difference in 'photoshifts' between the two cyclopyrrolones binding and BZD agonists binding. If we confirm that BZD agonists exhibit a high affinity shift in photolabelling membranes, in our hands the two cyclopyrrolones agonists have a low 'photoshift' (Tables 2 and 3). Low photoshifts for ZPC were also found by Möhler and Karobath [4, 5].

Our results clearly suggest that, contrarily to a recent hypothesis, the existence of a pharmacological agonist activity is not necessarily linked to a reduced affinity for photolabelled membranes. It must indeed be stressed that ZPC and SRC behave pharmacologically and clinically as agonists [10, 11]. It should also be noted that these findings with the cyclopyrrolone family agree well with the results reported by Brown [8] for the pyrazoloquinolinones.

Moreover, we confirm our previous data [2], indicating the existence of differences in the recognition of BZD agonists and SRC by BZD receptors. The BZD receptor model of Crippen [12] offers a tentative interpretation of these differences. According to this model, a ligand interacts with the receptor through 'binding site points' computed from structural and energetical data characterizing various agents able to bind to the BZD receptor site. In this model, ZPC interacts with less than half the number of binding site points associated with diazepam or FLU. This suggests that ZPC possesses a higher flexibility for binding to different conformations of BZD receptors induced by photolabelling.

In summary, we find that, in contrast to BZD agonists but like pyrazoloquinolinones, the two cyclopyrrolones agonists SRC and ZPC have a low photoshift whatever [$^3$H] ligand of BZD receptors is used.

These results raise a question about the possibility of generalizing beyond the BZD family the recent hypothesis linking a pharmacological agonist activity and a reduced affinity for photolabelled membranes.

*Acknowledgements*—We wish to thank Mr. Carruette for his skillful technical assistance and Mr. Long for his help with English in the writing of the paper.

*Rhône-Poulenc Recherches*
*Centre de Recherches de Vitry*
*13 quai Jules Guesde*
*F 94407 Vitry sur Seine*
*Cedex, France*

J. C. BLANCHARD
J. L. ZUNDEL
L. JULOU

## REFERENCES

1. J. C. Blanchard, A. Boireau, C. Garret and L. Julou, *Life Sci.* **24**, 2417 (1979).
2. J. C. Blanchard and L. Julou, *J. Neurochem.* **40**, 601 (1983).
3. H. Möhler and J. G. Richards, *Nature, Lond.* **249**, 763 (1981).
4. H. Möhler, *Eur. J. Pharmac.* **80**, 435 (1982).
5. M. Karobath and P. Supavilai, *Neurosci. Lett.* **31**, 65 (1982).
6. K. W. Gee and H. Y. Yamamura, *Eur. J. Pharmac.* **82**, 239 (1982).
7. P. Skolnick, M. M. Schweri, E. F. Williams, V. Y. Moncada and S. M. Paul, *Eur. J. Pharmac.* **78**, 133 (1982).
8. C. L. Brown and I. L. Martin, *Neurosci. Lett.* **35**, 37 (1983).
9. N. Yokoyama, B. Ritter and A. D. Neubert, *J. med. Chem.* **25**, 337 (1982).
10. C. Garret, M. C. Bardone, J. C. Blanchard, J. M. Stutzmann and L. Julou, 13th C.I.N.P. Congress, Jerusalem, 20–25 June, Abstract 1, 244 (1982).
11. L. Julou, M. C. Bardone, J. C. Blanchard, C. Garret and J. M. Stutzmann, *Int. Pharmacopsychiat.* (1983) in press.
12. G. M. Crippen, *Molec. Pharmac.* **22**, 11 (1982).

