and deprenyl, respectively, as selective MAO A and B inhibitors, their activity pattern has been defined and compared with that of human liver. PEA had a much higher V_{max} with placental MAO A than did Bz; it behaved largely as an A substrate in placenta, and partly as an A substrate in lung. At commonly used substrate concentrations, deamination of Bz (sensitive to 10^{-7} M deprenyl) was a better indicator of MAO B activity than deamination of PEA. The divergence between PEA and Bz as MAO A and B substrates may be one reason for some of the apparent discrepancies in the behaviour of MAO A and B noted in a variety of tissues in the literature.

However, Bz reacts with benzylamine oxidase (BzAO) as well as MAO B. Depending on the tissue, deprenyl-resistant Bz activity may indicate the presence of BzAO rather than MAO A. As there is a widespread distribution of BzAO in man and rat, BzAO should be considered among the alternatives of enzyme activity when Bz is used as substrate.

Johnston's classification [1] of monoamine oxidase [MAO; monoamine: oxygen oxidoreductase (deaminating) EC 1.4.3.4] into A and B forms, based on the selective inhibitory action of clorgyline, was to some extent an oversimplification and the premises on which it was founded have latterly come under increasing scrutiny [2–6]. Although, by definition, the classification is based on the action of a particular selective inhibitor, it has often been more convenient to identify the different forms by their preferential action on particular substrates. This practice has brought difficulties in its train. One major problem has been that two substrates commonly assumed (e.g. ref. 7) to distinguish between the two forms, 5-hydroxytryptamine (5-HT) for MAO A, and β-phenylethylamine (PEA) for MAO B, are not completely specific. Both can act as substrates for either form, and any specificity they possess lies in differences in K_m and V_{max} [17, 19]. Moreover, benzylamine (Bz), another substrate for MAO B, is also deaminated by benzylamine oxidase (BzAO) [9, 14]. If one enzyme form is present in great excess, it may contribute the larger part of the activity observed with any of a range of substrates. This could be one explanation of some of the apparent anomalies and differences in the behaviour of MAO A and B recorded for different tissues.

We have recently been engaged in a large-scale investigation of the distribution of BzAO and the two forms of MAO in human tissues [8], using Bz, PEA and 5-HT as substrates. Our criterion for MAO B activity with Bz as substrate is that part of the total suppressed by a deprenyl concentration selective for MAO B, i.e. the deprenyl-sensitive moiety of the activity observed with Bz (D*Bz). The ratio of this moiety to the uninhibited activity registered with PEA as substrate (D*Bz/PEA ratio) tended to be fairly constant and was used by us as a check of the results obtained with either substrate. However, in the course of our study we have come across some notable exceptions. Placenta, lung and some blood vessels have a D*Bz/PEA ratio quite distinct from that observed in the great majority of tissues. We report here the results of a study of MAO A and B in human placenta, lung and liver.

MATERIALS AND METHODS

Lung and liver samples, obtained at autopsy, were dissected, cleaned and freed from blood by rinsing in cold 0.9% saline, quick-frozen in solid CO_2 (−80°), and stored at −20°. The interval between death and autopsy varied between 12 and 24 hr, during which time the bodies were kept at 2–4°. Placentae, obtained from the Labour Ward at Queen Charlotte's Maternity Hospital within 1–6 hr of delivery, were treated similarly. All other procedures involving tissues up to time of assay, and the chemicals used, were as outlined in our previous report [9]. In addition, [^{14}C]5-HT, sp.act. 58 mCi/mmole, was purchased from the Radiochemical Centre Ltd., Amersham, U.K., and clorgyline was kindly donated by May & Baker Ltd., Dagenham, U.K.

The basic assay procedure was the radiometric microassay described in detail in our previous paper [9], with the following modifications: the extraction method described for [^{14}C]Bz was also used for [^{14}C]PEA and [^{14}C]5-HT, and the reaction product of the PEA assay was extracted into toluene, whilst that of the 5-HT assay was extracted into a 1:1 mixture of ethyl acetate and toluene.

For the assays shown in Table 1, final concentrations of substrate in the assay mixture were: Bz, 42 μM; PEA, 150 μM; and 5-HT, 371 μM.

Most assays using Bz as substrate were carried out

Table 1. Activity of BzAO, MAO B and MAO A against Bz, PEA and 5-HT. 4×10^{-7} M deprenyl was used with Bz to select for MAO B activity. Except where otherwise stated, values represent specific activity (nmoles/mg protein/30 min). For molar concentrations of substrates, see Materials and Methods. Bz assays were carried out at pH 9.0, PEA and 5-HT assays at pH 7.2. Homogenates were 10 per cent (w/v) in 0.1 M potassium phosphate buffer, pH 7.2

	Bz			Ratio		Ratio	
Tissue (N)	BzAO	D*Bz	PEA	D*Bz/PEA	5-HT	5-HT/PEA	5-HT/D*Bz
Placenta (4)	5.2	5.1	20	0.26	140	7.0	27
Lung (3)	25	1.8	4.1	0.44	17	4.1	9.7
Liver (6)	6.0	80	33	2.4	92	2.8	1.1

with 0.1 M Tris buffer (pH 9.0); this pH has been found by others [10] and by us (unpublished observations) to produce far more activity than pH 7.2, which we had used in our previous experiments [9]. The specific activities recorded in our previous report [9] and those in Table 1 cannot, therefore, be considered directly comparable. As shown by Fowler *et al.* [22], oxidation of tyramine, 5-HT and PEA is inhibited by Tris buffer, but Bz oxidation is not. These findings were confirmed by our experiments with PEA as substrate and Tris buffer (pH 9.0), which produced substantial inhibition (unpublished results). On the other hand, in parallel experiments with potassium phosphate and Tris buffers at pH 7.2 and 9.0, respectively, using the same homogenates, we found that while the higher pH produced far higher activity, the sensitivity of MAO B to deprenyl was the same at pH 7.2 and 9.0 (unpublished results). We therefore considered it justified to use the optimum pH for both Bz (9.0) and PEA (7.2) in these experiments.

The final concentrations of substrate in the assay mixture of the experiments illustrated by Figs. 1 and 2, and Table 2, were as follows: Bz, 42 μM; PEA, 88 μM; 5-HT, 217 μM. To ensure uniformity of results, all these experiments (including those with Bz as substrate) were carried out at pH 7.2 with 0.1 M K_2HOP_4/KH_2PO_4 buffer.

Substrate concentrations used in the assays to determine MAO A V_{max} and K_m (Table 3) were as follows: Bz and 5-HT, 500, 250, 125, 62.5, 31.25 μM; PEA, 250, 125, 62.5, 31.5, 15 μM.

Several experiments were carried out with a full range of inhibitor concentrations (4×10^{-3} to 4×10^{-9} M) and various 10 per cent (w/v) homogenates, the tissue concentration used throughout these experiments. 4×10^{-7} M (–)-deprenyl was found to be optimal at distinguishing BzAO from MAO B.

Total tissue protein was determined by the method of Lowry *et al.* [11] with bovine serum albumin as standard.

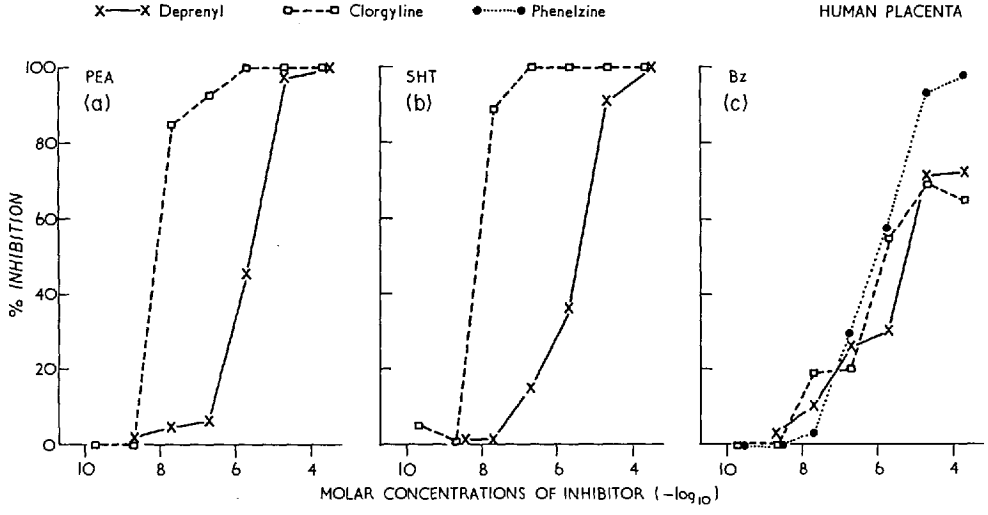

Fig. 1. Effect of different concentrations of clorgyline and (−)-deprenyl on the inhibition of PEA, 5-HT and Bz deamination in human placenta, 10 per cent (w/v) homogenate in 0.1 M potassium phosphate buffer, pH 7.2. All assays carried out at pH 7.2. For substrate concentrations see Materials and Methods. (a) PEA, (b) 5-HT, (c) Bz. □----□ clorgyline, ×——× (−)-deprenyl, ●····● phenelzine. Three separate experiments gave similar curves.

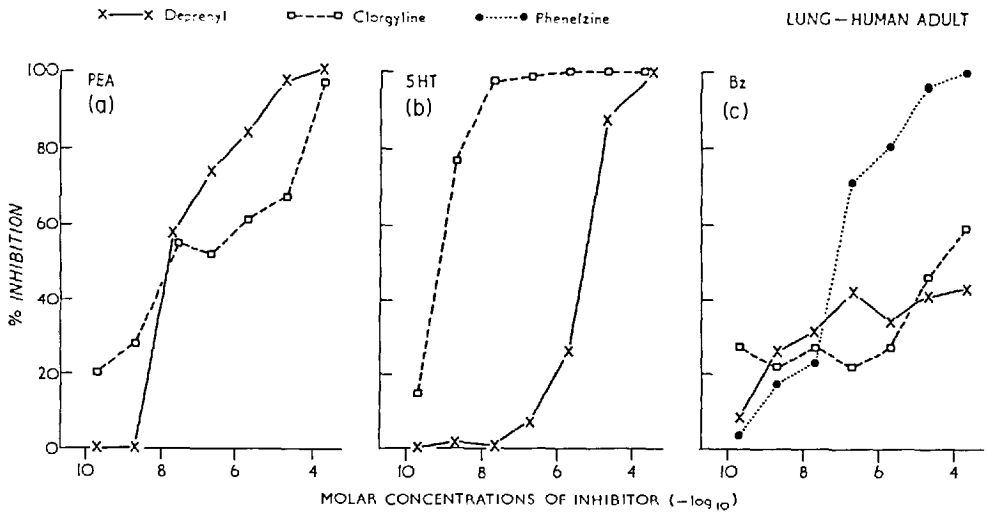

Fig. 2. Effect of different concentrations of clorgyline and (−)-deprenyl on the inhibition of PEA, 5-HT and Bz deamination in human lung. Assay conditions and symbols as in Fig. 1. Three separate experiments gave similar curves.

