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A comparison of amine oxidase activity in human skin, rat skin and rat liver: relevance to collagen cross-linking

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AMINE oxidases are present in many mammalian tissues and their properties are known to vary from tissue to tissue.[1, 2] Although amine oxidases are thought to function primarily in the metabolism of physiologically active amines, this type of enzyme has recently been reported to participate in the cross-linking reactions of collagen[3] and elastin.[4] The cross-linking reaction is presumably initiated by oxidation of specific epsilon amino groups of polypeptide-bound lysine to the corresponding aldehydes which then undergo aldol condensation reactions.[3]

Since skin is a major source of collagen, it was of interest to find that this tissue contains significant amine oxidase activity. The present communication describes the partial purification of this enzyme from rat skin and the comparison of its substrate and inhibitor characteristics with the enzyme from rat liver. The inhibitor characteristics of the amine oxidase in rat skin and human skin were also compared by using tissue minces. Of particular interest was the finding that β-aminoproprionitrile (BAPN), which inhibits collagen cross-linking *in vivo* by blocking the oxidation of epsilon amino groups of lysine,[3] is not an effective inhibitor of skin amine oxidase.

The source of materials and the enzyme assay techniques have been described recently.[5] Initial studies as well as the comparison of amine oxidase activity in rat skin and human skin were done with minces of skin as enzyme source, whereas the substrate kinetics and inhibitor studies of rat skin and liver amine oxidase were done with partially purified enzymes. Rat skin amine oxidase was prepared as follows: male NIH strain Sprague–Dawley rats were decapitated, the hair was removed by electric clippers, and the skin was stripped from the carcase. Epidermis and s.c. fat were removed. The dermis was sliced finely, placed in a mortar containing liquid nitrogen and pulverized with a chilled pestle. The pulverized dermis was suspended in 0·2 M phosphate buffer, pH 7·5 (5 ml/g) containing 0·2 mg/ml bacterial collagenase (type CLS from Worthington Biochemical Corp.). This suspension was incubated for 17 hr at 25° and then homogenized in a glass homogenizer at 0°. This procedure caused no appreciable loss of amine oxidase activity. The homogenate was stirred at 4° for 4 hr and then centrifuged at 20,000 *g* for 15 min. The supernatant fraction, which contained most of the amine oxidase activity, was brought to 55 per cent saturation with ammonium sulfate. This suspension was allowed to stand for 2 hr at 0° before centrifugation. The sediment, which now contained the majority of the activity (approximately 80 per cent), was resuspended in 0·2 M phosphate buffer, pH 7·5 (10–13 mg protein/ml), and dialyzed for 12 hr against 0·02 M phosphate buffer, pH 7·5. The dialysis was repeated twice against fresh buffer solutions. The enzyme was apparently insoluble at this stage although fully active. Because of the insolubility, no further attempts were made to purify the enzyme which was about 10-fold purified over the crude tissue homogenate.

Rat liver enzyme activity was studied in whole liver homogenates and in purified enzyme solution, prepared through Fraction III as described by Nara *et al.*[6] Fraction III was taken to 55 per cent saturation with $(NH_4)_2SO_4$, centrifuged, and the precipitate was resuspended in 0·2 M $NaKPO_4$ buffer. This was dialyzed for 50 hr in 0·02 M phosphate buffer, pH 7·5, centrifuged, and the supernatant fraction was used as the enzyme source.

Minces of human and of rat skin catalyze the oxidation of benzylamine to benzaldehyde at the rate of about 1–2 μmole/g skin/hr. This activity is appreciably less than that found in many rat tissues,[1, 7] particularly liver homogenates (33 μmole/g liver/hr), but does represent a significant amount of enzyme activity. It has been reported that skin is devoid of amine oxidase activity.[1] However, manometric techniques used in earlier studies may not have detected this level of activity. In view of the recent implication of amine oxidase in cross-linking of collagen and elastin, it seemed of importance to characterize the enzyme of skin and to define its possible role in the cross-linking reaction.

