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MONOAMINE OXIDASE ACTIVITIES IN BROWN ADIPOSE TISSUE OF THE RAT: SOME PROPERTIES AND SUBCELLULAR DISTRIBUTION

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Abstract—Amine oxidase activity towards 5-hydroxytryptamine (5HT), tyramine (TYR), 2-phenylethylamine (PEA) and benzylamine (BZ) was studied in homogenates of interscapular brown adipose tissue of the rat. By the use of clorgyline, an irreversible inhibitor of MAO, it was established that 5HT was deaminated solely by MAO-A, and TYR and PEA mainly by MAO-A and clorgyline-resistant semicarbazide-sensitive amine oxidase (CRAO). BZ appeared to be oxidized almost entirely by CRAO. A very small amount of MAO-B activity was detectable with PEA and BZ as substrates. A variety of amines, amino acids and known amine oxidase inhibitors were tested for their ability to inhibit the deamination of BZ by CRAO. BZ metabolism by the enzyme was not affected by any secondary amines, unlike enzymes of the flavin type, but it was inhibited by carbonyl reagents, like the pyridoxal phosphate and copper-dependent amine oxidases described in plasma and connective tissue. Unlike these enzymes, however, CRAO in brown adipose tissue was resistant to KCN and unaffected by the amines, histamine, mescaline and some polyamines but it was inhibited by cuprizone. It was found to have a low K_m ($<5\ \mu M$) for BZ and showed the greatest similarity to a clorgyline-resistant enzyme described in rat blood vessels. Cell fractionation studies revealed that CRAO, being associated with the particulate fractions, was mainly membrane-bound. The distribution of CRAO activity between various cell fractions was different from that of the mitochondrial enzymes assayed and was more like that of either the plasma membrane or microsomal enzymes. When microsomal and plasma membrane vesicles were separated CRAO activity appeared distributed equally between the two fractions, suggesting that the enzyme may have a dual location within the cell. The specific activity of CRAO was higher in brown adipose tissue from obese animals than in tissue from lean animals. The significance of these findings is discussed in relation to the possible physiological function of this enzyme.

The amine oxidases that deaminate monoamines in mammalian tissues constitute a large group of enzymes with widely differing substrate and inhibitor specificities [1]. The group includes flavin-dependent monoamine oxidase (monoamine O_2:oxidoreductase, EC 1.4.3.4: MAO) a mitochondrial enzyme which plays a role in regulating intraneuronal levels of monoamines; the pyridoxal-dependent, copper-containing enzymes of connective tissue which are thought to be involved in collagen cross-linkage [2] and a number of other amine oxidases of less certain function, which may also be pyridoxal-dependent and have been found in blood plasma [3], in rabbit and bovine aorta [4, 5], in rat cardiovascular tissue [6–8] and also in human vascular smooth muscle [9].

It is possible that many of these enzyme activities may be divided further into more than one enzyme or form. This has already been shown for MAO which is now generally accepted to exist in at least two forms commonly called MAO-A and MAO-B [10].

Many of these enzymes metabolize the same substrates. Therefore, to identify which types of enzymes are present in a tissue, it is essential to employ several potential substrates together with a variety of appropriate inhibitors. Benzylamine (BZ), for example, is a substrate not only for amine oxidases in plasma and connective tissue but also for the B form of MAO [2, 10] and in some tissues [11] or under certain circumstances it may be deaminated by MAO-A also [12]. A convenient way of distinguishing between A and B forms of MAO is by the use of the acetylenic irreversible inhibitor clorgyline. Since MAO-A is about 1000 times more sensitive than MAO-B, in tissue homogenates preincubated for 20 min, by careful choice of suitable inhibitor concentrations it is possible to distinguish one form of activity from the other. However, since the action of clorgyline is exerted through the FAD moiety, partial or total lack of inhibition of deaminating activity in the presence of 10^{-4} M clorgyline may be used as presumptive evidence for the presence of an amine oxidase other than MAO. This activity has been referred to as 'clorgyline-resistant amine oxidase', since its physiological substrate is yet to be identified and its character established [13]. However, since clorgyline at concentrations between 10^{-3} and 10^{-4} M has been found to inhibit bovine plasma amine oxidase [14], the name semicarbazide-sensitive amine oxidase may be more suitable. Alternatively, since BZ appears to be the preferred substrate in many tissues [9], benzylamine oxidase could be used. At present this problem of nomenclature is unresolved. However, in this paper the term clorgyline-resistant amine oxidase (CRAO) is used to refer to that amine oxidase activity remaining after preincubation with 2×10^{-4} M clorgyline. We report here the finding of both MAO-A, -B and CRAO in the brown adipose tissue of the rat.