All assays were carried out in duplicate; values given represent the means of multiple determinations.

RESULTS

Table 1 shows the specific activities of BzAO, MAO A and MAO B in human placenta, lung and liver, with Bz, 5-HT and PEA as substrates. The D*Bz/PEA ratio, as defined above, is seen to differ substantially in placenta and lung from that observed in liver. In most adult human tissues we have studied [8], the ratio is greater than 2.0, but in lung it is 0.44. The other striking feature of this table is the different pattern of 5-HT/PEA ratios compared with 5-HT/D*Bz ratios.

Figure 1 shows the effect of different concentrations of clorgyline and deprenyl on the inhibition of 5-HT, PEA and Bz deamination in human placenta. It will be seen that 5-HT and PEA gave similar curves (Figs. 1a and b), indicating that a major portion of PEA was metabolized by MAO A. With Bz (Fig. 1c) the pattern was more complicated: a proportion of the activity was not inhibited by either deprenyl or clorgyline and presumably derived from BzAO. This interpretation is borne out by an inhibition curve with phenelzine which, at 4×10^{-5} M, blocked 93 per cent of the activity observed with Bz, whereas a tenfold greater concentration of deprenyl and clorgyline achieved only 72 and 65 per cent inhibition, respectively. The remaining activity with these two inhibitors shows a double sigmoid curve, suggesting that some Bz acts as a substrate for MAO A, and some for MAO B.

Figure 2 shows the results of similar experiments on human lung homogenate (10% w/v). With 5-HT as substrate (Fig. 2b), a simple sigmoid curve is seen with both clorgyline and deprenyl, resembling the placental response. The response with Bz and PEA, however, was more complex. Most of the Bz activity was both clorgyline and deprenyl-resistant, but sensitive to 10^{-6} M phenelzine, suggesting that it was due to BzAO. The remaining activity was somewhat more sensitive to deprenyl than clorgyline, both inhibitors showing a plateau. With PEA also, a plateau was found with clorgyline, suggesting that the substrate was metabolized by both MAO A and B in this tissue.

When similar experiments were performed with human liver homogenate (10% w/v), 5-HT behaved as a typical MAO A substrate, and Bz and PEA as typical B substrates, all giving single sigmoid plots with both clorgyline and deprenyl.

I_{50} values for the three substrates with deprenyl

Table 2. PI_{50} values for deprenyl and clorgyline inhibition of MAO A and MAO B in human placenta, liver and lung. Values are neg.log10 molar concentrations of inhibitors. For molar concentrations of substrates, see Materials and Methods

	Placenta		Liver		Lung	
Substrate	Dep.	Clorg.	Dep.	Clorg.	Dep.	Clorg.
Bz	5.3	5.8	8.7	5.7	3.7*	4.4
PEA	5.6	8.1	7.3	4.9	7.8	7.8
5-HT	5.5	8.2	5.3	9.0	5.3	9.1

* Only 42 per cent inhibition was achieved with the highest concentration used, 4×10^{-4} M.

Table 3. K_m and V_{max} values for 5-HT, PEA and Bz with placental MAO A, defined as activity sensitive to 10^{-7} M clorgyline. These parameters were determined with a mitochondrial preparation from human placenta. V_{max} values are a percentage of MAO activity with 5-HT

	5-HT	PEA	Bz
V_{max}	100	13	0.7
K_m (μM)	200	222	400

and clorgyline against human placenta, lung and liver are shown in Table 2. These values depend on the protein concentration of the homogenate examined.

Table 3 shows the K_m and V_{max} for 5-HT, PEA and Bz with placental MAO A, which is defined as the activity sensitive to 10^{-7} M clorgyline. It will be seen that with human placenta, PEA has a much higher V_{max} than Bz and a K_m very close to that of 5-HT; it is therefore a considerably more active substrate of MAO A than Bz.

DISCUSSION

The striking differences between the D*Bz/PEA ratios of human placenta, lung and liver (Table 1) are at first sight surprising, as both PEA and Bz are generally considered to be substrates for MAO B. Two explanations are possible: (1) MAO B in placenta and adult human lung may differ from that in adult liver and other human tissues; (2) either Bz or PEA, or both, may be acting as substrate(s) for MAO A also and the different contribution of MAO A to total activity in placenta, lung and liver may account for the observed differences in ratios.

The present study shows that it is possible to explain the different D*Bz/PEA ratios (Table 1) without postulating the existence of variant forms of MAO B in the three tissues. In the liver, Bz and PEA are metabolized predominantly by MAO B, and the D*Bz/PEA ratio indicates the relative activity of these two substrates with MAO B. In placenta PEA is deaminated overwhelmingly by MAO A. Relatively little D*Bz activity is seen, both because there is very little MAO B present, compared with MAO A, and because Bz is a much less active substrate for MAO A than PEA (Table 3).

In the lung, the situation is intermediate between placenta and liver, with a significant proportion of activity towards PEA contributed both by MAO A and B. Table 1 shows that in the lung, the A/B ratio expressed by 5-HT/D*Bz is much higher than in the liver, but not so high as in placenta. In the experimental conditions used here, therefore, D*Bz would appear to be a much better measure than PEA of MAO B activity. This conclusion is emphasized by the difference in the A/B ratio expressed by 5-HT/PEA as compared with 5-HT/D*Bz (Table 1).

One of the observations described here has, in fact, been noted previously. The relative activities we observed for 5-HT, PEA and Bz in human placenta are very similar to those found by Egashira [12] who failed, however, to study inhibitor responses with the different substrates. In the human placenta, Bz is oxidized to a slight extent by MAO A (Fig. 1c). Lyles and Callingham [13–15] reported some clorgyline-sensitivity in rat heart, when Bz was employed as substrate, concluding that Bz deamination in that site is achieved, in part, by MAO A. No corresponding activity was detected by Parkinson and Callingham in human heart, however [16]. Working with rat heart, vas deferens and liver, Dial and Clarke [17] claim that PEA acts as a substrate for MAO A, MAO B or a mixture of both, depending on the organ. However, other authors have drawn different conclusions from similar results. For example, Lyles and Callingham find that in rat heart, Bz is a substrate for both MAO A and B, whereas tyramine and PEA are substrates for the A form only; it appears that in this organ, MAO A and B exist in a different form from that of rat liver. Although MAO A or MAO B heterogeneity in a single individual or species cannot be ruled out, the contributions of MAO A, MAO B and BzAO all have to be taken into account before such an inference can be drawn. The biphasic curve found by Lyles and Callingham in rat heart with Bz as substrate [14] resembles that shown here with Bz in human placenta. It seems possible that their results are explicable in terms of different ratios rather than different types of MAO A or B, as rat heart MAO consists predominantly of the A form, whilst liver contains a higher proportion of B. Dial and Clarke's findings with rat vas deferens [17] resemble those reported here with human lung and may have a similar explanation.

The activity obtained with different substrates will depend both on the tissue and on substrate concentrations used. The K_m for PEA with MAO A and MAO B in rat liver has been reported as 6.2×10^{-5} M and 4×10^{-6} M, respectively [18]. In human platelet MAO B, Edwards and Chang [19] have shown the V_{max} for 5-HT, PEA and Bz to be 7.3, 19 and 41 nmoles, respectively, and the K_m, 540, 3 and 130 μM, respectively. A comparison of these findings with ours (Table 3) for human placental MAO A illuminates our point. Assuming these forms of MAO A and B to be similar to those in other human tissues, we can see that PEA has a much higher affinity for MAO B, so that it will be a more specific substrate at low than at higher concentrations. Bz is more specific than PEA for the B form, in that its V_{max} with MAO B is much higher than that of PEA; conversely, with MAO A, the V_{max} of Bz is much lower than that of PEA. This may account for our results using PEA and Bz at high concentrations in a histochemical study [20], where we found PEA to act as a substrate for both MAO A and B, whereas Bz was specific for MAO B and BzAO.

In defining BzAO and MAO B, respectively, as the deprenyl-resistant and deprenyl-sensitive (D*Bz) moieties of total Bz activity, we are assuming that all deprenyl-resistant activity in the tissues we studied derives from BzAO. A small amount may be due to MAO A, but the present results point to this component being negligible. It is, however, still possible that this deprenyl-resistant BzAO itself consists of more than one enzyme.

Phenelzine is seen to be a potent inhibitor of BzAO (Figs. 1c and 2c). A study of various human tissues at inhibitor concentrations from 4×10^{-3} to 4×10^{-10} M with Bz as substrate furnished the optimum concentration, 4×10^{-6} M, for blocking this enzyme (unpublished results). Unfortunately, in the larger study on human tissues carried out by us (in preparation), it was found that phenelzine is less able than deprenyl or clorgyline to distinguish between the different enzyme forms; at the concentration used, it merely serves to confirm the presence of BzAO, but cannot be considered a selective differentiator by itself.

The data presented here reinforce the view that care must be taken in judging the activities of MAO

A and B in a particular tissue merely from measurements of the oxidation of 5-HT and PEA. At the substrate concentrations generally used, Bz is a more specific substrate for MAO B than PEA, but it also reacts with BzAO. The position can be clarified by using the different substrates with selective concentrations of clorgyline or deprenyl to distinguish the contribution to the total activity of MAO A, MAO B or BzAO.

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Addendum—The report of Suzuki *et al.* [23] on the effect of PEA concentration on substrate specificity for MAO A and B, was published after the present paper was written, and is largely confirmed by our findings.

