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*From the Department of Pharmacology,
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AMINE OXIDASES IN DENTAL PULP

*A study with special regard
to a semicarbazide-sensitive amine
oxidase with catalytic potency
towards serotonin*

by

ASTRID NORQVIST-SJÖBERG

Umeå 1988

To my family

UMEÅ UNIVERSITET

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Department of Pharmacology

Unfortunately in paper no. V the references given in the text have lost their numbers in the reference list. This is thus a completion given for pages 137-139.

- 16 Andree
- 5 Blaschko
- 9 Callingham
- 13 Callingham and Barrand
- 17 Callingham and Laverty
- 22 Dixon
- 8 Ekstedt
- 18 Fowler, Ekstedt
- 19 Fowler, Orelan
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Hälsningar

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A study with special regard to a
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catalytic potency towards serotonin

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ASTRID NORQVIST-SJÖBERG

Umeå 1988

ABSTRACT

The amine oxidizing capacity of piglet dental pulp tissue has been investigated. At least six separate enzyme activities could be distinguished:

- a soluble semicarbazide-sensitive (SSAO) activity towards benzylamine, most likely to be derived from the content of blood plasma within the pulp tissue;
- a soluble pulp tissue SSAO activity with most properties in common with the plasma enzyme, however with great efficiency not only regarding benzylamine but also tryptamine;
- a tissue bound SSAO activity with activity towards benzylamine, tryptamine, tyramine and β -phenylethylamine;
- a tissue bound semicarbazide-sensitive activity towards serotonin (SSAO-5HT) (a combination of properties never reported before);
- a mitochondrially bound and clorgyline-sensitive monoamine oxidase-A (MAO-A) activity with activity towards serotonin;
- a mitochondrially bound and deprenyl-sensitive MAO-B activity with activity towards e.g. benzylamine, tryptamine, tyramine and β -phenylethylamine.

The relative importance of the various activities for the metabolism of some monoamines has been investigated. Although the SSAO enzymes had lower K_m values with most substrates than the MAO, the bulk of the compounds was likely to be metabolized by MAO-B because of higher V_{max} values. K_m values and V_{max} values with serotonin, however, were rather similar for the MAO and the SSAO membrane-bound enzyme(s).

The thermal sensitivity of membrane-bound SSAO activities was found to be considerably lower than that for MAO-B.

The SSAO-5HT activity was found to be localized to the plasma membrane with a greater activity towards the periphery of the pulp tissue than towards the centre.

No great difference in the various amine oxidase activities was found when pulp tissues of varying degrees of maturity were compared.

The occurrence of membrane-bound SSAO and MAO activities was investigated and compared between pig, ox and human dental pulp. All activities were found to be present in about the same order of magnitude in all three species with the exception of a higher MAO-A activity in the ox pulp and the absence of this enzyme activity in the human dental pulp.

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10.	PAPERS	

1. PAPERS DISCUSSED

This thesis is based on the following five papers:

- I. Norqvist, A., Fowler, C.J. and Oreland, L. (1981): The deamination of monoamines by pig dental pulp. *Biochem. Pharmacol.* 30, 403-409.
- II. Norqvist, A., Oreland, L. and Fowler, C.J. (1982): Some properties of monoamine oxidase and a semicarbazide-sensitive amine oxidase capable of the deamination of 5-hydroxytryptamine from porcine dental pulp. *Biochem. Pharmacol.* 31, 2739-2744.
- III. Norqvist, A. and Oreland, L. (1988): Localization of a semicarbazide-sensitive serotonin-oxidizing enzyme from porcine dental pulp. Submitted.
- IV. Norqvist, A. and Oreland, L. (1988): Semicarbazide-sensitive serotonin-oxidizing activity in porcine dental pulp in relation to dental maturity. Submitted.
- V. Norqvist, A. and Oreland, L. (1988): Studies of the effects of some inhibitors on semicarbazide-sensitive serotonin-oxidizing activity in dental pulp. Submitted.

In the text, the above papers are referred to by the Roman numerals I-V.

2. ABBREVIATIONS

β -APN	-	β -aminopropionitrile
DAO	-	diamine oxidase
FAD	-	flavin adenine dinucleotide
5-HT	-	5-hydroxytryptamine (=serotonin)
LO	-	lysyl oxidase
MAO	-	monoamine oxidase
MAO-A	-	monoamine oxidase, type A
MAO-B	-	monoamine oxidase, type B
MEK	-	methyl ethyl ketone
Mg^{2+} -ATPase	-	magnesium dependent adenosinetriphosphatase
SSAO	-	semicarbazide-sensitive amine oxidase
SSAO-5HT	-	semicarbazide-sensitive amine oxidase with the ability to deaminate 5-hydroxy- tryptamine

3. INTRODUCTION

3.1 History and general background

The history of amine oxidases goes back to the late nineteen twenties when an enzyme responsible for the oxidative deamination of tyramine was discovered (Hare, 1928). Another amine oxidase deaminating histamine was discovered later (Best, 1929). These enzymes differed, however, in several respects which lead to a decision to name the first enzyme, MAO and the second one DAO, also called histaminase (Zeller, 1938). The next major step occurred when it was discovered that the MAO activity could be catalyzed by two forms of the enzyme, MAO-A and MAO-B (Johnston, 1968). Furthermore, histaminase turned out to consist of a whole family of amine oxidases whose special characteristic was a cofactor which differed from the flavin-adenine dinucleotide (FAD) of MAO (see e.g. Blaschko, 1974). Several of these latter enzymes have been found to be responsible for the deaminating activity (including also monoamines) remaining after complete inactivation of MAO by an irreversible inhibitor such as clorgyline (Coquil et al., 1973; Lyles and Callingham, 1975). These enzymes are also characterized by being inhibited by semicarbazide and other so-called carbonyl reagents (see Kapeller-Adler, 1970; Blaschko, 1974; Lewinsohn, 1984). There is, however, considerable uncertainty concerning the identity of the cofactor for semicarbazide-sensitive amine oxidases. For many years pyridoxal appears to have been the favoured candidate, but in many cases there was little supporting evidence. Recently it has been discovered that the amine oxidase from bovine serum contains a covalently bound prosthetic group identified as pyrrolo quinoline quinone (Lobenstein-Verbeek et al., 1984), which raises the possibility that several other SSAOs may contain this or a similar group. Another characteristic feature of these enzymes is their need for copper, (Blaschko, 1974) although

this may not be true for all. Such a dependence for a metal has not been shown for MAO.

In this thesis SSAO (Semicarbazide-Sensitive Amine Oxidase) has been used as a designation for this family of enzymes which, however, still defy systematic classification and proper naming (Lewinsohn, 1984).

3.2 Classification, distribution and localization of amine oxidases

Classification

Monoamine oxidases are flavo-(FAD)-proteins which, like DAO, catalyze the oxidative deamination of primary amines and, usually, also secondary and some tertiary amines. DAO, MAO and SSAO all catalyze the same general reaction: $RCH_2NH_2 + H_2O + O_2 = RCHO + NH_3 + H_2O_2$; where R- represents a fatty-aromatic or aliphatic residue. Since MAO and DAO and most, if not all, SSAOs use oxygen as their second substrate they are classified as EC 1.4.3.4 (MAO) and EC 1.4.3.6 (DAO, SSAO), although the latter classification originally included DAO alone (Dixon and Webb, 1979; Banchelli et al., 1983).

