2. Krause BR and Newton RS. ACAT Inhibitors: preclinical pharmacological profiles and implications for plasma lipid regulation and prevention of atherosclerosis. In: *Atherosclerosis VIII, Proceedings of the 8th International Symposium on Atherosclerosis,* (Eds. Gepaldi G, Gotto AM, Manzato E and Baggio G), pp. 707–710. Excerpta Medica, Amsterdam, 1989.
3. Ross CA, Go KJ, Heider JG and Rothblat GH, Selective inhibition of acyl-CoA:cholesterol acyltransferase by compound 58-035. *J Biol Chem* **259**: 815–819, 1984.
4. De Vries VG, Schaffer SA, Largis EE, Dutia MD, Wang CH, Bloom JD and Katocs AS, Potential antiatherosclerotic agents. 5. An acyl-CoA:cholesterol *O*-acyltransferase inhibitor with hypocholesterolemic activity. *J Med Chem* **29**: 1133–1134, 1986.
5. Rucker W, Prop G and Huther AM, Antiatherosclerotic and antihyperlipidemic effects of octimibate sodium in rabbits. *Atherosclerosis* **69**: 155–160, 1988.
6. Sampson WJ, Suffolk RA, Bowers P, Houghton JD, Botham KM and Suckling KE, The role of acyl-CoA:cholesterol acyltransferase in the metabolism of free cholesterol to cholesteryl esters or bile acids in primary cultures of rat hepatocytes. *Biochim Biophys Acta* **920**: 1–8, 1987.
7. Ochoa B and Suckling KE, Short-term metabolism of cholesteryl ester from low-density lipoprotein in primary monolayers of bovine adrenal cortical cells. *Biochim Biophys Acta* **918**: 159–167, 1987.
8. Suckling KE and Stange EF, Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* **26**: 647–671, 1985.
9. Dietschy JM, Spady DK and Meddings JB, A quantitative approach to low density lipoprotein metabolism in man and in various experimental animals. In: *Hyperlipidaemia and Atherosclerosis* (Eds. Suckling KE and Groot PHE), Chapter 2. Academic Press, London, 1988.

Biochemical Pharmacology, Vol. 39, No. 9, pp. 1489–1491, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Inhibition of tyrosine-3-monooxygenase by benserazide

(*Received* 12 *July* 1988; *accepted* 16 *November* 1989)

One procedure frequently used to estimate catecholamine synthesis *in vivo* is to measure the accumulation of 3,4-dihydroxyphenylalanine (DOPA) after inhibition of L-aromatic amino acid decarboxylase (3,4-dihydroxy-L-phenylalanine carboxy-lyase; EC 4.1.1.26; AAAD) [1]. In this procedure, the linear accumulation of DOPA in tissues, following a single, large dose of an inhibitor of AAAD, is considered to be a measure of the rate of tyrosine hydroxylation. The method makes at least three major assumptions: (1) AAAD is completely and immediately inhibited; (2) the DOPA formed is not metabolized further and does not diffuse out of the tissue; and (3) the inhibitor of AAAD does not change, directly or indirectly, the hydroxylation of tyrosine. The two most commonly used inhibitors of AAAD, for the *in vivo* estimation of catecholamine synthesis, are 3-hydroxybenzyl hydrazine (NSD-1015) and benserazide (Ro4-4602; DL-serine, 2-[(2,3,4-trihydroxyphenyl)methyl]hydrazide). The structures of these compounds are presented in Fig. 1. Examination of the structure of benserazide reveals a catechol (trihydroxyphenyl) structure. Since catechols have been known to inhibit tyrosine-3-monooxygenase (L-tryosine, tetrahydropteridine: oxygen oxidoreductase [3-hydroxylating]; EC 1.14.16.2; TH) [2], we examined NSD-1015 and benserazide as inhibitors of TH. We now report that benserazide, but not NSD-1015, is an inhibitor of TH, complicating its use for determining tyrosine hydroxylation *in vivo*.

Materials and methods

Benserazide was furnished by Hoffmann-LaRoche (Nutley, NJ), while 3-hydroxybenzyl hydrazine was purchased from the Aldrich Chemical Co. (Milwaukee, WI). The 6(*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) was obtained from Dr B. Schircks (Jona, Switzerland). All other chemicals were obtained from the Sigma Chemical Co. (St Louis, MO).