Brown fat has in recent times been the subject of much interest not only in rats and other small mammals but also in human subjects [15] as an organ important in thermogenesis [16]. Noradrenaline released from sympathetic nerve endings as well as some circulating amines not only increase thermogenic activity but also modify blood flow through this tissue. It was of interest, therefore, to see whether this tissue could deaminate such amines not only in normal animals but also in those where thermogenesis is thought to be defective, i.e. in the obese state [17].

MAO activity has been described before in this tissue [18–21] but as far as it is known, no CRAO activity has ever been reported before.

Attempts were made to investigate the nature and properties of this enzyme and also to gain some idea of its subcellular localization.

MATERIALS AND METHODS

Male Sprague–Dawley rats, ranging in weight from 150 to 250 g, were used for the majority of studies. For comparative observations on tissue from lean and obese animals, male littermate pairs of the Zucker fa/fa strain were used (kindly supplied by Dr. P. Trayhurn, Dunn Nutritional Laboratory, Cambridge). The animals were killed by stunning followed by decapitation, and interscapular brown fat tissue removed and weighed.

For whole homogenate studies, the tissue was homogenized 1:20 (w/v) in 0.001 M potassium phosphate buffer at pH 7.8, centrifuged at 600 *g* for 10 min, and the resultant supernatant divided into suitable aliquots and stored at −20° until assay. Homogenates were diluted further as required to ensure that the enzyme–substrate reactions remained linear during the time of incubation and that the amount of substrate metabolized was never more than 20% of that available.

For subcellular fractionation, brown adipose tissue from male rats 300–800 g in weight was homogenized 1:10 (w/v) in either of: (i) 0.25 M sucrose in 0.001 M potassium phosphate buffer, pH 7.8, centrifuged at 600 *g* for 10 min to remove cell nuclei, debris and unbroken cells. This supernatant was then centrifuged at 11,000 *g* for 30 min, the pellet washed in buffer/sucrose, recentrifuged and finally suspended in buffer to obtain the 'mitochondrial' fraction. The supernatant from the 11,000 *g* spin was centrifuged at 100,000 *g* for 2 hr and the pellet from this suspended in buffer to obtain the 'microsomal' fraction. The remaining supernatant was taken as the 'soluble' fraction; or (ii) 0.25 M sucrose in 0.01 M Tris–HCl, pH 7.8, containing 0.001 M EDTA, centrifuged at 600 *g* for 10 min and the supernatant then centrifuged at 11,000 *g* for 30 min. The pellet from the 11,000 *g* spin was washed in sucrose/buffer and recentrifuged to obtain the 'mitochondrial' fraction. The supernatant from the 11,000 *g* spin was centrifuged at 100,000 *g* for 2 hr, the pellet from this was resuspended in 0.001 M Tris–HCl, pH 7.8, containing 0.001 M $MgSO_4$ and dialysed for 1 hr against 200 vol. buffer/$MgSO_4$ [22]. Some was taken for assay as the 'microsomal' fraction. The remainder was then layered on to 26% dextran (relative density 1.089–1.096) made up in buffer/$MgSO_4$ and centrifuged in a swing-out rotor at 200,000 *g* for 6 hr. The two resultant bands of material which could be identified, one at the dextran-supernatant interface, the other the pellet at the bottom of the tube, were dispersed and diluted with buffer, recentrifuged at 100,000 *g* for 2 hr and taken up in buffer. All high speed spins were done in an MSE Prepspin 50 refrigerated ultracentrifuge.

Samples of supernatant from the high speed centrifugation were dialysed overnight against 200 vol. 0.001 M Tris–Cl, pH 7.8, to remove sucrose, then concentrated in the dialysis bags with Aquacide (CalBiochem, Los Angeles, CA) to around 20–40 mg/ml protein and 1 ml aliquots applied to a Sepharose 4B column (fractionation range 6×10^4–2×10^7 mol. wt.). Blue Dextran 200 and Phenol red were used to calculate the void and bed volumes. Two-millilitre aliquot fractions were collected and concentrated approximately five times with Aquacide for subsequent amine oxidase assay.