The SSAO enzyme activities have variously been referred to as "clorgyline-resistant amine oxidase" (Callingham et al., 1983), "pargyline-resistant amine oxidase" (Del Carmen Urdin and Fuentes, 1983) or "benzylamine oxidase" (Buffoni, 1983). These names, however, do not characterize the enzyme(s) unambiguously consequently, "semicarbazide-sensitive amine oxidase" (Callingham and Barrand, 1987) has been used in the present study. They are copper-containing proteins, and as their action depends on a carbonyl group they are sensitive to inhibition by carbonyl reagents such as semicarbazide.

Diamine oxidases is a group of enzymes within the family of SSAO enzymes involved in the oxidative deamination of primary amines, including monoamines, but preferred substrates are diamines such as putrescine and cadaverine.

Lysyl oxidase (LO) is an enzyme with some properties in common with the class of DAO (EC 1.4.3.4): type of enzymatic reaction (oxidative deamination), carbonyl-containing cofactor and inhibition by cupric copper-chelating agents and carbonyl reagents. Lysyloxidase has been assigned an EC class of its own (EC 1.4.3.13), defined by its unusual substrate specificity for lysyl residues in peptide linkage in collagen and elastin and the lack of activity with the free lysyl residues or other amines (Pinnell and Martin, 1968).

Distribution and localization

There is widespread distribution of DAO, SSAO, MAO and LO through species and organs.

Thus, DAO has a high activity in kidney (Zeller, 1963), intestine (Watson, 1956) and placenta (Zeller, 1963) but it is also found in body fluids such as blood plasma. The enzyme belongs to a category of soluble enzymes which can generally be found in the cytoplasm (Kapeller-Adler, 1970). In fresh kidney homogenates, however, approximately 10 per cent of the total diamine oxidase activity was in the mitochondrial fraction (Kapeller-Adler, 1970).

In mammals SSAO has been detected in tissues such as rat heart (Lyles and Callingham, 1975), rat aorta (Coquil et al., 1973), rat mesenteric arteries and veins (Callingham et al., 1983), rat and human placenta (Emerole, 1982; Kinemuchi et al., 1982) and human vascular smooth muscle (Lewinsohn et al., 1978). It has been suggested that this enzyme activity is similar, if not identical, to plasma benzylamine oxidase (Buffoni, 1983), which thus means that some of the SSAO activity belongs to the soluble enzymes. It is strongly

indicated, however, that some part of the SSAO activity is localized to the plasma membrane (Wibo et al., 1980; Barrand and Callingham, 1982, 1984).

In mammals MAO has been found in most cells and tissues with the exception of erythrocytes and blood plasma (Blaschko, 1952). In the cells MAO is mainly localized to the mitochondria where it is firmly bound to the outer membrane (Schnaitman and Greenawalt, 1968). About 25 per cent of the enzyme can, however, be found in the microsomal fraction (Hawkins, 1952) and histochemical studies have confirmed that a proportion of the MAO is associated with the endoplasmatic reticulum (Muller and Delage, 1977; Shannon et al., 1974).

Lysyl oxidase is richly represented in e.g. pig aorta (Buf-foni, 1980) and it is suggested that it is an extracellular tissue enzyme (Layman et al., 1972) of connective tissue origin. Like plasma SSAO it is thought to be a secretion product of fibroblasts and smooth muscle cells.

3.3 Physiological role of amine oxidases

Monoamine oxidase is known to be important in the metabolism of biogenic monoamines (adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine) and in protecting the organism from the action of exogenous, biologically active amines such as tyramine (Blaschko, 1974).

The DAO of the intestine and kidney has been shown to be involved in the protection of the organism from certain of the toxic effects of histamine (Zeller, 1972). Furthermore, these enzymes are involved in the regulation of the metabolism of putrescine which is a key compound in the biosynthesis of polyamines, important in the regulation of protein synthesis (Baylin et al., 1978).

The physiological role of SSAO is not well established, although it has been demonstrated (Lewinsohn 1977, 1984) that plasma SSAO activity (named benzylamine oxidase) was greatly reduced in patients suffering from severe burns or from a variety of malignant disorders. The enzyme activity was also shown to be closely related to the severity of the disease with the activity, however, becoming normalized after healing of the wound or after removal of a tumor. Plasma SSAO may also be involved in more normal events such as pregnancy (Lewinsohn, 1984). It has been suggested that tissue bound SSAO is important for cell membrane function, since the plasma lemma of vascular smooth muscle and brown adipose fat tissue (BAT) have been shown to provide rich sources of SSAO (Callingham and Barrand, 1987). One of the products in the enzyme reaction, at least in the BAT of the rat, is hydrogen peroxide (Barrand and Callingham, 1984) and it has been suggested that this peroxide may be involved in transmembrane signalling (Mukherjee and Mukherjee, 1982). Furthermore, the peroxide generated by MAO has also been shown to be important in e.g. the brain where it may influence the synthesis of prostaglandins (Seregi et al., 1982, 1983). The possible involvement of SSAO in the transmembrane signalling system may impart a role to this enzyme, in some specific aspect, for maintenance and control of vasomotor tone as well as for the responses of adipose tissue to some physiological stimuli (Callingham and Barrand, 1987).

Lysyl oxidase is the enzyme that initiates the biosynthesis of cross-links in collagen and elastin (Pinnell and Martin, 1968). Although there is evidence for separating the tissue SSAO from lysyl oxidase there may, nevertheless, be some relationship between them. SSAO may thus be involved in the metabolism of collagen and elastin, since it has been found in e.g. rat articular cartilage (Lyles and Berthie, 1986).

3.4 Substrates and inhibitors

Substrates

Amine oxidases may exhibit considerable differences in substrate specificity if they come from different organs, or from the same organ in different species (Gorkin, 1983). In order to compare the efficiency of the deamination of different substrates by amine oxidases from different tissues and organs, it is thus necessary to consider such things as the degree of purification of the enzyme and conditions under which the deamination rate is measured (primarily substrate concentrations and pH). If a generalization is made about the substrate selectivities for amine oxidases, identified in human and animal tissues by means of inhibitor analysis, the following can be said:

Monoamine oxidase, type A (MAO-A) substrates are the important neurotransmitters, dopamine, noradrenaline and 5-HT. Dopamine is an equally preferred substrate for MAO-B which is why the ratio of MAO-A/MAO-B activity is a function of the ratio of concentration of each enzyme form and the dopamine concentration exposed to them. Exogenous substrates for MAO-B are e.g. benzylamine and β -phenylethylamine, while others e.g. tyramine are deaminated by both forms.

Among the best substrates for DAO are the aliphatic diamines, putrescine and cadaverine, which are not oxidized by monoamine oxidases.