The Michaelis constants and the relative rates of oxidation of four common amine oxidase substrates were determined for the partially purified preparations of rat skin and rat liver. The results are shown in Table 1. Although slight differences in the relative rates of oxidation of the various

substrates are apparent, benzylamine was most actively deaminated by both enzymes; at the same time, the most striking difference noted between the two enzymes is the approximately 25-fold difference in the K_m value for benzylamine. In addition to the substrates shown in Table 1, the skin enzyme also catalyzed the oxidation of kynuramine and BAPN at relative rates analogues to the rat liver

TABLE 1. SUBSTRATE CHARACTERISTICS OF AMINE OXIDASE IN RAT SKIN AND LIVER*

Substrate	Rat skin		Rat liver	
	V_{max}	K_m	V_{max}	K_m
Benzylamine	100	2×10^{-5}	100	5×10^{-4}
Tryptamine	80	3×10^{-5}	62	5×10^{-5}
Tyramine	69	9×10^{-5}	92	6×10^{-5}
Serotonin	45	2×10^{-5}	36	4×10^{-5}

* The V_{max} and K_m values were calculated from double reciprocal plots of substrate concentrations vs. rate. The V_{max} values are expressed relative to that obtained for benzylamine with the enzyme from each source.

enzyme. The skin did not exhibit any measurable diamine oxidase activity with cadaverine as a substrate in the assay procedure of Okuyama and Kobayashi.[8]

The rat skin enzyme was inhibited by many of the classical inhibitors of liver amine oxidase (Table 2) at concentrations similar to those which are effective for the latter enzyme. One exception, however

TABLE 2. INHIBITION OF AMINE OXIDASE ACTIVITY OF RAT SKIN AND LIVER*

Inhibitor†	Concentration producing 50 per cent inhibition	
	Rat liver	Rat skin
Pargyline · HCl	5×10^{-7}	3×10^{-4}
JB-516	2×10^{-6}	6×10^{-6}
Tranylcypromine · HCl	5×10^{-6}	5×10^{-6}
Iproniazid	5×10^{-5}	2×10^{-5}
Isoniazid	$> 10^{-3}$	1×10^{-3}
KCN	$> 10^{-3}$	1×10^{-3}
BAPN	$> 10^{-3}$	$> 10^{-3}$
Penicillamine	$> 10^{-3}$	$> 10^{-3}$
Tris	$> 10^{-1}$	1×10^{-2}

* These studies were done with partially purified enzymes from each tissue with tryptamine-^{14}C (3×10^{-6} dpm/μmole), $1{\cdot}2 \times 10^{-4}$ M, as substrate. The inhibitors were preincubated with the enzyme for 20 min at 37° prior to addition of the substrate.

† Pargyline = *N*-benzyl-*N*-methyl-2-propynylamine; JB 516 = phenylisopropylhydrazine; tranylcypromine = 2-phenylcyclopropylamine.

is pargyline, which causes 50 per cent inhibition of liver amine oxidase at a concentration 1/600 of that required for equal inhibition of skin amine oxidase. Penicillamine and BAPN, which inhibit collagen cross-linking when administered to animals *in vivo*,[9,10] do not appear to be significant inhibitors of amine oxidase from skin. Tris buffer also inhibits the skin enzyme, whereas little or no effect is observed with the liver enzyme. Human skin amine oxidase activity is inhibited by the same inhibitors that inhibit the enzyme from rat skin, and to about the same extent (Table 3). The skin enzyme from either human or rat is strongly inhibited by aminoguanidine suggesting the possibility that this enzyme is of the pyridoxal type. Conversely, cyanide, which is a strong inhibitor of the pyridoxal-containing plasma amine oxidase,[5] is a weak inhibitor of the skin enzyme, and in several different partially purified enzyme preparations no stimulation of activity could be obtained by the

addition of pyridoxal phosphate to the incubation media. The nature of the cofactor for skin amine oxidase therefore remains obscure.

Preliminary experiments on the effects *in vivo* of BAPN on rat skin amine oxidase were done by feeding rats a standard diet containing 1% BAPN. The rats were sacrificed after 6 weeks when they

TABLE 3. INHIBITORS OF AMINE OXIDASE ACTIVITY OF RAT SKIN AND HUMAN SKIN*

Inhibitor†	Per cent inhibition at 10^{-3} M	
	Rat skin	Human skin
Pargyline	34	42
JB-516	100	100
Tranylcypromine · HCl	75	56
Isoniazid	33	56
Aminoguanidine	100	100
KCN	18	70
BAPN	30	16
D-Penicillamine	40	26
N-acetyl-D-penicillamine	21	3

* Minces of skin (50 mg) were used as the enzyme source, and benzylamine-^{14}C ($1{\cdot}2 \times 10^5$ dpm/μmole), $1{\cdot}3 \times 10^{-3}$ M as the substrate. The inhibitors were preincubated for 30 min with the enzyme at 37° prior to addition of the substrate.