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DEVELOPMENT OF BENZYLAMINE OXIDASE AND MONOAMINE OXIDASE A AND B IN MAN

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Abstract—We describe the widespread distribution of the enzymes benzylamine oxidase (BzAO) and monoamine oxidase A and B (MAO A, MAO B) in human tissues at three stages of development: fetal, neonatal and adult. Relatively low activity of each is present in fetal tissues, but the specific activities of BzAO and MAO B in fetal liver are similar to those in the adult, suggesting that these enzyme systems are functionally mature in the liver at 20 weeks gestation. Specific activity of BzAO in the lung, twice as high in the adult as in the fetus, reaches adult value at birth. In fetal brain, lung, aorta and digestive tract, MAO A emerges before MAO B. Estimation of total activity developed by tissue or organ sheds a new light on the importance of each enzyme in body economy and shows skeletal muscle to be by far the most active whole body source of MAO B, whilst the liver has the highest total and specific activity of MAO A. A similar approach clearly demonstrates that BzAO is essentially a tissue rather than a plasma enzyme, which tends to predominate in blood vessel walls.

In an earlier paper [1], we described the widespread distribution of benzylamine oxidase (BzAO) and monoamine oxidase (MAO) B in human and rat tissues. This study was undertaken in an attempt to elucidate the significance of the finding by one of us [2] of low serum BzAO activity in patients with severe burns or cancer. It then seemed reasonable to investigate the activity of BzAO in rapidly-growing tissues. In this paper, we describe the distribution of BzAO in human tissues and compare activities in fetus, neonate and adult. The development of MAO A and MAO B activity was investigated in parallel.

Despite considerable research effort in recent years [3–9], the physiological role of the amine oxidases in the degradation of a multiplicity of endogenous and exogenous substrates remains poorly understood. Benzylamine (Bz) is the best substrate for both MAO B and BzAO [1] and, indeed, is the only known substrate for human BzAO. This compound, together with the MAO A substrate, 5-hydroxytryptamine (5-HT, serotonin) [7] and the putative MAO B substrate, phenylethylamine (PEA) [10], has been used to chart the development of the amine oxidases in a wide variety of tissues.

MATERIALS AND METHODS

Chemicals

Benzylamine hydrochloride methylene [^{14}C], sp. act. 56 mCi/mmole, radiochemical purity 98%, and 5-hydroxy[*side-chain*-2-^{14}C] tryptamine sulphate, sp. act. 58 mCi/mmole, radiochemical purity 98%, were purchased from Radiochemical Centre Ltd., Amersham, U.K. Phenylethylamine hydrochloride, beta-[ethyl-1-^{14}C], sp. act. 50.98 mCi/mmole, radiochemical purity 97%, was purchased from New England Nuclear, Boston, MA, U.S.A. (-)-Deprenyl was generously donated by Professor J. Knoll, Budapest, clorgyline by May & Baker Ltd., Dagenham, U.K., and phenelzine by William R. Warner & Co. Ltd., Eastleigh, Hants, U.K. All other reagents were obtained from commercial sources or as described earlier [1].

Tissues

(a) *Fetal.* Six human fetuses (gestational age 19–21 weeks) were obtained from the fetal tissue bank of the Royal Marsden Hospital, London, through the kind offices of Dr. Sylvia D. Lawler; one fetus was obtained from Chelsea Hospital for Women, London (Mr. John Shepherd). Abortion in all cases had been induced by intra-amniotic injection of 80 g urea in 200 ml Hartmann's solution, followed by 5 mg prostaglandin E_2 [11]. Six fetuses were male and one female; the mean crown–rump length was 16.3 cm (range 15–17.7 cm) and the mean weight 343 g (range 259–450 g). Two fetuses were moderately macerated, with the abdominal cavity full of blood clot, but the livers were intact. In one of them, one lung was grossly haemorrhagic, and tissue was taken from the other lung, which was spottily haemorrhagic. Skeletal muscles used were pectoral and thigh, assayed separately; activities shown are means of pooled values. Tissues of the digestive tract were split open and washed thoroughly in running water before treatment as described below. The fetuses were kept at 2–4° up to the time of dissection, which was carried out by one of us (R.L.) within 12 hr of extraction. Except for lung, liver, skin, scalp and muscle, the organs were homogenized in their entirety. The entire brain was coarsely mixed and an aliquot taken for homogenization and assay.

(b) *Neonatal.* In this group were two stillbirths (one male, one female) and one male neonate, a

premature of 34 weeks gestational age who died at 7 days. In the interval between death and dissection (post-mortem lag, 50–64 hr), the bodies were kept at 2–4°. Birth weights were 2845, 4160 and 2060 g and crown–rump lengths 34, 38 and 32.3 cm, respectively. The brain region assayed was the frontal cortex; heart tissue was a section of the left ventricle. Renal cortex and some medulla were used. The data for skeletal muscle are means of pooled values of the pectoral and thigh muscles, assayed individually. Tissues of the digestive tract were split open and their entire thickness used, after washing as described above. Subsequent treatment was as described under "Freezing and homogenizing procedures".

(c) *Adult.* Tissues from adults were obtained at autopsy from three Coroner's cases, two male and one female, aged 64, 55 and 68 yr, respectively. Liver and aorta were used from three and one additional autopsies, respectively. All died from coronary heart disease; no evidence of neoplastic or infectious disease was found in any of them. Post-mortem lag was 12–36 hr; in the interval the bodies were kept at 2–4°. As with the neonatal tissues, frontal cortex and left ventricular region represented brain and heart; renal cortex, wedge-shaped sections of the adrenals and the sharp edge of liver and lung were used. Atherosclerotic lesions were seen in all aortae; these were avoided and tissue specimens taken from apparently normal regions. Abdominal skin was freed from fat and subcutaneous tissue at dissection. Tissues of the digestive tract were whole wall thickness of all but the colon, where taenia coli were avoided. These tissues were scraped on the mucosal side and thoroughly washed in running cold water before treatment as described under "Freezing and homogenizing procedures".

It is well known that autolysis sets in immediately after death and sometimes earlier. It would not, of course, occur uniformly throughout the digestive tract and this may, indeed, provide a partial explanation for the variability in activity of the parts of the digestive tract examined. Nevertheless, in order to get some approximate idea of activity, it was decided to proceed with the assay as though breakdown of tissues occurred uniformly. Any effect of the intestinal flora must also be taken into account. However, since all the tissues were treated similarly, emptied of contents and the mucosal surfaces scraped and thoroughly washed, we hoped that any enzyme activity deriving from bacteria would be reduced to a minimum.

Pectoral and psoas muscles were examined individually and the values presented are means of the two.

Freezing and homogenizing procedures

All tissues were treated in identical fashion. At dissection, they were freed from blood by rinsing in cold 0.9% saline, dried between layers of filter paper, coarsely minced with scissors or scalpel blade, and quick-frozen in solid CO_2 (−80°). Within a few days they were pulverized as described earlier [1] and homogenized at 0° in an Ultra-Turrax homogenizer with a 10-N shaft (Sartorius Instruments, Surrey, U.K.) for 5–10 sec at top speed. A 10% (w/v) homogenate was prepared with 0.1 M potassium phosphate buffer (K_2HPO_4/KH_2PO_4, pH 7.2), divided into aliquots and stored at −20° until required.

Assay procedures

The basic procedure employed was the radiometric microassay (extraction method) described earlier [1]. With Bz as substrate, the assay tube contained, in a total volume of 240 μl: 0.1 M Tris buffer (pH 9.0), 100 μl; inhibitor solution or water, 100 μl; tissue homogenate, 20 μl; and substrate, 20 μl. With PEA or 5-HT as substrates, the assay tube contained, in a total volume of 140 μl: 0.1 M potassium phosphate buffer (pH 7.2), 100 μl; tissue homogenate, 20 μl; and substrate, 20 μl. The final concentration of substrate in the assay tube was 42 μM for Bz, 150 μM for PEA and 371 μM for 5-HT. Inhibitors used were (-)-deprenyl (final concentration 4×10^{-7}M) or phenelzine (final concentration 2.2×10^{-6}M). Water blanks were prepared by substituting water for tissue homogenate. Where inhibitors were used, the mixture was preincubated at room temperature for 20 min before labelled substrate was added. Incubation was carried out for 30 min in a shaking water bath at 37°, and the reaction stopped by adding 2 M citric acid (100 μl) to the solution, which was immediately mixed on a Vortex mixer at top speed. Three millilitres of toluene (Bz, PEA) or ethyl acetate/toluene 1:1 (5-HT) was used for extraction. The remaining steps were as described previously [1]. Blanks gave radioactivity counts representing the following percentages of total substrate activity: Bz≤0.5 per cent, PEA≤3 per cent, 5-HT≤0.01 per cent. Extraction efficiency with the three substrates, tested in 4–6 different human tissues, gave closely similar values for all tissues with each substrate. The mean extraction efficiency was 95, 96.2 and 91.2 per cent, respectively, for Bz, PEA and 5-HT, and has been corrected for in the specific enzyme activities quoted in this paper.

Both Bz and PEA were used to determine MAO B activity in each tissue tested. Phenelzine, used in all experiments as an inhibitor of BzAO, was potent, but relatively non-selective, with poor replication of results. Whilst it served to confirm the presence of BzAO at the concentration employed, it was not able to separate the different enzyme forms as successfully as deprenyl or clorgyline [12].

All assays were carried out at least in duplicate; results express the means of these replicates. All assays on every series of tissues from a particular subject were carried out on the same day. The Lowry method [13] was used to determine total tissue protein, with bovine serum albumin as standard.

Estimation of total tissue or organ activity

This was based on total protein content of the tissue, multiplied by specific activity of the particular enzyme. The values for organ or tissue percentages of total body weight used in these calculations (Table 5) were taken from various sources [14–19]. Where such data were unobtainable (e.g. for the digestive tract), they were supplemented by our own measurements and those kindly supplied for some fetal tissues by the fetal tissue bank of the Royal Marsden Hospital (Dr. L. Wong).

Total body weights used in these calculations were as follows: fetus, 300 g; neonate, 3000 g; adult, 70 kg.

Variability of method

Replicate determinations on aliquots of 2 neonatal tissues (lung = 11; liver = 10) provided additional controls of substrate and inhibitor efficacy. One previously unthawed aliquot of each of these tissue homogenates was assayed together with a series of tissues. Mean specific activity of lung was 22.7 (±6%), 2.5 (±6%), 5.3 (±5%) and 25.8 (±8%) nmoles/mg protein/30 min for deprenyl-resistant Bz-oxidizing activity, D*Bz (deprenyl-sensitive Bz-oxidizing activity), PEA and 5-HT, respectively. The corresponding values for liver were 4.1 (±5%), 77.4 (±5%), 22.9 (±5%) and 56.3 (±6%), respectively. Percentages between brackets represent the standard error of the mean.