No generalizations can be made concerning substrate selectivity for the various SSAO enzymes and, in addition, there is no clear idea about the nature of the preferred physiological substrate, although many amines have been shown to function as substrates (Callingham and Barrand, 1987). The

SSAO enzymes which are tissue bound or soluble in the plasma, in contrast to the "soluble" tissue enzyme, usually possess a comparatively high affinity for benzylamine. Although benzylamine is not a compound which occurs physiologically in human plasma and smooth muscle, benzylamine is an outstanding substrate (Lewinsohn et al., 1978; Lewinsohn, 1984). The situation is further complicated by the fact that the substrates for SSAO are also deaminated by MAO; sometimes even more efficiently and with a greater capacity. The SSAOs, having a high affinity (low K_m) but a low capacity (low V_{max}), are thus unlikely to have any major function as scavengers of unnecessary or potentially harmful amines (a role effectively fulfilled by MAO). Instead they may play a part in the supply of different products from the deamination process such as hydrogen peroxide (Callingham and Barrand, 1987). In such a case saturating concentrations of some suitable amine might well be required, with control being exercised by some other endogenous substance. It is against this background that the substrate selectivity of SSAO should be examined. In pig plasma the SSAO enzyme has been shown to be more effective against mescaline than benzylamine but it will also deaminate histamine, tyramine and dopamine (Buffoni and Blaschko, 1964). In human plasma, benzylamine is deaminated at a higher rate than any other amine (Lewinsohn, 1984).

Lysyl oxidase activity can be defined by its strict selectivity for lysyl residues in collagen and elastine. Thus, benzylamine is not a substrate for lysyl oxidase (e.g. Buffoni, 1980). Distinctions between SSAO and LO deaminating enzymes are not always easy to establish and they may share common substrates. For example, the ox dental pulp enzyme can metabolize both benzylamine and lysyl-vasopressin (Nakano et al., 1974).

Apart from amines, oxygen is a substrate for MAO (Tipton, 1968; Oi et al., 1970) and DAO (Finazzi Agro, 1969) and oxygen is also likely to be the second substrate for other amine oxidases (Fowler and Callingham, 1978).

Inhibitors

Inhibitors are used to be able to separate the oxidizing activities of MAO-A and -B, SSAO, DAO and LO from each other. In 1968 Johnston demonstrated that the irreversible acetylenic inhibitor clorgyline, was able to cause a sigmoid enzyme inhibition pattern in tissue homogenates containing a mixture of MAO-A and -B. It was shown that MAO-A was inhibited by nanomolar concentrations of clorgyline while MAO-B was inhibited by micromolar concentrations. In a tissue containing a mixture of MAO (MAO-A and -B) and SSAO it is thus possible to inhibit completely the MAO activity by using millimolar concentrations of clorgyline. The remaining amine oxidase activity will be due to the SSAO, since this enzyme(s) is insensitive to clorgyline. Deprenyl is considered to be a selective MAO-B inhibitor (Knoll and Magyar, 1972).

SSAOs are classified according to their susceptibility to the slowly reversible inhibitor semicarbazide and related compounds (Kapeller-Adler, 1970; Blaschko, 1974). The DOPA-decarboxylase inhibitor benzerazide, with no effect on MAO activity, is a documented SSAO inhibitor (Andree and Clarke, 1982; Lyles and Callingham, 1982). Benzerazide, used in in vivo experiments in pig can induce the connective tissue disease, lathyrism (Levene, 1971) and a similar condition in rats (Theiss and Schärer, 1970). A symptom in the pig is aorta rupture because of incomplete formation of collagen and elastin molecules in the connective tissues. The mechanism of inhibition probably involves inactivation of the carbonyl group of SSAO. (Blaschko, 1974).

One of the few inhibitors with the reported selected effect of LO is β -APN. This inhibitor can also cause the connective tissue disease lathyrism in experimental situations.

3.5 Histology of the dental pulp and the physiological role of 5-HT

The histological structure of the dental pulp is similar to that of loose connective tissues elsewhere in the body. There are, however, several differences, which can be of importance for pulpal reaction in physiological and pathological situations. Thus pulp contains no fat cells or elastic fibers, few or no mast cells (Seltzer and Bender, 1984) and, as far as is known, maybe no lymphatic vessels. Pulpal connective tissue therefore consists of collagen and reticulin fibers, proteoglycans, glycoproteins and other ground substance components, fibroblasts undifferentiated mesenchymal cells, blood-vessels and nerves. Furthermore, dental pulp contains a specific cell element: the odontoblasts, which are involved in the production of dentin. A study of the metabolism of the pulp is therefore a study of the dynamic aspects of these components and their interactions.

The circulation system of the pulp tissue consists of arterioles, capillaries and venules (Kim et al., 1981). The teeth are innervated by the second and third division of the fifth cranial (trigeminal) nerve. Sympathetic nerve fibres release norepinephrine, which constricts the blood vessels and parasympathetic nerves release acetylcholine, which causes a relaxation of the smooth muscle of the vessels. There are also however other chemical mediators, like bradykinin and substance P, involved in the control of the blood flow. The pulp is completely surrounded by mineralized hard tissue, which probably limits its capacity to tolerate edemic changes. Moreover the fact that there is no collateral circulation as a whole and that all the blood vessels of

the pulp are led into the root canal via the foramen apicale must make the organ more vulnerable.

The neurotransmitter, serotonin has recently been detected in the dental pulp (Olgart, 1979). The presence of 5-HT in dental pulp is interesting in view of the function of the vascular system. Small amounts of this monoamine can be found in blood, most of it stored in the platelets, and hence 5-HT can obviously reach all parts of the body including the dental pulp. In the dental pulp, which can be regarded as a microcirculatory system, it is suggested that the potential role of serotonin is as illustrated in Fig. 1 (modified from Vanhoutte and Cohen, 1983). The combined effect of arteriolar vasoconstriction, increase in capillary and venular permeability and venoconstriction favours local edema and stasis. Apart from its direct action, serotonin can considerably potentiate the responses to other neurohumoral mediators such as norepinephrine or angiotensin II (Van Nueten et al., 1982). Serotonin has also effects on cells of the blood vessel wall other than smooth muscle cells (Vanhoutte, 1983), which is probably a major factor in the heterogeneity of the responsiveness, particularly in the intact organism. The endothelial cells, however, seemingly form an effective barrier against the vasoconstrictor action of serotonin, since the presence of endothelium markedly reduces the contractions evoked by platelets and exogenous 5-HT (Cohen et al., 1983). This protective role may result, at least partially, from an enzymatic destruction of serotonin by endothelial monoamine oxidase (Vanhoutte, 1983).

Fig. 1. Potential role of serotonin at the microcirculatory level. The release of 5-HT from aggregating platelets would tend to: left - cause vasodilatation at the arteriolar level; middle - increase capillary and venular permeability, and right - cause venoconstriction. The combination of these effects favours local edema and stasis.

Rat dental pulps have been used in an experimental model of traumatic pulpitis in order to investigate the mechanisms involved in the changes in vascular permeability in the inflamed pulp (Araujo, 1980). It was found that histamine and 5-HT mediated an increased vascular permeability in the traumatised teeth at least during the first 30 minutes after the trauma. Prostaglandins are chemical mediators, known to be potent inflammatory agents and recently rat pulp tissue has been shown to contain an appreciable amount of prostaglandins (Hirafuji et al., 1980). In non-inflamed rat dental pulp 5-HT selectively stimulates the biosynthesis of prostacyclin (Hirafuji and Ogura, 1987) but not thromboxane A₂.