*Biochemical Pharmacology*, Vol. 32, No. 23, pp. 3653–3656, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00


# Characterization of the amine oxidase activities of liver microsomes of different vertebrate and invertebrate species

(*Received* 13 *December* 1982; *accepted* 4 *July* 1983)

A clorgyline-resistant amine oxidase has been described in different tissues of several mammals [1–9]. This enzyme shows some similarities to the benzylamine oxidase activity which has been described in the blood plasma of non ruminant mammals [Blaschko, 10]. It clearly differs from the classical mitochondrial monoamine oxidase (MAO) (E.C. 1.4.3.4.) in its sensitivity to the inhibitors such as clorgyline and deprenyl [Buffoni, 11] and it is very similar, in its sensitivity to the inhibitors, to the diamine oxidase (DAO) (E.C.1.4.3.6.). Discrimination between clorgyline-resistant amine oxidase and DAO is possible only by means of competition experiments with different substrates. Because the physiological significance of the clorgyline-resistant amine oxidase is still unknown, it appeared interesting to us to characterize the amine oxidase activties of liver or digestive gland microsomes in vertebrate and invertebrate species in order to better understand their distribution in nature.

## *Materials and methods*

The maintenance conditions of rat (Wistar), quail (*Coturnix coturnix japonica*), trout (*Salmo gairdneri*) and mussel (*Mytilus galloprovincialis*) are reported in Table 1.

The animals were killed by decapitation or by a blow to the head. The liver was removed, weighed, then perfused with an ice-cold solution of KCl 0.15 M at pH 7.4 and homogenized in a glass–Teflon homogenizer at 1:4 (w/v) ratio with ice-cold sucrose 0.25 M containing tris [Tris(hydroxymethyl)amino-methan] 0.05 M at pH 7.4. The homogenates were centrifuged at 9000 *g* for 20 min; the resulting supernatant was centrifuged at 105,000 *g* for 60 min. The pellets were resuspended by hand in a glass–Teflon homogenizer in KCl 0·15 M at pH 7.4 and centrifuged at 105,000 *g* for 50 min; the final pellets were stored at −70° until the enzymatic assays (less than 2 weeks) were performed.

Table 1. Maintenance conditions and characteristics of the animals

| | Animal | | | |
|---|---|---|---|---|
| | Rat | Quail | Trout | Mussel |
| Temperature (°C) | 19–21 | 19–21 | room temp. | 14–15 |
| Acclimatization (days) | 8 | 20 | * | 7 |
| Diet | Commercial pellets | Poultry feed† | Commercial pellets | Synthetic sea water‡ |
| Sex | Males | Males | Mixed | Mixed |
| Age (days) | 60 | 60 | — | — |
| Weight (g) or dimension (cm) | 200–250 | 120–160 | 200–300 | 10–11 cm |
| Light/dark | 12/12 | 16/8 | 14/10 | 12/12 |

The microsomal fractions of mussel were kindly supplied by Dr. A. Viarengo of the Institute of General Physiology of the University of Genova.

* The trouts were kept in fresh stream water until sacrifice.

† Plata cornflour 43%, barley flour 10%, oatmeal 4%, soya extract flour 35%, dehydrated lucern flour 4%, $CaCO_3$ 1.5%, $CaPO_4$ 2%, vitamin additive 0.5%; to this were added: trace mineral premixed in NaCl 0.25% and methionine 0.02%.

‡ Prepared according to La Roche *et al.* (12); made up to 35%.

The mussel digestive glands were minced and homogenized in ice-cold sucrose 0.9 M containing 20% glycerol, 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethansulfonic acid) at pH 7.6 and EDTA (ethylenediaminetetracetic acid) 0.001 M, dithiotreitol 0.005 M, cysteine 0.01 M, PMSF (phenylmethylsulfonilfluoride) 0.66 mM and trypsin inhibitor (Boehringer, from soybean) 120 mg/ml and were centrifuged at 30,000 *g* for 20 min. The resulting supernatant was centrifuged as described before; all the centrifugations were made at 0–4°.