RESULTS

We have interpreted the enzyme activities observed with Bz, PEA and 5-HT as follows:

(a) Bz—activity insensitive to 4×10^{-7}M deprenyl = BzAO;
(b) Bz—activity sensitive to 4×10^{-7}M deprenyl = MAO B;
(c) PEA—activity in all human tissues except vessels, placenta and (partly) lung = MAO B;
(d) 5-HT—activity in all human tissues = MAO A.

Bz is a substrate for both BzAO and MAO B [1], but is a very poor substrate for MAO A [12]. Both PEA and 5-HT can be substrates for MAO A and B, and at higher substrate concentrations each becomes less specific [20]. The use of a particular substrate at a single concentration may therefore give only an approximate indication of the activity of a particular enzyme form.

Concerning (a) above, it is true that 10^{-4}M deprenyl inhibits both MAO A and B activity, whereas 10^{-7}M will inhibit the latter but not the former. We have chosen the lower concentration, however, because fetal tissues seem to be more sensitive to the inhibitor than neonatal or adult tissues: in several experiments on fetal tissues with 10^{-4}M deprenyl, Bz-oxidizing activity was inhibited in all tissues to a similar degree. Moreover, in the adult human lung, rich in MAO A, there was no difference in Bz activity at 10^{-7} and 10^{-4}M deprenyl [12]. The question arises, however, whether the deprenyl-insensitive Bz activity we detect could be partly catalyzed by MAO A. We think it very unlikely that MAO A contributes more than a trace of activity because we have shown that Bz is a very poor substrate for human MAO A, e.g. in placenta (K_m = 400 μM; V_{max} = 0.7 per cent of that for 5-HT) [12]. Moreover, we have found no relation between the distribution patterns of MAO A and deprenyl-insensitive Bz activity; in fetal brain and adult cerebral vessels, both totally insensitive to deprenyl, the ratio of Bz to 5-HT-oxidizing activity was 0.03 and 5.8, respectively (see also Discussion).

As for (c) above, both our work and that of others [12, 21–23] has produced evidence for major or total deamination of PEA by MAO A in a few tissues only. In the vast majority of tissues examined by us, the bulk of activity elicited with PEA as substrate

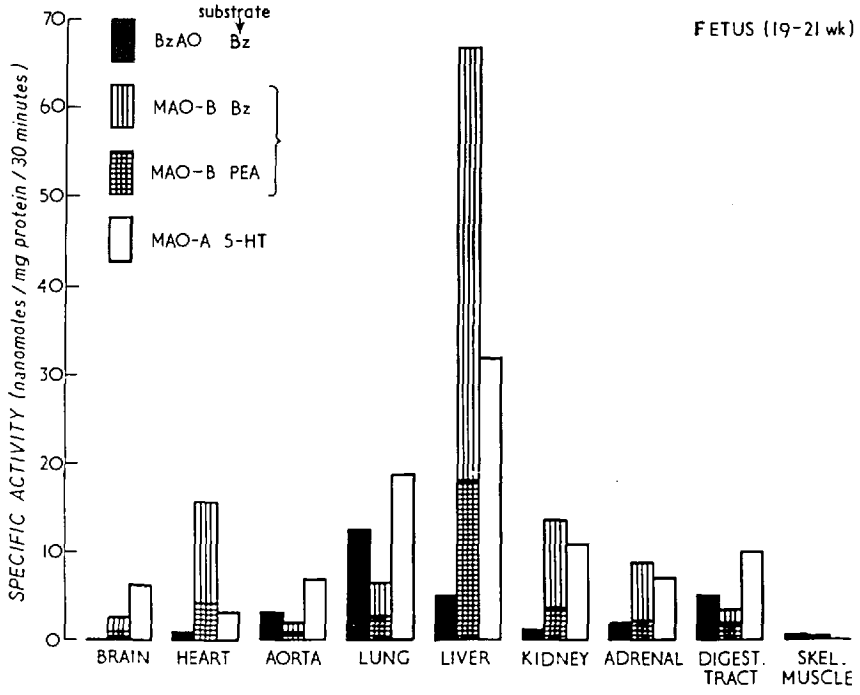

Fig. 1. Distribution of BzAO, MAO B and MAO A in the human fetus (19–21 weeks). For assay conditions, see Materials and Methods. Middle column shows MAO B activity assayed with Bz and PEA; total height of column = D*Bz, deprenyl-sensitive moiety of benzylamine-oxidizing activity; cross-hatched portion = activity registered with PEA as substrate. For interpretation of PEA activity, see text.

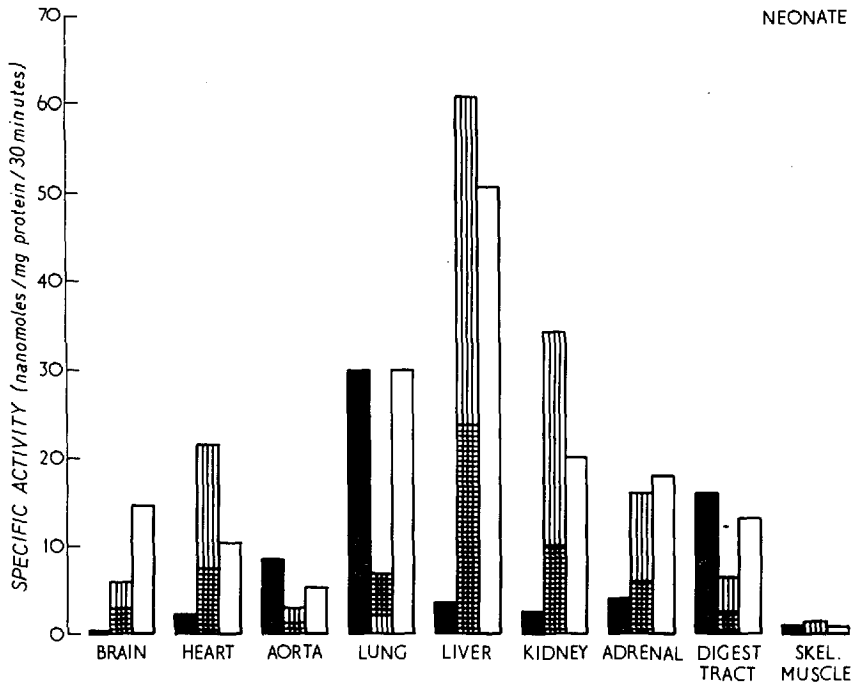

Fig. 2. Distribution of BzAO, MAO B and MAO A in the human neonate. Legends and observation as in Fig. 1.

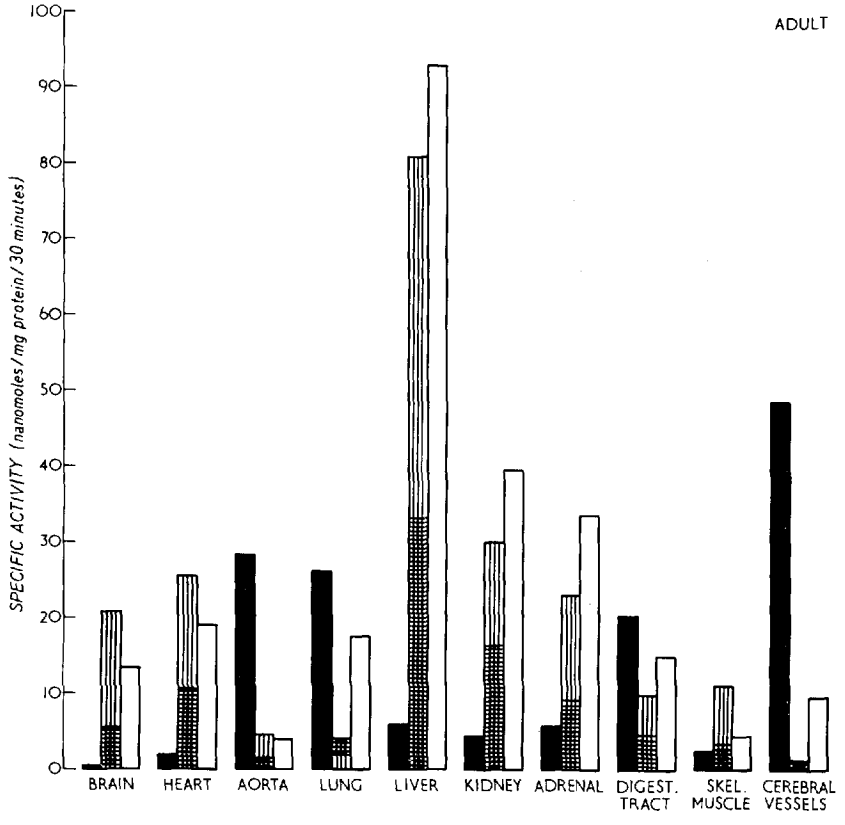

Fig. 3. Distribution of BzAO, MAO B and MAO A in the human adult. Legends and observation as in Fig. 1.

was in all likelihood predominantly due to MAO B. Though variable in the various tissues, the D*Bz/PEA ratio [mean 2.74 ± 0.24 (S.E. of mean), 2.66 ± 0.31 and 2.48 ± 0.21 for fetal, neonatal and adult tissues, respectively] was significantly different only in umbilical vessels (0.4), placenta (0.26), lung (0.4) and one or two other tissues (see ref.12). A low specific activity was found in cerebral vessels with PEA as substrate, but D*Bz gave no detectable activity. On the other hand, no activity could be registered with PEA as substrate in several tissues with low D*Bz values, such as fetal tongue, fetal and neonatal muscle and skin, adult skin and a few others. Oxidation of PEA is undoubtedly less sensitive or reliable than D*Bz as a measure of MAO B activity; nevertheless we feel justified in using PEA-deamination in most human tissues as a rough indication of the activity of MAO B.

The specificity of 5-HT as a substrate for MAO A, on the other hand, is well established, notwithstanding several reports on the deamination of the substrate by MAO B. Edwards and Chang [20] considered 5-HT a poor substrate for MAO B in the human platelet, since its K_m was much higher than that observed for either rabbit platelets or rabbit brain regions. Other authors have shown 5-HT oxidation by MAO B in beef heart [24], certain circumventricular regions of the rat [25] and rat area postrema [26]. From all these reports it seems obvious, however, that the contribution of MAO B to the deamination of 5-HT is slight, unless high concentrations of 5-HT are employed. At the substrate concentration used in our experiments, it is likely that 5-HT was deaminated almost exclusively by MAO A.