4. THE PURPOSE OF THE PRESENT INVESTIGATION

Knowledge about amine oxidase activities and their cellular localization in the dental pulp is sparse. Dental pulp was thought to contain not only classical mitochondrial MAO but also other amine oxidizing activities (e.g. that of lysyl oxidase) associated with the connective tissue. The original purpose of the present investigation was to study the various amine oxidase activities in dental pulp. However, our finding that this tissue contains a semicarbazide-sensitive amine oxidase with the capacity to oxidize 5-HT, raised many questions, since such an enzyme activity had never been described before. Therefore, the main purpose of the present investigation became:

- 1) To investigate the biochemical properties of the different amine oxidase activities in pulp tissue with special regard to SSAO-5HT.
- 2) To investigate the possibility of obtaining the membrane bound SSAO-5HT activity in a soluble form for further purification.
- 3) To study the anatomical and cellular localization of SSAO-5HT.
- 4) To study the connection between SSAO-5HT activity and degree of maturation of pulp tissue.

5. METHODS

5.1 Rationale for the use of pig and ox teeth

Many basic questions about mammalian metabolism and development can be studied in small laboratory rodents such as the

rat, hamster, or guinea pig. For this investigation, however, a large amount of pulp tissue was needed and that is one reason why the pig was used. Furthermore, we would be able to relate our results to human teeth if pig teeth (permanent dentition) were used as a source of pulp tissue because the embryology of the pig is well known and resembles that of man in those aspects that are the most important (Weaver et al., 1962). Pigs' teeth grow in a similar way to human teeth, with the exception of the canine tooth, which can grow continuously. Ox teeth (permanent dentition) have the ability to grow continuously until the animal reaches the age of 14 years - a quality that distinguishes ruminants from humans.

5.2 Tissue collection and classification

Pig teeth were the main sources of pulp tissue but ox and human teeth have also been used. Pig and ox teeth, deriving from the lower jaws, were obtained not more than one day after slaughter. The jaws were frozen (-20°) until the pulps were collected. The frozen pig and ox jaws were separated with a band saw and the teeth dissected. The pulp tissue was always taken from the permanent teeth and therefore the pig and ox teeth remained embedded in the jaws (pig: 3-4 months old; ox: 5-6 months old). The ox teeth constituted a selection of all types of permanent molars and premolars in the lower jaw. Human pulp tissue was taken from extracted third molars which were free from caries lesions, parodontitis or fillings. The teeth were collected (at the Department of Surgery) and put into physiological sodium chloride (4°) until they were frozen (-80°) 2-3 hours later. Before removal of the pulp the teeth were wrapped in parafilm and crushed in a vice.

Pig molar and canine tooth pulps were used in the cellular distribution experiments. In order to divide the pulps into three parts the tissue was loosened from the mineralized dentin/enamel tissue with a pair of tweezers. It was then further divided into a central and a periferal part, equal in weight. A third portion consisted of the cell residues (scraped out with an excavator) from the inside of the mineralized dentin tissue. To be able to verify the tissue constituents of the third portion of the dental pulp, histological dyes were produced. In Fig. 2 a pig tooth pulp is seen, loosened as described, with an intact odontoblastic layer on the periphery. Since there is an unbroken odontoblast layer, the constituents scraped out ("third portion"), together with the predentin, should mainly consist of odontoblast residues.

Fig. 2. Pig tooth pulp (P), with an intact odontoblastic layer (Odl) x 100.

The tooth pulp from the pig alone was classified into different stages of dental development. This classification was made according to a dental maturity system by Demirjian based on the biological criteria of human teeth (Demirjian, 1978). The immature group corresponded to stage A-B (Fig. 3) according to Demirjian and a mature group to stage E in a scale from A to H with stage H being a fully developed tooth. A third group, contained canine teeth. Since pig canines are capable of continuous growth but are without roots in the strict sense, these teeth cannot be classified according to criteria for human teeth. The development of the vascular system in growing human teeth has been studied and a positive correlation was found with the mineralization of the teeth (Tobin, 1972). Since the pig canines had a richer vascularization as well as a developed dentin - enamel layer, a more mature class was suggested for these teeth. Differences in vascularization were confirmed by histological dyeing for vessels of immature and mature pulps (Fig. 4).

Fig. 3. The developmental status of human molars is defined for stages A to H according to Demirjian (1978). The definition of stage A-B ("immature" pulp tissue) and stage E ("mature" pulp tissue) is based on the following criteria: Stage A-B: a beginning of calcification is seen at the superior level of the crypt, in the form of inverted cone or cones; no fusion, or fusion of the calcified points form one or several cusps which unite to give a regularly outlined occlusal surface; no dentinal deposit is seen. Stage E: initial formation of the radicular bifurcation is seen in the semilunar shape; the root length is still less than the crown height.

Pig: "immature" molar
pulp tissue

"mature" molar
pulp tissue

"mature" canine
pulp tissue

Human: mature molar pulp tissue

Fig. 4. Pig pulp tissue classified into different stages of dental development (immature and "mature") compared with human pulp tissue (mature; from a tooth with completed root formation) x 40.

5.3 Tissue preparation

5.3.a Solubilization of SSAO-5HT

As with most membrane bound enzymes, the main problem in the purification of SSAO-5HT was the liberation of the enzyme from the membranes. We did, however, attempt purification but failed to obtain any significant amount in solubilized form. Attempts to release the enzyme activity from pig pulp homogenate either by sonication, treatment with Triton X-100 (0.1 % v/v) or urea (up to 4 M) were thus unsuccessful. Our laboratory has, however, succeeded in solubilizing and purifying pig liver MAO by means of an extraction procedure with MEK (Hollunger and Oreland, 1967). It was possible to extract 10-15% of the original SSAO-5HT activity from the crude homogenate with MEK. Unfortunately the SSAO-5HT activity was lost after storage for eight hours in 4⁰, the interpretation of these results was that the SSAO-5HT enzyme(s) probably needed the membrane to maintain its original qualities or that MEK inactivated the enzyme. Subsequent investigations of SSAO-5HT were therefore carried out with the enzyme connected to the membranes.

5.3.b Homogenization and centrifugation aimed at obtaining a high specific SSAO-5HT activity

The tissue was homogenized in either 50 mM potassium phosphate, pH 7.8 (I and II) or buffered sucrose (0.25 M), buffered at pH 7.4 with 3 mM imidazole-HCl (III, IV and V).

Homogenization procedures were always performed on ice. The homogenization was carried out in buffer (1:4; (w/v), in an Ultra-Turrax homogenizer (type 18/2) at 0⁰. The homogenate was centrifuged at 13,000 x g, for 15 min and the pellet rehomogenized in the same buffer added to the original

volume, recentrifuged and the supernatants combined. This combined supernatant was centrifuged at $60,000 \times g$, for 75 min to give a high-speed supernatant ("soluble fraction") and a pellet ("membrane fraction"). Expected constituents of this high-speed supernatant are microsomes and other membrane fragments with the same density (Fig. 5). When we used immature tooth pulps (IV) the high-speed supernatant was divided into two fractions; one was fluid and the other gelatinous because its content was rich in highly polymerized ground substance (Seltzer and Bender, 1984). All membrane fractions were set to 8 mg protein/ml before further assays. All fractions were stored at -20° until used for assay of activity.

Fig. 5. Homogenization and centrifugation procedures.