Amine oxidase activity was determined radiochemically by a method slightly modified from that described by McCaman *et al.* [23] and by Callingham and Laverty [24] in which samples were incubated in the presence of 100 mM potassium phosphate buffer, pH 7.8, and either [^{3}H]tyramine, TYR (sp. act. 1, 5 or 10 μCi/μmole and concentration range 0.05–1 mM) or [^{14}C]benzylamine, BZ (sp. act. either 2 or 10 μCi/μmole and concentration range 0.004–0.5 mM) or [^{14}C]2-phenylethylamine, PEA (sp. act. 0.5, 2 or 5 μCi/μmole and concentration range 0.005–0.5 mM) or [^{3}H]5-hydroxytryptamine, 5HT (sp. act. 2 or 10 μCi/μmole and concentration range 0.025–0.5 mM).

Inhibition studies were carred out by preincubating the samples at 37° for 30 min with appropriate dilutions of the inhibitors before addition of the substrate. Reversibility of inhibition was investigated using the dilution, washing and recentrifugation technique described by Lyles and Shaffer [25].

Protein content was measured by the method of Lowry *et al.* [26].

After subcellular fractionation of brown adipose tissue, each fraction was assayed for MAO-A activity with 0.5 mM 5HT as substrate and for CRAO activity with 0.2 mM BZ as substrate in the presence of 2×10^{-4} M clorgyline. As markers of various cell components [27] the following enzymes were also assayed by spectrophotometric methods; succinic dehydrogenase [28], an inner mitochondrial membrane enzyme; fumarase [29], a soluble mitochondrial matrix enzyme; NADPH-cytochrome *c* reductase [30] and glucose-6-phosphatase [31], microsomal enzymes; phosphodiesterase I [32] and 5′-nucleotidase (kit supplied by BDH Chemicals Ltd., Poole, U.K.), cell membrane enzymes.

2-[Ethyl-1-^{14}C]phenylethylamine hydrochloride and [G-^{3}H]tyramine were purchased from New England Nuclear (Dreieich, F.R.G.). 5-Hydroxy[G-^{3}H]tryptamine creatinine sulphate and [7-^{14}C]benzylamine hydrochloride were purchased from Amersham International Ltd. (Amersham, U.K.). (+) and (−)-Mexiletine and desmexiletine were gifts from Boehringer Ingelheim (Bracknell, U.K.) and clorgyline hydrochloride was a gift from May and

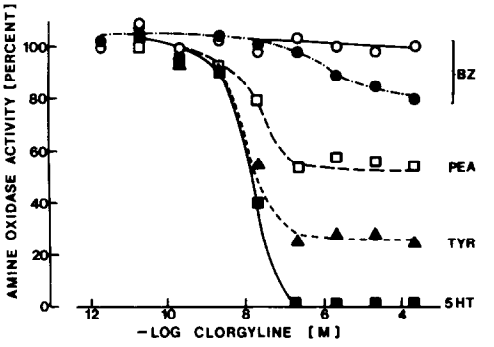

Fig. 1. Inhibition by clorgyline of amine oxidase activity in brown adipose tissue of the rat. Substrates used were BZ at 50 μM (○) and 500 μM (●); PEA at 500 μM (□); TYR at 500 μM (▲); and 5HT at 500 μM (■). Each point is the mean of quadruplicate determinations. In the absence of clorgyline, specific activities (in nmoles/hr/mg protein) were 20.6 for BZ at 500 μM, 14.86 for PEA at 500 μM, 30.7 for TYR at 500 μM and 18.0 for 5HT at 500 μM.

Baker Ltd. (Dagenham, U.K.). Debrisoquine sulphate and benserazide hydrochloride were gifts from Roche Products Ltd. (Welwyn Garden City, U.K.). (−)-Deprenyl hydrochloride was a gift from Professor M. Sandler (Queen Charlotte's Hospital, London, U.K.). All other compounds were standard laboratory reagents of analytical grade. Isoamylamine and isobutylamine, obtained as the free base, were converted to the hydrochloride salt before use.

RESULTS

Homogenates of brown fat were found to contain amine oxidase activity towards all four substrates tested (see Fig. 1). However, the degree to which clorgyline was able to inhibit this activity depended on both the type and concentration of the substrate used.

With BZ there was no significant inhibition at 0.005 and 0.05 mM substrate concentration; an enzyme resistant to clorgyline was therefore involved with metabolism. At 0.2 mM and higher substrate concentrations, a small amount (around 10%) of activity was inhibited by clorgyline concentrations at 2×10^{-5} M and above. This clorgyline-sensitive activity was probably due to the presence of a small amount of MAO-B.