Enzyme distribution

The distribution of BzAO, MAO A and MAO B in human tissues at three stages of development is shown in Tables 1–3. All three enzyme forms were detectable in every tissue studied, apart from MAO B in cerebral vessels. It is clear from these tables and Figs. 1–3 that each has its distinctive distribution and developmental pattern. In almost all tissues, the specific activity is lowest in the fetus, intermediate in the neonate and highest in the adult. In certain instances, such as BzAO in aorta or MAO B in brain and skeletal muscle, there is a 10-fold or greater increase in specific activity between fetus and adult. In no case (except for MAO B in lung) is there any substantial decrease with age. The developmental pattern varies from tissue to tissue, however, and there is relatively little change, for example, in the specific activity of the three enzyme forms between fetus and adult in the heart, and even less between the specific activity of BzAO and MAO B in fetal and adult liver.

Figures 1–3 show activity obtained with PEA, compared with deprenyl-sensitive activity using Bz as substrate (D*Bz) as a measure of MAO B. D*Bz gives a higher specific activity than PEA throughout (see above).

BzAO (Table 1). The findings in the present study confirm and extend our earlier data [1]. BzAO is most active in adult cerebral vessels (not done in fetus or neonate), by far the highest value for this enzyme in any human tissue. Activity is also high in aorta, lung and digestive tract. BzAO activity in blood vessels, which we commented upon earlier [1], is the subject of a separate study (in preparation). The extremely low activity in brain, the lowest of any adult tissue studied, may derive from traces of vascular tissue. Specific activity is also low in liver, heart, kidney and skeletal muscle, as well as fetal skin. Values for fetal scalp (not done in neonate or adult) are similar to those for other skin areas.

Table 1. Specific activity of BzAO in human tissues (nmoles/mg protein/30 min). Residual activity with Bz as substrate (42 μM) after inhibition with deprenyl (4×10^{-7}M)

Tissue	Fetal		Neonatal		Adult	
	Mean	Range	Mean	Range	Mean	Range
Brain	0.2	0.1–0.4	0.4	0.36–0.44	0.5	0.4–0.6
Cerebral vessels					47.8	33.2–55.3
Heart	1.0	0.3–1.5	2.1	1.6–2.8	1.9	1.5–2.8
Aorta	2.9	0.6–5.7	8.0	4.0–11.0	27.9	20.0–41.1
Lung	12.5	7.4–19.2	29.7	22.9–38.1	25.4	19.8–31.0
Liver	5.2	3.4–6.8	3.4	2.2–5.0	6.0	3.4–13.8
Kidney	0.8	0.1–1.3	2.4	1.4–3.2	4.6	3.5–5.3
Adrenal	2.1	0.5–3.9	3.9	2.1–6.8	5.2	2.5–7.9
Spleen	0.5	0.3–0.8	1.0	0.8–1.3	2.8	2.3–3.2
Tongue	0.6	0.1–1.6	1.3	0.6–2.1	4.1	3.8–4.4
Oesophagus	9.7	6.8–15.6	11.3	7.0–16.8	23.0	22.1–24.0
Stomach	5.3	0.7–12.3	13.5	7.1–21.5	19.7	16.8–24.1
Ileum	2.7	1.7–4.2	17.8	8.7–24.2	15.6	9.6–20.6
Colon	2.5	0.5–4.7	20.1	14.0–29.5	19.2	11.4–26.9
Mesentery and mesocolon*	5.0	1.9–8.2	6.5	2.8–16.6	23.5	22.6–24.3
Pancreas	0.8	0.4–1.3	2.8	1.2–4.0	4.5	N=1
Skeletal muscle*	0.6	0.4–0.7	0.9	0.5–1.4	2.0	1.4–2.9
Diaphragm	0.7	0.1–1.4	2.3	1.8–2.8	3.3	1.5–4.1
Skin	0.5	0.02–1.3	2.7	1.3–4.6	1.7	1.1–2.4

* Pooled values from individual assays (see Materials and Methods, Tissues). For assay procedure, see Materials and Methods.

Table 2. Specific activity of MAO B in human tissues (nmoles/mg protein/30 min)*

Tissue	Fetal Mean	Fetal Range	Neonatal Mean	Neonatal Range	Adult Mean	Adult Range
Brain	2.7	1.6–4.1	6.1	5.4–7.0	21.1	13.8–29.4
Cerebral vessels					‡	‡
Heart	15.4	7.1–19.8	21.4	20.5–22.6	25.2	23.5–28.3
Aorta	2.2	0.8–3.9	3.1	2.0–4.2	4.4	1.8–7.0
Lung	6.6	4.2–9.1	2.4	0–4.9	1.8	0–3.5
Liver	66.4	50.4–99.2	59.8	51.4–66.4	80.0	49.4–123.6
Kidney	14.1	6.0–24.8	33.5	22.6–49.2	29.5	24.1–32.8
Adrenal	7.8	4.9–10.1	16.6	10.1–20.2	22.6	22.4–22.8
Spleen	1.3	0.1–2.8	0.5	0.5–0.6	3.4	2.6–4.3
Tongue	0.6	0.3–0.9	3.5	2.0–4.7	8.2	7.2–8.7
Oesophagus	1.8	0.5–2.6	6.6	4.7–8.9	8.5	6.7–10.0
Stomach	3.0	1.1–5.9	3.4	1.1–6.2	8.4	5.9–12.7
Ileum	2.8	2.7–3.0	10.1	4.6–17.5	18.1	12.0–27.3
Colon	3.1	1.2–6.1	3.4	3.1–3.9	11.8	9.7–13.9
Mesentery and mesocolon†	8.1	3.4–10.8	3.9	2.3–6.0	12.1	11.1–13.1
Pancreas	4.4	2.4–5.7	3.4	2.3–4.7	3.0	N = 1
Skeletal muscle†	0.4	0.2–0.7	1.6	0.9–2.2	11.2	10.9–11.8
Diaphragm	0.8	0.1–1.7	9.3	7.5–11.2	10.9	8.4–13.3
Skin	0.5	0.2–0.9	2.7	1.9–3.2	1.7	1.4–1.9

* Activity expresses moiety sensitive to 4×10^{-7}M deprenyl (D*Bz), with Bz as substrate (42 μM). For assay procedure, see Materials and Methods.
† See footnote, Table 1.
‡ No detectable activity.

MAO B (Table 2). Liver, at all stages of development, showed the highest specific activity for MAO B of any human tissue studied. While there is virtually no change in specific activity of cardiac MAO B from fetus to adult, activity in kidney and adrenal increases sharply. In brain, MAO A predominates at the fetal and neonatal stages, but MAO B becomes dominant in the adult. Adult kidney, heart, adrenal and brain are relatively rich in MAO B; considerable activity is also found in adult digestive tract and skeletal muscle.

MAO A (Table 3). In fetal brain, MAO A emerges before MAO B. Specific activity of hepatic MAO A rises steadily from fetal to adult stage. Excepting only the term human placenta [12], adult liver is the human tissue showing by far the highest activity of MAO A. Although all fetal MAO A specific activity is relatively low, compared with the adult, fetal liver, lung, kidney, adrenal, aorta and digestive tract show relatively high values. Peak MAO A activity in brain, lung and colon is seen in the neonate.

Ratio MAO A/B (Table 4). The relative tissue pro-

Table 3. Specific activity of MAO A in human tissues (nmoles/mg protein/30 min)*

Tissue	Fetal Mean	Fetal Range	Neonatal Mean	Neonatal Range	Adult Mean	Adult Range
Brain	6.7	3.6–13.5	14.5	11.5–18.9	12.9	8.7–16.9
Cerebral vessels					8.3	6.4–11.3
Heart	2.9	2.1–4.0	10.2	8.7–10.9	19.0	15.7–23.8
Aorta	6.8	2.4–15.8	5.1	4.0–6.0	4.1	3.2–5.6
Lung	18.2	14.2–21.8	29.9	25.8–36.9	17.4	9.4–25.8
Liver	31.4	14.6–55.0	50.2	27.4–74.8	92.0	29.8–215.4
Kidney	11.0	7.5–14.3	19.6	9.3–25.7	38.8	37.1–41.9
Adrenal	7.1	4.8–8.0	17.5	5.1–28.3	33.4	30.3–36.5
Spleen	3.8	2.9–5.1	0.8	0.3–1.2	1.8	1.2–2.4
Tongue	1.8	0.7–2.5	3.9	1.6–6.2	12.9	12.4–13.1
Oesophagus	15.6	10.3–24.0	7.8	4.4–12.5	18.6	10.2–21.6
Stomach	11.2	8.9–13.4	9.1	8.0–11.1	8.2	7.1–9.7
Ileum	4.3	3.8–5.2	13.5	11.6–16.4	15.8	12.7–17.7
Colon	6.8	2.5–10.9	19.8	15.5–25.2	13.5	13.1–13.8
Mesentery and mesocolon†	11.7	6.7–19.4	4.9	3.5–5.7	28.0	21.9–34.0
Pancreas	17.9	11.2–20.3	12.4	7.3–19.6	26.3	N = 1
Skeletal muscle†	0.3	0.2–0.7	0.6	0.2–1.3	4.3	1.8–6.4
Diaphragm	1.1	0.6–3.1	3.6	1.5–5.7	6.8	5.6–7.9
Skin	2.0	0.3–3.0	4.5	3.8–5.9	0.7	0.7–0.8

* Substrate, 5-HT (371 μM). No inhibitor. For assay procedure, see Materials and Methods.
† See footnote, Table 1.

Table 4. Ratio MAO A/B (5-HT/D*Bz)*

Tissue	Fetal	Neonatal	Adult
Brain	2.43	2.39	0.61
Cerebral vessels			‡
Heart	0.19	0.47	0.75
Aorta	3.15	1.65	0.93
Lung	2.77	12.27	9.78
Liver	0.47	0.84	1.15
Kidney	0.78	0.59	1.31
Adrenal	0.91	1.06	1.48
Spleen	2.98	1.44	0.52
Tongue	2.88	1.12	1.58
Oesophagus	8.46	1.17	2.56
Stomach	3.78	2.65	1.0
Ileum	1.53	1.35	0.87
Colon	2.22	5.77	1.14
Mesentery and mesocolon†	1.45	1.26	2.31
Pancreas	4.08	3.62	8.89
Skeletal muscle†	0.75	0.41	0.39
Diaphragm	1.31	0.39	0.62
Skin	4.39	1.70	0.44

* Substrates as in Tables 2 and 3.
† See footnote, Table 1.
‡ No detectable MAO B activity.

portions of MAO A and B, as determined by the 5-HT/D*Bz ratio, are shown in Table 4. In general, the A/B ratio is higher in fetus than adult. This phenomenon is particularly noticeable in brain and oesophagus, where the A/B ratio in the fetus is 4 times greater than that of the adult; the ratio in skin is ten times greater in fetus than adult. In the adult, with the exception of placenta (see ref. 12), the lung has the highest A/B ratio.