5.3.c Homogenization aimed at obtaining a fraction with SSAO-5HT, suitable for isopychnic gradient centrifugation

The tissue was homogenized and centrifuged according to a method described by Wibo et al. (1980). The pulps were chopped in ice-cold, buffered sucrose and portions of these chopped pulps (approximately 1 g) were homogenized ice cold, in 10 ml of buffered sucrose, with a 15 ml Dounce homogenizer. The homogenization was carried out by six strokes with a type-A pestle (glass). The total suspension obtained was brought to a volume of 150 ml and then centrifuged at $600 \times g$ for 10 min. The supernatant fluid was removed by suction and the resulting pellet further homogenized with four strokes of the pestle in a total volume of 75 ml. This suspension was centrifuged as above and the pellet subjected to a third cycle of homogenization in the Dounce homogenizer (four strokes) and centrifugation. This pellet was suspended and homogenized briefly in a 15 ml Potter S tube with one stroke of a Teflon pestle (Braun, Melsungen, FRG), rotating at 1500 rpm in a total volume of 50 ml of buffer. The combined supernatants from four homogenization - centrifugation cycles (about 300 ml) constituted our cytoplasmic extract.

The cytoplasmic extract was kept in a refrigerator for one night ($+4^0$) and then centrifuged at $55,000 \times g$ for 6 min. The supernatant fluid was removed by suction and the pellets suspended in 100 ml of buffered sucrose. This suspension was centrifuged as above and the washed pellet, suspended in approximately 8 ml of buffered sucrose, was set to a protein concentration of 4 mg protein/ml. This constitutes the mitochondrial (F_2) fraction (Wibo et al., 1980).

To prepare the plasma membrane fraction (F_3 according to Wibo et al., 1980) the post-mitochondrial supernatant (washing included) was centrifuged at $45,000 \times g$ for 135 min. The resulting microsomal pellet, including plasma membrane

fragments, was washed once in 50 ml of buffered sucrose and then suspended in approximately 6 ml of the buffered sucrose and set to 4 mg protein/ml.

5.4 Plasma preparation

Plasma from several species contains an SSAO activity with benzylamine as a preferred substrate. Pig plasma obtained by centrifugation of citrated blood, was centrifuged at 25,000 x g for 20 minutes. The plasma proteins were precipitated with ammonium sulfate and after standing overnight the precipitate was removed by centrifugation and then dissolved in 0.01 M phosphate buffer, pH 7.0. The solution was dialyzed overnight against 0.003 M phosphate buffer, pH 7.0. An inactive precipitate which was formed was removed by centrifugation. The dialyzed enzyme solution was subjected to DEAE-cellulose chromatography. The adsorbent was packed into a column and equilibrated with 0.003 M phosphate buffer, pH 7.0. The enzyme was eluted with 0.1 M phosphate buffer, pH 7.0. The elution of the protein was followed both by the measurement of the absorbancy at 280 nm and by radiochemical determination of activity (described in 5.6). The fractions containing the highest specific benzylamine oxidase activity were pooled and then concentrated by the addition of solid ammonium sulphate to 55% saturation. The precipitate obtained by centrifugation was dissolved in 0.01 M phosphate buffer, pH 7.0, and dialyzed overnight against 0.003 M phosphate buffer, pH 7.0. This procedure resulted in a four-fold purification with regard to the specific activity towards benzylamine.

In order to obtain a preparation of a tissue "soluble" SSAO activity the same procedure was followed for the high-speed supernatant of the pulp homogenate (5.3 b). The purification of this supernatant was two-fold with respect to the specific activity towards benzylamine.

5.5 Centrifugation procedures

Most of the centrifugation (and homogenization) procedures were performed in isotonic (0.25 M) sucrose buffer. Any tissue passing through the gentle grinding procedure (described in 5.3 c) can be expected to have its subcellular particles distributed according to the process chart in Table 1. The fraction containing the "microsomes" may also include fragments of e.g. plasma membrane. The degree to which organelles and subcellular particles are fragmented during the homogenization procedure as well as the density of the medium and the conditions of centrifugation strongly influence the "typical" distribution of subcellular particles (White et al., 1973).

Table 1

Distribution of typical cellular components achieved by fractional centrifugation of a broken cell preparation of liver (White et al., 1973).

Fraction	Centrifugal field for separation, x gravity	Time of centrifugation (minutes)
Cellular debris; nuclei	1,000 - 6,000	10
Mitochondria	10,000 - 15,000	30
Lysosomes; fragments of microtubules; peroxisomes	15,000 - 20,000	30
Microsome (fragmented endoplasmatic reticulum); ribosomes; reticulum; Golgi membrane	100,000	60
"Soluble fraction" or supernate (cytosol)	...	..

In Paper III an "isopycnic gradient centrifugation" technique was employed which makes use of fairly steep gradients extending over the range of densities of the particles to be separated. The centrifugation procedure is pursued until each particle has reached a position corresponding to its own density. In combination with this centrifugation, digitonin was added in order to change the distribution of the plasma membrane fragments towards a higher equilibrium density. Digitonin is a glycoside of a steroid with high affinity to cholesterol and, by adding small amounts (digitonin to cholesterol molar ratio of approximately 1) which do not alter the density of endoplasmic reticulum elements and of mitochondrial membranes, the equilibrium density of plasmalemmal elements was markedly increased.

5.6 Estimation of amine oxidase activities

Monoamine oxidase activity has been assayed radiochemically by a method developed by Wurtman and Axelrod (1963) as modified by Fowler et al., (1979) with ^{14}C -[serotonin], ^{14}C -[tryptamine] and ^{14}C -[benzylamine) as substrates. Aliquots of the diluted homogenate were suspended in potassium phosphate buffer and incubated at 37° in the presence of the appropriate concentrations of the radiolabelled substrates. Under these conditions initial reaction velocities were always measured. The incubations were terminated by the addition of HCl (1 M) to the test tubes. The deaminated metabolites were then extracted into an organic solvent (ethyl-acetate-toluene; 1:1 v/v, saturated with water) (Fowler and Oreland, 1980). After centrifugation an aliquot of the organic phase was removed for liquid scintillation counting. Separation of the part of oxidation carried out by MAO and SSAO, respectively, was achieved by the inclusion of e.g. the MAO inhibitor clorgyline (1mM e.c.) or the SSAO inhibitor semicarbazide (1 mM e.c.) during a prein-

cubation period (30 min). The same preincubation procedure was carried out for all the inhibitors used in this study.

Diamine oxidase activity, if present, was detected by a photometric assay for tissue deaminating activity. This method was based on the measurement of the mono- and diamine oxidase-dependent production of hydrogen peroxide (Köchli and von Wartburg, 1978).

5.7 Estimation of membrane markers

Mg²⁺-ATPase (oligomycin-insensitive) and phosphodiesterase I activities were selected as markers of the plasma membranes. Both of these markers are localized primarily in cell particulates highly enriched with 5'nucleotidase, which is the most commonly accepted plasma membrane marker, and therefore should themselves be assigned a plasma membrane localization (Wibo et al., 1980; Touster et al., 1970).

Mg²⁺-ATPase activities were determined by measuring the rate of inorganic phosphate (P_i) liberation as described by Godfraind et al. (1976). The incubation medium contained 2.5 mM ATP, 3 mM MgCl₂, 100 mM NaCl, 1 mM EGTA and 20 mM maleic acid adjusted to pH 7.4 with Tris buffer. After 30 minutes of incubation at 37⁰, the reaction was stopped by adding 1 ml of 10% TCA (trichloroacetic acid). After centrifugation, inorganic phosphate was determined in the protein-free supernatant by a modified Fiske and SubbaRow method (1925). To 1 ml of sample were added 1.6 ml of 1.25% ammonium-molybdate in 0.88 M H₂SO₄ and 0.4 ml of the Fiske and SubbaRow reagent. Absorbance was measured at 660 nm, exactly 15 min later. Corrections were always made for acid ATP hydrolysis, measured in samples treated as above but in the absence of

tissue homogenate. The Mg^{2+} -ATPase activity in plasma membranes is considered to be insensitive to inhibition by oligomycin (e.g. Wibó et al., 1980). Therefore, the effect of 2 μ g oligomycin/ml on the Mg^{2+} -ATPase activity was tested whenever this estimation was used. In no case did the degree of inhibition exceed 18%.