In order to check the purity of the preparations, the microsomal fractions were examined by electronic microscopy; mitochondrial contaminations were measured by the determination of cytochrome c oxidase activity [Wharton and Tzagoloff, 13] and cytoplasmic contaminations by the determination of lactate dehydrogenase activity according to Bergmeyer [14].

The amine oxidase activities were determined radiochemically by the method of Buffoni and Ignesti [15] and the diamine oxidase activity according to Kusche *et al.* [16].

The protein content was determined according to Lowry *et al.* [17]. Tyramine-(7-$^{14}$C)-hydrochloride (56 mCi/mmol), 5-hydroxy-(side-chain-2-$^{14}$C)-tryptamine creatinine sulphate (53 mCi/mmol) and (1,4-$^{14}$C) putrescine dihydrochloride (113 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, U.K.; benzylamine (methylene-$^{14}$C) hydrochloride (14 mCi/mmol) from ICN Pharmaceuticals, Irvine, CA. All the other reagents were analytical grade products of Merck Darmstad, F.R.G.

*Results and discussion*

The amine oxidase activities of the microsomal fractions, assayed with different substrates, are reported in Table 2. Electronic microscopy and enzymatic assays showed that cytoplasmic and mitochondrial contaminations were less than 1% (except in the mussel) and recovery of amine oxidase activity (with benzylamine as substrate) was 30% in the trout and quail, 17% in the rat and 5% in the mussel.

Table 2. Amine oxidase activity in microsomal fraction (nmoles/mg/hr) (mean ± S.E.)

| | Benzylamine | Tyramine | 5-Hydroxy tryptamine | Putrescine |
|---|---|---|---|---|
| Rat (N = 5) | 44.8 ± 4.6 | 108.4 ± 10.8 | 53.6 ± 4.8 | 0 |
| Trout (N = 5) | 2.4 ± 0.2 | 29.6 ± 9.6 | — | 0 |
| Quail (N = 5) | 18.2 ± 2.2 | 116.4 ± 16.4 | 65.6 ± 7.6 | 12.0 ± 1.3 |
| Mussel (N = 2) | 8.1 – 17.1 | — | — | 15.9 – 16.1 |

*MAO activity assay.* 0.1 ml of microsomes resuspended in M/15 phosphate buffer (2–5 mg of protein/ml) and 0.15 ml of M/15 phosphate buffer at pH 7.4 were preincubated by 10 min at 37° (a); 0.05 ml of substrate 4 mM ($^{14}$C) was then added. The reaction was stopped, after 30 min, with 0.1 ml of HCl 3 N and the aldehyde extracted with 1 ml of ethylacetate; 0.5 ml of this was counted in 15 ml of Instagel.

*DAO activity assay.* 0.2 ml of microsomes (2–5 mg of protein/ml) and 0.7 ml of M/15 phosphate buffer at pH 7.4 were preincubated for 10 min at 37° (a); 0.1 ml of putrescine ($^{14}$C) was then added. After 1 hr of incubation 0.1 ml of perchloric acid 2 M and 1 ml of strong alkaline buffer at pH 12.2 were added. Reaction products were extracted with 6 ml of butyl PBD 0.4% in toluene, 3 ml of which were counted in 12 ml of the same phase.

(a) Trout and mussel microsomes were incubated at 25°. Benzylamine, tryamine, 5-hydroxytryptamine were used at 0.66 mM concentration, putrescine at 1 mM concentration.