The TH, used in these studies, was purified in its native form from bovine adrenal medulla by modifications to the method of Togari *et al.* [3]. The modifications were: (1) addition of 10 μg/mL of leupeptin and pepstatin-A to all of the buffers, except the homogenization buffer which additionally contained 0.1 mg/mL of aprotinin, soybean trypsin inhibitor and 1 mM diisopropylfluorophosphate; (2) replacement of the ion exchange column step with a 30–40% ammonium sulfate fractionation; (3) replacing the Biogel A-1.5 M column with a Sephacryl S-400 column (Pharmacia, Piscataway NJ); and (4) washing the heparin agarose affinity column at a flow rate of 3 mL/min. The enzyme, using BH_4 as cofactor, had a pterin K_m of 112 ± 11 μM, a tyrosine K_m of 6.4 ± 2.2 μM, a pH optimum of 6.55, and a specific activity of 37.45 units/mg protein. One unit is presently defined as the amount of enzyme which forms 1 nmol of product in 1 min.

The TH assay was performed using a recent modification of the ^3HOH release assay, where the unreacted isotopic substrate is adsorbed and precipitated with charcoal [4]. Each assay tube contained, in a final volume of 0.1 mL: $NaPO_4$, pH 6.55, 10 μmol; catalase, 30 μg; superoxide dis-

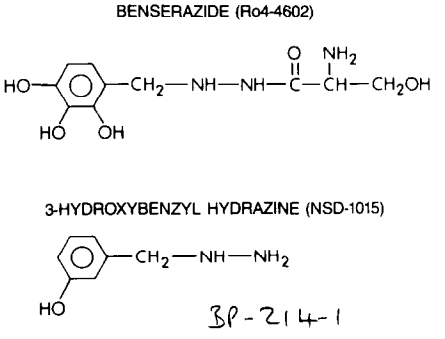

Fig. 1. Structures of benserazide and 3-hydroxybenzyl hydrazine.

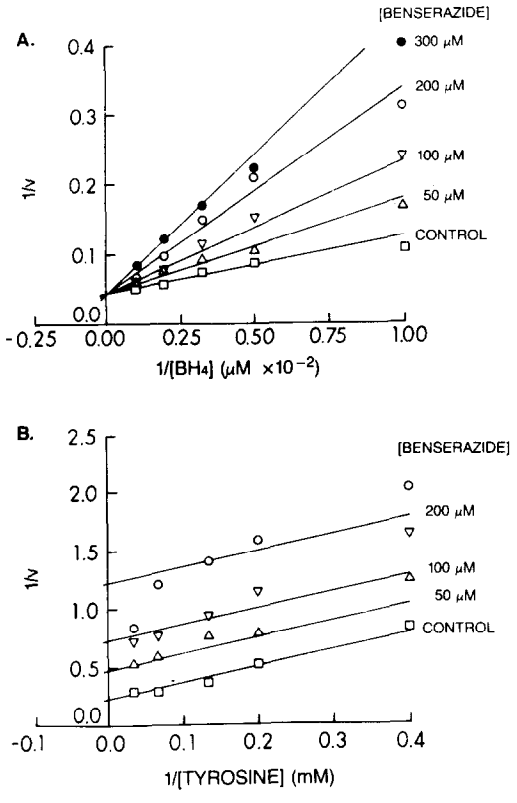

Fig. 2. Tyrosine-3-monooxygenase inhibition by benserazide. (A) The plot was obtained at a fixed tyrosine concentration of 30 μM, while varying the BH_4 (B) The BH_4 concentration was fixed at 0.1 mM, while the tyrosine concentration was varied.

mutase, 0.1 μg; dithiothreitol, 500 nmol; L-[3,5-^{3}H]tyrosine, 3.0 nmol (1–2 μCi); BH_4, 10 nmol, or as otherwise indicated; and 15 mUnits of the TH preparation. Incubations were for 10 min at 37°. Blanks were prepared by omitting the BH_4 and were found to equal those blanks resulting from the deletion of enzyme.

The activity of AAAD was measured on cell-free extracts of rat striatum using a $^{14}CO_2$ trapping method [5].

Enzyme kinetic data were analyzed by the method of Cleland [6], with statistical comparisons by the F-test, as described by Spector and Hajian [7]. A probability of 0.05 was considered as significant.

The *in vivo* accumulation of DOPA was measured 30 min after either benserazide (800 mg/kg, i.p.), or NSD-1015 (100 mg/kg, i.p.). Tissues were assayed for DOPA using liquid chromatography with electrochemical detection [8], as modified [9].