Phosphodiesterase I activity was assayed at 37⁰ in a total volume of 0.5 ml containing 0.001 M para-nitrophenyl thymidine-5'-phosphate and 0.02 M tris buffer, pH 9.0. After 10-15 minutes the reaction was stopped by the addition of 1.5 ml 8% TCA. The protein precipitate was removed by centrifugation and aliquots (1.0 ml) of the supernatant fluid added to 3.0 ml of an alkaline buffer (0.133 M glycine, 0.083 M Na_2CO_3 , and 0.067 M NaCl), adjusted to pH 10.7 with NaOH. The optical density at 400 nm was measured and compared with standard para-nitrophenol in order to determine the amount of substrate hydrolyzed.

5.8 Protein

Protein concentrations have been assayed by the method of Lowry et al. (1951) and by a modified method by Markwell et al. (1978), with human (I and II) and bovine (III, IV and V) serum albumin as standards. The Lowry method has been modified by the addition of sodium dodecyl sulphate to the alkali reagent and by increasing the amount of copper tartrate reagent to allow this method to be used on membrane and lipoprotein preparations without prior solubilization or lipid extraction and with samples containing about 200 mM sucrose.

5.9 Statistical procedures

Values are always given as means $\pm$ S.E.R. or S.E.M. Probability levels of random difference were determined by Student's t-test.

6. RESULTS AND DISCUSSION OF THE PRESENT INVESTIGATIONS

6.1 Amine oxidizing activities in pig and ox dental pulp (I, II, IV, V)

The nature of "soluble" and membrane bound SSAO activity.

It is known that in pig plasma there is a soluble SSAO benzylamine activity. Dental pulp tissue is richly vascularized. Soluble plasma SSAO might therefore be expected to be present in a homogenate of pulp tissue. The high-speed centrifugation resulted in a supernatant likely to contain the soluble tissue SSAO enzyme as well as plasma SSAO, and a pellet likely to contain membrane bound SSAO enzyme (60,000 x g for 75 minutes). To be able to investigate the relationship between tissue "soluble" and plasma SSAO activities, the high-speed supernatant of the pig pulp tissue homogenate and pig blood plasma were partly purified (see 5.4) with regard to SSAO activity towards benzylamine. The sensitivity of the "soluble" and plasma soluble benzylamine oxidizing SSAO activity to inhibition by a variety of compounds in both preparations was investigated and found to be very similar. This similarity would suggest that the tissue "soluble" activity and the plasma activity are composed by very similar or identical proteins. The finding of a soluble tissue SSAO activity in pig aorta, connective tissue free from plasma SSAO activity (Buffoni et al., 1976), raises the possibility that the tissue soluble pig pulp SSAO activity obtained from the high-speed supernatant derives not only from plasma but also from the connective tissue. Support for

this assumption is given by the finding that the tissue "soluble" SSAO besides benzylamine could also efficiently oxidize tryptamine (I, table 1), while plasma SSAO is reported to only oxidize benzylamine (Lewinsohn, 1984). The membrane bound SSAO activity, on the other hand, showed an inhibitor sensitivity that differed from both of the soluble benzyl-amine oxidizing activities. This finding is in agreement with previous reports about the existence of other apparently insoluble tissue bound SSAOs, which differ from the soluble tissue enzyme in various respects (e.g. Buffoni et al., 1976).

Deamination of monoamines by dental pulp.

The "soluble" and membrane bound MAO and SSAO activities from pig dental pulp were compared with regard to monoamine oxidizing activity. It was found that both the "soluble" and the membrane bound fractions could deaminate tryptamine, benzylamine and tyramine but the "soluble" fraction deaminated 5-HT and β -phenylethylamine very poorly (I; Table 1), indicating that the membrane bound fraction was responsible for the bulk of the deamination of 5-HT and β -phenylethylamine.

It is possible to separate the membrane bound monoamine deaminating activity in dental pulp into two main groups; the classical mitochondrially bound MAO and the membrane bound SSAO. With all monoamine substrates tested (benzylamine, tryptamine, tyramine, β -phenylethylamine and 5-HT), the inhibitions produced by the MAO inhibitor clorgyline (0.33 mM) and the SSAO inhibitor semicarbazide (0.33 mM) were additive.

In order to investigate the proportion to which the enzymes, MAO-B or SSAO are responsible for the metabolism of benzylamine and tryptamine in the membrane fraction, K_m and

$V_{\max}$ values were calculated. The K_m and $V_{\max}$ values of both MAO-B and the semicarbazide-sensitive enzyme towards benzylamine were similar (II; Table 4), indicating that each enzyme is responsible for the oxidation of about 50 per cent of the oxidizing rate. Tryptamine appears to be preferentially to be metabolized by MAO-B, since this enzyme metabolizes tryptamine with a higher $V_{\max}$ and a lower K_m than the semicarbazide-sensitive enzyme.

The activity of MAO in the pig pulp membranes was investigated by clorgyline inhibition studies in the presence of the SSAO inhibitor semicarbazide. MAO-A is inhibited at nanomolar concentrations of clorgyline while MAO-B needs considerably higher concentrations to be inhibited (Johnston, 1968). Therefore, according to the results shown in I; Fig 3A and B, the monoamines benzylamine, tryptamine, tyramine and β -phenylethtylamine all appeared to be substrates for MAO-B rather than MAO-A.

Most pig tissues have been reported to contain only the B-form of MAO (see e.g. Lyles and Greenawalt, 1978; Stanton et al., 1975). Nevertheless 5-HT has been found to be a substrate for both forms of the enzyme in pig brain and liver (Ekstedt and Oreland, 1976). The oxidation of 5-HT by pig dental pulp was to a large extent sensitive to inhibition by low concentrations of clorgyline (MAO-A inhibitor). Furthermore, the selective MAO-A inhibitor FLA 336(+) (4-dimethylamino- α , 2-dimethylphenethylamine) was similarly able to inhibit about half of the MAO activity towards 5-HT. Thus, it can be concluded that MAO-A is present in the pig dental pulp tissue and is of considerable importance for the oxidation of 5-HT.

Homogenates from pig and ox dental pulps were compared with regard to MAO and SSAO activities towards the monoamines benzylamine and 5-HT (V; Table 1). In both species the total enzyme activity per mg protein activity was found to

be of approximately the same order of magnitude and the activity towards benzylamine about ten times higher than that towards 5-HT. When the activities were divided into a portion catalyzed by classical, mitochondrial MAO and another catalyzed by SSAO enzymes, both the MAO activity towards 5-HT (MAO-A and -B) and towards benzylamine (MAO-B) were about four times higher in the ox pulp than in the pig pulp. With regard to SSAO activities, with both substrates, the difference between the two species was less pronounced with an activity towards serotonin (SSAO-5HT) in the ox pulp about twice as high.