Total activity in tissue or organ (Table 5). Although specific enzyme activity in a given tissue may be low, the activity of the enzyme in the total tissue or organ mass—and, therefore, in the total body economy—may be considerable, as shown in Table 5. Obviously, estimates of total activity such as those shown can, at best, be only a rough approximation and should be approached as indicating orders of magnitude rather than precise values.

DISCUSSION

It is evident from the data presented here that the distribution and development of the copper enzyme [4] BzAO, and the flavin enzyme forms [4] MAO A and B differ in every tissue.

By demonstrating a series of ontogenetic differences not only between BzAO and monoamine oxidase, but also between MAO A and MAO B, support may be provided for the view that MAO A and MAO B are different functional enzymic entities. We have also produced evidence to show that Bz (D*Bz) is a far more sensitive and reliable substrate for MAO B than PEA (Figs. 1–3). In general, D*Bz measurement gives higher absolute values for MAO B specific activity than PEA. An exception is the lung in neonate and adult (Figs. 2 and 3). As we have shown [12], PEA in adult lung preparations is metabolized partly by MAO A and partly by MAO B. This experiment was not carried

Table 5. Total enzyme activity of organ or tissue (μ moles)*

Tissue		Per cent of body weight	Increase in total protein (F = 1)	BzAO	MAO B (D*Bz)	MAO B (PEA)	MAO A
Skeletal muscle	F	25		4	3	0	2
	N	25	40	264	438	0	180
	A	43	730	10,602	59,370	16,857	23,006
Liver	F	4		10	133	36	63
	N	4.2	14	94	1634	651	1386
	A	2.1	150	1764	23,520	9643	27,048
Digestive tract	F	3.8		3	3	1	6
	N	3.5	9	145	69	38	142
	A	4.2	300	5027	4259	1728	4136
Lungs	F	2		6	3	1	9
	N	2	16	235	19	55	237
	A	1.2	255	3293	231	530	2258
Brain	F	13.4		0.6	7	2	18
	N	11.7	19	20	308	149	734
	A	2.3	85	119	4667	1216	2850
Kidneys	F	0.7		0.2	3	0.8	2
	N	0.8	17	9	121	35	71
	A	0.4	250	242	1552	859	2040
Heart	F	0.6		0.2	3	1	0.6
	N	0.7	13	6	59	20	28
	A	0.5	260	109	1416	582	1069
Skin	F	13		2	2	0	7
	N	15	10	108	105	0	179
	A	7	120	757	739	0	326
Aorta	A	0.14		251	39	13	37
Plasma	A	4.2		37	—	—	—

* For assay procedure and calculations, see text. F = fetus; N = neonate; A = adult.

out with neonatal lung, although a similar situation seems likely to prevail (Fig. 2). As for adult cerebral vessels (Fig. 3), no D*Bz activity was registered, and the deamination of PEA is undoubtedly catalyzed by MAO A, as in the case of placenta [12].

The D*Bz/PEA ratio varies in the tissues studied (Figs. 1–3). This may be explained in part by MAO B sensitivity towards deprenyl with Bz as substrate, which in most tissues differs with the developmental stage. The main reason, however, may well be that MAO A contributes to a variable extent to PEA deamination in tissues other than the lung and vessels.

The argument that Bz might be oxidized to some extent by MAO A is mainly based on the work of Lyles and Callingham [27–29]. These authors found that rat heart (but not the human heart, ref. 30) has a Bz-oxidizing activity which gives a biphasic curve when clorgyline is used as inhibitor, suggesting the presence of mitochondrial MAO in all the fractions studied. Similar results were obtained in our experiments with human placenta and lung [12]. Since all these tissues are very rich in MAO A, it is not impossible that this enzyme contributed to the deamination of Bz in these experiments. Another explanation, however, is equally plausible: clorgyline may be less selective for MAO A than has been postulated, and in certain tissues it may inhibit the activity of BzAO. Lyles and Callingham [28] do not entirely dismiss this possibility, although they point out that the clorgyline-sensitive moiety of Bz-oxidizing activity, whilst sensitive to semicarbazide, was insensitive to cyanide, a selective inhibitor of plasma amine oxidase. A similar sensitivity pattern, with tyramine as substrate, has been shown by Coquil *et al.* [31] in a component of rat artery amine oxidase, suggesting that rat tissues in general may be insensitive to cyanide.

Support for our interpretation of inhibition of BzAO by clorgyline is afforded by Houslay and Tipton's experiments with a purified preparation of beef plasma and Bz as substrate, in which clorgyline was shown to be a reversible inhibitor of BzAO [32]. These authors stress the need for care in avoiding blood-contamination of the tissues studied. As pointed out in our previous paper, however [1], contamination with blood is unlikely to be a cause of substantial error in the interpretation of results, since the specific activity of plasma BzAO is negligible, compared with that of most tissues. If there is contamination, it is far more likely to be due to the presence of vascular fragments, particularly in crude homogenates, a possibility pointed out by Lyles and Callingham [28].

BzAO. Fragments of vascular tissue may account for the relatively high BzAO value in fetal liver, where specific activities of BzAO and MAO B (with Bz but not PEA as substrates—see Fig. 1) are similar to those found in the adult (Tables 1 and 2, Fig. 3), suggesting that at 19–21 weeks gestation, these enzyme systems have reached a comparable level of maturity. Nor is this surprising, in view of the intense metabolic activity of the fetal liver. Vascularization is well advanced at this stage, when the need for blood vessels must be similar to, if not greater than, that of the adult organ [33, 34]. In the fetus, indeed, total BzAO activity in the liver equals that of all the other main tissues together (Table 5).

Tongue, diaphragm and skeletal muscle show values sufficiently similar at all three stages of development to indicate that BzAO activity in striated muscle does not differ, whatever the origin of the muscle. Conversely, the wide scatter of BzAO values noted in the different parts of the fetal digestive tract narrows with development and, in the adult, specific activities appear to belong to a homogeneous system.

Except for lung, liver, aorta and digestive tract, specific activity of BzAO is very low in the fetus. Evidence continues to mount suggesting an association of this enzyme with blood vessels. The localization of BzAO in blood vessel walls of human placenta and rat liver has recently been demonstrated histochemically in these laboratories: intense staining was present in the media, whilst the endothelium appeared unstained [35]. One possible explanation for the increase in specific activity of BzAO from fetal to adult stage might be the increase in vascularity of the tissues examined. This interpretation may be particularly relevant to the gastric musculature of the fetus, which is somewhat deficient, and the fetal intestine with its relatively greater thickness of mucosa compared to muscle [36].

The virtual absence of BzAO in brain parenchyma is of particular interest, for another copper enzyme of parallel function, diamine oxidase, has also not been demonstrated in this tissue in rabbit, guinea-pig and rat [45].

MAO A and B. Like BzAO, MAO A and B are widely distributed in the fetus. Although MAO B activity in fetal brain is barely demonstrable, specific activity of MAO A at 20 weeks' gestation is already about one-half that of the adult. The emergence of MAO A before MAO B in the fetal brain has also been demonstrated in rat [37] and mouse [38], and seems to cut across the view [39] that MAO B is a pure protein bound in a lipid environment and capable of being converted into MAO A with an additional lipid complement. If this hypothesis were correct, MAO B might have been expected to precede MAO A ontogenetically.

Our results agree with those of Suzuki and Yagi [40], who found MAO A values similar to those shown by us in human fetal and adult brain. However, their values for MAO B using PEA as substrate are higher than ours, perhaps reflecting the higher substrate concentration they employed, which might have resulted in some deamination by MAO A.

Studies of human fetal liver with a variety of substrates [41] have revealed some fluctuation of activity between the 16th and 32nd week of gestation; from then on there was a steady increase until birth. Since Bz, 5-HT and tyramine were among the substrates employed, the enzymes involved were, presumably, MAO B and A.

Our study shows that, in human tissues, the development of the activity of the three enzymes investigated is related to functional rather than morphological maturity. Differing rates of functional and morphological ontogenesis of enzyme systems are well described in man [36, 42] and rat [33, 43, 44] and seem likely to represent a widespread phenomenon.

Specific activity of MAO A in fetal liver is one third that of the adult, whilst that of pulmonary MAO A, similar in fetus and adult, shows a rise in the neonate. Perhaps the fetal lung and digestive tract contribute to the deamination of 5-HT to a relatively greater extent than in the adult, with the lung playing a particularly prominent role.

The widespread distribution of MAO B is perhaps more puzzling. Because of the absence of diamine oxidase in brain [45], mentioned above, one of its major functions may be related to the metabolism of *tele-N*-methylhistamine, the major histamine metabolite in this tissue. Recent research has shown that *tele*-methylhistamine is a specific substrate for MAO B in both rat [46, 47] and man (J. D. Elsworth, V. Glover and M. Sandler, in preparation). It would be of interest to determine the extent to which peripheral MAO B, for example in skeletal muscle, is present to inactivate local *tele*-methylhistamine produced from the decarboxylation of muscle *tele*-methylhistidine [48, 49].

Total enzyme activity. The estimated total activity of the three enzymes (Table 5), most striking in skeletal muscle, deserves comment. The very large increase in activity between fetus and adult seems to shed new light on the importance of these enzymes, and, in particular, of skeletal muscle amine oxidase, in the total body economy. No doubt the increase in mass from accretional growth accounts in part for the increase from total fetal to adult activity seen e.g. in skeletal muscle and digestive tract.† Increase in vascular tissue probably also contributes substantially to the increase both in specific and total activity of BzAO between fetus and adult. The total activity of MAO B (D*Bz) in adult skeletal muscle is about twice the total activity of this enzyme in all the other major organs. Even with PEA as substrate, the total value for MAO B in skeletal muscle exceeds the activity of all the other tissues together (Table 5). Estimated total activity of MAO A in skeletal muscle is second only to that in the liver, in which the specific activity of MAO A is so high that the 1.5 kg mass of the adult produces a greater total activity than the 20-fold larger mass of skeletal muscle. On the other hand, the entire digestive tract develops only about 15 per cent of the MAO A activity of the adult liver.