With PEA as substrate 2×10^{-7} M clorgyline and higher caused 45, 35 and 25% inhibition of enzyme activity at 500, 50 and 5 μM substrate concentrations, respectively. Two separate enzymes, MAO-A and a clorgyline-resistant enzyme (CRAO), were therefore involved in PEA metabolism. Also at the lowest (5 μM) substrate concentration only, an additional small decrease in enzyme activity (<5%) was evident with clorgyline concentrations between 2×10^{-6} and 2×10^{-5} M. This suggested that a very small fraction of PEA may be metabolized by MAO-B providing the substrate concentration remains low. It is known that high concentrations of PEA inhibit the B form of MAO [33, 34].

With TYR as substrate, 2×10^{-7} M clorgyline and higher caused 75, 60 and 40% inhibition at 500, 50 and 5 μM substrate concentrations, respectively. The two enzymes MAO-A and CRAO were therefore involved in metabolizing TYR with MAO-A clearly having a greater effect on TYR at each substrate concentration than on PEA.

With 0.5 mM 5HT clorgyline at 2×10^{-7} M and above caused total inhibition indicating that MAO-A alone was involved in deaminating this substrate.

In brown fat samples taken from obese and lean littermates, the clorgyline-resistant component of amine oxidase activity was found to be significantly greater in the homogenates from the obese than in those from the lean animals in all three age groups studied. The clorgyline-sensitive MAO activity, however, was not significantly different at 5–10 weeks; it became slightly but significantly greater in the samples from obese animals at 16 weeks and slightly but significantly smaller at 30 weeks (Table 1). The relative proportions of the two components of amine oxidase activity are thus altered in the obese animals, a change which was reflected in the different shapes of the clorgyline inhibition curves for TYR in the samples from lean and obese animals.

Since BZ, at least in μmolar concentrations, appeared to be metabolized solely by the clorgyline-resistant enzyme, this was the substrate used in further studies on the enzyme.

With various concentrations of substrate, activity increased to a maximum around 0.2 mM substrate

Table 1. CRAO and MAO-A activities in homogenates of brown adipose tissue from lean and obese (littermate) Zucker rats

No. of animals in each group	Age range in weeks	Specific activity (nmoles/hr/mg protein) CRAO Lean	CRAO Obese	MAO-A Lean	MAO-A Obese
7	5–10	25.84 ± 2.15	52.97 ± 4.70†	46.75 ± 2.93	54.39 ± 7.68
5	16	27.39 ± 1.14	59.45 ± 6.66†	26.52 ± 0.79	35.03 ± 1.65*
4	30	40.61 ± 4.29	113.65 ± 25.25*	29.50 ± 0.89	25.85 ± 1.25*

Each value is the mean (± S.E.M.) derived from assays performed in quadruplicate on homogenates of brown adipose tissue from individual animals in each group. For CRAO activity, BZ (2×10^{-4} M) was used as substrate while for MAO-A, 5HT (5×10^{-4} M) was used. Statistical significance between lean and obese groups was calculated by paired Student's *t*-test.

* $P < 0.05$, † $P < 0.001$.

Table 2. K_m values for the deamination of four substrates by CRAO and MAO-A in homogenates of brown adipose tissue of the rat

Substrate	K_m values in μM	
	CRAO	MAO-A
BZ	2.98 ± 0.47 (5)	
PEA	10.0 (1)	250 (1)
TYR	40.2 ± 4.17 (14)	171 ± 29.6 (7)
5HT		146 ± 10.1 (5)

Each value is the mean (± S.E.M.) of a number (shown in parentheses) of separate determinations of K_m. Six different substrate concentrations were used and these varied from 2×10^{-6} M to 2×10^{-4} M with BZ and from 2×10^{-5} M to 2×10^{-3} M with TYR, 5HT and PEA. For K_m determinations of PEA and TYR deamination by MAO-A, homogenates were preincubated with semicarbazide (2×10^{-4} M). For K_m determinations of BZ, PEA and TYR deamination by CRAO, homogenates were preincubated with clorgyline (2×10^{-4} M). Up to 6 different homogenates were used in each experiment and triplicate determinations were made of each point.

concentration. Inhibition was seen at higher substrate concentrations, i.e. 0.5 mM and above.

In Table 2 are given K_m values for 5HT and BZ metabolism and for the clorgyline-resistant and clorgyline-sensitive components of TYR and PEA metabolism. Low and similar K_m values were obtained for CRAO in samples from lean and from obese animals.