† See Table 2 for chemical names of inhibitors. D-Penicillamine and *N*-acetyl-D-pencillamine were obtained from Merck & Co.

had become grossly lathyritic. The amine oxidase was prepared from the skin of these animals and controls which had received the same diet without BAPN. In separate experiments (2 animals each of the experimental and control groups) with assays done in duplicate, no significant difference was observed in the skin amine oxidase levels of control and lathyritic animals.

It is now well established[1, 2] that amine oxidase of various mammalian tissues exhibits varying properties, suggesting the possibility of numerous molecular species within a single animal. Although skin amine oxidase appears to be very similar to the enzyme extracted from liver mitochondria, three characteristics clearly distinguish the enzyme from these two sources. The skin enzyme exhibits a K_m value for benzylamine which is about 1/25 that observed for liver, and the sensitivity of the skin enzyme to inhibtion by pargyline is appreciably less than liver. Furthermore, Tris buffer significantly inhibits the skin enzyme while exerting no appreciable effect on the liver enzyme.

It was not possible in the current line of experimentation to implicate or eliminate the role of skin amine oxidase in collagen cross-linking. The finding that neither BAPN nor penicillamine inhibits this enzyme significantly *in vitro*, and that lathyritic rats appear to have normal skin amine oxidase activity, would tend to eliminate this enzyme as the initiator of the cross-linking reaction. Recent reports[11, 12] indicate that BAPN is a weak competitive inhibitor of chick aorta and pig plasma amine oxidase respectively. With human plasma amine oxidase purified through the second ammonium sulfate step of McEwen,[13] we have found that BAPN is a typical competitive inhibitor with regard to the substrate benzylamine. A concentration of about 2 to 10^{-3}M is required for 50 per cent inhibition. It is therefore possible that the plasma enzyme participates in cross-linking in some fashion. It should be pointed out, however, that benzylamine is an artificial substrate for both the skin and plasma enzymes and inhibition is usually studied at close to optimal substrate concentration. The situation may be entirely different in tissue where a polypeptide chain containing epsilon amino groups of lysine is present in low concentration and may exhibit markedly different affinity for the enzyme. The final answer to this question must await the isolation of highly purified skin amine oxidase which can be used with polypeptide-bound lysine as the substrate.

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Thyroid inhibition by γ-amino-β-hydroxybutyric acid

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BENEDETTI *et al.* (1964) have found that in patients affected by Grave's disease the neuro-psychic symptoms improve following administration of γ-amino-β-hydroxybutyric acid (GABOB). They believe this improvement is due to a central action of GABOB. We have resolved to investigate if this drug may modify thyroid activity too. The results shown in this preliminary paper point out that large doses of GABOB depress the function of thyroid gland both in animals and in men.

EXPERIMENTAL AND RESULTS

In the rat 600–1000 mg/kg per day of GABOB orally administered for 5 days decreased the thyroid uptake of ^{131}iodine (I^{131}). A similar result was obtained in the rabbit by s.c. daily administration of 600 mg/kg during 23 days (Table 1).

TABLE 1. EFFECT OF GABOB ON I^{131} THYROID UPTAKE IN THE ANIMAL. THE DIFFERENCE FROM THE NORMAL IS ALWAYS SIGNIFICANT

Species	No	Drug	mg/kg /day	Route	I^{131} uptake after 24 hr (%)	
					mean	S.D.
rat	10	—	—	—	30·53	7·34
rat	10	GABOB	600	oral	21·76	8·72
rat	10	propyl-thiouracil	10	oral	7·49	3·17
rat	10	—	—	—	30·79	6·09
rat	10	GABOB	1000	oral	20·73	5·29
rat	10	propyl-thiouracil	3	oral	2·27	1·33
rabbit	10	—	—	—	20·04	3·53
rabbit	9	GABOB	600	s.c.	9·18	4·11
rabbit	9	propyl-thiouracil	0·5	s.c.	4·96	1·87