Table 3. Percent of inhibition of the amine oxidase activity or rat, trout, quail liver and mussel digestive gland microsomes by some inhibitors

| | Inhibitors | Substrates | | | |
|---|---|---|---|---|---|
| | | 5-Hydroxy tryptamine | Tyramine | Benzilamine | Putrescine |
| Rat | Clorgyline | 98 ± 0.2 | 93 ± 4 | 78 ± 2 | — |
| | Semicarbazide | 9 ± 6 | 15 ± 4 | 7 ± 2 | — |
| Trout | α-Aminoguanidine | — | 60 ± 4 | — | — |
| | Iproniazid | — | 100 | — | — |
| Quail | Semicarbazide | — | — | 44 ± 3 | 100 |
| | Putrescine | — | — | 0 | — |
| Mussel | Semicarbazide | — | — | 88–96 | 60–66 |

Activity assay as in Table 2, with 0.05 ml of inhibitor instead of 0.05 ml of buffer. The final inhibitors concentration was $10^{-4}$ M; putrescine concentration was in all cases 1 mM. The substrates concentration was 0.66 mM.

Results are mean ± S.E. of 5 different microsomes preparations from rat, trout and quail and from 2 different preparations of mussel microsomes.

In the rat liver microsomes no DAO activity was found, whereas amine oxidase activity is very high; this enzymatic activity is almost wholly inhibited by clorgyline, as shown in Table 3 and Fig. 1. The activity of rat liver microsomes is of E.C.1.4.3.4. type; it is in fact completely inhibited by clorgyline, with only a trace of semicarbazide-sensitive benzylamine oxidase. The existence of MAO activity in rat liver microsomes has been previously described by different authors [18–21].

No DAO activity is present in the trout liver microsomes, whereas the amine oxidase activity is partially inhibited by α-aminoguanidine and completely by iproniazid, as is shown in Table 3. In the trout a semicarbazide-sensitive benzylamine oxidase is thus present.

Strong DAO activity exists together with the amine oxidase activities in quail liver microsomes, but DAO is not responsible for the benzylamine oxidation, as is shown in Table 3.

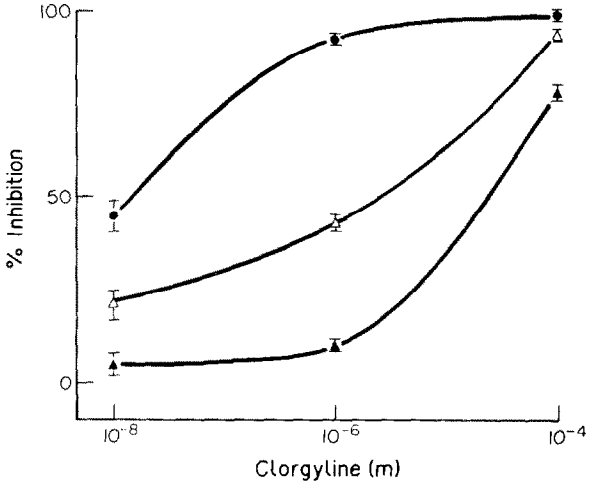


Fig. 1. Effect of different concentrations of clorgyline on amine oxidase activity of rat liver microsomes (mean ± S.E.) (N = 5). (●) 5-Hydroxytryptamine 0.66 mM, (△) tyramine 0.66 mM, (▲) benzylamine 0.66 mM.

Table 3 is also indicates that in the mussel digestive gland is present a benzylamine oxidase activity inhibited by semicarbazide and DAO activity.

Quail liver microsomes oxidize benzylamine, tyramine and serotonin in the same way as rat liver microsomes do, but they possess the added activity of oxidizing putrescine. The benzylamine oxidation does not depend on the presence of DAO because there is no competition between putrescine and benzylamine, and the fact that this activity is partially inhibited by semicarbazide suggests the presence of a semicarbazide-sensitive benzylamine oxidase. This activity is also present in mussel digestive gland alongside the DAO activity.

The comparison of these four different animals reveals that a mitochondrial type MAO activity is present in all of them, albeit in different amounts. A semicarbazide-sensitive benzylamine oxidase seems to be widespread. The fact that mussel is very rich in connective tissue as well as in benzylamine oxidase activity may add further support to the observations that this enzyme is of connectival origin [Buffoni *et al.*, 22; Papini *et al.*, 23]. DAO activity is present only in quail and mussel. The absence of DAO activity in the assay of rat and trout liver microsomes is not dependent on the presence of enzymes able to metatolize the aldehyde formed, because the addition of $10^{-2}$M acetaldehyde, which inhibits such enzymes, does not vary the results.