With phosphate as buffer, the optimum pH for BZ metabolism was 7.8–8.0.

A range of potential inhibitors was tested against BZ with or without preincubation (Table 3). Deprenyl, the preferred inhibitor for MAO-B, was without effect. So also was KCN, an inhibitor of plasma amine oxidases [3, 5]. In addition, β-aminopropionitrile, an inhibitor of lysyl oxidase [35] produced no inhibition at 10^{-4} M and even at 10^{-2} M inhibited CRAO activity by no more than 30%.

Certain of the compounds found to inhibit the deamination of BZ were tested against 2×10^{-4} M BZ over a range of concentrations to compare their potency. Phenelzine appeared to be one of the most potent (approximate IC_{50} values were for phenelzine 2×10^{-9} M compared with 5×10^{-7} M for benzyloxylamine, 10^{-7} M for iproniazid, 10^{-6} M for semicarbazide and 5×10^{-4} M for isoniazid). It is interesting to note that (+)-mexiletine is a much more potent inhibitor than (−)-mexiletine which is in contrast to the situation found with MAO (Callingham, unpublished observations). Phenelzine was found to cause irreversible inhibition, whereas kynuramine showed partial reversibility. To check the effectiveness of the washing technique, both BZ itself and amphetamine, which is known to be totally reversible [13], were tested alongside kynuramine.

The CRAO activity towards BZ was inhibited by preincubation with cuprizone for 30 min over the range 10^{-4}–10^{-7} M (IC_{50} under these conditions approximately 10^{-5} M). At these concentrations there was little or no inhibition of MAO activity. If the homogenates were washed and centrifuged twice after preincubation with cuprizone, there was virtually no recovery of CRAO activity, indicating that the inhibition produced by cuprizone was probably irreversible. Addition of copper in the form of copper sulphate to the incubation medium did not restore enzyme activity.

A series of compounds which from their structure might be considered possible substrates for the enzyme were tested for their capacity to interfere with BZ metabolism (Table 4). None of the compounds with substituted amine groups, e.g. adrenaline, isoprenaline, phenylephrine, or metanephrine, showed any inhibitory effect on metabolism, nor did any of the amino acids, not even those closely related to amines which are known substrates for the enzyme. TYR, PEA, dopamine and kynuramine were inhibitory whereas their amino acid analogues tyrosine, phenylalanine, L-dopa and kynurenine were not.

The presence of one or more –OH groups on the benzene ring decreased the inhibitory effectiveness

Table 3. Effect of some potential inhibitors on CRAO activity in brown adipose tissue of the rat

Inhibitor	Percentage inhibition	Inhibitor	Percentage inhibition
Phenelzine	100	Iproniazid	100
β-Phenylisopropylhydrazine	100	Benzyloxylamine	100
Hydrazine	100	Semicarbazide	100
Aminoguanidine	96 ± 1	Kynuramine	87 ± 3
Benserazide	84 ± 3	Isoniazid	87 ± 3
Debrisoquine	69 ± 4	(+)-Mexiletine	58 ± 6
Desmexiletine	57 ± 3	Nialamid	31 ± 8
(−)-Mexiletine	10 ± 3	β-Aminopropionitrile	0
KCN	0	Deprenyl	0

Each potential inhibitor at a concentration of 2×10^{-4} M was preincubated for 30 min at 37° with homogenates of brown adipose tissue. 10^{-5} M BZ was then added as substrate and incubated for 30 min during which time the reaction was still linear. Each assay was performed in quadruplicate. Mean values ± S.E.R. from three separate experiments are given in the table. Kynuramine was included with the potential inhibitors since its effect on CRAO activity was only partially reversible. It is not known whether any compound included in Table 4 has a similar effect.

Table 4. Effect of some potential substrates on CRAO activity in brown adipose tissue of the rat

Compound	Percentage inhibition	Compound	Percentage inhibition
Phenylethanolamine	75 ± 2	TYR	37 ± 1
PEA	67 ± 2	Isobutylamine	33 ± 2
Octopamine	48 ± 1	Dopamine	16 ± 1
Tryptamine	45 ± 3	Normetanephrine	19 ± 2
Isoamylamine	43 ± 3	Methoxytyramine	12 ± 1
5HT	41 ± 2	Noradrenaline	16 ± 2

The following compounds were without effect: adrenaline, metanephrine, histamine, putrescine, spermine, spermidine, mescaline, isoprenaline, phenylephrine, adenosine, methyltyrosine, phenylalanine, tyrosine, L-dopa, histidine, methionine, lysine, cysteine, glycine, leucine, kynurenine, arginine, γ-aminobutyric acid, glutamine, serine, β-alanine, threonine, citrulline, asparagine, ornithine.