Despite their semiquantitative nature, these figures may provide a more realistic insight into the relative importance of enzyme activity in the round, compared with the more limited perspective provided by specific activity alone.

Far from simplifying the issue, these observations seem to add a further dimension of complexity to our knowledge of the amine oxidases in the human body. One conclusion, however, is implicit in the relatively low specific activity of all three enzymes in fetal tissues: neither BzAO nor MAO A or B can be related to rapid growth in tissues *per se*.

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† Including oesophagus, stomach, intestine and mesentery.

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A Coupled Peroxidatic Oxidation Technique for the Histochemical Localization of Monoamine Oxidase A and B and Benzylamine Oxidase

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Summary. A coupled peroxidatic oxidation technique is presented which employs benzylamine and tyramine as substrates and clorgyline, deprenyl, phenelzine and pargyline as specific inhibitors. Using this technique with frozen sections of human term placenta and rat liver, the histochemical localization of monoamine oxidase A and B and benzylamine oxidase has been demonstrated.

Introduction

There are many questions about the flavine enzyme, monoamine oxidase (MAO) (see Costa and Sandler, 1972; Wolstenholme and Knight, 1976) and the copper-dependent benzylamine oxidase (BzAO) (Lewinsohn et al., 1978) for which histochemical studies may help to provide answers. MAO is thought to be concerned with the degradation of potentially toxic dietary amines and with inactivation of certain endogenous neurotransmitter substrates. Thus, its high activity in intestine, liver and brain are easy to explain, but its function in kidney or muscle is quite unclear. Little, too, is known of the precise in vivo role of the multiple forms of MAO, which may be classified into two types, A and B, according to their sensitivity to the selective inhibitor, clorgyline (Johnston, 1968). MAO A which, in general, preferentially metabolises 5-hydroxytryptamine and noradrenaline, is specifically inhibited by 10^{-7} M clorgyline. β-Phenylethylamine and benzylamine are preferred substrates for MAO B (Houslay and Tipton, 1974) which is relatively insensitive to clorgyline but specifically inhibited by 10^{-7} M deprenyl (Knoll, 1976). 10^{-3} M pargyline inhibits both MAO A and MAO B. The substrate specificity of these forms varies from species to species and the distribution from tissue to tissue (Fowler et al., 1978). Some knowledge of the exact localization of the two forms might well help to shed light on their function.

If our knowledge of MAO function is scanty, the role of BzAO in the body is virtually unknown. This enzyme is quite distinct from MAO B with which it shares its major substrate, benzylamine (Lewinsohn et al., 1978). Bioche-

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mically the two can be distinguished by the use of 10^{-7} M deprenyl, which selectively inhibits MAO B, and 5×10^{-5} M phenelzine which inhibits BzAO.

The histochemical localization of MAO has been widely achieved with the tetrazolium method of Glenner et al. (1957) which Williams et al. (1975) have adapted in an attempt to localize the two forms of MAO. There are several problems with tetrazolium methods, however: on gels, the intensity of staining does not correspond with the known enzyme activity (Youdim and Lagnado, 1972); in skin, it has been shown that formazan dye generation can proceed without added substrate (Scheidegger, 1967); most unfortunately of all it is not possible to use either phenylethylamine or benzylamine, the "standard" MAO B substrates, in a tetrazolium method. For these reasons we felt there was need for some alternative histochemical technique. Such an alternative is the coupled peroxidatic oxidation approach of Graham and Karnovsky (1965). When originally proposed, it was thought inferior to the tetrazolium method since its reaction product is more difficult to visualise and photograph. A recent publication by Williams et al. (1979) has demonstrated that the substrate benzylamine can be used effectively with this technique. We demonstrate here too, that a modification of the Graham and Karnovsky procedure, with appropriate substrates and inhibitors, can be used both to localize and to discriminate between MAO A, MAO B and benzylamine oxidase in rat liver and in human term placenta. These tissues were chosen to illustrate the present study as we have a detailed biochemical knowledge of the enzymes they contain.

Methods and Materials

Fresh rat liver and human term placenta were quenched in liquid nitrogen and then allowed to equilibrate at −25° C prior to cutting 18–20 μm frozen cryostat sections. The sections were mounted on glass slides and allowed to dry in air at room temperature for 15–60 min. They were incubated in the following media (modified from the method of Graham and Karnovsky, 1965).

Standard incubation medium:
3-Amino-9-ethyl carbazole (Sigma), 2 mg.
Dimethyl formamide (Analar), 0.5 ml.
Phosphate buffer 0.05 M, pH 7.6, 9.5 ml.
Mixture shaken, filtered and added to:
Peroxidase (Sigma type II), 10 mg.
Substrate (either tyramine HCl (Sigma), β-phenylethylamine (Sigma), tryptamine HCl (Sigma) or benzylamine HCl (prepared from free base)), 12 mg
Incubation was for 45–90 min at either 22° C or 37° C.

Inhibitor Controls: Sections were preincubated for 15 min in each of the following inhibitors made up in 0.05 M phosphate buffer, pH 7.6. The same concentration of inhibitor was incorporated into the standard medium.
Clorgyline: 10^{-7} M, 10^{-8} M, 10^{-9} M (final concentration)
Deprenyl: 10^{-6} M, 10^{-7} M, 10^{-8} M
Pargyline: 10^{-3} M
Phenelzine (placental sections only): 5×10^{-5} M.

No Substrate Control: Sections were incubated in a substrate-free medium.

Cold Acetone Control: To avoid the effect of the solubility of the reaction product in lipid, a series of sections was treated for 15 min in cold acetone and washed in 0.9% (w/v) NaCl solution before incubation.

After incubation, sections were washed in 0.9% (w/v) NaCl solution and post-fixed for approximately 2 h in 10% formalin. They were mounted in glycerol jelly.

Results

With both tissues investigated, the red/brown reaction product was visible only in the presence of substrate. The results are summarized in Table 1. In placenta, the staining was highly localized; using benzylamine as substrate, reaction product was present in the tunica media of the blood vessels in the larger villi (Fig. 1) and appeared to be associated with the smooth muscle. This activity was inhibited by 5×10^{-5} M phenelzine, but was insensitive to 10^{-7} M deprenyl and 10^{-3} M pargyline. With tyramine, phenylethylamine and tryptamine as substrates, reaction was confined to the trophoblastic layer of the villi (Fig.

Table 1. Effect of different substrates and inhibitors on the deposition of reaction product

Substrate	Inhibitor	Rat liver parenchyma	Placenta trophoblast	Placenta vessels
Benzylamine	–	+++	0	+++
,,	Deprenyl, 10^{-6} M	0	–	+++
,,	Deprenyl, 10^{-7} M	0	–	+++
,,	Deprenyl, 10^{-8} M	+	–	+++
,,	Clorgyline, 10^{-7} M	+++	–	+++
,,	Clorgyline, 10^{-8} M	+++	–	+++
,,	Clorgyline, 10^{-9} M	+++	–	+++
,,	Pargyline, 10^{-3} M	0	–	+++
,,	Phenelzine, 5×10^{-5} M	–	0	0
Tyramine	–	+++	+++	0
,,	Deprenyl, 10^{-6} M	++	++	–
,,	Deprenyl, 10^{-7} M	++	+++	–
,,	Deprenyl, 10^{-8} M	+++	+++	–
,,	Clorgyline, 10^{-7} M	+	0	–
,,	Clorgyline, 10^{-8} M	++	+	–
,,	Clorgyline, 10^{-9} M	+++	++	–
,,	Pargyline, 10^{-3} M	0	0	–
,,	Clorgyline, 10^{-7} M and Deprenyl, 10^{-7} M	0	0	–
β-Phenylethylamine	–	+++	+++	0
,,	Deprenyl, 10^{-6} M	++	+++	–
,,	Clorgyline, 10^{-6} M	+	0	–
,,	Pargyline, 10^{-3} M	0	0	–
Tryptamine	–	+++	+++	0
No substrate	–	0	0	0

Key: +++ = strong reaction, ++ = moderate reaction, + = slight reaction, 0 = no reaction, – = not tested