*Acknowledgements*—We thank Dr. F. M. Casini and Mr. P. Guasti of the Institute of Histology of the University of Florence for the electron microscopy observations of the microsomal fractions.

This work was supported by C.N.R. grant CT 81.0013704.

*Department of Pharmacology and Toxicology*
*University of Florence*
*Italy*

G. IGNESTI
G. BANCHELLI
C. FALAI
R. PIRISINO
L. RAIMONDI
F. BUFFONI*

* To whom correscondence should be addressed.

REFERENCES

1. J. E. Coquil, C. Goridis, G. Mack and N. H. Neff, *Br. J. Pharmac.* **48**, 590 (1973).
2. F. Buffoni, R. Pirisino and P. Marino, *Agents and Actions* **5**, 429 (1975).
3. G. A. Lyles and B. A. Callingham, *J. Pharm. Pharmac.* **27**, 682 (1975).
4. J. A. Fuentes and N. H. Neff, *Biochem. Pharmac.* **26**, 2107 (1977).

5. R. Lewinsohn, H. K. Bohm, V. Glover and M. Sandler, *Biochem. Pharmac.* **27**, 1857 (1978).
6. M. Wibo, A. G. Duong and T. Godfraind, *Eur. J. Biochem.* **112**, 87 (1980).
7. D. E. Clarke, G. A. Lyles and B. A. Callingham, *Biochem. Pharmac.* **31**, 27 (1982).
8. M. A. Barrand and B. A. Callingham, *Biochem. Pharmac.* **31**, 2177 (1982).
9. C. Shakhar, K. Mayanie and N. Z. Baquez, *Biochem. Pharmac.* **31**, 3925 (1982).
10. H. Blaschko, *Adv. Comp. Physiol. Biochem.* **1**, 67 (1962).
11. F. Buffoni, In *Monoamine oxidase* (Eds. K. Kamijo, E. Usdin and T. Nagatsu), p. 584. Excerpta Medica, Amsterdam (1981).
12. G. La Roche, R. Eisler and J. Tarzwell (1970). *J. Water Pollution Fed.* **42**, 1982 (1970).
13. Wharton D. C. and A. Tzagoloff, in *Methods in Enzymology* (Eds. R. W. Eastbrook and M. E. Pullman), vol. 10, p. 245. Academic Press, New York (1967).
14. H. U. Bergmeyer, in *Methods of Enzymatic Analysis* (Eds. H. U. Bergmeyer and K. Gawehn), p. 481. Verlag Chemie, Weinheim; Academic Press. New York (1974).
15. F. Buffoni and G. Ignesti, *Biochem. J.* **145**, 369 (1975).
16. J. Kusche, M. Pichter, R. Hersterberger, J. Schmidt and W. Lorenz, *Agents and Actions* **3**, 148 (1973).
17. O. H. Lowry, N. J. Rosebrough, A. Lewis Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. E. O. Ostwald and C. F. Strittmater, *Proc. Soc. exp. Biol. Med.* **114**, 668 (1963).
19. P. Bandhuin, H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques and C. De Duve, *Biochem. J.* **92**, 179 (1964).
20. B. Jarrott and L. Iversen, *Biochem. Pharmac.* **17**, 1619 (1968).
21. C. De Duve, *J. Cell. Biol.* **50**, 20D (1971).
22. F. Buffoni, L. Della Corte and D. B. Hope, *Proc. R. Soc., Ser. B*, **195**, 417 (0000).
23. M. Papini, M. G. Martinetti, F. Buffoni, G. Ignesti and F. Franconi, *Ital. J. Neurol. Sci.* **2**, 129 (1981).