Each potential substrate was added to homogenates of brown adipose tissue at a final concentration of 2×10^{-4} M and CRAO activity assayed, without any preincubation, with 10^{-5} M BZ as substrate. Each assay was performed in quadruplicate. Mean values ± S.E.R. from three separate experiments are given in the table.

of the compound (PEA > TYR) whereas an –OH group at the β position on the side chain increased its effectiveness (phenylethanolamine > PEA; octopamine > TYR). Compounds without the phenyl group, isoamylamine and isobutylamine, also showed inhibitory effects. These compounds have been shown to be substrates for MAO [36].

In Fig. 2 are shown the results of three initial subcellular fractionation experiments in which fractionation scheme (i) was used. Only 9–15% of the CRAO activity appeared to be associated with the soluble fraction. However, since it is possible for soluble material to become trapped within the membrane vesicles during preparation and thereby separate with the particulate fractions, mitochondrial fractions were further subjected to freezing, thawing and osmotic shock followed by centrifugation. No significant amount of CRAO activity was found in the resulting supernatant even though the soluble enzyme fumarase was released from the mitochondrial matrix by such treatment. It seems, therefore, that CRAO activity is largely associated with membranes.

When aliquots of the soluble fraction were passed down a Sepharose 4B column, the small amount of CRAO activity detectable in this fraction appeared in and near the void volume. (Recovery was found to be at least 30% of the CRAO activity in the original fraction applied to the column.) Since particles of molecular weight above 2×10^7 are excluded from the gel, the possibility therefore exists that the small amount of CRAO activity detected in the 'soluble' fraction is still membrane-bound and associated with vesicles, too light to spin down at 100,000 *g* but too large to enter the gel. It has previously been reported that particulate material containing MAO can remain suspended after centrifugation at 100,000 *g* in the supernatant in the form of micelles [38].

CRAO activity was detected in both particulate

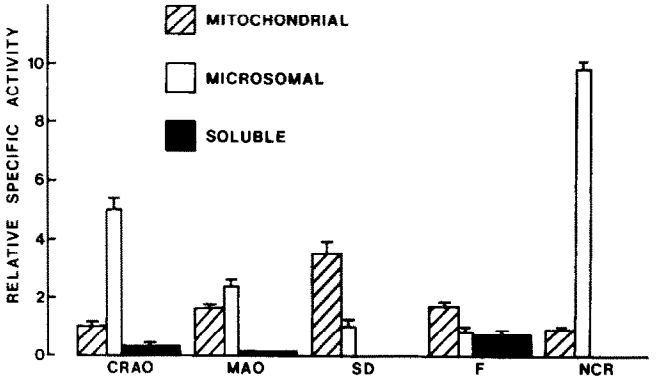

Fig. 2. Distribution of CRAO, succinic dehydrogenase (SD), MAO-A, NADPH cytochrome *c* reductase (NCR), and fumarase (F) within the 'mitochondrial', 'microsomal' and 'soluble' fractions obtained after cell fractionation (method i) of brown adipose tissue of the rat. Substrate for MAO-A was 5HT at 500 μM and for CRAO was BZ at 200 μM in the presence of 4×10^{-4} M clorgyline. Values are the mean ± S.E.R. of results taken from three separate preparations. Relative specific activities [37] were calculated from (% of total homogenate enzyme activity in each fraction/% of total homogenate protein in each fraction) and are represented by the heights of the columns. The column width represents the proportion of protein in each fraction.

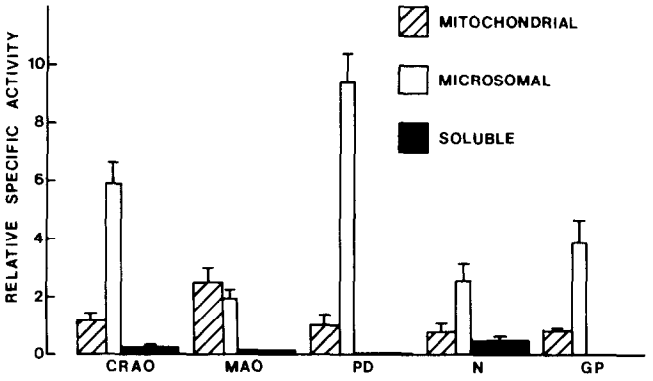

Fig. 3. Distribution of CRAO, MAO-A, of the cell membrane enzymes phosphodiesterase (PD) and 5'-nucleotidase (N), and of the microsomal enzyme glucose-6-phosphatase (GP) in the 'mitochondrial, 'microsomal' and 'soluble' fractions obtained after cell fractionation (method ii) of brown adipose tissue of the rat. Values are the mean ± S.E.R. of results from four separate preparations, and are expressed as described in Fig. 2.