The oxidative deaminating activity of diamines and histamine by dental pulp.

No deamination of the three DAO substrates cadaverin, putrescine and histamine could be detected in the membrane bound fractions(II). Neither cadaverine nor putrescine, even at concentrations of 10 mM, inhibited the oxidation of benzylamine or tryptamine by either MAO or SSAO to any great extent (II; Table 2). Histamine, at a concentration of 10 mM, however, was inhibitory towards MAO (II; Table 2) which agrees with a previous result (Lyles and Shaffer, 1979). In conclusion, therefore, it seems that the membrane bound dental pulp SSAO activity is different from the classical diamine oxidase.

Oxygen as a second substrate for MAO and the membrane bound SSAO activities in dental pulp.

Monoamine oxidase is thought to follow a ping-pong, or double displacement reaction in a variety of tissues (Tip-ton, 1968). A similar mechanism is also thought to occur for diamine oxidase (Finazzi Agro, 1969). As a consequence of such a mechanism, the enzyme activity is increased in an uncompetitive manner as the concentration of the second

substrate, oxygen, is raised (Tipton, 1972). Membrane bound, benzylamine and tryptamine oxidizing activities from porcine dental pulp were investigated and appeared to be increased in an uncompetetive manner with an increase in oxygen concentration (I; Fig. 2). Since both tryptamine and benzylamine oxidations are catalyzed by both MAO and SSAO, the effect of changed oxygen concentration upon the activity of the membrane preparations towards these two substrates was determined after prior preincubation with clorgyline and semicarbazide (I; Table 4). In both cases the interaction between the enzymes and oxygen appeared to be uncompetetive by nature. Thus, the SSAO enzymes as well as MAO in dental pulp follow the ping-pong or double displacement reaction.

Influence of the degree of maturity of the dental pulp for its amine oxidizing activities.

The oxidative deaminating activities of monoamines by semicarbazide-sensitive amine oxidases (SSAO) and classical monoamine oxidases (MAO) in porcine dental pulp, were investigated with special regard to the degree of maturity of the tissue (IV). The pig dental pulps were classified as to maturity in three groups (immature molar, mature molar and mature canine) before subcellular fractionation and estimation of enzyme activities towards benzylamine, tryptamine and 5-HT. The rates of oxidative deamination of benzylamine (IV; Table 3) seem to differ more between canine and molar tooth pulp than between mature and immature pulp tissue. Thus a slightly greater activity was found in the canine pulp tissue both with regard to MAO-B and SSAO activities in the homogenates as well as in the supernatants after 60,000 x g for 75 minutes. It is notable that the canine pulp tissue, in contrast to the other two, contained no MAO-A activity (IV; Table 4). Estimation of the deamination rates

of 5-HT revealed that immature molar pulp homogenate contained more SSAO-5HT activity than mature molar and canine homogenates.

6.2 Evidence for an SSAO enzyme with activity towards 5-HT in dental pulp

6.2.a Biochemical properties (I, II, V)

Pig dental pulp appears to be the only tissue so far reported where a 5-HT metabolizing, semicarbazide-sensitive enzyme has been found (I; Table 3). The only other mammalian tissue reported to contain the oxidative deaminating capacity of 5-HT not catalyzed by MAO-A is giraffe liver (Callingham, 1983). It has not yet been investigated, however, whether this giraffe liver enzyme activity is sensitive to semicarbazide.

The SSAO-5HT enzyme protein(s) is membrane bound. The possibility that this activity could be due to tightly bound plasma or soluble tissue SSAO is unlikely, since the soluble enzymes do not appear to be able to deaminate 5-HT to any significant degree (I; table 1).

In order to investigate the proportion to which MAO or SSAO-5HT activities, are responsible, for the metabolism of serotonin, K_m and V_{max} values were calculated. The K_m value of the SSAO-5HT was very much lower than that of the MAO (I; Fig. 1 and II; Table 4) and the V_{max} values of approximately the same order of magnitude. The higher affinity for SSAO-5HT in comparison with MAO ascribes to SSAO-5HT the responsibility for the metabolism of the bulk of 5-HT occurring in dental pulp.

Benserazide is a selective SSAO inhibitor in e.g. the rat heart, aorta and in some instances brown adipose tissue (Lyles and Callingham, 1982) which was confirmed by a high degree of inhibition of the SSAO activity towards benzylamine in the membrane fraction from both pig and ox pulps. The degree of inhibition of the SSAO-5HT activity however, at the same concentration of benserazide (10 μ M) was considerably lower (V; Table 3).

β -aminopropionitrile is an irreversible lysyl oxidase inhibitor in a concentration range of 4-10 μ M (Siegel, 1979). This is approximately 100 times less than the concentrations required for competitive inhibition of SSAO (Tang et al., 1983). β -Aminopropionitrile is also known to be a reversible competitive inhibitor of SSAO in rat aorta vascular smooth muscle cells (Lyles and Singh, 1985). Pig pulp membrane semicarbazide-sensitive benzylamine oxidizing activity was not completely inhibited until 10^{-2} M β -APN was used (I; Table 2).

The results from the inhibitor experiments with β -APN also strongly indicate that the deamination of serotonin in the dental pulp is likely to be catalyzed by an enzyme (or enzymes) other than those catalyzing the deamination of e.g. benzylamine. Thus β -APN in the concentrations required for irreversible inhibition of lysyl oxidase did not inhibit the semicarbazide-sensitive oxidation of benzylamine, but caused a partial inhibition (non-competitive) of the oxidation of serotonin. Thus, in the pig pulp membrane preparation, β -APN (10 μ M) inhibited about 75% (18% out of 24%) of the SSAO-5HT activity but none of the SSAO activity towards benzylamine (V; Table 2).

In order to further investigate the possible heterogeneity of SSAO-5HT, a mixed substrate experiment was conducted.

For the clorgyline inhibited membrane preparations, i.e. where the activity of semicarbazide-sensitive enzyme(s) is being assayed, the K_i values of both 5-HT and tryptamine, used as inhibitors for the oxidation for the other compound, are similar to their K_m values. This result strongly indicates that these monoamines are deaminated at the same catalytic center. The K_m value of benzylamine, however, is considerably lower than the K_i values of benzylamine as an inhibitor of the oxidation of 5-HT and tryptamine (II; Table 4). This result means that it is likely that the semicarbazide-sensitive deaminating activity may be composed of more than one enzyme species with one form active towards benzylamine and the other form active towards tryptamine and 5-HT.

The sensitivity to thermal inactivation of pig dental pulp MAO and SSAO enzymes differs considerably (II; Table 3 and Fig. 2). The semicarbazide-sensitive enzymes were extremely thermostable, with little or no inhibition of enzyme activity being found even after 100 minutes of incubation at 50⁰. This result was found regardless of the amine substrate used to assay for activity. The monoamine oxidase component was very much more thermosensitive; the degree of thermal inhibition depending to some extent on the substrate used to assay for enzyme activity. For example, the activity of MAO towards benzylamine was more thermosensitive than the activity towards tryptamine, despite the fact that both substrates are metabolized by the same form of MAO (I). However, such differences in the thermal inactivation pattern of an enzyme with different substrates do not constitute definite proof of enzyme heterogeneity, since the thermal denaturation is likely to involve changes in the enzyme reaction mechanism as well as involving inactivation of the enzyme.