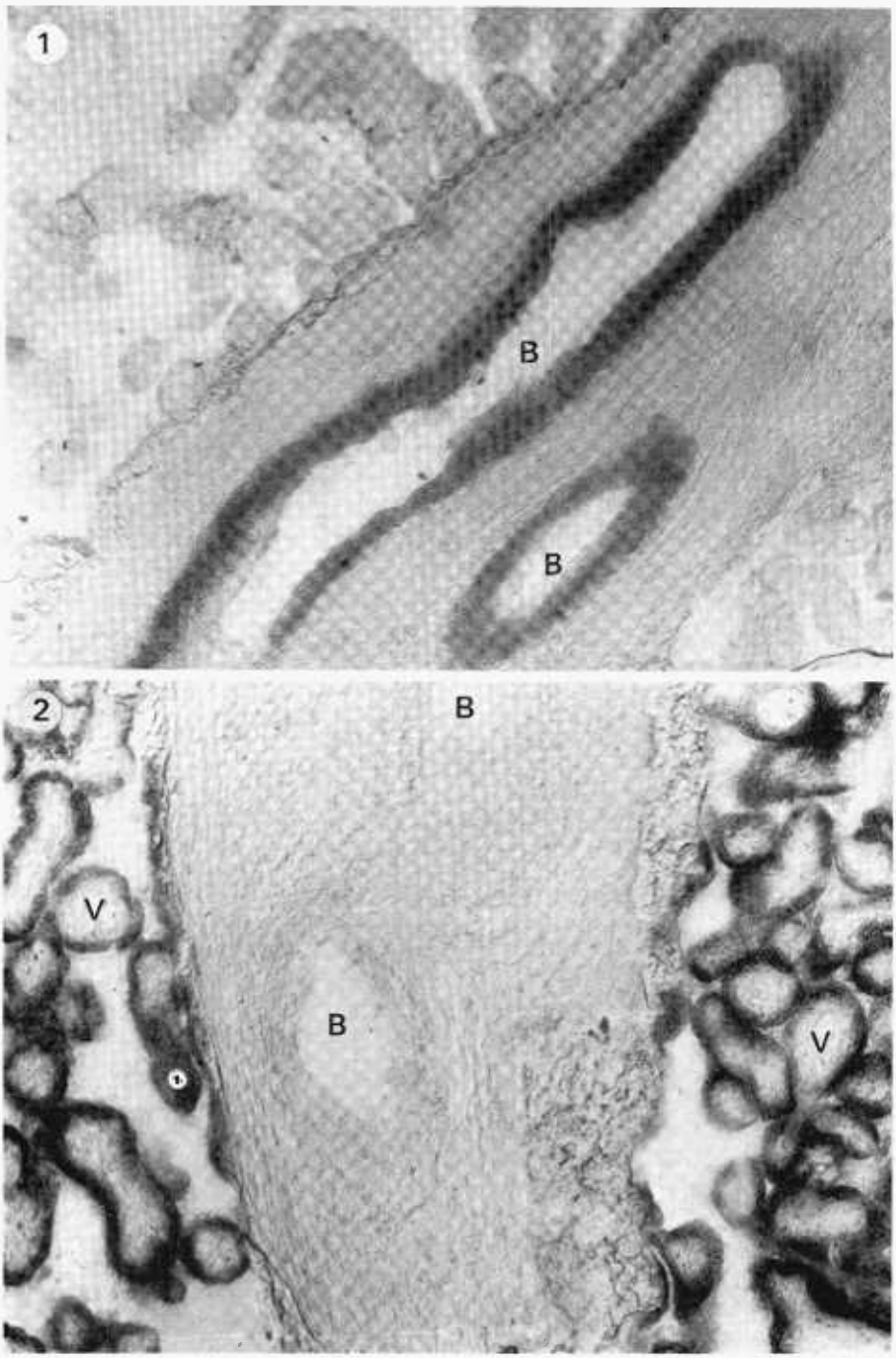

Fig. 1. Histochemical localization of benzylamine oxidase activity in a cryostat section of human term placenta. Reaction product strongly stains the tunica media of the blood vessels (*B*) in the larger villi. The smaller villi are unstained. Substrate: benzylamine. Magnification × 110

Fig. 2. The localization of monoamine oxidase A in the trophoblastic layer of the chorionic villi (*V*) in human placenta. The blood vessels (*B*) are unstained. Substrate: tyramine. Magnification × 140

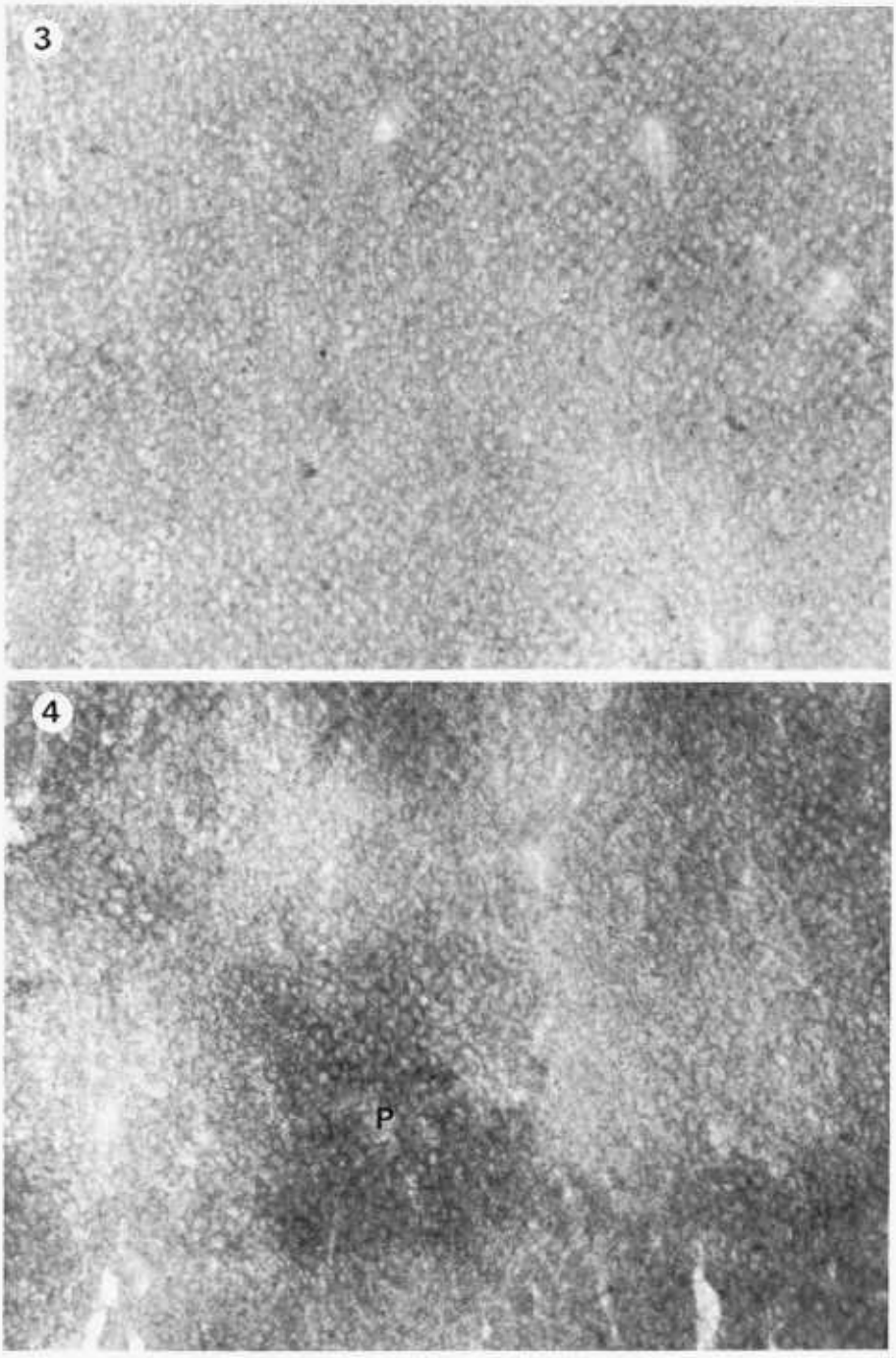

Fig. 3. The localization of monoamine oxidase in rat liver. In this instance the reaction product is evenly distributed throughout the parenchyma. Substrate: tyramine. Magnification × 110

Fig. 4. The histochemical localization of monoamine oxidase in rat liver using benzylamine as substrate. The characteristic "mottled" appearance is due to increased reaction product deposits in parenchymal cells around portal areas (*P*). Magnification × 110

2). This activity was sensitive to 10^{-7} M clorgyline and unaffected by 10^{-7} M deprenyl.

In the rat liver and using tyramine as substrate, staining was evenly distributed throughout the cytoplasm of the parenchymal cells (Fig. 3). However with benzylamine as substrate, or with tyramine in the presence of 10^{-7} M clorgyline, staining was not uniform, the sections having a "mottled" appearance. Activity in this instance appeared stronger in parenchymal cells lying in the vicinity of the portal areas (Fig. 4). Staining with tyramine was partially inhibited by 10^{-7} M deprenyl, more substantially so by 10^{-7} M clorgyline and completely inhibited by a combination of the two. When benzylamine was used as substrate, all staining was abolished with 10^{-7} M deprenyl but it was unaffected by 10^{-7} M clorgyline. With phenylethylamine a similar staining and inhibitor pattern was obtained to that with tyramine.

Acetone pretreatment had no effect on histochemical localization, indicating that lipid solubility of the reaction product is not a problem in this instance.

Discussion

The present work has shown that the substrate, benzylamine, can be successfully incorporated into a coupled peroxidatic oxidation technique for the localization of MAO B and thus confirms the recent study of Williams et al. (1979). We further show that with an appropriate choice of specific inhibitors, the method is an effective differential stain for MAO A, MAO B and benzylamine oxidase. The localization and nature of the staining in the human term placenta correlated completely with the known biochemistry of the enzymes in this tissue; the staining of the trophoblastic layer behaved as if it were pure MAO A (Egashira, 1976). Activity was demonstrated with tyramine but not benzylamine and was inhibited by 10^{-7} M clorgyline but not 10^{-7} M deprenyl. Phenylethylamine also gave a clorgyline-sensitive staining pattern and was thus a substrate for MAO A in this tissue. Reaction in the placenta, using benzylamine as substrate, was confined to the tunica media of the blood vessels and this staining was completely inhibited by 5×10^{-5} M phenelzine but was unaffected by deprenyl or pargyline. These results correlate well with our biochemical studies of the placenta (Lewinsohn et al., in preparation). Apart from the immunofluorescent study of Buffoni et al. (1977), to our knowledge this is the first time that BzAO has been localized histochemically.

In the liver, tyramine and phenylethylamine both showed activity which was partially sensitive to 10^{-7} M deprenyl and more so to 10^{-7} M clorgyline. This finding suggests that MAO A is present in a greater concentration and agrees with our biochemical studies (unpublished observations). Benzylamine stained MAO B only, in that all the activity was abolished by 10^{-7} M deprenyl. This amine appears, therefore, to be considerably more specific as a substrate for MAO B, in both rat liver and human placenta than phenylethylamine (Lewinsohn et al., in preparation)

The substrates and inhibitors we propose for the selective staining of MAO A, MAO B and BzAO are shown in Table 2. It may well be that different concentra-

Table 2. Staining methods for MAO A, B and BzAO

Enzyme	Substrate	Insensitive to	Sensitive to
MAO A	Tyramine	10^{-7} M deprenyl	10^{-7} M clorgyline
MAO B	Tyramine Benzylamine	10^{-7} M clorgyline	10^{-7} M deprenyl 10^{-7} M deprenyl
Benzylamine oxidase	Benzylamine	10^{-7} M deprenyl 10^{-3} M pargyline	5×10^{-5} M phenelzine

tions of the inhibitors are needed for selective inhibition in species or tissues other than those investigated in the present study.

One unexpected observation, when benzylamine was used as substrate, was the apparent concentration of activity around portal areas in liver parenchymal cells. This effect was due to MAO B activity since it also occurred with tyramine in the presence of 10^{-7} M clorgyline. Tyramine used alone, and with 10^{-7} M deprenyl, produced a fairly uniform staining pattern throughout the material. An ability to distinguish subtle differences in activity of this sort may be useful in elucidating the physiological role of these two enzyme variants.

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