fractions, 'mitochondrial' and 'microsomal'. K_m values for BZ metabolism with the CRAO enzyme obtained in the three fractions were identical. However, the distribution did not follow that of the mitochondrial enzymes; either the inner membrane-bound succinic dehydrogenase or the outer membrane-bound MAO (Fig. 2).

A second series of subcellular fractionation experiments was undertaken (fractionation scheme ii) in which sucrose in Tris buffer was used as the homogenizing medium. Although Tris buffer is known to inhibit MAO activity [39], it clearly did not affect the subcellular distribution of this enzyme (see Figs 2 and 3). CRAO activity appeared to be distributed in a manner similar to both the plasma membrane and microsomal enzymes (Fig. 3). Attempts were made, therefore, to separate the plasma membranes from the microsomal membranes. The technique employed made use of the property of divalent cations to cause shrinkage and an increase in density of microsomal vesicles but not of vesicles derived from the cell surface [22]. Centrifugation of the 'microsomal' fraction through dextran (relative density 1.095) after dialysis against buffer containing magnesium then resulted in separation of the material into two bands, an upper layer of material at the interface between the Dextran and the buffer containing the lighter plasma membrane vesicles, and a pellet at the bottom of the tube containing the heavier microsomal vesicles. After such treatment it was found (Fig. 4) that plasma membrane and microsomal enzymes were separately distributed in the two bands of material. However, CRAO activity appeared almost equally in the two bands.

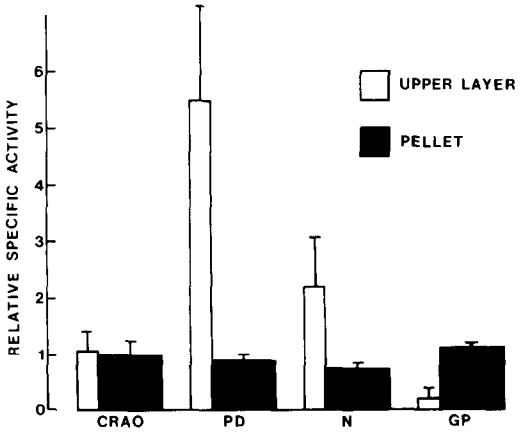

Fig. 4. Distribution of CRAO, phosphodiesterase (PD), 5'-nucleotidase (N) and glucose-6-phosphatase (GP) in the upper layer and pellet obtained after separation of the 'microsomal' fraction of brown adipose tissue by centrifugation through Dextran (see method ii). Values are the mean ± S.E.R. of results from four separate preparations. Relative specific activities were calculated from (% of enzyme activity in total 'microsomal' fraction present in each band/% of protein in total 'microsomal' fraction present in each band) and expressed as described in Fig. 2.

DISCUSSION

From the present study it is apparent that in brown adipose tissue of the rat there are at least two distinct amine oxidases, one metabolizing 5HT and almost totally inhibited by clorgyline at concentrations of 2×10^{-7} M and above, characteristic of MAO-A [10], and another which preferentially metabolizes benzylamine and is insensitive to clorgyline at 10^{-4} M. A small percentage of BZ metabolism was inhibited by clorgyline at concentrations between 2×10^{-6} and 2×10^{-4} M, indicating the presence of a minute amount of MAO-B activity.

MAO activity has been identified previously in brown adipose tissue of ground squirrels [18] and of mice [20]. In these cases tryptamine was used as substrate. Activity towards TYR, 5HT and BZ has also been described in the brown fat of lean and obese mice [21]. Inhibitor studies using deprenyl and clorgyline indicated that the activity towards TYR could be separated into two components presumed to be MAO-A and MAO-B. There were no indications from the data for a clorgyline-resistant component in the mouse tissue. However, it should be noted that only the mitochondrial fraction of brown adipose tissue was studied in these experiments and in brown fat of the rat at least, as shown in the

present study, CRAO does not appear to be a mitochondrial enzyme.