Results

Benserazide was found to be an inhibitor of TH of moderate potency (Fig. 2). The mechanism of TH inhibition by benserazide was examined at a fixed tyrosine concentration of 30 μM, while varying the BH_4. The data in Fig. 2A reveal a competitive mechanism with the pterin cofactor, and a K_i of 75.0 ± 8.8 μM. In contrast, when tyrosine was varied at a fixed BH_4 concentration of 0.1 mM, an uncompetitive pattern of inhibition was observed with an apparent K_i of 68.6 ± 7.2 μM (Fig. 2B). In contrast to benserazide, the non-catechol NSD-1015 was ineffective at concentrations as high as 0.3 mM (data not shown).

Lastly, the accumulation of DOPA in rat striata following injections of either benserazide or NSD-1015 revealed a significant difference in DOPA levels, the NSD-1015 value being 34% higher than that obtained with benserazide (Table 1). We also assessed the degree of inhibition of AAAD produced by these two decarboxylase inhibitors and found essentially complete inhibition by either compound (Table 1).

Discussion

The present results showing inhibition of TH by benserazide are not surprising since many catechols inhibit TH [2] and benserazide possesses a catechol within its structure. Again, the lack of inhibition of TH by the non-catechol, NSD-1015, is consistent with these structural requirements for TH inhibitors. Earlier studies had shown that the kinetic pattern of inhibition of TH by catechols was competitive with the pterin cofactor and non-competitive with the tyrosine substrate [10, 11]. The present studies are in agreement with the earlier findings. *In vivo*, NSD-1015 promoted a higher accumulation of DOPA than did benserazide. Alternatively, these *in vivo* results might be attributed to incomplete inhibition of cerebral AAAD by benserazide. However, we measured striatal AAAD activity in rats following the i.p. injection of either compound and observed complete inhibition by benserazide and nearly complete inhibition by NSD-1015 (Table 1), consistent with an earlier report [12]. Additionally NSD-1015, but not benserazide, inhibits monoamine oxidase (MAO) at the 100 mg/kg dose used [13, 14]. If the inhibition of MAO were to reduce DOPA formation we would, in the present study, *underestimate* the differences between NSD-1015 and benserazide. However, inhibition of MAO by pargyline did not affect the accumulation of DOPA following NSD-1034, an AAAD inhibitor with much lower effects on MAO [13]. The effects of six different inhibitors of AAAD were

Table 1. Effects of NSD-1015 and benserazide on tyrosine hydroxylation in rat striata *in vivo*

Inhibitor	DOPA [nmol·(g·30 min)$^{-1}$]	AAAD activity [nmol·(min·mg protein)$^{-1}$]
Control	NS*	0.244 ± 0.032
NSD-1015	8.67 ± 0.89†	0.055 ± 0.046
Benserazide	6.43 ± 0.12	ND‡

Data are the means ± SE of five animals per group. The inhibitors were administered at doses described in the text in injection volumes of 5 mL/kg; animals were killed 30 min later.

* NA denotes that the tissue was not assayed.

† The groups differ significantly ($t = 2.21$, 8 df, $P < 0.05$).

‡ ND denotes that the activity was too low to be detected.

compared recently for their effects on DOPA accumulation. However, in that study, no direct comparisons were made between the two inhibitors used here [13].

Earlier studies had shown benserazide to be a potent inhibitor of AAAD which, *in vivo*, inhibits brain AAAD at doses higher than NSD-1015 [12, 14]. From a comparison of the doses of benserazide required to inhibit brain and heart AAAD, we can estimate the brain penetration of benserazide to be only 5.6% [15]. Assuming the 2.71 mmol/kg dose to equilibrate with body water (approximately 75% of body mass) and brain entry of 5.6%, we calculate that the levels of benserazide in the brain would be about 200 μM. *In vitro*, these concentrations of benserazide can inhibit TH by greater than the 26% reduction in DOPA formation which we observed. Several possibilities can account for this difference. First, the inhibition of TH by benserazide was competitive with tetrahydrobiopterin, whose levels in the dopamine neuronal cytosol are unknown. High levels of the endogenous cofactor would reduce the efficacy of an inhibitor which was competitive at this site. A second possibility is that catechol inhibitors of TH are less effective against the high-affinity, phosphorylated form of the enzyme [16] which is thought to be the form responsible for catecholamine biosynthesis [17]. Thus, the potency of benserazide on activated TH *in vivo* may have been less than on the low-affinity form used in the present studies. Nevertheless, the difference in DOPA formation between NSD-1015 and benserazide suggests that functional inhibition of TH does occur at the doses of benserazide used to promote DOPA accumulation.