6.2.b Localization (III, IV)

The subcellular localization of SSAO-5HT seems to be plasma membranes (plasma lemma). After gentle homogenization (described in 5.3 c) and differential centrifugation the distribution of SSAO-5HT activity was found to be about 40% and 60% respectively in the mitochondrial (F_2) fraction and the microsomal (F_3) fraction. The microsomal fraction is, besides microsomes, expected to contain the plasma membrane fragments but also some fragmented mitochondria. The distribution of the mitochondrial markers MAO-A and -B turned out to be about 80% and 20% within F_2 and F_3 fractions. The MAO activity found in the microsomal fraction can be explained either by contamination of mitochondrial fragments or by the presence of some MAO in the endoplasmatic reticulum (see 5.5). The distribution of SSAO-5HT activity between the mitochondrial and the microsomal fraction was 1:1.8. A similar distribution was found for Mg^{2+} -ATPase, which indicated that SSAO-5HT is bound to plasma membranes.

In order to further study the localization of SSAO-5HT in pig dental pulp, analytical fractionation experiments based on isopycnic gradient centrifugation were performed. Particular advantage was taken of a procedure whereby the equilibrium density of plasma lemmal elements is markedly increased after addition of small amounts of digitonin. This density shift of plasma lemmal elements is thought to result from the binding of digitonin to the cholesterol present in these membranes. Plasma membrane markers (Mg^{2+} -ATPase and phosphodiesterase I) as well as SSAO-5HT activity moved towards an environment with a higher density (III; Fig. 1 and 2). This is in contrast to the MAO-B activity where digitonin did not displace the activity. These results support the notion that SSAO-5HT is bound to plasma membra-

nes. The membrane markers Mg^{2+} -ATPase (oligomycin-insensitive) and phosphodiesterase I did not respond in an identical way to digitonin treatment. This raised the question about the possibility of the plasma membranes being heterogeneous. The phosphodiesterase I activity pattern after digitonin treatment resembled the SSAO-5HT more than Mg^{2+} -ATPase. Wibo et al. (1980) investigated the effect of digitonin on the distribution of the established membrane marker 5'-nucleotidase in rat aorta homogenate. The low 5'-nucleotidase activity in pig pulp tissue, however, made estimation of this enzyme activity impossible.

The cellular distribution of SSAO-5HT within the pig dental pulp was found to be uneven, with greater activity (per mg protein) towards the periphery (III; Table 1). Since a concentration of cells (odontoblasts) is seen towards the periphery of the unmineralized tissue (5.2; Fig. 2) it could be speculated that the SSAO-5HT was localized to those cells. However, since SSAO-5HT activity was found also in the central part (free of odontoblasts) of the pulp, this amine oxidase activity also has to be connected also with other cells, e.g. the closely related fibroblasts or smooth muscle cells. The peripheral part of the pulp is also rich in lysyl oxidase activity, demonstrated for bovine pulp (Numata and Hayakawa, 1986), which indicates that this part of the pulp has a high turnover of tissue components.

6.3 Amine oxidase activities in human dental pulp

It was thought to be of interest to investigate the occurrence of SSAO-5HT in human tooth pulp. Therefore, a material of pulp tissue from 150 third molars with complete root formation, was collected. The tissue was homogenized in buffered sucrose, as described for pig and ox pulp tissue

(5.3 a), omitting, however, the rehomogenization and centrifugation. The MAO-B activity in human pulps was similar to that of pig pulps, while MAO-A activity was lacking (Table 2). Semicarbazide-sensitive amine oxidase activity both with serotonin and benzylamine as substrates was found to be of the same order of magnitude as that for pig and ox. Similarly to the pig and ox pulp tissue homogenates, the deamination rate of benzylamine per mg protein was ten times higher than that of 5-HT. The rate of oxidation of benzylamine catalyzed by MAO-B was about three times lower than that catalyzed by SSAO. When the human pulp amine oxidase activities were compared with those of the pig pulp of different degrees of maturity, the pattern mostly resembled that of the mature canine pulp tissue (IV; Tables 3 and 4). The histological picture of the human third molar pulps is also more reminiscent of that of pig canine tooth pulps than the immature molar of the pig, especially with regard to the occurrence of vessels.

Table 2

Amine oxidase activities in homogenate from human dental pulp*

5-hydroxytryptamine oxidizing activity (%)			Benzylamine oxidizing activity (%)		
MAO-A + SSAO-5HT 1)	MAO-A 2)	SSAO-5HT 3)	MAO-B + SSAO 1)	MAO-B 2)	SSAO 3)
100 ⁴⁾	0	110	100 ⁵⁾	21	65

* All figures are the means from 3 different preparations.

1) no enzyme inhibition

2) 1mM semicarbazide present

3) 1 mM clorgyline present

4) 4 pmole 5-hydroxytryptamine/mg protein/min

5) 32 pmole benzylamine/mg protein/min

6.4 Amine oxidase activities in pig aorta

Aorta tissues from e.g. pig (Buffoni et al., 1976) and rat (Coquil et al., 1973) have been reported to be rich sources of SSAO activity, both of a soluble and a particulate, insoluble nature. Homogenization and centrifugation were carried out by the method described in 5.3 b. Surprisingly no SSAO-5HT was detectable in the pig aorta membranes (pellet after 60,000 x g for 75 minutes). However, compared with pig pulp membranes, aorta membranes had about 20 times greater SSAO activities towards the substrates used (benzylamine, tryptamine, tyramine and β -phenylethylamine).

7. SUMMARY

The amine oxidizing capacity of piglet dental pulp tissue has been investigated. At least six separate enzyme activities could be distinguished:

- a soluble semicarbazide-sensitive (SSAO) activity towards benzylamine, most likely to be derived from the content of blood plasma within the pulp tissue;
- a soluble pulp tissue SSAO activity with most properties in common with the plasma enzyme, however with great efficiency not only regarding benzylamine but also tryptamine;
- a tissue bound SSAO activity with activity towards benzylamine, tryptamine, tyramine and β -phenylethylamine;
- a tissue bound semicarbazide-sensitive activity towards serotonin (SSAO-5HT) (a combination of properties never reported before);
- a mitochondrially bound and clorgyline-sensitive monoamine oxidase-A (MAO-A) activity with activity towards serotonin;
- a mitochondrially bound and deprenyl-sensitive MAO-B activity with activity towards e.g. benzylamine, tryptamine, tyramine and β -phenylethylamine.

The relative importance of the various activities for the metabolism of some monoamines has been investigated. Although the SSAO enzymes had lower K_m values with most substrates than the MAO, the bulk of the compounds was likely to be metabolized by MAO-B because of higher V_{max} values. K_m values and V_{max} values with serotonin, however, were rather similar for the MAO and the SSAO membrane-bound enzyme(s).

The thermal sensitivity of membrane-bound SSAO activities was found to be considerably lower than that for MAO-B.

The SSAO-5HT activity was found to be localized to the plasma membrane with a greater activity towards the periphery of the pulp tissue than towards the centre.

No great difference in the various amine oxidase activities was found when pulp tissues of varying degrees of maturity were compared.

The occurrence of membrane-bound SSAO and MAO activities was investigated and compared between pig, ox and human dental pulp. All activities were found to be present in about the same order of magnitude in all three species with the exception of a higher MAO-A activity in the ox pulp and the absence of this enzyme activity in the human dental pulp.

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