BZ metabolism by the clorgyline-resistant enzyme in the brown adipose tissue of the rat appears to be affected by a variety of primary amines but not by the secondary amines, adrenaline, metanephrine, phenylephrine and isoprenaline, unlike the amine oxidases of the flavin type but like the copper-dependent enzymes [1]. Further, it is sensitive to inhibition by carbonyl reagents, e.g. semicarbazide and iproniazid, as are a number of amine oxidases which are thought to use pyridoxal phosphate and copper as their co-factors. The direct involvement of pyridoxal phosphate in the deaminating action of the plasma amine oxidases has, however, been questioned [40, 41]. Brown fat CRAO is not affected by histamine or mescaline, which are substrates for pig plasma benzylamine oxidases [3], nor by polyamines which are substrates for the amine oxidases of bovine serum, aorta [5] and bovine dental pulp [42]; neither is it inhibited by KCN, unlike the amine oxidases found both in pig plasma and also rabbit and bovine aorta [4, 5]. However, its inhibition by cuprizone would indicate the possible involvement of copper in its activity.

It would seem that this clorgyline-resistant enzyme found in brown fat most closely resembles an enzyme with a low K_m for BZ metabolism which has been found in rat arterial vessels [6, 7], in human aorta [43] and in rat heart [8]. Thermostability studies have indicated, however, that the heart enzyme may perhaps be different from that in the aorta [13]. CRAO in brown adipose tissue is unlikely to resemble lysyl oxidase since it is largely resistant to inhibition by β-aminopropionitrile.

The pH optimum of this enzyme with BZ as substrate and phosphate as buffer was found to be 7.8–8.0. Clearly this could vary for other substrates, a fact that has already been noted with MAO [44]. Thus a change in pH could well influence the relative inhibitory potencies reported here of various other amines towards BZ metabolism. The naturally occurring substrates for CRAO activity have not yet been identified. BZ is the preferred substrate *in vitro* at pH 7.8 but at the physiological pH of the microenvironment of the cell, conditions could well favour other amine structures and BZ is not generally thought to be an endogenous amine.

In this context it is important to note the evidence from the subcellular fractionation studies reported here on brown adipose tissue of the possible location of the enzyme in the cell. The CRAO enzyme appears to be associated with the particulate fractions and is therefore highly likely to be a membrane-bound enzyme. In a previous study on rat arterial tissue, CRAO activity was found mainly in the supernatant [6]. However, differences in preparative technique might well influence the apparent solubility of the enzyme. Recent studies [45] have shown that the clorgyline-resistant amine oxidase activity present in rat artery is membrane-bound, and furthermore probably bound to the plasma membrane. In our own fractionation studies it was found that CRAO activity, like the activity of the plasma membrane and microsomal enzymes, was highest in the 'microsomal' fraction. However, when plasma membrane and microsomal membranes were separated, CRAO activity appeared equally in the two fractions. It is possible, therefore, that the enzyme may be present at two separate locations within the cell. Preliminary observations (unpublished) have indicated that the proportion of CRAO activity in the plasma membrane fraction may be higher in summer than in winter.

The enzyme does not share the same properties as those amine oxidases which have been described in bovine and rabbit aortae, and bovine and pig dental pulp [4, 42, 46, 47] and are thought to be associated with connective tissue metabolism. However, it cannot be stated categorically that CRAO in brown adipose tissue of the rat is not associated with connective tissue, bearing in mind the possibility of interspecies variation in inhibitor and substrate specificities.

In the present study, CRAO activity in brown fat was found to be significantly higher in obese animals than in lean animals, whereas the levels of MAO activity in the two groups were not consistently elevated in the three age groups studied. Previous observations in tissues of lean and obese mice have failed to find any significant differences in MAO activity in brown adipose tissue. In obese mice MAO activity was enhanced in white adipose tissue [21]. This was said to correlate well with the decrease in lipolysis noted in white adipose tissue in obese animals [20]. It has been observed that the obese Zucker rat is much more susceptible to body cooling than its lean littermate and is unable to maintain its body temperature even with noradrenaline infusion [48]. A defective response to noradrenaline was suggested. Defective responses to NA infusion have also been reported in obese human subjects [49]. The possibility exists that the greater CRAO activity seen in brown adipose tissue of obese animals might well be involved in decreasing the thermogenic response of this tissue to effector amines either derived from the circulation or locally released.

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