The data demonstrate that benserazide is an inhibitor of TH. Consequently, a major assumption of AAAD inhibitor specificity is not met with benserazide and *in vivo* estimates of catecholamine synthesis may be inaccurate. Since NSD-1015 did not inhibit TH, it is a better choice for studies on DOPA formation.

Department of Medicinal Biochemistry
The Wellcome Research Laboratories
Research Triangle Park
NC 27709, U.S.A.

JOHN F. REINHARD, JR.*
MARY D. SHEARIN

REFERENCES

1. Carlsson A, Kehr W, Lindquist M, Magnusson T and Atack CV, Regulation of monoamine metabolism in the central nervous system. *Pharmacol Rev* **24**: 371–383, 1972.
2. McGeer EG and McGeer PL, *In vitro* screen of inhibitors of rat brain tyrosine hydroxylase. *Can J Biochem* **45**: 115–145, 1967.
3. Togari A, Kano H, Oka K and Nagatsu T, Simultaneous simple purification of tyrosine hydroxylase and dihydropteridine reductase. *Anal Biochem* **132**: 183–189, 1983.
4. Reinhard JF Jr, Smith GK and Nichol CA, A rapid and sensitive assay for tyrosine-3-monooxygenase based upon the release of ^3H_2O and adsorption of ^3H-tyrosine by charcoal. *Life Sci* **39**: 2185–2189, 1986.
5. Sourkes TL, Aromatic-L-amino acid decarboxylase. In: *Methods in Enzymology* (Ed. Kaufman S), Vol. 142, pp. 170–178. Academic Press, New York, 1987.
6. Cleland WW, Statistical analysis of enzyme kinetic data. In: *Methods in Enzymology* (Ed. Parich DL,) Vol. 63, pp. 103–138. Academic Press, New York, 1979.
7. Spector T and Hajian G, Statistical methods to distinguish competitive, noncompetitive, and uncompetitive enzyme inhibitors. *Anal Biochem* **115**: 403–409, 1981.
8. Reinhard JF Jr and Roth RH, Noradrenergic modulation of serotonin synthesis and metabolism. I. Inhibition by clonidine *in vivo*. *J Pharmacol Exp Ther* **221**: 541–546, 1981.
9. Reinhard JF Jr and Perry JA, Fast analysis of tissue catechols using a short, high-efficiency (3 μM) LC column and amperometric detection. *J Liquid Chromatogr* **7**: 1211–1220, 1984.
10. Ikeda M, Fahien LA and Udenfriend S, A kinetic study of bovine adrenal tyrosine hydroxylase. *J Biol Chem* **241**: 4452–4456, 1966.
11. Bullard WP and Capson TL, Steady-state kinetics of bovine striatal tyrosine hydroxylase. *Mol Pharmacol* **23**: 104–111, 1983.
12. Bartholini G and Pletscher A, Effect of various decarboxylase inhibitors on the cerebral metabolism of dihydroxyphenylalanine. *J Pharm Pharmacol* **21**: 323–324, 1969.
13. Nissbrandt H, Engberg G, Wikström H, Magnusson T and Carlsson A, NSD-1034: An amino acid decarboxylase inhibitor with a stimulatory action on dopamine synthesis not mediated by classical dopamine receptors. *Naunyn Schmiedebergs Arch Pharmacol* **338**: 148–161, 1988.
14. Burkhard WP, Gey KF and Pletscher A, Inhibition of decarboxylase of aromatic amino acids by 2,3,4-trihydroxybenzylhydrazine and its seryl derivative. *Arch Biochem Biophys* **107**: 187–196, 1964.
15. Bartholini G, Burkhard WP, Pletscher A and Bates HM, Increase of cerebral catecholamines caused by 3,4-dihydroxyphenylalanine after inhibition of peripheral decarboxylase. *Nature* **215**: 852–853, 1967.
16. Bennett DB and Coscia CJ, Differential inhibition of activated tyrosine hydroxylase. *Arch Biochem Biophys* **227**: 562–569, 1983.
17. Levine RA, Miller LP and Lovenberg W, Tetrahydrobiopterin in striatum: Localization in dopamine nerve terminals and role in catecholamine synthesis. *Science* **214**: 919–921, 1981.

* Address correspondence to: Dr John F. Reinhard, Jr., Department of Medicinal Biochemistry, The Wellcome Research Laboratories, 3030 Cornwallis Rd., Research Triangle Park, NC 27709.